Changes in Pain Sensitivity in the Amygdala Kindling Model of Temporal Lobe Epilepsy

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ABSTRACT

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Pain conditions occur at an increasing rate alongside people with temporal lobe epilepsy (TLE) and can include chronic headaches, migraines, and neuropathic pain. In order to begin to understand the concurrence, this experiment aimed to investigate the effect of long-term amygdala kindling, a model of TLE, on the affective and nociceptive components of pain in rats. Formalin-induced affective avoidance was investigated using the conditioned place aversion (CPA) test and found aversion in kindled, but not sham rats. Nociceptive behaviours were observed using the formalin test and found a peripheral reduction of pain, that persisted one-week following the last stimulation in kindled rats. Lower activation of c-Fos in the periaqueductal gray was seen in kindled rats, while no changes in protein kinase C δ activation was found. Amygdala kindling contributed to pain sensitivity changes that persisted into the interictal period, and male and female pain trends were found, requiring further investigation.

Keywords: amygdala, amygdala kindling, affective pain, central amygdala, formalin, formalin-induced conditioned place aversion, formalin test, interictal, nociceptive pain, paraventricular hypothalamus

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LIST OF ABBREVIATIONS

ACC	Anterior cingulate cortex
AD	After discharge
ADT	After discharge threshold
ANOVA	Analysis of variance
BLA	Basolateral amygdala
CeA	Central amygdala
CeL	Lateral division of the central amygdala
CeM	Medial division of the central amygdala
CNS	Central nervous system
CPA	Conditioned place aversion
Egr1	Early growth response 1
F-CPA	Formalin-induced conditioned place aversion
GABA	γ-amino butyric acid
IASP	International Association on the Study of Pain
mPFC	Medial pre-frontal cortex
PAG	Periaqueductal gray
PKCδ	Protein kinase calcium δ
PNS	Peripheral nervous system
PVN	Paraventricular hypothalamic nuclei
ROI	Region of interest
SEM	Standard error of the mean

TLE	Temporal lobe epilepsy
PWE	Persons with epilepsy

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Epilepsy is a chronic non-communicable neurological condition that is characterized by recurrent seizures (Fisher et al., 2014). In Canada, epilepsy is reported to affect approximately 300,000 individuals, with 54 new cases reported each day (Government of Canada, 2018). Epilepsy can affect people of all ages, sexes, ethnic backgrounds, income groups and geographical locations, making it a significant cause of global neurological disability and morbidity. Due to the unpredictable nature of seizures, epilepsy can be highly disruptive and can interfere with a person's daily function. For example, unlike other chronic diseases, the clinical manifestations of epilepsy are often, at times, highly visible. Indeed, several studies have consistently found that people with epilepsy (PWE) report reduced social support and face higher rates of unemployment and underemployment compared to individuals with other chronic neurological conditions (Strzelczyk et al., 2023; Gaitatzis et al., 2012). Consequently, the experience of epilepsy is significant, permeating across all areas of person's life and significantly contributing to a reduced quality-of-life.

Beside the experience of seizures, almost half of people with epilepsy (PWE) are also diagnosed with one or more comorbid disorders, which can include cognitive, psychiatric, and physical disorders (Strzelczyk et al., 2023). Among these comorbidities, pain conditions—such as chronic headaches, migraines, and neuropathic pain—are

increasingly prevalent alongside epilepsy. Despite the high frequency of pain-related conditions among PWE, these issues are often underreported or not reported at all. Furthermore, few studies have attempted to investigate the potential neurobiological mechanisms or substrates that may underlie the close association between epilepsy and pain.

In mesial temporal lobe epilepsy (TLE), the most common form of adult focal epilepsy, seizures arise from limbic forebrain regions, such as the amygdala, entorhinal cortex, hippocampus and parahippocampal gyrus. These seizures are often associated with a host of behavioural, cognitive, and psychiatric disturbances that can persist into the seizure-free or interictal period for some patients (Keezer et al., 2016). In particular, long-term changes in emotionality are frequently reported by individuals with TLE with disturbances in fear and anxiety being the most prominent problem (Melo et al., 2021). Similar changes involving interictal fear and anxiety-related behaviours have also been reported after experimentally evoking seizures through daily electrical stimulation or kindling of the amygdala in rodents (Kalynchuk et al., 2000; Fournier et al., 2020) and felines (Adamec & Stark-Adamec, 1983). These findings suggest that epilepsy-associated emotional disturbances and symptoms may be linked to structural and functional changes in limbic circuitry, particularly those involving the amygdala. However, given that much of the neural circuitry underlying fear and anxiety appears to overlap closely with those involved in the processing of pain, then chronic stimulation of these circuits by seizure discharges may produce neuroplastic modifications that lead to heightened physiological and affective responses to painful stimuli. This potential mechanism could help explain

the high comorbidity of pain symptoms and pain disorders among PWE. Thus, my thesis research aims to investigate the effect of long-term amygdala kindling in rats on the affective dimension of pain by using a conditioned place avoidance task. In addition, I will also explore potential neurobiological mechanisms that may underlie the relationship between pain and epilepsy.

In this introductory chapter of my thesis, I will first begin by characterizing epilepsy and its related symptoms. I will then discuss behavioural and affective comorbidities associated with epilepsy focusing specifically on pain and pain-related symptoms. Next, I will review literature highlighting the use of the amygdala kindling model to study the neural circuitry of emotionality and pain in animals. Finally, I will conclude the chapter with a discussion of the overarching objectives and hypotheses of my thesis research.

1.2 Epilepsy and Seizures

1.2.1 Characteristics of Epilepsy

The clinical diagnostic criteria for epilepsy emphasizes the characteristics of the seizures and their localization within the brain (Scheffer et al., 2017; Katyayan & Diaz-Medina, 2021). Seizures involve a sudden synchronized discharge of neurons that results in abnormalities in the function of the neural network (Stafstrom & Carmant, 2021). Seizures have two generally defined types: focal and generalized (Fisher et al., 2017). Focal seizures are characterized by an area of abnormal neural activity that is associated with a specific brain region in one brain hemisphere, while generalized seizures can arise

from multiple points of origin within either one or across both cerebral hemispheres (Stafstrom & Carmant, 2021; Fisher et al., 2017).

Focal onset (or partial) seizures can occur with intact alertness and awareness of the person at the time of the seizure event (formerly simple partial seizure) or can involve a change in awareness or consciousness (formerly complex partial seizures). These focal onset seizures can differ in the presence of motor symptoms, characterized by involuntary muscular spasms or tension, or non-motor symptoms, which may also involve behavioural arrest and cognitive, emotional, and sensory symptoms. In addition, the location of the abnormal neural activity can also determine the types of symptoms that are exhibited. For example, seizure discharges affecting the occipital cortex are typically associated with visual sensations or visual hallucinations, whereas the seizure discharges impacting the pre-central gyrus can result in tonic or clonic movements. Focal onset seizures that are initially localized to a specific area in one hemisphere can propagate to affect the contralateral side. These focal to bilateral tonic-clonic seizures differ from generalized onset tonic-clonic seizures in which the seizure discharge begins simultaneous on both sides of the brain (Fisher et al., 2017). Generalized seizures can include myoclonic seizures, absence seizures, generalized tonic-clonic (GTC) seizures, myoclonic seizures, and atonic seizures (Stafstrom & Carmant, 2021). Myoