Mechanisms of Chronic Migraine and the Development of Novel Therapeutic Targets for this Disorder

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THESIS

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Dedicated to my wife, Alison Bertels

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This thesis contains concepts, figures, and methods that have been previously published. Specifically Chapter 1 draws on concepts that were previously published in 2 review articles. Chapter 2 is currently under review for publication at *eLife*. Chapter 4 has been previously peer-reviewed and published in *Headache*. Briefly, the title, authors, and contribution of each author follows.

Chapter 1

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Chapter 2

Bertels, Z., Singh, H., Dripps, I., Siegersma, K., Tipton, A., Witkowski, W., Sheets, Z., Shah, P., Conway, C., Petukhova, V., Karumudi, B., Petukhov, P, Baca, S., Rasenick, M., Pradhan, A. A. Neuronal complexity is attenuated in chronic migraine and restored by HDAC6 inhibition. bioRxiv, 2020: p. 2020.04.21.053272.

Chapter 4

Bertels Z, Witkowski WD, Asif S, Siegersma K, van Rijn RM, Pradhan AA. A non-convulsant delta-opioid receptor agonist, KNT-127, reduces cortical spreading depression and nitroglycerin-induced allodynia. Headache. 2020 Dec 16.

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LIST OF ABBREVIATIONS

+TIPS	plus end tracking proteins.
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
αTAT1	α-tubulin N-acetyltransferase 1
ARRIVE	Animal Research: reporting of *In Vivo* Experiments
CALCA	calcitonin-CGRP gene
cAMP	cyclic adenosine monophosphate
CCP	cytosolic carboxypetidase
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
CK1δ	casein kinase 1δ
CMT	Charcot-Marie-Tooth
CREB	cAMP response element-binding protein
CRLR	calcitonin receptor-like receptor
CRPS	Complex Regional pain syndrome
CSD	Cortical Spreading Depression
CT	Threshold cycle
DC	Direct current
DHE	dihydroergotamine
DiI	dialkylcarbocyanine
DOR	Delta Opioid Receptor
DRG	Dorsal root ganglia
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GRK2	GPCR kinase 2
GSK-3β-	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HCN	Hyperpolarization-activated cyclic nucleotide-gated channel

HDAC6	Histone deacetylase 6
Hypo	Hypothalamus
IASP	International association for the study of pain
ICHD	International Classification of Headache Disorders
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
LFP	Local field potentials
LSC	Lumbar Spinal Cord
MAP	Microtubule associated proteins
MEC-17	Mechanosenory abnormality 17
MOH	Medication overuse headache
MOR	Mu Opioid Receptor
NAc	Nucleus accumbens
NDS	Normal donkey serum
NO	Nitric oxide
NOS	Nitric oxide synthase
NTG	Nitroglycerin
OIS	Optical intrinsic imaging
PDE5	Phosphodiesterase 5
PFA	Paraformaldehyde
PKCs	Protein Kinase Cs
PKG	cGMP dependent protein kinase
RAMP1	Receptor activity-modifying protein 1
RAS	Renin-angiotensin system
RCP	Receptor component protein
SC	Subcutaneously
SCx	Somatosensory Cortex
SD	Spreading Depolarization
sGC	Soluble guanylyl cyclase
SIRT2	Sirtuin 2

smFPs	Spaghetti monster fluorescent proteins
SSNRIs	Selective serotonin and norepinephrine reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
TG	Trigeminal Ganglia
TNC	Trigeminal Nucleus Caudalis
TSA	Trichostatin A
TTL	Tubulin tyr ligase
TTLL1	TTL-Like 1
vlPAG	Ventrolateral periaqueductal gray
VPM	Ventral posteromedial thalamic nuclei

SUMMARY

Chronic migraine is an extremely common disorder that greatly impacts the quality of life of the sufferer. Chronic migraine affects up to 2% of the general population and within the United States alone accounts for almost 3 millions Americans. Despite the high prevalence for this disorder and the recent breakthroughs that have been achieved, therapeutic strategies for the treatment of migraine are still limited. With the recent addition of the calcitonin gene related peptide (CGRP) antibodies some patients with chronic migraine who previously had poor response to available migraine pharmaceutics finally experienced a reduction in headache days per month. While these advancements are great and show the therapeutic potential that can be gained from exploring migraine pathophysiology there is still not a migraine therapeutic that is effective in all people nor is there a cure to migraine. Gaining a better understanding about the pathophysiology of chronic migraine would allow for more effective therapeutic options to treat this population. In this thesis I have uncovered a greater understanding of migraine pathophysiology and characterized novel treatment options for chronic migraine. Under the scope of discovering the pathophysiology of migraine I have characterized a neuronal cytoarchitectural basis for migraine chronification. I further established the use of histone deacetylase 6 (HDAC6) inhibitors as a novel treatment target for chronic migraine and migraine aura. I further expanded these findings to reveal other cytoarchitectural changes associated with chronic migraine and another chronic pain disorder, chronic regional pain syndrome (CRPS). I also demonstrated that a non-convulsant delta opioid receptor agonist could effectively relieve chronic migraine associated symptoms and reduce cortical spreading depression events.

Many chronic neuropsychiatric conditions are implicated as having alterations in neuroplasticity. Chronic pain disorders have been found to not be an exception and many have changes in the cytoarchitecture in both humans and rodent models. In this thesis I demonstrate that chronic migraine is correlated with changes in neuronal cytoarchitecture. Following a chronic intermittent nitroglycerin (NTG) model I observed reduced neuronal complexity in the trigeminal nucleus caudalis, periaqueductal gray, and somatosensory cortex, regions that are important for migraine processing. These results are the first of their kind to demonstrate altered neuronal cytoarchitecture correlating with a chronic migraine model. Furthermore, the chronic NTG model resulted in chronic basal hyperalgesia that was reversible through treatment with an HDAC6 inhibitor. HDAC6 inhibition also caused increased neuronal complexity correlating with reduced allodynia. A second mechanistically distinct model examining migraine aura, cortical spreading depression, also showed decreased neuronal complexity in the somatosensory cortex and trigeminal nucleus caudalis. HDAC6 inhibition was able to prevent these cytoarchitectural changes and reduce susceptibility to cortical spreading depression. Building on these findings we found further disruption of the cytoarchitecture in other key migraine and pain related brain regions. Changes in cytoarchitecture were also detected in another model, chronic regional pain syndrome.

Finally in this thesis I further demonstrated the promising potential of using delta opioid receptor agonists for the treatment of migraine. The non-convulsant delta opioid receptor agonist, KNT-127, reduced the number of cortical spreading depression events and reversed established basal hyperalgesia following the chronic NTG model.

Collectively, the results presented in this thesis demonstrate a better understanding of the pathophysiology of chronic migraine as well as further development of therapeutic targets for treatment of this disorder.

1. Introduction

1.1 Preface

In ancient Greek mythology Poena was the goddess of punishment and it is from her we derive our word for pain. The origins of pain being a direct punishment from the gods highlights the magnitude of suffering that surrounds chronic pain disorders. However, despite its intrusive and disruptive nature we cannot live without pain. Physiologically pain is necessary to alert ourselves to injury and dangers in our surroundings. Disruptions in the pain circuitry, resulting in chronic pain conditions, greatly decrease the quality of life for the sufferer. Gaining a better understanding of what causes chronic pain conditions and how these disorders hijack the pain pathway is necessary to develop better therapeutic options for the sufferer.

One especially common form of chronic pain is chronic migraine. Chronic migraine effects up to 2% of the American population [1]. Patients with chronic migraine have a greatly decreased quality of life and are in need of much better treatment options. While much knowledge has been recently gained about how we treat chronic migraine there is still much left unknown. Over half of patients with migraine are dissatisfied with their current therapeutic options [2]. This leaves a wide window of opportunity to develop better treatment options and learn more about the underlying pathophysiology of chronic migraine. As of now there is no cure for migraine and no one treatment is effective in all cases. The development of novel therapeutic targets and a better understanding of the pathophysiology of migraine is necessary to better treat chronic migraine. Gaining a better understanding of the mechanisms that drive migraine

from an acute to a chronic disorder and the characterization of therapeutic targets for the treatment of chronic migraine is the primary goal of this thesis. Following the Introduction I will present data supporting this proposal in manuscript form. This will include 1 published peer-reviewed publication, 1 publication under revision, and 1publication that is in preparation]. These data include the detailed characterization of the cytoarchitecture of neurons within key migraine pain processing regions following a chronic nitroglycerin migraine model. It will also include evidence for the development of histone deacetylase 6 inhibitors as targets for reversing chronic migraine cytoarchitectural changes. Further this thesis will show how cortical spreading depression, a model of migraine aura, can also result in alterations in cytoarchitecture prevented by histone deacetylase 6 treatments. I will also show how these cytoarchitectural alerations are not unique to migraine and are present in another chronic pain disorder. I will finally demonstrate how a novel non-convulsant delta opioid receptor agonist can effectively treat many migraine-associated symptoms. Collectively, the data shows a greater understanding for the pathophysiology of migraine and lays the groundwork for the development of migraine treatments in the future.

This Introduction will cover background information concerning current knowledge on migraine, the effect of microtubule dynamics in regulating migraine, and the potential of delta opioid receptors (DOR) as a migraine therapeutic. Following chapters will each have a short introduction specific to the project discussed. In the thesis Introduction I will begin by discussing the current knowledge surrounding migraine including symptoms, anatomical regions implicated in migraine, and current migraine therapeutic options. I will then discuss the phenomenon of migraine aura and

its physiological correlate cortical spreading depression (CSD). Following this, I will then demonstrate the use of DOR agonists in treating migraine and variations in this treatment. Finally, I will finish this introduction by discussing the cytoarchitecture of neurons and the role that microtubules play in cellular dynamics. I will then briefly conclude by stating the aims and further organization of the thesis.

1.2 Defining Migraine

Migraine is an extremely common neurological disorder affecting upwards of 14% of the world population. This makes migraine the sixth most prevalent disease worldwide [3]. Migraine generally has peak effects during the most productive years of a patient's life, between the ages 20—60 [4]. This has resulted in substantial economic burden of migraine with an estimated $36 billion in productivity lost annually in the United States [5]. Migraine is often found to greatly lower the quality of life of sufferers and with its widespread prevalence it presents a critical problem that must be remedied.

To begin understanding how to better treat migraine disorders it is necessary to first define what a migraine is. The International Classification of Headache Disorders (ICHD) identifies migraine as a primary headache disorder. That is, a headache that is not due to a secondary symptom of another disorder [6]. The hallmark symptom of migraine is the headache, which typically lasts between 4-72 hours [6]. To be classified as a migraine the attack also must fulfill two of the following four phenomenon; headache has a unilateral location, is pulsating in quality, causes moderate or severe pain, and/or can be aggravated by routine physical activity [6]. Within these broad categories there can be a good deal of heterogeneity into how a sufferer experiences their migraine. Additionally, a migraine diagnosis must have at least one of the following

3 criteria accompanying the headache: nausea, photophobia, and/or phonophobia. This array of symptoms help to demonstrate the different characteristics that can define a migraine disorder and further hint at the complex pathophysiology [6].

Given that migraine is a very complex and varied disorder subdivisions into migraine categories have been made to allow for better classification and treatment. One large division is migraines with or without aura. Migraine with aura is less common, but still accounts for approximately 1/3rd of total migraine cases [6]. A migraine with aura patient will have the migraine attack phase preceded, usually 30 minutes to 72 hours before, by specific neurological symptoms, which are categorized as the aura [6]. The ICHD defines migraine with aura as having reversible aura symptoms that affect one of the following areas: visual, sensory, motor, brainstem, retinal, speech and/or language. Of these listed symptoms visual disturbances are the most common. To fit the definition of a migraine with aura, the aura symptoms must spread gradually over a 5-minute interval. In the case of a visual aura, the visual disturbances slowly pass across the field of vision for the 5-minute period. Furthermore, the aura must have two or more symptoms occur in succession, with at least one symptom being positive and unilateral. In the case of visual aura, the positive symptom often presents as a scintillating scotoma that moves across the field of vision and is followed by a longer lasting blurring of the vision. The blurring of the vision is referred to as the negative symptom. Finally, as stated before, the aura must be followed by head pain [6]. If these symptoms are present then the migraine is diagnosed as migraine with aura, if not it is diagnosed as the more common migraine without aura.

Another common distinction in migraine diagnosis derives from the frequency of migraine attacks and can be classified as either episodic or chronic migraine. Chronic migraine is defined as having 15 or more headache days a month, in which at least 8 of the days per month have features of a migraine headache. This criteria must be met for a minimum of 3 months before being considered chronic migraine [6]. Chronic migraine has an estimated prevalence of approximately 2% of the total population [1, 7]. Due to the frequency of attacks, chronic migraine is an especially disabling category of migraine and is notoriously difficult to treat. Chronic migraine is further complicated as patients are found to be at much higher risk of comorbidity with other neurological disorders [8]. Chronic migraine is a growing problem as the transition of migraine from an episodic migraine condition to a chronic one is estimated to occur at a rate of 2.5% per year [8, 9]. The mechanism responsible for the progression of migraine from an episodic to a chronic case is still unclear. However, there are some known factors that can increase the transition, including frequent use of triptans and the use of μ-opioid receptor (MOR) agonists [10, 11].

While these are just a subset of the categories of migraine classification, they highlight some important distinctions within migraine. Properly diagnosing patients with their specific type of migraine is important to aide in proper therapy as different migraine types have been found to respond differently to various therapeutic interventions. The division in migraine classification also impacts preclinical research as various animal models can reflect certain aspects and subtypes of migraine. The diversity in classification of migraine really highlights its heterogeneity and underlying complex pathophysiology. While there are a variety of specific classifications of migraine many

follow the same pattern of attacks. Below I will first be discussing the neuroanatomy implicated in migraine and then go on to discuss how these regions are implicated in the phases of migraine.

1.2.1 Neuroanatomy of Migraine

Migraine has a complex pathophysiology that is comprised of both the central and peripheral nervous system. Migraine attacks are thought to begin in the trigeminovascular complex, a summary of the pathways can be found in Figure 1. The trigeminovascular pathway receives nociceptive information from the meninges and projects these signals onto the brain [12]. The trigeminovascular pathway begins within the trigeminal ganglia (TG). These trigeminal ganglia have peripheral axons that innervate, among other things, the pia, dura, and large cerebral arteries [13]. The TG then projects to the brain through the dorsal horn laminae of the trigeminal nucleus caudalis (TNC) [14]. Within the TNC other signals from the periorbital skin and pericranial muscles are also innervated [15]. These signals are integrated and then project to multiple parts of the central nervous system. The TNC has monosynaptic connections to brainstem nuclei including the ventrolateral periaqueductal gray (vlPAG), as well as the superior salivatory, parabrachial cuneiform, and the nucleus of the solitary tract. The TNC also directly connects to the hypothalamic nuclei, and the basal ganglia including the caudate-putamen, globus pallidus, and substantia innominata [16]. The TNC neurons innervated by the trigeminovascular pathway also project to the ventral posteromedial (VPM), posterior, and parafascicular nuclei of the thalamus [16]. Finally these projections can be relayed from the thalamic nuclei and on to several cortical regions including somatosensory, insular, motor, auditory, visual, and olfactory

cortices. Collectively these various projections and connections account for the variety of symptoms that accompany a migraine. Selective activation of different areas of the pathway contributes to the various phases of the migraine attack that are detailed below.

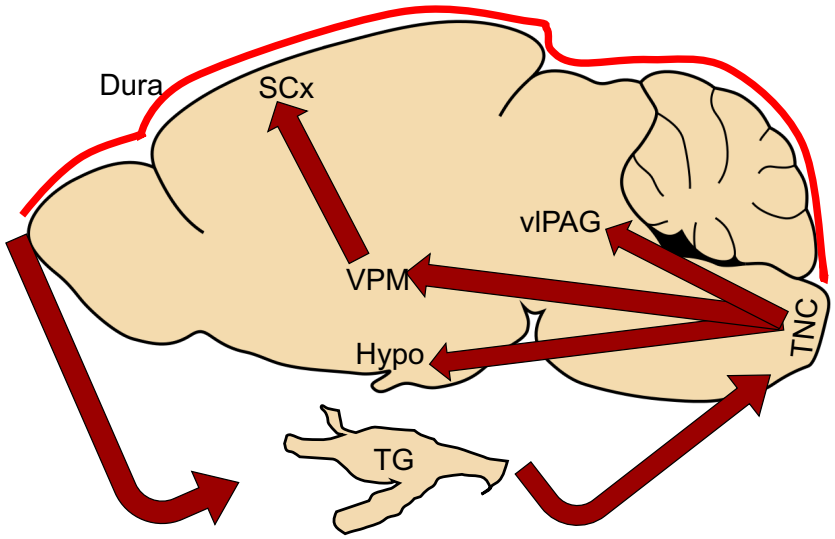

Figure 1 Schematic of trigeminovascular pathway The trigeminal ganglia (TG) is outside of the central nervous system and innervates the pia, dura, and large cerebral arteries. The TG then projects to the outer lamina of the trigeminal nucleus caudalis (TNC). From here the signal can be sent to multiple places including various hypothalamic (Hypo) nuclei, the ventrolateral periaqueductal gray (vlPAG), and the ventral posteromedial (VPM) nucleus of the thalamus. The thalamus, among other regions, can project to the somatosensory cortex (SCx). These projections account for many of the symptoms accompanying a migraine attack including the headache pain.

1.2.2 Migraine Prodrome/ Premonitory Phase

The migraine prodrome phase encompasses symptoms that precede the headache. The occurrence of these symptoms allows astute patients with migraine to predict a migraine attack up to 12 hours in advance [17]. The most common symptoms of the prodrome phase are fatigue, mood changes, food cravings, yawning, muscle tenderness, and photophobia. This wide array of symptoms give further evidence to the complexity of migraine and suggest differential neuronal circuitry [18]. The hypothalamus is likely involved in several of the more common prodrome symptoms

including fatigue, depression, irritability, food cravings, and yawning. Evidence for this comes from an fMRI study in which a patient with migraine was monitored for 30 days, and revealed hypothalamus activation up to 48 hours before the migraine onset [19]. Other brain regions such as the brainstem are thought to be responsible for the muscle tenderness and neck stiffness that can precede the migraine attack. The cortex is thought to be responsible for sensitivity to light, sound, and smell, while, the limbic system is implicated in driving the mood changes [18]. These data highlight that migraine is not just a single region neurological disorder; rather it can affect many regions across the central nervous system.

Many of the migraine prodrome symptoms hint at dysregulation of homeostasis. Homeostasis is classically thought to be regulated by the hypothalamus. There are two leading theories as to how the hypothalamic neurons may be involved in triggering prodromal symptoms. The hypothalamus neurons can activate meningeal nociceptors and shift the sympathetic tone to more parasympathetic [20, 21]. Hypothalamic neurons can activate preganglionic parasympathetic neurons in the superior salivatory nucleus and sympathetic preganglionic neurons in the spinal intermediolateral nucleus [22-26]. Subsequently, the superior salivatory nucleus can release acetylcholine, vasoactive intestinal peptide, and nitric oxide (NO) into meningeal terminals. The release of these molecules and peptide can result in dilation and further release of inflammatory molecules that can activate the meningeal nociceptors. Furthermore, the superior salivatory nucleus can have direct effects on the TG, which could drive the migraine prodromal and eventual headache phase [27].

The other leading theory suggests that hypothalamic and brainstem neurons regulate responses to changes in homeostasis and can lower the threshold of transmission from trigeminovascular signals from the thalamus to the cortex [28]. The thalamus is known to select, amplify, and prioritize information that is sent to the cortex and the hypothalamus and brainstem nuclei regulate relay thalamocortical neurons allowing them control over this circuitry [29-33]. These trigeminothalamic neurons were found to receive direct input from the hypothalamic neurons [28, 34]. These hypothalamic input neurons were found to contain dopamine, histamine, orexin, and melanin concentrating hormone. Theoretically these neuropeptides and neurotransmitters can shift the activity of thalamic neurons from burst to tonic firing or vice versa depending on the inhibitory or excitatory potential. The various factors that surround the hypothalamic firing demonstrate how various external and internal conditions can trigger migraines in some prone individuals but not all. This theory demonstrates how hypothalamic and brainstem neurons projecting on the thalamus can alter different set points for the migraine brain [34]. The variations in the amount of brain activity required to emotional or physiological stress can explain why some are more prone to migraine at different points in their circadian rhythms. Collectively these data demonstrate how neuronal populations can interact with one another in complex circuitry to produce a premonitory phase in some individuals. The premonitory phase is followed directly by the headache phase.

1.2.3 Migraine Headache Phase

The headache phase of a migraine attack is what is most often associated with migraine, as it is the throbbing, pulsating, unilateral headache. As mentioned, the headache can present alone, but to be classified as a migraine it must be accompanied by nausea, photophobia, and/or phonophobia. The attack itself is relatively long lasting and usually takes 4-72 hours to subside. The trigeminovascular pathway is highly implicated in driving the migraine headache phase as it conveys nociceptive information from the meninges to the central nervous system. As discussed earlier the trigeminovascular pathway begins outside of the central nervous system, with the TG. The TG neurons bifurcate, such that one part of the axon innervates the pia, dura, and large cerebral arteries and the other projects onto the dorsal horn of the TNC [13, 14]. This projection is responsible for the headache pain associated with migraine. The TNC neurons receive additional input from the periorbital skin and pericranial muscles, which contribute to the substantial cephalic allodynia that accompanies migraine [15]. As discussed above the TNC projections connect to brainstem, hypothalamic, and basal ganglia nuclei. [16]. These various projections are implicated in a number of other symptoms that accompany migraine besides the direct headache pain [20]. The TNC was also found to project to the thalamic VPM, posterior, and parafascicular nuclei [16]. The projection of the TNC onto the thalamus and the various projections from the thalamus to the various cortical areas are believed to regulate a number of other migraine symptoms including allodynia, photophobia, and phonophobia [35].

It is believed that a key factor in why some people are more susceptible to migraine triggers than others is hypersensitization of the migraine brain [36]. Sensitization is the phenomenon in which response thresholds decrease while the

subsequent response magnitude increases [37]. In a migraine conducive environment peripheral trigeminovascular neurons can become sensitized. The sensitization of the trigeminovascular neurons generates responses from dura stimuli that would normally show minimal or no response [38]. This sensitization not only directly affects the trigeminovascular neurons; the TNC and thalamic nuclei also become sensitized. This spreading sensitization results in spontaneous activity and increases the responsive fields, such that innocuous stimulation of cephalic and extracephalic areas are now noxious [39, 40]. The central sensitization of the TNC, which normally takes 30-60 minutes to develop and 2 hours to peak following a migraine attack, is thought to be the cause of cephalic allodynia [8, 41, 42]. Similarly, the extracephalic allodynia is linked to sensitization of the thalamus, which normally takes 2-4 hours to occur after a migraine attack [8, 41, 42]. The cephalic and extracephalic allodynia contribute to the severity of migraine attacks and demonstrate how sensitization can lead to a greater disability of patients with migraine. There is evidence that the common migraine abortive therapies, triptans, can disrupt communication between peripheral and central trigeminovascular neurons in the dorsal horn [43]. Disruption of this circuit is thought to contribute to decrease in sensitization or prevention of the sensitization. Ending the spread of sensitization early in the chronological pathway is thought to mitigate migraine and is likely why abortive therapies are most effective when taken earlier rather than later in the attack [20, 44]. After the headache phase many patients with migraine will then enter the postdrome phase.

1.2.4 Postdrome Phase

Postdrome is the final stage of the migraine attack and many of the symptoms that are seen in the postdrome are also seen during the premonitory phase [45, 46]. The most common symptoms of postdrome include fatigue, difficulty concentrating, and a stiff neck [46]. Usually the postdrome lasts less than 24 hours after the migraine pain has resolved [46]. Interestingly, the severity of the migraine is not associated with duration of the postdrome [47]. Given the similar symptomology that is seen between the prodromal and postdromal phase it is suggested that many of the same brain regions are involved. It is believed that there is diffuse cortical and subcortical involvement driving the postdromal phase based on symptomology [37]. It has been suggested that the postdrome is driven by the frontal lobes and the hypothalamus. There has also been MRI studies that show reduction in brain blood flow in the postdrome [37]. Similarly, the phenomenon that is believed to drive migraine aura, CSD, has been implicated in the postdrome symptoms again highlighting the similarity between the pro- and post-drome phases [37]. There is evidence that there is persistent hypoperfusion following CSD events and this may link to the pathophysiology seen in the postdrome phase [48].

Collectively these data show the impact that migraine can have on a sufferer outside of the actual headache attack phase. When including the prodrome and postdrome phase with the migraine attack the patient can have decreased productivity and quality of life for multiple days from just a single episode. Migraine is a pervasive neurological disorder and affects a large amount of the central nervous system resulting in a host of symptoms. Gaining a better understanding of the pathophysiology of

migraine would allow for better treatment of all phases of migraine. To this end I will discuss factors that have been found to increase the risk of migraine.

1.3 Migraine Susceptibility

There is likely a genetic component that regulates the enhanced susceptibility to migraine. Earlier age of onset and severity of migraine attacks were both found to be higher in those who had familial history of migraine [49]. Genome wide association studies investigating gene variants in migraine have revealed 13 susceptible gene variants that occur in migraine with and without aura [50-53]. These genes were found to regulate different aspects of the nervous system and in general cause neuronal hyperexcitability of the migraine brain [12]. Some of the identified genes increase glutamatergic neurotransmission which can result in increased NMDA receptor occupation, which is implicated in the development of allodynia and central sensitization [41]. The neuronal hyperexcitability of the migraine brain has also been implicated as dysfunction in thalamocortical dysrhythmia [54-57], or modulatory brainstem circuit dysfunctions responsible for regulation of excitability along the neuroaxis [58]. There is also evidence that there is improper regulation and habituation of cortical, thalamic and brainstem neurons form the limbic system [39, 59-62]. Collectively these data demonstrate the role that hyperexcitability of the brain can play in the development of migraine and how genetic factors may play a role in enhancing migraine susceptibility. Some studies have shown enhanced brain activation in patients with migraine compared to control subjects in the PAG [61], red nucleus, and substantia nigra [63]; hypothalamus [64]; posterior thalamus [39]; cerebellum, insula, cingulate and prefrontal

cortices, anterior temporal pole, and the hippocampus [65, 66]. The increases in activity were found after non-repetitive stimuli and strengthen the idea that migraine brains lack the ability to properly habituate incoming stimuli resulting in hyperexcitability [67, 68].

These changes in excitability are accompanied by morphological changes in migraine patients. Compared to healthy controls, patients with migraine were found to have thickening of the somatosensory cortex [69-71]; increased gray matter density in the caudate [72]; and gray matter volume loss in the superior temporal gyrus, inferior frontal gyrus, precentral gyrus, anterior cingulate cortex, amygdala, parietal operculum, middle and inferior frontal gyrus, inferior frontal gyrus, and bilateral insula [69, 73]. Interestingly, there is evidence that changes in cortical regions are dependent on the frequency of the migraine attacks [72, 74]. There is some evidence to suggest that these changes are adaptive following migraine pain as there are similar changes in other chronic pain conditions [75-77]. Furthermore, there is evidence to indicate that the changes are reversible, and magnitude of changes is correlated with duration of disease [78]. Some evidence also shows that the change in brain morphology is sex dependent [79, 80].

Although there is not a single gene mutation that has been identified to be the cause of most migraine cases, there are subsets of migraine patients who suffer from a genetically inheritable form of migraine known as familial hemiplegic migraine (FHM). Three genes are commonly mutated in FHM and they all regulate glutamate availability in the synapse. FHM1, caused by a mutation in CACNA1A, encodes pore-forming α1 subunit of the P/Q type calcium channel [81, 82]. FHM2, caused by a mutation in ATP1A2, encodes the α2 subunit of the Na/K ATPase pump [83]. The third FHM3,

caused by mutation in SCN1A encodes the α1 subunit of the neuronal voltage gated $Nav_{1.1}$ channel [84]. While these 3 gene mutations are linked to FHM they are not seen in most typical migraine patients. Additionally, a distinct missense mutation of the casein kinase 1δ (CK1δ) gene was identified in two migraine families [85]. This mutation was accompanied by both the presence of migraine and advanced sleep phase. Mice engineered to have the same mutation were found to be more sensitive to nitroglycerin and have a reduced threshold for CSD, two mechanistically distinct models of migraine [85].

To gain a better understanding of how the migraine brain has increased susceptibility it is necessary to perform preclinical research to unlock the pathophysiological underpinnings that result in migraine. Below I will discuss some of the most common preclinical models of migraine and how these can be used to gain a better understanding of the pathophysiology of this disorder.

1.4 Models of Migraine

Preclinical animal models are necessary to gain a better understanding of the pathophysiology of a disease and to screen novel therapeutics. Many different models of migraine have been developed in a host of animals. While no one model is perfect and can capture the full extent of migraine different models can be used to investigate various aspects of migraine.

1.4.1 Nitroglycerin and Migraine

One of the most common models of migraine, and the one primarily utilized in our research, is treatment with nitroglycerin (NTG). NTG in relation to migraine, was

discovered by Ascanio Sobrero in 1847 who reported, “a very minute quantity put upon the tongue produces a violent headache for several hours”[86]. Intravenous injection of NTG reliably triggers headache in healthy subjects and in patients with migraine produced a delayed migraine attack in a dose dependent manner [87-90]. Since these original findings NTG has been commonly used to trigger migraine attacks to study migraine in both human and animal experiments [90]. Upon administration, NTG is rapidly metabolized into NO within cells through both enzymatic and non-enzymatic processes. NO has a host of effects once metabolized including acting as an oxygen free radical on smooth muscle resulting in relaxation and vasodilation. NO also acts as a neuronal messenger with diverse signaling tasks in both the central and peripheral nervous system [91]. Other NO donors have also been used to trigger attacks in patients with migraine. Inhibition of nitric oxide synthase (NOS) has been studied as a possible therapeutic option further highlighting the importance of NO in the migraine pathway [87, 92, 93].

The treatment of NTG produces robust acute effects within 2 hours of injection, but importantly it also establishes basal hypersensitivity to mechanical stimuli even several days after the NTG treatment [94]. This, among other data, has led researchers to conclude that NTG has a biphasic activation that drives the pro-migraine effects. In humans NTG administration results in not only the immediate headache phase, but also has a delayed component implicated to involve production of new proteins [95]. Activation of trigeminal neurons in the early phase is thought to result from peripheral release of NO, which in turn dilates cranial blood vessels or stimulates sensory fibers. However, direct application of an NO donor to neurons in the TNC enhances facial

receptive fields stimulation indicating it is not solely a vascular effect [96]. The secondary component is induced by central effect upon neuronal elements responsible for signals from cranial structures. NTG can produce alterations through secondary messengers, such as cGMP, and release of calcitonin gene-related peptide (CGRP) following activation of the trigeminovascular system [97-100]. NTG infusion induces sustained elevations of cortical NO release that far exceeds the half-life of NTG implicating that it may drive synthesis of NO [101, 102]. NTG administration was also found to increase levels of NOS in neurons in dura mater, TG, TNC and perivascular nerve fibers supplying the meninges [103-107]. Furthermore, cGMP is increased in the cortical arteries as well as neuronal fibers in the TNC implicating a central effect as well as the changes in the periphery [99]. Increased cGMP levels can also have a direct effect on the hyperpolarization-activated cyclic nucleotide-gated channel (HCN) and a cGMP dependent protein kinase (PKG). PKG can regulate several proteins through phosphorylation and lower intracellular calcium. PKG can also directly affect phosphorylation of cAMP response element-binding protein (CREB), further altering transcriptional and epigenetic mechanisms [108]. A summary of NO pathway can be seen in Figure 2. These data collectively demonstrate the importance that NO has on migraine pathophysiology and how much can be learned from using NTG as a model system.

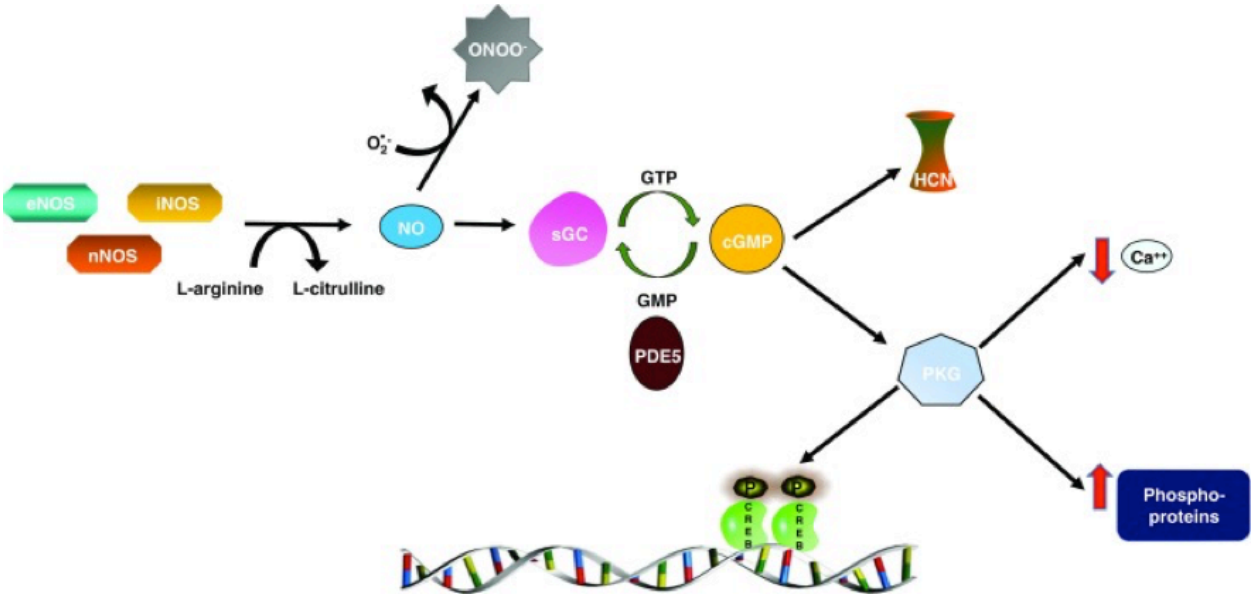

Figure 2. Signaling and synthesis of nitric oxide (NO) The three synthases for NO are nNOS, eNOS, and iNOS. These 3 produce NO as a byproduct of the oxidation of L-arginine to L-citrulline. NO can form oxygen radicals or bind to the high affinity receptor soluble guanylyl cyclase (sGC). sGC converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The cGMP can then activate the cell membrane bound ion channels, hyperpolarization-activated cyclic nucleotide-gated channel (HCN). cGMP can also activate the cGMP-dependent protein kinase (PKG), which can phosphorylate a number of different proteins and regulate them. CREB is one of these proteins and this can result in altered gene expression. PKG also inhibits intracellular calcium stores. Phosphodiesterase 5 (PDE5) breaks down cGMP to GMP which can act as a negative regulator of this pathway [108].

NTG is used in rodents to model several aspects of migraine. NTG can produce photophobia, phonophobia, and increased meningeal blood flow in rodents [109, 110]. Most commonly studied is the ability of NTG to examine allodynia and hyperalgesia associated with the migraine attack [94, 109, 111]. NTG treatment was found to produce both thermal and mechanical allodynia in mice that could be reversed through migraine treatments including sumatriptan and CGRP receptor antagonists [111, 112]. Repeated intermittent injections of NTG can be used to study the progression of migraine from an acute to chronic state, as repeated injections produce basal chronic

hypersensitivity (Figure 3) [94]. Acute treatments for migraine, such as sumatriptan, were previously found to block post-treatment hyperalgesia when given after NTG treatment [113] Many migraine preventives, including topiramate and propranolol, were seen to block NTG-induced basal hypersensitivity [114, 115]. NTG was also found to produce other migraine like symptoms in rodents including light-aversive behavior, a reflection of photophobia, and increased meningeal blood flow [109, 110]. Systemic NTG induces activation of many brain regions including the PAG and TNC, a similar pattern of activation that is seen in noxious stimulation of facial afferents [116, 117]. Further supporting the similarities between humans and rodents response to NTG, a transgenic mouse model of CK1δ showed greater sensitivity to NTG induced hyperalgesia compared to wild type animals [118]. Endogenously phosphodiesterase type 5 breaks down cGMP to GMP, which can act to inhibit the nitric oxide molecular mechanism. Phosphodiesterase type 5 inhibitors, such as sildenafil, were found to increase migraine frequency as these inhibitors promote cGMP signaling [108].

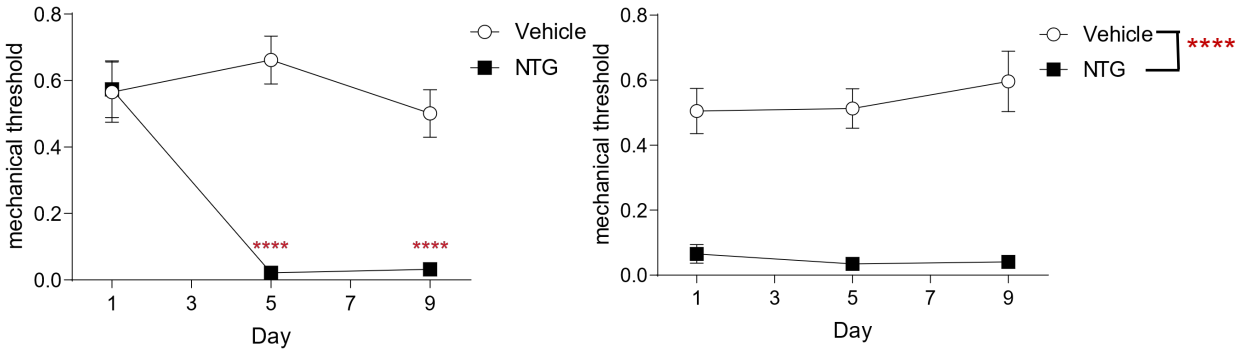

Figure 3 Nitroglycerin produces both basal and 2-hour post treatment hyperalgesia A) Nitroglycerin (NTG) given every other day for 9 days produces robust basal mechanical hyperalgesia detectable on day 5 and sustained through day 9 ****p<0.0001 Two-way ANOVA. B) 2-hours after injection of NTG, mice have decreased mechanical thresholds Two-way ANOVA ****p<0.0001

1.4.2 Vascular Models of Migraine

Migraine has a complex pathophysiology that affects many aspects of the nervous system and as such models focus on a subset of the changes induced in migraine. One of these changes is examining alterations in vasculature [119]. Many studies examined if pharmacological agents could produce vascular constriction. In pigs, arteriovenous oxygen differences have been studied as this is also thought to be reduced in humans during migraine [120]. Similar studies have been done investigating the constriction of the external carotid bed in canines [119]. While there have been some migraine therapeutics developed through this model it is limited as it can only investigate medications that have a primarily vascular mechanism. Similar models can be used *in vitro* through investigation of the dissected vascular segments. Vascular segments are mounted in organ baths and contraction or relaxation is measured isometrically. Response curves are then generated to determine the potency and efficacy of anti-migraine agents on their ability to reduce changes in the vasculature [121]. These studies have also been done to measure second messengers or intracellular calcium concentrations [122]. The vasculature can be stimulated chemically or electrically to monitor endogenous neuropeptides that are released in the vasculature including CGRP [123, 124].

In vivo studies investigating the dural vasculature have demonstrated considerable utility in identifying receptors capable of modulating nociceptive stimuli that may be clinically relevant. This model is performed by thinning a closed cranial window and using microscopy to visualize and measure changes in diameter of cranial, dural and pial blood vessels [125, 126]. Following electrical stimulation of the cranial window there is dural and pial blood vessel dilation and activation of the trigeminal nerve that is

thought to be due to the release of CGRP form presynaptic trigeminal nerve endings [125, 127, 128]. Examining the dural vasculature in this way has successfully been used to screen for potential migraine therapies, as inhibition of neurogenic dural vasodilation has been seen after treatment using triptans, dihydroergotamine, and CGRP receptor antagonists [125, 128-130]. Interestingly, other less successful but still used migraine therapies including MOR agonists and neuronal NOS inhibitors have reduced neurogenic dural vasodilation [127, 131]. Not all migraine treatments are successful in this model as propranolol, valproate, and flunarizine were all unsuccessful at inhibiting neurogenic dural vasodilation [132-134]. Importantly though, NO and CGRP were both able to induce dural blood vessel dilation strengthening the use of this model [127, 135]. While the vasculature component plays an important role in migraine, research has indicated that it is not the entire story and other factors account for migraine pathophysiology [136].

1.4.3 c-Fos Stimulation

c-Fos is an immediate-early response gene whose activation occurs within 5 minutes and continues for 15-20 minutes after stimuli. c-Fos protein immunoreactivity can be used as a marker of nociception as peripheral noxious stimulation induced Fos immunoreactivity within the spinal dorsal horn [137]. c-Fos protein expression within the TNC following mechanical, electrical, or chemical stimuli in the extracranial or intracranial tissues serves as a marker to examine facial nociception. Studies have investigated c-Fos immunoreactivity for identifying subpopulation of neurons activated in response to noxious stimuli and thus identifying related nociceptive pathways [121]. Electrical stimulation of the TG has shown increased c-Fos expression in the ipsilateral

TNC [138]. Similarly, chemical stimulation of the meninges, with the use of capsaicin or other nociception activators, has revealed c-Fos expression in a dose dependent manner in the TNC [139]. These studies demonstrate the importance of the trigeminovascular pathway and have allowed for a greater structural understanding of the pathophysiology of migraine [140-144]. From the original TNC studies work has further mapped neuronal activation of higher structures involved in ascending and descending modulatory control of migraine [145-147].

1.4.4 Inflammatory Soup

One of the most common preclinical models relies on chemical provocation that uses different vasodilatory agents. The administration of a mix of inflammatory mediators known commonly as “inflammatory soup”, which is often a mixture of prostaglandin, histamine, serotonin, bradykinin, can be used to stimulate meningeal trigeminovascular nociceptors [148]. The inflammatory soup is often administered by injection using a micro-catheter placed in the cisterna magna. Alternatively direct topical application on the dura mater of rats is used as it causes reversible cephalic mechanical sensitivity [149-151]. Similarly, there are several models that rely on stimulation of the trigeminal nerve to produce this cephalic nociceptive activation. Release of neuropeptides including, CGRP, Substance P, and neurokinin A are released from the perivascular nerve fibers [152-154]. These peptides promote vasodilation and mast cell activation [155, 156]. This model has yielded some extremely valuable evidence into the mechanisms of migraine including the role of histamine and/or mast cell degranulation [157].

While many of the models outlined above can effectively mimic various symptoms associated with migraine, no one model is all encompassing. Each have their own individual strengths and weaknesses. Furthermore, some models can show promising results when using a novel therapeutic, but these findings are not replicated in other models. Therefore, it is always important to consider using multiple migraine models to confirm findings and test new treatment options. While no one model is perfect the information we have gained about the mechanisms of migraine and the development of novel therapeutics highlights the importance of animal models to further advance the field.

1.5 Cortical Spreading Depression

Cortical spreading depression (CSD) was first discovered in 1944 by Aristides Leão, a Brazilian biologist who studied epilepsy in the cerebral cortex of rabbits [158]. The initial publications by Leão described a spreading suppression of spontaneous electroencephalogram activity that lasted for several minutes following electrical tetanic stimulation of the brain surface [158]. Following this hallmark study Leão went on to publish 3 more papers investigating this newly discovered electrophysiological phenomenon [158-161]. In these papers he discovered that the wave of suppression that gave CSD its namesake is preceded by a wave of hyperpolarization of the cells, which can be detected as a large shift in the direct current (DC) potential [159]. The DC shift was found to be a wave of hyperpolarization, which is then followed by a large depolarization and depression of activity, making the name of CSD somewhat of a misnomer. Leão also discovered vascular changes associated with the CSD wave, such that as it passed across the brain there was a dilation followed by a constriction of the

pial vessels [159-161]. Since these original finding CSD has continued to be investigated for its physiological components and its potential impact on various disease states most notably its effect on migraine.

Since Leão's original discovery of the DC potential shift, much work has been conducted to gain a greater understanding of the molecular mechanisms that regulate the DC shift and drive CSD. The DC potential shift normally lasts between 1 and 2 minutes and occurs as either a rapid peak followed by a plateau effect or as a second brief decline forming a second peak [161]. A depiction of a typical CSD shift induced through KCl stimulation can be seen in Figure 4. The DC potential shift is followed by changes in neuronal potential, as intracellular recordings of cortical neurons reveled an almost entire neuronal depolarization and a dramatic drop in membrane resistance [162]. The depolarization following CSD can be divided into an early phase, main phase, and a late phase. The early phase sees apical dendrites depolarize through activation of local ion channels localized on the dendrite [163, 164]. The early phase depolarization is also accompanied by a sharp increase in extracellular potassium [K^+] as well as decreases in extracellular sodium [Na^+], chloride ions [Cl^-], and calcium [Ca^{2+}] [162, 165, 166]. The main phase sees the depolarization move down the neuron and activation of ion channels on the somatodendritic membrane. Finally, the late phase concludes the rapid depolarization of the neuron and sees the closures of ion channels in the somatobasal zone [163, 164]. These changes in ionic concentration are accompanied by an increase in pH, which is followed by a sustained decreased pH [166]. There are also effluxes of anions including glutamtate and aspartate released during CSD, further altering the surrounding tissue [162, 167, 168]. Neuronal swelling

as well as dendritic morphology changes, including loss of spines and dendritic beading, accompany these drastic shifts in ionic concentrations and pH [169, 170]. All of these factors comprise a standard CSD wave and after initiation the wave can rapidly self-propagate throughout the surrounding tissue approximately 2-5mm/min [171, 172].

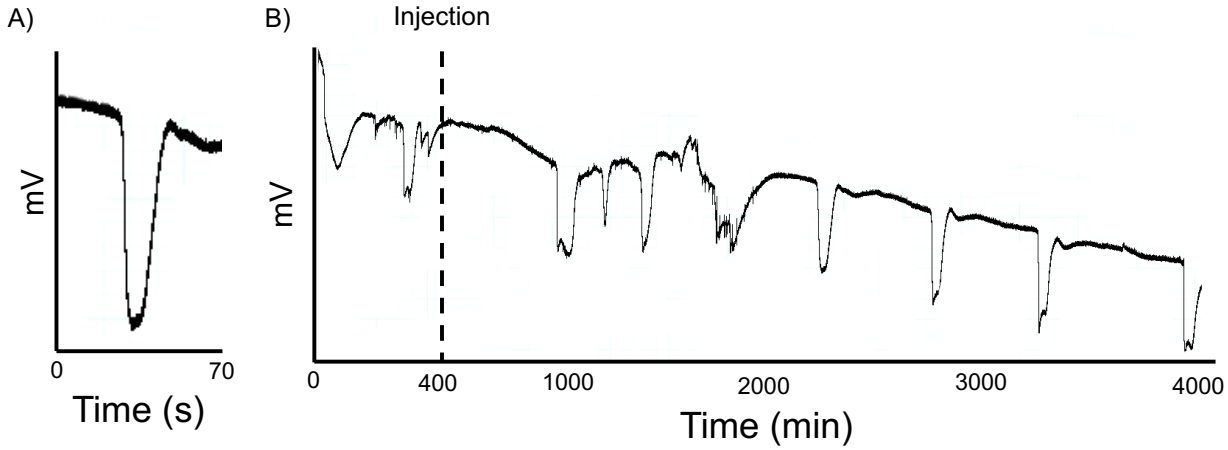

Figure 4 Representative line tracings of cortical spreading depression A) Line tracing of a single cortical spreading depression (CSD) wave depicting the sharp decrease in direct current followed by recovery. B) Line tracing of a full recording session. Compounds/Vehicle were injected 400 seconds after initial KCl drip. Continual KCl pool produced CSD events that were recorded for one hour following treatment.

As the CSD wave passes it has impact not just on neurons, but also greatly affects the surrounding vasculature. There is an initial vasodilation that is followed by constriction of the cerebral vesicles [173]. The wave further results in dramatic shifts in metabolism that are righted by large increase in cerebral blood flow [174, 175]. The demand for oxygen following CSD is so severe that the tissue may have oxygen levels decrease to the point of anoxia for up to 2 minutes following a CSD event [169, 175]. Following a CSD wave the vasculature quickly recovers or is slightly dilated, this is then followed by a longer lasting sustained constriction that can last up to an hour [176]. The constriction is often accompanied by a prolonged oligaemia that normally also lasts

between 1 and 2 hours and is a characteristic phenomenon associated with the second phase of CSD [175-179]. This second phase is also characterized by a prolonged direct current shift that is lower in amplitude than the initial CSD wave [175]. In addition, the second phase often results in hypersensitization and even activation of the trigeminal nociceptive neuronal pathway that may trigger migraine pain [180, 181]. The later hypersensitization is thought to result from the release of ATP, glutamate, CGRP, and NO by the depolarized neurons [101, 177, 182]. These peptides are thought to diffuse through the surface of the cortex where they then activate pial nociceptors [183, 184]. Upon activation the pial nociceptors trigger neurogenic inflammation and persistent activation of dural nociceptors [183, 184]. The activation of the dural nociceptors led some to believe that CSD plays a role in both the migraine headache and the aura phase [37, 185].

1.5.1 Cortical Spreading Depression and Migraine Aura

CSD is widely accepted to be the correlate of migraine aura. Mapping of CSD events onto the occipital cortex reveals a phenomenon with similar temporal and spatial features to the most common migraine aura symptoms, visual disturbances [159, 186]. The timing and spatial resolution has led to the almost universally accepted hypothesis that CSD is the cause of migraine aura [187]. However, there are those with reservations about CSD and migraine aura. One leading argument against CSD and aura, is that the classic electrophysiological correlates associated with CSD have not been observed in migraine patients [185]. However, there have been magnetoencephalography studies that show spatial patterns of DC shift in patients with migraine aura [188]. Conversely, there has been no evidence of traditional surface

EEG recordings using scalp electrodes to show these same changes during an aura event [189]. Functional imaging studies have investigated the blood-flow in patients with evoked migraine and found widespread propagation of cortical oligaemia, followed by hyperaemia, in migraine aura relevant areas of the cortex [190]. Interestingly, a PET study of a single patient with spontaneous migraine without aura showed a propagating wave of oligaemia associated with the migraine headache [191]. Additionally MRI BOLD studies found that in both spontaneous and trigged migraine there was a propagation of suppression of visually evoked BOLD signal [192, 193]. While these signals indicate correlates of the CSD they are not without flaw as there is still not the ideal EEG suppression in a spontaneous migraine aura patient. Hopefully, with future studies this discrepancy will be resolved.

Migraine aura most commonly presents as visual disturbances. The timing of the visual aura is correlated with the spreading of the CSD wave [6, 194]. The aura is most often lead by an edge of scintillating zigzag pattern, which has been linked to the leading edge of the propagation wave of depolarization associated with CSD. Following the scintillating zigzag there is often a scotoma or blurring of vision suggestive of the inhibition of neuronal activity in the occipital cortex that follows the initial wave of CSD [195]. While these features are the most common there are those that have variations in the migraine visual aura [196]. Some patients do not experience the positive zigzag pattern, but only the blurry vision and others do not show movement of the zigzag pattern, but rather see it as a stationary event. These differences in visual aura have been suggested as CSD propagation in different regions of the visual cortex or possibly due to heterogenous brain physiology of patients [196, 197].

While visual aura is the most common presentation, others symptoms have also been categorized as part of the migraine aura [6]. There has been positive sensory phenomenon in face and upper extremities, language disturbances, and in some cases motor weakness. In an extreme case of hemiplegic migraine there is often a pattern of progressive symptoms starting visual that then move to sensory, language, and motor symptoms that would closely follow the spreading of a CSD event from occipital lobe to the parietal, motor, and frontal lobe [185]. Not all symptoms occur in order and some can occur in the presence or absence of others further demonstrating heterogeneity to the migraine aura phenomenon. There is some evidence suggesting that there is migraine aura without a subsequent headache phase in some patients indicating that it is possible that CSD is a completely separate phenomena [198]. However, it is also suggested that the common promonitory symptoms of migraine, nausea and light sensitivity, are a result of a CSD event occurring in these patients that are just not classified as migraine aura [199]. CSD has also been purported to be part of a pathological brain state that accompanies migraine attack and may not be the direct cause, rather just another symptom of the hyperexcitable migraine brain [200]. While there is some controversy over the role of CSD and migraine, it is still widely accepted to be the cause of aura and possibly even extend to the migraine headache phase. Much controversy still exists in the field surrounding the importance of CSD and the idea of silent auras; further studies are needed to clarify the relation of CSD to migraine pathophysiology

1.5.2 Cortical Spreading Depression and Migraine Head Pain

As mentioned, while it is still a hotly debated topic CSD has been implicated in causing migraine associated head pain. An increase in c-Fos expression in the TNC, following CSD, has led many to conclude that CSD results in activation of the trigeminal nociceptive neurons [201, 202]. Strengthening this finding direct electrophysiological data found activation of meningeal nociceptors as well as delayed activation of neurons within the TNC following a CSD event [180, 181]. CSD has also been linked with increased blood flow in the middle meningeal artery that is dependent on trigeminal and parasympathetic activation, further indicating noxious stimulation as a result of CSD [201, 203].

How CSD activates the nociceptors is not entirely clear, but the leading hypothesis suggests that CSD causes opening of neuronal Panx1 megachannels, which can result in the release of the proinflammatory molecules into the meninges [203, 204]. Apart from these molecular alterations there is also behavioral evidence in awake rodents suggesting that CSD can cause nociception. A study using KCl to stimulate CSD events found changes in facial expression consistent with pain [203]. Another study found that in awake animals, tactile hypersensitivity in the face and hindpaw as well as cellular activation in the TNC after KCl initiated CSD [205]. While these studies indicate that CSD does have an impact on the migraine pain there is some evidence to the contrary. Awake rats had a CSD event induced through N-methyl-d-aspartate (NMDA) and found the rats underwent a freezing behavior, but did not show the characteristic ultrasonic vocalizations normally associated with rodents in pain states [206]. Another study using awake rodents and KCl stimulated CSDs investigated if CSD would produce avoidance behavior. They found no avoidance behavior when CSD

was paired with a dark chamber indicating that the CSD event itself is not aversive [207]. There is evidence that KCl itself is the necessary component for nociceptive neuronal activation, as KCl can cause nociceptive activation without causing a CSD. Furthermore, not all models of CSD produce the pain responses discussed above [205].

A common argument against CSD being crucial to the migraine headache is only 1/3rd of migraine patients actually experience the aura that is widely accepted to be correlated with CSD [6]. Some suggest that there are CSD events in the other 2/3rd of patients who have migraine without aura, but they have “silent aura”, an aura event with no notable symptoms [208, 209]. Silent auras could be contributed to CSD events in a cortical area where the activation and subsequent suppression of the neurons would not produce obvious symptoms [208, 209]. While there is strong conclusive evidence that CSD can result in some correlates associated with nociception associated with migraine it is still very controversial as to its overall role in migraine pain and more evidence will be needed to conclude this in the future.

1.5.3 Methods to Induce Cortical Spreading Depression Events

There are several commonly used models to induce CSD in animals each with various pros and cons. Spreading depolarization (SD) experiments can be performed *in vitro* as well as *in vivo*. *In vitro* models commonly use, isolated retina or brain slices which allow for the testing of pharmacological agents without having to struggle with the blood-brain barrier or interference from anesthesia [210]. However, *in vitro* models do not allow for analysis of hemodynamics, pharmacokinetics, or for systemic physiological factors greatly limiting the applicability of their results. Additionally, given that tissue oxygenation, metabolism, and blood flow play such a large role in CSD these models

are disadvantaged as they exclude these factors [211, 212]. However, these models do allow for high throughput screening of compounds because of their relative ease of set up.

In vivo studies have been done on a number of different animal models, most commonly rodents, but pigeons, cats, and primates, has also been used [213, 214]. CSD can be evoked in both gyrencephalic and lissenscephalic organisms, however the threshold to induce CSD is much lower in lissenscephalic organism, which needs to be considered when choosing a potential animal model [210]. Measuring the speed and changes in LFP is substantially easier in lissenscephalic organisms. Most of the models *in vivo* are conducted on anesthetized animals. This can potentially produce a problem as CSD has shown to easily be affected by anesthesia. Therefore, selection of which anesthetic and the dosage used must be considered as they can affect CSD susceptibility [215-221]. Isoflurane, nitrous oxide, and ketamine all have been found to suppress CSD induction and propagation. Barbiturates have less of an impact, but often produces respiratory depression which can confound the data [210]. All anesthetics have drawbacks, but if used at lower doses inhalants can produce the necessary anesthetic depth without inhibiting CSD induction. To overcome the drawbacks of anesthetics in CSD newer models are able to perform CSD in awake rodents [206, 222, 223]. These models allow for behavioral data following CSD events and have revealed hyperalgesia and increased anxiety measure from CSD [222]. Some models in awake rodents can use optogenetic approaches to induce CSD, which allow for the experiments to leave an intact skull, avoiding any surgery confounders [223].

CSD events can be triggered when there is intense depolarization that raises extracellular potassium [K+] levels [224, 225]. One common way to trigger the depolarization is direct electrical stimulation [180, 224, 226]. Commonly eca square pulse of stepwise escalating cathodal charges is applied directly to the tissue until a CSD event is triggered. A charge intensity threshold can be calculated to determine what was the minimum necessary charge that was needed to induce CSD events. This method allows for determination of what alterations result in increased or decreased susceptibility to CSD [210]. Electrical stimulation often shows higher variability than other methods, such as chemical, primarily due to issues with consistency of tissue contact as excessive bleeding or scar tissue build up are common.

The most common method used for CSD experiments is the use of chemical depolarizing agents. Several chemical solutions can be used for this, of which, KCl is most common [210]. Other chemicals can also be used including NMDA, Ca^{2+} channel openers or ionophores, and Na^{+} channel activators [210]. The depolarizing agent of choice can be applied topically or intraparenchymally to induce the CSD event [226-229]. To access changes in threshold needed to elicit a CSD event it is common to alter either volume of solute or increase the concentration of the depolarizing agent [230, 231]. Another method to investigate CSD is to use a suprathreshold concentration applied continuously and determine the number of events that occur in that time period [226, 229]. As mentioned above different depolarizing agents can cause different reactions in CSD and as such it can be difficult to extrapolate results. Cranial window size used to view the CSD event and the presence or absence of dura can affect a depolarizing agents' effectiveness. To overcome these disadvantages it is paramount

that parameters are accurately maintained across experiments and control conditions are always used [210].

The third and least common method for inducing CSD is direct mechanical pinprick stimulation of the cortex [232]. Given that it is difficult to titrate a force it is usually used to see how often a CSD occurs after stimulation [232]. Mechanical stimulation has the most drawbacks as it is difficult to reproduce the exact force, stimulus can easily produce traumatic injury, and mechanical induced CSD seem to have different pharmacological profiles compared to electrical or chemical induction [232-234]. However, some of Leão's original work was found through mechanical stimulation demonstrating it is a potential method.

1.5.4 Methods to Detect Cortical Spreading Depression

There are a few methods that are commonly used to detect CSD events or the correlates of the CSD event. Electrophysiologically, the extracellular negative slow potential shift is seen as the gold standard for detection of CSD events [210]. Measurements of the depolarization duration and amplitude can be gathered from this method. If multiple electrodes are used it is also possible to determine the speed of the CSD wave as it passes across the cortex. A less common, but still valid method is using MRI to see changes in diffusion weight imaging [235]. Using t-statistic mapping technique a passing event can be detected through single image pixels allowing for finer resolution than through region of interest based studies [235]. This method benefits from being non-invasive, however given its low spatial and temporal resolution it is frequently only used in larger gyrencephalic species. Another common method for CSD detection is the characteristic optical intrinsic signal transients that are associated with a

CSD wave [230]. The changes are caused by differences in light absorption and scattering properties of the tissue due to transmembrane ionic water shifts and changes in hemoglobin concentration. These changes allow for an easily identifiable wave that can be used to measure speed and frequency of the CSD wave [114, 173]. Blood flow changes can also be used to measure CSD [233, 236]. However, the changes in blood flow are simply correlates of CSD and should therefore only be used as a secondary measure. Given the numerous methods available to both induce and detect the CSD events it is important to consider the pros and cons of each method when beginning a new experiment.

1.5.5 Cortical Spreading Depression and Migraine

The evidence for CSD in human migraine is not as clear as it is in rodents and other animal models [185, 237]. While CSD can easily be evoked in lissencephalic animals like rodents it is more difficult, yet not impossible, to evoke them in gyrencephalic animals like cats and primates [158, 238]. While rare there have been some studies where CSD was evoked in conscious humans. Injection of KCl into the caudate nucleus or hippocampus produced the direct current shift associated with CSD that was seen to spread [239]. In epileptic patients, who were prepared to undergo lesion surgery experimenters failed to induce CSD with either mechanical deformation or electrical stimulation [240]. Similar studies used mechanical, electrical, and chemical stimulation in conscious patients did not produce a CSD event, despite these same phenomenon being able to produce a CSD event in rats [241]. These data indicate that while difficult, it is possible to induce CSD in some humans.

CSD has been heavily linked to animal models of migraine. Familial hemiplegic migraine (FHM) is a rare monogenetic disorder that causes unilateral weakness associated with migraine. A transgenic FHM mouse line has been developed and shows increased propensity to CSD versus wildtype controls [226, 242, 243]. Mutations in the enzyme casein kinase 1δ, a mutation identified in families with a more-common type of migraine, are also shown to have increased propensity to CSD [118]. Women have a higher prevalence for migraine than men and this too is reflected in animal models of CSD as female mice were found to have a lower threshold necessary for CSD induction [230]. Furthermore, female mice mutated with human migraine-associated genes have shown increased susceptibility to CSD compared to similarly mutated male counterparts [118, 226]. These data further indicate a link between sex hormones and CSD susceptibility. Strengthening the link between sex and CSD estrogen exposure results in increased CSD susceptibility and testosterone exposure results in decreased susceptibility [226, 244]. These data demonstrate how susceptibility factors in humans translate to increased susceptibility to CSD in rodent models further strengthening the relationship between migraine and CSD.

The strongest evidence indicating a link between CSD and migraine are studies that show that migraine preventive therapies can reduce the susceptibility to CSD. Chronic treatment with several migraine preventives that have diverse mechanism of action reduced the frequency of repetitive CSD evoked events [229]. Acute administration of the migraine preventive, topiramate, has been reported to inhibit CSD events [232]. Acute administration of an acid-sensing ion channel blocker, amilioride, was also shown to inhibit CSD events [245]. Similarly, drugs that have similar

mechanism to migraine preventives, but themselves are not migraine preventives like, D-propranolol, oxcarbazepine and carbamazepine show no inhibitory effects on CSD [229, 246, 247]. However, reducing susceptibility to CSD events is not a universal characteristic of all migraine preventives. A study examining repeated administration of lamotrigine, valproate, and riboflavin, found that only lamotrigine consistently inhibited CSD while valproate's effects were variable and riboflavin had no effect [248]. Previously inhibiting CSD events has been used to screen potential migraine treatments such as δ opioid receptor agonists [114]. Collectively, these data strengthen the link between CSD and migraine and help to validate the use of animal models of CSD for use in discovering novel migraine therapeutics.

1.5.6 Cortical Spreading Depression Outside of Migraine

CSD is not only seen in migraine cases. There is extensive work showing that following traumatic brain injury, subarachnoid hemorrhages, and stroke CSD events can occur [249-251]. While there has not been the direct electrocoritcography recording of CSD in migraine aura patients there has been in patients who had traumatic brain injuries that exposed the cortex. These recordings have shown repetitive CSD events that start at the site of injury and propagate. Importantly, the CSD events that are seen in these human cases are essentially identical to those observed in animal models. The CSD events observed after brain injuries and stroke have shown different vasculature changes than what has been observed in the other CSD events and include hyperaemia and oligaemia. Importantly CSD events have been correlated with worse clinical outcomes in these patients [249-251]. Furthermore, following ischemia, spreading

depolarizations (SDs) in general have been shown to contribute to lesion development [252].

While there is a distinction between migraine aura CSD and CSD induced by tissue damage there is evidence that those who are genetically susceptible to CSD have more SD events that accelerate the onset and size of stroke [253-255]. Women who have migraine with aura are at a higher risk for stroke [256]. While similar, anoxic depolarization, spreading depolarization, and CSD have been thought to exist on a spectrum [257]. Some researchers believe that anoxic depolarization can become spreading depolarization and then CSD events, when coming in contact with healthy tissue. CSD has been shown to be converted to more severe spreading depolarization after the addition of vasoconstrictors [258]. These studies show a potential link between these two disorders, but more research needs to be conducted as the mechanism linking the role of CSD and stroke is still not entirely understood.

1.6 Current Migraine Therapeutics

Current migraine treatments can be distributed between two broad categories, those that are used to stop a migraine attack while it is happening, known as abortive therapies, or therapeutics that prevent the migraine attack from occurring, known as preventives. Regardless of the class, the goal of migraine therapies is to relieve pain, restore function to the sufferer, and reduce the frequency of headache [259]. While there have been advances in migraine medication that will be discussed below many medications are still flawed. The most prescribed migraine abortive therapy, triptans, can have a paradoxical effect where upon repeated use they can result in development of a second headache diagnosis, medication overuse headache (MOH). While migraine

prevention would be the most ideal to improve a patient's quality of life, preventing migraine has proven to be a daunting task. Current migraine preventives have vastly diverse mechanisms of action and while some have effectively reduced headache days in patients many have harsh side effects that discourage their wide use. As mentioned previously, those with chronic migraine have a particularly debilitating migraine diagnosis and despite growing research many are still dissatisfied with current treatment options [2]. Recent advances on the pro-migraine peptide CGRP have been translated to the clinic. CGRP monoclonal antibodies targeting the peptide or the receptor have been successfully used in human patients [260-263]. Additionally, the use of small molecule CGRP receptor antagonists have also been recently approved for treatment of migraine [264] .

As was stated above, some migraine abortive therapies can result in a paradoxical increase in frequency of migraine attacks. Additionally, some medications can even result in a transition of migraine from an episodic state to a chronic one. Approximately only 1/3rd of patients who talk to a physician about migraines are given specific therapeutic interventions [265]. Instead, patients are often prescribed nonpharmacologic strategies including avoiding light, applying cold compresses, and avoiding things that are known to be migraine triggers. Outside of migraine-specific medications it is very common to use over the counter analgesics such as NSAIDS or acetaminophen. One study found that 98% of patients with migraine use some form of acute migraine treatment and of those 49% use over the counter medications, 20% use prescription medication, and 29% use both [266, 267]. As with other migraine therapeutics even overuse of NSAIDS can result in MOH and are recommended to not

be used more than 15 times per month [268]. Usually only after having poor responses to these kinds of treatments are patients advanced to migraine specific medications [269].

In an emergency room setting there are often different medications prescribed than would be by a headache specialist or primary physician. Diuresis can present as part of the premonitory phase and this in combination with nausea and potential vomiting can result in loss of fluids [270]. Fluids are usually given in the emergency room setting and are potentially helpful in reducing migraine severity and many intravenous migraine drug studies first prescribe fluids prior to administration of drugs [271]. MOR agonists are still frequently given as first-line treatment of acute migraines in the emergency room setting [272, 273]. MOR agonists are only partially effective at reducing migraine pain and comes with a host of deleterious side effects including reduced effectiveness of other migraine medications [274], promotion of chronic migraine [275], and promotion of more comorbid disorders [276].

Often not just a single drug will be prescribed for the treatment of migraine. Some physicians will use a combination of therapies known as the “migraine cocktail”. In the emergency room the cocktail is commonly comprised of NSAID agents, dopamine antagonist, and/or antihistamines [277]. Some doctors will also prescribe other medications to treat various common symptoms of migraine including anti-nausea medication. Despite the frequent use and perception of positive response by emergency department physicians typically the patients do not equate the cocktail to a good response 24 hours later [277].

1.6.1 Abortive Therapies

Ergotamine was first introduced in 1926 and was the first drug used for acute migraine treatment [278]. Ergotamine and ergot derivatives exert an anti-migraine effect through agonist action on serotonin receptors. Ergotamine has a long duration of action and is best used on patients who have long-lasting attacks or attacks that reoccur [279]. In both rodents and humans ergotamine was shown to act as a vasoconstrictor [280-283]. A disadvantage of ergot derivatives is their complex pharmacology that interacts with many receptors including α-adrenoceptors and dopamine D2 receptors. These interactions can result in frequent adverse effects including nausea, vomiting, cramps, and tiredness [268]. Dihydroergotamine (DHE) is a derivative that is often better tolerated than ergotamine, but is often less effective because of poor bioavailability [268]. Attempts to increase bioavailability by differential administration including intravenous injection has shown to increase effectiveness, but also increase side effects [284]. Ergotamine also has a high rate of MOH and is often only prescribed when paired with a preventive medication [268].

Triptans are the most prescribed drug class for the acute treatment of migraine and were the first drug developed specifically for migraine [285, 286]. Triptans are selective serotonin agonists at certain 5-HT_1 receptors. Activation of the 5-HT_{1B} receptor peripherally was shown to reduce pain, likely through vasoconstriction properties [287]. Triptans also work at the 5-HT_{1D} receptor and blockade at this receptor prevents the release of vasoactive peptides, which can trigger neurogenic inflammation. Triptans can also work by reducing afferent return of nociceptive signals to the TNC [288, 289]. Sumatriptan was the first drug of its class and was specifically designed to have ergot-like properties, without the harsh side effects [290-292]. Sumatriptan is regarded as the

gold standard in migraine abortive medications [293, 294]. A potential mechanism of action of the triptans is through CGRP modulation, as CGRP positive neurons in the rat TG were found to be co-expressed with 5-HT_{1B} and 5-HT_{1D} receptors [295]. In cultured TG neurons sumatriptan was able to inhibit the secretion of CGRP from sensory neurons [296]. Triptan treatment at dural CGRP positive nociceptors caused inhibition in the amplitude of action potentials [297]. Sumatriptan has some drawbacks as it has relatively low bioavailability and a short half-life [268]. However, many second-generation triptans solve these problems. These medications are available in several different agents with various forms of availability including oral, nasal, and injectable. Regardless of the type, triptans are most effective when they are taken early in the attack phase of migraine [298]. While often effective, triptans are recommended to not be used more than two days per week in order to reduce the risk of MOH [259]. To better learn what causes the transition to MOH rodent models have been developed by repeated administration of drugs that induce long lasting sensitization [299]. One model utilized by our lab has mice receive sumatriptan every day for 11 days and results in significant basal hyperalgesia demonstrating the risk of repeated sumatriptan use [113]. Triptans can produce vasoconstriction, which can potentially increase the risk of serious ischemic events [300]. Triptans are contradicted in patients with cardiovascular disease and during pregnancy and breastfeeding. Additionally, younger patients, under the age of 18, are generally also contradicted from triptan use; and some monoamine oxidase inhibitors are contradicted to be used in combination with triptans [301].

1.6.2 Migraine Preventives

Migraine prevention is usually considered when the attacks significantly affect quality of life; this is the case in approximately 1/3rd of migraine patients [267]. The most commonly prescribed migraine preventives are CGRP blockers, onabotulinum toxin A, propranolol, metoprolol, flunarizine, topiramate, and valproate [302]. While there have been advancements in migraine prevention, only about half of patients will even see a 50% reduction in migraine attacks requiring the need for better therapeutic options [303]. Most of the migraine preventives were repurposed for migraine treatment and were initially developed to treat other disease states including, arterial hypertension, epilepsy, and depression. While many of the migraine preventives have differential receptors and pathways they target, many have similar end results such as reduced CSD events, reduced levels of CGRP, or decreased neuronal sensitization [229, 304, 305].

Beta-adrenergic blockers are competitive inhibitors of β-receptors, and many of the different pharmacological agents differ in receptor binding and pharmacokinetic properties [306]. Not all beta-adrenergic blockers are effective in migraine, those with sympathomimetic activity have not been effective in migraine prevention [307]. Propranolol, timolol, bisoprolol, and metoprolol have all been used as effective migraine preventive treatments [308-310]. The full mechanism of how these compounds work to reduce migraine events is not fully understood. Blockade of β1 receptors could inhibit noradrenaline synthesis [311]. Propranolol was shown to reduce firing of noradrenergic neurons in the locus coeruleus [312]. Beta-adrenergic blockers also reduce firing in the PAG [313]. Propranolol, preclinically, was found to reduce sensitization in the rostral ventromedial medulla and locus coeruleus as well as reducing sensitization in the

trigeminocervical complex [305]. Beta-adrenergic blockers were also seen to reduce auditory cortex potentials further indicating its ability to decrease sensitization [314]. There are also some studies indicating that beta-adrenergic blockers can interact with serotonin receptors and may impact serotonin synthesis [315-318]. Propranolol was also seen to inhibit NO production through blocking inducible nitric oxide synthase (iNOS) [319]. In a model of migraine aura, propranolol reduced sensitivity to CSD in rats without affecting cerebral blood flow [320]. While the data is promising beta-blockers for migraine prevention use is limited by the host of adverse behavioral side effects including drowsiness, fatigue, sleep disorders, depression, memory disturbances and even hallucinations. Beta-blockers can also have other physiological side effects including gastrointestinal symptoms, decreased exercise tolerance, hypotension, and impotence [321].

Antiepileptics acting at different synaptic sites have been found to be effective in migraine prevention with the most success coming from topiramate and valproate [302]. Similar to beta-blockers, not all antiepileptics make effective migraine preventive agents. Interestingly, the same agents that are effective in reducing migraine attacks are also effective in reducing CSD events, indicating a possible similar mechanism [229]. Topiramate blocks multiple channels such as voltage-dependent sodium channels and high-voltage-activated L-type calcium channels [322]. Furthermore, it can also inhibit glutamate-mediated excitatory neurotransmission and facilitate GABA-A mediated inhibition and inhibit carbonic anhydrase activity [323]. Topiramate was also shown to reduce CGRP secretion from trigeminal neurons following depolarizing stimuli [324]. Valproate also has a complicated mechanism and can increase GABAergic

inhibition and block excitatory ion channels [322]. It also blocks several channels including voltage-dependent sodium and low-threshold T-type calcium ion channels. Valproate can also suppress protein kinase C and down regulates the expression of CGRP in brain tissue indicating a common mechanism between topiramate and valproate [325]. Some antiepileptics are also limited by undesirable side effects including weight gain and hypoandrogenism [321]. However, this is not true for all as topiramate actually causes a decrease in weight, but it can also cause cognitive problems discouraging its widespread use [326]

There are some calcium antagonists that are also used for migraine prevention including flunarizine and verapamil [327]. Flunarizine is a nonselective calcium entry blocker, which can also block voltage gated sodium channels [328, 329]. It is thought that these effects reduce neuronal excitability and normalize cortical hyperexcitabilty in migraine. Flunarizine has also been found to reduce the number and duration of CSD waves [330]. Unfortunately, flunarizine may increase leptin levels, which results in treatment related weight gain [331, 332].

Antidepressants have been commonly used as migraine preventives including the use of tricyclic antidepressants (TCAs), selective serotonin and norepinephrine reuptake inhibitors (SSNRIs; SSRIs) [333]. Interestingly, these compounds are effective in the absence of depression and usually at doses lower than effective in depression treatment. TCAs exert their antidepressant effects by modulating the reuptake of norepinephrine and serotonin as well as having an anticholinergic effect [334]. It is thought that they affect migraine through modulation of the serotonergic and norepinephrine tone. TCAs can inhibit norepinephrine and serotonin reuptake which can

modulate the pain systems and may also enhance endogenous opioids collectively decreasing signaling of nociceptive pathways [335-338]. Fluoxetine works to selectively block serotonin reuptake from the synaptic cleft leading to increased serotonin levels. Fluoxetine also competitively and reversibly blocks the 5-HT_{2C} receptor [339]. Proper modulation of serotonergic tone could result in a protective role and explain how antidepressants prevent migraine [334, 340]. SSRIs are also found to downregulate β-adrenergic receptors, and increase the number of $GABA_B$ receptors resulting in decreased excitatory tone [341-345]. Interestingly repeated SSRI use has been implicated to modulate serotonin at various receptors and this continual activation may result in headache through a similar mechanism that is thought to occur after repeated triptan use. Similarly, repeated fluoxetine use has been implicated to result in headache [346].

Angiotensin has several effects that are relevant to migraine including increased sympathetic discharge and adrenal medullary catecholamine release. The renin-angiotensin system (RAS) is thought to play a role in migraine pathogenesis [347]. RAS system modulates cerebrovascular flow and influences homeostasis of fluids, autonomic pathways and neuroendocrine system [348]. Angiotensin II modulates potassium channels and calcium activity in cells, which can increase concentration of dopamine and serotonin metabolites. These metabolites can further activate nuclear factor κB and increase expression of iNOS [104, 349]. Lisinopril, angiotensin-converting enzyme inhibitor, has shown to be an effective migraine prophylactic [350]. Angiotensin-converting enzyme inhibitors modulate vasoreactivity, alter sympathetic tone, and

promote degradation of proinflammatory factors [350, 351]. These molecules have also been implicated in modulation of the endogenous opioid system [352].

An interesting often effective migraine preventive, is onabotulinumtoxin A; which has been found to be efficacious in chronic migraine and in several countries is marketed as a chronic migraine preventive [353-355]. Onabotulinumtoxin A is a protein complex produced by the gram-positive anaerobic bacterium *Clostridium botulinum* [356]. There are 7 serotypes of botulinum toxin, and serotype A has been used in human therapy since 1980 [357]. In the 1990s it was found that onabotulinumtoxin A treatment for hyperfunctional lines of the face was also effective in improving migraine [358]. Onabotulinumtoxin A was found to significantly reduce headache and migraine days and cumulative headache hours on headache days in patients as well as improving overall health-related quality of life [353-355]. The exact mechanism for how onabotulinumtoxin A functions to reduce migraine is unknown, but it is thought to reduce antinociceptive action via peripheral mechanisms [359]. Onabotulinumtoxin A directly inhibits peripheral sensitization by attenuating neuropeptide and neurotransmitter exocytosis from peripheral sensory neurons indirectly reducing central sensitization [359]. This may indicate why it is more effective in chronic versus episodic migraine. Onabotulinumtoxin A works by interfering with the fusion and release of neuropeptides and neurotransmitter. The process is normally controlled by interaction of proteins on the vesicle and vesicle associated membrane proteins, such as VAMP and synaptobrevin and on the synaptosomal associated protein, SNAP-25, which when combined form the N-ethylmaleimide-sensitive factor attachment protein receptor known as the SNARE complex [360-362]. SNARE is necessary for proper vesicular trafficking

and vesicle fusion with the membrane, onabotulinumtoxin A adheres to the nerve cells and inhibits fusion of intracellular vesicles with the nerve membrane by cleaving SNAP-25 [363, 364]. This results in inhibition of neuropeptide release and receptors become down regulated [360, 362]. Botulinum toxin was found to inhibit the release of many neurotransmitters [365-367]. It is held that reducing the neurotransmitter release is capable of disrupting cascades of events that lead to peripheral and central sensitization and block the release of inflammatory neuropeptides from stimulated trigeminal sensory neurons [359-361, 368]. Among these is CGRP, which has been found to be reduced following botulinum toxin administration, providing a possible mechanism for how this treatment works [369]. While onabotulinumtoxin A treatment is effective for many it still has a delayed onset of effect and is often not the first choice for treatment options.

1.6.3 CGRP and Migraine Treatment

CGRP basal therapies are new and exciting options for migraine patients [370, 371]. CGRP was first discovered in 1982 [372] and has since revealed a critical role in regulating the trigeminovascular system as a potent vasodilator [370, 373]. CGRP is a peptide neuromodulator and is produced both in peripheral sensory neurons and in central nervous system. CGRP comes in both α and β components, with most research focusing on the α form. The α form of CGRP is a 37 amino acid peptide that is transcribed from the calcitonin related peptide alpha-CGRP gene (CALCA) [372, 374]. CGRP is released from stored vesicles through calcium-dependent exocytosis [375, 376]. Presynaptic receptors located on trigeminal neurons regulate CGRP release and activation of 5-HT1B and 1D was shown to inhibit the release of CGRP [377]. CGRP

has a long half-life due to amidation of the carboxyl terminus which allows for wider release [378]. Trigeminal CGRP has been found in rat jugular vein and in human blood collected from external jugular vein following trigeminal ganglion stimulation demonstrating its ability to circulate throughout the body [379, 380].

CGRP receptor is a complex of several proteins, most importantly the G-protein coupled receptor (GPCR) calcitonin receptor-like receptor (CRLR) [381]. To become functional the CRLR must form a heterodimer with receptor activity-modifying protein 1 (RAMP1) and through an intracellular component called the receptor component protein (RCP) [382, 383]. The ligand-binding domain for CGRP is located at the interface between RAMP1 and CRLR [384, 385]. The CRLR is coupled to a Gαs pathway [374, 386]. RCP is responsible for the coupling of Gαs proteins and adenylate cyclase to the intracellular effects of CGRP [387] Upon activation increases in cAMP result in activation of protein kinase A, which can phosphorylate many downstream targets including potassium ion channels and extracellular signal-related kinases. It has been shown that increased cAMP after CGRP activation results in vasorelaxation and dilation [388]. Interestingly, PDE inhibition thus driving greater cAMP, can cause migraine like attacks, further implicating this pathway, but complicating the story [389] CGRP continual stimulation also results in phosphorylation of CRLR and β-arrestin recruitment causing internalization of the receptor and upon chronic exposure results in degradation [262]. The amylin receptor, AMY, which is formed by RAMP1 and calcitonin receptor has also shown response to CGRP [381]. AMY1 has been found in the trigeminal ganglia but is likely to play a lesser role in CGRP activity [390].

CGRP is known as a pro-migraine peptide as patient blood samples from external jugular veins reveal increased CGRP levels during the headache phases [391]. Additionally CGRP was found to be increased in chronic migraine patients interictally [392]. In other primary headache disorders, like cluster headache, there have been similar findings of increased CGRP [393, 394]. Increased CGRP levels have also been found in other regions including plasma, saliva, and CSF samples [394-397]. CGRP infusion intravenously into migraine prone individuals produced a lasting, migraine-like headache suggesting a causal role of CGRP in migraine generation [398, 399]. Based on this data researchers sought to reduce the effect of CGRP and believed this would limit the severity of migraine.

Small-molecule antagonists, known as gepants, were found to potently and selectively block CGRP, both preclinically and clinically [400, 401]. The gepants have a high affinity for the CGRP receptor of humans relative to receptor affinity of other species. This selectivity is thought to be due to species-specific residue located at the interface between RAMP1 and CRLR [384]. Olcegepant was the first non-peptide CGRP receptor antagonist to be discovered [402]. Olcegepant is thought to block the binding of CGRP to its receptor as it decreased cAMP production, vasodilation, and caused a rightward shift of CGRP concentration response curves [402, 403]. In vivo experiments in rats showed that olcegepant blunted the CGRP response, further indicating gepants effectiveness [124]. Since this discovery gepants have been developed that have greater potency and improved oral bioavailability [404-407]. However, development of first generation gepants was largely stopped because of increased liver toxicity after repeated use [408, 409]. Two new gepants, ubrogepant and

rimegepant, without the liver toxicity have recently been approved for migraine treatment [410, 411].

The first antibodies against CGRP were discovered in 1982. The antibodies were used for radioimmunoassay and liquid chromatography to measure CGRP in tissue and plasma as well as for immunohistochemistry assays in tissue [13, 412]. Since then three humanized monoclonal anti-CGRP antibodies (galcanezumab, eptinezumab, and fremanezumb) have successfully completed clinical trials for migraine prevention and are now commonly prescribed to migraine patients [370, 371, 413-415]. Erenumab is a human monoclonal antibody that targets a fusion protein on the extracellular domains of human CRLR and RAMP1 that includes the binding pocket [416]. Erenumab is 5,000 times more selective for CGRP receptor than other related receptors in the calcitonin receptor family [415]. Erenumab has been shown to be a full antagonist of CGRP receptor complex both *in vitro* and *in vivo* [416]. As with the monoclonal antibodies for CGRP, erenumab is also an effective prophylactic therapy for episodic and chronic migraine [401, 413]. The antibody treatments have several advantages over other treatment options. They have a prolonged serum half-life, which allows for infrequent dosing. Importantly the antibodies avoid liver processing, preventing liver toxicity related side effects [262]. The strongest disadvantage of antibodies is they are not orally active and must be injected and injection-site reactions are somewhat common [408, 414, 417]. Additionally CGRP antibodies have been found to produce constipation in some patients as well as being contradicted for the treatment of some heart patients [283, 418, 419]. Despite these drawbacks, the CGRP antibodies have been hailed as a miracle treatment option for those who were previously unresponsive to other

medication. However, more work still needs to be done as CGRP antibodies do not work on everyone and largely only reduce migraine days, but do not eliminate them.

1.7 Opioid Receptors

MOR agonists such as morphine, hydrocodone, or oxycodone are still commonly prescribed to people with migraine [420, 421]. These agonists can provide short-term relief, but this comes at a high cost as they can be accompanied by a host of deleterious side effects including the paradoxical worsening of headache or MOH [420, 421]. While MOR agonists are the most common they are not the only opioid receptor that can be targeted. The opioid receptor family is comprised of 4 different receptors μ, δ, κ, and nociceptin/orphanin FQ [422, 423]. Each receptor interacts with a family of endogenous opioid peptides, the endorphins, enkephalins, dynorphins, and nociceptin [422]. The opioid receptors are GPCRs that primarily act through $G\alpha_{i/o}$ [424]. Each of the receptors can have different properties and efficacies in regulating mood and nociception. Specifically, for the treatment of migraine, only MOR agonists are currently available. However, clinical trials and extensive preclinical research is being conducted to investigate the effects of δ opioid receptor (DOR) agonists as well. Below I will briefly discuss the effects DOR agonists can have on pain, mood, and the effect of DOR biased agonists. I will then go on to discuss what is currently known about DOR and migraine and the signaling cascades thought to drive these effects.

1.7.1 DOR Signaling

DORs were originally thought to exist primarily in intracellular stores [425-427]. While this idea is still held by some it has undergone considerable controversy as

fluorescently tagged DOR reporter mice show a much higher membrane localization than was previously observed using antibody staining [428]. Regardless, it is still held that DOR can be easily trafficked in response to agonists. As mentioned above, all 4 opioid receptors are seven-transmembrane GPCRs and are largely coupled to $G\alpha_{i/o}$. As such, following activation the Gα functionally separates from the Gβγ subunits and following separation produce varying intracellular effects [429, 430]. As part of the G protein family, opioid agonists can encourage the exchange of GDP for GTP within the receptor [431]. $G\alpha_{i/o}$, following agonist stimulation, can have an inhibitory effect and decrease cyclic adenosine monophosphate (cAMP) production [432]. Studies using pertussis toxin revealed the cAMP inhibitory effects were through $G\alpha_i$ rather than Gβγ [433]. Apart from inhibiting cAMP levels activation of DOR regulates calcium and potassium ion channels. Upon separation the Gα component directly interacts with the G-protein gated inwardly rectifying potassium channel, Kir3 [434, 435]. This causes cellular hyperpolarization and inhibits neural activity. Reductions of calcium current are sensitive to P/Q-type, N-type, and L-type channel blockers [436]. The Gβγ component directly binds to the channel, which can reduce voltage activation of channel pore openings [437, 438]. The Gβγ DOR subunit decreases the release of neurotransmission through presynaptic calcium channel effects. Activation of the outward potassium channels decreases post-synaptic firing and the inhibition of the calcium channel lead to decreased neurotransmitter release presynaptic ally, collectively resulting in net inhibitory effects following activation of the post-synaptic opioid receptor.

Upon activation the DOR, and other opioid receptors, are desensitized through phosphorylation of the receptor at the Ser363 residue [439, 440]. The phosphorylation is

largely regulated by GPCR kinase 2 (GRK2) [440, 441]. Following phosphorylation arrestin 2 or 3 are recruited [442]. Arrestin binding is key in regulating desensitization, traffic, and receptor sorting. The C-terminal tail of DOR is necessary for arrestin binding. Following arrestin recruitment the DOR can be internalized in clathrin-coated pits, where it is predominately degraded, but can be recycled back to the cell membrane [443]. Transport to degradation is not the only process that occurs following arrestin recruitment. Recent evidence has highlighted the importance of actuation of signaling cascades following arrestin recruitment. These signaling cascades largely function through the MAPK family [444]. MAPKs cover many signaling processes including proliferation, differentiation, apoptosis, transcription regulation, channel phosphorylation and protein scaffolding [445]. Within the MAPK family ERK, JNK, and P38 are the most consistently affected. ERK 1 and 2 were found to be the most frequent opioid induced MAP kinases. Additionally, work has shown that there can be a second wave of cAMP signaling from intracellular vesicles [446]. Recently, how different agonists can affect different signaling cascades have garnered a lot of attention as differential pathway activation has demonstrated different signaling cascades and behavioral outcomes.

1.7.2 Behavioral Effects of DOR Stimulation

Much has been learned about the endogenous role that the various opioid receptors play in reward and mood through knockout mouse models [447]. Knockout of the DOR results in increased anxiety and depressive like behavior [448]. To better understand how DOR regulate mood, was agonists were investigated to determine the potential as antidepressant and anxiolytic agents. Some of the original findings demonstrated anti-depressant like effects of enkephalin and endorphins when

administered to rats [449]. Additionally, inhibiting the opioid receptors by administering the general opioid antagonist naloxone showed depressant like effects [450]. DOR agonists stand out as antidepressant and anxiolytic agents stemming from the promising knocked out mice data. DOR activation by several different agonists has shown positive effects at reducing depression like behaviors in both rats and mice [450, 451]. These data indicate the potential benefits of DOR agonists as possible antidepressants or anxiolytics.

MOR agonists are known to produce sedation as well as euphoria in humans [422, 452]. Similarly in rodents, MOR agonists can produce conditioned place preference and will be self-administered, both indicators of a drugs addictive potential [452]. The highly rewarding effects of MOR activation in part have led to the national opioid crisis in America. Conversely KOR agonists have been found to produce dysphoria and psychomimetic effects upon administration in humans [453, 454]. Similarly, in rats use of KOR agonists were found to produce conditioned place aversion further indicating unpleasant feelings after administration [455]. While KOR agonists have low abuse potential the dysphoria limits their use as therapeutic targets. DOR agonists also seem to have low abuse liability [456]. DOR agonists do not produce significant self-administration or rewarding behavior in animal models [457, 458]; but are also not anhedonic like KOR agonists. Collectively these data suggest that DOR agonists can positively regulate mood without having intense euphoric effects driving abuse potential.

The opioid receptors are widely known for their importance in regulating analgesia, especially the MOR. MORs are distributed across both central and peripheral

nervous systems and are especially densely expressed in pain related areas such as the periaqueductal gray and rostroventral medulla [459]. MOR agonists are widely known to be antinociceptive and are among the most highly prescribed therapeutics for pain treatment [460]. Interestingly, repeated use of MOR agonists to treat migraine can result in MOH [461]. Like the MOR, agonists to the KOR have been shown to produce pain relieving effects, but many of these findings are compounded by a stress response limiting wide use of KOR agonists [462]. Compared to the MOR agonist DOR agonists are relatively ineffective in treating acute pain states [463]. However, DOR agonists have shown great promise at treating chronic pain states. DOR agonists have shown efficacy in assays of chronic inflammatory pain [464, 465]. DOR also show promise in treating notoriously difficult pain states including neuropathic pain models, such as peripheral nerve injury [457, 465-467]. However, many DOR agonists also produce convulsions at higher doses, limiting their therapeutic window [456]. Specifically, in treating migraine, the DOR has emerged as a potential treatment option and these findings will be discussed below

1.7.3 DOR and Migraine

DOR has recently been identified as a target for treating migraine [468]. DOR is expressed in the TG, TNC, and the cortex all of which are key migraine pain regulatory regions [469-471]. DORs are also expressed in regions that regulate emotion such as the amygdala, hippocampus, and striatum [451, 469], which could in part explain the DOR agonists' antidepressant results and process emotional responses to pain. Given the expression of the DOR it is not surprising that agonists have been found to be particularly effective in models of headache [468]. Previously our lab has demonstrated

that 3 different DOR agonists can block mechanical hyperalgesia following repeated injections of NTG [468]. Furthermore, the DOR agonist, SNC80, was found to reduce thermal hyperalgesia following NTG treatment [472]. SNC80 also inhibited cephalic allodynia in models of chronic migraine and MOH [113]. In a model of posttraumatic headache acute SNC80 blocked established allodynia and was found to prevent chronification of the post-traumatic headache associated pain [113, 473]. In a model of CSD SNC80 also significantly reduce the number of CSD events induced by KCl [114]. In humans DOR agonists have been developed and tested for treatment in pain, anxiety and depression [474, 475]. A recent phase 1 clinical trial performed using TRV250 was completed for the treatment of acute migraine [476]. This compound is currently recruiting for a Phase II trial for the treatment of migraine further indicating the potential for DOR agonists in treating this disorder. To date most of the work in DOR has relied on balanced agonism, with limited success. Recent studies have shown the promise of using biased agonists for DOR to have a better therapeutic profile. I will be adding to this work in Chapter 4 of my thesis.

1.7.4 Biased Agonism of DOR

Ligand directed signaling or biased agonism is the idea that different agonists at a receptor can induce different signaling cascades and receptor trafficking events. These variations in signaling can cause immediate behavioral effects and changes in long-term adaptions through chronic drug treatment. Signaling can occur primarily through the G-protein signaling pathways or through G protein independent cascades either at the cell membrane or in subcellular compartments following receptor internalization [477]. Of the G-protein independent effects arrestin signaling cascades

are the most common [478, 479]. Arrestin can act as a scaffold for other signaling proteins to begin a secondary wave of signaling [480]. The use of biased agonism allows for preferential signaling at either the G-protein dependent or independent pathways, which could correlate with desired biological effect with decreased undesired side effects.

SNC80, the prototypic DOR agonist, and met-enkephalin are both relatively balanced agonists for DOR [481]. Most small molecule DOR ligands appear to have low efficacy for arrestin 3 binding and therefore have biased agonism to the G-protein signaling while only partial or weak agonist activity towards arrestin recruitment [482]. Additionally, different ligands can cause changes in the internalization of the DOR and the receptors eventual fate. SNC80 was found to promote the degradation pathway and met-enkephalin more receptor recycling [483]. Further evidence derived from fluorescent tagged DOR reporter mice, DOR-eGFP, found that SNC80 induced rapid internalization of the DOR, while another agonist, ARM390, did not produce detectable receptor sequestration [484, 485]. These variations in signaling cascades can have differential behavioral effects as well.

In acute tolerance studies SNC80, a high internalizing agonist, and ARM390, a low internalizing agonist, were both given and tolerance to the anti-hyperalgesic effects were measured. In this study SNC80 produced acute tolerance, along with receptor internalization, while ARM390 maintained its anti-hyperalgesic effect, while remaining on the cell membrane [485]. This study demonstrates that internalization of the DOR directly impacts behavioral effects of receptor activation. Other evidence to support the variation in behavioral outcomes from low versus high internalizing agonists comes from

arrestin recruitment data [457]. As mentioned previously DOR activation results in phosphorylation and subsequent internalization by either arrestin 2 or 3. Recent evidence found that SNC80 has preferential recruitment of arrestin 2 rather than 3, which is thought to be responsible for the desensitization and acute tolerance [442, 486]. Knockout of arrestin 2 results in increased potency and decreased tolerance to high internalizing agonist [442, 486]. In contrast a low internalizing DOR agonist was found to preferentially recruit arrestin 3, which has been found to facilitate the rate of receptor resensitization discouraging acute tolerance. Similarly, knockout of arrestin 3 resulted in increased tolerance.

Some DOR agonists can produce convulsions, which have greatly limited the development and wide use of these compounds [487, 488]. These convulsions are ligand specific. Convulsion following DOR activation is a form of biased agonism as not all agonists produce these effects and activation of different signaling cascades can prevent convulsions [457, 489]. ARM390 and KNT-127 were both found to not produce convulsions. These DOR agonists are both low internalizing further supporting that high internalization of the DOR agonist may lead to some of the deleterious effects associated with DOR agonism and biased ligands can circumvent these alterations [481, 490].

1.8 Neuronal Cytoarchitecture

Many chronic neuropathic pain states are shown to produce changes in brain cytoarchitecture [491, 492]. Morphological changes have been seen following neuropathic pain states and result in alterations in grey and white matter [493, 494]. Changes in cytoarchitecture have also been seen at the cellular level including

alterations in dendritic density and neurite outgrowth [495, 496]. To gain a better understanding of the pathophysiology driving migraine progression, I sought to investigate the neuronal cytoarchitecture and how that changes following chronic migraine model. Below I will give background information on the regulation of cytoskeletal dynamics.

1.8.1 Microtubules and the Cytoskeleton

The cytoskeleton is made up of a network of polymeric filaments that include actin filaments, intermediate filaments, and microtubules. The cytoskeleton controls the internal organization, shape, motility, and life cycle of eukaryotic cells. Microtubules are the largest of the cytoskeletal components. Microtubules are more rigid than actin filaments and are therefore more persistent and longer in length. Microtubules are commonly found to be around 5000 μm, while actin filaments are around 20 μm [497, 498].

Microtubules are built from heterodimers of α and β-tubulin. Nucleation is the de novo formation of microtubules and it is an energy unfavorable process and depends largely on tubulin concentration [499]. γ- tubulin is generally used to nucleate the growth of new microtubule structures, but is not required by all microtubules [500]. γ-tubulin ring processes, are lock-washer-shaped structures that act as a template for microtubule assembly and a cap for the minus end. The tubulin components bind in a head-to-tail fashion and form polarized linear protofilaments, which then associate together to form a hollow tube measuring approximately 25 nm at the outer diameter [501]. As the tube is hollow diffusion of small molecules through the microtubule is possible [502]. Microtubules are often 13 linear protofilaments that associate laterally together. The

formation of microtubules can be seen in Figure 5. The surface of the microtubule is negatively charged as the carboxy-terminal tails of the tubulins are located on the outer surface. These carboxy-terminal tails are key sites for many microtubule-binding proteins [503]. The microtubules are also polarized with one end having exposed β-tubulin, the plus end, and the other having exposed α-tubulin, minus end.

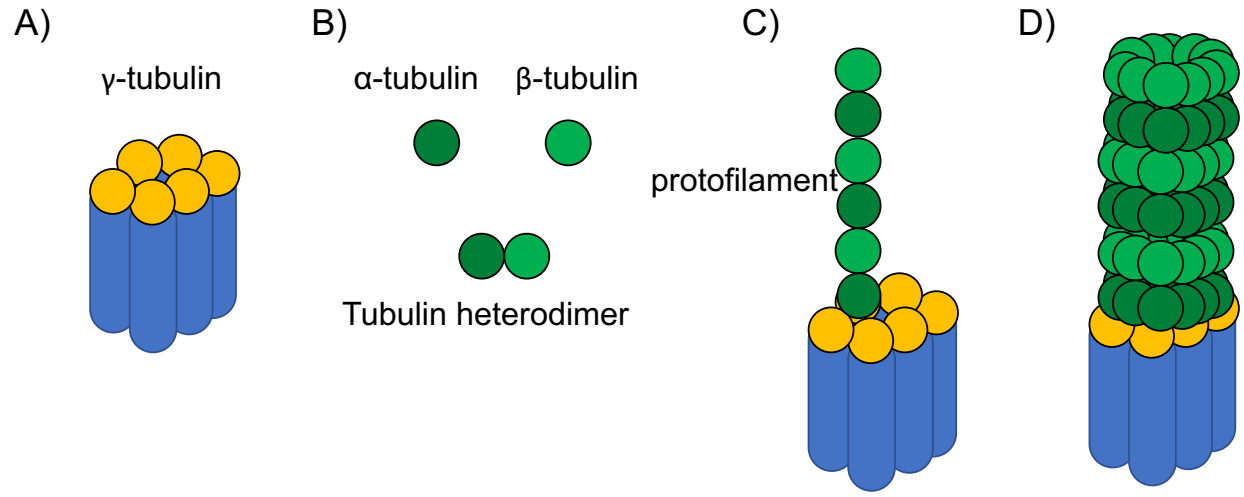

Figure 5 De novo synthesis of microtubules A) γ-tubulin molecules form a helix which serves as an anchor for tubulin binding. B) α tubulin and β tubulin form tubulin heterodimers. C) These dimers are connected into a protofilaments. D) 13 protofilaments combine to form a hollow microtubule

While both ends can grow and depolymerize there are still differences between them [504]. The β-tubulin positive end grows faster and is more likely to catastrophe and is therefore seen as more crucial for microtubule dynamics [505]. Microtubules are regulated by the properties of the tubulin dimer. Both the α and β tubulin subunit can bind GTP, but only the β-tubulin can hydrolyze the GTP and incorporate into the microtubule lattice. Assembly of the tubulin promotes hydrolysis of the GTP bound subunit because the incoming subunit acts as a GTPase-activating protein for the subunit completing its nucleotide active site [506]. At high enough concentrations GTP-

tubulin will assemble into microtubules, but GDP-tubulin will not assemble into more than oligomers [507]. GDP tubulin destabilizes the lattice, so a stable growth is dependent on the cap of GTP-tubulin at the microtubule plus end. Glycerol or MAPs, along with in some cases Gα can potentiate tubulin GTPase activity. The GTP cap is formed when a new subunit adds to the growing microtubule tip. Since there is a short delay in hydrolysis and phosphate release a GTP-rich region forms at the growing end. The GTP cap is thought to have strong lateral bonds, which can maintain the tubular structure and allows for continued polymerization. Upon loss of a GTP cap the microtubule will rapidly disassemble, known as catastrophe [508, 509]. These factors drive the constant state of growth and disassembly of the microtubule collectively known as dynamic instability [510]. While this is the most common method assembly and catastrophe can occur at both ends. There is also GTP tubulin scattered throughout the microtubule not just at the plus end cap [511]. The assembly of GTP-tubulin into microtubules is a spontaneous process that is driven primarily by the hydrophobic effect [512]. Disassembly primarily happens at the plus end but can happen at uncapped minus ends. The dynamic instability is necessary for several cellular processes. It allows cells to change shape to rapidly adapt to their environment. The instability allows microtubules to explore an environment and selective stabilization of microtubules play fundamental roles in many cellular processes [513].

1.8.2 Microtubules and Neurons

Microtubules are essential for many aspects of neuronal function throughout their development. The nature of neurons necessitates that active transport is needed to properly distribute many different cellular components and establish signaling pathways

from synapse to soma and vice versa. The transport of neurotransmitters and polarization of the neuron is heavily reliant on the microtubule [514, 515]. The microtubule based motors of kinesin and dynein families are necessary to transport the neuronal cargo throughout the cell [514, 516]. The microtubule cytoskeleton organization provides selective transport routes for sorting of cargo into axons or dendrites [517, 518]. Axon selectivity is mediated by specific properties of stabilized or modified microtubules as treatment with stabilizing agent result in non-polarized targeting of both axons and dendrites [519].

Microtubules along with actin filaments are driving components in the neuronal migration process [520]. After development a model has been proposed in which neurite initiation and outgrowth depends on increases in actin and stabilization of the microtubules [521]. Stabilization of the microtubule plays an important role in initial specification of the axon during neuronal polarization [521]. Kinesin-1 shows higher affinity for stabilized and/or modified microtubules and could select axon-specific tracks required for polarized trafficking [519, 522, 523]. Microtubules also play key roles after neuron formation, as axon regrowth is critically dependent on microtubule cytoskeleton [524-527]. Microtubules also contribute to the formation of the synaptic terminal [528] and shaping of the dendritic spine [529, 530]. Within axons and dendrites microtubules have different pattern orientations [531]. Axons appear to be more uniformly plus-end out-oriented microtubules whereas dendrites contained non-uniformly oriented microtubules [532, 533]. The differences in the microtubule cytoskeleton contributes to polarized trafficking to axons and dendrites [517, 518].

Microtubule dynamics and function are modulated through interaction with microtubule motor proteins and non-motor microtubule associate proteins (MAPs). Kinesins and dyneins are the two major microtubule motors [534, 535]. These motor proteins upon interaction with the microtubule produce force, which among other things, can be used for various intracellular functions including transport. The non-motor MAPs compromise many proteins that can both stabilize or destabilize microtubules [536]. Plus-end tracking proteins (+TIPs) accumulate at the ends of growing microtubules and control different aspects of neuronal development. [530]. +TIPs also control microtubule dynamics and interactions with other cellular organelles [505]. Collectively, these data demonstrate that many functions of microtubules are mediated by complex and diverse microtubule-interacting proteins.

While microtubules are in a dynamic state there are parts of the neurons that have stable microtubules resistant to polymerizing drugs [537]. It is thought that stable microtubules might still undergo growing and shrinkage at the plus end. However, the rest of the lattice makeup of the microtubule are protected against depolymerization. This protection can be seen in many axonal microtubules through post translation modifications near the minus end [537]. The post translation modification segments near the minus end suggest these stretches are longer lived than the rest of the microtubule. While there are many post translational modifications of microtubules, detyrosination and acetylation of α-tubulin are often cited to correlate with microtubule stability in many systems.

1.8.3 Post translational Modification of Microtubules

Post translational modifications of microtubules have recently garnered attention for their ability to control microtubule properties and functions. There are many modifications on the microtubule that can occur. The post translational modifications do not all occur at the same spot within the microtubule [538]. Some primarily take place on the α-tubulin component, such as detyrosination and acetylation, while others can take place on the carboxy tail, such as glutamylation [538]. The differences in where these post translational modifications occur and what the modifications are can cause differing effects on the function and stabilization of the microtubule. Below I will briefly discuss some of the post translational modifications and how these alterations can have an impact on the microtubule and further function of the cytoarchitecture.

Tyrosination and detyrosination of the tubulin were the first post translational modification discovered [538]. Tubulin tyrosination involves enzymatic addition of Tyr to the α-tubulin [539]. Importantly, tyrosination of the tubulin is reversible [540]. Endogenously it was found that most α-tubulin are tyrosinated so removal of the tyrosine is seen as the modification [541]. The carboxypeptidase responsible for detyrosination is still unknown, but the enzyme responsible for the reverse is known as tubulin Tyr ligase (TTL) [542]. Detyrosinating enzymes favor polymerized microtubule state while TTL works exclusively on soluble dimers [542, 543]. Detyrosination of tubulin can be further modified by removal of the C-terminal glutatmatic acid (Glu) residue to form Δ2-tubulin [544]. Formation of the Δ2-tubulin is performed by deglutamylase enzymes of the CCP family and in contrast to detyrosination is irreversible [545, 546].

The progressive addition of Glu residues on the γ-carboxyl group of one or more Glu residues near the C-terminus of polymerized tubulin, in a process known as

polyglutamylation [547-549]. There is a similar polyglycylation as well, which extends Gly side chains also from the Glu residues near the accessible C-terminus of tubulin within the microtubules [550]. Both of these processes are somewhat heterogeneous as they can affect either the α or β subunit of the tubulin and can form various lengths of chains [547, 548, 550, 551]. The modifications use similar sites and can compete with one another [552, 553]. Polyglutamylation is particularly frequent within neuronal tissue [554, 555]. Polyglutamylation was found to be caused by the TTL-like 1 (TTLL1) family and comprises glutamylating and glycylating enzymes [552, 553, 556-559]. The different enzymes within the family have preferences for either glutamylation or glycylation and preferences for α or β tubulin [545, 558]. This is also a reversible process, which can be done by a degluatmylaitng enzyme in the CCP family [545, 560].

Ubiquitin and SUMO are a small protein that can be added covalently [561, 562]. Ubiquitylation and Sumoylation are found as post translational modifications on many proteins, but have only been found to occur on soluble tubulin dimers [563-565]. The ubiquitin E3 ligase, parkin, binds and ubiquitylates tubulin dimers increasing tubulin degradation and recycling [563]. The ubiquitinated α-tubulin can accumulate in aggregates of Parkinson's disease and ALS and the inhibition of proteasomes enhance the aggregation [566-568]. Sumoylation is associated with cellular function that involve microtubules including trafficking and plasticity and may be changed in neurological disease states [561].

Acetylation of the Lys40 residue on α-tubulin is another common post translational modification [569]. Acetylation was found to take place on the microtubule polymer [570]. This is also found to be a reversible process as there are two known

deacetylating enzymes, histone deacetylase 6 (HDAC6) and sirtuin 2 (SIRT2) [571-573]. α-tubulin acetylation is affected by several acetyltransferases ARD1-NAT1 [574] and elongator protein complex. Elongator protein complex was shown to be necessary and sufficient to increase acetylation of the α-tubulin [575, 576]. The C. elegans protein mechanosensory abnormality 17 (MEC-17) and its mammalian counterparts, α-tubulin N-acetyltransferase 1 (αTAT1) were shown to unambiguously be the acetyltransferase for the α-tubulin [577, 578]. αTAT1 is perfectly correlated with microtubule acetylation, but not all organisms that have acetylated microtubule have αTAT1 or a homologous protein. This suggests that the other proteins discussed above may play a larger role in the acetylation of tubulin in these organisms.

1.8.4 Role of Post Translational Modifications

One important aspect of microtubule dynamics is stability of the microtubule. Many post translational modifications have been associated with increased longevity of microtubules. Detyrosination is found to be increased on long-lived microtubules, but the modification itself does not necessarily cause the increased longevity [579, 580]. A contributing factor to the stability may be the association of tyrosinated microtubules with depolymerizing proteins. Microtubule-depolymerization can be carried out by the Kinesin-13 family of proteins, these proteins have been found to preferentially associate with tyrosinated microtubules [581]. Furthermore, mice that are lacking Ttl, the enzyme responsible for tyrosination of tubulin, had overgrowth of microtubules. The overgrowth of microtubules was presumably due to the lack of depolymerized by the kinesin-13 family [582]. Δ2-tubulin has been seen as an irreversible lock on microtubules to permanently stabilize them [583]. Δ2-tubulin also generally occurs at the final stages of

differentiation where microtubules are no longer undergoing any dynamic changes [583]. Acetylation of microtubules are also commonly linked with stability of the microtubule. Overexpression of HDAC6 led to decrease in tubulin acetylation accompanied by increased susceptibility of microtubules to drug induced depolymerization [571]. Additionally, HDAC6 knockout or inhibition significantly decreased parameters of microtubule dynamics [584]. There is some evidence that preliminarily suggests that acetylation of the tubulin may induce enzymatic microtubule severing, which in turn can destabilize the acetylated microtubule [585]. However, there is no definitive information to show this impact on how acetylation negatively impacts stability. Polyglutamylation has also been linked to microtubule stability through microtubule-severing enzymes katanin and spastin interaction [586]. Long Glu side chains were found to activate microtubule severing while short has little effect [587].

Outside of stability post translational modifications of microtubules have many other effects on cellular function. Detyrosination of microtubules has been shown to regulate binding and activity of ubiquitous Kinesin-1 [588-591]. This increased affinity could have large implications for the movement of kinesin along neurons. Similarly acetylation has been found to increase the binding of KIF5 and dynein [592, 593]. However, these studies are confounded as they saw the increase only after inhibiting HDAC6, which has other targets besides tubulin. TTLL1 knockout mice showed decreased neuronal polyglutamylation that showed some effects on minor kinesin motors, but no effect on the conventional kinesin KIF5 [594].

Post translational modifications play an especially key role in neurons, as compared to other cell types, as microtubules in neurons have a much higher rate of

modification [544, 595-598]. Within neurons, detyrosination has been found to accumulate specifically within the axon and is thought to guide polarization of the neuron. Increased KIF5 binding on detyrosinated microtubules have been implicated to aide in navigating the neuron and establishing the axon [588, 591, 595, 599]. Further strengthening the importance of detyrosination in neurons, TTL-null mice have no tyrosinated tubulin detected in the neurons. TTL null mice show major developmental defects and culturing of their neurons displays premature axonal differentiation [582]. Acetylation follows a very similar pattern and is often enriched in the same microtubule populations that are detyrosinated [595]. Acetylation of microtubules in neurons accompanies neuronal differentiation [597, 600]. Silencing of the elongator protein complex correlated with decreased microtubule acetylation and impaired neuronal migration and branching [576]. Research in C. elegans revealed that reduced microtubule acetylation resulted in impaired touch receptor function [578]. Polyglutamylate activity was seen highest during development and at later stages microtubules are highly polyglutamylated while there is lower activity indicating stable microtubules [554]. TTLL1 knockout mice showed decreased levels of α-tubulin polyglutamylation resulting in altered synaptic vesicle transport [594]. Together these studies demonstrate the importance of post translational modifications on the microtubule and the effects in neurons. Post translational modifications on tubulin are summarized in Table 1.

Table 1:Tubulin Post Translational Modifications

Modification	Place of Action	Enzyme	Reversible	Consequence
Tyrosination	α-tubulin C-terminus tail	Tublin Tyr Ligase (TTL)	Yes	Associated with decreased longevity

Deytrosination	α-tubulin C-terminus tail	Unknown	Yes	Associated with increased longevity Binding of Kinesin-1
Δ2-tubulin	α-tubulin C-terminus tail	cytosolic carboxypeptidase (CCP)	No	Permanently stabilize microtubuels
Polyglycylation	γ-carboxyl group of polymerized dimers	TTL-like (TTLL)	Yes	Kinesin motor binding
Deglycylation	γ-carboxyl group of polymerized dimers	CCP	YEs	Decreased kinesin motor binding
Polyglutamylation	γ-carboxyl group of polymerized dimers	TTL-like (TTLL)	Yes	Associated with increased longevity Katnin and Spastin binding
Deglutamylation	γ-carboxyl group of polymerized dimers	Unknown	Yes	Associated with decreased longevity
Acetylation	Lys 40 residue on α-tubulin	α-tubulin N-acetyltransferase 1 (αTAT1)	Yes	Increased flexibility and stability
Deacetylation	Lys 40 residue on α-tubulin	Histone Deacetylase 6 (HDAC6)	Yes	Stiff and decreased flexibility
Ubiquitin	Soluble tubulin dimers	parkin	Yes	Tubulin accumulation

1.8.5 Acetylation of Microtubules

Acetylation of the microtubule is unique compared to other post translational modifications as it is only found on the microtubule lattice and not on cytosolic tubulin. This means the enzyme must enter the lumen of microtubules to acetylate the tubulin. In support of this αTAT1 affinity is 100 times higher towards tubulin in polymeric state compared to unpolymerized tubulin [570, 601-603]. It is still unknown how αTAT1 enters the lumen, but several models have been proposed. αTAT1 may enter the

microtubule lumen through irregularities or breaks in the microtubule wall [604]. The model proposes that αTAT1 scans the microtubule for defects and upon finding one enters the lumen. Entering the lumen when cracks are detected can explain a lack of complete damage to the microtubule that would necessitate αTAT1 activity [605-607]. Bending of the microtubules has shown increased incorporation of new dimers, demonstrating a repair mechanism in place after cracks are present [605]. The main drawback of this model is that it suggests there are many irregularities and damage throughout the lumen to allow for the amount of acetylation seen in mature microtubules [608].

Another leading model suggests that αTAT1 enters the lumen of microtubules from the ends. Several studies have shown preferential entry at open ends and increased acetylation of microtubules at the ends [604, 607, 609-611]. This model is complicated by the number of MAPs and other enzymes that localize around the ends of the microtubule, which could make it difficult for αTAT1 to enter the lumen. Similarly, questions of how microtubules are deacetylated by HDAC6 or SIRT2 still persist as this mechanism is also largely unknown. Some evidence suggests that tubulin deacetylation corresponds with depolymerization of microtubule meaning it likely affects cytosolic dimers [602]. However, this finding was contradicted by a study that found *in vitro* polymerized tubulin was deacetylated [571, 572]. Newer models suggest that HDAC6 may function similarly to αTAT1 and enter the lumen through breaks or the open site [612].

As discussed earlier it has been suggested that tubulin acetylation is important for stabilizing microtubules [570, 602, 613]. There is an ongoing debate on whether

microtubules are acetylated because they are stable or whether the stability is due to acetylation. Early studies found that when tubulin acetylation is artificially increased it does not stabilize microtubules [614]. Another model suggests that microtubules undergo structural changes after acetylation. Acetylation of the Lys40 residue was shown to weaken lateral interactions between protofilaments, which in turn softens the microtubule [615]. Given the stress that normally accompanies microtubules in cellular life, softened microtubules would be more flexible and therefore more resilient to breakage [616]. Furthermore, it was found that acetylated microtubule hotspots are around the curved ends of microtubule, which are where the most breakage and openings occur [616, 617]. This strengthens the hypothesis that αTAT1 can enter the microtubule through these cracks and repair the microtubule lumen making it less likely to be damaged in the future resulting in longer lived more stable microtubules.

αTAT1 and HDAC6 manipulation has given functional insight into the importance of proper tubulin acetylation. Acetylation of microtubules has been linked to cell migration, autophagy, neuronal dependent touch in C. elegans and mice, intracellular trafficking, and cell adhesion, [592, 593, 609, 610, 618-620]. While the role of acetylation in regulating these cellular functions is largely accepted the mechanism for how acetylated tubulin functions is still controversial. Some studies have shown that molecular motors run preferentially on acetylated tracks, while studies with purified microtubules have shown that this may not be the case [592, 593, 621, 622]. It has been suggested that the finding of improved motor movement is due to the longer lasting microtubule rather than the actual acetylation. αTAT1 knockout mice have deformations in the dentate gyrus [623], touch sensation, and sperm motility [619, 624].

The cells from these mice showed reduced levels of contact inhibition during cell proliferation possibly explaining the role of acetylation in these various functions.

1.8.6 Histone Deacetylase 6

HDAC6 was the first HDAC discovered to be actively maintained in the cytoplasm [625]. HDAC6, in rodents, is actively retained in the cytoplasm and only under specific circumstances can be partially found in the nucleus [625]. HDAC6 has a strong nuclear export signal on the N terminus preventing the accumulation of the protein in the nucleus. The nuclear export signal is maintained in humans, but an additional cytoplasmic anchoring domain is also present in rodents [626]. HDAC6 has been found to primarily interact with two cellular signaling systems, ubiquitination and acetylation [627]. HDAC6 possesses 2 catalytic activity domains [628] and the duplication has been argued to demonstrate its overall importance in function. There have been conflicting reports as to the importance of both domains as some studies find that the ability of HDAC6 to deacetylase tubulin needs both domains [629, 630] others showing that only the second domain is necessary [631, 632]. Between the two catalytic domains is a spacer domain that was also found to be critical for tubulin deacetylation activity as an amino acid addition or deletion dramatically affected the activity [629]. Despite the controversial findings around the duplicated domain, the second catalytic domain has always been found to be necessary for the tubulin deacetylase activity [629, 631, 632]. HDAC6 has a ubiquitin binding domain and participates in cellular functions depending on protein ubiquitination [633, 634]. HDAC6 has a conserved cysteine and histidine rich domain in the C-terminus, which is also present in a group of ubiquitin-specific proteases, known as ZnF-UBP [627, 635]. This domain was found to bind to

monomeric ubiquitin with the highest affinity of all known ubiquitin interacting proteins [634]. The domain was also found to bind polyubiquitin chains [634, 636].

1.8.7 Physiological Roles of Histone Deacetylase 6

The first identified acetylation substrate for HDAC6 was α-tubulin [571, 572, 630]. As previously discussed, tubulin acetylation has been linked to increase kinesin-1 activity and inhibiting HDAC6 can promote transport [593]. The increased activity of motor proteins may explain the ability of HDAC6 to transport protein aggregates to aggresomes [633]. HDAC6 also has deacetylase activity on the chaperone protein Hsp90 [637-639]. Hsp90 was found to interact with up to 10% of the total yeast proteome [640] demonstrating its importance for cellular function. Hsp90 can stabilize metastable regions of specific factors by keeping them in a 'holding' position. An example of this is the glucocorticoid receptor; the hormone-binding activity of the receptor is dependent on its association with Hsp90 [641]. HDAC6 acetylation of Hsp90 was shown to induce dissociation of its co-chaperone p23 and result in accumulation of glucocorticoid receptors that were defective in hormone binding [638, 639]. Acetylation of Hsp90 was further found to be a major cause of instability of many of its client proteins [642].

HDAC6 has been shown to regulate Hsp90 and glucocorticoid receptor chaperoning [637, 638]. There is a direct physical interaction between HDAC6 and Hsp90. Activity of both deacetylation and the ubiquitin binding are required for interaction with Hsp90 [638, 643]. Overexpression of HDAC6 led to hypoacetyalation of Hsp90 and inhibition with trichostatin A (TSA), a selective Class I and II HDAC inhibitor, or HDAC6 siRNA knockdown led to hyperacetylation [638]. The glucocorticoid receptor

in HDAC6 knockdown cells exhibits reduced ligand binding and was defective in translocating to the nucleus to activate transcription of glucocorticoid regulated genes. Further demonstrating a link between HDAC6 and glucocorticoids, cells that have HDAC6 knockdown and were treated with a synthetic glucocorticoid results in an increase in acetylation of Hsp90 [638, 643]. Hsp90 when hyperacetylated has disrupted assembly with co-chaperones that are necessary for normal glucocorticoid receptor maturation and assembly. To this end, co-chaperone p23 affinity for Hsp90 was dramatically reduced in both HDAC6 knockdown and TSA-treated cells. Improper Hsp90 co-chaperone interaction leads to destabilizing of Hsp90 and glucocorticoid receptor interaction leading to deficits in signaling [638]. However, Hsp90 has multiple acetylation sites and it is likely that HDAC6 does not have affinity for them all [644]. Deletion of HDAC6 in serotonin positive neurons reduced acute anxiety-like effects of the glucocorticoid hormone corticosterone [645]. Furthermore, depletion of HDAC6 in this neuronal population prevented electrophysiological and morphological changed induced by traumatic stress implicated HDAC6 inhibitors as potential anxiolytics and antidepressants [645].

HDAC6 has been found to be specifically involved in the fate of ubiquitinated proteins. The ubiquitin proteasome system is the main pathway for protein degradation. It functions by multiproteic proteolytic complexes that degrade short-lived ubiquitin marked proteins [646]. A model of proteasome-inhibition in *Drosophila* was found to cause deterioration, but an overexpression of HDAC6 can suppress the degenerative phenotype. This data strongly demonstrates that HDAC6 can suppress cytotoxicity [646]. Furthermore, knockdown of endogenous HDAC6 increases tissue degeneration.

When the ubiquitin proteasome system is overwhelmed HDAC6 can regulate toxicity through the macroautophagic system via microtubules and dynein-mediated transport ensuring delivery of the autophagosomes to lysosomes [647]. Aggresomes are inclusion bodies in which misfolded proteins are processed and are formed in conditions of proteasome deficiency. The ZnF-UBP within HDAC6 plays a critical role in clearance of cytotoxic aggregates [633]. HDAC6 acts as a linker between the dynein motors and the polyubiquitinated proteins and is crucial for dynein to carry HDAC6 associated polyubiquitinated aggregates from the cytoplasm and move them to the aggresome. Interestingly both the deacetylase and the ZnF-UBP domains are required for aggresome formation. In HDAC6 silenced cells accumulation of ubiquitin-misfolded proteins occurs and results in two-fold higher levels of apoptosis.

Cortactin is an F-actin-binding protein and promotes polymerization and branching and is found in areas of dynamic actin assembly including leading edge of cell [648, 649]. HDAC6 deacetylates cortactin through the deacetylase domains [648]. Deacetylated cortactin has been shown to increase ability to bind to F-actin, which then promotes F-actin-dependent cell movement [648]. Conversely, when cortactin is highly acetylated it is less likely to translocate to the cell periphery and bind with F-actin resulting in decreased cell motility [648].

1.8.8 Knockout and Inhibition of Tubulin Acetylation Components

There are knockout mouse lines of both HDAC6 and αTAT1. HDAC6 gene was targeted through homologous recombination in embryonic stem cells and used to generate knockout mice [650]. The embryonic fibroblasts of these knockout mice showed normal microtubule organization and stability but had increased tubulin and

Hsp90 acetylation and impaired Hsp90 function. These mice are viable and mature to adulthood. Interestingly, SIRT2 inhibition or genetic deletion in mouse embryonic fibroblasts failed to alter the levels of acetylated tubulin. Within embryonic fibroblasts, HDAC6 is the only tubulin deacetylase with detectable *in vivo* activity [650].

Knockout of αTAT1 in C. elegans resulted in almost complete loss of α-tubulin acetylation [578]. Furthermore, in C. Elegans there was reduced touch sensitivity and disruption of microtubule structure and organization in touch receptor neurons [651, 652]. Furthermore, the depletion of the αTAT1 orthologue, mec17, in zebra fish resulted in developmental defects and neuromuscular deficiencies [578]. Homozygous αTAT1 knockout mice had no detectable tubulin acetylation in embryonic or adult mice in any tissue examined [624]. Subcellular investigation also revealed no tubulin acetylation in the cytoplasm or cilia. They found that tubulin in the αTAT1 knockout mice were more resilient to depolymerization. These mice were viable, despite some spermatozoa morphology and motility issues [624]. There were also limited behavioral alterations noted, except mild anxiety like changes in the elevated plus maze [624]. αTAT1 was found to be ubiquitously expressed in all mouse sensory neurons and the sensory neurons have the highest level of α-tubulin acetylation [624, 653]. A study found that αTAT1 knockout in mouse peripheral sensory neurons results in loss of mechanical sensitivity to both light touch and painful stimuli, but no alteration in hot and cold touch sensitivity [619].

One study found that after chronic social defeat stress there was lower acetylated Hsp90, higher glucocorticoid receptor and Hsp90 association, and enhanced glucocorticoid translocation in the vulnerable mice. HDAC6 inhibitor, ACY-738 led to

Hsp90 hyperacetylation. Furthermore, ACY-738 administration resulted in resilience to chronic social defeat stress [654]. Similarly, the same group found that ACY-738 resulted in a dramatic increase in α-tubulin acetylation in the brain. They also found decreased depression measures in tail suspension test and a social defeat paradigm [655]. Plasma-membrane associated tubulin showed significant decreases in acetylation from depressed-suicide, depressed-no suicide, versus control in human postmortem tissue [656]. HDAC6 inhibition has also shown some promise in treating pain. HDAC6 inhibition increased acetylated α-tubulin and was shown to prevent electrophysiological and behavioral induced neurological symptoms of vincristine based chemotherapy [657]. Another group found that cisplatin-induced neuropathy was prevented through HDAC6 inhibition and even reversed existing mechanical allodynia, spontaneous pain, and numbness [658]. Furthermore, HDAC6 inhibition was able to relieve hyperalgesia following a model of spared nerve injury [659]

1.9 Summary and Dissertation Organization

In summary, migraine is a complex and undertreated disorder that requires more research to gain a better understanding about its complex pathophysiology and development of more effective therapeutic options. Investigation into cytoarchitectural basis for chronic migraine and other chronic pain disorders could potentially unlock novel treatments for chronic pain sufferers. Additionally, the DOR has anti-hyperalgesia effects and was previously shown to have promise in treating headache disorders. In this thesis, I carry out a thorough behavioral and molecular experiment detailing the impact of neuronal cytoarchitecture in regulating chronic migraine as well as compare these finding to a peripheral pain disorder,. I further highlight the effectiveness of a

biased DOR agonist, KNT-127, in regulating migraine associated models of pain and aura. This dissertation is presented in a manuscript format in which each chapter embodies experiments that gain a better understanding of the pathophysiology of chronic pain and preclinical screening of compounds for the future treatment of migraine. Chapter 2 has been peer reviewed initially at *eLife* and is currently in revision. This work demonstrates a cytoarchitectural basis for chronic migraine and shows how HDAC6 inhibition could be used as a novel therapeutic option. Chapter 3 expands on these findings and investigates other cytoarchitectural changes in chronic migraine models as well as in a model of CSD. Within chapter 3 I further demonstrate that cytoarchitectural changes can accompany chronic regional pain syndrome, a peripheral neuropathic pain state. Chapter 4 was previously peer-reviewed and published in *Headache*, and details the use of a biased DOR agonist as potential therapy for treating chronic migraine as well as migraine aura. Collectively these works provide a better understanding of chronic migraine and identifies novel therapeutic targets for this disorder.

Chapter 2: Neuronal complexity is attenuated in chronic migraine and restored by HDAC6 inhibition

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2.1 Introduction

Migraine is an extremely common neurological disorder that is estimated to affect 14% of the world population, making it the third most prevalent disease worldwide [3, 660]. One particularly debilitating subset of migraine patients are those with chronic migraine, which is defined as having more than 15 headache days a month [661]. Despite its high prevalence, migraine therapies are often only partially effective or are poorly tolerated, creating a need for better pharmacotherapies [662]. Recent clinical success of antibodies against calcitonin gene related peptide (CGRP) and CGRP receptor demonstrate the effectiveness of targeted migraine therapeutics. While there has been more research into understanding the molecular mechanisms of migraine, there remains much to be discovered.

Neuroplastic changes play an important role in a variety of chronic neuropsychiatric conditions [663], and epigenetic alterations through histone deacetylases (HDACs) are frequently investigated. HDACs are best characterized for their ability to deacetylate histones, promoting chromatin condensation and altered gene expression [664]. Intriguingly, some HDACs can also deacetylate non-histone targets,

including proteins involved in the regulation of cytoarchitecture. Due to its cytoplasmic retention signal, HDAC6 is primarily expressed in the cytosol [646], and one of its primary targets for deacetylation is α-tubulin [646, 665]. α- and β-tubulin form heterodimers that make up microtubules, which are a major component of the cytoskeleton, and regulate intracellular transport, cell morphology, motility, and organelle distribution [538, 608]. Microtubules undergo multiple cycles of polymerization and depolymerization, thus rendering them in a constant state of dynamic instability [538]. Tubulin displays a variety of post translational modifications, including α-tubulin acetylation, which occurs endogenously through α-tubulin N-acetyltransferase I (αTAT1) and it is correspondingly deacetylated by HDAC6 [608]. Tubulin acetylation is associated with increased flexibility and stability of microtubules [616]. In contrast, deacetylated microtubules are more fragile and prone to breakage [616]. Microtubules are important for cellular response to injury and play a role in neurite branching [666]; and microtubule dynamics influence neuronal signaling and mediate axonal transport [657, 667-670]. Importantly, changes in cellular structure such as alterations in dendritic spine density, have been implicated in disease chronicity [654, 671-673].

The aim of this study was to determine if altered neuronal cytoarchitecture facilitates the chronic migraine state. We observed decreased neuronal complexity in headache-processing brain regions in the nitroglycerin (NTG) model of chronic migraine-associated pain. We further demonstrated that treatment with HDAC6 inhibitor reversed these cytoarchitectural changes and correspondingly decreased cephalic allodynia. These studies were extended to a mechanistically distinct model of migraine, cortical spreading depression (CSD), which is thought to be the electrophysiological

correlate of migraine aura. Again, we observed decreased neuronal complexity in migraine related sites, which was reversed by HDAC6 inhibitor. To investigate the translational implication, we also tested the effect of olcegepant, a CGRP receptor inhibitor, and found that it alleviated chronic allodynia induced by NTG, and restored cytoarchitectural changes associated with chronic migraine-associated pain. These results suggest a novel mechanism for migraine pathophysiology and establish HDAC6 as a novel therapeutic target for this disorder.

2.2 Materials and Methods

Animals: Experiments were performed on adult male and female C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME. USA) weighing 20-30g. Mice were group housed in a 12h-12h light-dark cycle, where the lights were turned on at 07:00 and turned off at 19:00. Food and water were available ad libitum. All experiments were conducted in a blinded fashion by 1-3 experimenters. Weight was recorded on each test day for all experiments. All experimental procedures were approved by the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety Committee, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines and the Animal Care Policies of the University of Illinois at Chicago. All results are reported according to Animal Research: reporting of *In vivo* Experiments (ARRIVE) guidelines. No adverse effects were observed during these studies, and all animals were included in statistical analysis.

Sensory Sensitivity testing: Different groups of animals were used for each experiment. Mice were counter-balanced into groups following the first basal test for mechanical thresholds. Mice were tested in a behavior room, separate from the vivarium, with low

light (~35-50 lux) and low-noise conditions, between 09:00 and 16:00. Mice were habituated to the testing racks for 2 days before the initial test day, and on each subsequent test days were habituated for 20 min before the first test measurement. For cephalic measures mice were tested in 4 oz paper cups. The periorbital region caudal to the eyes and near the midline was tested. For experiments testing peripheral mechanical responses, the intraplantar region of the hind paw was assessed. Testing of mechanical thresholds to punctate mechanical stimuli was tested using the up-and-down method. The selected region of interest was stimulated using a series of manual von Frey hair filaments (bending force ranging from 0.008 g to 2 g). A response of the head was defined as shaking, repeated pawing, or cowering away from the filament. In the hind paw a response was lifting of the paw, shaking, or licking the paw after stimulation. The first filament used was 0.4 g. If there was no response a heavier filament (up) was used, and if there was a response a lighter filament (down) was tested. The up-down pattern persisted for 4 filaments after the first response.

Nitroglycerin model of chronic migraine: Nitroglycerin (NTG) was purchased at a concentration of 5 mg/ml, in 30% alcohol, 30% propylene glycol and water (American Reagent, NY, USA). NTG was diluted on each test day in 0.9% saline to a concentration of 1 mg/ml for a dose of 10 mg/kg. Mice were administered NTG or vehicle every other day for 9 days. Animals used in cephalic experiments were tested on days 1, 5, and 9. On test days a basal threshold was measured then animals were treated with either NTG or vehicle and then put back in the testing racks and subsequently tested 2 h later for the post-treatment effect.

Cortical Spreading Depression Model: The procedure for the cortical spreading depression (CSD) model is based on work previously published by Ayata [229] that is commonly used to screen potential migraine preventives and further used in our own work Pradhan and colleagues [114]. Mice were grouped into sham and CSD groups and then further subdivide into ACY-738 (50 mg/kg, IP) or vehicle (i.e., Sham-ACY, Sham-Veh, CSD-ACY, CSD-Veh). To make the thinned skull cortical window, mice were anesthetized with isoflurane (induction 3-4%; maintenance 0.75 to 1.25%; in 67% N_2 / 33% O_2) and placed in a stereotaxic frame on a homoeothermic heating pad. Core temperature (37.0 ± 0.5℃), non- peripheral oxygen saturation (~ 99%), heart rate, and respiratory rate (80–120 bpm) were continuously monitored (PhysioSuite; Kent Scientific Instruments, Torrington, CT, USA). Mice were frequently tested for tail and hind paw reactivity to ensure that the anesthesia plane was maintained.

To verify CSD events, optical intrinsic imaging (OIS) and electrophysiological recordings were performed as previously described[114]. Briefly, following anesthesia, the skin from the skull was detached and a rectangular region of ~2.5 x 3.3 mm^2 (~0.5 mm from sagittal, and ~1.4 from coronal and lambdoid sutures) of the right parietal bone was thinned to transparency with a dental drill (Fine Science Tools, Inc., Foster City, CA, USA). Mineral oil application improved transparency of cortical surface parenchyma and vasculature for video recording. A green LED (530 nm) illuminated the skull throughout the experiment (1-UP; LED Supply, Randolph, VT, USA). Cortical surface reflectance detected by OIS was collected with a lens (HR Plan Apo 0.5 × WD 136) through a 515LP emission filter on a Nikon SMZ 1500 stereomicroscope (Nikon Instruments, Melville, NY, USA). Images were acquired at 1–5 Hz using a high-sensitivity USB

monochrome CCD (CCE-B013-U; Mightex, Pleasanton, CA, USA) with 4.65-micron square pixels and 1392 × 1040 pixel resolution.

Lateral to the thinned window two burr holes were drilled around the midpoint of the rectangle. These burr holes were deeper than the previously drilled skull region such that the dura was exposed but not broken. To record local field potentials (LFPs) an electrode (in a pulled glass pipette filled with saline) was inserted into one burr hole and attached to an amplifier. A separate ground wire, placed underneath the skin caudal to the skull, grounded this set up and LFPs were recorded for an hour to ensure a stable baseline and recovery from any surgically induced CSDs. After an hour of stabilization, a second pulled glass pipette was filled with 1 M KCl and placed into the more rostral burr hole, avoiding contact with the brain or the surrounding skull. An initial flow of KCl was pushed to begin and then an even flow was held so that there was a constant small pool of KCl that filled the burr hole. Excess liquid was removed with tissue paper applied next to the burr hole. Regardless of grouping the CSD recording continued for 3600s after the initial drip of KCl. Mice were euthanized by anesthetic overdose followed by decapitation.

Golgi Staining: Golgi staining was performed according to the FD Rapid Golgi Stain kit (FD Neurotechnologies). For NTG or Veh treated mice, they underwent the chronic NTG model and on day 10, 4 h after ACY-738 treatment or vehicle, mice were anesthetized with isoflurane and then euthanized. After euthanizing the brains were removed rapidly. The tissue was then rinsed briefly in double distilled water. Tissue was then placed in the impregnation solution that was an equal amount of solutions A and B that was prepared at least 24 h in advance. After the first 24 h the brain was

placed in new impregnation solution and then stored for 1 week in the dark. The brains were then transferred to solution C, which was also replaced after the first 24 h. After replacing solution C the brains were stored at room temperature for 72 h more. Following solution C, brains were flash frozen in 2-methyl butane and cryostat cut at -20°C into 100 μm slices. The slices were mounted onto gelatin coated slides and secured by a drop of solution C placed onto each slice. These slides were then left to dry naturally in the dark.

Neurite Tracing: After processing images were taken at 20x magnification and a Z-stack was created based on different levels of focal plane. After the Z-stack was created the FIJI program Simple Neurite Tracer was used to trace the processes of the neuron. Furthermore, after tracing the neurons were analyzed using Simple Neurite Tracer [674] software to assess the number of branch points from each neuron, overall length of the neuron, and Sholl Analysis. Sholl Analysis was performed by placing a center ROI point at the center of the soma and producing consecutive circles every 20 pixels for the entire body of the neuron. Intersections were counted based on the number of times a neurite crossed each of these consecutive circles. These data were compiled per neuron and then brought into one Masterfile.

Neuron Selection: Throughout tracing all tracers were blinded to which group the images belonged to. For all brain regions analyzed, six to eight relatively isolated neurons were randomly chosen per mouse. The selected neurons were fully impregnated with Golgi stain and relatively complete. An atlas was used along with clear anatomical markers to ensure the neurons were being taken from their described region of interest. Neurons characterized for the trigeminal nucleus caudalis region were

taken only from the outer lamina of caudal sections. Neurons analyzed for somatosensory cortex were all taken from layer IV of the primary somatosensory barrel cortex. To ensure a homogenous cell population only pyramidal cells were selected. The most complex neurons were chosen for analysis in all regions. Previously, it was shown that dendritic complexity was directly correlated to soma size. To ensure that the NTG group where not just smaller in size we directly compared soma diameter of neurons in the NTG and Veh group. There was no significant difference in soma size between these two groups (Veh 9.258 ± .2515 and NTG 9.192 ± .2782, student run t-test p= 0.8608). Two individuals traced all cells. Interrater reliability was determined by having each tracer trace 5 neurons in their entirety. Pearson product correlations were accessed in three measures; number of branches, total dendritic length, and total intersection number through Sholl analysis and found to be 0.91, 0.94, and 0.95 respectively. All tracings of neurons were re-examined by the primary tracer (Z.B.) to assure quality control.

Drug Injections: All injections were administered at 10 ml/kg volume, intraperitoneally (IP), unless otherwise indicated. ACY-738 was dissolved in a 5% DMSO saline solution, which was used as the vehicle control. RN-73 was dissolved in 10% DMSO, 10% Tween-80, and 80% saline and was injected 1 mg/kg or 10 mg/kg, this mixture was also used as the vehicle control group. ASV-85 was dissolved in 15% DMSO, 15% Tween-80, and then 70% saline, this mixture was also used as the vehicle control group. ASV-85 was injected at 1 mg/kg dose. TSA was dissolved in 20% DMSO solution in 80% 0.01M PBS and injected at a dose of 2 mg/kg, this same solution was used for the vehicle. Olcegepant was dissolved in saline solution and was injected at a 0.1 mg/kg

dose. For the CSD experiments ACY-738 was injected 3 hours before starting the surgery so that it would reach its peak efficiency of 4 h by the time the CSD event started.

Quantitative RT-PCR: Total RNA was isolated from flash frozen brain punches using the RNeasy Plus Mini kit from Quiagen. RNA samples were reverse transcribed to single-stranded cDNA. cDNA transcription was used following the protocol from Superscript III (Life Technologies) and the TaqMan Gene Expression Assay system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991_g1) was used as a housekeeping gene. The threshold cycle (CT) of each target product was determined and CT values between HDAC6 transcripts and housekeeping genes were calculated (ΔCT). The fold change ($2^{-\Delta\Delta CT}$) for each was calculated relative to the median ΔCT from the saline control animals.

Immunohistochemistry: Mice were anesthetized with Somnasol (100 µl/mouse; 390 mg/mL pentobarbital sodium; Henry Schein) and perfused intracardially with 15 ml of ice-cold phosphate-buffered saline (0.1 M PBS, pH 7.2) and subsequently 50 mL of ice-cold 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). Whole brain and trigeminal ganglia (TG) were harvested and overnight left to post-fix in 4% PFA/0.1M PBS at 4°C. Brain and TG were then cryoprotected in 30% sucrose in 0.1M PBS until they sunk. Brains were then flash frozen using 2-methyl butane over dry ice. Coronal sections of the trigeminal nucleus caudalis (TNC) and the somatosensory cortex were sliced at 20 µM and TG at 16 µM. All slices were immediately mounted onto slides after slicing in the cryostat. Slides were washed with PBST. Then a blocking solution with 5% normal donkey serum with PBST for 1 h at room temperature. Slides were then incubated

overnight at RT with the primary rabbit anti-HDAC6 antibody (1:500, courtesy of Tso-Pang Yao at Duke University) diluted in 1% NDSDT. Slides were subsequently washed with 1% NDST and then the secondary antibody was added for 2 hours at room temperature (donkey anti rabbit IgG, 1:2000). Slides were washed with 0.1 M phosphate buffer, and cover slipped with Mowiol-DAPI mounting medium. Images were taken by in a blinded manner using the EVOS FL Auto Cell Imaging system, using a 40 x objective.

Western Blots: Samples were taken from chronically treated NTG or Vehicle mice, which received an injection of ACY-738 or Vehicle on day 10. Samples were collected 4h post-ACY/VEH. Protein concentrations were assessed using a Nanodrop 2000c spectrophotometer and equal quantities were loaded onto each Stain-Free acrylamide gel for SDS-PAGE (Bio-Rad, Hercules, CA, USA). The gels were subsequently transferred to Nitrocellulose membranes (Bio-Rad, Hercules, CA USA) for western blotting. The membranes were blocked with 5% non-fat dry milk diluted in TBS-T (10 mM Tris-HCl, 159 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h. Following the blocking step, membranes were washed with Tris-buffered saline/Tween 20 and then incubated with an anti-acetyl-α-tubulin antibody (Lysine-40) (Sigma Clone 6-11B1), α-tubulin (Sigma), overnight at 4 °C. Membranes were washed with TBS-T and incubated with a secondary antibody [HRP-linked anti-mouse antibody IgG F(ab′)2 or HRP-linked anti-rabbit antibody IgG F(ab′)2] (Jackson ImmunoResearch, West Grove, PA, USA, catalog #115-036-072 for mouse, and catalog #111-036-047 for rabbit, RRID) for 1 h at room temperature, washed, and developed using ECL Luminata Forte chemiluminescent reagent (Millipore, Billerica, MA, USA). Blots were imaged using a Chemidoc computerized densitometer (Bio-Rad, Hercules, CA, USA) and quantified by

ImageLab 3.0 software (Bio-Rad, Hercules, CA, USA). In all experiments, the original gels were visualized using BioRad stainfree technology to verify protein loading.

Statistical Analysis: Sample size was calculated by power analysis and previous experience. Since we investigated changes at the cellular level, an individual neuron represented a single sample [645, 675]. Data analysis was performed using GraphPad Prism version 8.00 (GraphPad, San Diego, CA). The level of significance (α) for all tests was set to 0.05. Post hoc analysis was conducted using Holm-Sidak post hoc test to correct for multiple comparisons. Post hoc analysis was only performed when F values achieved $p < 0.05$. All values in the text are reported as mean ± SEM. Detailed statistical analysis can be found in Supplementary Table 2.

2.3 Results

2.3.1 Exposure to chronic NTG induces cytoarchitectural changes in key pain processing regions

Changes in the structural plasticity of neurons have been observed in a number of neuropsychiatric disorders and can serve as a marker of disease chronicity [672, 673]. To investigate if this was also the case in migraine, we treated male and female C57BL6J mice every other day for 9 days with NTG or vehicle and tested for mechanical periorbital responses on days 1, 5 and 9 (Figure 6A). NTG induced severe and sustained cephalic allodynia as measured by von Frey hair stimulation of the periorbital region as compared to vehicle animals on the same day (Figure 6B). Mice were sacrificed on day 10, 24 h after the final NTG/VEH treatment, and neuronal size and arborization were examined through a Golgi staining procedure in a key cephalic pain processing region, the trigeminal nucleus caudalis (TNC) (Figure 6C) [676]. We

observed a dramatic decrease in neuronal complexity after NTG treatment (Figure 6D). Neurons of chronic NTG-treated mice had significantly fewer branch points (Figure 6E), and shorter neurites resulting in decreased overall length of the neurons (Figure 6F). Further examination of the complexity of the neurons using Scholl Analysis, showed a significant decrease in the number of intersections following NTG treatment (Figure 6 G-I). In addition to the TNC we also determined if other brain regions related to central pain processing were affected by chronic NTG treatment. We examined the somatosensory cortex (SCx) and periaqueductal grey (PAG) of these mice and found similar results, where neurons from NTG-treated mice had fewer branch points, were shorter in length, and had fewer intersections. To ensure that this effect was associated with migraine-pain processing and not a non-specific effect of NTG we also analyzed neuronal complexity in the nucleus accumbens shell (NAc), a region more commonly associated with reward, and found no alteration in number of branches, total neuron length, or Sholl analysis for cells in this region (Figure 7). Furthermore, we also examined the dorsal horn of the lumbar spinal cord, an important site for peripheral but not head-pain processing; and found no differences in NTG versus vehicle controls in number of branches, total neuron length, or Sholl analysis (Figure 7). These results suggest that decreased neuronal complexity may be a feature that maintains the chronic migraine state; a previously undiscovered phenomenon.

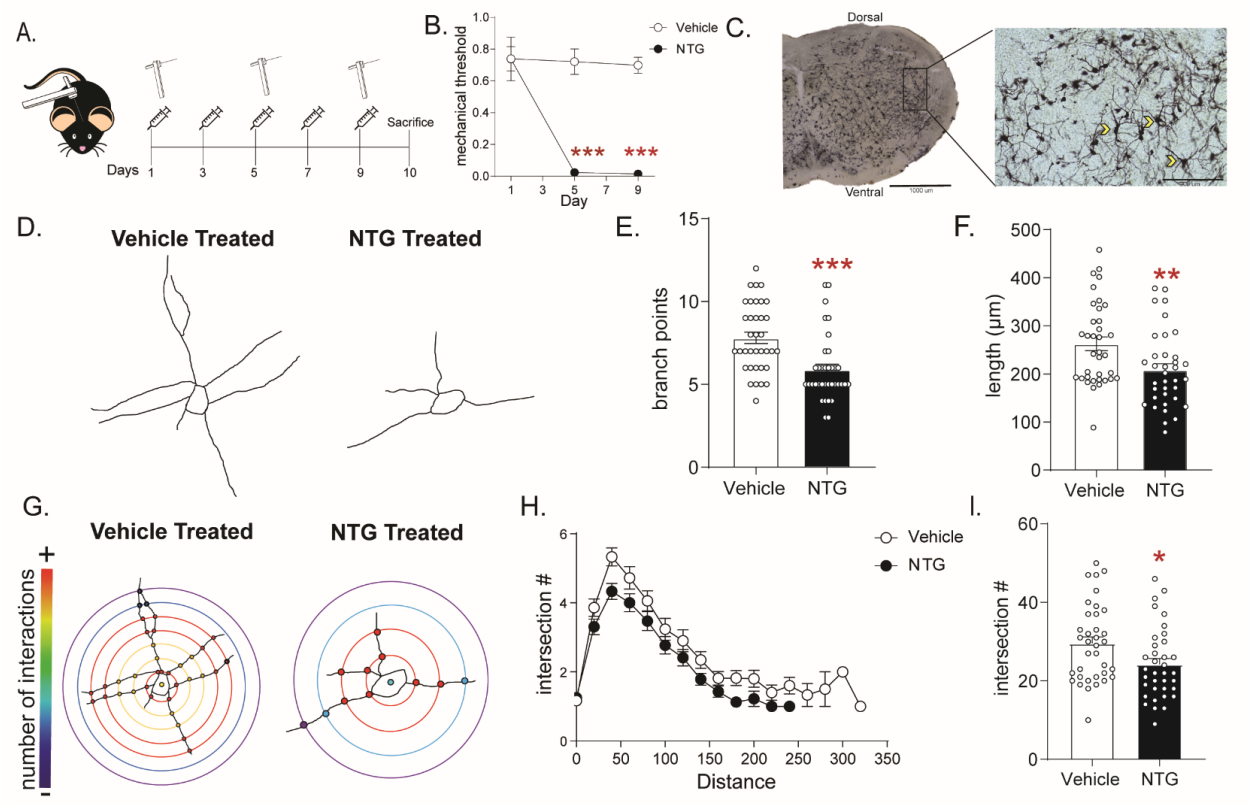

Figure 6. The NTG model of chronic migraine produces cytoarchitectural changes in a cephalic pain processing region (A) Schematic of testing schedule, M&F C57Bl6/J mice were treated with vehicle or nitroglycerin (10 mg/kg, IP; NTG) every other day for 9 days. (B) Periorbital mechanical thresholds were accessed prior to Vehicle/NTG administration on days 1, 5 and 9. NTG produced severe cephalic allodynia $p<0.001$ effect of drug, time, and interaction, two-way RM ANOVA and Holm-Sidak post hoc analysis. ***$p<0.001$ relative to vehicle on day 1 n=8/group. (C) Representative image taken of Golgi stained TNC at 4x (left) and 20x (right). Chevrons indicate neurons traced from the selected image and demonstrate type of neurons selected from this region. (D) Representative tracing of neurons from mice treated with chronic vehicle (left) or NTG (right) demonstrating reduced neural processes after chronic NTG treatment. (E) The number of branch points/neuron was decreased following chronic NTG. Unpaired t-test. ***$p<0.001$ (F) Total neuron length was also compromised after NTG treatment. Unpaired t-test. **$p<0.01$. (G) Representative Sholl plots of vehicle (left) and NTG (right) treated mice. (H) Sholl analysis broken up by 20 voxel distances from the center of the cell showing differences between groups. (I) Sholl analysis revealed a significant decrease in total intersections after chronic NTG treatment. Unpaired t-test. *$p<.05$ n=6 mice/group, 6 neurons per mouse.

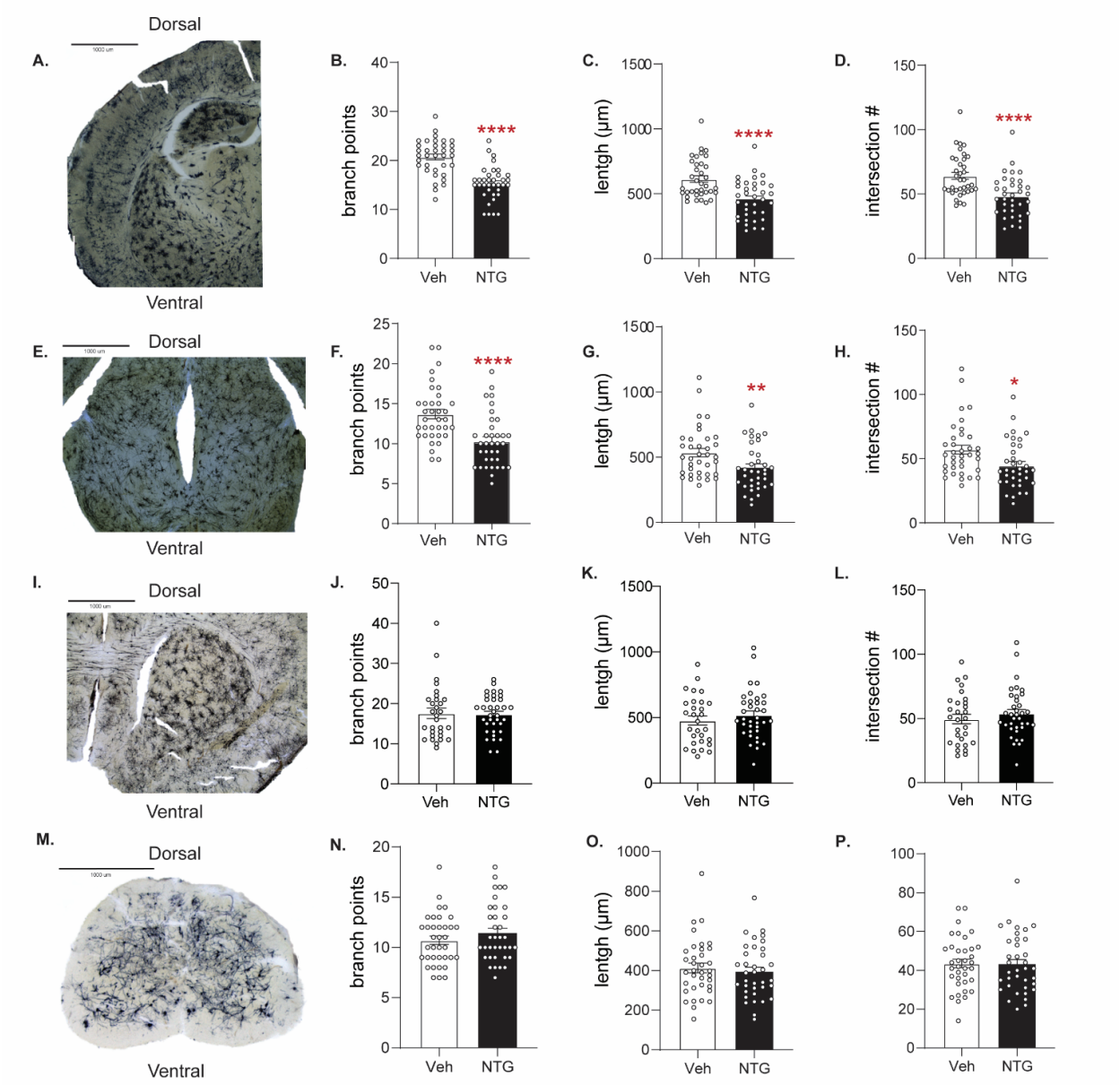

Figure 7. Chronic NTG treatment causes cytoarchitectural changes in the somatosensory cortex (SCx) and periaqueductal grey (PAG) but not the nucleus accumbens (Nac) or lumbar spinal cord (LSC) Mice were treated chronic intermittently with either NTG or Vehicle for 9 days and on day 10 tissue was collected for Golgi staining. (A,E,I,M) Representative image showing Golgi staining in the SCx ,PAG, Nac, and LSC. (B,F,J,N) Neurons were analyzed for number of branch points, (C,G,K,O) combined neuronal length and (D,H,L,P) total number of intersections using Sholl analysis. In the SCx (A-D) and PAG (E-H). chronic NTG resulted in significantly fewer branches, neurite length, and interactions compared to vehicle treated controls; unpaired t-test. *p<0.05, ** p<0.01, ****p<.0001. In the Nac (I-L) and LSC (M-P), chronic NTG did not produce significant changes in the number of branch points, neurite length, or interactions compared to vehicle controls. n=6 mice/group, 6 neurons/mouse.

2.3.2 HDAC6 inhibition increases acetylated α-tubulin and neuronal cytoarchitectural complexity

We hypothesized that if we could restore migraine-compromised cytoarchitectural complexity, that this might also relieve cephalic pain. Recent studies indicate that increased tubulin acetylation facilitates microtubule flexibility and prevents breakage [608, 615, 677]. Thus, we hypothesized that inhibiting HDAC6 to promote microtubule stability may restore the neuronal complexity observed following chronic NTG. We tested the selective HDAC6 inhibitor, ACY-738, in the chronic NTG model. Mice were treated chronically with NTG or VEH for 9 days. On day 10, mice were injected with ACY-738, after 4h, tissue was processed and analyzed by western blot. ACY-738 treatment resulted in a significant increase in the ratio of acetylated α-tubulin to total tubulin in three key migraine processing regions; the trigeminal ganglia (TG), TNC, and SCx (Figure 8). A separate group of mice were analyzed by neuronal tracing in the TNC, 4h post-ACY-738 (Figure 9A). Again, chronic NTG treatment caused a decrease in branch points (Figure 9B), combined neurite length (Figure 9C), and number of intersections (Figure 9D-F). In contrast, treatment with ACY-738 led to a significant increase in these measures in both chronic vehicle and NTG groups (Figure 9A-F).

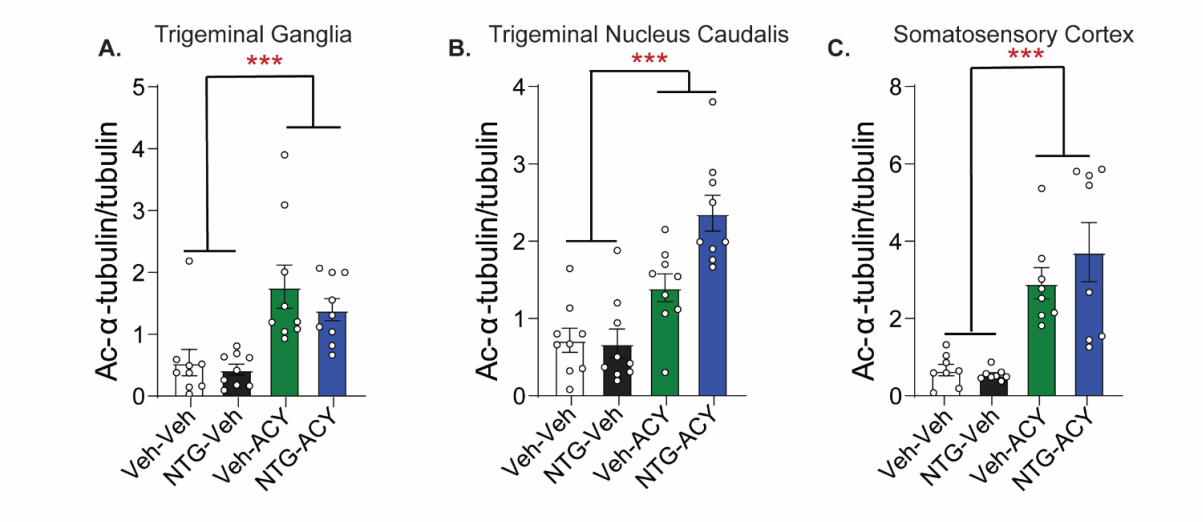

Figure 8. ACY-738 increased levels of acetylated α-tubulin Mice were chronically treated with NTG or vehicle for 9 days. On day 10 mice were injected with ACY-738 (50 mg/kg IP) or vehicle (5% DMSO, 0.9% NaCl, IP) and tissue was collected 4h later. (A) Trigeminal ganglia (B) somatosensory cortex, and (C) trigeminal nucleus caudalis of mice treated with ACY-738 showed a significant increase in the ratio of acetylated α-tubulin/total α-tubulin compared to the vehicle treated mice ***p<.0001 n=8-9/group effect drug treatment two-way ANOVA.

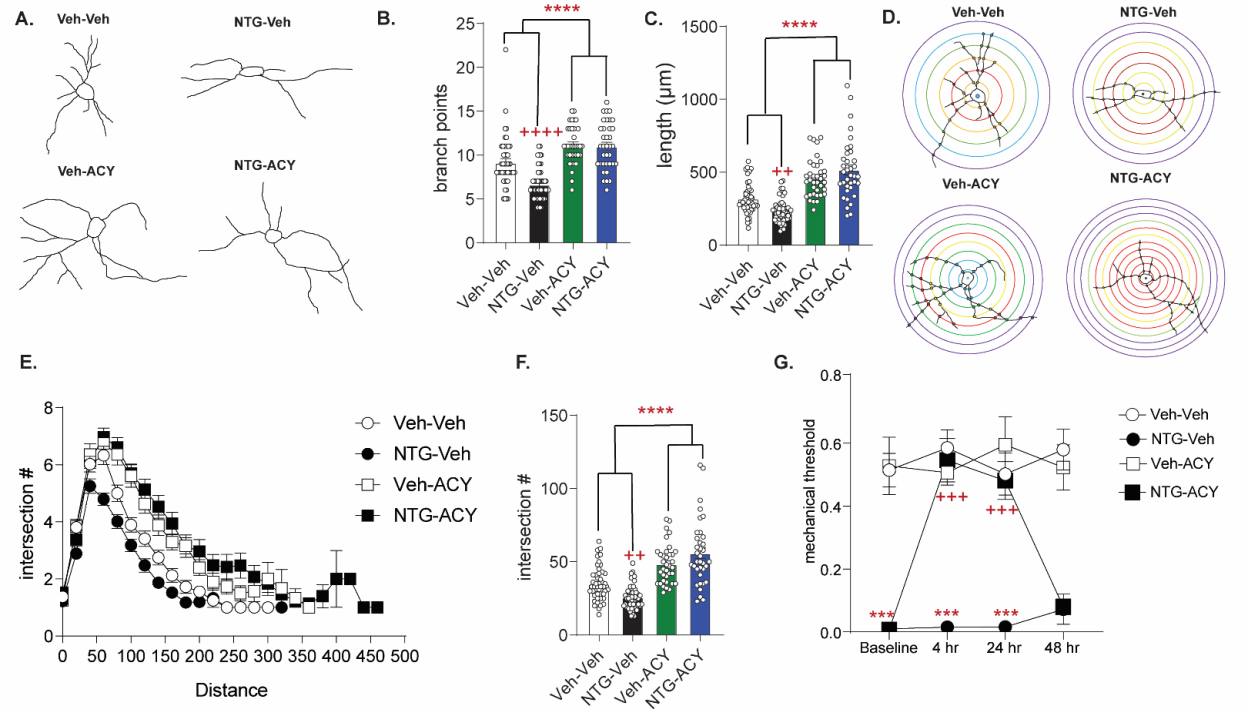

Figure 9. Treatment with HDAC6 inhibitor restores blunted neuronal complexity and inhibits migraine-associated pain. (A) Representative neuron tracing of mice that were chronically treated with vehicle or NTG (10 mg/kg, IP) every other day for 9 days and on day 10 were injected with ACY-738 (50 mg/kg, IP) or vehicle (5 % DMSO

in 0.9% NaCl, IP) and sacrificed 4h later for Golgi staining. (B) The number of branch points/neuron were significantly decreased following NTG treatment (NTG-Vehicle); while ACY-738 treated mice showed increased branching irrespective of pretreatment. $p<0.0001$ effect of chronic treatment, drug treatment, and interaction, two-way ANOVA and Holm-Sidak post hoc analysis. ****$p<0.001$ effect of ACY-738; ++++$p<.001$ effect of NTG. (C) NTG also decreased total pixel length/neuron, while ACY-738 treatment increased it. $p<0.0001$ effect of NTG treatment, drug treatment, and interaction, two-way ANOVA and Holm-Sidak post hoc analysis. ****$p<0.0001$ effect of ACY-738; ++$p<0.01$ effect of NTG. (D) Representative Sholl analysis image of neuronal complexity in the four groups. (E) Sholl analysis broken up by 20 voxel distances reveal differences between NTG and ACY-738 treatment. (F) Chronic NTG results in significantly fewer total interactions relative to vehicle treatment. ACY-738 increases total interactions in both vehicle and NTG groups. $p<0.0001$ effect chronic treatment, drug treatment, and interaction, two-way ANOVA and Holm-Sidak post hoc analysis. ****$p<0.001$ effect of ACY-738; ++$p<0.01$ effect of NTG. For all analysis n=6 mice/group, 6-8 neurons per mouse. (G) C57Bl6/J mice underwent chronic intermittent NTG/Veh treatment for 9 days, on day 10 basal mechanical thresholds were assessed, and mice were subsequently injected with ACY-738 (50 mg/kg IP) or Vehicle and tested 4, 24, or 48 h later. Chronic NTG treatment caused severe cephalic allodynia (Baselines); which was significantly inhibited by ACY- 738 at 4h and 24h post-injection. $p<.001$ drug, time, and interaction, two-way RM ANOVA, Holm- Sidak post hoc analysis, ***$p<.001$ as compared to Veh-Veh treated mice; +++$p<.001$ as compared to the NTG-Veh treated mice; n=12 mice/group.

2.3.3 HDAC6 inhibition reverses NTG-induced allodynia

Next, we determined if this restored neuronal complexity would affect behavioral outcomes. Mice were treated with chronic intermittent NTG or vehicle for 9 days. On day 10, baseline cephalic allodynia was observed in mice treated chronically with NTG but not vehicle (Figure 9G, baselines). Mice were then injected with ACY-738 or vehicle and tested 4, 24, or 48h later. ACY-738 significantly reversed cephalic allodynia in NTG treated mice for up to 24h post-injection. Mechanical responses in vehicle-ACY-738 treated animals were unaffected (Figure 9G), which suggests that HDAC6-augmented neuronal complexity in a pain-free animal does not alter endogenous pain processing. Interestingly, the half-life of ACY-738 is only 12 minutes [678], thus short-term inhibition of HDAC6 still produced long-lasting behavioral and cytoarchitectural changes.

We confirmed that this behavioral effect was due specifically to changes in HDAC6 inhibition. We first tested two pan-HDAC inhibitors: the well characterized inhibitor trichostatin A (TSA, Figure 10A); and a novel brain-penetrant pan-HDAC inhibitor, RN-73 [679] (Figure 10B). Both significantly reversed chronic NTG-induced allodynia, albeit for a much shorter duration than ACY-738. In contrast, when we tested the Class I, HDAC1 and 2 selective inhibitor, ASV-85, we did not observe any change in NTG-induced chronic allodynia relative to vehicle controls (Figure 10C). These data further support our finding that chronic migraine-associated pain can be blocked, specifically, by HDCA6 inhibition, and that this effect is due to increased acetylation of cytosolic HDAC6 substrates, rather than histone acetylation in the cell nucleus.

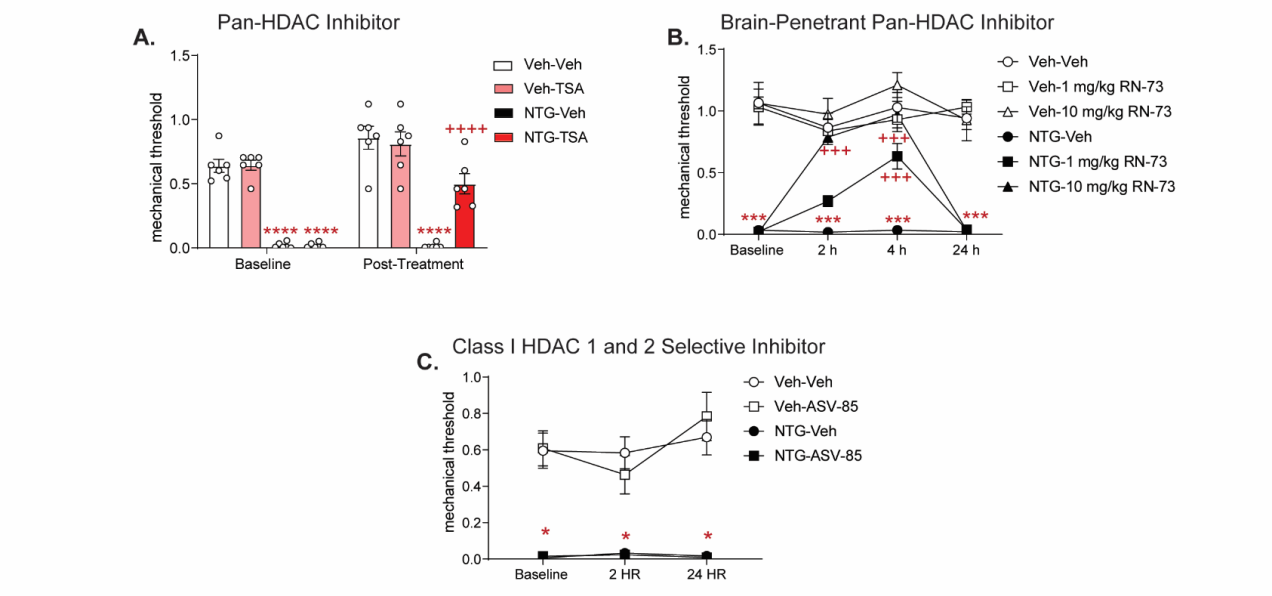

Figure 10. Pan-HDAC inhibitors, but not Class I-selective HDAC inhibitor block chronic migraine-associated pain Male and female C57BL6J mice were treated with chronic intermittent NTG (10 mg/kg IP) or Vehicle for 9 days. On day 10 mice were subsequently tested for baseline responses (Baseline) and then injected with various HDAC inhibitors. Baselines were always lower for NTG-treated mice demonstrating chronic cephalic allodynia. Separate groups of mice were tested for each drug. (A) Mice were treated with the pan-HDAC6 inhibitor Trichostatin A (TSA, 2 mg/kg IP) or Vehicle

(20% DMSO in 0.01 M PBS IP) and subsequently tested 2 h later. TSA significantly inhibited chronic cephalic allodynia. $p<0.05$ effect of treatment, drug, time, and interaction, Three-way ANOVA and Holm-Sidak post hoc analysis. ****$p<0.0001$ relative to Vehicle-Vehicle, ++++$p<0.0001$ relative to NTG-Vehicle at same time point n=6-10/group (B) Mice were treated with the novel brain-penetrant pan-HDAC inhibitor, RN-73 at 1 or 10 mg/kg (IP) or vehicle (10% DMSO, 10%Tween-80, and 0.9% NaCl). Mice were subsequently tested in the hind-paw region 2, 4, and 24 h post treatment. RN-73 at the 10 mg/kg dose had a significant effect at the 2 and 4 h time point, and the 1 mg/kg dose had a significant effect only at the 4 h time point compared to the NTG-Veh group. Neither dose of RN-73 produced any effect 24 h after treatment compared to NTG-Veh. Three-way RM ANOVA and Holm-Sidak post hoc analysis, $p<0.05$ effect of treatment, drug, time and interaction, ****$p<0.001$ relative to Vehicle-Vehicle at matching time point, +++$p<.001$ NTG- RN-73 relative to NTG-Vehicle at same time point, n=8/group. (C) Following chronic NTG/VEH treatment, mice were treated with the Class I specific HDAC inhibitor, ASV-85 (1 mg/kg IP) or Vehicle (6.25% DMSO, 5.625% Tween- 80, and 0.9% NaCl, IP) and had subsequent mechanical thresholds taken at 2 and 24 h post-treatment. ASV-85 failed to inhibit NTG-induced pain. NTG-ASV-85 and NTG-Veh treated mice both were significantly different than the Vehicle control groups at both 2 and 24 h time points, Three-way ANOVA and Turkey post hoc analysis $p<.05$ effect of treatment, *$p<.05$ NTG treated mice compared to Vehicle treated mice at same time point.

2.3.4 HDAC6 mRNA and protein is found ubiquitously in key migraine processing regions

HDAC6 expression is enriched in certain brain regions, such as the dorsal raphe [645] and, to the best of our knowledge, HDAC6 expression in head pain processing regions is not well characterized. In situ hybridization using RNAScope and immunohistochemical analysis (Figure 11A,B) revealed abundant expression of HDAC6 transcripts in TG, TNC, and SCx. Gene expression analysis revealed that, of these regions, chronic NTG treatment increased HDAC6 expression in the TG (Figure 11C), which are the first order cells regulating cephalic pain processing. Thus, HDAC6 is expressed and regulated dynamically in regions that are critical for migraine-associated pain processing.

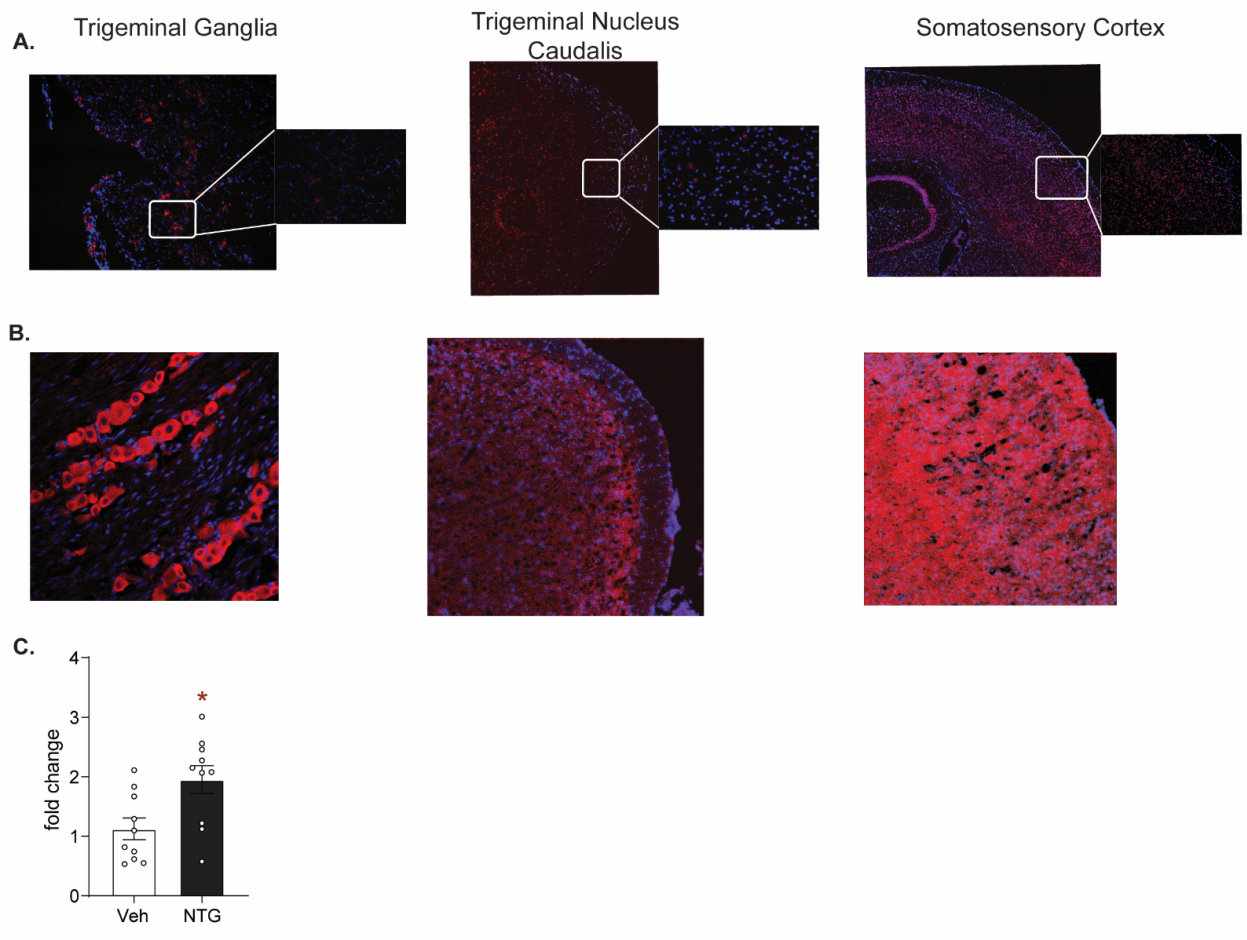

Figure 11. HDAC6 is expressed in migraine-processing regions and is dynamically regulated (A.) In situ hybridization by RNAScope reveals abundant HDAC6 transcripts (red) in trigeminal ganglia, trigeminal nucleus caudalis, and somatosensory cortex (B.) Immunohistochemical staining also reveals HDAC6 expression in these regions. (C.) Mice were treated chronically with vehicle or NTG for 9 days and tissue was analyzed for HDAC6 gene expression on day 10. HDAC6 transcript levels were significantly increased following NTG treatment in the TG, unpaired t-test, *p<0.05, n=10 mice/group.

2.3.5 HDAC6 inhibitor results in reduced CSD events

CSD is an electrophysiological property thought to underlie migraine aura. It is mechanistically and etiologically distinct from the NTG model of migraine pain, and reduction of CSD events is a feature of many migraine preventives [680]. Thus, we

examined whether CSD propagation was also affected by HDAC6 inhibition. Briefly, the skull was thinned in an anesthetized animal to reveal the dural vasculature and cortex underneath (Figure 12A). Two burr holes were made, and the more rostral was used to continuously drip KCl onto the dura to induce CSD, while local field potentials (LFPs) were recorded from the caudal burr hole. The somatosensory/barrel cortex was targeted, as it is more sensitive to CSD induction [681]. Throughout the 1h recording, CSDs were identified by visual shifts in light and sharp decreases in the LFP (Figure 12B-C). Pretreatment with ACY-738 resulted in significantly fewer CSD events relative to vehicle controls (Figure 12D), indicating that HDAC6 inhibition also effectively blocks this separate migraine mechanism.

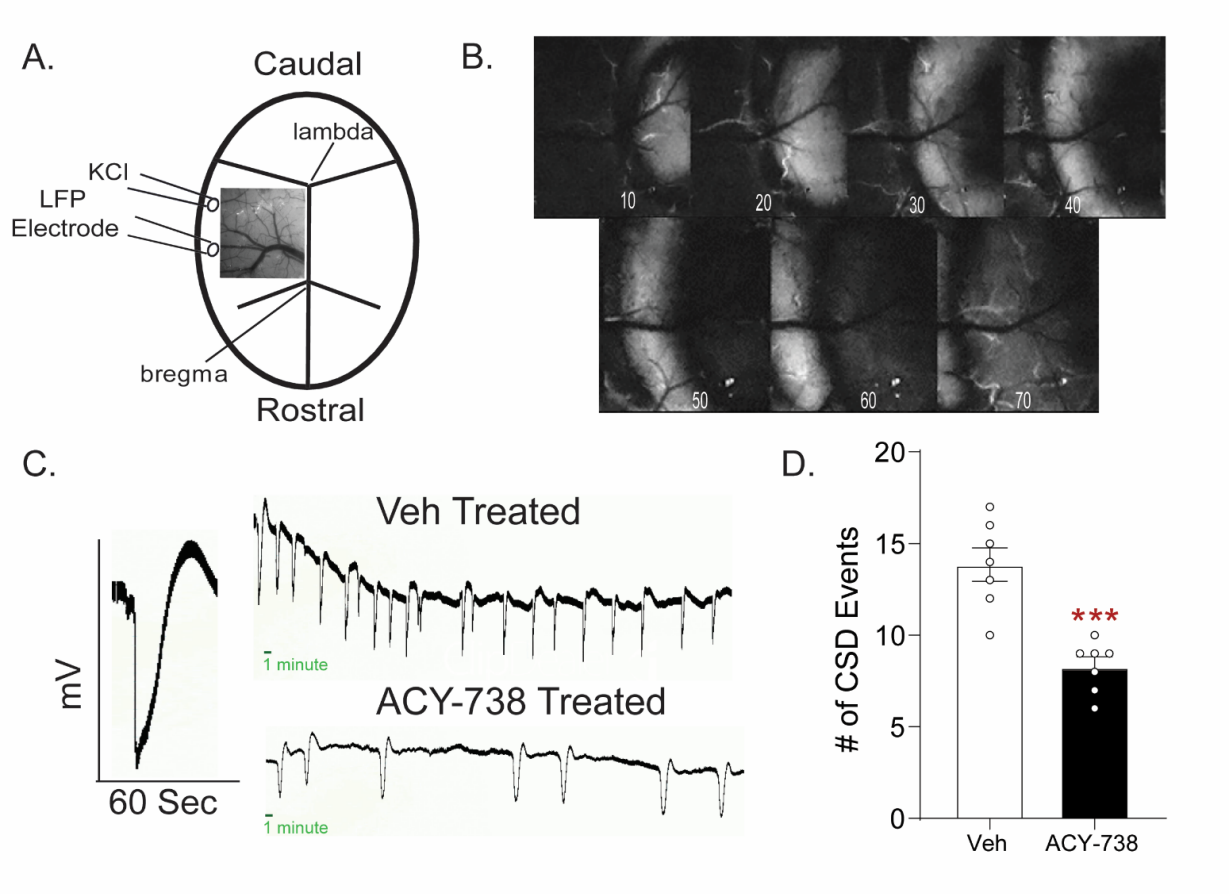

Figure 12. ACY-738 reduces cortical spreading depression events (A) Schematic of the thinned skull preparation used to visualize CSD and placement of KCl infusion and LFP recording. (B) Image sequence shows the wave of change in reflectance associated with a CSD event. (C) Representative tracing of a single CSD event of voltage change versus time. Representative line tracing of CSDs in a Vehicle (Top) vs. ACY-738 (Bottom) treated mouse over a 1h period. (D) Animals pretreated with ACY-738 (50 mg/kg IP) 4h before CSD recordings began showed a significant reduction in the average number of CSD events recorded over an hour. Unpaired t-test. ***p<0.001 n=7/group.

2.3.6 CSD results in decreased neuronal complexity in the somatosensory cortex that is restored by HDAC6 inhibition

We next examined the neuronal complexity of pyramidal neurons within the somatosensory cortex following CSD induction. Sham mice that underwent anesthesia and surgery, but did not receive KCl, were used as controls. Mice were pretreated with ACY-738 or vehicle, underwent CSD or sham procedure, and were immediately sacrificed for Golgi staining of the SCx (Figure 13A-B). In the somatosensory cortex, CSD evoked a significant decrease in branch points (Figure 13C) and total length of neurons (Figure 13D). In addition, CSD also resulted in a significant reduction in the number of branches in neurons of the TNC (Figure 14), a region that is known to be activated following CSD events [180]. In contrast, ACY-738 increased neuronal complexity in the cortex in both sham and CSD groups. Sholl analysis demonstrated a dramatic decrease in neuronal complexity after CSD, while ACY-738 treatment had the opposite effect (Figure 13E-G). These results demonstrate that decreased neuronal complexity is also observed in a second, mechanistically distinct model of migraine, and that HDAC6 inhibition can prevent these changes in neuronal cytoarchitecture and decrease CSD events.

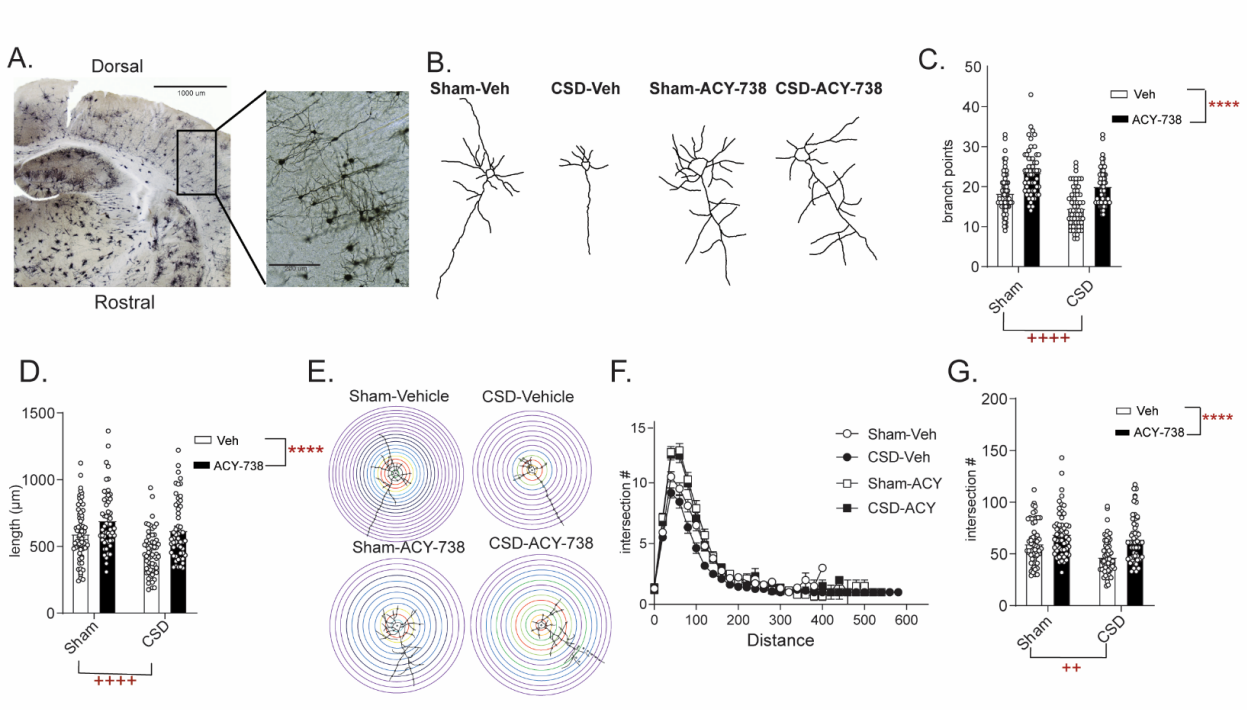

Figure 13. CSD induces decreased neuronal complexity that is prevented by treatment with ACY-738 (A) Representative image of Golgi stained sensory/barrel cortex at 4x (left) and 20x (right). (B) Representative neuronal tracing for mice that underwent pretreatment with Vehicle or ACY-738 and underwent Sham or CSD procedures. (C) Analysis of number of branch points/neuron reveal a significant effect of CSD and of ACY-738. Two-way ANOVA with Holm-Sidak post hoc analysis. ++++p<0.0001 effect of CSD; ****p<0.0001 effect of ACY-738. (D) Neurons were further analyzed for pixel length per neuron and CSD significantly decreased overall length, while ACY-738 significantly increased length. Two-way ANOVA and Holm-Sidak post hoc analysis. ++++ p<0.0001 effect of CSD, **** p<0.0001 effect of ACY-738 (E) Representative Sholl analysis plot of a neuron demonstrating CSD reduces and ACY-738 increases neuronal complexity. (F) Sholl analysis broken up by 20 voxel distances showing differences between groups. (G) Sholl analysis revealed a significant decrease in total intersections after CSD compared to Sham mice; and pretreatment with ACY-738 increased total intersections compared to vehicle treated groups. Two-way ANOVA and Holm-Sidak post hoc analysis ++p<0.01 effect of CSD, **** p<0.0001 effect of ACY-738. n=6 mice/group, 9 neurons/mouse

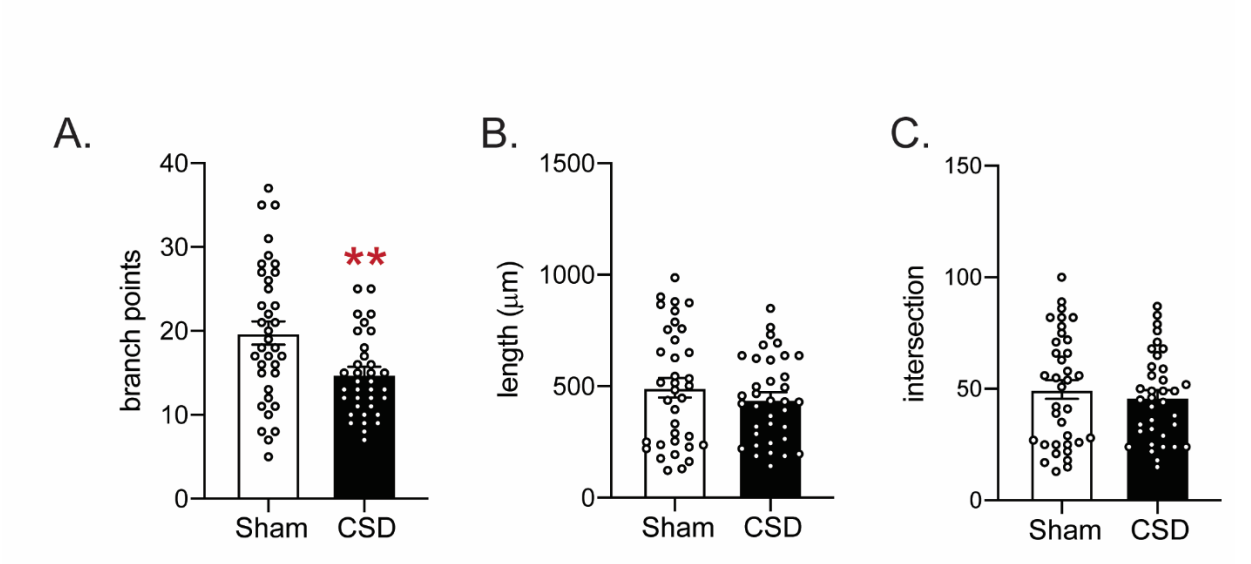

Figure 14. Cortical spreading depression (CSD) results in blunted neuronal complexity in the trigeminal nucleus caudalis (TNC) Mice underwent the previously described CSD procedure or a sham surgery in which no KCl was dripped onto the dura. Mice were sacrificed immediately after an hour of recording. (A) Neurons were analyzed for number of branch points, and CSD resulted in significantly fewer branch points relative to sham controls. (B) Neurons were further analyzed for combined neuronal length and no significant difference was observed. (C) Total number of intersections were analyzed using Sholl analysis and there was no significant difference. Unpaired t-test ** $p<0.01$ n=6/group n=6 mice/group.

2.3.7 CGRP receptor blockade reverses NTG-induced chronic allodynia and cytoarchitectural alterations

We next sought to determine if migraine-selective therapies could influence neuronal cytoarchitecture; and we tested the small molecule CGRP receptor antagonist, olcegepant, in the chronic NTG model [682]. Mice developed a sustained allodynia to repeated NTG treatment (Figure 15A). On day 10, 24 hours after the final NTG injection, baseline mechanical responses were assessed and mice were treated with olcegepant or vehicle. Olcegepant significantly inhibited NTG-induced cephalic allodynia (Figure 15B), similar to previously published reports [683]. Subsequent golgi analysis of

TNC revealed cytoarchitectural alterations in this cohort of animals (Figure 15C). As was observed previously, chronic NTG treatment decreased the number of branch points (Figure 15D), combined neurite length (Figure 15E), and number of intersections using Sholl analysis (Figure 15F-H). Interestingly, olcegepant treatment restored neuronal complexity induced by chronic NTG, but had no effect in chronic vehicle treated mice (Figure 15C-H). These data demonstrate that altered neuronal complexity could be a feature of chronic migraine, and that restoration of these changes may be a marker of effective migraine treatment.

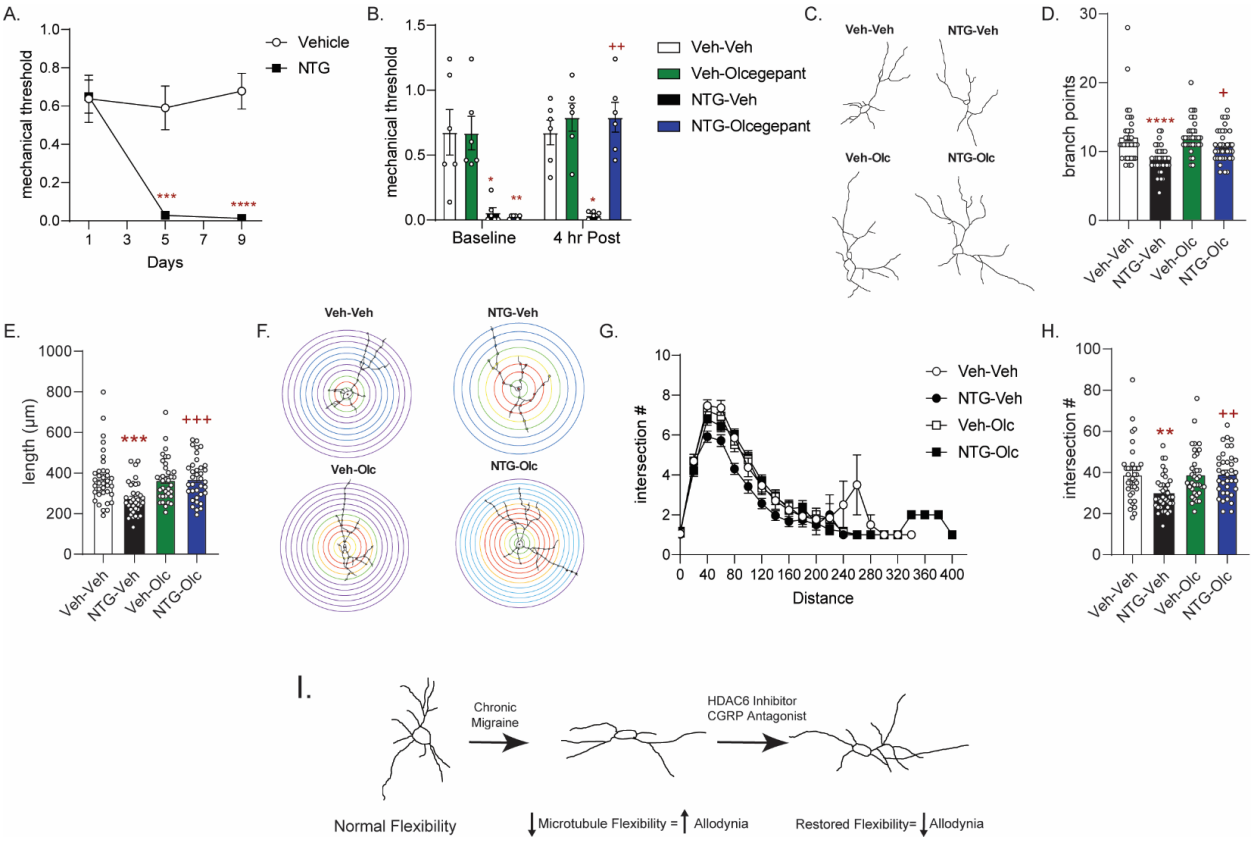

Figure 15. Treatment with the CGRP receptor antagonist, olcegepant, blocks NTG-induced chronic allodynia and reverses blunted cytoarchitecture (A) Periorbital mechanical thresholds were accessed prior to Vehicle/NTG administration on days 1, 5 and 9. NTG produced cephalic allodynia; $p<0.001$ effect of drug, time, and interaction, two-way RM ANOVA and Holm-Sidak post hoc analysis. ***$p<0.001$, ****$p<0.0001$, relative to vehicle on same day 1 n=12/group. (B) Mice were treated with olcegepant (0.1 mg/ml, IP) or vehicle (0.9% NaCl) and tested 4 hours later. Olcegepant significantly

reversed chronic cephalic allodynia. p <0.05 effect of treatment, drug, time, and interaction. Three-way ANOVA and Holm-Sidak post hoc analysis. *p<0.05, **p<0.01 relative to Vehicle-Vehicle, ++p<0.01 relative to NTG-Vehicle at the same time point n=6/group. (C) Representative neuron tracing of mice that were chronically treated with Vehicle or NTG (10 mg/kg, IP) every other day for 9 days and on day 10 were treated with olcegepant or vehicle and sacrificed 4 hours later for Golgi staining. (D) The number of branch points/neuron were significantly decreased following NTG treatment (NTG-Vehicle); while NTG-olcegepant treated mice showed increased branching compared to the NTG-Vehicle treatment alone. p<0.05 effect of chronic treatment, drug treatment, and interaction, two-way ANOVA and Holm-Sidak post hoc analysis. ****p<0.0001 NTG-Veh compared to Veh-Veh, +p<0.05 NTG-olcegepant compared to NTG-Veh. (E) NTG also decreased total length/neuron; while NTG-olcegepant treated mice showed restored length compared to the NTG-Vehicle treatment alone p<0.01 effect of NTG treatment, drug treatment, and interaction, two-way ANOVA and Holm-Sidak post hoc analysis. ***p<0.001 NTG-Veh compared to Veh-Veh, +++p<0.001 NTG-olcegepant compared to NTG-Veh. (F) Representative Sholl analysis image of neuronal complexity in the four groups. (G) Sholl analysis broken up by 20 voxel distances reveal differences between NTG-Veh treatment relative to the other groups. (H) NTG-Veh results in significantly fewer total interactions relative to the Veh-Veh treatment and NTG-olcegepant mice had significantly more total interactions compared to NTG-Veh p<0.05 effect chronic treatment, drug treatment, and interaction, two-way ANOVA and Holm-Sidak post hoc analysis. **p<0.01 NTG-Veh compared to Veh-Veh; ++p<0.01 NTG-Veh compared to NTG-olcegepant. For all analysis n=6 mice/group, 6 neurons per mouse. (I) Schematic summary of findings. Endogenously there is a balance of acetylated and deacetylated α-tubulin which regulates optimal neuronal complexity. In the case of chronic migraine, there is a disbalance resulting in decreased neuronal complexity. HDAC6 or CGRP receptor inhibition restores tubulin dynamics and neuronal complexity and correspondingly decreases chronic migraine-associated symptoms.

2.4 Discussion

Our results indicate that in models of chronic migraine there is a dysregulation of cellular plasticity resulting in decreased neuronal complexity. We found that following the establishment of chronic cephalic allodynia in the NTG model of migraine-associated pain, there was a decrease in the number of branch points, combined neurite length, and interactions of neurons within the TNC, PAG, and somatosensory cortex. With this newly discovered phenomenon we sought to mitigate this decrease through inhibition of HDAC6, which we found to restore neuronal complexity and

correspondingly, inhibit allodynia. We found that the cytoarchitectural changes were not just NTG induced but were also prominent following CSD. Reduction in neuronal complexity was also observed in this model of migraine aura, and again HDAC6 inhibition restored neuronal plasticity and decreased the number of CSD events. The latter effect is a hallmark of migraine preventive drugs. Furthermore, we found that a migraine specific treatment, CGRP receptor inhibition, also restored cytoarchitectural changes. Together our results demonstrate a novel mechanism of chronic migraine and reveal HDAC6 as a novel therapeutic target for this disorder (Figure 15I).

We used the NTG model in this study as it is a well-validated model of migraine [684]. NTG is a known human migraine trigger and has been used as a human experimental model of migraine [685]. Similar to humans, NTG produces a delayed allodynia in mice [686], as well as photophobia and altered meningeal blood flow [110, 687]. Chronic intermittent administration of NTG is used to model chronic migraine [683, 688-691]. Although, compared to humans, much higher doses of NTG are required in rodents, the allodynia induced in mice is inhibited by migraine-specific medications, such as sumatriptan [114, 686, 688] and CGRP targeting drugs [683], as well as the migraine preventives propranolol and topiramate [115, 692]. Further, mice with human migraine gene mutations are more sensitive to NTG [85]. Systemic administration of NTG also causes cellular activation throughout nociceptive pathways including in the TNC and brainstem [116, 692-694]. Correspondingly, we also observed changes in neuronal complexity in the TNC, as well as in the PAG and somatosensory cortex, regions heavily involved in pain processing. Alterations in these regions could contribute to allodynia or interictal sensitivity observed in chronic migraine patients. We did not

observe any alterations in neuronal complexity in the nucleus accumbens, which is commonly associated with processing of reward and motivation. RNA-Seq experiments from our lab have also showed that the nucleus accumbens shows very different responses to chronic NTG relative to parts of the trigeminovascluar system [695]. Importantly, we observed no change in neuronal cytoarchitecture in the lumbar spinal cord, a region involved in peripheral but not cephalic pain processing. These findings suggest that neuronal cytoarchitectural changes may be a common maladaptive mechanism driving migraine chronicity.

While these results are the first of their kind to demonstrate cytoarchitectural changes in models of chronic migraine, alterations in neuronal plasticity have been described previously in models of neuropathic pain. A mouse model of chronic constriction injury of the sciatic nerve reduced neurite length in GABA neurons within lamina II of the spinal cord [496]. Another group observed that following spared nerve injury, there were decreases in the number of branches and neurite length of hippocampal neurons but increases in spinal dorsal horn neurons [696]. Together these studies, along with those reported here, suggest adaptations of the central nervous system to chronic pain culminate in alteration of the neuronal cytoarchitecture.

We also observed decreased neuronal complexity in CSD, a migraine model mechanistically distinct from NTG. CSD is thought to underlie migraine aura and reflects changes in cortical excitability associated with the migraine brain state [185, 697]. Previous studies also support the idea of cytoarchitectural alterations accompanying spreading depression/depolarization events, and have mainly focused on dendritic morphology. Neuronal swelling [169] and dendritic beading [698] have been observed

following spreading depression events. CSD also resulted in alterations in dendritic structure [169, 699] and volumetric changes [169]. Further, Steffensen et al. also showed decreased microtubule presence in dendrites following spreading depression in hippocampal slices, again implying alterations in cytoarchictural dynamics [698]. Microtubules have been shown to disassemble in response to increased intracellular calcium [700]; and the increased calcium influx following spreading depolarization [701] may facilitate this breakdown. Tubulin acetylation is associated with increased flexibility and stability of microtubules [616]. One way in which HDAC6 inhibitors could attenuate CSD is through increased tubulin acetylation, thus counteracting cytoarchitectural changes produced by CSD events. Furthermore, multiple reports indicate that CSD can activate the trigeminovascular complex, and evoke cephalic allodynia in rodents [205, 702-706]. We also observed decreased neurite branching in the TNC following CSD, further linking CSD to head pain processing. Combined, these data suggest that CSD has an impact on neuronal morphology that contributes to migraine pathophysiology.

Proper acetylation of microtubules is necessary for a variety of cellular functions including appropriate neurite branching [666], cell response to injury [666], mitochondrial movement [667, 668], anchoring of kinesin for microtubule mediated transport [707] and regulation of synaptic G protein signaling [670, 708, 709]. Knockout of the α-tubulin acetylating enzyme, α-TAT1, in sensory neurons results in profound deficits in touch [619]. Furthermore, Charcot-Marie-Tooth (CMT) disease is a hereditary axonopathy that affects peripheral nerves resulting in damage to both sensory and motor function. Mouse models of CMT reveal deficits in mitochondrial transport in the dorsal root ganglia (DRG) due to reduced acetylation of α-tubulin, while HDAC6

inhibition ameliorates CMT-associated symptoms [710, 711]. HDAC6 inhibitors were also found to be effective in models of chemotherapy induced neuropathic pain [657, 658]. In mice, HDAC6 inhibitors effectively reduced chemotherapy-induced allodynia following treatment with vincristine [657] or cisplatin [658]. In addition, both groups found that chemotherapy blunted mitochondrial transport in sensory neurons, an effect that was restored by HDAC6 inhibition. A recent study also found that HDAC6 inhibition was effective in models of inflammatory and neuropathic pain [659]. Together with our study, these data further demonstrate the importance of HDAC6 in regulating acetylated α-tubulin and how tubulin acetylation state can regulate sensory dysfunction; thus supporting the potential of HDAC6 inhibitors for the treatment for chronic pain conditions.

We observed that chronic migraine resulted in decreased neuronal complexity and therefore focused on the role of HDAC6 in tubulin acetylation and cytoarchitectural dynamics [608, 666]. However, HDAC6 also regulates Hsp90 and cortactin [646]. HDAC6 deacetylates Hsp90, which plays an important role in glucocorticoid receptor maturation and adaptation to stress [638]. A previous study using a model of social defeat stress showed that HDAC6 knockout or inhibition decreased Hsp90-glucocorticoid receptor interaction and subsequent glucocorticoid signaling, thus encouraging resilience [645]. In line with these findings, HDAC6 inhibitors also show antidepressant-like effects [669, 678], and membrane-associated acetylated tubulin is decreased in humans with depression [709]. Changes in neuronal complexity may serve as a common mechanism underlying the chronicity of psychiatric and neurological diseases. Further, HDAC6 also directly deacetylates cortactin, a protein that regulates

actin dependent cell motility [648]. Future studies will focus on identifying the precise mechanism by which HDAC6 impacts neuronal complexity in the chronic migraine state.

We investigated whether a current migraine treatment strategy, CGRP receptor inhibition, could ameliorate the cytoarchitectural changes induced by chronic migraine-associated pain. We found a good correlation between the anti-allodynic effects of olcegepant and its ability to restore neuronal complexity in the chronic NTG model. In contrast to ACY-738, olcegepant had no effect on vehicle treated mice and only recovered but did not increase neuronal branching, length, or intersections in the NTG treated group. These results, along with the finding that NTG did not alter complexity in the lumbar spinal cord or nucleus accumbens, help to confirm that the changes in neuronal cytoarchitecture following NTG are associated with migraine mechanisms. In addition, this study suggests a possible mechanism in which recovered neuronal complexity is a marker of effective migraine medication. Our findings also open up research on the signaling mechanisms that link migraine relief with tubulin dynamics. One possibility is that decreased neuronal cytoarchitecture is driven through a CGRP related mechanism and direct antagonism of the CGRP receptor reverses these alterations. NTG treatment was previously found to upregulate CGRP in the blood, TNC, and dura mater demonstrating a direct link between NTG and CGRP [712-714]. Future studies will explore the relationship between CGRP and other migraine therapies with neuronal cytoarchitecture.

Our results reveal a novel cytoarchitectural mechanism underlying chronic migraine, and imply that this disorder may result from attenuation of neurite outgrowth and branching. This blunted complexity would consequently decrease neurnoal

plasiticity and the ability of the cell to respond to its environment. We propose that this decreased flexibility could also encourage the strengthening of maladaptive synapses and prevent the formation of potentially beneficial synapses. Human imagining studies reveal decreased cortical thickness [715], and grey matter reductions in the insula, anterior cingulate cortex, and amygdala of migraine patients [73]. Interestingly, a significant correlation was observed between gray matter reduction in anterior cingulate cortex and frequency of migraine attacks [73]. These structual changes could reflect decreased neuronal complextity in combination with other factors.

We propose that strategies targeted towards pathways regulating neuronal cytoarchicture may be an effective approach for the treatment of chronic migraine. Although HDAC6 inhibition increased neuronal complexity in vehicle or sham animals, this did not affect normal pain processing or cortical excitability, respectively. Additionally, constitutive knockout of HDAC6 produces viable offspring with few phenotypic changes other than resilience to stress [650]. Together these results suggest that HDAC6 inhbitors may restore cellular adaptations induced by chronic disease states but may not otherwise affect healthy physiological function. Chronic migraine may result from attenuation of neurite outgrowth and branching and compounds that reverse this adaptation, such as HDAC6 inhibitors, may contribute to the migraine therapeutic armamentarium.

Chapter 3: Altered Neuronal Complexity in chronic nitroglycerin, cortical spreading depression, and complex regional pain syndrome

Chapter 3.1 Introduction

Chronic pain syndromes are debilitating conditions that affect approximately 20% of American adults, greatly impacting their quality of life [716]. The International Association for the Study of Pain (IASP) defines chronic pain as, “pain without apparent biological value that has persisted beyond the normal tissue healing time” [717]. Despite its wide prevalence, the pathophysiology that drives the transition of pain from an acute state to a chronic one is still not entirely known [718]. One possible mechanism implicated in many neuropsychiatric conditions, including chronic pain, is alterations in neuroplasticity [663]. Within the broad range of neuroplasticity, recent evidence has suggested the importance of cytoarchitectural alterations in the regulation of many psychiatric conditions [671, 709]. A key component of the cytoarchitecture is microtubules, which are in a constant state of dynamic instability [538]. Microtubules facilitate cellular responses to injury and regulate many neuronal functions, including neurite branching, axonal transport, and signaling [666, 667]. Importantly, alterations in neurite structure, including gross changes in neuronal complexity and dendritic spine density are observed in animal models of neuropathic pain disorders [491, 496]. Therefore, it is possible that alterations in neuronal cytoarchitecture may be fundamental for initiating and/or maintaining pain chronicity.

Chronic pain can encompass disorders effecting both central and peripheral processes [719]. An especially common centrally regulated pain disorder is chronic migraine. Chronic migraine affects up to 2% of the general population and is defined as having at least 15 headache days a month for a minimum of 3 months [7, 661]. While much has been recently learned about the pathophysiology responsible for the transition of migraine from an acute to chronic state the underlying mechanisms are still being explored [720]. A chronic migraine model, which uses the human migraine trigger nitroglycerin (NTG), as well as a model of cortical spreading depression (CSD) were recently shown to result in alterations in cytoarchitecture within key migraine regulating regions [721]. This, along with human research showing alterations in grey matter volume following chronic migraine further indicates a possible causal mechanism between headache chronicity and alterations in cytoarchitectural dynamics [722, 723]. Other chronic pain disorders have also been shown to have alterations in neuronal complexity in humans and animal models including, chronic regional pain syndrome (CRPS) [492, 493, 495, 724]. CRPS originally presents itself as an acute/peripheral disorder, but over time can transition to a chronic/centrally regulated pain disorder [725]. The acute phase is characterized by warmth of the affected skin, while in the chronic phase there is no peripheral limb warmth or inflammation, but there is chronic pain in humans and allodynia in animal models as well as debilitating cognitive deficits [726, 727]. As with chronic migraine much is still unknown about the transition from the acute to chronic CRPS [718]. Gaining a better understanding of the changes in the cytoarchitecture following chronic pain disorders could allow for the development of novel therapeutics that are affective in multiple pain conditions.

The aim of this study was to further investigate alterations that occur following chronic migraine and assess if similar alterations in cytoarchitecture are observed in a peripheral model of CRPS. We previously showed that there were neuronal cytoarchitectural differences in key migraine pain processing regions following the NTG model of chronic migraine [721]. These results revealed a novel mechanism responsible for the transition of migraine from an episodic to a chronic condition. We sought to build on these original findings by exploring other brain regions related to migraine and homeostatic dysregulation and determine how far reaching these cytoarchitectural changes were. In the NTG model of chronic migraine, we observed an increase in cytoarchitectural complexity within the thalamus. Additionally, we also investigated alterations in areas that are found to regulate the emotional and affective aspects of pain, specifically the central amygdala and the caudate putamen [728, 729]. However, we found that our chronic migraine model showed no significant changes in these regions. We further expanded on previous CSD results and found that within the periaqueductal gray (PAG) there is also decreased neuronal complexity, similar to the SCx and TNC (Chapter 2). We further investigated if another chronic pain model, CRPS, also resulted in cytoarchitectural alterations. Within the hippocampus we observed decreased neuronal complexity. However, there was an increase in the complexity of neurons within the PAG. These results suggest that altered neuronal complexity could be an important hallmark of the transition of pain from an acute to chronic state.

3.2 Materials and Methods

Animals: Experiments were performed on adult C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME. USA). The chronic migraine experiments used both male and female mice, while the cortical spreading depression work used onle females. All experiments performed with the chronic regional pain syndrome model used only male mice. All mice weighed between 20-30 grams for the length of the study. To ensure health, weight was recorded on each day for all experiments. Mice were group housed in a 12h-12h light-dark cycle, where the lights were turned on at 07:00 and then turned off at 19:00. Both food and water were available ad libitum. All experiments were conducted in a blinded fashion. The procedures for all studies were approved by the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety Committee, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines and the Animal Care Policies of the University of Illinois at Chicago. The results are reported according to Animal Research: reporting *In vivo* Experiments (ARRIVE). The complex regional pain syndrome experiments were done in collaboration with Dr. Vivianne Tawfik at Stanford University. The *in vivo* experiments modeling complex regional pain syndrome were done at Stanford University and in accordance of their animal care committees. No animals were seen to have adverse effects and all animals were included in the statistical analysis.

Chronic migraine sensory sensitivity testing: Different groups of animals were used for each experiment. The mice for the chronic migraine experiment were counter-balanced on the first day based upon their original basal threshold. Mice were tested in a behavior room, which was completely separated from the vivarium. The testing room had low light and low noise conditions. All tests were performed between 08:00-16:00. For

cephalic testing animals were habituated to the testing rack for 2 days prior to the original test day. The rack also contained 4 oz paper cups that the mice also habituated to. On subsequent test days mice were placed on the rack with the cups 20 minutes prior to that day's basal measurement. The up and down method was used to assess punctate mechanical stimuli [730]. Manual von Frey hair filaments with a bending force between 0.008 to 2 g were used in these experiments. A response in the cephalic measures was defined as repeated shaking, pawing at the face, or cowering from the filament following stimulation. The first filament used was 0.4 g and following no response a heavier filament (up) was used. Alternatively if there was a response a lighter filament (down) was used next. This up and down pattern was repeated for 4 filaments following the initial response.

Complex regional pain syndrome sensitivity testing: *In vivo* experiments and testing were conducted by Dr. Vivianne Tawfik's lab at Stanford University. Logarithmically increasing set of von Frey filaments were used in a range of 0.007 to 6.0 g. The filaments were applied to the plantar hind paw until a bend occurred. A positive response was a withdrawal from the filament within 4 seconds. The up and down method was also used for these experiments and a 50% withdrawal mechanical threshold were calculated for the mice.

Nitroglycerin model of chronic migraine: Nitroglycerin (NTG) was purchased at a concentration of 5 mg/ml, in 30% alcohol, 30% propylene glycol and water (American Reagent, NY, USA). For the chronic NTG experiment, the NTG was diluted prior to testing on each day with 0.9% saline to a concentration of 1 mg/ml and a dose of 10 mg/kg. The 0.9% saline served as the vehicle control. Mice were injected with NTG IP

every other day for 9 days i. On days 1, 5, and 9 mice were tested before and 2h following NTG injection.

Cortical Spreading Depression Model: The cortical spreading depression model used in this study, is based off previous work by Ayata [210, 229] that is commonly used to screen potential migraine therapeutics. For these studies only female mice were used and mice were randomly assigned to sham or CSD groups. The skulls of the mice were thinned to form a cortical window. For the surgery the mice were anesthetized with isoflurane induction 3-4%; maintenance 0.75 to 1.25%; in 67% N2 / 33% O2). Once adequate anesthetic level was assessed the mice were placed on a stereotaxic frame on a homoeothermic-heating pad. Life signs were monitored throughout the experiment including core temperature, non-peripheral oxygen saturation, heart rate, and respiratory rate (PhysioSuite; Kent Scientific Instruments, Torrington, CT, USA). To ensure proper anesthetic depth mice were frequently tested for tail and hind paw reactivity.

CSD events were verified using optical intrinsic imaging (OIS) and electrophysiological recordings as previously described [465]. Following anesthesia the skin was cleared from the skull and a rectangular region of ~2.5 x 3.3 mm2 (~0.5 mm from sagittal, and ~1.4 from coronal and lambdoid sutures) of the right parietal bone was thinned to transparency with a dental drill (Fine Science Tools, Inc., Foster City, CA, USA). Following successful window creation mineral oil was applied to the surface to improve transparency for video recording. A green LED (530 nm) was further used to illuminate the skull to aid in recording (1-UP; LED Supply, Randolph, VT, USA). Cortical surface reflectance detected by OIS was collected with a lens (HR Plan Apo 0.5 × WD 136)

through a 515LP emission filter on a Nikon SMZ 1500 stereomicroscope (Nikon Instruments, Melville, NY, USA). Images were acquired at 1–5 Hz using a high-sensitivity USB monochrome CCD (CCE-B013-U; Mightex, Pleasanton, CA, USA) with 4.65-micron square pixels and 1392 × 1040 pixel resolution.

Lateral to the window 2 burr holes were drilled. These burr holes were drilled deeper than the thinned window such that exposure to the dura was achieved, but not so deep as to damage the dura. Local field potentials were recorded using an electrode filled with saline that was inserted into the dorsal burr hole and subsequently attached to an amplifier. Placing a silver wire beneath the skin grounded the animals. Basal electrophysiological measurements were measured for 1h prior to KCl application to induce CSD. KCl (1M) was dripped into the rostral burr hole at a rate that ensured a continual pool, but not so much that excess spilled over onto the thinned skull. The pool of KCl was maintained for an hour of recording. Sham mice had the skull thinned and the burr holes drilled, but they did not receive KCl drip or have an electrode placed as punctate stimuli can produce a CSD itself. Following CSD recording mice were euthanized and brains were collected for Golgi staining.

Complex regional pain syndrome model: Dr. Vivianne Tawfik's lab at Stanford University performed the CRPS model. Mice were anesthetized using isflourane and had the closed right distal tibial fracture followed by casting [731]. The right hind limb was wrapped in gauze and a hemostat was used to fracture the distal tibia. The hind limb was wrapped using casting tape from the metatarsals to the spica formed around the abdomen. The cast was applied only to the plantar surface and a window was left to prevent constriction. 21 days following casting the casts were removed. Mice had a

basal measure prior to fracture, one 21 days after fracture (acute phase), and then at 7 weeks (chronic phase). Following completion of the 7th week the mice were sacrificed and their brains underwent the Golgi staining technique. Afterwards they were shipped to the University of Illinois at Chicago for tissue processing and analysis.

Golgi Staining: All Golgi staining was done using the FD Rapid Golgi Stain Kit (FD Neurotechonlogies). Chronic NTG mice were sacrificed on day 10 following anesthesia using isoflourane and then decapitation. CSD/Sham mice were similarly sacrificed following the hour of CSD recordings. CRPS animals were sacrificed 7 weeks after casting and were subsequently used in the same Golgi staining behavior. Brains were removed and then rinsed in distilled water. An impregnation solution of A and B was prepared in advance and brains were placed in this solution for 1 week in the dark. Following this they were placed into solution C for 72 hours. Brains were then flash frozen in 2-mehty butane and cut on a cryostat to 100 μm slices. The slices were then put on slides and stained using the kit procedure.

Neurite Tracing: After tissue processing all images were taken at 20x magnification and a Z-stack was created through different focal planes. FIJI program Simple Neurite Tracer was used to process al the neurons [674]. The software was also used to count the number of branch points, overall length, and Sholl analysis. Sholl analysis used the soma as the center point and 20 pixels as the consecutive circles.

Neuron Selection: The tracers were blinded to which group the images belonged. 6 to 8 relatively isolated neurons were randomly chosen per mouse. The neurons were fully impregnated with Golgi stain. An atlas and clear anatomical markers were used to take

images from the region of interest. Somatosensory cortex neurons were taken from layer IV of the primary somatosensory barrel cortex.

Statistical analysis: Sample sizes were calculated by power analysis and based on previous experiments. Data analysis was performed using GraphPad Prsim version 8.00 (Graphpad, San Diego, CA). The level of significance (α) for all tests was set to 0.05. Post hoc analyses were conducted using Holm-Sidak post hoc test. Post hoc analysis was only conducted when F values achieved significance of $p<0.05$. All values in text and in figures are reported as mean ± SEM.

3.3 Results

3.3.1 Chronic NTG treatment produces cytoarchitectural changes in pain relay circuitry

We used a previously established model of chronic migraine by treating male and female C57BL6J mice every other day for 9 days with NTG or vehicle (Figure 16A) [94]. We measured periorbital response to mechanical stimulation on days 1, 5, and 9 (Figure 16A). This model produced a significant decrease in the basal cephalic mechanical threshold within the NTG treatment group (Figure 16B). These data represent the development of chronic hypersensitivity, as is often seen in migraine patients [39]. Following chronic NTG/Veh treatment mice were sacrificed on day 10, 24 hours after the final treatment. The brains then underwent the Golgi staining procedure to investigate possible neuronal cytoarchitectural alterations. We chose to investigate the ventral posteromedial nucleus of the thalamus (VPM) as it has been implicated as a key player in regulating allodynia and sensitization from the trigeminal circuit (Figure

16C) [15, 39, 732, 733]. Examination of the number of branch points per neuron revealed a dramatic increase in the total number of branches in the NTG treated mice (Figure 16D). Interestingly, this stark increase in the number of branch points did not correlate with an increase in the overall length of neurons (Figure 16E) or change in the complexity as assessed through Sholl analysis (Figure 16F-G). While there was not a significant change in all measures, the increase of branching of neurons within the VPM are an interesting contrast to the previous findings of decreased branching and complexity in the trigeminal nucleus caudalis (TNC), ventrolateral periaqueductal gray (vlPAG), and the somatosensory cortex (SCx) following chronic NTG [721].

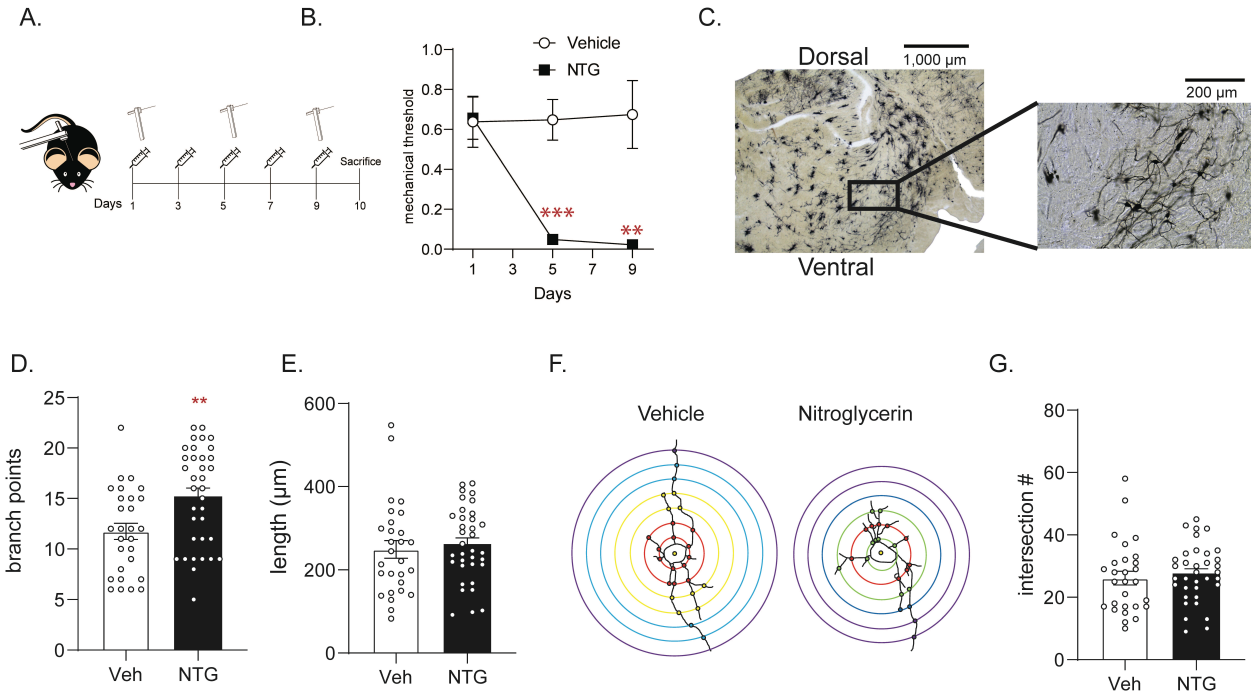

Figure 16. Chronic NTG Treatment resulted in cytoarchitectural changes within the ventral posteromedial nucleus

(A) Schematic of testing and injections, M&F C57Bl6/J mice were treated with chronic intermittent Nitroglycerin (10 mg/kg, IP; NTG) or Vehicle for 9 days and on day 10 tissue was collected for Golgi staining (B) Periorbital mechanical thresholds were accessed prior to Vehicle/NTG administration on days 1, 5 and 9. NTG produced severecephalic allodynia $p<0.001$ effect of drug, time, and interaction, two-way RM ANOVA and Holm-

Sidak post hoc analysis. ***p<0.001, **p<0.01 relative to vehicle on same day n=5-6/group (C) Representative image taken of the Golgi stained Ventral posteromedial nucleus of the thalamus at 4x(left) and 20x (right). (D) The number of branch points per neuron were significantly increased following treatment with chronic NTG. Unpaired t-test **p<0.01 (E) Total neuron length was also measured, but showed no significant change. Unpaired t-test. (F) Representative Sholl image of Vehicle (left) and CSD (right). (G) Sholl analysis showed no significant changes between the NTG and vehicle groups with in the thalamus. Unpaired t-test. n=5-6mice per group. 6 neurons per mouse

3.3.2 No difference following chronic NTG in central amygdala and caudate putamen, areas that regulate affective and emotional responses

To further build on our current and previous [721] findings we sought to determine if there were any other regions implicated in migraine that also showed cytoarchitectural alterations. Migraine results in a host of symptoms outside of pain, including alterations in emotional regulation and cognitive dysfunction [6, 12]. The amygdala and the basal ganglia have both been highly implicated in migraine pathophysiology [72, 734]. We first investigated if there were any changes within the central amygdala following chronic NTG treatment (Figure 17A). There was no significant change in the number of branch points (Figure 17B), total neuronal length (Figure 17C), or in the complexity of the neurons assessed through Sholl analysis (Figure 17D-E). We had very similar findings when investigating the caudate putamen (Figure 17F). Again, There was no significant difference in branches (Figure 17G), total neuronal length (Figure 17H) or complexity (Figure 17I-J). These data show that the alterations in cytoarchitecture following chronic NTG, while widespread, do not affect every region.

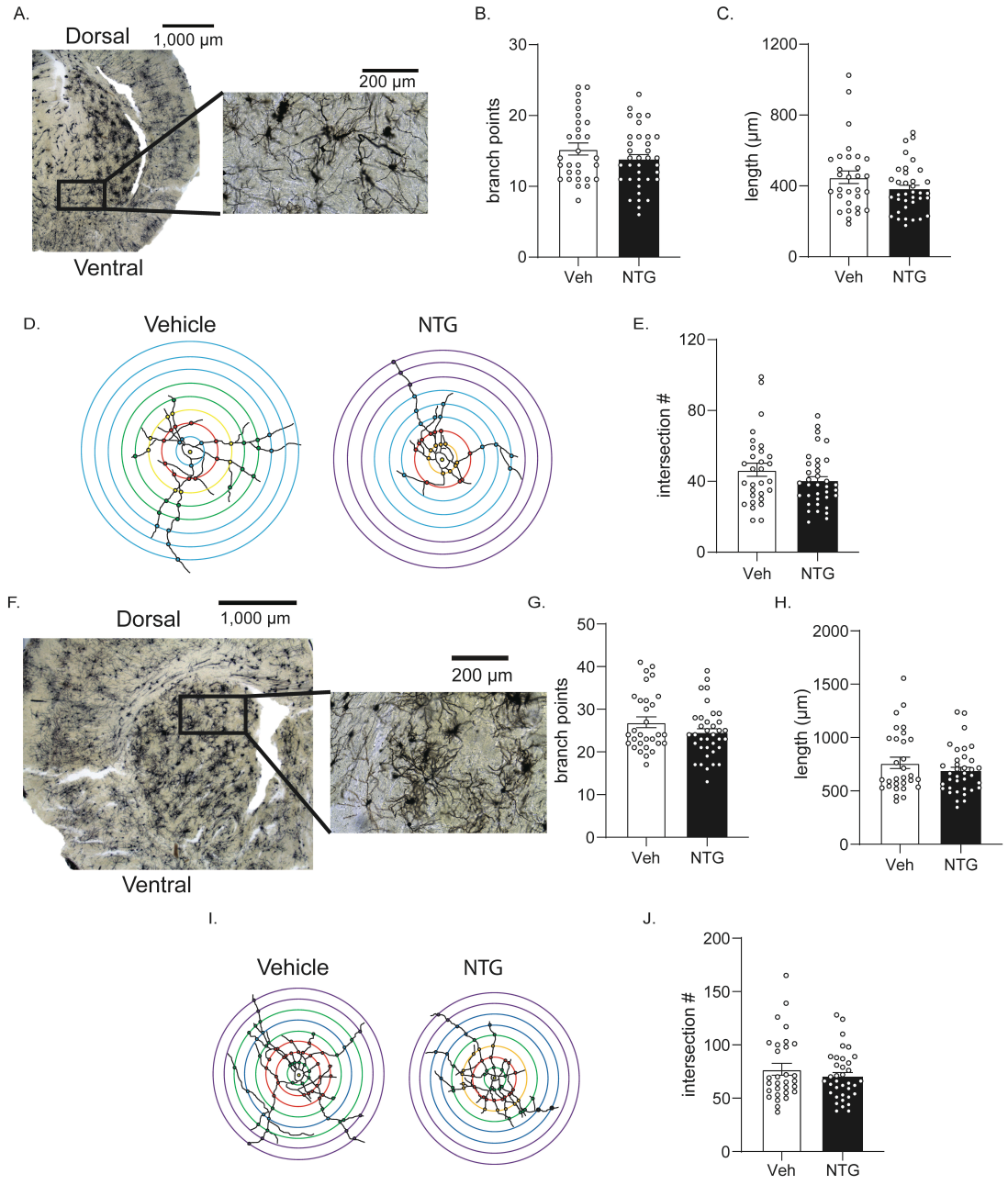

Figure 17. Chronic NTG does not produce cytoarchitectural changes in brain regions that regulate emotional regulation Mice were treated with chronic intermittent NTG or Vehicle for 9 days and no day 10 tissue was collected for Golgi staining. (A) Representational image of central amygdala 4x(left) and 20x (right) (B) Neurons from this region were analyzed for number of branch points and showed a decreasing trend, but no significant change. Unpaired t-test (C) Total neuron length was also found to trend towards a decrease, but no significant change. Unpaired t-test. (D) Representational Sholl analysis image of Vehicle (Left) and NTG (Right). (E) Sholl analyses were conducted and there was no significant change in the number of intersections within the central amygdala. Unpaired t-test (F) Representational image of

the Caudate Putamen 4x(left) and 20x (right) (G) Neurons from this region were analyzed for number of branch points and showed a decreasing trend, but no significant change. Unpaired T-test (H) Total neuron length was also found to be trend towards a decrease, but no significant change. Unpaired t-test. (I) Representational Sholl analysis image of Vehicle (Left) and NTG (Right) (J) Sholl analyses were conducted and there was no significant change in the number of intersections within the Caudate Putamen. Unpaired t-test. n=5-6/mice/group, 6 neurons per mouse

3.3.3 Cortical spreading depression results in alterations in the PAG

CSD is an electrophysiological phenomenon long held to be the cause of migraine aura [185]. CSD is mechanistically distinct from the NTG model of migraine pain, and migraine preventives have been shown to decrease CSD events [229]. We examined mice that underwent multiple KCl stimulated CSDs or Sham procedures (Figure 18A-B). These mice had their skulls thinned and then had continual KCl dripped onto the dura for an hour. Sham mice were used as controls and underwent the same skull thinning procedure, were anesthetized for the same duration of time, but did not receive KCl. Following the CSD/sham procedure, brains of these mice underwent Golgi staining and the vlPAG was examined (Figure 18C). Neurons within the vlPAG were found to have significantly decreased number of branch points following repeated CSD events (Figure 18C). Neurons in this region also showed decreased length (Figure 18D). Sholl analysis was conducted to investigate the overall complexity of these neurons, and while there was no significant change there was a trend towards decreased complexity within the vlPAG (Figure 18E-G). These data show that a primarily cortical driven phenomenon can have widespread impact on other brain regions, which could represent the relationship between aura and headache as well as representing chronification of migraine.

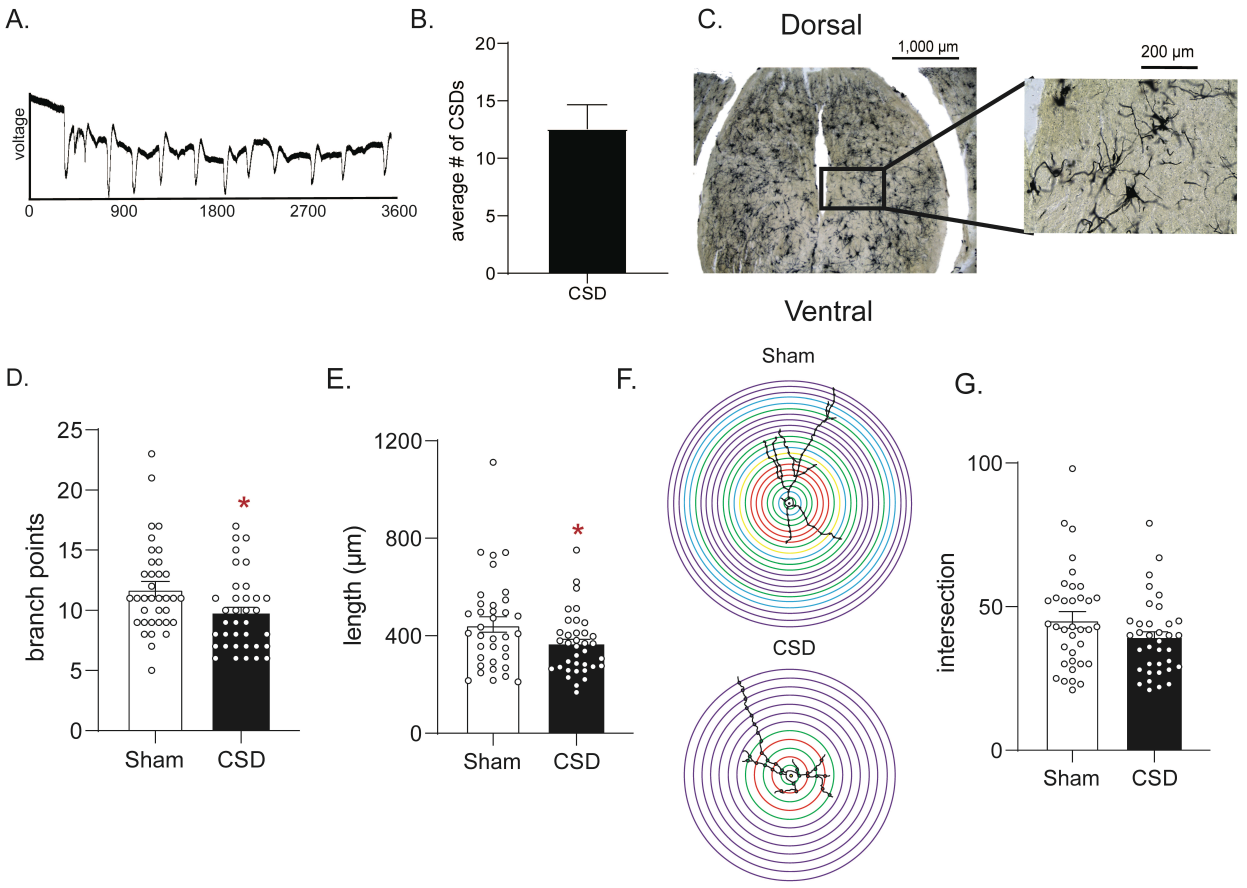

Figure 18. CSD results in decreased cytoarchitectural complexity in the periaqueductal gray (A) Representative line tracing of CSD events over a 3600 second period (B) Graph shows the average number of CSD events that occurred in the hour of recording. n=7 mice (C) Representational image taken of Golgi stained PAG at 4x (left) and 20X (right). (D) The number of branch points/neuron was significantly decreased in the CSD group compared to Sham surgery counterparts. Unpaired t-test *p<0.05 (E) Total neuron length was also found to be significantly decreased following CSD. Unpaired t-test *p<0.05. (F) Representative Sholl Image of Sham (left) and CSD (right). (G) Sholl analysis revealed a trend towards decrease, but not statistically significant. Unpaired t-test. n=6-7/mice/group, 6 neurons per mouse

3.3.4 Complex regional pain syndrome resulted in varying alterations in neuronal complexity depending on the brain region examined

Given our findings that widespread alterations in cytoarchitecture are present in two distinct models of migraine, we sought to find if these changes could be observed in a peripheral chronic pain state. To do this we used an established model of CRPS [731, 735]. Briefly, animals underwent a distal tibial fracture followed by 3 weeks of casting. As casting and fracture on their own can cause CRPS, uninjured mice were used as control. Following removal of the cast at 3 weeks, animals have sustained basal hypersensitivity that persists through 20 weeks post fracture [731]. These mice were sacrificed at week 7, which is considered well within the chronic/central phase, and had their tissue processed through Golgi staining. While the pain is the primary symptom associated with CRPS, cognitive impairment and memory deficits are also common affecting upwards of 50% of chronic CRPS patients [736, 737]. Previously another lab using a similar model showed alterations in hippocampal complexity. We sought to replicate these results and examined cytoarchitectural alterations in the dorsal hippocampus in CRPS and control mice (Figure 19A). Investigation of branching revealed a significant decrease in the total number of branches following CRPS (Figure 19B). Changes in branches also correlated with decreased neuronal length (Figure 19C); and Sholl analysis also revealed a significant decrease in the complexity of these neurons (Figure 19D-E). These results show a possible mechanism for cognitive alteration accompanying chronic pain.

Previous findings showed alterations within the vlPAG following chronic migraine and CSD, we sought to investigate if CRPS produced any similar changes in this key pain circuitry region (Figure 20A). We investigated the number of branch points per neuron, and while we saw a trend towards increased branching, the trend did not reach

significance (Figure 20B). However, examining total neuronal length revealed significantly increased neuronal length following CRPS in the PAG (Figure 20C). Furthermore, we also observed an increase in the complexity of neurons within the PAG following CRPS using Sholl analysis (Figure 20D-E).

We also investigated if CRPS altered neuronal complexity in pyramidal cell neurons in the SCx (Figure 21A), as this region was shown to be dramatically altered following chronic NTG or CSD [721]. No change was observed in branching (Figure 21B), length (Figure 21C), or analysis complexity (Figure 21D-E). These data indicate that different brain regions show distinct cytoarchitectural alterations in response to CRPS.

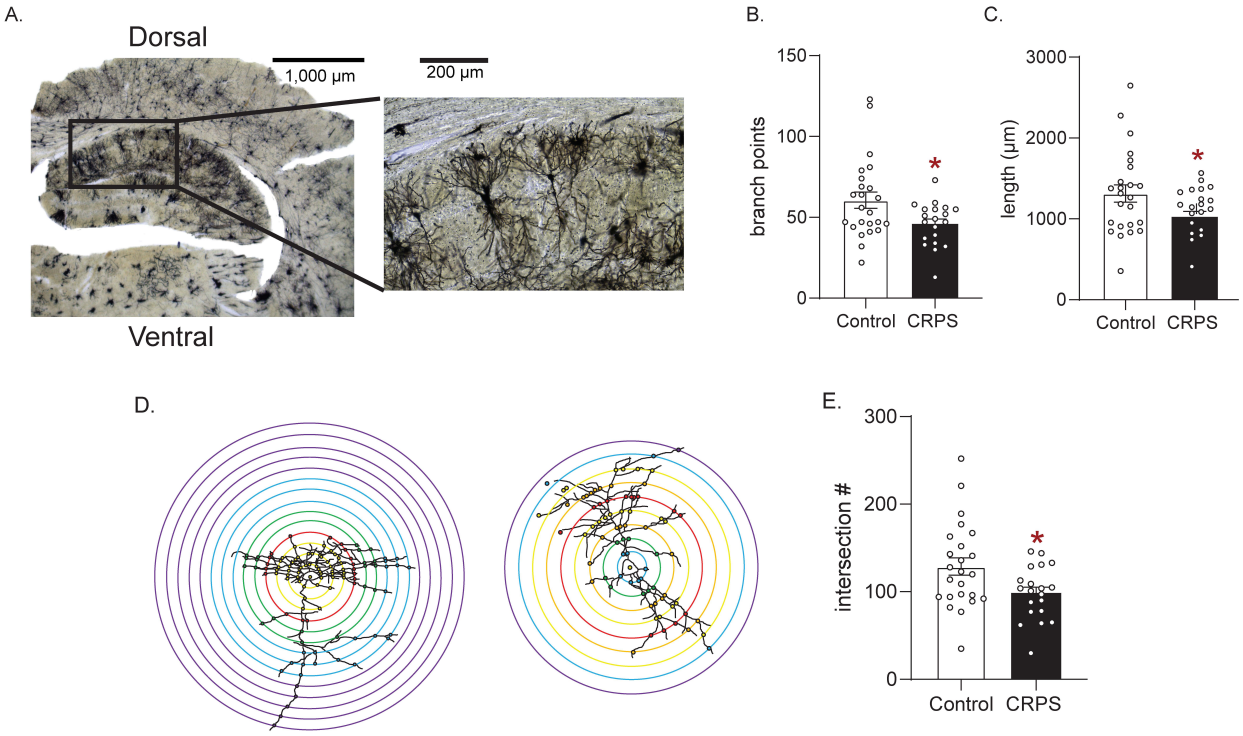

Figure 19. CRPS results in decreased hippocampal neuronal complexity (A) Representation image taken of the Dorsal Hippocampus 4x(left) and 20x (right). (B)

Number of branch points per neuron were analyzed and found to be significantly decreased in the CRPS group. *p<0.05 unpaired t-test. (C) Similarly, total neuron length was found to be significantly decreased in the Hippocampus following CRPS. *p<0.05. Unpaired t-test. (D) Representational neurons of Control (Left) and CRPS (Right). (E) Sholl analysis also revealed a significant decrease in the number of intersections following CRPS. *p<0.05 Unpaired t-test.

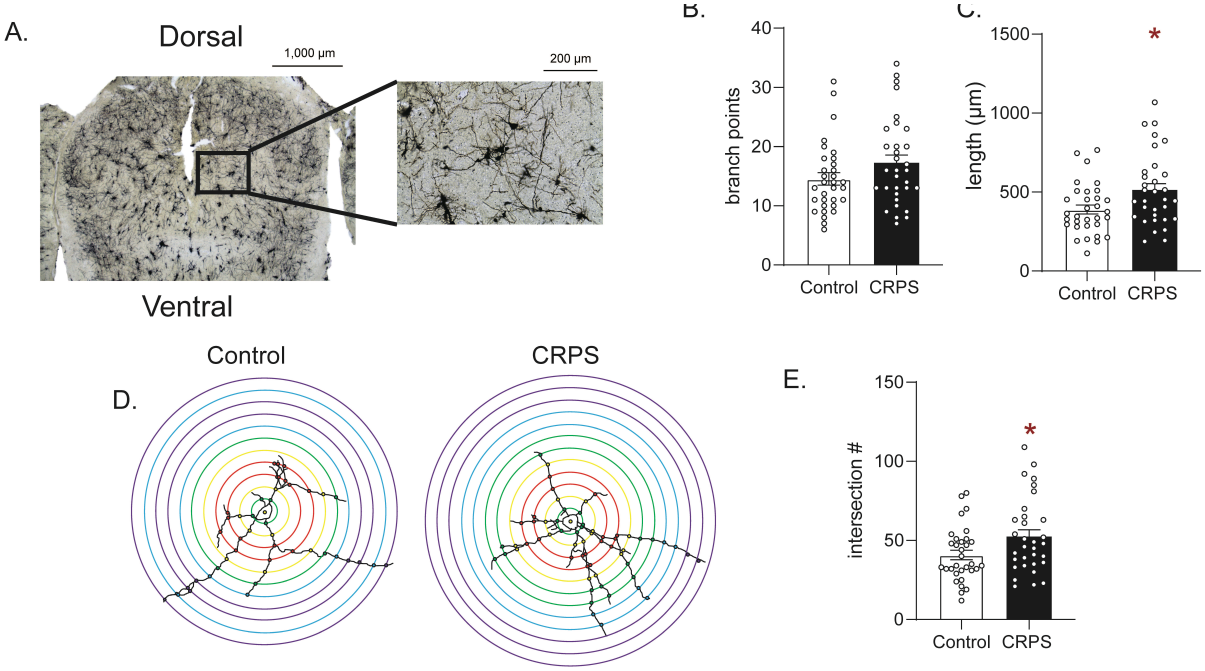

Figure 20. CRPS produces a significant increase in cytoarchitectural complexity in the periaqueductal gray (A)Representation image taken of the Golgi stained Periaqueductal gray at 4x(left) and 20x (right). (B) Number of branch points per neuron were analyzed and found no significant difference between the control and the CRPS mice. Unpaired t-test. (C) Total neuron length was found to be significantly increased in the PAG following CRPS. *p<0.05. Unpaired t-test. (D) Representational neurons of Control (Left) and CRPS (Right). (E). Sholl analysis also revealed a significant increase in the number of intersections following CRPS. *p<0.05 Unpaired t-test.

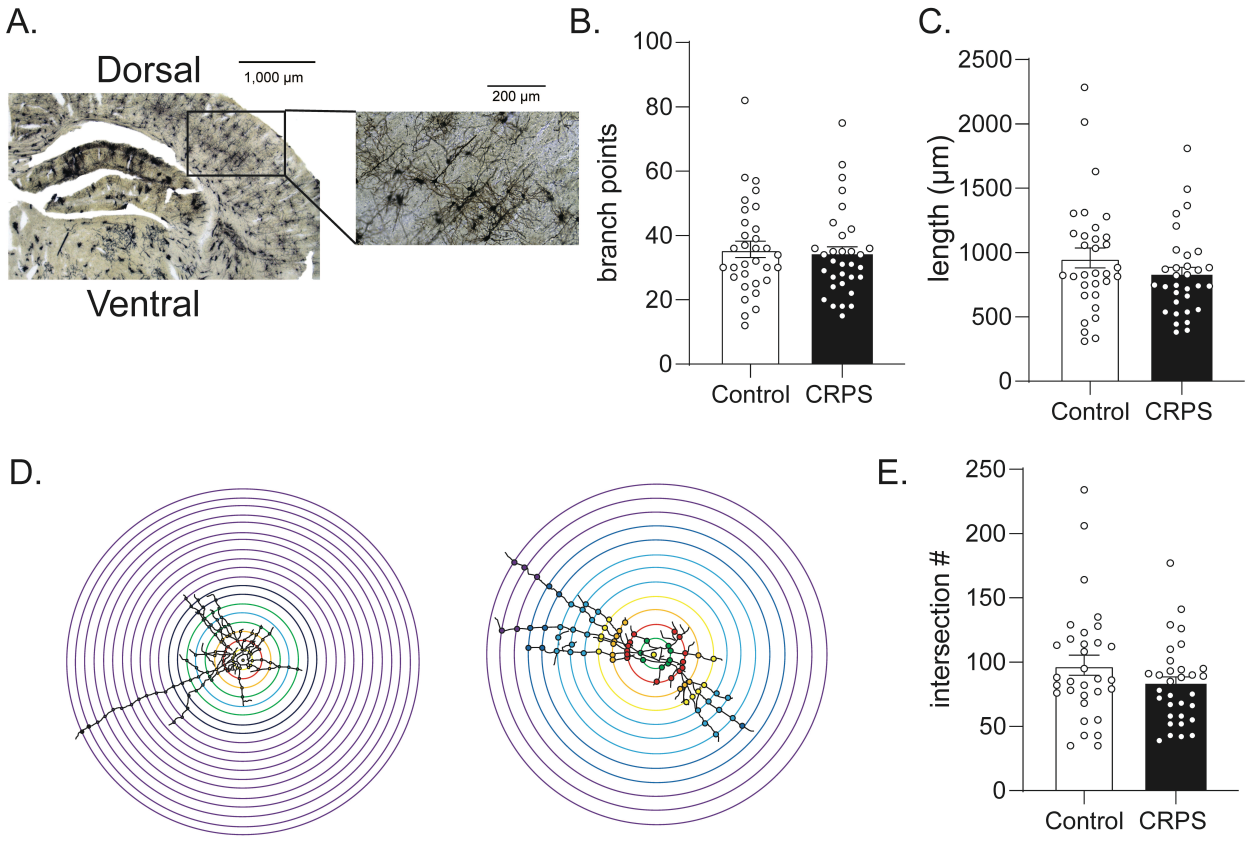

Figure 21. CRPS resulted in no change in pyramidal neurons in the somatosensory cortex (A) Representational image taken of the Golgi stained Somatosensory Cortex 4x (left) and 20x (right). (B) No statistical difference was found after examining number of branch points per neuron. Unpaired T-test (C) No statistical difference was seen after examining overall neuron length. Unpaired T-test (D) Representational neurons form the somatosensory cortex Control (Left) and CRPS (Right) (E) Similarly there was no significant change in the number of intersections within the somatosensory cortex following CRPS. Unpaired t-test. n=4/group, 8 neurons per mouse.

3.4 Discussion

Our results build on previous studies that support the notion that chronic pain is characterized by alterations in neuronal plasticity. A summary of the cytoarchitectural changes in this study and our previous work can be found in Table 2. We continue this

work by first examining alterations following our chronic NTG model [94]. NTG has long been used as a human migraine trigger and has been used experimentally to induce migraines in humans and migraine like symptoms in rodents [738]. Rodent models using NTG have been able to produce several human associated symptoms including delayed allodynia, photophobia, and altered meningeal blood flow [110, 111, 684]. NTG has further been demonstrated to activate nociceptive pathways [692, 693]. We previously demonstrated that chronic NTG decreased cytoarchitectural complexity in many brain regions important for migraine pain processing including the TNC, SCx, and the PAG [721].

Table 2 Summary of cytoarchitecture changes

Model	TNC	SCx	vlPAG	Dorsal Hippocampus	LSC	VPM	CPu	NAc	CeA
NTG	↓ **Decrease**	↓**Decrease**	↓**Decrease**	N/A	= No Change	↑ Increase	= No Change	= No Change	= No Change
CSD	↓ **Decrease**	↓**Decrease**	↓**Decrease**	N/A	N/A	N/A	N/A	N/A	N/A
CRPS	N/A	= No Change	↑ **Increase**	↓ **Decrease**	N/A	N/A	N/A	N/A	N/A

We built on these previous findings by revealing alterations in the VPM of the thalamus. Interestingly, in stark contrast to the decreased neuronal complexity observed within the TNC, PAG, and SCx, we saw an overall increase in the number of branches within the VPM neurons. There are known dura-sensitive neurons within the

VPM that receive direct projections from the TNC [733]. The VPM was shown to become sensitized following repeated activation of the trigeminocervical pathway [39]. Following sensitization innocuous stimuli produced activation to the level of noxious stimuli within the VPM, indicating this region is important for driving allodynia [39]. Increased branching within the VPM could drive the allodynia associated with chronic migraine. Increased volume changes within the thalamus have been observed in humans with chronic migraine [723, 739]. One study found chronic migraine patients had increased left thalamus size and that this size positively correlated with the frequency of migraine attacks [739]. Similarly, in a model of medication overuse headache the whole thalamus and each subnuclei also had increased volume [723]. While increased volume in humans cannot be directly compared to alterations in branching, our data are in line with the idea that augmentation of thalamic nuclei could be a driving mechanism for chronic migraine. Additionally, given our previous observations of decreased complexity in the TNC, PAG, and SCx this data suggests an overall imbalance in the migraine brain. Decreased branching from TNC could result in disruption of the usual connection between the TNC, VPM, and SCx. This could explain why we see increased complexity within the VPM, but see decreased complexity within the TNC and SCx. Future studies will be needed to better understand the connections between these regions and how changing cytoarchitecture in one region affects projections to another.

The amygdala has been implicated in regulating the affective aspects of pain and more recently to also have a role in regulating analgesia [740]. Furthermore, human imaging studies of patients with migraine have shown alterations in the amygdala, as

whole brain functional connectivity of the amygdala was increased [740]. Chronic migraine patients were also found to have decreased left amygdala volume [73]. Based on these studies we investigated if chronic NTG would also produce changes in neuronal cytoarchitecture of the amygdala. While our studies did not reveal any significant alterations following chronic NTG, it is possible that functional alterations in this region are present that are missed through our gross morphological analysis.

The caudate putamen is a major site of cortical and subcortical input into the basal ganglia [741]. While, the caudate putamen is frequently seen activated during pain, it was thought to primarily be due to its role in motor function [742, 743]. However more recent findings suggest it is important in processing the sensory aspects of pain [744]. Functional imaging of the brain of a migraine patient showed decreased activation within the caudate putamen following non-repetitive stimuli [72]. Alterations in caudate signaling show the inability of the migraine brain to properly habituate itself to stimuli [72, 729]. Interestingly, researchers also found increased gray matter density in the caudate of migraine patients compared to healthy controls [72]. In our study we did not observe any alteration in neuronal complexity within the caudate. In our previous work, we also did not observe any changes in the anatomically related nucleus accumbens following chronic NTG [721]. These results in combination with our central amygdala data suggest that regions important in regulating the affective aspects of pain do not undergo the same cytoarchitectural changes that we have previously observed in the TNC, PAG, and SCx.

In this study we also built upon our previous findings using the CSD model of migraine aura. CSD is thought to underlie the hyperexcitable brain state of migraine

patients, and to underlie migraine aura [185, 697]. CSD has been shown to result in neuronal swelling, alterations in dendritic structure, and even volumetric change [169]. While CSD is primarily a cortical phenomenon it has been shown to cause sensitization of other brain regions, primarily the TNC, along with activation of meningeal nociceptors [37, 181]. These data indicate that the cortical phenomenon of CSD could have other distant effects in pain circuitry. In previous work conducted in our lab, we found that CSD correlated with a dramatic decrease in the cytoarchitecture of the SCx as well as the TNC [721]. While these two regions are the most highly implicated in CSD and migraine other connected regions may also show alterations following CSD events. The PAG is part of the pain circuitry and receives a direct projection from the TNC [12, 745, 746]. Based on the role of the PAG in regulating pain we looked at cytoarchitectural changes following CSD in the PAG. We found decreased neuronal complexity within the PAG, demonstrating that CSD events can cause cytoarchitectural alterations in the pain matrix more broadly. Growing evidence further suggests the effect that CSD has on activation of the pain circuitry. Activation of Pannexin1 channels was seen following CSD. The activation and subsequent signaling cascade has been implicated in promoting the trigeminal afferents and promoting the headache phase that follows migraine aura [203]. Furthermore, a recent study found that optogenetic stimulated cortical spreading depression events produced sustained periorbital mechanical allodynia as well as increased anxiety measures [222]. These studies in combination with our cytoarchitectural findings provide further support for the link between CSD and pain.

We finally wanted to determine if alterations in neuronal complexity were a feature of chronic pain conditions more broadly, or limited to chronic migraine. We chose to study CRPS, as this type of pain clearly transitions from an acute peripheral pain to a centrally mediated chronic pain state [725]. This transition is accompanied by a host of changes including altered immune response, DNA methylation, and even some alterations in cytoarchitecture [495, 724, 735, 747]. In humans CRPS was found to result in shrinkage of cortical mapping of affected limb index fingers [493]; and these alterations in cortical representation were positively correlated with severity of pain in the affected limb [493]. A previous mouse model of CRPS showed alterations of dendritic architecture in the amygdala and perirhinal cortex [495]. This study also investigated the hippocampus and while they did not see any changes in dendrite density, they did find a decrease in synaptophysin indicating alterations in hippocampal processing [495]. A more recent study showed decreased dendritic complexity within the hippocampus following injury and casting [724]. We sought to replicate these findings using the Golgi staining technique and we also observed decreased neuronal complexity within the hippocampus following CRPS. These results further strengthen the connection between altered hippocampal neurons and the resulting changes in cognitive function associated with CRPS.

The PAG is an important region in regulating pain, and stimulation of the PAG was found to produce analgesia [748]. Since this initial finding much more has been learned about the role of the PAG in the pain circuitry [749]. Specifically, the vlPAG is a key regulator of endogenous opioid mediated pain suppression as this region is rich in mu opioid receptors and enkephalin [459, 470, 750]. Previous research has shown that

different populations of GABAergic and glutamatergic neurons can divergently modulate pain signals and either promote or inhibit analgesia [751]. We found an overall increase in neuronal complexity within the vlPAG following CRPS. This is interesting as it is in sharp contrast to the decreased complexity we and others have observed in the hippocampus. While we saw decreased cytoarchitecture in the PAG following both chronic NTG and repeated CSD events it is not too surprising we see differential findings following CRPS. One factor that could contribute to variation in our findings is the length of time animals underwent each condition. CSD was done over an hour of repeated stimulation and chronic migraine exposed mice for 9 days. This is in contrast to CRPS, in which mice were in pain for 7 weeks before they were sacrificed and neuronal cytoarchitecture examined. It is possible that if we continued the chronic migraine experiments longer we would see a similar increase in cytoarchitecture complexity in the PAG rather than the decrease we see. Additionally, CRPS begins as a peripheral chronic pain state and eventually transitions to a central one. Chronic migraine and CSD, both affect the central nervous system from the beginning, which could cause the differences we see in cytoarchitecture. These results still collectively show the importance that cytoarchitectural dynamics play in regulating chronic pain states.

The cytoarchitectural analysis that was conducted in these studies is limited by the Golgi staining technique, as it largely does not allow for co-staining with immunohistochemical markers [752]. Without this information it is difficult to determine how different cellular populations respond to chronic pain. In mice activation of a subsection of GABAergic cells within the central amygdala was found to decrease both

mechanical and thermal allodynia, while inhibition resulted in increased allodynia [753]. Our findings in the amygdala showed no significant changes. It is possible given the importance of the GABAergic subpopulation in controlling pain signals focusing solely on the GABAergic population would reveal changes in neuronal complexity that were lost when looking at gross morphological changes. Additionally, differentiation in cellular populations could give greater meaning to our cytoarchitectural changes as it could reveal more about how these changes cause alterations in signaling.

Many human anatomical studies have shown alterations in cytoarchitecture in psychiatric disorders including chronic pain [492, 656, 721, 754]. These alterations can have wide impact on function of these areas and in many cases are also correlated with altered functional readings. Our findings give greater understanding to the possible molecular basis for these alterations. While more work needs to be conducted in the future to further investigate why some of these regions result in decreased, increased, or no change in complexity our findings make it clear that cytoarchitectural changes are a key part of chronic pain. This knowledge could be used in the future to identify neuronal signatures of chronic pain, and could result in the development of novel therapeutics targeting signaling mechanisms that govern neuronal plasticity.

Chapter 4: A non-convulsant delta opioid receptor agonist, KNT-127, reduces cortical spreading depression and nitroglycerin-induced allodynia

(Previously published as Bertels Z, Witkowski WD, Asif S, Siegersma K, van Rijn RM, Pradhan AA. A non-convulsant delta-opioid receptor agonist, KNT-127, reduces cortical spreading depression and nitroglycerin-induced allodynia. Headache. 2021 Jan;61(1):170-178.)

4.1 Introduction:

Migraine is an extremely prevalent neurological disorder that disproportionately affects women and people between the ages of 15-60[755]. While there have been recent advances in migraine therapeutics there are still a large number of patients who are not fully satisfied with their current therapies[662, 756, 757]. Effective acute and preventive migraine treatments target a wide range of molecular mechanisms[758, 759] and this emphasizes the complex pathophysiology of migraine. The delta opioid receptor (DOR) has recently emerged as a promising therapeutic target for migraine[468, 760]. Three different DOR agonists effectively reduced chronic migraine-associated pain induced by the known human migraine trigger, nitroglycerin (NTG)[114]. Subsequent studies also showed that the agonist, SNC80, also blocked allodynia in models of post-traumatic headache and triptan- and opioid-induced medication overuse headache[761, 762]. SNC80 also reversed NTG induced condition place aversion a model reflecting the negative emotional state induced by migraine[114]. Furthermore, in a KCl stimulated model of cortical spreading depression (CSD), SNC80 decreased CSD events a result predictive of migraine preventives[114]. These studies highlight the potential of DOR agonists for the treatment of migraine.

A major limitation to the development of DOR as a target for clinical use is the seizurogenic properties of some DOR agonists[469, 763]. Some of the early small molecule DOR ligands such as SNC80 and BW373U86 were found to produce short non-lethal convulsions followed by a brief cataleptic period in rodents[764-768], as well as pro-convulsant effects in rhesus monkeys[769]. Although this pro-convulsant activity is dependent on activation of DOR[766, 770], not all DOR agonists produce these effects[771]. For example, ARM390[770], PN6047[772], and ADL5859[773] do not show seizurogenic properties yet maintain pain-relieving effects characteristic of DOR agonists. This ligand specific effect is thought to be due to activation of distinct intracellular signaling cascades by pro-convulsant DOR agonists, potentially linked to their ability to internalize DOR upon binding[771]. Both convulsant (SNC80) and non-convulsant (ARM390, JNJ20788560) DOR agonists were shown to effectively block allodynia in a NTG model of migraine[114], suggesting that this anti-migraine property is not ligand specific but universal to DOR agonists. However, only SNC80 has been tested in CSD models. The ability of DOR agonists to affect cortical activity may be dependent on the same signaling mechanisms that regulate pro-convulsant effects, and the aim of this study was to explore this relationship further.

The selective DOR agonist, KNT-127, was recently reported to have a wide therapeutic margin, and even at 10X the physiologically relevant dose did not show pro-convulsant properties[774]. Similar to SNC80, administration of KNT-127 reduces hyperalgesia in chronic peripheral pain models and produces anti-depressant effects[490, 775]. In this study we determined if KNT-127 was effective in the CSD model of migraine aura. In addition, we also tested KNT-127 in the NTG model of

chronic migraine-associate pain[688]. The ability of an agonist to produce internalization of DOR has been correlated with pro-convulant properties[770, 771]. Previous publications show that KNT-127 does not cause internalization of DOR in forebrain, lumbar spinal cord, and dorsal root ganglia (DRG)[442]. We therefore sought to determine if this was also true in key migraine processing regions: trigeminal ganglia (TG), trigeminal nucleus caudalis (TNC), and somatosensory cortex (SCx). We hypothesized that, despite KNT-127 being a low internalizing agonist, it would still reduce CSD events and reverse established NTG allodynia. These studies provide valuable information on whether the anti-migraine effects of DOR agonists can be separated from the pro-convulsant effects, which can subsequently inform drug development of this target.

4.2 Materials and Methods

Animals: Adult male and female C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME. USA) from 8-15 weeks of age were used in the behavioral experiments, and DOR-eGFP knockin mice (C57BL6/J background) were used for internalization studies. Mice were group housed in a 12h-12h light-dark cycle, in which the lights were on from 07:00-19:00. Mice had access to food and water ad libitum. When possible, all experiments were conducted in a blinded fashion by the experimenters. All experiments were approved by the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety Committee, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International guidelines and the Animal Care Policies of the University of Illinois at Chicago. Weights were recorded on each test day of experiments and no adverse effects were observed during the studies.

Sensory Sensitivity Testing: In order to minimize differences between groups, mice were counter-balanced into groups based on the basal cephalic mechanical thresholds assessed on day 1 using Excel software. All mice were tested in a behavioral room that was separate from the vivarium. The behavioral room had low light (~35-50 lux) and maintained low-noise conditions. All behavioral tests were done between 09:00-16:00. Mice were habituated 2 days prior to the first test day and 30 minutes before each test [113, 688, 776, 777]. To assess cephalic measure mice were placed in 4 oz paper cups. The periorbital region caudal to the eyes and near the midline was tested to assess cephalic allodynia. To test mechanical thresholds, we stimulated the periorbital region with von Frey hair filaments (bending force ranging from 0.008g to 2g) using the up-and-down method. A positive response was defined as a shaking, repeated pawing, or cowering following a full bend from the filament. The first filament used in all cases was 0.4 g. If there was no response a heavier filament (up) was used, and if there was a response a lighter filament (down) was tested[778]. The up-down pattern continued for 4 filaments after the first response. All animals tested were used in the final analysis.

Cortical Spreading Depression: The procedure for cortical spreading depression (CSD) is based on work previously published by Ayata and colleagues [229]. Previously, this model has been used to screen for potential novel migraine preventive therapeutics including work previously done in our lab [94]. Female mice in the first experiment were randomly grouped into Vehicle or SNC80 (10 mg/kg, IP) and in the second experiment Vehicle or KNT-127 (5 mg/kg, SC). For the CSD procedure mice were anesthetized with isoflurane (induction 3-4%; maintenance 0.75 to 1.25%; in 67% N2 / 33% O2) and placed in a stereotaxic frame on a homoeothermic heating pad. Core temperature

(37.0 ± 0.5℃), oxygen saturation (~ 99%), heart rate, and respiratory rate (80–120 bpm) were continuously monitored (PhysioSuite; Kent Scientific Instruments, Torrington, CT, USA). Mice were repeatedly tested with tail and hind paw pinch to ensure proper anesthetic levels were maintained.

CSD was verified in two ways, optical intrinsic signal (OIS) and electrophysiological recordings, as previously described [94]. For OIS a thinned rectangular region of the skull was made about 2.5 x 3.3 mm^2 (~0.5 mm from sagittal, and ~1.4 from coronal and lambdoid sutures) of the right parietal bone. A dental drill (Fine Science Tools, Inc., Foster City, CA, USA) was used to thin the skull. To increase transparency mineral oil was applied to the thinned region, which allowed for further visualization of the parenchyma and vasculature. For video recording a green LED (520 nm) light illuminated the skull throughout the experiment (1-UP; LED Supply, Randolph, VT, USA). Reflectance was collected with a lens (HR Plan Apo 0.5 × WD 136) through a 515LP emission filter on a Nikon SMZ 1500 stereomicroscope (Nikon Instruments, Melville, NY, USA). Images were acquired at 1–5 Hz using a high-sensitivity USB monochrome CCD (CCE-B013-U; Mightex, Pleasanton, CA, USA) with 4.65-micron square pixels and 1392 × 1040 pixel resolution.

Two burr holes were drilled lateral to the thinned window around the midpoint of the rectangle. The burr holes were drilled deeper than the thinned skull portion such that the dura was exposed, but not so deep that the dura was broken. Local field potentials (LFPs) were recorded using a pulled glass pipette filled with saline and attached to an electrode, which was further connected to an amplifier. The electrode was placed inside

of the lateral burr hole such that it was inside of the cortical tissue. A separate ground wire was placed underneath the skin caudal to the skull, which was used to ground the LFPs. After set up the LFP was recorded for an hour to allow for stabilization in the case that a CSD occurred during the placement of the electrode or the thinning surgery. After stabilization a second pulled glass pipette was filled with 1M KCl and placed into the rostral bur hole, ensuring there was no direct contact with the brain or surrounding skull. Once placed, an initial flow of KCl was started and an even flow was maintained so that a constant small pool of KCl filled the burr hole. Any excess liquid was removed with tissue paper. After initial KCl administration mice were recorded for 400 seconds. Following which mice were treated with the appropriate compound (vehicle, SNC80, or KNT-127) and then recorded for a remaining 3600 seconds, for a total recording time of 4000 seconds. Animals were only included in the final analysis if at least 2 CSD events occurred within the first 400s of recording. No animals were excluded in this study. Following the recording, video and LFP were analyzed and used to count the number of CSD events that occurred within the hour recording. Following the procedure mice were euthanized by anesthetic overdose followed by decapitation.

Drug Injections: All injections were administered at 10ml/kg volume, intraperitoneally (IP) or subcutaneously (SC), unless otherwise indicated. KNT-127 was custom synthesized and purified by ChemPartner (Shanghai, China) as has been previously reported [779]. 1H NMR and HPLC-MS were employed to confirm identity and ensure the purity was >95%.

Immunohistochemistry: Male and female DOR-eGFP mice were anesthetized with Somnasol 100 µl/mouse; 390 mg/mL pentobarbital sodium; Henry Schein) and perfused

intracardially with 20 mL of ice-cold phosphate-buffered solution (0.1 M PB, pH 7.2) and subsequently 50 mL of Ice cold 4% paraformaldehyde (PFA) in 0.1M PB (pH 7.4). After perfusion, whole brain and trigeminal ganglia (TG) were harvested and left in 4% PFA/0.1M PB at 4°C overnight to post-fix. The next day tissue was cyroprotected in 30% sucrose in 0.1M PB. Brain and TG were flash frozen using 2-methylbutane over dry ice. Coronal sections of somatosensory cortex and trigeminal nucleus caudalis were sliced at 20 μM while the TG were sliced at 16 μM. Sections were immediately mounted onto slides after slicing. Slides were then washed with PBST. A blocking solution with 5% normal donkey serum (NDS) with PBST was used to block slides for 1h at room temperature. Slides were then incubated overnight at RT with the primary chicken anti-GFP antibody (1:500 AB_13970) diluted in 1% NDST. Slides were subsequently washed with 1% NDST and then the secondary antibody was added for 2 hours at room temperature (donkey anti chicken IgG, 1:2000). Slides were washed with 0.1M PB, and cover slipped with Mowiol-DAPI mounting medium. A blinded investigator then took images using the EVOS FL Auto Cell Imaging system, using a 40x objective. Quantification of intracellular mean fluorescence intensity was determined with ImageJ software. Nuclear fluorescence was used to calculate background optical density and was subtracted from intracellular fluorescence. No samples were excluded from this analysis.

Statistical Analysis: The sample size needed for each experiment was either based on similar previous experiments or calculated through power analysis where the minimal detectable difference in means =0.3, expected standard deviation of residuals= 0.14, desired power=0.8, alpha=0.05, n=6/group. Each experiment was replicated with

separate cohorts of animals to ensure reproducibility. All data were analyzed using GraphPad Prism (GraphPad, San Diego, CA). The level of significance (α) for all tests was set to $p<0.05$. Unpaired, two-tailed, t-tests were performed to determine significance of CSD data. 2-way RM ANOVA was performed on NTG-Veh Basal and Post-Treatment cephalic mechanical allodynia. The factors were treatment, time, and interaction. 3-way ANOVA was conducted to determine the effect of KNT-127 on day 10. Treatment (Vehicle-Nitroglycerin), Drug (Vehicle-KNT-127), and time were used as the 3 factors for the ANOVA. Post hoc analysis was conducted using Holm-Sidak analysis to correct for multiple comparisons. Post hoc analysis was only performed when F values achieved $p < 0.05$. All values in the text are reported as mean ± SD.

Data availability: All data are available upon reasonable request.

4.3 Results

4.3.1 KNT-127 decreases cortical spreading depression events

CSD is an electrophysiological phenomenon that is considered to be the physiological correlate of migraine aura, and reduction of CSD events is also a good predictor of migraine preventives[229]. We have previously shown that the DOR agonist SNC80 can inhibit CSD events[114], and in this experiment we repeated these findings both as a positive control and a test of internal reproducibility. We used the CSD model as previously described[114]. Briefly, in an anesthetized mouse the skull was thinned to reveal the dural vasculature and cortex underneath (Figure 22A). Two burr holes were made, and one was used to continuously drip KCl onto the dura to induce CSD, and local field potentials (LFPs) were recorded from the other burr hole. The

somatosensory/barrel cortex was targeted, as it is more sensitive to CSD induction[681]. CSDs were identified by visual shifts in light (Figure 22B) and sharp decreases in the LFP (Figure 22C). Four hundred seconds after KCl application mice were injected IP with SNC80 or vehicle. SNC80 treated mice showed significantly fewer CSD events relative to vehicle controls (Figure 22D; $t_{(10)}$=3.400, p=0.0068, Difference between means 2.833; ~71.2% of Vehicle). In a separate group of mice we tested the non-convulsant DOR agonist, KNT-127 or vehicle. Similar to SNC80, KNT-127 also significantly reduced CSD events (Figure 22E; $t_{(10)}$=3.570, p=0.0051, Difference between means -5.167; 55.7% of Vehicle). These data demonstrate that the inhibition of CSD by DOR agonists is not dependent on signaling related to pro-convulsant effects.

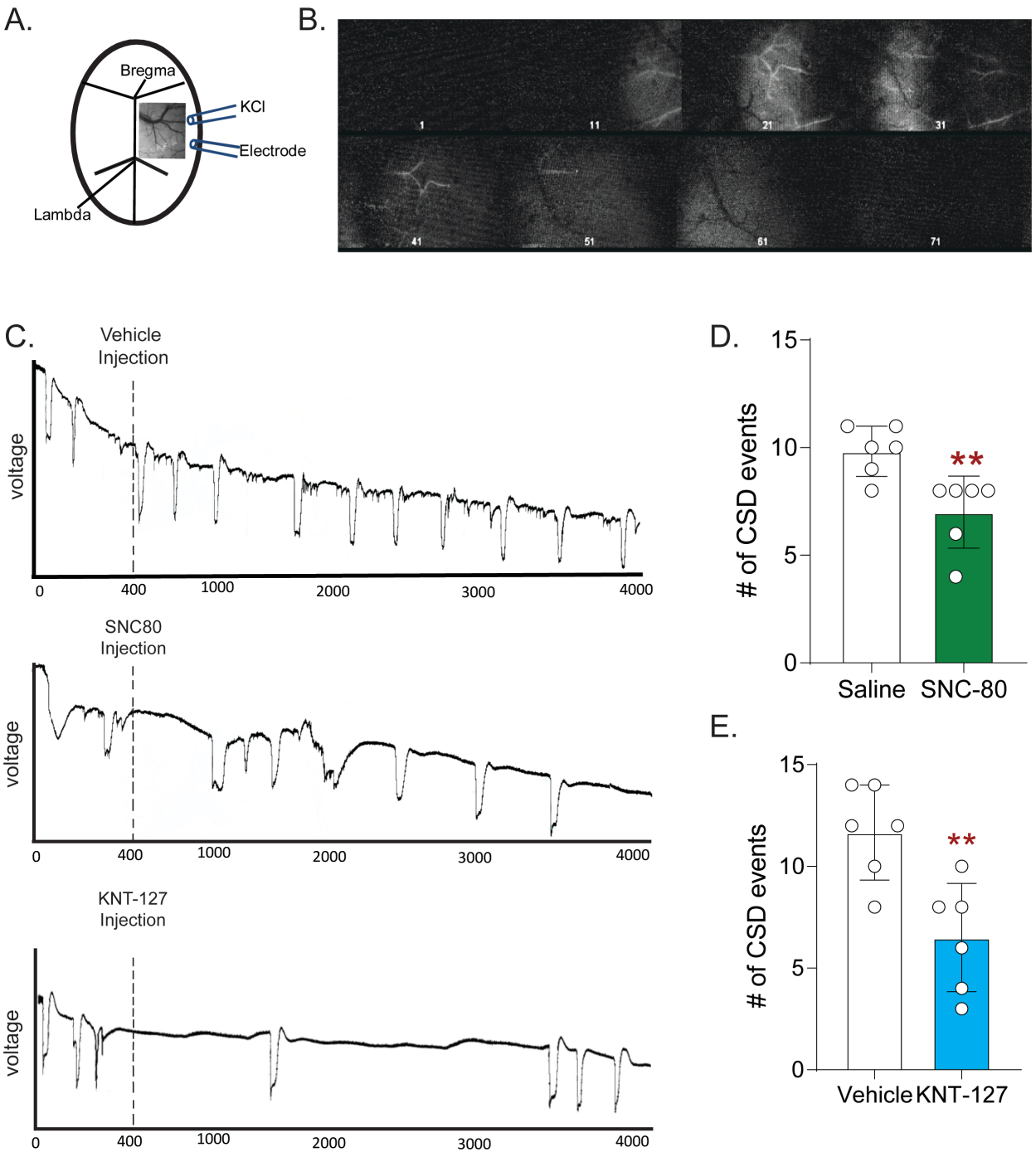

Figure 22 SNC80 and the non-convulsant DOR agonist, KNT-127, both reduce cortical spreading depression events (A) Schematic illustrating the thinned skull preparation that allows for the visualization of CSD waves. Two burr holes were used for KCl infusion (rostral) and to record LFP (caudal). (B) Image sequence demonstrating a typical change in reflectance associated with a single CSD event. (C) Representative line tracing of CSD events over a 4000-second period Vehicle (Veh; Top), SNC80 (Middle), and KNT-127 (Bottom). KCl infusion was initiated and video recording began,

after 400 seconds the mice were treated with either Veh, SNC80, or KNT-127. (D) Animals treated with SNC80 (5 mg/kg, ip) had a significant reduction in the average number of CSD events recorded in the remaining 3600 seconds of recording compared to Veh. Unpaired t-test **p < 0.01. (E) Similarly, animals treated with KNT-127 (5 mg/kg, sc) had a significant reduction in the average number of CSD events recorded in the remaining 3600 seconds compared to Veh treatment. Data are represented as mean ± SD, unpaired t-test **p < 0.01 n = 6/group

4.3.2 KNT-127 inhibits chronic migraine-associated allodynia

We next determined if KNT-127 was effective in the NTG model of chronic migraine[688]. To this end we treated male and female C57BL/6J mice every other day with NTG or vehicle for 9 days and assessed cephalic mechanical threshold on days 1, 5, and 9 (Figure 23A). Chronic NTG treatment resulted in a significant decrease in basal mechanical thresholds over time (Figure 23B; Two-Way ANOVA NTG-Veh: F (1, 22) = 26.93, Interaction: F(2, 44)=13.07, Difference between means Day 5=0.6177, p<0.0001; 11.27% of Vehicle, Difference between means Day 9=0.6179, p<0.0001; 0.928% of Vehicle). On day 10, 24 hours after the final NTG/vehicle injection, NTG-treated mice continued to show significant cephalic allodynia (Figure 23C, baseline; Three-Way ANOVA NTG-Veh: F(1,20)=45.81, Vehicle-KNT-127: F(1, 20) = 2.356, Time: F(1, 20) = 15.42, Interaction: F(1, 20) = 12.80). Mice were subsequently injected with KNT-127 (5 mg/kg, SC) or vehicle and tested 30 min later. KNT-127 did not alter mechanical responses in vehicle pre-treated mice, but significantly inhibited NTG-induced chronic allodynia (Figure 23C, post-treatment; Difference between NTG-VEH and NTG-KNT means=0.5515, p=0.0011. Responses in the NTG-KNT group were comparable to Veh-Veh controls; differences between means=0.1882, p=0.8605).

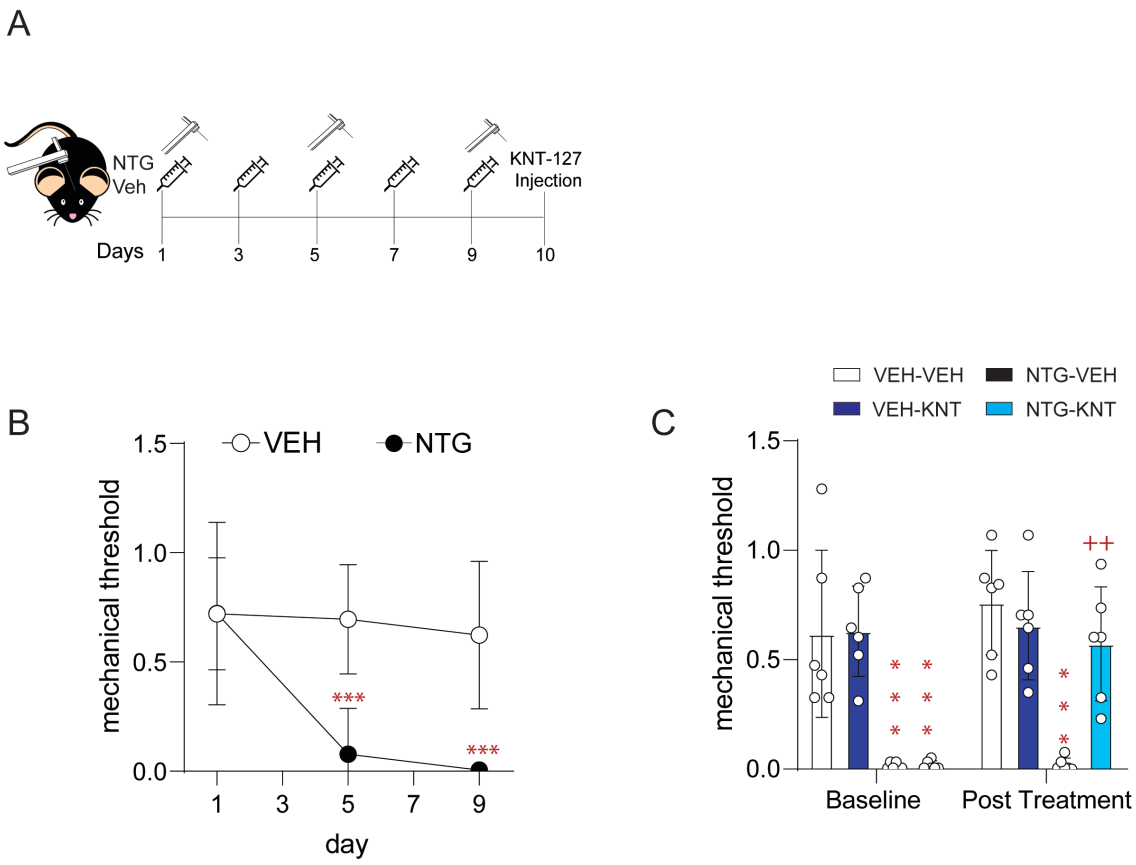

Figure 23 KNT-127 effectively reverses established cephalic allodynia induced by chronic nitroglycerin (NTG) treatment (A) Schematic of testing schedule, Male and female C57BL6/J mice were treated with intermittent chronic NTG (10 mg/kg, ip) or vehicle (Veh; 0.9% NaCl, ip) every other day for 9 days. On day 10, 24 hours after the last NTG/Veh injection, mice received KNT-127 or Veh treatment. (B) On days 1, 5, and 9 mice had their basal periorbital mechanical threshold assessed prior to the NTG/Veh injection. NTG produced persistent and severe cephalic allodynia $p < 0.001$ effect of treatment, time, and interaction, two-way RM ANOVA, and Holm–Sidak post hoc analysis. ****$p < 0.001$ relative to Veh on day 1, n = 12/group. (C) On day 10, baseline responses were determined in the morning (baseline), and NTG-treated animals continued to show severe allodynia compared to Veh controls (***$p < 0.001$). In the afternoon, mice were injected with KNT-127 (5 mg/kg, sc) or Veh (0.9% NaCl, sc) and tested 30 minutes later. KNT-127 significantly reversed chronic cephalic allodynia, $p < 0.05$ effect of treatment, drug, time, and interaction, three-way ANOVA and Holm–Sidak post hoc analysis. ****$p < 0.001$ relative to Veh–Veh at the same time point, ++$p < 0.01$ relative to NTG/Veh at the same time point, n = 6/group. Data are represented as mean ± SD

4.3.3 KNT-127 is a low-internalizing DOR agonist

Some but not all DOR agonists induce internalization of DOR *in vivo*[780]. Previous studies showed that KNT-127 produced little or no internalization of DOR in the DRG, lumbar spinal cord, hippocampus, and striatum[490]. We next determined if KNT-127 was also a low-internalizing agonist in key migraine pain processing regions; trigeminal ganglia (TG), trigeminal nucleus caudalis (TNC), and somatosensory cortex (SCx). DOR-eGFP mice were used to examine DOR internalization state, and we used the same dose and timing of KNT-127 that was effective in behavioral experiments outlined above. KNT-127 did not induce detectable DOR internalization (Figure 24A,B,C). Intracellular expression of DOR was quantified in TG and SCx as a measurement of internalized receptors, and there was no significant difference between KNT-127 and vehicle injected mice (Figure 24D; $t_{(6)}$=0.7827, p=0.7827, Difference between means -3.210, Figure 3E; $t_{(6)}$=0.1900, p=0.8556, Difference between means -3.331). In the TNC, DOR internalization helps to identify the individual cell bodies from the fibers and background (see Figure 24F, SNC80). As neither vehicle nor KNT-127 induced substantial internalization we could not quantify intracellular fluorescence in cells in this region.

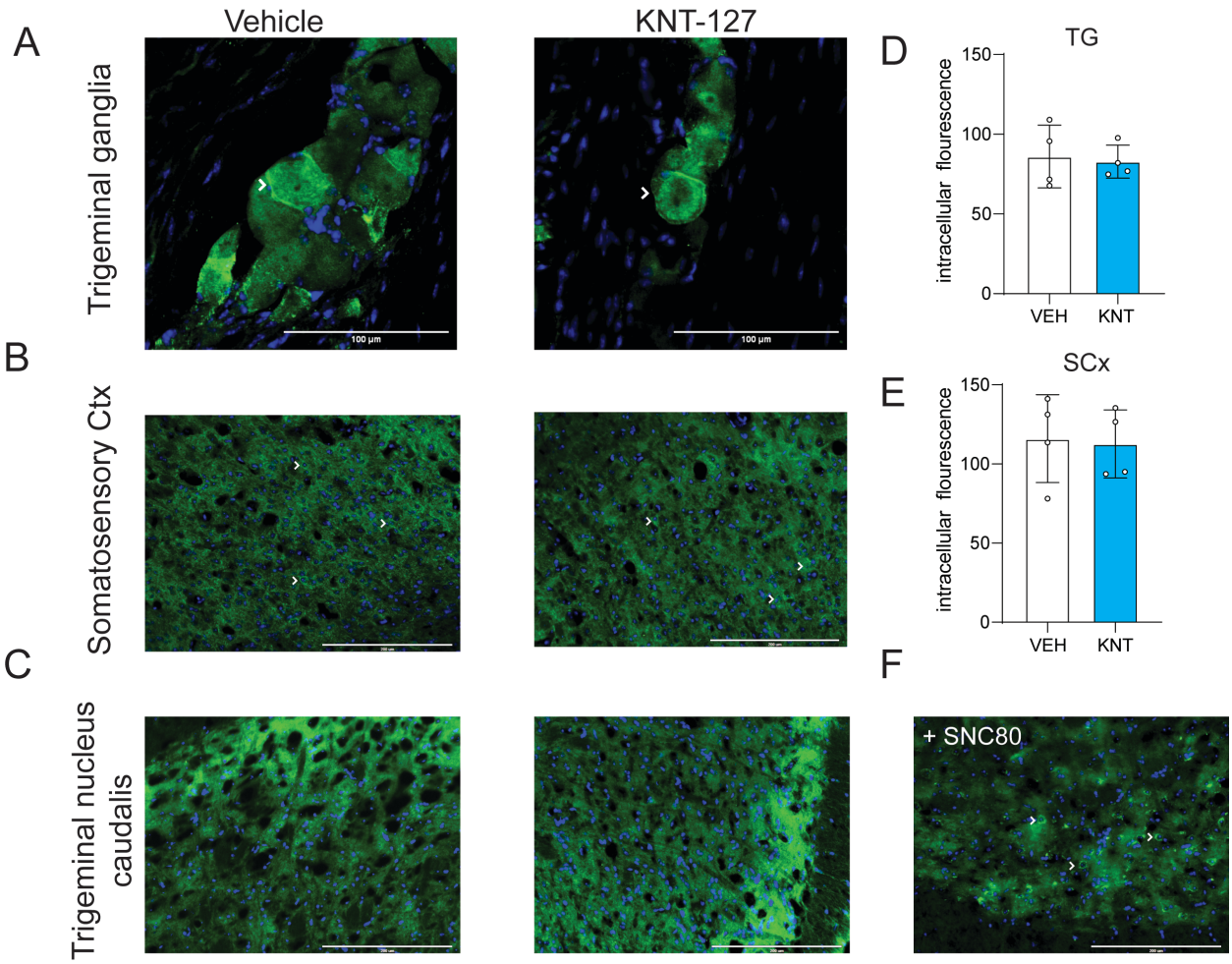

Figure 24 KNT-127 produces low-internalization of DOR-eGFP in key migraine pain processing regions DOR-eGFP mice were treated with vehicle (Veh) or KNT-127 (5 mg/kg, sc) and tissue was subsequently harvested 30 minutes post-injection. Representative images of (A) trigeminal ganglia (TG) (B) somatosensory cortex (SCx), and (C) trigeminal nucleus caudalis (TNC) of Veh (left), KNT-127 (middle)-treated mice. White arrowheads indicate DOR+cells. Mean intracellular fluorescence was quantified for TG (D) and SCx (E); and revealed no significant difference between Veh and KNT-127-treated groups, unpaired t-test. Due to the low internalization by Veh or KNT-127 it was difficult to identify clear DOR+cell bodies in the TNC; therefore, this region could not be quantified. Data are represented as mean ± SD. (F) Representative image of DOR-eGFP mouse treated with SNC80 (10 mg/kg, ip), a high-internalizing DOR agonist. Internalization of DOR reveals cell bodies in the TNC that is not apparent without receptor internalization (Veh, KNT-127)

4.4 Discussion

Our findings demonstrate that the non-convulsant DOR agonist KNT-127 effectively decreases established cephalic allodynia induced by chronic NTG and inhibits CSD evoked by KCl stimulation. Furthermore, we confirm that KNT-127 is a low-internalizing DOR agonist and does not produce significant receptor internalization in migraine processing regions. These results show that the signaling mechanisms that produce seizurogenic activity of DOR agonists are not necessary for the mechanisms that inhibit migraine-associated symptoms.

DOR agonists have been investigated for migraine as they are highly expressed in key migraine processing regions such as the dura, TNC, TG, and SCx [114, 470, 471, 781]. DOR agonists effectively inhibit allodynia in multiple models of headache disorders [113, 114]. In an NTG model of migraine SNC80, ARM390, and JNJ20788560 were all able to inhibit migraine-associated allodynia[114]. Furthermore, SNC80 blocked established allodynia in models of chronic migraine, post-traumatic headache, and medication overuse headace[113, 473]. Compared to the mu opioid receptor (MOR) agonist, morphine, and sumatriptan, DOR agonists produced limited medication overuse headache or opioid induced hyperalgesia[113]. The G protein biased DOR agonist, TRV250, has been developed for the treatment of migraine[782]; and is currently undergoing Phase II clinical trial (https://clinicaltrials.gov/ct2/show/NCT04201080). Our data build on these previous findings and demonstrate that KNT-127, a G protein biased DOR agonist, is also able to reverse established migraine-associated allodynia.

DOR agonists have previously been demonstrated to effectively relieve hyperalgesia/allodynia associated with models of chronic inflammatory and neuropathic

pain[114, 466, 783, 784]. KNT-127 has also been shown to be pain-relieving in preclinical models. In acute pain models, this compound effectively inhibited acetic acid induced writhing and formalin paw-licking[775]. Further, in the Complete Freund's Adjuvant (CFA) model of chronic inflammatory pain, KNT-127, blocked both thermal and mechanical hyperalgesia in a dose dependent manner[785]. In addition, KNT-127 also shows anti-depressant and anxiolytic like effects[490, 775, 786], similar to many other DOR agonists including SNC80[469, 787]. Systemic administration of KNT-127[490, 775], as well as injection specifically into the basolateral amygdala[788] produced anxiolytic effects which were blocked by the DOR antagonist naltrindole. We found that KNT-127 was also effective at inhibiting preclinical correlates of migraine-associated pain and aura. These data support the idea that anti-migraine effects are a commonly shared effect of DOR agonists, similar to the pain-relieving and anti-depressant/anxiolytic effects. Furthermore, there is a high co-morbidity between migraine, chronic pain, and/or emotional disorders, and delta agonists may therefore benefit migraine patients on multiple levels.

Approximately a third of migraine patients have their migraine attacks preceded by an aura[6], which is believed to be due to an electrophysiological phenomenon known as cortical spreading depression. CSD is a slowly propagating wave of depolarization that is followed by inhibition of brain activity[158, 160, 161]. Preclinically, models of CSD have been used to predict the validity of potential migraine therapies[210] as many migraine preventive drugs were found to reduce the number of CSD events[229, 789]. We had previously shown that SNC80 could decrease CSD[114], and our current results confirm that this effect is shared by other DOR agonists. Modulation of CSD by

DOR may be occurring at cortical or sub-cortical regions. There is high expression of DOR in the cortex[469, 790, 791]; and in mice ~15-25% of DOR+ cells in the somatosensory cortex are GABAergic[792]. Future studies will focus on identifying the cellular and anatomical sites through which DOR agonists regulate CSD.

Some, but not all, DOR ligands produce receptor internalization[485, 793-795]. SNC80 and a few other DOR agonists have pro-convulsant effects at higher doses, and this effect may be correlated with the ability of an agonist to induce receptor internalization[469, 771]. This ligand specific effect appears to be biased towards engagement of specific arrestin populations [771, 795]; and SNC80 has been shown to have more potent convulsant activity in arrestin 2 knockout mice[472, 795]. While this data may seem to be contradictory, some studies have found that the factor important for driving convulsions and receptor internalization is dependent on which arrestin is engaged. In comparison, KNT-127 does not produce convulsant activity[774, 775], produces little DOR internalization[785], and is a low arrestin recruiter[779]. Our study demonstrated that DOR agonist ability to decrease CSD events was not linked to the internalization of DOR or signaling pathways that are related to pro-convulsant effects, thus suggesting that the DOR modulation of CSD is likely also a G protein mediated effect.

Overall, our data further support the development of DOR agonists for the treatment of migraine. Many small molecule DOR agonists are non-convulsant, and our study shows that the mechanisms that regulate seizurogenic activity are separate from the anti-migraine effects of DOR activation. Importantly, DOR agonists represent a novel

therapeutic target for migraine that is mechanistically distinct; and may not alter endogenous vascular responding.

5. Conclusion

5.1 Introduction

In this thesis I investigated the pathophysiology of chronic migraine disorders to develop better therapeutic targets for their treatment. To do so I used preclinical models of migraine to screen and gain a better understanding of how these conditions progressed and the effectiveness of novel therapeutic targets. Overall, I demonstrated a novel cytoarchitectural mechanism of migraine chronification. Chronic migraine and CSD events induced cytoarchitectural changes in key migraine pain processing regions that could be reversed through HDAC6 inhibition. Interestingly, cytoarchitectural changes were also found in a neuropathic pain model, CRPS indicating the importance of cytoarchitectural regulation in regulating pain. Furthermore, I showed that the biased DOR agonist, KNT-127, could be used to reverse established hyperalgesia induced by chronic NTG treatment as well as reduce CSD events. In this last chapter of the thesis, I will give a more in depth summary of each individual chapter's key discoveries, discuss methodological limitations, and possible future directions to continue building on the established findings.

5.2 Summary

In chapter 2 I discovered and characterized a novel cytoarchitectural modification that correlates with migraine chronification. To achieve this, I used a previously established model of chronic migraine [94]. This model entails repeated intermittent treatment with NTG and produces chronic hypersensitivity. Following completion of the treatment mice were sacrificed and Golgi stain was used to examine the neuronal

cytoarchitecture within some key migraine pain regions, primarily the TNC, vlPAG, and SCx. NTG treated mice had decreased neuronal complexity within these regions. Importantly these changes did not extend to every region as the LSC and NAc were unchanged, demonstrating that these alterations were not due to whole brain NTG toxicity, but rather chronic migraine specific effects. Following these changes I demonstrated that HDAC6 inhibition could be used to increase acetylated α-tubulin and reverse the alterations in cytoarchitecture resulting in an overall increase in neuronal complexity. Interestingly, this recovery of neuronal complexity also coincided with a recovery of the basal hyperalgesia as a single HDAC6 inhibitor dose reversed mechanical threshold changes for over 24 hours.

To further explore these changes in cytoarchitectural complexity we also investigated a mechanistically distinct model of migraine aura. Many migraine preventives have been found to reduce frequency of CSD events in a continual KCl stimulation model [229]. I demonstrated that HDAC6 inhibition could reduce CSD events compared to vehicle controls. This finding indicated that tubulin acetylation and neuronal cytoarchitecture could play a role in regulating CSD as well. Therefore, we examined the cytoarchitecture of mice following repeated CSD events. We found that CSD produced decreased cytoarchitectural complexity of pyramidal neurons in the SCx. Importantly, pretreatment with HDAC6 inhibitor prevented these changes and decreased number of CSDs further demonstrating HDAC6 as a potential migraine therapeutic target. Given these novel findings of cytoarchitecture as a part of migraine pathophysiology, we sought to determine if a common migraine treatment option, olcegepant, could also restore neuronal complexity. We found that NTG again resulted

in decreased cytoarchitecture and that olcegepant treatment reversed these changes. This study demonstrated a novel mechanism for migraine and established HDAC6 inhibition as a promising therapeutic target.

Chapter 3 of this thesis further explored the cytoarchitectural changes that accompany chronic migraine related symptoms. We further explored other brain regions linked to migraine in NTG treated mice. Firstly I found that within the VPM nuclei of the thalamus, a direct projection from the TNC [12], there was increased branch points of neurons. This is especially interesting as it is in sharp contrast to the decrease in the TNC, PAG, and SCx that we observed. I also investigated regions that could be responsible for the affective aspects of pain, including the central amygdala and the basal ganglia, and found that these regions were unchanged following chronic NTG. To further investigate if CSD events had cytoarchitectural effects outside of the SCx, I examined the vlPAG and saw that following CSD this region had decreased neuronal complexity, similar to what was seen in the SCx and TNC. This further demonstrated that the CSD affects are not entirely cortical and can impact other parts of the pain pathway, further implicating the role of CSD outside of strictly migraine aura. This adds to previous data implicating CSD as an important factor in migraine pain outside of just migraine aura.

After building on these novel discoveries in migraine models I wanted to investigate if these changes were seen in a peripheral chronic pain state as well. We investigated CRPS, as it has a very interesting transition from an entirely peripheral pain state to one that is centrally regulated [725]. We first investigated the hippocampus, as neuronal complexity in this region was previously shown to be down regulated following

CRPS [495]. We successfully reproduced these findings showing decreased complexity of hippocampal neurons. We further investigated the vlPAG and found a contrasting increase in neuronal complexity similar to what was seen in the VPM following NTG. Throughout the 2nd and 3rd chapter I demonstrated a variety of cytoarchitectural changes following different models.

Chapter 4 investigated if a non-convulsant biased agonist of DOR would still be effective in reducing chronic migraine associated symptoms. DOR agonists were previously shown to reduce migraine-associated symptoms in preclinical models including reversing established allodynia as well as reducing CSD events. Many DOR agonists have potential to cause convulsions at physiological relevant doses. A biased DOR agonist, KNT-127, was previously shown to not produce convulsions even at 10 times the a behaviorally effective dose [775]. I demonstrated that KNT-127 was also able to reverse the established basal allodynia following chronic migraine. Additionally, KNT-127 was able to reduce CSD events following KCl stimulation. Finally we showed that KNT-127 treatment did not cause internalization of the DOR in key migraine regions, which correlates with previous findings of low internalizing DOR agonists being non-convulsants. These data further establish KNT-127 and non-convulsant DOR agonists in general as therapeutic targets for the treatment of chronic migraine and other pain disorders.

While these chapters all focus on identifying novel treatments for migraine the overall concepts may seem somewhat disparate. I first began investigating the novel chronic migraine model and repeated CSD events and found there is decreased cytoarchitecture in key migraine processing regions. Following this we found that both

HDAC6 inhibition and CGRP receptor antagonism can reverse these changes in cytoarchitecture. Additionally this thesis built on previous work in the Pradhan lab and demonstrated the effectiveness of non-convulsant DOR agonists, KNT-127, to treat both the allodynia following chronic migraine and reduce CSD events. While these concepts all revolve around development of chronic migraine treatments, a model demonstrating a common mechanism has not been presented. A theoretical model linking the delta opioid receptor work to the changes in cytoarchitecture is presented (Figure 25). CGRP activates the CRLR-RAMP1 heterodimer complex, which upon activation can cause increased activity of $G\alpha_s$ [383, 385]. Previous work has shown that activation of Gα can cause destabilization of the GTP bound microtubule cap and this can result in catastrophe, thus decreasing microtubule length [796]. Collectively these data give a possible mechanism through which CGRP inhibitors could reverse changes seen in cytoarchitecture (Figure 25A). DOR agonists have also been shown to reduce the level of CGRP in migraine models indicating a possible mechanism for how they could also be related to cytoarchitectural changes (Figure 25B). Finally our work with HDAC6 inhibitors demonstrates that increased cytoarchitecture is possible through increased acetylation of tubulin (Figure 25C). Collectively these studies outline a possible mechanism for how all of the work in this thesis could regulate microtubule dynamics and in turn chronic migraine mechanisms. More work in the future will be needed to unravel the exact mechanism of these findings.

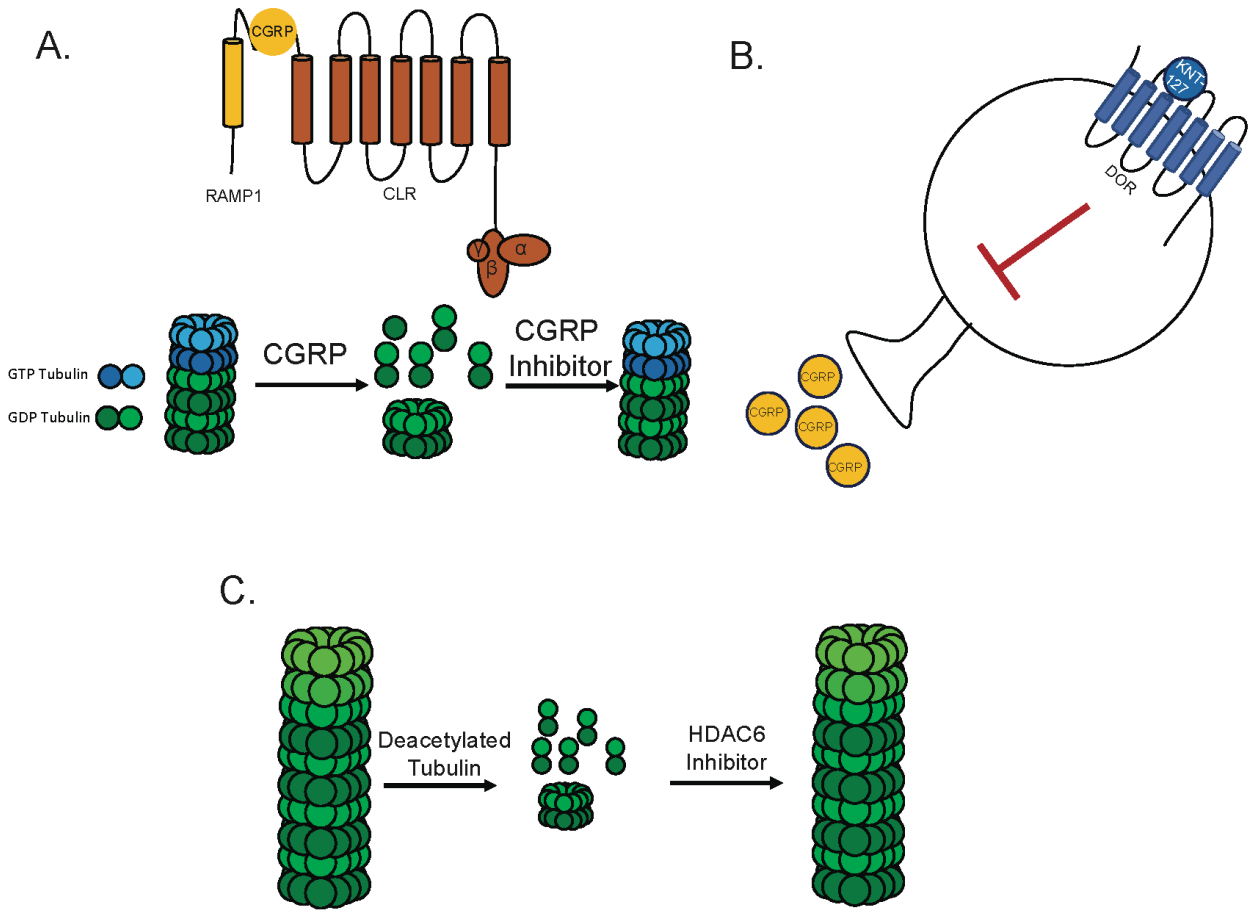

Figure 25 Regulatory mechanisms through CGRP, DOR agonists, and HDAC6 inhibition. Collectively my findings demonstrate a novel cytoarchitectural basis for chronic migraine and show the effectiveness of CGRP antagonist, DOR agonists, and HDAC6 inhibition. (A) CGRP receptor activation could lead to destabilization of the GTP microtubule cap leading to catastrophe. This could be reversed through inhibition of CGRP. (B) DOR agonist, KNT-127, could function to decrease the amount of CGRP that is released which would then further limit the effect that CGRP could have on microtubule disassembly. (C) HDAC6 inhibition was shown to increase tubulin acetylation which in turn could result in stabilized microtubules.

5.3 Limitations

Measuring pain in an animal model presents several difficulties and limitations. In the studies above we use von Frey hair filaments to detect thresholds to mechanical

stimuli in periorbital and hind paw tests. We use the measure of decreased thresholds as a representation of the allodynia that accompanies migraine in patients. However, the decreased mechanical threshold does not necessarily correlate to a migraine attack, or pain in general. Pain is a complex state and cannot be measured by simple von Frey hair mechanical threshold testing [797]. However, the mechanical assay used in this thesis does allow for the detection of basal allodynia, which is a common chronic migraine symptom and has been previously used to model migraine [39, 41]. While the mechanical threshold testing can give us a lot of information, it cannot assess thermal hyperalgesia, which does not always correlate exactly with mechanical hyperalgesia. It is possible that looking solely at mechanical measures could miss some important information that would be unlocked by also looking at other endpoints.

As mentioned previously migraine symptoms extend far beyond the headache phase [661]. While one of these symptoms is frequent whole body and cephalic allodynia there are other symptoms that frequently accompany migraine including photophobia. Future work should integrate animal models to detect more symptoms including a light dark box chamber experiment to examine photophobia or the use of the facial grimace scale to better understand the affective aspects of migraine [798, 799]. A larger understanding of the other aspects would allow for more effective treatment of migraine and a more efficient transition of compounds from a preclinical to a clinical state.

Throughout the study we primarily used the NTG based models to induce migraine like symptomology. While the NTG model has previously been used to gain information about migraine pathophysiology [100], the method is not without flaws. In

these studies the dose of 10 mg/kg was used to induce the chronic migraine state. This dose is substantially higher than the equivalent dose used in humans [90]. This could mean that the NTG is having other effects outside of migraine induction. However, previous work has shown that migraine preventives at human relevant doses have been able to ameliorate the mechanical hyperalgesia following NTG injection [94]. As discussed in Chapter 1 of this thesis, there are many models of migraine which each target different aspects of the disorder. While NTG has been previously established and shown to produce many migraine-associated symptoms, it still does not induce every aspect of a migraine attack. To gain a more well-rounded picture of migraine it is important in future studies to use additional models to ensure that similar pathophysiological changes accompany other migraine models.

CSD was used throughout this thesis to model migraine aura. As discussed above there are several ways to induce CSD events including electrical or mechanical stimulation. We chose to use the KCl model as it previously has been successfully used to screen preclinical compounds [114, 229]. However, like the NTG model, CSD induction through KCl has some unique properties that are not necessarily seen in other models. Additionally we used an anesthetized model and anesthesia has been previously shown to potentially effect CSD induction [210]. To avoid this, we always used vehicle treated animals as controls under the same conditions, but still this could be a confounding variable. Previous studies have used a threshold model to establish susceptibility to migraine [230]. We used a suprathreshold continuous supply of KCl and examined the total number of CSD events that occurred in both our HDAC6 and

KNT-127 experiments. Future studies should also examine how these compounds affect the threshold to induce a CSD event to gain a more complete picture.

Golgi staining method was used in many of the experiments to examine the cytoarchitecture of the neurons in the regions of interest. While Golgi method has been used for centuries to study cytoarchitecture it is not without limitations. While there have been some modern adaptations of the Golgi method; the overall principles remain the same from when the method was first used [800-803]. The method selectively visualizes the entire architecture of a neuron and gives a clear background while thousands of neurons next to it remain unstained. The reaction works through chromium salts binding to proteins in the neuron that then are transformed to black mercuric sulfide deposits upon alkali treatment [804-806]. The result is clearly stained soma, dendrites, axons, and spines that can be easily visualized under a bright field microscope. The Golgi method stains neurons sporadically and it is still unclear why some neurons are stained and others are not [807]. Since it randomly selects neurons and only stains a small subsection of the population it is difficult to selectively stain a minority population of neurons [807]. The Golgi staining process makes the tissue extremely light sensitive and can quickly become damaged after just a brief amount of light. Additionally, while it has occasionally been done, using immunohistochemical techniques in combination with Golgi stain has largely been unsuccessful [752]. Since the immunohistochemical staining cannot be combined with the Golgi stain it is difficult to gather more information about the cellular population being examined aside from purely morphological analysis. This greatly limits the information that can be gained using the Golgi method. In the

future directions I will discuss other techniques that could be used to overcome this disadvantage and alternatives for future studies.

5.4 Future Directions

Alternatives to Golgi Stain

Golgi staining method while widely used is not without its limitations. One major limitation discussed throughout the thesis is the inability of Golgi method to be used with immunohistochemical techniques. This limits the information about the particular cell population and makes it difficult to gather deeper knowledge on how cell morphology changes may interact the surrounding neuronal environment. I will briefly discuss other staining methods that can be used to effectively stain neuronal populations and how these could be implicated in future studies.

An alternative method to Golgi staining that is commonly used is intracellular injection of staining agent. Lucifer Yellow or biocytin can be delivered into the cell following electrical recording [808]. Injection of the labeling agent allows for a morphological reconstruction to accompany the previously recorded electrophysiological data. This technique also has disadvantages as it can only be done in slice preparations, which can sever the axon fibers limiting the full arborization morphological data. This technique is very time consuming and only a single neuron can be done at once making it very difficult to label large populations.

Electroporation and transfection methods allow for neuronal morphological labeling that does not have many of the disadvantages seen in the Golgi staining method [805, 809]. Electroporation is the application of an electric pulse that disrupts

the plasma membrane and allows for DNA or other molecules to be forced inside the cell allowing for labeling [810]. Electroporation and transfection is commonly done by administering dye to individual cells via microinjection through intracellular or patch pipettes. This method allows for comprehensive labeling of a single cell, but it is very technically demanding and can result in sampling bias [809, 811-813]. Another commonly used option is neuronal transfection, which uses DNA constructs to target cells and tissue. Biolistic delivery uses a gene gun and a pressurized release of gas to shoot the DNA-coated micro-particles into tissue that cross the plasma membrane and targets the cell [814]. This method is much simpler than the conventional intracellular injections [815]. However, it must be done in living tissue samples to allow for replication of the DNA and transfection. One way around this is the use of diolistic labeling of live or fixed neurons [816]. Diolistic labeling uses the lipophilic fluorescent dye dialkylcarbocyanine (Dil). Dil was first used as an anterograde and retrograde tracer, but it can also be used to fluorescently label neuronal cell membranes [816]. Dil coated micro-carriers are delivered in a ballistic way to fixed or cultured tissue slices where it is incorporated in the cellular membrane. The lateral diffusion of the Dil allows for movement into the plasma membrane providing fluorescent labeling of the entire cell. Often ballistically delivered using gas Dil-coated particles are embedded in various neurons of tissue [817]. The micro-carriers can travel into the soma while the Dil stays in the neuronal membrane. This allows Dil to diffuse through the cellular membrane of a single neuron fluorescently labeling the neuronal architecture [817].

Virus vectors are another common approach to selectively label neurons. Genetically encoded viral dyes can be transgenically labeled that are non-invasive and

can provide stable labeling of neurons in reproducible patterns across animals. Green fluorescent protein (GFP) was first used to label sensory neurons of the C. elegans [818]. This has been expanded to fruit flies and the use of Flp-out allows for sparse labeling of neurons by exposing some to heat shock producing Flp expression which then expresses GFP in a small subset of neurons [819, 820]. In mice a similar method using position effect variegation can cause line-to-line variations in transgene expression [821]. This allows for mice expressing GFP under a modified protein and has a small subset of neurons labeled [822]. Another common way is to use the GFP under an inducible form of Cre recombinase. This can allow for dose-dependent labeling of cells [823, 824]. While these viral vectors allow for mass labeling of neurons, the clarity of the neurons are lost compared to Golgi stain.

Labeling using multiple distinct labels distributed to different cells allows for labeling and analysis of many cells even when they are in close proximity to one another [825]. Fluorescent proteins (FPs) use genetically encoded labels that can be restricted to specific cell types or areas that can rely on promoters or sequences. FPs can be amplified using a number of approaches including antibodies, fusion proteins, or chromogenic enzymes [823, 826, 827]. To induce specific gene modifications site-specific recombinases like Cre and Flp are commonly used [828]. The recombinases catalyze DNA recombination between a pair of short specific sequences. These can be used to arrange different target sequences and result in excision, inversion, or intermolecular exchange [825]. The site-specific recombinase described here is an all or none approach but use of ligand-activated CreER recombinase allows for alteration of the expression level, providing a more fine tune labeling [828]. In general, this approach

combines the advantages of genetic targeting and random labeling creating sparse expression within a genetically defined domain.

Spaghetti monster fluorescent proteins (smFPs) are highly antigenic molecules based on GFP like fluorescent proteins that contain numerous copies of peptide epitopes and bind IgG antibodies [829]. smFPs were found to be fully distributed into neurons including the axons, dendrites, and even spines. Furthermore, by varying epitope and scaffolds a diverse family of mutually orthogonal antigens can be generated [829]. smFPs were found to perform much better than standard labeling techniques to investigate low abundance proteins. They also allow for increased number of simultaneous imaging channels that can be used at once [829]. Use of several of these techniques would allow for cell population specific information to be gained. Future studies could be conducted to investigate how specific populations change following chronic pain states.

HDAC6 Modulation

Throughout the second chapter of the thesis I highlighted the importance of HDAC6 in regulating cytoarchitectural dynamics. I further showed how HDAC6 inhibition can be used to reverse or prevent some cytoarchitectural changes associated with chronic migraine models. Building on these findings it would be interesting to examine factors that endogenously regulate HDAC6 and how manipulating these factors can affect tubulin dynamics and cytoarchitecture. While HDAC6 can interact and deacetylate several other proteins within the cell, it can also be regulated. Glycogen synthase kinase 3β (GSK-3β) is a cytoplasmic serine/threonine kinase and is important for many cellular processes including survival and apoptosis [830]. GSK-3β is widely distributed

in the brain [831]. Many of GSK-3β substrates are cytoskeleton-related proteins [832] and inhibition of GSK-3β results in increased acetylated tubulin as well as kinesin-1 association with mitochondria [833]. GSK-3β inhibition resulted in a slight decrease in HDAC6 protein, but a much larger decrease of deacetylase activity. Conversely enhancement of GSK-3β resulted in increased activity of HDAC6 [833]. HDAC6 and GSK-3β were found to co-localize in hippocampal neurons. Further HDAC6 can be phosphorylated at the serine-22 residue [834]. Importantly, inhibition of GSK-3β resulted in decreased phosphorylation of this residue and resulted in increased acetylated microtubules [833]. This data demonstrates that HDAC6 phosphorylation can have direct impact on its acetylation capability. Examining GSK-3β following chronic pain models could reveal a molecular basis for how chronic pain states may alter cytoarchitecture.

GPCR kinase 2 (GRK2) has also been found to modulate a number of signaling sensors in phosphorylation dependent and independent manner [835]. Decreased expression of GRK2 in MEFs have enhanced tubulin acetylation compared to wildtype MEFs, however these changes were not accompanied by changes in expression of HDAC6 or SIRT2 [836]. HDAC6 was found to co-immunoprecipitate with GRK2 in the cytoplasm [836]. GRK2 was further found to phosphorylate the second catalytic domain of HDAC6 and mutants of GRK2 that cannot phosphorylate HDAC6 showed increased acetylated microtubules [836]. Protein Kinase Cs (PKCs) are known to play roles in cytoskeleton regulation and are involved with cell polarization, directional sensing, and cell motility [837]. Multiple PKCs including PKC α and PKC ζ were found to form protein complexes with HDAC6 and phosphorylate HDAC6 [838]. Increased phosphorylation of

HDAC6 through PKC ζ was found to result in increased deacetylase activity and reduced acetylated tubulin levels [838]. Protein kinase CK2 was also found to phosphorylate HDAC6 and increase HDAC6 deacetylase activity within the cytoplasm and the phosphorylation through CK2 was found to be necessary for proper aggresome formation [839]. Collectively these data demonstrate the importance of phosphorylation of HDAC6 on its proper function. Future studies examining the relationship between HDAC6 phosphorylation and alterations in cytoarchitecture could produce effective therapeutic treatment options for chronic pain.

Chapter 1 revealed that a common migraine therapeutic, Olcegepant, was not only able to relieve allodynia from the chronic NTG model, but was also able to reverse cytoarchitectural changes. This opens up some very interesting possibilities for how migraine therapeutics may work. It is possible that migraine therapeutics could produce undiscovered cytoarchitectural changes and this could be key to their effectiveness in treating migraine. Future work could be done to examine if this trend holds true for other therapeutics or if this is solely a HDAC6 inhibitor and Olcegepant effect. In this thesis we showed that KNT-127 was capable of reversal of allodynia as well as reduction of CSD events. Further work should be conducted to further explore the relation of DOR agonists and other migraine therapeutics on the effect on tubulin acetylation and cytoarchitectural changes.

5.5 Concluding Remarks

The overarching aim of this thesis was to investigate novel therapeutic targets for the treatment of chronic pain disorders. Chapter 2 demonstrated a cytoarchitectural basis for chronic migraine as well as the use of HDAC6 inhibitors as a novel therapeutic

target. Chapter 3 built on these findings and showed that cytoarchitectural changes are widespread across the mouse brain following chronic migraine and can also be seen in another chronic pain state, CRPS. Chapter 4 used a non-convulsant DOR agonist and revealed that a biased agonist with a greater therapeutic window can still effectively be used to reverse migraine-associated symptoms. Overall the work in this thesis establishes the use of HDAC6 inhibitors for migraine treatment, that cytoarchitectural changes persist in multiple pain states, and that a non-convulsant DOR agonist can still effectively treat migraine. Future experiments could be conducted to better understand the neuronal population changes associated with chronic pain disorders. Further studies could also examine the changes in HDAC6 regulation and use these as targets for treatment for chronic pain disorders

References

1. Castillo, J., et al., *Kaplan Award 1998. Epidemiology of chronic daily headache in the general population.* Headache, 1999. **39**(3): p. 190-6.
2. Blumenfeld, A.M., et al., *Patterns of use and reasons for discontinuation of prophylactic medications for episodic migraine and chronic migraine: results from the second international burden of migraine study (IBMS-II).* Headache, 2013. **53**(4): p. 644-55.
3. *Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016.* Lancet, 2017. **390**(10100): p. 1211-1259.
4. Stewart, W.F., A. Shechter, and B.K. Rasmussen, *Migraine prevalence. A review of population-based studies.* Neurology, 1994. **44**(6 Suppl 4): p. S17-23.
5. Bonafede, M., et al., *Direct and Indirect Healthcare Resource Utilization and Costs Among Migraine Patients in the United States.* Headache, 2018. **58**(5): p. 700-714.
6. Headache Classification Committee of the International Headache, S., *The International Classification of Headache Disorders, 3rd edition (beta version).* Cephalalgia, 2013. **33**(9): p. 629-808.
7. Scher, A.I., et al., *Prevalence of frequent headache in a population sample.* Headache, 1998. **38**(7): p. 497-506.
8. Bigal, M.E., et al., *Chronic migraine in the population burden, diagnosis, and satisfaction with treatment.* Neurology 2008. **71**: p. 559-566.
9. Stovner, L.J., et al., *The global burden of headache: a documentation of headache prevalence and disability worldwide.* Cephalalgia, 2007. **27**: p. 193-210.
10. Lipton, R.B., *Tracing transformation: chronic migraine classification, progression, and epidemiology.* Neurology, 2009. **72**(5 Suppl): p. S3-7.
11. Dodick, D.W., et al., *Assessing Barriers to Chronic Migraine Consultation, Diagnosis, and Treatment: Results From the Chronic Migraine Epidemiology and Outcomes (CaMEO) Study.* Headache, 2016. **56**(5): p. 821-834.
12. Burstein, R., R. Noseda, and D. Borsook, *Migraine: multiple processes, complex pathophysiology.* J Neurosci, 2015. **35**(17): p. 6619-29.
13. Uddman, R., et al., *Innervation of the feline cerebral vasculature by nerve fibers containing calcitonin gene-related peptide: trigeminal origin and co-existence with substance P.* Neuroscience Letters, 1985. **62**: p. 131-136.
14. Liu, Y., J. Broman, and L. Edvinsson, *Central projections of sensory innervation of the rat superior sagittal sinus.* Neuroscience, 2004. **129**(2): p. 431-7.
15. Davis, K.D. and J.O. Dostrovsky, *Responses of Feline Trigeminal Spinal Tract Nucleus Neurons to Stimulation of the Middle Meningeal Artery and Sagital Sinus.* journal of Neurophsyiology, 1988. **59**(2): p. 648-666.
16. Malick, A., R.M. Strassman, and R. Burstein, *Trigeminohypothalamic and reticulohypothalamic tract neurons in the upper cervical spinal cord and caudal medulla of the rat.* J Neurophysiol, 2000. **84**(4): p. 2078-112.
17. Giffin, N.J., et al., *Premonitory symptoms in migraine: an electronic diary study.* Neurology, 2003. **60**(6): p. 935-40.
18. Maniyar, F.H., et al., *Brain activations in the premonitory phase of nitroglycerin-triggered migraine attacks.* Brain, 2014. **137**(Pt 1): p. 232-41.
19. Schulte, L.H., A. Allers, and A. May, *Hypothalamus as a mediator of chronic migraine: Evidence from high-resolution fMRI.* Neurology, 2017. **88**(21): p. 2011-2016.

20. Burstein, R. and M. Jakubowski, *Unitary hypothesis for multiple triggers of the pain and strain of migraine.* J Comp Neurol, 2005. **493**(1): p. 9-14.
21. Shechter, A., et al., *Migraine and autonomic nervous system function: a population-based, case-control study.* Neurology, 2002. **58**(3): p. 422-7.
22. Hosoya, Y., M. Matsushita, and Y. Sugiura, *A direct hypothalamic projection to the superior salivatory nucleus neurons in the rat. A study using anterograde autoradiographic and retrograde HRP methods.* Brain Res, 1983. **266**(2): p. 329-33.
23. Hosoya, Y., M. Matsushita, and Y. Sugiura, *Hypothalamic descending afferents to cells of origin of the greater petrosal nerve in the rat, as revealed by a combination of retrograde HRP and anterograde autoradiographic techniques.* Brain Res, 1984. **290**(1): p. 141-5.
24. Tucker, D.C. and C.B. Saper, *Specificity of spinal projections from hypothalamic and brainstem areas which innervate sympathetic preganglionic neurons.* Brain Res, 1985. **360**(1-2): p. 159-64.
25. Loewy, A.D. and K.M. Spyer, *Central regulation of autonomic functions*. 1990: Oxford University Press.
26. Dampney, R., *The hypothalamus and autonomic regulation: an overview.* Central regulation of autonomic functions, 2011: p. 47-61.
27. Akerman, S., et al., *A translational in vivo model of trigeminal autonomic cephalalgias: therapeutic characterization.* Brain, 2012. **135**(Pt 12): p. 3664-75.
28. Noseda, R., et al., *Neurochemical pathways that converge on thalamic trigeminovascular neurons: potential substrate for modulation of migraine by sleep, food intake, stress and anxiety.* PLoS One, 2014. **9**(8): p. e103929.
29. McCormick, D.A., *Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity.* Prog Neurobiol, 1992. **39**(4): p. 337-88.
30. Sherman, S.M. and R.W. Guillery, *On the actions that one nerve cell can have on another: distinguishing "drivers" from "modulators".* Proc Natl Acad Sci U S A, 1998. **95**(12): p. 7121-6.
31. Sherman, S.M., *Thalamic relays and cortical functioning.* Prog Brain Res, 2005. **149**: p. 107-26.
32. Saper, C.B., et al., *Sleep state switching.* Neuron, 2010. **68**(6): p. 1023-42.
33. Saper, C.B., T.E. Scammell, and J. Lu, *Hypothalamic regulation of sleep and circadian rhythms.* Nature, 2005. **437**(7063): p. 1257-63.
34. Borsook, D., et al., *Understanding migraine through the lens of maladaptive stress responses: a model disease of allostatic load.* Neuron, 2012. **73**(2): p. 219-34.
35. Noseda, R., et al., *Cortical projections of functionally identified thalamic trigeminovascular neurons: implications for migraine headache and its associated symptoms.* J Neurosci, 2011. **31**(40): p. 14204-17.
36. Coppola, G., F. Pierelli, and J. Schoenen, *Is the cerebral cortex hyperexcitable or hyperresponsive in migraine?* Cephalalgia, 2007. **27**(12): p. 1427-39.
37. Noseda, R. and R. Burstein, *Migraine pathophysiology: Anatomy of the trigeminovascular pathway and associated neurological symptoms, cortical spreading depression, sensitization, and modulation of pain.* Pain, 2013. **154**: p. S44-S53.
38. Strassman, A.M., S.A. Raymond, and R. Burstein, *Sensitization of meningeal sensory neurons and the origin of headaches.* Nature, 1996. **384**(6609): p. 560-4.
39. Burstein, R., et al., *Thalamic sensitization transforms localized pain into widespread allodynia.* Ann Neurol, 2010. **68**(1): p. 81-91.
40. Burstein, R., et al., *Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons.* J Neurophysiol, 1998. **79**(2): p. 964-82.
41. Burstein, R., M.F. Cutrer, and D. Yarnitsky, *The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine.* Brain, 2000. **123 (Pt 8)**: p. 1703-9.

42. Lipton, R.B., et al., *Cutaneous allodynia in the migraine population.* Ann Neurol, 2008. **63**(2): p. 148-58.
43. Levy, D., M. Jakubowski, and R. Burstein, *Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT 1B/1D receptor agonists.* Proc Natl Acad Sci U S A, 2004. **101**(12): p. 4274-9.
44. Burstein, R., B. Collins, and M. Jakubowski, *Defeating migraine pain with triptans: a race against the development of cutaneous allodynia.* Ann Neurol, 2004. **55**(1): p. 19-26.
45. Blau, J.N., *Migraine postdromes: symptoms after attacks.* Cephalalgia, 1991. **11**(5): p. 229-31.
46. Giffin, N.J., et al., *The migraine postdrome: An electronic diary study.* Neurology, 2016. **87**(3): p. 309-13.
47. Kelman, L., *The postdrome of the acute migraine attack.* Cephalalgia, 2006. **26**(2): p. 214-20.
48. Lauritzen, M., *Long-lasting reduction of cortical blood flow of the brain after spreading depression with preserved autoregulation and impaired CO2 response.* J Cereb Blood Flow Metab, 1984. **4**(4): p. 546-54.
49. Stewart, W.F., et al., *Familial risk of migraine: variation by proband age at onset and headache severity.* Neurology, 2006. **66**(3): p. 344-8.
50. Anttila, V., et al., *Genome-wide association study of migraine implicates a common susceptibility variant on 8q22.1.* Nat Genet, 2010. **42**(10): p. 869-73.
51. Anttila, V., et al., *Genome-wide meta-analysis identifies new susceptibility loci for migraine.* Nat Genet, 2013. **45**(8): p. 912-917.
52. Chasman, D.I., et al., *Genome-wide association study reveals three susceptibility loci for common migraine in the general population.* Nat Genet, 2011. **43**(7): p. 695-8.
53. Freilinger, T., et al., *Genome-wide association analysis identifies susceptibility loci for migraine without aura.* Nat Genet, 2012. **44**(7): p. 777-82.
54. Llinas, R.R., *The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function.* Science, 1988. **242**(4886): p. 1654-64.
55. Steriade, M. and R.R. Llinas, *The functional states of the thalamus and the associated neuronal interplay.* Physiol Rev, 1988. **68**(3): p. 649-742.
56. Steriade, M., D.A. McCormick, and T.J. Sejnowski, *Thalamocortical oscillations in the sleeping and aroused brain.* Science, 1993. **262**(5134): p. 679-85.
57. Fuggetta, G. and N.A. Noh, *A neurophysiological insight into the potential link between transcranial magnetic stimulation, thalamocortical dysrhythmia and neuropsychiatric disorders.* Exp Neurol, 2013. **245**: p. 87-95.
58. Bahra, A., et al., *Brainstem activation specific to migraine headache.* Lancet, 2001. **357**(9261): p. 1016-7.
59. Coppola, G. and J. Schoenen, *Cortical excitability in chronic migraine.* Curr Pain Headache Rep, 2012. **16**(1): p. 93-100.
60. Coppola, G., et al., *Habituation and sensitization in primary headaches.* J Headache Pain, 2013. **14**: p. 65.
61. Weiller, C., et al., *Brain stem activation in spontaneous human migraine attacks.* Nat Med, 1995. **1**(7): p. 658-60.
62. Moulton, E.A., et al., *Interictal dysfunction of a brainstem descending modulatory center in migraine patients.* PLoS One, 2008. **3**(11): p. e3799.
63. Cao, Y., et al., *Functional MRI-BOLD of brainstem structures during visually triggered migraine.* Neurology, 2002. **59**(1): p. 72-8.
64. Denuelle, M. and N. Fabre, *Functional neuroimaging of migraine.* Rev Neurol (Paris), 2013. **169**(5): p. 380-9.

65. Afridi, S.K., et al., *A positron emission tomographic study in spontaneous migraine.* Arch Neurol, 2005. **62**(8): p. 1270-5.
66. Moulton, E.A., et al., *Painful heat reveals hyperexcitability of the temporal pole in interictal and ictal migraine States.* Cereb Cortex, 2011. **21**(2): p. 435-48.
67. Coppola, G., F. Pierelli, and J. Shoenen, *Is the cerebral cortex hypereexcitable or hyperresponsive in migraine?* Cephalalgia, 2007. **27**: p. 1429-1439.
68. Coppola, G., F. Pierelli, and J. Schoenen, *Habituation and migraine.* Neurobiol Learn Mem, 2009. **92**(2): p. 249-59.
69. DaSilva, A.F., et al., *Thickening in the somatosensory cortex of patients with migraine.* Neurology, 2007. **69**(21): p. 1990-5.
70. Hadjikhani, N., *Relevance of cortical thickness in migraine sufferers.* Expert Rev Neurother, 2008. **8**(3): p. 327-9.
71. Maleki, N., et al., *Concurrent functional and structural cortical alterations in migraine.* Cephalalgia, 2012. **32**(8): p. 607-20.
72. Maleki, N., et al., *Migraine attacks the Basal Ganglia.* Mol Pain, 2011. **7**: p. 71.
73. Valfre, W., et al., *Voxel-based morphometry reveals gray matter abnormalities in migraine.* Headache, 2008. **48**(1): p. 109-17.
74. Maleki, N., et al., *Common hippocampal structural and functional changes in migraine.* Brain Struct Funct, 2013. **218**(4): p. 903-12.
75. Apkarian, A.V., et al., *Chronic back pain is associated with decreased prefrontal and thalamic gray matter density.* J Neurosci, 2004. **24**(46): p. 10410-5.
76. Baliki, M.N., et al., *Beyond feeling: chronic pain hurts the brain, disrupting the default-mode network dynamics.* J Neurosci, 2008. **28**(6): p. 1398-403.
77. Baliki, M.N., et al., *Brain morphological signatures for chronic pain.* PLoS One, 2011. **6**(10): p. e26010.
78. Rodriguez-Raecke, R., et al., *Brain gray matter decrease in chronic pain is the consequence and not the cause of pain.* J Neurosci, 2009. **29**(44): p. 13746-50.
79. Maleki, N., et al., *Her versus his migraine: multiple sex differences in brain function and structure.* Brain, 2012. **135**(Pt 8): p. 2546-59.
80. Borsook, D., et al., *Sex and the migraine brain.* Neurobiol Dis, 2014. **68**: p. 200-14.
81. Ophoff, R.A., et al., *Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4.* Cell, 1996. **87**(3): p. 543-52.
82. Ducros, A., et al., *The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel.* N Engl J Med, 2001. **345**(1): p. 17-24.
83. De Fusco, M., et al., *Haploinsufficiency of ATP1A2 encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2.* Nat Genet, 2003. **33**(2): p. 192-6.
84. Dichgans, M., et al., *Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine.* Lancet, 2005. **366**(9483): p. 371-7.
85. Brennan, K.C., et al., *Casein kinase idelta mutations in familial migraine and advanced sleep phase.* Sci Transl Med, 2013. **5**(183): p. 183ra56.
86. Hughes, R., *A Cyclopaedia of drug pathogenesy*. Vol. 1. 1886: Gould.
87. Iversen, H.K., J. Olesen, and P. Tfelt-Hansen, *Intravenous nitroglycerin as an experimental model of vascular headache. Basic characteristics.* Pain, 1989. **38**: p. 17-24.
88. Christiansen, I., et al., *Glyceryl trinitrate induces attacks of migraine without aura in sufferers of migraine with aura.* Cephalalgia, 1999. **19**: p. 660-607.
89. Afridi, S.K., H. Kaube, and P.J. Goadsby, *Glyceryl trinitrate triggers premonitory symptoms in migraineurs.* Pain, 2004. **110**(3): p. 675-80.

90. Olesen, J., *The role of nitric oxide (NO) in migraine, tension-type headache and cluster headache.* Pharmacol Ther, 2008. **120**(2): p. 157-71.
91. Moncada, S., R.M. Palmer, and E.A. Higgs, *Nitric oxide: physiology, pathophysiology, and pharmacology.* Pharmacol Rev, 1991. **43**(2): p. 109-42.
92. Thomsen, L.L., et al., *A nitric oxide donor (nitroglycerin) triggers genuine migraine attacks.* Eur J Neurol, 1994. **1**(1): p. 73-80.
93. Lassen, L. and M. Ashina, *Nitric oxide synthase inhibition in migraine.* The Lancet, 1997. **349**(9049): p. 401-402.
94. Pradhan, A.A., et al., *Characterization of a Novel Model of Chronic Migraine.* Pain, 2014. **155**(2): p. 269-274.
95. Koulchitsky, S., et al., *Biphasic response to nitric oxide of spinal trigeminal neurons with meningeal input in rat--possible implications for the pathophysiology of headaches.* J Neurophysiol, 2004. **92**(3): p. 1320-8.
96. Pardutz, A., et al., *Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis.* NeuroReport, 2000. **11**(14): p. 3071-3075.
97. Pardutz, A., et al., *Effect of systemic nitroglycerin on CGRP and 5-HT afferents to rat caudal spinal trigeminal nucleus and its modulation by estrogen.* Eur J Neurosci, 2002. **15**(11): p. 1803-9.
98. Tassorelli, C., et al., *Nitroglycerin-induced activation of monoaminergic transmission in the rat.* Cephalalgia, 2002. **22**(3): p. 226-32.
99. Tassorelli, C., et al., *Nitroglycerin enhances cGMP expression in specific neuronal and cerebrovascular structures of the rat brain.* J Chem Neuroanat, 2004. **27**(1): p. 23-32.
100. Aissa, M.B., et al., *Soluble guanylyl cyclase is a critical regulator of migraine-associated pain.* Cephalalgia, 2017. **0**(0): p. 1-14.
101. Read, S.J., et al., *Enhanced nitric oxide release during cortical spreading depression following infusion of glyceryl trinitrate in the anaesthetized cat.* Cephalalgia, 1997. **17**(3): p. 159-65.
102. Read, S.J., et al., *Effects of sumatriptan on nitric oxide and superoxide balance during glyceryl trinitrate infusion in the rat. Implications for antimigraine mechanisms.* Brain Res, 1999. **847**(1): p. 1-8.
103. Reuter, U., et al., *Delayed inflammation in rat meninges: implications for migraine pathophysiology.* Brain, 2001. **124**: p. 2490-2502.
104. Reuter, U., et al., *Nuclear factor-kappaB as a molecular target for migraine therapy.* Ann Neurol, 2002. **51**(4): p. 507-16.
105. Knyihár-Csillik, E., et al., *Effects of eletriptan on the peptidergic innervation of the cerebral dura mater and trigeminal ganglion, and on the expression of c-fos and c-jun in the trigeminal complex of the rat in an experimental migraine model.* Eur J Neurosci, 2000. **12**(11): p. 3991-4002.
106. Srikiatkhachorn, A., et al., *2002 Wolff Award. 5 -HT2A receptor activation and nitric oxide synthesis: a possible mechanism determining migraine attacks.* Headache, 2002. **42**(7): p. 566-74.
107. Suwattanasophon, C., P. Phansuwan-Pujito, and A. Srikiatkhachorn, *5-HT(1B/1D) serotonin receptor agonist attenuates nitroglycerin-evoked nitric oxide synthase expression in trigeminal pathway.* Cephalalgia, 2003. **23**(8): p. 825-32.
108. Pradhan, A.A., Z. Bertels, and S. Akerman, *Targeted Nitric Oxide Synthase Inhibitors for Migraine.* Neurotherapeutics, 2018. **15**(2): p. 391-401.
109. Markovics, A., et al., *Pituitary adenylate cyclase-activating polypeptide plays a key role in nitroglycerol-induced trigeminovascular activation in mice.* Neurobiol Dis, 2012. **45**(1): p. 633-44.

110. Greco, R., et al., *Temporal profile of vascular changes induced by systemic nitroglycerin in the meningeal and cortical districts.* Cephalalgia, 2011. **31**(2): p. 190-8.
111. Bates, E.A., et al., *Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice.* Cephalalgia, 2010. **30**(2): p. 170-8.
112. Capuano, A., et al., *Correlation between algogenic effects of calcitonin-gene-related peptide (CGRP) and activation of trigeminal vascular system, in an in vivo experimental model of nitroglycerin-induced sensitization.* European journal of pharmacology, 2014. **740**: p. 97-102.
113. Moye, L.S., et al., *Delta opioid receptor agonists are effective for multiple types of headache disorders.* Neuropharmacology, 2019. **148**: p. 77-86.
114. Pradhan, A.A., et al., *delta-Opioid receptor agonists inhibit migraine-related hyperalgesia, aversive state and cortical spreading depression in mice.* Br J Pharmacol, 2014. **171**(9): p. 2375-84.
115. Tipton, A.F., et al., *The effects of acute and preventive migraine therapies in a mouse model of chronic migraine.* Cephalalgia, 2016. **36**(11): p. 1048-1056.
116. Tassorelli, C. and S.A. Joseph, *NADPH-diaphorase activity and Fos expression in brain nuclei following nitroglycerin administration.* Brain Res, 1995. **695**(1): p. 37-44.
117. Strassman, A.M. and B.P. Vos, *Somatotopic and laminar organization of fos-like immunoreactivity in the medullary and upper cervical dorsal horn induced by noxious facial stimulation in the rat.* J Comp Neurol, 1993. **331**(4): p. 495-516.
118. Brennan, K.C., et al., *Casein Kinase Id Mutations in Familial Migraine and Advanced Sleep Phase.* Science Translational Medicine, 2013. **5**(183): p. 1-11.
119. De Vries, P., C.M. Villalón, and P.R. Saxena, *Pharmacological aspects of experimental headache models in relation to acute antimigraine therapy.* Eur J Pharmacol, 1999. **375**(1-3): p. 61-74.
120. Heyck, H., *Pathogenesis of Migraine1*, in *Pain and Headache*. 1969, Karger Publishers. p. 1-28.
121. Bergerot, A., et al., *Animal models of migraine: looking at the component parts of a complex disorder.* Eur J Neurosci, 2006. **24**(6): p. 1517-34.
122. Ishida, T., et al., *Serotonin-induced hypercontraction through 5-hydroxytryptamine 1B receptors in atherosclerotic rabbit coronary arteries.* Circulation, 2001. **103**(9): p. 1289-95.
123. Franco-Cereceda, A., A. Rudehill, and J.M. Lundberg, *Calcitonin gene-related peptide but not substance P mimics capsaicin-induced coronary vasodilation in the pig.* Eur J Pharmacol, 1987. **142**(2): p. 235-43.
124. Petersen, K.A., et al., *Presence and function of the calcitonin gene-related peptide receptor on rat pial arteries investigated in vitro and in vivo.* Cephalalgia, 2005. **25**(6): p. 424-32.
125. Williamson, D.J., et al., *Intravital microscope studies on the effects of neurokinin agonists and calcitonin gene-related peptide on dural vessel diameter in the anaesthetized rat.* Cephalalgia, 1997. **17**(4): p. 518-24.
126. Williamson, D.J., et al., *Sumatriptan inhibits neurogenic vasodilation of dural blood vessels in the anaesthetized rat--intravital microscope studies.* Cephalalgia, 1997. **17**(4): p. 525-31.
127. Akerman, S., et al., *Nitric oxide synthase inhibitors can antagonize neurogenic and calcitonin gene-related peptide induced dilation of dural meningeal vessels.* Br J Pharmacol, 2002. **137**(1): p. 62-8.
128. Petersen, K.A., et al., *Inhibitory effect of BIBN4096BS on cephalic vasodilatation induced by CGRP or transcranial electrical stimulation in the rat.* Br J Pharmacol, 2004. **143**(6): p. 697-704.
129. Williamson, D.J., et al., *The novel anti-migraine agent rizatriptan inhibits neurogenic dural vasodilation and extravasation.* Eur J Pharmacol, 1997. **328**(1): p. 61-4.
130. Williamson, D.J., et al., *The anti-migraine 5-HT(1B/1D) agonist rizatriptan inhibits neurogenic dural vasodilation in anaesthetized guinea-pigs.* Br J Pharmacol, 2001. **133**(7): p. 1029-34.

131. Williamson, D.J., et al., *Role of opioid receptors in neurogenic dural vasodilation and sensitization of trigeminal neurones in anaesthetized rats.* Br J Pharmacol, 2001. **133**(6): p. 807-14.
132. Akerman, S., et al., *The effect of adrenergic compounds on neurogenic dural vasodilatation.* Eur J Pharmacol, 2001. **424**(1): p. 53-8.
133. Williamson, D.J. and R.J. Hargreaves, *Neurogenic inflammation in the context of migraine.* Microsc Res Tech, 2001. **53**(3): p. 167-78.
134. Akerman, S., et al., *The effect of anti-migraine compounds on nitric oxide-induced dilation of dural meningeal vessels.* Eur J Pharmacol, 2002. **452**(2): p. 223-8.
135. Akerman, S., H. Kaube, and P.J. Goadsby, *Anandamide acts as a vasodilator of dural blood vessels in vivo by activating TRPV1 receptors.* Br J Pharmacol, 2004. **142**(8): p. 1354-60.
136. Jacobs, B. and G. Dussor, *Neurovascular contributions to migraine: Moving beyond vasodilation.* Neuroscience, 2016. **338**: p. 130-144.
137. Hunt, S.P., A. Pini, and G. Evan, *Induction of c-fos-like protein in spinal cord neurons following sensory stimulation.* Nature, 1987. **328**(6131): p. 632-4.
138. Clayton, J.S., P.J. Gaskin, and D.T. Beattie, *Attenuation of Fos-like immunoreactivity in the trigeminal nucleus caudalis following trigeminovascular activation in the anaesthetised guinea-pig.* Brain Res, 1997. **775**(1-2): p. 74-80.
139. Mitsikostas, D.D., et al., *The NMDA receptor antagonist MK-801 reduces capsaicin-induced c-fos expression within rat trigeminal nucleus caudalis.* Pain, 1998. **76**(1-2): p. 239-48.
140. Kaube, H., et al., *Expression of c-Fos-like immunoreactivity in the caudal medulla and upper cervical spinal cord following stimulation of the superior sagittal sinus in the cat.* Brain Res, 1993. **629**(1): p. 95-102.
141. Strassman, A.M., Y. Mineta, and B.P. Vos, *Distribution of fos-like immunoreactivity in the medullary and upper cervical dorsal horn produced by stimulation of dural blood vessels in the rat.* J Neurosci, 1994. **14**(6): p. 3725-35.
142. Goadsby, P.J. and K.L. Hoskin, *The distribution of trigeminovascular afferents in the nonhuman primate brain Macaca nemestrina: a c-fos immunocytochemical study.* J Anat, 1997. **190 (Pt 3)**(Pt 3): p. 367-75.
143. Sugimoto, T., et al., *c-fos induction in the subnucleus oralis following trigeminal nerve stimulation.* Brain Res, 1998. **783**(1): p. 158-62.
144. Hoskin, K.L., A.S. Zagami, and P.J. Goadsby, *Stimulation of the middle meningeal artery leads to Fos expression in the trigeminocervical nucleus: a comparative study of monkey and cat.* J Anat, 1999. **194 (Pt 4)**(Pt 4): p. 579-88.
145. Hoskin, K.L., et al., *Fos expression in the midbrain periaqueductal grey after trigeminovascular stimulation.* J Anat, 2001. **198**(Pt 1): p. 29-35.
146. Keay, K.A. and R. Bandler, *Vascular head pain selectively activates ventrolateral periaqueductal gray in the cat.* Neurosci Lett, 1998. **245**(1): p. 58-60.
147. Keay, K.A. and R. Bandler, *Distinct central representations of inescapable and escapable pain: observations and speculation.* Exp Physiol, 2002. **87**(2): p. 275-9.
148. Munro, G., I. Jansen-Olesen, and J. Olesen, *Animal models of pain and migraine in drug discovery.* Drug Discov Today, 2017. **22**(7): p. 1103-1111.
149. Oshinsky, M.L. and S. Gomonchareonsiri, *Episodic dural stimulation in awake rats: a model for recurrent headache.* Headache, 2007. **47**(7): p. 1026-36.
150. Melo-Carrillo, A. and A. Lopez-Avila, *A chronic animal model of migraine, induced by repeated meningeal nociception, characterized by a behavioral and pharmacological approach.* Cephalalgia, 2013. **33**(13): p. 1096-105.

151. Boyer, N., et al., *General trigeminospinal central sensitization and impaired descending pain inhibitory controls contribute to migraine progression.* Pain, 2014. **155**(7): p. 1196-205.
152. Liu-Chen, L.Y., M.R. Mayberg, and M.A. Moskowitz, *Immunohistochemical evidence for a substance P-containing trigeminovascular pathway to pial arteries in cats.* Brain Res, 1983. **268**(1): p. 162-6.
153. Jansen, I., et al., *Tachykinins (substance P, neurokinin A, neuropeptide K, and neurokinin B) in the cerebral circulation: vasomotor responses in vitro and in situ.* J Cereb Blood Flow Metab, 1991. **11**(4): p. 567-75.
154. Edvinsson, L. and P.J. Goadsby, *Neuropeptides in headache.* European Journal of Neurology, 1998. **5**(4): p. 329-341.
155. Dimitriadou, V., et al., *Trigeminal sensory fiber stimulation induces morphological changes reflecting secretion in rat dura mater mast cells.* Neuroscience, 1991. **44**(1): p. 97-112.
156. Dimitriadou, V., et al., *Ultrastructural evidence for neurogenically mediated changes in blood vessels of the rat dura mater and tongue following antidromic trigeminal stimulation.* Neuroscience, 1992. **48**(1): p. 187-203.
157. Theoharides, T.C., et al., *The role of mast cells in migraine pathophysiology.* Brain Res Brain Res Rev, 2005. **49**(1): p. 65-76.
158. Leao, A., *Spreading Depression of Activity in the Cerebral Cortex.* Journal of Neurophsyiology, 1944. **7**: p. 359-390.
159. Leo, A.A.P. and R.S. Morison, *PROPAGATION OF SPREADING CORTICAL DEPRESSION.* Journal of Neurophysiology, 1945. **8**(1): p. 33-45.
160. Leao, A., *Pial Circulation and Spreading Depression of Activity in the Cerebral Cortex.* Journal of Neurophsyiology, 1944. **7**: p. 391-396.
161. Leao, A., *Further Observations on the Spreading Depression of Activity in the Cerebral Cortex.* Journal of Neurophsyiology, 1947: p. 409-414.
162. Somjen, G., *Mechanisms of Spreading Depression and Hypoxic Spreading Depression-Like Depolarization.* Physiol Rev, 2001. **81**(3): p. 1065-1096.
163. Makarova, J., M. Gomez-Galan, and O. Herreras, *Variations in tissue resistivity and in the extension of activated neuron domains shape the voltage signal during spreading depression in the CA1 in vivo.* Eur J Neurosci, 2008. **27**(2): p. 444-56.
164. Canals, S., et al., *Longitudinal depolarization gradients along the somatodendritic axis of CA1 pyramidal cells: a novel feature of spreading depression.* J Neurophysiol, 2005. **94**(2): p. 943-51.
165. Hansen, A.J. and T. Zeuthen, *Extracellular ion concentrations during spreading depression and ischemia in the rat brain cortex.* Acta Physiol Scand, 1981. **113**(4): p. 437-45.
166. Mutch, W.A. and A.J. Hansen, *Extracellular pH changes during spreading depression and cerebral ischemia: mechanisms of brain pH regulation.* J Cereb Blood Flow Metab, 1984. **4**(1): p. 17-27.
167. Davies, J.A., et al., *A comparison between the stimulated and paroxysmal release of endogenous amino acids from rat cerebellar, striatal and hippocampal slices: a manifestation of spreading depression?* J Neurol Sci, 1995. **131**(1): p. 8-14.
168. Fabricius, M., L.H. Jensen, and M. Lauritzen, *Microdialysis of interstitial amino acids during spreading depression and anoxic depolarization in rat neocortex.* Brain Res, 1993. **612**(1-2): p. 61-9.
169. Takano, T., et al., *Cortical spreading depression causes and coincides with tissue hypoxia.* Nat Neurosci, 2007. **10**(6): p. 754-62.
170. Zhou, N., et al., *Transient swelling, acidification, and mitochondrial depolarization occurs in neurons but not astrocytes during spreading depression.* Cereb Cortex, 2010. **20**(11): p. 2614-24.
171. Herreras, O. and G.G. Somjen, *Propagation of spreading depression among dendrites and somata of the same cell population.* Brain Res, 1993. **610**(2): p. 276-82.

172. Vilagi, I., N. Klapka, and H.J. Luhmann, *Optical recording of spreading depression in rat neocortical slices.* Brain Res, 2001. **898**(2): p. 288-96.
173. Brennan, K.C., et al., *Distinct vascular conduction with cortical spreading depression.* J Neurophysiol, 2007. **97**(6): p. 4143-51.
174. Shinohara, M., et al., *Cerebral glucose utilization: local changes during and after recovery from spreading cortical depression.* Science, 1979. **203**(4376): p. 188-90.
175. Piilgaard, H. and M. Lauritzen, *Persistent increase in oxygen consumption and impaired neurovascular coupling after spreading depression in rat neocortex.* J Cereb Blood Flow Metab, 2009. **29**(9): p. 1517-27.
176. Chang, J.C., et al., *Biphasic direct current shift, haemoglobin desaturation and neurovascular uncoupling in cortical spreading depression.* Brain, 2010. **133**(Pt 4): p. 996-1012.
177. Wahl, M., et al., *Involvement of calcitonin gene-related peptide (CGRP) and nitric oxide (NO) in the pial artery dilatation elicited by cortical spreading depression.* Brain Research, 1994. **367**: p. 204-210.
178. Wahl, M., M. Lauritzen, and L. Schilling, *Change of cerebrovascular reactivity after cortical spreading depression in cats and rats.* Brain Res, 1987. **411**(1): p. 72-80.
179. Seitz, I., U. Dirnagl, and U. Lindauer, *Impaired vascular reactivity of isolated rat middle cerebral artery after cortical spreading depression in vivo.* J Cereb Blood Flow Metab, 2004. **24**(5): p. 526-30.
180. Zhang, X., et al., *Activation of central trigeminovascular neurons by cortical spreading depression.* Ann Neurol, 2011. **69**(5): p. 855-65.
181. Zhang, X., et al., *Activation of meningeal nociceptors by cortical spreading depression: implications for migraine with aura.* J Neurosci, 2010. **30**(26): p. 8807-14.
182. Reuter, U., et al., *Perivascular nerves contribute to cortical spreading depression-associated hyperemia in rats.* Am J Physiol, 1998. **274**(6): p. H1979-87.
183. Ayata, C., *Cortical Spreading Depression Triggers Migraine Attack: Pro.* Headache, 2010: p. 725-730.
184. Moskowitz, M.A. and R. Macfarlane, *Neurovascular and molecular mechanisms in migraine headaches.* Cerebrovasc Brain Metab Rev, 1993. **5**(3): p. 159-77.
185. Charles, A.C. and S.M. Baca, *Cortical spreading depression and migraine.* Nat Rev Neurol, 2013. **9**(11): p. 637-44.
186. LASHLEY, K.S., *PATTERNS OF CEREBRAL INTEGRATION INDICATED BY THE SCOTOMAS OF MIGRAINE.* Archives of Neurology & Psychiatry, 1941. **46**(2): p. 331-339.
187. Tfelt-Hansen, P.C., *History of migraine with aura and cortical spreading depression from 1941 and onwards.* Cephalalgia, 2010. **30**(7): p. 780-92.
188. Bowyer, S.M., et al., *Magnetoencephalographic fields from patients with spontaneous and induced migraine aura.* Ann Neurol, 2001. **50**(5): p. 582-7.
189. Drenckhahn, C., et al., *Correlates of spreading depolarization in human scalp electroencephalography.* Brain, 2012. **135**(Pt 3): p. 853-68.
190. Olesen, J., et al., *Timing and topography of cerebral blood flow, aura, and headache during migraine attacks.* Ann Neurol, 1990. **28**(6): p. 791-8.
191. Woods, R.P., M. Iacoboni, and J.C. Mazziotta, *Brief report: bilateral spreading cerebral hypoperfusion during spontaneous migraine headache.* N Engl J Med, 1994. **331**(25): p. 1689-92.
192. Hadjikhani, N., et al., *Mechanisms of migraine aura revealed by functional MRI in human visual cortex.* Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4687-92.
193. Cao, Y., et al., *Functional MRI-BOLD of visually triggered headache in patients with migraine.* Arch Neurol, 1999. **56**(5): p. 548-54.

194. Schott, G.D., *Exploring the visual hallucinations of migraine aura: the tacit contribution of illustration.* Brain, 2007. **130**(Pt 6): p. 1690-703.
195. Queiroz, L.P., et al., *Characteristics of migraine visual aura.* Headache, 1997. **37**(3): p. 137-41.
196. Queiroz, L.P., et al., *Characteristics of migraine visual aura in Southern Brazil and Northern USA.* Cephalalgia, 2011. **31**(16): p. 1652-8.
197. Charles, A. and K. Brennan, *Cortical spreading depression-new insights and persistent questions.* Cephalalgia, 2009. **29**(10): p. 1115-24.
198. Jensen, K., et al., *Classic migraine. A prospective recording of symptoms.* Acta Neurol Scand, 1986. **73**(4): p. 359-62.
199. Hansen, J.M., et al., *Migraine headache is present in the aura phase: a prospective study.* Neurology, 2012. **79**(20): p. 2044-9.
200. Charles, A., *Migraine: a brain state.* Curr Opin Neurol, 2013. **26**(3): p. 235-9.
201. Bolay, H., et al., *Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model.* Nature Medicine, 2002. **8**(2): p. 136-142.
202. Moskowitz, M.A., K. Nozaki, and R.P. Kraig, *Neocortical spreading depression provokes the expression of c-fos protein-like immunoreactivity within trigeminal nucleus caudalis via trigeminovascular mechanisms.* J Neurosci, 1993. **13**(3): p. 1167-77.
203. Karatas, H., et al., *Spreading Depression Triggers Headache by Activating Neuronal Panx1 Channels.* Science, 2013. **339**: p. 1092-1095.
204. Kosaras, B., et al., *Sensory innervation of the calvarial bones of the mouse.* J Comp Neurol, 2009. **515**(3): p. 331-48.
205. Fioravanti, B., et al., *Evaluation of cutaneous allodynia following induction of cortical spreading depression in freely moving rats.* Cephalalgia, 2011. **31**(10): p. 1090-100.
206. Akcali, D., et al., *Does single cortical spreading depression elicit pain behaviour in freely moving rats?* Cephalalgia, 2010. **30**(10): p. 1195-206.
207. Koroleva, V.I. and J. Bures, *Rats do not experience cortical or hippocampal spreading depression as aversive.* Neurosci Lett, 1993. **149**(2): p. 153-6.
208. Hadjikhani, N. and M. Vincent, *Neuroimaging clues of migraine aura.* J Headache Pain, 2019. **20**(1): p. 32.
209. Maurice-Williams, R.S. and G. Dunwoody, *Late diagnosis of frontal meningiomas presenting with psychiatric symptoms.* Br Med J (Clin Res Ed), 1988. **296**(6639): p. 1785-6.
210. Ayata, C., *Pearls and pitfalls in experimental models of spreading depression.* Cephalalgia, 2013. **33**(8): p. 604-13.
211. Turner, D.A., et al., *Differences in O2 availability resolve the apparent discrepancies in metabolic intrinsic optical signals in vivo and in vitro.* Trends Neurosci, 2007. **30**(8): p. 390-8.
212. Galeffi, F., et al., *Simultaneous monitoring of tissue PO2 and NADH fluorescence during synaptic stimulation and spreading depression reveals a transient dissociation between oxygen utilization and mitochondrial redox state in rat hippocampal slices.* J Cereb Blood Flow Metab, 2011. **31**(2): p. 626-39.
213. Van Harreveld, A., J.S. Stamm, and E. Christensen, *Spreading depression in rabbit, cat and monkey.* Am J Physiol, 1956. **184**(2): p. 312-20.
214. Shima, I., E. Fifkova, and J. Bures, *LIMITS OF SPREADING DEPRESSION IN PIGEON STRIATUM.* J Comp Neurol, 1963. **121**: p. 485-92.
215. Kudo, C., et al., *The impact of anesthetics and hyperoxia on cortical spreading depression.* Exp Neurol, 2008. **212**(1): p. 201-6.
216. Sonn, J. and A. Mayevsky, *Effects of anesthesia on the responses to cortical spreading depression in the rat brain in vivo.* Neurol Res, 2006. **28**(2): p. 206-19.

217. Kitahara, Y., et al., *The effects of anesthetics on cortical spreading depression elicitation and c-fos expression in rats.* J Neurosurg Anesthesiol, 2001. **13**(1): p. 26-32.
218. Saito, R., et al., *Halothane, but not alpha-chloralose, blocks potassium-evoked cortical spreading depression in cats.* Brain Res, 1995. **699**(1): p. 109-15.
219. Saito, R., et al., *Reduction of infarct volume by halothane: effect on cerebral blood flow or perifocal spreading depression-like depolarizations.* J Cereb Blood Flow Metab, 1997. **17**(8): p. 857-64.
220. Guedes, R.C. and J.M. Barreto, *Effect of anesthesia on the propagation of cortical spreading depression in rats.* Braz J Med Biol Res, 1992. **25**(4): p. 393-7.
221. Piper, R.D. and G.A. Lambert, *Inhalational anesthetics inhibit spreading depression: relevance to migraine.* Cephalalgia, 1996. **16**(2): p. 87-92.
222. Harriott, A.M., et al., *Optogenetic Spreading Depression Elicits Trigeminal Pain and Anxiety Behavior.* Ann Neurol, 2021. **89**(1): p. 99-110.
223. Takizawa, T., et al., *Non-invasively triggered spreading depolarizations induce a rapid pro-inflammatory response in cerebral cortex.* J Cereb Blood Flow Metab, 2020. **40**(5): p. 1117-1131.
224. Reid, K.H., et al., *Strength-duration properties of cathodal pulses eliciting spreading depression in rat cerebral cortex.* Brain Research, 1987. **404**: p. 361-364.
225. Matsuura, T. and J. Bures, *The minimum volume of depolarized neural tissue required for triggering cortical spreading depression in rat.* Exp Brain Res, 1971. **12**(3): p. 238-49.
226. Eikermann-Haerter, K., et al., *Genetic and hormonal factors modulate spreading depression and transient hemiparesis in mouse models of familial hemiplegic migraine type 1.* J Clin Invest, 2009. **119**(1): p. 99-109.
227. Wang, M., T.P. Obrenovitch, and J. Urenjak, *Effects of the nitric oxide donor, DEA/NO on cortical spreading depression.* Neuropharmacology, 2003. **44**(7): p. 949-57.
228. Obrenovitch, T.P., J. Urenjak, and E. Zilkha, *Intracerebral microdialysis combined with recording of extracellular field potential: a novel method for investigation of depolarizing drugs in vivo.* Br J Pharmacol, 1994. **113**(4): p. 1295-302.
229. Ayata, C., et al., *Suppression of Cortical Spreading Depression in Migraine Prophylaxis.* Annals of Neurology, 2006. **59**: p. 652-661.
230. Brennan, K.C., et al., *Reduced threshold for cortical spreading depression in female mice.* Ann Neurol, 2007. **61**(6): p. 603-6.
231. Ayata, C., et al., *Pronounced hypoperfusion during spreading depression in mouse cortex.* J Cereb Blood Flow Metab, 2004. **24**(10): p. 1172-82.
232. Akerman, S. and P.J. Goadsby, *Topiramate inhibits cortical spreading depression in rat and cat: impact in migraine aura.* Neuroreport, 2005. **16**(12): p. 1383-7.
233. Akerman, S., P.R. Holland, and P.J. Goadsby, *Mechanically-induced cortical spreading depression associated regional cerebral blood flow changes are blocked by Na+ ion channel blockade.* Brain Res, 2008. **1229**: p. 27-36.
234. Holland, P.R., S. Akerman, and P.J. Goadsby, *Cortical spreading depression-associated cerebral blood flow changes induced by mechanical stimulation are modulated by AMPA and GABA receptors.* Cephalalgia, 2010. **30**(5): p. 519-27.
235. Bockhorst, K.H., et al., *A quantitative analysis of cortical spreading depression events in the feline brain characterized with diffusion-weighted MRI.* J Magn Reson Imaging, 2000. **12**(5): p. 722-33.
236. Yuzawa, I., et al., *Cortical spreading depression impairs oxygen delivery and metabolism in mice.* J Cereb Blood Flow Metab, 2012. **32**(2): p. 376-86.
237. Borgdorff, P., *Arguments against the role of cortical spreading depression in migraine.* Neurol Res, 2018. **40**(3): p. 173-181.

238. Marshall, W.H. and C.F. Essig, *RELATION OF AIR EXPOSURE OF CORTEX TO SPREADING DEPRESSION OF LEAO.* Journal of Neurophysiology, 1951. **14**(4): p. 265-273.
239. Sramka, M., et al., *Functional ablation by spreading depression: possible use in human stereotactic neurosurgery.* Appl Neurophysiol, 1977. **40**(1): p. 48-61.
240. Gloor, P., *Migraine and regional cerebral blood flow.* Trends in Neurosciences, 1986. **9**: p. 21.
241. McLachlan, R.S. and J.P. Girvin, *Spreading depression of Leao in rodent and human cortex.* Brain Res, 1994. **666**(1): p. 133-6.
242. van den Maagdenberg, A.M., et al., *A Cacna1a Knockin Migraine Mouse Model with Increased Susceptibility to Cortical Spreading Depression.* Neuron, 2004. **41**: p. 701-710.
243. Leo, L., et al., *Increased susceptibility to cortical spreading depression in the mouse model of familial hemiplegic migraine type 2.* PLoS Genet, 2011. **7**(6): p. e1002129.
244. Eikermann-Haerter, K., et al., *Androgenic suppression of spreading depression in familial hemiplegic migraine type 1 mutant mice.* Ann Neurol, 2009. **66**(4): p. 564-8.
245. Holland, P.R., et al., *Acid-sensing ion channel 1: a novel therapeutic target for migraine with aura.* Ann Neurol, 2012. **72**(4): p. 559-63.
246. Hoffmann, U., et al., *Oxcarbazepine does not suppress cortical spreading depression.* Cephalalgia, 2011. **31**(5): p. 537-42.
247. Tozzi, A., et al., *Critical role of calcitonin gene-related peptide receptors in cortical spreading depression.* Proc Natl Acad Sci U S A, 2012. **109**(46): p. 18985-90.
248. Bogdanov, V.B., et al., *Migraine preventive drugs differentially affect cortical spreading depression in rat.* Neurobiol Dis, 2011. **41**(2): p. 430-5.
249. Dreier, J.P., et al., *Cortical spreading ischaemia is a novel process involved in ischaemic damage in patients with aneurysmal subarachnoid haemorrhage.* Brain, 2009. **132**(Pt 7): p. 1866-81.
250. Hartings, J.A., et al., *Spreading depolarizations have prolonged direct current shifts and are associated with poor outcome in brain trauma.* Brain, 2011. **134**(Pt 5): p. 1529-40.
251. Woitzik, J., et al., *Propagation of cortical spreading depolarization in the human cortex after malignant stroke.* Neurology, 2013. **80**(12): p. 1095-102.
252. Dreier, J.P., *The role of spreading depression, spreading depolarization and spreading ischemia in neurological disease.* Nat Med, 2011. **17**(4): p. 439-47.
253. Eikermann-Haerter, K., et al., *Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy syndrome mutations increase susceptibility to spreading depression.* Ann Neurol, 2011. **69**(2): p. 413-8.
254. Eikermann-Haerter, K., et al., *Migraine mutations increase stroke vulnerability by facilitating ischemic depolarizations.* Circulation, 2012. **125**(2): p. 335-45.
255. Arboleda-Velasquez, J.F., et al., *Hypomorphic Notch 3 alleles link Notch signaling to ischemic cerebral small-vessel disease.* Proc Natl Acad Sci U S A, 2011. **108**(21): p. E128-35.
256. Kurth, T., H. Chabriat, and M.G. Bousser, *Migraine and stroke: a complex association with clinical implications.* Lancet Neurol, 2012. **11**(1): p. 92-100.
257. Pietrobon, D. and M.A. Moskowitz, *Chaos and commotion in the wake of cortical spreading depression and spreading depolarizations.* Nat Rev Neurosci, 2014. **15**(6): p. 379-93.
258. Dreier, J.P., et al., *Nitric oxide scavenging by hemoglobin or nitric oxide synthase inhibition by N-nitro-L-arginine induces cortical spreading ischemia when K+ is increased in the subarachnoid space.* J Cereb Blood Flow Metab, 1998. **18**(9): p. 978-90.
259. Lipton, R.B. and S.D. Silberstein, *Episodic and chronic migraine headache: breaking down barriers to optimal treatment and prevention.* Headache, 2015. **55 Suppl 2**: p. 103-22; quiz 123-6.
260. Diener, H.C., *CGRP as a new target in prevention and treatment of migraine.* Lancet Neurol, 2014. **13**(11): p. 1065-1067.

261. Bigal, M.E., S. Walter, and A.M. Rapoport, *Therapeutic antibodies against CGRP or its receptor.* Br J Clin Pharmacol, 2015. **79**(6): p. 886-95.
262. Edvinsson, L., et al., *CGRP as the target of new migraine therapies - successful translation from bench to clinic.* Nat Rev Neurol, 2018. **14**(6): p. 338-350.
263. Russo, A.F., *Calcitonin gene-related peptide (CGRP): a new target for migraine.* Annu Rev Pharmacol Toxicol, 2015. **55**: p. 533-52.
264. Mohanty, D. and S. Lippmann, *CGRP Inhibitors for Migraine.* Innov Clin Neurosci, 2020. **17**(4-6): p. 39-40.
265. Lipton, R.B., et al., *Barriers to the diagnosis and treatment of migraine: effects of sex, income, and headache features.* Headache, 2013. **53**(1): p. 81-92.
266. Diamond, S., et al., *Patterns of diagnosis and acute and preventive treatment for migraine in the United States: results from the American Migraine Prevalence and Prevention study.* Headache, 2007. **47**(3): p. 355-63.
267. Lipton, R.B., et al., *Migraine prevalence, disease burden, and the need for preventive therapy.* Neurology, 2007. **68**(5): p. 343-9.
268. Antonaci, F., et al., *Recent advances in migraine therapy.* Springerplus, 2016. **5**: p. 637.
269. Gilmore, B. and M. Michael, *Treatment of acute migraine headache.* Am Fam Physician, 2011. **83**(3): p. 271-80.
270. Giffin, N.J., et al., *Premonitory symptoms in migraine An electronic diary study.* Neurology, 2003. **60**: p. 935-940.
271. Gelfand, A.A. and P.J. Goadsby, *A Neurologist's Guide to Acute Migraine Therapy in the Emergency Room.* Neurohospitalist, 2012. **2**(2): p. 51-59.
272. Vinson, D.R., *Treatment patterns of isolated benign headache in US emergency departments.* Ann Emerg Med, 2002. **39**(3): p. 215-22.
273. Colman, I., et al., *Use of narcotic analgesics in the emergency department treatment of migraine headache.* Neurology, 2004. **62**(10): p. 1695-700.
274. Ho, T.W., A. Rodgers, and M.E. Bigal, *Impact of recent prior opioid use on rizatriptan efficacy. A post hoc pooled analysis.* Headache, 2009. **49**(3): p. 395-403.
275. Bigal, M.E., et al., *Assessment of migraine disability using the migraine disability assessment (MIDAS) questionnaire: a comparison of chronic migraine with episodic migraine.* Headache, 2003. **43**(4): p. 336-42.
276. Buse, D.C., et al., *Opioid use and dependence among persons with migraine: results of the AMPP study.* Headache, 2012. **52**(1): p. 18-36.
277. Blumenthal, H.J., et al., *Treatment of primary headache in the emergency department.* Headache, 2003. **43**(10): p. 1026-31.
278. Stoll, A. and A. Hofmann, *Zur Kenntnis des Polypeptidteils der Mutterkornalkaloide II. (partielle alkalische Hydrolyse der Mutterkornalkaloide). 20. Mitteilung über Mutterkornalkaloide.* Helvetica Chimica Acta, 1950. **33**(6): p. 1705-1711.
279. *A randomized, double-blind comparison of sumatriptan and Cafergot in the acute treatment of migraine. The Multinational Oral Sumatriptan and Cafergot Comparative Study Group.* Eur Neurol, 1991. **31**(5): p. 314-22.
280. JOHNSTON, B.M. and P.R. SAXENA, *THE EFFECT OF ERGOTAMINE ON TISSUE BLOOD FLOW AND THE ARTERIOVENOUS SHUNTING OF RADIOACTIVE MICROSPHERES IN THE HEAD.* British Journal of Pharmacology, 1978. **63**(3): p. 541-549.
281. De Vries, P., et al., *Characterization of 5-HT receptors mediating constriction of porcine carotid arteriovenous anastomoses; involvement of 5-HT1B/1D and novel receptors.* Br J Pharmacol, 1998. **123**(8): p. 1561-70.

282. Muller-Schweinitzer, E. and A. Fanchamps, *Effects on arterial receptors of ergot derivatives used in migraine.* Adv Neurol, 1982. **33**: p. 343-56.
283. MaassenVanDenBrink, A., et al., *Coronary side-effect potential of current and prospective antimigraine drugs.* Circulation, 1998. **98**(1): p. 25-30.
284. Colman, I., et al., *Parenteral dihydroergotamine for acute migraine headache: a systematic review of the literature.* Ann Emerg Med, 2005. **45**(4): p. 393-401.
285. Smith, T.R., et al., *Sumatriptan and naproxen sodium for the acute treatment of migraine.* Headache, 2005. **45**(8): p. 983-91.
286. Winner, P., et al., *A double-blind study of subcutaneous dihydroergotamine vs subcutaneous sumatriptan in the treatment of acute migraine.* Arch Neurol, 1996. **53**(2): p. 180-4.
287. Saxena, P.R. and M.D. Ferrari, *5-HT(1)-like receptor agonists and the pathophysiology of migraine.* Trends Pharmacol Sci, 1989. **10**(5): p. 200-4.
288. Hargreaves, R. and S. Shepheard, *Pathophysiology of migraine—new insights.* Canadian journal of neurological sciences, 1999. **26**(3): p. 12-19.
289. Ahn, A.H. and A.I. Basbaum, *Where do triptans act in the treatment of migraine?* Pain, 2005. **115**(1-2): p. 1.
290. Humphrey, P.P., et al., *The pharmacology of the novel 5-HT1-like receptor agonist, GR43175.* Cephalalgia, 1989. **9 Suppl 9**: p. 23-33.
291. Humphrey, P.P., et al., *Serotonin and migraine.* Ann N Y Acad Sci, 1990. **600**: p. 587-98; discussion 598-600.
292. Humphrey, P.P., W. Feniuk, and M.J. Perren, *Anti-migraine drugs in development: advances in serotonin receptor pharmacology.* Headache, 1990. **30**(1 Suppl): p. 12-6; discussion 24-8.
293. McCrory, D.C. and R.N. Gray, *Oral sumatriptan for acute migraine.* Cochrane Database of Systematic Reviews, 2003(3).
294. Winner, P., et al., *Early intervention in migraine with sumatriptan tablets 50 mg versus 100 mg: a pooled analysis of data from six clinical trials.* Clin Ther, 2005. **27**(11): p. 1785-94.
295. Ma, Q.P., R. Hill, and D. Sirinathsinghji, *Colocalization of CGRP with 5-HT1B/1D receptors and substance P in trigeminal ganglion neurons in rats.* Eur J Neurosci, 2001. **13**(11): p. 2099-104.
296. Durham, P.L. and A.F. Russo, *Regulation of calcitonin gene-related peptide secretion by a serotonergic antimigraine drug.* J Neurosci, 1999. **19**(9): p. 3423-9.
297. Baillie, L.D., A.H. Ahn, and S.J. Mulligan, *Sumatriptan inhibition of N-type calcium channel mediated signaling in dural CGRP terminal fibres.* Neuropharmacology, 2012. **63**(3): p. 362-7.
298. Thompson, M.R., *The active constituents of ergot. A pharmacological and chemical study.* Journal of the American Pharmaceutical Association, 1935. **24**(3): p. 185-196.
299. De Felice, M., et al., *Triptan-induced enhancement of neuronal nitric oxide synthase in trigeminal ganglion dural afferents underlies increased responsiveness to potential migraine triggers.* Brain, 2010. **133**(Pt 8): p. 2475-88.
300. Roberto, G., et al., *Adverse cardiovascular events associated with triptans and ergotamines for treatment of migraine: systematic review of observational studies.* Cephalalgia, 2015. **35**(2): p. 118-31.
301. Ferrari, M., et al., *Triptans (Serotonin, 5-HT1B/1D Agonists) in Migraine: Detailed Results and Methods of A Meta-Analysis of 53 Trials.* Cephalalgia, 2002. **22**(8): p. 633-658.
302. Evers, S., et al., *EFNS guideline on the drug treatment of migraine--revised report of an EFNS task force.* Eur J Neurol, 2009. **16**(9): p. 968-81.
303. Sprenger, T., M. Viana, and C. Tassorelli, *Current Prophylactic Medications for Migraine and Their Potential Mechanisms of Action.* Neurotherapeutics, 2018. **15**(2): p. 313-323.
304. Dolly, J.O. and K.R. Aoki, *The structure and mode of action of different botulinum toxins.* Eur J Neurol, 2006. **13 Suppl 4**: p. 1-9.

305. Boyer, N., et al., *Propranolol treatment prevents chronic central sensitization induced by repeated dural stimulation.* Pain, 2017. **158**(10): p. 2025-2034.
306. Bangalore, S., et al., *Cardiovascular protection using beta-blockers: a critical review of the evidence.* J Am Coll Cardiol, 2007. **50**(7): p. 563-72.
307. Limmroth, V. and M.C. Michel, *The prevention of migraine: a critical review with special emphasis on beta-adrenoceptor blockers.* Br J Clin Pharmacol, 2001. **52**(3): p. 237-43.
308. Tfelt-Hansen, P., *Efficacy of beta-blockers in migraine. A critical review.* Cephalalgia, 1986. **6 Suppl 5**: p. 15-24.
309. Schellenberg, R., et al., *Nebivolol and metoprolol for treating migraine: an advance on beta-blocker treatment?* Headache, 2008. **48**(1): p. 118-25.
310. van de Ven, L.L., C.L. Franke, and P.J. Koehler, *Prophylactic treatment of migraine with bisoprolol: a placebo-controlled study.* Cephalalgia, 1997. **17**(5): p. 596-9.
311. Hanbauer, I., et al., *Induction of tyrosine hydroxylase elicited by beta adrenergic receptor agonists in normal and decentralized sympathetic ganglia: role of cyclic 3',5' - adenosine monophosphate.* J Pharmacol Exp Ther, 1975. **193**(1): p. 95-104.
312. Hieble, J.P., *Adrenoceptor subclassification: an approach to improved cardiovascular therapeutics.* Pharm Acta Helv, 2000. **74**(2-3): p. 163-71.
313. Xiao, C., et al., *Labetalol facilitates GABAergic transmission to rat periaqueductal gray neurons via antagonizing beta1-adrenergic receptors--a possible mechanism underlying labetalol-induced analgesia.* Brain Res, 2008. **1198**: p. 34-43.
314. Sandor, P.S., et al., *Prophylactic treatment of migraine with beta-blockers and riboflavin: differential effects on the intensity dependence of auditory evoked cortical potentials.* Headache, 2000. **40**(1): p. 30-5.
315. Koella, W.P., *CNS-related (side-)effects of beta-blockers with special reference to mechanisms of action.* Eur J Clin Pharmacol, 1985. **28 Suppl**: p. 55-63.
316. Ablad, B. and C. Dahlof, *Migraine and beta-blockade: modulation of sympathetic neurotransmission.* Cephalalgia, 1986. **6 Suppl 5**: p. 7-13.
317. Kalkman, H.O., *Is migraine prophylactic activity caused by 5-HT2B or 5-HT2C receptor blockade?* Life Sci, 1994. **54**(10): p. 641-4.
318. Chugani, D.C., et al., *Increased brain serotonin synthesis in migraine.* Neurology, 1999. **53**(7): p. 1473-9.
319. !!! INVALID CITATION !!! {}.
320. Richter, F., et al., *Noradrenergic agonists and antagonists influence migration of cortical spreading depression in rat-a possible mechanism of migraine prophylaxis and prevention of postischemic neuronal damage.* J Cereb Blood Flow Metab, 2005. **25**(9): p. 1225-35.
321. Silberstein, S.D., *Preventive Migraine Treatment.* Continuum (Minneap Minn), 2015. **21**(4 Headache): p. 973-89.
322. Cutrer, F.M., *Antiepileptic drugs: how they work in headache.* Headache, 2001. **41 Suppl 1**: p. S3-10.
323. Shank, R.P., et al., *Plasma and whole blood pharmacokinetics of topiramate: the role of carbonic anhydrase.* Epilepsy Res, 2005. **63**(2-3): p. 103-12.
324. Durham, P.L., C. Niemann, and R. Cady, *Repression of stimulated calcitonin gene-related peptide secretion by topiramate.* Headache, 2006. **46**(8): p. 1291-5.
325. Li, Y., et al., *Valproate ameliorates nitroglycerin-induced migraine in trigeminal nucleus caudalis in rats through inhibition of NF-small ka, CyrillicB.* J Headache Pain, 2016. **17**: p. 49.
326. Silberstein, S.D., *Topiramate in Migraine Prevention: A 2016 Perspective.* Headache, 2017. **57**(1): p. 165-178.

327. Solomon, G.D., *Comparative efficacy of calcium antagonist drugs in the prophylaxis of migraine.* Headache, 1985. **25**(7): p. 368-71.
328. Ye, Q., et al., *Flunarizine blocks voltage-gated Na(+) and Ca(2+) currents in cultured rat cortical neurons: A possible locus of action in the prevention of migraine.* Neurosci Lett, 2011. **487**(3): p. 394-9.
329. Ye, Q., et al., *Flunarizine inhibits sensory neuron excitability by blocking voltage-gated Na+ and Ca2+ currents in trigeminal ganglion neurons.* Chin Med J (Engl), 2011. **124**(17): p. 2649-55.
330. Li, F., et al., *Protection of flunarizine on cerebral mitochondria injury induced by cortical spreading depression under hypoxic conditions.* J Headache Pain, 2011. **12**(1): p. 47-53.
331. Hirfanoglu, T., et al., *Prophylactic drugs and cytokine and leptin levels in children with migraine.* Pediatr Neurol, 2009. **41**(4): p. 281-7.
332. Berilgen, M.S., et al., *Comparison of the effects of amitriptyline and flunarizine on weight gain and serum leptin, C peptide and insulin levels when used as migraine preventive treatment.* Cephalalgia, 2005. **25**(11): p. 1048-53.
333. Jackson, J.L., et al., *A Comparative Effectiveness Meta-Analysis of Drugs for the Prophylaxis of Migraine Headache.* PLoS One, 2015. **10**(7): p. e0130733.
334. Silberstein, S.D., *Preventive treatment of migraine.* Trends Pharmacol Sci, 2006. **27**(8): p. 410-5.
335. Sawynok, J., M.J. Esser, and A.R. Reid, *Antidepressants as analgesics: an overview of central and peripheral mechanisms of action.* J Psychiatry Neurosci, 2001. **26**(1): p. 21-9.
336. Colombo, B., P.O. Annovazzi, and G. Comi, *Therapy of primary headaches: the role of antidepressants.* Neurol Sci, 2004. **25 Suppl 3**: p. S171-5.
337. Ramadan, N.M., *Prophylactic migraine therapy: mechanisms and evidence.* Curr Pain Headache Rep, 2004. **8**(2): p. 91-5.
338. Garza, I. and J.W. Swanson, *Prophylaxis of migraine.* Neuropsychiatr Dis Treat, 2006. **2**(3): p. 281-91.
339. Gray, A.M., D.M. Pache, and R.D. Sewell, *Do alpha2-adrenoceptors play an integral role in the antinociceptive mechanism of action of antidepressant compounds?* Eur J Pharmacol, 1999. **378**(2): p. 161-8.
340. Punay, N.C. and J.R. Couch, *Antidepressants in the treatment of migraine headache.* Curr Pain Headache Rep, 2003. **7**(1): p. 51-4.
341. Pilc, A. and K.G. Lloyd, *Chronic antidepressants and GABA "B" receptors: a GABA hypothesis of antidepressant drug action.* Life Sci, 1984. **35**(21): p. 2149-54.
342. Lloyd, K.G., F. Thuret, and A. Pilc, *Upregulation of gamma-aminobutyric acid (GABA) B binding sites in rat frontal cortex: a common action of repeated administration of different classes of antidepressants and electroshock.* J Pharmacol Exp Ther, 1985. **235**(1): p. 191-9.
343. Suzdak, P.D. and G. Gianutsos, *Effect of chronic imipramine or baclofen on GABA-B binding and cyclic AMP production in cerebral cortex.* Eur J Pharmacol, 1986. **131**(1): p. 129-33.
344. Asakura, M., et al., *Role of serotonin in the regulation of beta-adrenoceptors by antidepressants.* Eur J Pharmacol, 1987. **141**(1): p. 95-100.
345. Pratt, G.D. and N.G. Bowery, *Repeated administration of desipramine and a GABAB receptor antagonist, CGP 36742, discretely up-regulates GABAB receptor binding sites in rat frontal cortex.* Br J Pharmacol, 1993. **110**(2): p. 724-35.
346. Ferguson, J.M., *The effects of antidepressants on sexual functioning in depressed patients: a review.* J Clin Psychiatry, 2001. **62 Suppl 3**: p. 22-34.
347. Tronvik, E., et al., *Involvement of the renin-angiotensin system in migraine.* J Hypertens Suppl, 2006. **24**(1): p. S139-43.

348. Nishimura, Y., T. Ito, and J.M. Saavedra, *Angiotensin II AT(1) blockade normalizes cerebrovascular autoregulation and reduces cerebral ischemia in spontaneously hypertensive rats.* Stroke, 2000. **31**(10): p. 2478-86.
349. Lorenzo, O., et al., *Angiotensin III activates nuclear transcription factor-kappaB in cultured mesangial cells mainly via AT(2) receptors: studies with AT(1) receptor-knockout mice.* J Am Soc Nephrol, 2002. **13**(5): p. 1162-71.
350. Schrader, H., et al., *Prophylactic treatment of migraine with angiotensin converting enzyme inhibitor (lisinopril): randomised, placebo controlled, crossover study.* Bmj, 2001. **322**(7277): p. 19-22.
351. Ito, H., K. Takemori, and T. Suzuki, *Role of angiotensin II type 1 receptor in the leucocytes and endothelial cells of brain microvessels in the pathogenesis of hypertensive cerebral injury.* J Hypertens, 2001. **19**(3 Pt 2): p. 591-7.
352. Montastruc, P., et al., *Naloxone reverses the effects of enalapril and enalaprilic acid on the pressor responses to afferent vagal stimulation.* Neuropeptides, 1985. **6**(6): p. 537-42.
353. Aurora, S.K., et al., *OnabotulinumtoxinA for treatment of chronic migraine: results from the double-blind, randomized, placebo-controlled phase of the PREEMPT 1 trial.* Cephalalgia, 2010. **30**(7): p. 793-803.
354. Diener, H.C., et al., *OnabotulinumtoxinA for treatment of chronic migraine: results from the double-blind, randomized, placebo-controlled phase of the PREEMPT 2 trial.* Cephalalgia, 2010. **30**(7): p. 804-14.
355. Dodick, D.W., et al., *OnabotulinumtoxinA for treatment of chronic migraine: pooled results from the double-blind, randomized, placebo-controlled phases of the PREEMPT clinical program.* Headache, 2010. **50**(6): p. 921-36.
356. Erbguth, F.J., *From poison to remedy: the chequered history of botulinum toxin.* J Neural Transm (Vienna), 2008. **115**(4): p. 559-65.
357. Scott, A.B., *Botulinum toxin injection into extraocular muscles as an alternative to strabismus surgery.* Ophthalmology, 1980. **87**(10): p. 1044-9.
358. Binder, W.J., A. Blitzer, and M.F. Brin, *Treatment of hyperfunctional lines of the face with botulinum toxin A.* Dermatol Surg, 1998. **24**(11): p. 1198-205.
359. Aoki, K.R., *Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A.* Neurotoxicology, 2005. **26**(5): p. 785-93.
360. Burstein, R., et al., *Selective inhibition of meningeal nociceptors by botulinum neurotoxin type A: therapeutic implications for migraine and other pains.* Cephalalgia, 2014. **34**(11): p. 853-69.
361. Aoki, K.R., *Evidence for antinociceptive activity of botulinum toxin type A in pain management.* Headache, 2003. **43 Suppl 1**: p. S9-15.
362. Whitcup, S.M., et al., *Development of onabotulinumtoxinA for chronic migraine.* Ann N Y Acad Sci, 2014. **1329**: p. 67-80.
363. Simpson, L.L., *The origin, structure, and pharmacological activity of botulinum toxin.* Pharmacol Rev, 1981. **33**(3): p. 155-88.
364. Blasi, J., et al., *Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25.* Nature, 1993. **365**(6442): p. 160-3.
365. Verderio, C., et al., *Traffic of botulinum toxins A and E in excitatory and inhibitory neurons.* Traffic, 2007. **8**(2): p. 142-53.
366. Drinovac, V., L. Bach-Rojecky, and Z. Lackovic, *Association of antinociceptive action of botulinum toxin type A with GABA-A receptor.* J Neural Transm (Vienna), 2014. **121**(6): p. 665-9.
367. Habermann, E., *Inhibition by tetanus and botulinum A toxin of the release of [3H]noradrenaline and [3H]GABA from rat brain homogenate.* Experientia, 1988. **44**(3): p. 224-6.

368. Aoki, K.R. and J. Francis, *Updates on the antinociceptive mechanism hypothesis of botulinum toxin A.* Parkinsonism Relat Disord, 2011. **17 Suppl 1**: p. S28-33.
369. Rapp, D.E., et al., *Botulinum toxin type a inhibits calcitonin gene-related peptide release from isolated rat bladder.* J Urol, 2006. **175**(3 Pt 1): p. 1138-42.
370. Edvinsson, L., *The Trigeminovascular Pathway: Role of CGRP and CGRP Receptors in Migraine.* Headache, 2017. **57 Suppl 2**: p. 47-55.
371. Schuster, N.M. and A.M. Rapoport, *Calcitonin Gene-Related Peptide-Targeted Therapies for Migraine and Cluster Headache: A Review.* Clin Neuropharmacol, 2017. **40**(4): p. 169-174.
372. Amara, S.G., et al., *Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products.* Nature, 1982. **298**(5871): p. 240-4.
373. Edvinsson, L., *The Journey to Establish CGRP as a Migraine Target: A Retrospective View.* Headache, 2015. **55**(9): p. 1249-55.
374. Russell, F.A., et al., *Calcitonin gene-related peptide: physiology and pathophysiology.* Physiol Rev, 2014. **94**(4): p. 1099-142.
375. Jansen-Olesen, I., A. Mortensen, and L. Edvinsson, *Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase.* Cephalalgia, 1996. **16**(5): p. 310-6.
376. Edvinsson, L., et al., *Cerebrovascular responses to capsaicin in vitro and in situ.* Br J Pharmacol, 1990. **100**(2): p. 312-8.
377. Goadsby, P.J. and L. Edvinsson, *Joint 1994 Wolff Award Presentation. Peripheral and central trigeminovascular activation in cat is blocked by the serotonin (5HT)-1D receptor agonist 311C90.* Headache, 1994. **34**(7): p. 394-9.
378. Russo, A.F., *Overview of Neuropeptides: Awakening the Senses?* Headache, 2017. **57 Suppl 2**(Suppl 2): p. 37-46.
379. Hoffmann, J., et al., *Primary trigeminal afferents are the main source for stimulus-induced CGRP release into jugular vein blood and CSF.* Cephalalgia, 2012. **32**(9): p. 659-67.
380. Goadsby, P.J., L. Edvinsson, and R. Ekman, *Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system.* Ann Neurol, 1988. **23**(2): p. 193-6.
381. Hay, D.L., et al., *Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25.* Br J Pharmacol, 2018. **175**(1): p. 3-17.
382. McLatchie, L.M., et al., *RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor.* Nature, 1998. **393**(6683): p. 333-9.
383. Seiler, K., et al., *Changes in calcitonin gene-related peptide (CGRP) receptor component and nitric oxide receptor (sGC) immunoreactivity in rat trigeminal ganglion following glyceroltrinitrate pretreatment.* J Headache Pain, 2013. **14**(1): p. 74.
384. Mallee, J.J., et al., *Receptor activity-modifying protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists.* J Biol Chem, 2002. **277**(16): p. 14294-8.
385. Hay, D.L. and A.A. Pioszak, *Receptor Activity-Modifying Proteins (RAMPs): New Insights and Roles.* Annu Rev Pharmacol Toxicol, 2016. **56**: p. 469-87.
386. Egea, S.C. and I.M. Dickerson, *Direct interactions between calcitonin-like receptor (CLR) and CGRP-receptor component protein (RCP) regulate CGRP receptor signaling.* Endocrinology, 2012. **153**(4): p. 1850-60.
387. Evans, B.N., et al., *CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors.* J Biol Chem, 2000. **275**(40): p. 31438-43.
388. Edvinsson, L., et al., *Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat.* Neurosci Lett, 1985. **58**(2): p. 213-7.

389. Khan, S., et al., *Reproducibility of migraine-like attacks induced by phosphodiesterase-3-inhibitor cilostazol.* Cephalalgia, 2018. **38**(5): p. 892-903.
390. Walker, C.S., et al., *A second trigeminal CGRP receptor: function and expression of the AMY1 receptor.* Ann Clin Transl Neurol, 2015. **2**(6): p. 595-608.
391. Goadsby, P.J., L. Edvinsson, and R. Ekman, *Vasoactive peptide release in the extracerebral circulation of humans during migraine headache.* Ann Neurol, 1990. **28**(2): p. 183-7.
392. Cernuda-Morollón, E., et al., *Interictal increase of CGRP levels in peripheral blood as a biomarker for chronic migraine.* Neurology, 2013. **81**(14): p. 1191-6.
393. Goadsby, P.J. and L. Edvinsson, *Human in vivo evidence for trigeminovascular activation in cluster headache. Neuropeptide changes and effects of acute attacks therapies.* Brain, 1994. **117 (Pt 3)**: p. 427-34.
394. Bellamy, J.L., R.K. Cady, and P.L. Durham, *Salivary levels of CGRP and VIP in rhinosinusitis and migraine patients.* Headache, 2006. **46**(1): p. 24-33.
395. Jang, M.U., et al., *Plasma and saliva levels of nerve growth factor and neuropeptides in chronic migraine patients.* Oral Dis, 2011. **17**(2): p. 187-93.
396. Cady, R.K., et al., *Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan.* Headache, 2009. **49**(9): p. 1258-66.
397. van Dongen, R.M., et al., *Migraine biomarkers in cerebrospinal fluid: A systematic review and meta-analysis.* Cephalalgia, 2017. **37**(1): p. 49-63.
398. Lassen, L.H., et al., *CGRP may play a causative role in migraine.* Cephalalgia, 2002. **22**(1): p. 54-61.
399. Hansen, J.M., et al., *Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura.* Cephalalgia, 2010. **30**(10): p. 1179-86.
400. Edvinsson, L., *Novel migraine therapy with calcitonin gene-regulated peptide receptor antagonists.* Expert Opin Ther Targets, 2007. **11**(9): p. 1179-88.
401. Edvinsson, L., *CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment.* Br J Clin Pharmacol, 2015. **80**(2): p. 193-9.
402. Doods, H., et al., *Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist.* Br J Pharmacol, 2000. **129**(3): p. 420-3.
403. Edvinsson, L., et al., *Effect of the CGRP receptor antagonist BIBN4096BS in human cerebral, coronary and omental arteries and in SK-N-MC cells.* Eur J Pharmacol, 2002. **434**(1-2): p. 49-53.
404. Ho, T.W., et al., *Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial.* Lancet, 2008. **372**(9656): p. 2115-23.
405. Connor, K.M., et al., *Randomized, controlled trial of telcagepant for the acute treatment of migraine.* Neurology, 2009. **73**(12): p. 970-7.
406. Paone, D.V., et al., *Potent, orally bioavailable calcitonin gene-related peptide receptor antagonists for the treatment of migraine: discovery of N-[(3R,6S)-6-(2,3-difluorophenyl)-2-oxo-1- (2,2,2-trifluoroethyl)azepan-3-yl]-4- (2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin- 1-yl)piperidine-1-carboxamide (MK-0974).* J Med Chem, 2007. **50**(23): p. 5564-7.
407. Edvinsson, L. and M. Linde, *New drugs in migraine treatment and prophylaxis: telcagepant and topiramate.* Lancet, 2010. **376**(9741): p. 645-55.
408. González-Hernández, A., et al., *Side effects associated with current and prospective antimigraine pharmacotherapies.* Expert Opin Drug Metab Toxicol, 2018. **14**(1): p. 25-41.
409. Bell, I.M., *Calcitonin gene-related peptide receptor antagonists: new therapeutic agents for migraine.* J Med Chem, 2014. **57**(19): p. 7838-58.
410. Dodick, D.W., et al., *Ubrogepant for the Treatment of Migraine.* N Engl J Med, 2019. **381**(23): p. 2230-2241.

411. Croop, R., et al., *Efficacy, safety, and tolerability of rimegepant orally disintegrating tablet for the acute treatment of migraine: a randomised, phase 3, double-blind, placebo-controlled trial.* Lancet, 2019. **394**(10200): p. 737-745.
412. Edvinsson, L., R. Ekman, and A. Ottosson, *Demonstration of perivascular peptides and changes in concentration with age in man.* Gerontology, 1986. **32 Suppl 1**: p. 50-2.
413. Schuster, N.M. and A.M. Rapoport, *New strategies for the treatment and prevention of primary headache disorders.* Nat Rev Neurol, 2016. **12**(11): p. 635-650.
414. Silberstein, S.D., et al., *Fremanezumab for the Preventive Treatment of Chronic Migraine.* N Engl J Med, 2017. **377**(22): p. 2113-2122.
415. Goadsby, P.J., et al., *A Controlled Trial of Erenumab for Episodic Migraine.* N Engl J Med, 2017. **377**(22): p. 2123-2132.
416. Shi, L., et al., *Pharmacologic Characterization of AMG 334, a Potent and Selective Human Monoclonal Antibody against the Calcitonin Gene-Related Peptide Receptor.* J Pharmacol Exp Ther, 2016. **356**(1): p. 223-31.
417. Bigal, M.E., et al., *Safety, tolerability, and efficacy of TEV-48125 for preventive treatment of chronic migraine: a multicentre, randomised, double-blind, placebo-controlled, phase 2b study.* Lancet Neurol, 2015. **14**(11): p. 1091-100.
418. Haanes, K.A., L. Edvinsson, and A. Sams, *Understanding side-effects of anti-CGRP and anti-CGRP receptor antibodies.* J Headache Pain, 2020. **21**(1): p. 26.
419. MaassenVanDenBrink, A., et al., *Wiping Out CGRP: Potential Cardiovascular Risks.* Trends Pharmacol Sci, 2016. **37**(9): p. 779-788.
420. Bigal, M.E. and R.B. Lipton, *Excessive opioid use and the development of chronic migraine.* Pain, 2009. **142**(3): p. 179-82.
421. Lipton, R.B., et al., *Characterizing opioid use in a US population with migraine: Results from the CaMEO study.* Neurology, 2020. **95**(5): p. e457-e468.
422. Le Merrer, J., et al., *Reward processing by the opioid system in the brain.* Physiol Rev, 2009. **89**(4): p. 1379-412.
423. Alexander, S.P.H., et al., *THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: G protein-coupled receptors.* Br J Pharmacol, 2019. **176 Suppl 1**(Suppl 1): p. S21-s141.
424. Dhawan, B.N., et al., *International Union of Pharmacology. XII. Classification of opioid receptors.* Pharmacol Rev, 1996. **48**(4): p. 567-92.
425. Cahill, C.M., et al., *Immunohistochemical distribution of delta opioid receptors in the rat central nervous system: evidence for somatodendritic labeling and antigen-specific cellular compartmentalization.* J Comp Neurol, 2001. **440**(1): p. 65-84.
426. Wang, H. and V.M. Pickel, *Preferential cytoplasmic localization of delta-opioid receptors in rat striatal patches: comparison with plasmalemmal mu-opioid receptors.* J Neurosci, 2001. **21**(9): p. 3242-50.
427. Gendron, L., et al., *Morphine and pain-related stimuli enhance cell surface availability of somatic delta-opioid receptors in rat dorsal root ganglia.* J Neurosci, 2006. **26**(3): p. 953-62.
428. Scherrer, G., et al., *Dissociation of the opioid receptor mechanisms that control mechanical and heat pain.* Cell, 2009. **137**(6): p. 1148-59.
429. Childers, S.R. and S.H. Snyder, *Guanine nucleotides differentiate agonist and antagonist interactions with opiate receptors.* Life Sci, 1978. **23**(7): p. 759-61.
430. Childers, S.R., et al., *Opiate receptor binding affected differentially by opiates and opioid peptides.* Eur J Pharmacol, 1979. **55**(1): p. 11-8.
431. Barchfeld, C.C. and F. Medzihradsky, *Receptor-mediated stimulation of brain GTPase by opiates in normal and dependent rats.* Biochem Biophys Res Commun, 1984. **121**(2): p. 641-8.

432. Minneman, K. and L.L. Iversen, *Enkephalin and opiate narcotics increase cyclic GMP accumulation in the slice of rat neostriatum.* Nature, 1976. **262**: p. 313-314.
433. Hsia, J.A., et al., *ADP-ribosylation of Adenylate Cyclase by Pertussis Toxin effects on inhibitory agonist binding.* Journal of Biological Chemistry, 1984. **259**(2): p. 1086-1090.
434. Torrecilla, M., et al., *Pre- and postsynaptic regulation of locus coeruleus neurons after chronic morphine treatment: a study of GIRK-knockout mice.* Eur J Neurosci, 2008. **28**(3): p. 618-24.
435. Torrecilla, M., et al., *G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons.* J Neurosci, 2002. **22**(11): p. 4328-34.
436. Rusin, K.I., et al., *Kappa-opioid receptor activation modulates Ca2+ currents and secretion in isolated neuroendocrine nerve terminals.* J Neurosci, 1997. **17**(17): p. 6565-74.
437. Zamponi, G. and T. Snutcht, *Modulation of voltage-dependent calcium channels by G proteins.* Current Opinion in Neurobiology, 1998. **8**: p. 351-356.
438. Zamponi, G.W. and T.P. Snutch, *Modulating modulation: crosstalk between regulatory pathways of presynaptic calcium channels.* Mol Interv, 2002. **2**(8): p. 476-8.
439. Bradbury, F.A., J.C. Zelnik, and J.R. Traynor, *G protein independent phosphorylation and internalization of the delta-opioid receptor.* J Neurochem, 2009. **109**(5): p. 1526-35.
440. Guo, J., et al., *Identification of G protein-coupled receptor kinase 2 phosphorylation sites responsible for agonist-stimulated delta-opioid receptor phosphorylation.* Mol Pharmacol, 2000. **58**(5): p. 1050-6.
441. Pei, G., et al., *Agonist-dependent phosphorylation of the mouse delta-opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C.* Mol Pharmacol, 1995. **48**(2): p. 173-7.
442. Pradhan, A.A., et al., *Agonist-Specific Recruitment of Arrestin Isoforms Differentially Modify Delta Opioid Receptor Function.* J Neurosci, 2016. **36**(12): p. 3541-51.
443. Lobingier, B.T. and M. von Zastrow, *When trafficking and signaling mix: How subcellular location shapes G protein-coupled receptor activation of heterotrimeric G proteins.* Traffic, 2019. **20**(2): p. 130-136.
444. Lefkowitz, R.J. and S.K. Shenoy, *Transduction of receptor signals by beta-arrestins.* Science, 2005. **308**(5721): p. 512-7.
445. Raman, M., W. Chen, and M.H. Cobb, *Differential regulation and properties of MAPKs.* Oncogene, 2007. **26**(22): p. 3100-12.
446. Tsvetanova, N.G. and M. von Zastrow, *Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis.* Nat Chem Biol, 2014. **10**(12): p. 1061-5.
447. Gavériaux-Ruff, C. and B.L. Kieffer, *Opioid receptor genes inactivated in mice: the highlights.* Neuropeptides, 2002. **36**(2-3): p. 62-71.
448. Filliol, D., et al., *Mice deficient for δ- and μ-opioid receptors exhibit opposing alterations of emotional responses.* Nat Genet, 2000. **25**: p. 195-200.
449. Kastin, A.J., et al., *Enkephalin and other peptides reduce passiveness.* Pharmacol Biochem Behav, 1978. **9**(4): p. 515-9.
450. Tejedor-Real, P., et al., *Implication of endogenous opioid system in the learned helplessness model of depression.* Pharmacol Biochem Behav, 1995. **52**(1): p. 145-52.
451. Lutz, P.E. and B.L. Kieffer, *Opioid receptors: distinct roles in mood disorders.* Trends Neurosci, 2013. **36**(3): p. 195-206.
452. Charbogne, P., B.L. Kieffer, and K. Befort, *15 years of genetic approaches in vivo for addiction research: Opioid receptor and peptide gene knockout in mouse models of drug abuse.* Neuropharmacology, 2014. **76 Pt B**(0 0): p. 204-17.

453. Roth, B.L., et al., *Salvinorin A: A potent naturally occurring nonnitrogenous κ opioid selective agonist.* Proceedings of the National Academy of Sciences, 2002. **99**(18): p. 11934-11939.
454. Pfeiffer, A., et al., *Psychotomimesis mediated by kappa opiate receptors.* Science, 1986. **233**(4765): p. 774-776.
455. Shippenberg, T.S. and A. Herz, *Differential effects of mu and kappa opioid systems on motivational processes.* NIDA Res Monogr, 1986. **75**: p. 563-66.
456. Negus, S.S., et al., *Behavioral Effects of the Delta-Selective Opioid Agonist SNC80 and Related Compounds in Rhesus Monkeys.* The journal of Pharmacology and Experimental Therapuetics 1998. **286**(1): p. 362-375.
457. Pradhan, A.A., et al., *Ligand-directed signalling within the opioid receptor family.* Br J Pharmacol, 2012. **167**(5): p. 960-9.
458. Brandt, M.R., et al., *Studies of tolerance and dependence with the delta-opioid agonist SNC80 in rhesus monkeys responding under a schedule of food presentation.* J Pharmacol Exp Ther, 2001. **299**(2): p. 629-37.
459. Mansour, A., et al., *Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications.* Trends in Neuroscience, 1995. **18**: p. 22-29.
460. Tseng, L.F. and J.M. Fujimoto, *Differential actions of intrathecal naloxone on blocking the tail-flick inhibition induced by intraventricular beta-endorphin and morphine in rats.* Journal of Pharmacology and Experimental Therapeutics, 1985. **232**(1): p. 74-79.
461. Hayhurst, C.J. and M.E. Durieux, *Differential Opioid Tolerance and Opioid-induced Hyperalgesia: A Clinical Reality.* Anesthesiology, 2016. **124**(2): p. 483-8.
462. Ji, G. and V. Neugebauer, *Kappa opioid receptors in the central amygdala modulate spinal nociceptive processing through an action on amygdala CRF neurons.* Molecular Brain, 2020. **13**(1): p. 128.
463. Gallantine, E.L. and T.F. Meert, *A comparison of the antinociceptive and adverse effects of the mu-opioid agonist morphine and the delta-opioid agonist SNC80.* Basic Clin Pharmacol Toxicol, 2005. **97**(1): p. 39-51.
464. Fraser, G.L., et al., *Antihyperalgesic effects of delta opioid agonists in a rat model of chronic inflammation.* Br J Pharmacol, 2000. **129**(8): p. 1668-72.
465. Pradhan, A.A., et al., *In vivo delta opioid receptor internalization controls behavioral effects of agonists.* PLoS One, 2009. **4**(5): p. e5425.
466. Cahill, M.C., et al., *Up-regulation and trafficking of δ opioid receptor in a model of chronic inflammation: implications for pain control.* Pain, 2003. **101**(1): p. 199-208.
467. Gaveriaux-Ruff, C. and B.L. Kieffer, *Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches.* Behav Pharmacol, 2011. **22**(5-6): p. 405-14.
468. Charles, A. and A.A. Pradhan, *Delta-opioid receptors as targets for migraine therapy.* Curr Opin Neurol, 2016. **29**(3): p. 314-9.
469. Pradhan, A.A., et al., *The delta opioid receptor: an evolving target for the treatment of brain disorders.* Trends Pharmacol Sci, 2011. **32**(10): p. 581-90.
470. Mansour, A., et al., *Anatomy of CNS opioid receptors.* Trends Neurosci, 1988. **11**(7): p. 308-14.
471. Mennicken, F., et al., *Phylogenetic changes in the expression of delta opioid receptors in spinal cord and dorsal root ganglia.* J Comp Neurol, 2003. **465**(3): p. 349-60.
472. Dripps, I.J., et al., *Role of signalling molecules in behaviours mediated by the delta opioid receptor agonist SNC80.* Br J Pharmacol, 2018. **175**(6): p. 891-901.
473. Moye, L.S., et al., *The development of a mouse model of mTBI-induced post-traumatic migraine, and identification of the delta opioid receptor as a novel therapeutic target.* Cephalalgia, 2019. **39**(1): p. 77-90.

474. Bertels, Z. and A.A.A. Pradhan, *Emerging Treatment Targets for Migraine and Other Headaches.* Headache, 2019. **59 Suppl 2**(Suppl 2): p. 50-65.
475. Spahn, V. and C. Stein, *Targeting delta opioid receptors for pain treatment: drugs in phase I and II clinical development.* Expert Opin Investig Drugs, 2017. **26**(2): p. 155-160.
476. Fossler, M.J., et al., *A Phase I, Randomized, Single-Blind, Placebo-Controlled, Single Ascending Dose Study of the Safety, Tolerability, and Pharmacokinetics of Subcutaneous and Oral TRV250, a G Protein-Selective Delta Receptor Agonist, in Healthy Subjects.* CNS Drugs, 2020. **34**(8): p. 853-865.
477. Costa-Neto, C.M., E.S.L.T. Parreiras, and M. Bouvier, *A Pluridimensional View of Biased Agonism.* Mol Pharmacol, 2016. **90**(5): p. 587-595.
478. Pierce, K.L., R.T. Premont, and R.J. Lefkowitz, *Seven-transmembrane receptors.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 639-50.
479. Reiter, E., et al., *Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors.* Annu Rev Pharmacol Toxicol, 2012. **52**: p. 179-97.
480. Sorkin, A. and M. von Zastrow, *Endocytosis and signalling: intertwining molecular networks.* Nat Rev Mol Cell Biol, 2009. **10**(9): p. 609-22.
481. Pradhan, A.A., et al., *Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance.* J Neurosci, 2010. **30**(49): p. 16459-68.
482. Molinari, P., et al., *Morphine-like opiates selectively antagonize receptor-arrestin interactions.* J Biol Chem, 2010. **285**(17): p. 12522-35.
483. Marie, N., et al., *Differential sorting of human delta-opioid receptors after internalization by peptide and alkaloid agonists.* J Biol Chem, 2003. **278**(25): p. 22795-804.
484. Scherrer, G., et al., *Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo.* Proc Natl Acad Sci U S A, 2006. **103**(25): p. 9691-6.
485. Pradhan, A.A., et al., *In vivo delta opioid receptor internalization controls behavioral effects of agonists.* PLoS One, 2009. **4**(5): p. e5425.
486. Mittal, N., et al., *Select G-protein-coupled receptors modulate agonist-induced signaling via a ROCK, LIMK, and β-arrestin 1 pathway.* Cell Rep, 2013. **5**(4): p. 1010-21.
487. Negus, S.S., et al., *Behavioral effects of the systemically active delta opioid agonist BW373U86 in rhesus monkeys.* J Pharmacol Exp Ther, 1994. **270**(3): p. 1025-34.
488. Jutkiewicz, E.M., et al., *The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid agonists in rats: comparison with pentylenetetrazol.* J Pharmacol Exp Ther, 2006. **317**(3): p. 1337-48.
489. Chung, P.C., et al., *Delta opioid receptors expressed in forebrain GABAergic neurons are responsible for SNC80-induced seizures.* Behav Brain Res, 2015. **278**: p. 429-34.
490. Nozaki, C., et al., *In vivo properties of KNT-127, a novel delta opioid receptor agonist: receptor internalization, antihyperalgesia and antidepressant effects in mice.* Br J Pharmacol, 2014. **171**(23): p. 5376-86.
491. Tan, A.M., et al., *Dendritic spine remodeling after spinal cord injury alters neuronal signal processing.* J Neurophysiol, 2009. **102**(4): p. 2396-409.
492. Smallwood, R.F., et al., *Structural brain anomalies and chronic pain: a quantitative meta-analysis of gray matter volume.* J Pain, 2013. **14**(7): p. 663-75.
493. Maihöfner, C., et al., *Patterns of cortical reorganization in complex regional pain syndrome.* Neurology, 2003. **61**(12): p. 1707-15.
494. Tatu, K., et al., *How do morphological alterations caused by chronic pain distribute across the brain? A meta-analytic co-alteration study.* Neuroimage Clin, 2018. **18**: p. 15-30.
495. Tajerian, M., et al., *Brain neuroplastic changes accompany anxiety and memory deficits in a model of complex regional pain syndrome.* Anesthesiology, 2014. **121**(4): p. 852-65.

496. Zhang, H., et al., *Morphological and Physiological Plasticity of Spinal Lamina II GABA Neurons Is Induced by Sciatic Nerve Chronic Constriction Injury in Mice.* Front Cell Neurosci, 2018. **12**: p. 143.
497. Gittes, F., et al., *Flexural rigidity of microtubules and actin filaments measured from thermal fluctuations in shape.* J Cell Biol, 1993. **120**(4): p. 923-34.
498. Hawkins, T., et al., *Mechanics of microtubules.* J Biomech, 2010. **43**(1): p. 23-30.
499. Caudron, N., et al., *Microtubule nucleation from stable tubulin oligomers.* J Biol Chem, 2002. **277**(52): p. 50973-9.
500. Kollman, J.M., et al., *Microtubule nucleation by gamma-tubulin complexes.* Nat Rev Mol Cell Biol, 2011. **12**(11): p. 709-21.
501. Akhmanova, A. and M.O. Steinmetz, *Control of microtubule organization and dynamics: two ends in the limelight.* Nat Rev Mol Cell Biol, 2015. **16**(12): p. 711-26.
502. Li, H., et al., *Microtubule structure at 8 A resolution.* Structure, 2002. **10**(10): p. 1317-28.
503. Roll-Mecak, A., *Intrinsically disordered tubulin tails: complex tuners of microtubule functions?* Semin Cell Dev Biol, 2015. **37**: p. 11-9.
504. Akhmanova, A. and C.C. Hoogenraad, *Microtubule minus-end-targeting proteins.* Curr Biol, 2015. **25**(4): p. R162-71.
505. Akhmanova, A. and M.O. Steinmetz, *Tracking the ends: a dynamic protein network controls the fate of microtubule tips.* Nat Rev Mol Cell Biol, 2008. **9**(4): p. 309-22.
506. Nogales, E., et al., *Tubulin and FtsZ form a distinct family of GTPases.* Nat Struct Biol, 1998. **5**(6): p. 451-8.
507. Howard, J., *Mechanics of Motor Proteins and the Cytoskeleton Sunderland*. 2001.
508. Alushin, G.M., et al., *High-resolution microtubule structures reveal the structural transitions in alphabeta-tubulin upon GTP hydrolysis.* Cell, 2014. **157**(5): p. 1117-29.
509. Goodson, H.V. and E.M. Jonasson, *Microtubules and Microtubule-Associated Proteins.* Cold Spring Harb Perspect Biol, 2018. **10**(6).
510. Mitchison, T. and M. Kirschner, *Dynamic instability of microtubule growth.* Nature, 1984. **312**(15): p. 237-242.
511. Dimitrov, A., et al., *Detection of GTP-tubulin conformation in vivo reveals a role for GTP remnants in microtubule rescues.* Science, 2008. **322**(5906): p. 1353-6.
512. Vulevic, B. and J.J. Correia, *Thermodynamic and structural analysis of microtubule assembly: the role of GTP hydrolysis.* Biophys J, 1997. **72**(3): p. 1357-75.
513. Kirschner, M. and T. Mitchison, *Beyond self-assembly: from microtubules to morphogenesis.* Cell, 1986. **45**(3): p. 329-42.
514. Hirokawa, N., S. Niwa, and Y. Tanaka, *Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease.* Neuron, 2010. **68**(4): p. 610-38.
515. Maday, S., et al., *Axonal transport: cargo-specific mechanisms of motility and regulation.* Neuron, 2014. **84**(2): p. 292-309.
516. Kardon, J.R. and R.D. Vale, *Regulators of the cytoplasmic dynein motor.* Nat Rev Mol Cell Biol, 2009. **10**(12): p. 854-65.
517. Kapitein, L.C. and C.C. Hoogenraad, *Which way to go? Cytoskeletal organization and polarized transport in neurons.* Mol Cell Neurosci, 2011. **46**(1): p. 9-20.
518. Rolls, M.M., *Neuronal polarity in Drosophila: sorting out axons and dendrites.* Dev Neurobiol, 2011. **71**(6): p. 419-29.
519. Kapitein, L.C., et al., *Mixed microtubules steer dynein-driven cargo transport into dendrites.* Curr Biol, 2010. **20**(4): p. 290-9.
520. Cooper, J.A., *Cell biology in neuroscience: mechanisms of cell migration in the nervous system.* J Cell Biol, 2013. **202**(5): p. 725-34.

521. Flynn, K.C., et al., *ADF/cofilin-mediated actin retrograde flow directs neurite formation in the developing brain.* Neuron, 2012. **76**(6): p. 1091-107.
522. Nakata, T. and N. Hirokawa, *Microtubules provide directional cues for polarized axonal transport through interaction with kinesin motor head.* J Cell Biol, 2003. **162**(6): p. 1045-55.
523. Nakata, T., et al., *Preferential binding of a kinesin-1 motor to GTP-tubulin-rich microtubules underlies polarized vesicle transport.* J Cell Biol, 2011. **194**(2): p. 245-55.
524. Baas, P.W. and F.J. Ahmad, *Beyond taxol: microtubule-based treatment of disease and injury of the nervous system.* Brain, 2013. **136**(Pt 10): p. 2937-51.
525. Bradke, F., J.W. Fawcett, and M.E. Spira, *Assembly of a new growth cone after axotomy: the precursor to axon regeneration.* Nat Rev Neurosci, 2012. **13**(3): p. 183-93.
526. Chisholm, A.D., *Cytoskeletal dynamics in Caenorhabditis elegans axon regeneration.* Annu Rev Cell Dev Biol, 2013. **29**: p. 271-97.
527. Hur, E.M., Saijilafu, and F.Q. Zhou, *Growing the growth cone: remodeling the cytoskeleton to promote axon regeneration.* Trends Neurosci, 2012. **35**(3): p. 164-74.
528. Roos, J., et al., *Drosophila Futsch regulates synaptic microtubule organization and is necessary for synaptic growth.* Neuron, 2000. **26**(2): p. 371-82.
529. Hu, X., et al., *BDNF-induced increase of PSD-95 in dendritic spines requires dynamic microtubule invasions.* J Neurosci, 2011. **31**(43): p. 15597-603.
530. Hoogenraad, C.C. and F. Bradke, *Control of neuronal polarity and plasticity--a renaissance for microtubules?* Trends Cell Biol, 2009. **19**(12): p. 669-76.
531. Baas, P.W. and S. Lin, *Hooks and comets: The story of microtubule polarity orientation in the neuron.* Dev Neurobiol, 2011. **71**(6): p. 403-18.
532. Baas, P.W., et al., *Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite.* Proc Natl Acad Sci U S A, 1988. **85**(21): p. 8335-9.
533. Burton, P.R., *Dendrites of mitral cell neurons contain microtubules of opposite polarity.* Brain Res, 1988. **473**(1): p. 107-15.
534. Verhey, K.J. and J.W. Hammond, *Traffic control: regulation of kinesin motors.* Nat Rev Mol Cell Biol, 2009. **10**(11): p. 765-77.
535. Vallee, R.B., et al., *Dynein: An ancient motor protein involved in multiple modes of transport.* J Neurobiol, 2004. **58**(2): p. 189-200.
536. Roll-Mecak, A. and F.J. McNally, *Microtubule-severing enzymes.* Curr Opin Cell Biol, 2010. **22**(1): p. 96-103.
537. Baas, P.W. and M.M. Black, *Individual microtubules in the axon consist of domains that differ in both composition and stability.* J Cell Biol, 1990. **111**(2): p. 495-509.
538. Janke, C. and J.C. Bulinski, *Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions.* Nat Rev Mol Cell Biol, 2011. **12**(12): p. 773-86.
539. Arce, C.A., et al., *Incorporation of L-tyrosine, L-phenylalanine and L-3,4-dihydroxyphenylalanine as single units into rat brain tubulin.* Eur J Biochem, 1975. **59**(1): p. 145-9.
540. Hallak, M.E., et al., *Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin.* FEBS Lett, 1977. **73**(2): p. 147-50.
541. Valenzuela, P., et al., *Nucleotide and corresponding amino acid sequences encoded by alpha and beta tubulin mRNAs.* Nature, 1981. **289**(5799): p. 650-5.
542. Raybin, D. and M. Flavin, *Enzyme which specifically adds tyrosine to the alpha chain of tubulin.* Biochemistry, 1977. **16**(10): p. 2189-94.
543. Kumar, N. and M. Flavin, *Preferential action of a brain detyrosino-lating carboxypeptidase on polymeric tubulin.* The Journal of biological chemistry, 1981. **256**: p. 7678-86.
544. Paturle-Lafanechere, L., et al., *Characterization of a major brain tubulin variant which cannot be tyrosinated.* Biochemistry, 1991. **30**(43): p. 10523-8.

545. Rogowski, K., et al., *A family of protein-deglutamylating enzymes associated with neurodegeneration.* Cell, 2010. **143**(4): p. 564-78.
546. Rudiger, M., J. Wehland, and K. Weber, *The carboxy-terminal peptide of detyrosinated alpha tubulin provides a minimal system to study the substrate specificity of tubulin-tyrosine ligase.* Eur J Biochem, 1994. **220**(2): p. 309-20.
547. Edde, B., et al., *Posttranslational glutamylation of alpha-tubulin.* Science, 1990. **247**(4938): p. 83-5.
548. Alexander, J.E., et al., *Characterization of posttranslational modifications in neuron-specific class III beta-tubulin by mass spectrometry.* Proc Natl Acad Sci U S A, 1991. **88**(11): p. 4685-9.
549. Rudiger, M., et al., *Class II tubulin, the major brain beta tubulin isotype is polyglutamylated on glutamic acid residue 435.* FEBS Lett, 1992. **308**(1): p. 101-5.
550. Redeker, V., et al., *Polyglycylation of tubulin: a posttranslational modification in axonemal microtubules.* Science, 1994. **266**(5191): p. 1688-91.
551. Mukai, M., et al., *Recombinant mammalian tubulin polyglutamylase TTLL7 performs both initiation and elongation of polyglutamylation on beta-tubulin through a random sequential pathway.* Biochemistry, 2009. **48**(5): p. 1084-93.
552. Rogowski, K., et al., *Evolutionary divergence of enzymatic mechanisms for posttranslational polyglycylation.* Cell, 2009. **137**(6): p. 1076-87.
553. Wloga, D., et al., *TTLL3 Is a tubulin glycine ligase that regulates the assembly of cilia.* Dev Cell, 2009. **16**(6): p. 867-76.
554. Audebert, S., et al., *Reversible polyglutamylation of alpha- and beta-tubulin and microtubule dynamics in mouse brain neurons.* Mol Biol Cell, 1993. **4**(6): p. 615-26.
555. Audebert, S., et al., *Developmental regulation of polyglutamylated alpha- and beta-tubulin in mouse brain neurons.* J Cell Sci, 1994. **107 (Pt 8)**: p. 2313-22.
556. Ikegami, K., et al., *TTLL10 is a protein polyglycylase that can modify nucleosome assembly protein 1.* FEBS Lett, 2008. **582**(7): p. 1129-34.
557. Ikegami, K., et al., *TTLL7 is a mammalian beta-tubulin polyglutamylase required for growth of MAP2-positive neurites.* J Biol Chem, 2006. **281**(41): p. 30707-16.
558. van Dijk, J., et al., *A targeted multienzyme mechanism for selective microtubule polyglutamylation.* Mol Cell, 2007. **26**(3): p. 437-48.
559. Ikegami, K. and M. Setou, *TTLL10 can perform tubulin glycylation when co-expressed with TTLL8.* FEBS Lett, 2009. **583**(12): p. 1957-63.
560. Kimura, Y., et al., *Identification of tubulin deglutamylase among Caenorhabditis elegans and mammalian cytosolic carboxypeptidases (CCPs).* J Biol Chem, 2010. **285**(30): p. 22936-41.
561. Flotho, A. and F. Melchior, *Sumoylation: a regulatory protein modification in health and disease.* Annu Rev Biochem, 2013. **82**: p. 357-85.
562. Varshavsky, A., *The ubiquitin system, an immense realm.* Annu Rev Biochem, 2012. **81**: p. 167-76.
563. Ren, Y., J. Zhao, and J. Feng, *Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation.* J Neurosci, 2003. **23**(8): p. 3316-24.
564. Panse, V.G., et al., *A proteome-wide approach identifies sumoylated substrate proteins in yeast.* J Biol Chem, 2004. **279**(40): p. 41346-51.
565. Rosas-Acosta, G., et al., *A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers.* Mol Cell Proteomics, 2005. **4**(1): p. 56-72.
566. Ardley, H.C., et al., *Inhibition of proteasomal activity causes inclusion formation in neuronal and non-neuronal cells overexpressing Parkin.* Mol Biol Cell, 2003. **14**(11): p. 4541-56.

567. Muqit, M.M., et al., *Parkin is recruited into aggresomes in a stress-specific manner: over-expression of parkin reduces aggresome formation but can be dissociated from parkin's effect on neuronal survival.* Hum Mol Genet, 2004. **13**(1): p. 117-35.
568. Hyun, D.H., et al., *Proteasomal inhibition causes the formation of protein aggregates containing a wide range of proteins, including nitrated proteins.* J Neurochem, 2003. **86**(2): p. 363-73.
569. L'Hernault, S.W. and J.L. Rosenbaum, *Chlamydomonas alpha-tubulin is posttranslationally modified by acetylation on the epsilon-amino group of a lysine.* Biochemistry, 1985. **24**(2): p. 473-8.
570. Maruta, H., K. Greer, and J.L. Rosenbaum, *The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules.* J Cell Biol, 1986. **103**(2): p. 571-9.
571. Matsuyama, A., et al., *In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation.* 2002. **21**(24): p. 6820-6831.
572. Hubbert, C., et al., *HDAC6 is a microtubule-associated deacetylase.* Nature, 2002. **417**(6887): p. 455-8.
573. North, B.J., et al., *The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase.* Mol Cell, 2003. **11**(2): p. 437-44.
574. Ohkawa, N., et al., *N-acetyltransferase ARD1-NAT1 regulates neuronal dendritic development.* Genes Cells, 2008. **13**(11): p. 1171-83.
575. Solinger, J.A., et al., *The Caenorhabditis elegans Elongator complex regulates neuronal alpha-tubulin acetylation.* PLoS Genet, 2010. **6**(1): p. e1000820.
576. Creppe, C., et al., *Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin.* Cell, 2009. **136**(3): p. 551-64.
577. Shida, T., et al., *The major alpha-tubulin K40 acetyltransferase alphaTAT1 promotes rapid ciliogenesis and efficient mechanosensation.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21517-22.
578. Akella, J.S., et al., *MEC-17 is an alpha-tubulin acetyltransferase.* Nature, 2010. **467**(7312): p. 218-22.
579. Webster, D.R., et al., *Differential turnover of tyrosinated and detyrosinated microtubules.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9040-4.
580. Khawaja, S., G.G. Gundersen, and J.C. Bulinski, *Enhanced stability of microtubules enriched in detyrosinated tubulin is not a direct function of detyrosination level.* J Cell Biol, 1988. **106**(1): p. 141-9.
581. Peris, L., et al., *Motor-dependent microtubule disassembly driven by tubulin tyrosination.* J Cell Biol, 2009. **185**(7): p. 1159-66.
582. Erck, C., et al., *A vital role of tubulin-tyrosine-ligase for neuronal organization.* Proc Natl Acad Sci U S A, 2005. **102**(22): p. 7853-8.
583. Paturle-Lafanechere, L., et al., *Accumulation of delta 2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies.* J Cell Sci, 1994. **107 (Pt 6)**: p. 1529-43.
584. Tran, A.D., et al., *HDAC6 deacetylation of tubulin modulates dynamics of cellular adhesions.* J Cell Sci, 2007. **120**(Pt 8): p. 1469-79.
585. Sudo, H. and P.W. Baas, *Acetylation of microtubules influences their sensitivity to severing by katanin in neurons and fibroblasts.* J Neurosci, 2010. **30**(21): p. 7215-26.
586. Sharma, N., et al., *Katanin regulates dynamics of microtubules and biogenesis of motile cilia.* J Cell Biol, 2007. **178**(6): p. 1065-79.
587. Lacroix, B., et al., *Tubulin polyglutamylation stimulates spastin-mediated microtubule severing.* J Cell Biol, 2010. **189**(6): p. 945-54.

588. Liao, G. and G.G. Gundersen, *Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. Selective binding of kinesin to detyrosinated tubulin and vimentin.* J Biol Chem, 1998. **273**(16): p. 9797-803.
589. Kreitzer, G., G. Liao, and G.G. Gundersen, *Detyrosination of tubulin regulates the interaction of intermediate filaments with microtubules in vivo via a kinesin-dependent mechanism.* Mol Biol Cell, 1999. **10**(4): p. 1105-18.
590. Dunn, S., et al., *Differential trafficking of Kif5c on tyrosinated and detyrosinated microtubules in live cells.* J Cell Sci, 2008. **121**(Pt 7): p. 1085-95.
591. Konishi, Y. and M. Setou, *Tubulin tyrosination navigates the kinesin-1 motor domain to axons.* Nat Neurosci, 2009. **12**(5): p. 559-67.
592. Dompierre, J.P., et al., *Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation.* J Neurosci, 2007. **27**(13): p. 3571-83.
593. Reed, N.A., et al., *Microtubule acetylation promotes kinesin-1 binding and transport.* Curr Biol, 2006. **16**(21): p. 2166-72.
594. Ikegami, K., et al., *Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3213-8.
595. Cambray-Deakin, M.A. and R.D. Burgoyne, *Posttranslational modifications of alpha-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum.* J Cell Biol, 1987. **104**(6): p. 1569-74.
596. Kim, H., *Depletion of acetylated alpha-tubulin during microtubule purification from bovine brain gray and white matter regions.* J Neurosci Res, 1991. **30**(1): p. 172-82.
597. Black, M.M. and P. Keyser, *Acetylation of alpha-tubulin in cultured neurons and the induction of alpha-tubulin acetylation in PC12 cells by treatment with nerve growth factor.* J Neurosci, 1987. **7**(6): p. 1833-42.
598. Wolff, A., et al., *Distribution of glutamylated alpha and beta-tubulin in mouse tissues using a specific monoclonal antibody, GT335.* Eur J Cell Biol, 1992. **59**(2): p. 425-32.
599. Jacobson, C., B. Schnapp, and G.A. Banker, *A change in the selective translocation of the Kinesin-1 motor domain marks the initial specification of the axon.* Neuron, 2006. **49**(6): p. 797-804.
600. Falconer, M.M., U. Vielkind, and D.L. Brown, *Establishment of a stable, acetylated microtubule bundle during neuronal commitment.* Cell Motil Cytoskeleton, 1989. **12**(3): p. 169-80.
601. L'Hernault, S.W. and J.L. Rosenbaum, *Chlamydomonas alpha-tubulin is posttranslationally modified in the flagella during flagellar assembly.* J Cell Biol, 1983. **97**(1): p. 258-63.
602. Piperno, G., M. LeDizet, and X.J. Chang, *Microtubules containing acetylated alpha-tubulin in mammalian cells in culture.* J Cell Biol, 1987. **104**(2): p. 289-302.
603. Bulinski, J.C., J.E. Richards, and G. Piperno, *Posttranslational modifications of alpha tubulin: detyrosination and acetylation differentiate populations of interphase microtubules in cultured cells.* J Cell Biol, 1988. **106**(4): p. 1213-20.
604. Coombes, C., et al., *Mechanism of microtubule lumen entry for the alpha-tubulin acetyltransferase enzyme alphaTAT1.* Proc Natl Acad Sci U S A, 2016. **113**(46): p. E7176-e7184.
605. Schaedel, L., et al., *Microtubules self-repair in response to mechanical stress.* Nat Mater, 2015. **14**(11): p. 1156-63.
606. Howes, S.C., et al., *Effects of tubulin acetylation and tubulin acetyltransferase binding on microtubule structure.* Mol Biol Cell, 2014. **25**(2): p. 257-66.
607. Ly, N., et al., *alphaTAT1 controls longitudinal spreading of acetylation marks from open microtubules extremities.* Sci Rep, 2016. **6**: p. 35624.
608. Janke, C. and G. Montagnac, *Causes and Consequences of Microtubule Acetylation.* Curr Biol, 2017. **27**(23): p. R1287-R1292.

609. Castro-Castro, A., et al., *ATAT1/MEC-17 acetyltransferase and HDAC6 deacetylase control a balance of acetylation of alpha-tubulin and cortactin and regulate MT1-MMP trafficking and breast tumor cell invasion.* Eur J Cell Biol, 2012. **91**(11-12): p. 950-60.
610. Montagnac, G., et al., *alphaTAT1 catalyses microtubule acetylation at clathrin-coated pits.* Nature, 2013. **502**(7472): p. 567-70.
611. Szyk, A., et al., *Molecular basis for age-dependent microtubule acetylation by tubulin acetyltransferase.* Cell, 2014. **157**(6): p. 1405-15.
612. Zilberman, Y., et al., *Regulation of microtubule dynamics by inhibition of the tubulin deacetylase HDAC6.* Journal of Cell Science, 2009. **122**: p. 3531-3541.
613. LeDizet, M. and G. Piperno, *Cytoplasmic microtubules containing acetylated alpha-tubulin in Chlamydomonas reinhardtii: spatial arrangement and properties.* J Cell Biol, 1986. **103**(1): p. 13-22.
614. Palazzo, A., B. Ackerman, and G.G. Gundersen, *Cell biology: Tubulin acetylation and cell motility.* Nature, 2003. **421**(6920): p. 230.
615. Portran, D., et al., *Tubulin acetylation protects long-lived microtubules against mechanical ageing.* Nat Cell Biol, 2017. **19**(4): p. 391-398.
616. Xu, Z., et al., *Microtubules acquire resistance from mechanical breakage through intralumenal acetylation.* Science, 2017. **356**: p. 328-332.
617. Schaap, I.A., et al., *Elastic response, buckling, and instability of microtubules under radial indentation.* Biophys J, 2006. **91**(4): p. 1521-31.
618. Geeraert, C., et al., *Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation.* J Biol Chem, 2010. **285**(31): p. 24184-94.
619. Morley, S.J., et al., *Acetylated tubulin is essential for touch sensation in mice.* Elife, 2016. **5**.
620. Aguilar, A., et al., *Alpha-tubulin K40 acetylation is required for contact inhibition of proliferation and cell-substrate adhesion.* Mol Biol Cell, 2014. **25**(12): p. 1854-66.
621. Kaul, N., V. Soppina, and K.J. Verhey, *Effects of alpha-tubulin K40 acetylation and detyrosination on kinesin-1 motility in a purified system.* Biophys J, 2014. **106**(12): p. 2636-43.
622. Walter, W.J., et al., *Tubulin acetylation alone does not affect kinesin-1 velocity and run length in vitro.* PLoS One, 2012. **7**(8): p. e42218.
623. Kim, G.W., et al., *Mice lacking alpha-tubulin acetyltransferase 1 are viable but display alpha-tubulin acetylation deficiency and dentate gyrus distortion.* J Biol Chem, 2016. **291**(48): p. 25279.
624. Kalebic, N., et al., *alphaTAT1 is the major alpha-tubulin acetyltransferase in mice.* Nat Commun, 2013. **4**: p. 1962.
625. Verdel, A., et al., *Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm.* Curr Biol, 2000. **10**(12): p. 747-9.
626. Bertos, N.R., et al., *Role of the tetradecapeptide repeat domain of human histone deacetylase 6 in cytoplasmic retention.* J Biol Chem, 2004. **279**(46): p. 48246-54.
627. Seigneurin-Berny, D., et al., *Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways.* Mol Cell Biol, 2001. **21**(23): p. 8035-44.
628. Grozinger, C.M., C.A. Hassig, and S.L. Schreiber, *Three proteins define a class of human histone deacetylases related to yeast Hda1p.* Proc Natl Acad Sci U S A, 1999. **96**(9): p. 4868-73.
629. Zhang, Y., et al., *Two catalytic domains are required for protein deacetylation.* J Biol Chem, 2006. **281**(5): p. 2401-4.
630. Zhang, Y., et al., *HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo.* Embo j, 2003. **22**(5): p. 1168-79.
631. Haggarty, S.J., et al., *Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4389-94.

632. Zou, H., et al., *Characterization of the two catalytic domains in histone deacetylase 6.* Biochem Biophys Res Commun, 2006. **341**(1): p. 45-50.
633. Kawaguchi, Y., et al., *The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress.* Cell, 2003. **115**(6): p. 727-38.
634. Boyault, C., et al., *HDAC6-p97/VCP controlled polyubiquitin chain turnover.* Embo j, 2006. **25**(14): p. 3357-66.
635. Amerik, A.Y., S.J. Li, and M. Hochstrasser, *Analysis of the deubiquitinating enzymes of the yeast Saccharomyces cerevisiae.* Biol Chem, 2000. **381**(9-10): p. 981-92.
636. Hook, S.S., et al., *Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13425-30.
637. Bali, P., et al., *Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors.* J Biol Chem, 2005. **280**(29): p. 26729-34.
638. Kovacs, J.J., et al., *HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor.* Mol Cell, 2005. **18**(5): p. 601-7.
639. Murphy, P.J., et al., *Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone.* J Biol Chem, 2005. **280**(40): p. 33792-9.
640. Zhao, R., et al., *Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone.* Cell, 2005. **120**(5): p. 715-27.
641. Pratt, W.B. and D.O. Toft, *Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery.* Exp Biol Med (Maywood), 2003. **228**(2): p. 111-33.
642. Caron, C., C. Boyault, and S. Khochbin, *Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability.* Bioessays, 2005. **27**(4): p. 408-15.
643. Aoyagi, S. and T.K. Archer, *Modulating molecular chaperone Hsp90 functions through reversible acetylation.* Trends Cell Biol, 2005. **15**(11): p. 565-7.
644. Scroggins, B.T., et al., *An acetylation site in the middle domain of Hsp90 regulates chaperone function.* Mol Cell, 2007. **25**(1): p. 151-9.
645. Espallergues, J., et al., *HDAC6 regulates glucocorticoid receptor signaling in serotonin pathways with critical impact on stress resilience.* J Neurosci, 2012. **32**(13): p. 4400-16.
646. Valenzuela-Fernandez, A., et al., *HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions.* Trends Cell Biol, 2008. **18**(6): p. 291-7.
647. Pandey, U.B., et al., *HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS.* Nature, 2007. **447**(7146): p. 859-63.
648. Zhang, X., et al., *HDAC6 modulates cell motility by altering the acetylation level of cortactin.* Mol Cell, 2007. **27**(2): p. 197-213.
649. Aldana-Masangkay, G.I. and K.M. Sakamoto, *The role of HDAC6 in cancer.* J Biomed Biotechnol, 2011. **2011**: p. 875824.
650. Zhang, Y., et al., *Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally.* Mol Cell Biol, 2008. **28**(5): p. 1688-701.
651. Topalidou, I., et al., *Genetically separable functions of the MEC-17 tubulin acetyltransferase affect microtubule organization.* Curr Biol, 2012. **22**(12): p. 1057-65.
652. Cueva, J.G., et al., *Posttranslational acetylation of alpha-tubulin constrains protofilament number in native microtubules.* Curr Biol, 2012. **22**(12): p. 1066-74.
653. Kalebic, N., et al., *Tubulin acetyltransferase alphaTAT1 destabilizes microtubules independently of its acetylation activity.* Mol Cell Biol, 2013. **33**(6): p. 1114-23.

654. Jochems, J., et al., *Enhancement of stress resilience through histone deacetylase 6-mediated regulation of glucocorticoid receptor chaperone dynamics.* Biol Psychiatry, 2015. **77**(4): p. 345-55.
655. Jochems, J., et al., *Antidepressant-like properties of novel HDAC6-selective inhibitors with improved brain bioavailability.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2014. **39**(2): p. 389-400.
656. Singh, H., et al., *Membrane-associated alpha-tubulin is less acetylated in postmortem prefrontal cortex from depressed subjects relative to controls: cytoskeletal dynamics, HDAC6 and depression.* J Neurosci, 2020.
657. Van Helleputte, L., et al., *Inhibition of histone deacetylase 6 (HDAC6) protects against vincristine-induced peripheral neuropathies and inhibits tumor growth.* Neurobiol Dis, 2018. **111**: p. 59-69.
658. Krukowski, K., et al., *HDAC6 inhibition effectively reverses chemotherapy-induced peripheral neuropathy.* Pain, 2017. **158**(6): p. 1126-1137.
659. Sakloth, F., et al., *HDAC6-selective inhibitors decrease nerve-injury and inflammation-associated mechanical hypersensitivity in mice.* Psychopharmacology (Berl), 2020.
660. *Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013.* Lancet, 2015. **386**(9995): p. 743-800.
661. ICHD, *The International Classification of Headache Disorders, 3rd edition (beta version).* Cephalalgia, 2013. **33**(9): p. 629-808.
662. Ford, J.H., et al., *A Real-World Analysis of Migraine: A Cross-Sectional Study of Disease Burden and Treatment Patterns.* Headache, 2017. **57**(10): p. 1532-1544.
663. Descalzi, G., et al., *Epigenetic mechanisms of chronic pain.* Trends Neurosci, 2015. **38**(4): p. 237-46.
664. Krishnan, H.R., et al., *The epigenetic landscape of alcoholism.* Int Rev Neurobiol, 2014. **115**: p. 75-116.
665. d'Ydewalle, C., E. Bogaert, and L. Van Den Bosch, *HDAC6 at the Intersection of Neuroprotection and Neurodegeneration.* Traffic, 2012. **13**(6): p. 771-9.
666. Gallo, G., *The cytoskeletal and signaling mechanisms of axon collateral branching.* Dev Neurobiol, 2011. **71**(3): p. 201-20.
667. Braun, G., et al., *TRESK background K(+) channel is inhibited by PAR-1/MARK microtubule affinity-regulating kinases in Xenopus oocytes.* PLoS One, 2011. **6**(12): p. e28119.
668. Jin, P., et al., *Electron cryo-microscopy structure of the mechanotransduction channel NOMPC.* Nature, 2017. **547**(7661): p. 118-122.
669. Covington, H.E., 3rd, et al., *Antidepressant actions of histone deacetylase inhibitors.* J Neurosci, 2009. **29**(37): p. 11451-60.
670. Schappi, J.M., A. Krbanjevic, and M.M. Rasenick, *Tubulin, actin and heterotrimeric G proteins: coordination of signaling and structure.* Biochim Biophys Acta, 2014. **1838**(2): p. 674-81.
671. Singh, H., et al., *Disruption of lipid-raft localized Galphas/tubulin complexes by antidepressants: a unique feature of HDAC6 inhibitors, SSRI and tricyclic compounds.* Neuropsychopharmacology, 2018.
672. Forrest, M.P., E. Parnell, and P. Penzes, *Dendritic structural plasticity and neuropsychiatric disease.* Nat Rev Neurosci, 2018. **19**(4): p. 215-234.
673. Nestler, E.J. and C. Luscher, *The Molecular Basis of Drug Addiction: Linking Epigenetic to Synaptic and Circuit Mechanisms.* Neuron, 2019. **102**(1): p. 48-59.
674. Longair, M.H., D.A. Baker, and J.D. Armstrong, *Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes.* Bioinformatics, 2011. **27**(17): p. 2453-2454.

675. Moonat, S., et al., *Aberrant histone deacetylase2-mediated histone modifications and synaptic plasticity in the amygdala predisposes to anxiety and alcoholism.* Biol Psychiatry, 2013. **73**(8): p. 763-73.
676. Goadsby, P.J., et al., *Pathophysiology of Migraine: A Disorder of Sensory Processing.* Physiol Rev, 2017. **97**(2): p. 553-622.
677. Xu, Z., et al., *Microtubules acquire resistance from mechanical breakage through intralumenal acetylation.* Science, 2017. **356**(6335): p. 328-332.
678. Jochems, J., et al., *Antidepressant-like properties of novel HDAC6-selective inhibitors with improved brain bioavailability.* Neuropsychopharmacology, 2014. **39**(2): p. 389-400.
679. Abdelkarim, H., et al., *Design, Synthesis, Molecular Modeling, and Biological Evaluation of Novel Amine-based Histone Deacetylase Inhibitors.* ChemMedChem, 2017. **12**(24): p. 2030-2043.
680. Ayata, C., et al., *Suppression of cortical spreading depression in migraine prophylaxis.* Ann.Neurol., 2006. **59**(4): p. 652-661.
681. Bogdanov, V.B., et al., *Susceptibility of Primary Sensory Cortex to Spreading Depolarizations.* J Neurosci, 2016. **36**(17): p. 4733-43.
682. Olesen, J., et al., *Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine.* N Engl J Med, 2004. **350**(11): p. 1104-10.
683. Christensen, S.L., et al., *Targeting CGRP via receptor antagonism and antibody neutralisation in two distinct rodent models of migraine-like pain.* Cephalalgia, 2019. **39**(14): p. 1827-1837.
684. Demartini, C., et al., *Nitroglycerin as a comparative experimental model of migraine pain: From animal to human and back.* Prog Neurobiol, 2019. **177**: p. 15-32.
685. Schytz, H.W., G.G. Schoonman, and M. Ashina, *What have we learnt from triggering migraine?* Curr.Opin.Neurol., 2010. **23**(3): p. 259-265.
686. Bates, E.A., et al., *Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice.* Cephalalgia, 2010. **30**(2): p. 170-178.
687. Markovics, A., et al., *Pituitary adenylate cyclase-activating polypeptide plays a key role in nitroglycerol-induced trigeminovascular activation in mice.* Neurobiol.Dis., 2012. **45**(1): p. 633-644.
688. Pradhan, A.A., et al., *Characterization of a novel model of chronic migraine.* Pain, 2014. **155**(2): p. 269-74.
689. Farajdokht, F., et al., *Ghrelin attenuated hyperalgesia induced by chronic nitroglycerin: CGRP and TRPV1 as targets for migraine management.* Cephalalgia, 2017: p. 333102417748563.
690. Long, T., et al., *Microglia P2X4 receptor contributes to central sensitization following recurrent nitroglycerin stimulation.* J Neuroinflammation, 2018. **15**(1): p. 245.
691. Zhang, J., et al., *Low-dose interleukin-2 reverses behavioral sensitization in multiple mouse models of headache disorders.* Pain, 2020.
692. Greco, R., et al., *Chronic and intermittent administration of systemic nitroglycerin in the rat induces an increase in the gene expression of CGRP in central areas: potential contribution to pain processing.* J Headache Pain, 2018. **19**(1): p. 51.
693. Tassorelli, C. and S.A. Joseph, *Systemic nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat.* Brain Res, 1995. **682**(1-2): p. 167-81.
694. Ramachandran, R., et al., *A naturalistic glyceryl trinitrate infusion migraine model in the rat.* Cephalalgia, 2011. **32**(1): p. 73-84.
695. Jeong, H., et al., *Gene Network Dysregulation in the Trigeminal Ganglia and Nucleus Accumbens of a Model of Chronic Migraine-Associated Hyperalgesia.* Front Syst Neurosci, 2018. **12**: p. 63.
696. Liu, Y., et al., *TNF-alpha Differentially Regulates Synaptic Plasticity in the Hippocampus and Spinal Cord by Microglia-Dependent Mechanisms after Peripheral Nerve Injury.* J Neurosci, 2017. **37**(4): p. 871-881.

697. Brennan, K.C. and D. Pietrobon, *A Systems Neuroscience Approach to Migraine.* Neuron, 2018. **97**(5): p. 1004-1021.
698. Steffensen, A.B., et al., *Chloride Cotransporters as a Molecular Mechanism underlying Spreading Depolarization-Induced Dendritic Beading.* J Neurosci, 2015. **35**(35): p. 12172-87.
699. Eikermann-Haerter, K., et al., *Abnormal synaptic Ca(2+) homeostasis and morphology in cortical neurons of familial hemiplegic migraine type 1 mutant mice.* Ann Neurol, 2015. **78**(2): p. 193-210.
700. Schliwa, M., et al., *Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins.* Proc Natl Acad Sci U S A, 1981. **78**(2): p. 1037-41.
701. Basarsky, T.A., et al., *Imaging spreading depression and associated intracellular calcium waves in brain slices.* J Neurosci, 1998. **18**(18): p. 7189-99.
702. Zhang, X., R. Burstein, and D. Levy, *Local action of the proinflammatory cytokines IL-1beta and IL-6 on intracranial meningeal nociceptors.* Cephalalgia, 2012. **32**(1): p. 66-72.
703. Noseda, R. and R. Burstein, *Migraine pathophysiology: anatomy of the trigeminovascular pathway and associated neurological symptoms, cortical spreading depression, sensitization, and modulation of pain.* Pain, 2013. **154 Suppl 1**: p. S44-53.
704. Melo-Carrillo, A., et al., *Selective Inhibition of Trigeminovascular Neurons by Fremanezumab: A Humanized Monoclonal Anti-CGRP Antibody.* J Neurosci, 2017. **37**(30): p. 7149-7163.
705. Bolay, H., et al., *Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model.* Nat Med, 2002. **8**(2): p. 136-42.
706. Filiz, A., et al., *CGRP receptor antagonist MK-8825 attenuates cortical spreading depression induced pain behavior.* Cephalalgia, 2019. **39**(3): p. 354-365.
707. Gibbs, K.L., L. Greensmith, and G. Schiavo, *Regulation of Axonal Transport by Protein Kinases.* Trends Biochem Sci, 2015. **40**(10): p. 597-610.
708. Singh, H., et al., *Disruption of lipid-raft localized Gαs/tubulin complexes by antidepressants: a unique feature of HDAC6 inhibitors, SSRI and tricyclic compounds.* Neuropsychopharmacology, 2018. **43**(7): p. 1481-1491.
709. Singh, H., et al., *Membrane-associated α-tubulin is less acetylated in postmortem prefrontal cortex from depressed subjects relative to controls: cytoskeletal dynamics, HDAC6 and depression.* J Neurosci, 2020.
710. d'Ydewalle, C., et al., *HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease.* Nat Med, 2011. **17**(8): p. 968-74.
711. Benoy, V., et al., *HDAC6 is a therapeutic target in mutant GARS-induced Charcot-Marie-Tooth disease.* Brain, 2018. **141**(3): p. 673-687.
712. Offenhauser, N., et al., *CGRP release and c-fos expression within trigeminal nucleus caudalis of the rat following glyceryltrinitrate infusion.* Cephalalgia, 2005. **25**(3): p. 225-36.
713. Ramachandran, R., et al., *Nitric oxide synthase, calcitonin gene-related peptide and NK-1 receptor mechanisms are involved in GTN-induced neuronal activation.* Cephalalgia, 2014. **34**(2): p. 136-47.
714. Di, W., et al., *Pregabalin alleviates the nitroglycerin-induced hyperalgesia in rats.* Neuroscience, 2015. **284**: p. 11-17.
715. Magon, S., et al., *Cortical abnormalities in episodic migraine: A multi-center 3T MRI study.* Cephalalgia, 2019. **39**(5): p. 665-673.
716. Dahlhamer, J., et al., *Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults - United States, 2016.* MMWR Morb Mortal Wkly Rep, 2018. **67**(36): p. 1001-1006.
717. Treede, R.D., et al., *A classification of chronic pain for ICD-11.* Pain, 2015. **156**(6): p. 1003-7.
718. Chapman, C.R. and C.J. Vierck, *The Transition of Acute Postoperative Pain to Chronic Pain: An Integrative Overview of Research on Mechanisms.* J Pain, 2017. **18**(4): p. 359.e1-359.e38.

719. Meacham, K., et al., *Neuropathic Pain: Central vs. Peripheral Mechanisms.* Curr Pain Headache Rep, 2017. **21**(6): p. 28.
720. May, A. and L.H. Schulte, *Chronic migraine: risk factors, mechanisms and treatment.* Nat Rev Neurol, 2016. **12**(8): p. 455-64.
721. Bertels, Z., et al., *Neuronal complexity is attenuated in chronic migraine and restored by HDAC6 inhibition.* bioRxiv, 2020: p. 2020.04.21.053272.
722. Chen, S.P. and C. Ayata, *Novel Therapeutic Targets Against Spreading Depression.* Headache, 2017. **57**(9): p. 1340-1358.
723. Chen, Z., et al., *Volumetric abnormalities of thalamic subnuclei in medication-overuse headache.* J Headache Pain, 2017. **18**(1): p. 82.
724. Tajerian, M., et al., *The hippocampal extracellular matrix regulates pain and memory after injury.* Mol Psychiatry, 2018. **23**(12): p. 2302-2313.
725. Wei, T., et al., *Acute versus chronic phase mechanisms in a rat model of CRPS.* J Neuroinflammation, 2016. **13**: p. 14.
726. Lenz, M., et al., *Local cytokine changes in complex regional pain syndrome type I (CRPS I) resolve after 6 months.* Pain, 2013. **154**(10): p. 2142-9.
727. Bruehl, S., et al., *Complex regional pain syndrome: evidence for warm and cold subtypes in a large prospective clinical sample.* Pain, 2016. **157**(8): p. 1674-81.
728. Neugebauer, V., *Amygdala pain mechanisms.* Handb Exp Pharmacol, 2015. **227**: p. 261-84.
729. Borsook, D., et al., *A key role of the basal ganglia in pain and analgesia - insights gained through human functional imaging.* Molecular Pain, 2010. **6**(1): p. 27.
730. Chaplan, S.R., et al., *Quantitative assessment of tactile allodynia in the rat paw.* J Neurosci Methods, 1994. **53**(1): p. 55-63.
731. Tajerian, M., et al., *Sex differences in a Murine Model of Complex Regional Pain Syndrome.* Neurobiol Learn Mem, 2015. **123**: p. 100-9.
732. Zagami, A.S. and G.A. Lambert, *Stimulation of cranial vessels excites nociceptive neurones in several thalamic nuclei of the cat.* Exp Brain Res, 1990. **81**(3): p. 552-66.
733. Shields, K.G. and P.J. Goadsby, *Propranolol modulates trigeminovascular responses in thalamic ventroposteromedial nucleus: a role in migraine?* Brain, 2005. **128**(Pt 1): p. 86-97.
734. Ren, W. and V. Neugebauer, *Pain-related increase of excitatory transmission and decrease of inhibitory transmission in the central nucleus of the amygdala are mediated by mGluR1.* Mol Pain, 2010. **6**: p. 93.
735. Cropper, H.C., et al., *Longitudinal translocator protein-18 kDa-positron emission tomography imaging of peripheral and central myeloid cells in a mouse model of complex regional pain syndrome.* Pain, 2019. **160**(9): p. 2136-2148.
736. McCracken, L.M. and G.L. Iverson, *Predicting complaints of impaired cognitive functioning in patients with chronic pain.* J Pain Symptom Manage, 2001. **21**(5): p. 392-6.
737. Landrø, N.I., et al., *The extent of neurocognitive dysfunction in a multidisciplinary pain centre population. Is there a relation between reported and tested neuropsychological functioning?* Pain, 2013. **154**(7): p. 972-7.
738. Schytz, H.W., G.G. Schoonman, and M. Ashina, *What have we learnt from triggering migraine?* Curr Opin Neurol, 2010. **23**(3): p. 259-65.
739. Chen, X.Y., et al., *Regional volume changes of the brain in migraine chronification.* Neural Regen Res, 2020. **15**(9): p. 1701-1708.
740. Chen, Z., et al., *Altered functional connectivity of amygdala underlying the neuromechanism of migraine pathogenesis.* J Headache Pain, 2017. **18**(1): p. 7.

741. Alexander, G.E., M.D. Crutcher, and M.R. DeLong, *Basal ganglia-thalamocortical circuits: parallel substrates for motor, oculomotor, "prefrontal" and "limbic" functions.* Prog Brain Res, 1990. **85**: p. 119-46.
742. Jones, A.K., et al., *Cortical and subcortical localization of response to pain in man using positron emission tomography.* Proc Biol Sci, 1991. **244**(1309): p. 39-44.
743. Peyron, R., B. Laurent, and L. García-Larrea, *Functional imaging of brain responses to pain. A review and meta-analysis (2000).* Neurophysiol Clin, 2000. **30**(5): p. 263-88.
744. Starr, C.J., et al., *The contribution of the putamen to sensory aspects of pain: insights from structural connectivity and brain lesions.* Brain, 2011. **134**(Pt 7): p. 1987-2004.
745. Li, Y.Q., et al., *Direct projections from the midbrain periaqueductal gray and the dorsal raphe nucleus to the trigeminal sensory complex in the rat.* Neuroscience, 1993. **54**(2): p. 431-43.
746. Knight, Y.E., et al., *P/Q-type calcium-channel blockade in the periaqueductal gray facilitates trigeminal nociception: a functional genetic link for migraine?* J Neurosci, 2002. **22**(5): p. Rc213.
747. Bruehl, S., et al., *DNA methylation profiles are associated with complex regional pain syndrome after traumatic injury.* Pain, 2019. **160**(10): p. 2328-2337.
748. Reynolds, D.V., *Surgery in the rat during electrical analgesia induced by focal brain stimulation.* Science, 1969. **164**(3878): p. 444-5.
749. Loyd, D.R. and A.Z. Murphy, *The Role of the Periaqueductal Gray in the Modulation of Pain in Males and Females: Are the Anatomy and Physiology Really that Different?* Neural Plasticity, 2009. **2009**: p. 462879.
750. Fields, H., *State-dependent opioid control of pain.* Nat Rev Neurosci, 2004. **5**(7): p. 565-75.
751. Samineni, V.K., et al., *Divergent Modulation of Nociception by Glutamatergic and GABAergic Neuronal Subpopulations in the Periaqueductal Gray.* eneuro, 2017. **4**(2): p. ENEURO.0129-16.2017.
752. Spiga, S., et al., *Simultaneous Golgi-Cox and immunofluorescence using confocal microscopy.* Brain Struct Funct, 2011. **216**(3): p. 171-82.
753. Hua, T., et al., *General anesthetics activate a potent central pain-suppression circuit in the amygdala.* Nat Neurosci, 2020. **23**(7): p. 854-868.
754. Santhanam, P., et al., *Age-Accelerated Reduction in Cortical Surface Area in United States Service Members and Veterans with Mild Traumatic Brain Injury and Post-Traumatic Stress Disorder.* J Neurotrauma, 2019. **36**(20): p. 2922-2929.
755. *Global, regional, and national burden of neurological disorders during 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015.* Lancet Neurol, 2017. **16**(11): p. 877-897.
756. Lipton, R.B., et al., *Discontinuation of Acute Prescription Medication for Migraine: Results From the Chronic Migraine Epidemiology and Outcomes (CaMEO) Study.* Headache, 2019. **59**(10): p. 1762-1772.
757. Dodick, D.W., *CGRP ligand and receptor monoclonal antibodies for migraine prevention: Evidence review and clinical implications.* Cephalalgia, 2019. **39**(3): p. 445-458.
758. Do, T.P., S. Guo, and M. Ashina, *Therapeutic novelties in migraine: new drugs, new hope?* J Headache Pain, 2019. **20**(1): p. 37.
759. Goadsby, P.J., *Bench to bedside advances in the 21st century for primary headache disorders: migraine treatments for migraine patients.* Brain, 2016. **139**(Pt 10): p. 2571-2577.
760. Bertels, Z. and A.A.A. Pradhan, *Emerging Treatment Targets for Migraine and Other Headaches.* Headache, 2019. **59 Suppl 2**: p. 50-65.
761. Moye, L.S., et al., *The development of a mouse model of mTBI-induced post-traumatic migraine, and identification of the delta opioid receptor as a novel therapeutic target.* Cephalalgia, 2018: p. 333102418777507.

762. Moye, L.S., et al., *Delta opioid receptor agonists are effective for multiple types of headache disorders.* Neuropharmacology, 2018. **148**: p. 77-86.
763. Gendron, L., et al., *Molecular Pharmacology of delta-Opioid Receptors.* Pharmacol Rev, 2016. **68**(3): p. 631-700.
764. Comer, S.D., et al., *Convulsive effects of systemic administration of the delta opioid agonist BW373U86 in mice.* J.Pharmacol.Exp.Ther., 1993. **267**(2): p. 888-895.
765. Negus, S.S., et al., *Behavioral effects of the systemically active delta opioid agonist BW373U86 in rhesus monkeys.* J.Pharmacol.Exp.Ther., 1994. **270**(3): p. 1025-1034.
766. Broom, D.C., et al., *Comparison of receptor mechanisms and efficacy requirements for delta-agonist-induced convulsive activity and antinociception in mice.* J.Pharmacol.Exp.Ther., 2002. **303**(2): p. 723-729.
767. Broom, D.C., et al., *Convulsant activity of a non-peptidic delta-opioid receptor agonist is not required for its antidepressant-like effects in Sprague-Dawley rats.* Psychopharmacology (Berl), 2002. **164**(1): p. 42-48.
768. Jutkiewicz, E.M., et al., *The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid agonists in rats: comparison with pentylenetetrazol.* J.Pharmacol.Exp.Ther., 2006. **317**(3): p. 1337-1348.
769. Danielsson, I., et al., *Electroencephalographic and convulsant effects of the delta opioid agonist SNC80 in rhesus monkeys.* Pharmacol Biochem Behav, 2006. **85**(2): p. 428-34.
770. Chu Sin Chung, P., et al., *Delta opioid receptors expressed in forebrain GABAergic neurons are responsible for SNC80-induced seizures.* Behav Brain Res, 2014. **278C**: p. 429-434.
771. Pradhan, A.A., et al., *Ligand-directed signalling within the opioid receptor family.* Br.J.Pharmacol., 2012. **167**(5): p. 960-969.
772. Conibear, A.E., et al., *A Novel G Protein-Biased Agonist at the δ Opioid Receptor with Analgesic Efficacy in Models of Chronic Pain.* J Pharmacol Exp Ther, 2020. **372**(2): p. 224-236.
773. Le Bourdonnec, B., et al., *Potent, orally bioavailable delta opioid receptor agonists for the treatment of pain: discovery of N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl)benzamide (ADL5859).* J.Med.Chem., 2008. **51**(19): p. 5893-5896.
774. Saitoh, A., et al., *Effects of the delta opioid receptor agonist KNT-127 on electroencephalographic activity in mice.* Pharmacol Rep, 2018. **70**(2): p. 350-354.
775. Saitoh, A., et al., *The novel delta opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions.* Behav Brain Res, 2011. **223**(2): p. 271-9.
776. Moye, L.S. and A.A.A. Pradhan, *Animal Model of Chronic Migraine-Associated Pain.* Curr Protoc Neurosci, 2017. **80**: p. 9.60.1-9.60.9.
777. Ben Aissa, M., et al., *Soluble guanylyl cyclase is a critical regulator of migraine-associated pain.* Cephalalgia, 2018. **38**(8): p. 1471-1484.
778. Chaplan , S.R., et al., *Quantitative assessment of tactile allodynia in the rat paw.* Journal of Neuroscience Methods, 1994. **53**: p. 55-63.
779. Chiang, T., K. Sansuk, and R.M. van Rijn, *Beta-arrestin 2 dependence of delta opioid receptor agonists is correlated with alcohol intake.* Br J Pharmacol, 2015.
780. Gastard, M., *Delta-opioid receptor endocytosis in spinal cord after dermenkephalin activation.* BMC Neurosci, 2000. **1**: p. 1.
781. Rice, F.L., et al., *Anatomy and immunochemical characterization of the non-arterial peptidergic diffuse dural innervation of the rat and Rhesus monkey: Implications for functional regulation and treatment in migraine.* Cephalalgia, 2017. **37**(14): p. 1350-1372.

782. Fossler, M.J., et al., *A Phase I, Randomized, Single-Blind, Placebo-Controlled, Single Ascending Dose Study of the Safety, Tolerability, and Pharmacokinetics of Subcutaneous and Oral TRV250, a G Protein-Selective Delta Receptor Agonist, in Healthy Subjects.* CNS Drugs, 2020.
783. Petrillo, P., et al., *Evidence for a selective role of the delta-opioid agonist [8R-(4bS*,8aalpha,8abeta, 12bbeta)]7,10-Dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro[3,2-e]pyrrolo[2,3-g]isoquinoli ne hydrochloride (SB-235863) in blocking hyperalgesia associated with inflammatory and neuropathic pain responses.* J Pharmacol Exp Ther, 2003. **307**(3): p. 1079-89.
784. Kabli, N. and C.M. Cahill, *Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain.* Pain, 2007. **127**(1-2): p. 84-93.
785. Nozaki, C., et al., *In vivo properties of KNT-127, a novel delta opioid agonist: receptor internalisation, antihyperalgesia and antidepressant effects in mice.* Br J Pharmacol, 2014.
786. Gotoh, L., et al., *Effects of repeated treatment with a delta opioid receptor agonist KNT-127 on hyperemotionality in olfactory-bulbectomized rats.* Behav Brain Res, 2017. **323**: p. 11-14.
787. van Rijn, R.M., J.N. Defriel, and J.L. Whistler, *Pharmacological traits of delta opioid receptors: pitfalls or opportunities?* Psychopharmacology (Berl), 2013. **228**(1): p. 1-18.
788. Sugiyama, A., et al., *Administration of a delta opioid receptor agonist KNT-127 to the basolateral amygdala has robust anxiolytic-like effects in rats.* Psychopharmacology (Berl), 2018. **235**(10): p. 2947-2955.
789. Bogdanov, V.B., et al., *Migraine preventive drugs differentially affect cortical spreading depression in rat.* Neurobiol.Dis., 2011. **41**(2): p. 430-435.
790. Pradhan, A.A. and P.B. Clarke, *Comparison between delta-opioid receptor functional response and autoradiographic labeling in rat brain and spinal cord.* J.Comp Neurol., 2005. **481**(4): p. 416-426.
791. Peckys, D. and G.B. Landwehrmeyer, *Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a 33P in situ hybridization study.* Neuroscience, 1999. **88**(4): p. 1093-135.
792. Chu Sin Chung, P., et al., *A novel anxiogenic role for the delta opioid receptor expressed in GABAergic forebrain neurons.* Biol Psychiatry, 2015. **77**(4): p. 404-15.
793. Pradhan, A.A., et al., *In vivo delta opioid receptor internalization controls behavioral effects of agonists.* PLoS.One., 2009. **4**(5): p. e5425.
794. Pradhan, A.A., et al., *Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance.* J.Neurosci., 2010. **30**(49): p. 16459-16468.
795. Vicente-Sanchez, A., et al., *Tolerance to high-internalizing delta opioid receptor agonist is critically mediated by arrestin 2.* Br J Pharmacol, 2018.
796. Roychowdhury, S., et al., *G protein alpha subunits activate tubulin GTPase and modulate microtubule polymerization dynamics.* J Biol Chem, 1999. **274**(19): p. 13485-90.
797. Turk, D.C. and H. Flor, *Pain greater than pain behaviors: the utility and limitations of the pain behavior construct.* Pain, 1987. **31**(3): p. 277-95.
798. Akintola, T., et al., *The grimace scale reliably assesses chronic pain in a rodent model of trigeminal neuropathic pain.* Neurobiol Pain, 2017. **2**: p. 13-17.
799. Targowska-Duda, K.M., et al., *NOP receptor agonist attenuates nitroglycerin-induced migraine-like symptoms in mice.* Neuropharmacology, 2020. **170**: p. 108029.
800. Cox, W.H., *Imprägnation des centralen Nervensystems mit Quecksilbersalzen.* Archiv für mikroskopische Anatomie, 1891. **37**(1): p. 16-21.
801. Kemali, M., *A Modification of the Rapid Golgi Method.* Stain Technology, 1976. **51**(3): p. 169-172.
802. Riley, J.N., *A reliable Golgi-Kopsch modification.* Brain Res Bull, 1979. **4**(1): p. 127-9.

803. Yuste, R., *The discovery of dendritic spines by Cajal.* Frontiers in Neuroanatomy, 2015. **9**(18).
804. Ramón-Moliner, E., *The Golgi-Cox Technique*, in *Contemporary Research Methods in Neuroanatomy*, W.J.H. Nauta and S.O.E. Ebbesson, Editors. 1970, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 32-55.
805. Spacek, J., *Dynamics of the Golgi method: a time-lapse study of the early stages of impregnation in single sections.* J Neurocytol, 1989. **18**(1): p. 27-38.
806. Rosoklija, G.B., et al., *Reliable and durable Golgi staining of brain tissue from human autopsies and experimental animals.* J Neurosci Methods, 2014. **230**: p. 20-9.
807. Kuramoto, E., *Method for labeling and reconstruction of single neurons using Sindbis virus vectors.* J Chem Neuroanat, 2019. **100**: p. 101648.
808. Horikawa, K. and W.E. Armstrong, *A versatile means of intracellular labeling: injection of biocytin and its detection with avidin conjugates.* J Neurosci Methods, 1988. **25**(1): p. 1-11.
809. Buhl, E.H. and J. Lubke, *Intracellular lucifer yellow injection in fixed brain slices combined with retrograde tracing, light and electron microscopy.* Neuroscience, 1989. **28**(1): p. 3-16.
810. Van Raay, T.J. and M.R. Stark, *Cell labeling and gene misexpression by electroporation.* Methods Mol Biol, 2002. **198**: p. 223-32.
811. de Lima, A.D., T. Voigt, and J.H. Morrison, *Morphology of the cells within the inferior temporal gyrus that project to the prefrontal cortex in the macaque monkey.* J Comp Neurol, 1990. **296**(1): p. 159-72.
812. Schmidt, M., et al., *Dendritic morphology of projection neurons in the cat pretectum.* J Comp Neurol, 1996. **369**(4): p. 520-32.
813. Surkis, A., et al., *Quantitative morphology of physiologically identified and intracellularly labeled neurons from the guinea-pig laterodorsal tegmental nucleus in vitro.* Neuroscience, 1996. **74**(2): p. 375-92.
814. Wu, C.C., et al., *High-throughput morphometric analysis of individual neurons.* Cereb Cortex, 2004. **14**(5): p. 543-54.
815. Bridgman, P.C., M.E. Brown, and I. Balan, *Biolistic transfection.* Methods Cell Biol, 2003. **71**: p. 353-68.
816. Gan, W.B., et al., *Multicolor "DiOlistic" labeling of the nervous system using lipophilic dye combinations.* Neuron, 2000. **27**(2): p. 219-25.
817. Gan, W.B., et al., *Ballistic delivery of dyes for structural and functional studies of the nervous system.* Cold Spring Harb Protoc, 2009. **2009**(4): p. pdb.prot5202.
818. Chalfie, M., et al., *Green fluorescent protein as a marker for gene expression.* Science, 1994. **263**(5148): p. 802-5.
819. Wong, A.M., J.W. Wang, and R. Axel, *Spatial representation of the glomerular map in the Drosophila protocerebrum.* Cell, 2002. **109**(2): p. 229-41.
820. Basler, K. and G. Struhl, *Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein.* Nature, 1994. **368**(6468): p. 208-14.
821. Festenstein, R., et al., *Locus control region function and heterochromatin-induced position effect variegation.* Science, 1996. **271**(5252): p. 1123-5.
822. Feng, G., et al., *Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP.* Neuron, 2000. **28**(1): p. 41-51.
823. Badea, T.C., Y. Wang, and J. Nathans, *A noninvasive genetic/pharmacologic strategy for visualizing cell morphology and clonal relationships in the mouse.* J Neurosci, 2003. **23**(6): p. 2314-22.
824. Buffelli, M., et al., *Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition.* Nature, 2003. **424**(6947): p. 430-4.

825. Jefferis, G.S. and J. Livet, *Sparse and combinatorial neuron labelling.* Curr Opin Neurobiol, 2012. **22**(1): p. 101-10.
826. Hampel, S., et al., *Drosophila Brainbow: a recombinase-based fluorescence labeling technique to subdivide neural expression patterns.* Nat Methods, 2011. **8**(3): p. 253-9.
827. Giepmans, B.N., et al., *The fluorescent toolbox for assessing protein location and function.* Science, 2006. **312**(5771): p. 217-24.
828. Birling, M.C., F. Gofflot, and X. Warot, *Site-specific recombinases for manipulation of the mouse genome.* Methods Mol Biol, 2009. **561**: p. 245-63.
829. Viswanathan, S., et al., *High-performance probes for light and electron microscopy.* Nat Methods, 2015. **12**(6): p. 568-76.
830. Chen, Y., et al., *OCT4B-190 protects against ischemic stroke by modulating GSK-3beta/HDAC6.* Exp Neurol, 2019. **316**: p. 52-62.
831. Rana, A.K. and D. Singh, *Targeting glycogen synthase kinase-3 for oxidative stress and neuroinflammation: Opportunities, challenges and future directions for cerebral stroke management.* Neuropharmacology, 2018. **139**: p. 124-136.
832. Jope, R.S. and G.V. Johnson, *The glamour and gloom of glycogen synthase kinase-3.* Trends Biochem Sci, 2004. **29**(2): p. 95-102.
833. Chen, S., et al., *HDAC6 regulates mitochondrial transport in hippocampal neurons.* PLoS One, 2010. **5**(5): p. e10848.
834. Linding, R., et al., *Systematic discovery of in vivo phosphorylation networks.* Cell, 2007. **129**(7): p. 1415-26.
835. Ribas, C., et al., *The G protein-coupled receptor kinase (GRK) interactome: Role of GRKs in GPCR regulation and signaling.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2007. **1768**(4): p. 913-922.
836. Lafarga, V., et al., *A novel GRK2/HDAC6 interaction modulates cell spreading and motility.* Embo j, 2012. **31**(4): p. 856-69.
837. Xiao, H. and M. Liu, *Atypical protein kinase C in cell motility.* Cell Mol Life Sci, 2013. **70**(17): p. 3057-66.
838. Du, Y., et al., *aPKC phosphorylation of HDAC6 results in increased deacetylation activity.* PLoS One, 2015. **10**(4): p. e0123191.
839. Watabe, M. and T. Nakaki, *Protein kinase CK2 regulates the formation and clearance of aggresomes in response to stress.* J Cell Sci, 2011. **124**(Pt 9): p. 1519-32.

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Education

University of Illinois at Chicago, Department of Psychiatry — Chicago, IL

- Ph.D., Graduate Program in Neuroscience — Aug. 2016-Present
- Expected defense January 2021

University of Illinois at Urbana-Champaign — Champaign, IL

- Bachelor of Science Degree with High Distinction May 2016 — May 2016
- Double Major in Molecular and Cellular Biology and Psychology

Research Experience

University of Illinois at Chicago — Chicago, IL

Graduate Researcher with Dr. Amynah Pradhan — 2016-Present

Novel mechanisms and therapeutic targets for chronic migraine

- Identified previously undiscovered alterations in neuron morphology in key migraine processing regions following chronic NTG model of migraine in mice
- Established cortical spreading depression in lab and revealed similar alterations in pyramidal neurons of the cortex following cortical spreading depression
- Demonstrated reversal of migraine allodynia, decreased susceptibility to cortical spreading depression events, and restored neuron morphology following HDAC6 inhibition
- Investigated the role of PACAP-38 peptides in opioid induced hyperalgesia
- Investigated the role of forebrain delta opioid receptors in regulation of cortical spreading depression

University of Illinois at Urbana-Champaign — Champaign, IL

Undergraduate Research Assistant with Dr. Florin Dolcos — 2013- 2016

- Undergraduate Thesis *Larger Volumes of Amygdala and Hippocampus Associated with Reduced Vulnerability to Affective Dysregulation in Healthy Participants*
- Participated in performing questionnaire for human psychological evaluation and testing of working memory tasks
- Analyzed MRI images of human brain and gathered volumetric data on amygdala and hippocampus

Skills and Techniques

- Use of several models of headache disorders including; chronic migraine, opioid induced hyperalgesia, medication overuse headache
- Use of Von Frey hair on mice to determine mechanical thresholds in the hindpaw and cephalic region
- Perform cortical spreading depression procedure including electrophysiological recording of local field potential *in vivo*
- Stereotaxic surgeries on mice for injection of AAVs/retrobeads

- Conditioned place preference and aversion paradigm with mice
- Statistical analysis of behavioral data using GraphPad Prism, SPSS, and SigmaStat
- Adept in intraperitoneal, subcutaneous, and intraplantar injections in mice
- Transcardial perfusion and fixation of mouse for tissue collection and analysis
- Golgi staining procedure
- Analysis of neuron morphology in several different brain regions including Sholl analysis
- Quantitative polymerase chain reaction (qPCR)
- Cryosectioning mouse tissue and subsequent immunohistochemistry protocol

Awards and Scholarships

- Winner of the International Headache Conference Trainees Excellence Tournament (Aug. 2019)
- 2nd place in Chicago Chapter of the Society for Neuroscience 2019 Graduate Student Symposium Competition (April 19, 2019)
- Winner of the Annual UIC Neuroscience Graduate Research Symposium (Feb. 2019)
- 3rd Place Poster Competition 8th Annual Psychiatry Research Forum/Extravaganza (Sept. 2017)
- Manny Donchin Award (April 2015)- Outstanding Undergraduate Student from the Cognitive Neuroscience Division
- James E. Spoor Scholarship (Sept. 2015)-for outstanding academic achievements

Teaching Experience

- **Lecturer,** 2 Lectures, Undergraduate *Neurobiology Seminar***,** University of Illinois at Chicago, (Spring 2019 and 2020)
- **Lecturer,** 4 lectures**,** Summer Pre-Matriculation Program, University of Illinois at Chicago, (Summer, 2019 and 2020)

Mentorship

- Wiktor Witkowski (2017-2020)
    - Assisted in training of *ex vivo* tissue processing
    - Aided in preparation of undergraduate thesis entitled: δ-Opioid Receptor Agonist, KNT-127, Produces Limited Internalization in Key Migraine Pain Processing Region
- Pal Shah (2016-2020)
    - Training of animal handling and behavior
    - Instructed on cortical spreading depression protocol
- Sarah Asif (2018-Present)
    - Trained in analysis of Golgi stained tissue
    - *Ex vivo* analysis of neuron structure and cytoarchitecture

Certifications

- Ethics Courses: Scientific Integrity & Responsible Research and Essentials for Animal Research
- Ethics training for faculty and staff completed every year

- Occupational Exposure to Blood-borne Pathogens Standard Certified
- Animal Research at UIC Certified
- Working with Mice and Rats at UIC Certified
- Radiation Safety Training Certified
- UIC Laboratory Safety Program

Invited Talks

- **Bertels, Z.** The Role of HDAC6 and the Cytoskeleton in Chronic Migraine-Associated Pain. Podium Presentation at: Next Generation Pain Therapeutics: From Discovery to the Clinic Conference; 2020 November 11th, 2020; Houston, Texas
- **Bertels, Z.** Differential Effects of Mu and Delta Opioid Receptor Agonists in Models of Chronic Migraine-Associated Pain and Aura. Oral presentation at: International Headache Conference trainees excellence tournament; 2019 September 6th, Dublin, Ireland
- **Bertels, Z.** Cytoarchitectural Changes Induced by Migraine Are Reversed by HDAC6 Inhibition. Oral presentation at: Chicago Chapter of the Society for Neuroscience 2019 Graduate Student Symposium Competition; 2019 April, Chicago, Illinois
- **Bertels, Z.** Cytoarchitectural Changes Induced by Migraine Are Reversed by HDAC6 Inhibition. Oral presentation at: Annual UIC Neuroscience Graduate Research Symposium; 2019 February, Chicago, Illinois

Poster Presentations

- **Bertels, Z.**, Singh, H., Dripps, I., Shah, P, Baca, S., Rasenick, M., Pradhan, A. Cephalic Allodynia and Cortical Spreading Depression results in decreased neuronal complexity, the restoration of which relieves migraine-associated symptoms. Poster presented at: Department of Psychiatry's 10th Annual Research Forum; 2019 September 17th; Chicago, Illinois
- **Bertels, Z.**, Siegersma, K., Tipton, A., Baca, S., Rijn, R., Pradhan, A. Differential Effects of Mu and Delta Opioid Receptor Agonists in Models of Chronic Migraine-Associated Pain and Aura. Poster presented at: Department of Psychiatry's 10th Annual Research Forum; 2019 September 17th; Chicago, Illinois
- **Bertels, Z.** Singh, H., Dripps, I., Shah, P, Baca, S., Rasenick, M., Pradhan, A. Cephalic Allodynia and Cortical Spreading Depression Results in Decreased Neuronal Complexity, the Restoration of Which Relieves Migraine-Associated Symptoms. Poster presentation at: International Headache Conference trainees excellence tournament; 2019 September 7th, Dublin, Ireland
- **Bertels, Z.**, Dripps, I., Moye, L., Tipton, A., Baca, S., Pradhan, A. The critical role of central delta opioid receptors in models of migraine and opioid-induced hyperalgesia. Poster presented at: International Narcotics Research Conference annual meeting; 2019 September 7th, Dublin, Ireland
- **Bertels, Z.**, Siegersma, K., Tipton, A., Baca, S., Rijn, R., Pradhan, A. Differential Effects of Mu and Delta Opioid Receptor Agonists in Models of Migraine. Poster presented at: International Narcotics Research Conference annual meeting; 2019 July 9th, New York, New York
- **Bertels, Z.**, Dripps, I., Moye, L., Tipton, A., Baca, S., Pradhan, A. The critical role of central delta opioid receptors in models of migraine and opioid-induced hyperalgesia.

Poster presented at: International Narcotics Research Conference annual meeting; 2019 July 8th, New York, New York

- **Bertels, Z.**, Tipton, A., Moye, L., Dripps, I., Shah, P., Karumudi, B., Petukhova, V., Petukhov, P., Thatcher, G., Pradhan, A. The effect of histone deacetylase inhibitors in a model of chronic migraine. Poster presented at: Society for Neuroscience Annual Meeting; 2018 November 3rd; San Diego, California
- **Bertels, Z.**, Singh, H., Dripps, I., Shah, P., Rasenick, M., Pradhan, A., HDAC6 inhibitors as novel therapeutic targets for migraine. Poster presented at: Department of Psychiatry's 9th Annual Research Forum; 2018 September 25th; Chicago, Illinois
- **Bertels, Z.**, Singh, H., Dripps, I., Shah, P., Rasenick, M., Pradhan, A., HDAC6 inhibitors as novel therapeutic targets for migraine. Poster presented at: CARE 3rd Annual Retreat; 2018 May 3rd; Chicago, Illinois
- **Bertels, Z.**, Tipton, A., Moye, L., Gandhi, R., Aissa, R., Thatcher, G., Pradhan, A., Soluble guanylate cyclase is a critical regulator of migraine induced cephalic pain. Poster presented at: Department of Psychiatry's 8th Annual Research Forum; 2017 September 28th; Chicago, Illinois

Publications in Preparation

- **Bertels, Z.**, Grewal, H., Dripps, I., Siegersma, K., Laboy, A., Witkowski, W., Sheets, Z., Shah, P., Conway, C., Petukhova, V., Karumudi, B., Petukhov, P., Baca, S., Rasenick, M., Pradhan, A.A. Neuronal complexity is attenuated in chronic migraine and restored by HDAC6 inhibition. In revision at elife, doi: https://doi.org/10.1101/2020.04.21.053272
- **Bertels, Z.**, Siegersma, K., Baca, S., Pradhan A.A. A translationally significant model of migraine facilitation through chronic morphine is attenuated by PAC1 receptor antagonism. In Preparation

Publications

- **Bertels Z**, Witkowski WD, Asif S, Siegersma K, van Rijn RM, Pradhan AA. A non-convulsant delta-opioid receptor agonist, KNT-127, reduces cortical spreading depression and nitroglycerin-induced allodynia. Headache. 2020 Dec 16. doi: 10.1111/head.14019. Epub ahead of print. PMID: 33326598.
- **Bertels, Z.** and Pradhan, A. A. (2019). Emerging Treatment Targets for Migraine and Other Headaches. Headache, doi: 10.1111/head.13585
- Dripps IJ, **Bertels Z**, Moye LS, Tipton AF, Siegersma K, Baca SM, Kieffer BL, Pradhan AA. Forebrain delta opioid receptors regulate the response of delta agonist in models of migraine and opioid-induced hyperalgesia. Sci Rep. 2020 Oct 19;10(1):17629. doi: 10.1038/s41598-020-74605-9. PMID: 33077757; PMCID: PMC7573615.
- Targowska-Duda KM, Ozawa A, **Bertels Z**, Cippitelli A, Marcus JL, Mielke-Maday HK, Zribi G, Rainey AN, Kieffer BL, Pradhan AA, Toll L. (2020) NOP receptor agonist attenuates nitroglycerin-induced migraine-like symptoms in mice. Neuropharmacology, DOI: 10.1016/j.neuropharm.2020.108029
- Pradhan, A. A., **Bertels, Z.**, & Akerman, S. (2018) Migraine Therapeutics: Current Practice, Recent Advances and Future Directions Targeted Nitric Oxide Synthase Inhibitors for Migraine. Neurotherapeutics, 29516436 doi: 10.1007/s13311-018-0614-7
- Ben Aissa M, Tipton AF, **Bertels Z**, Gandhi R, Moye LS, Novack M, Bennett BM, Wang Y, Litosh V, Lee SH, Gaisina IN, Thatcher GR, Pradhan AA. Soluble guanylyl cyclase is a critical regulator of migraine-associated pain. Cephalalgia. 2018 Jul;38(8):1471-1484.

doi: 10.1177/0333102417737778. Epub 2017 Oct 12. PMID: 29022756; PMCID: PMC5916516.

- Hu, Y., Moore, M., **Bertels, Z**., Phan, L., Dolcos, S., & Dolcos, F. (2017). Smaller Amygdala Volume and Increased Neuroticism Predict Anxiety Symptoms in Healthy Subjects: A Volumetric Approach Using Manual Tracing. Neuropsychologia, 29157997 DOI: 10.1016/j.neuropsychologia.2017.11.008

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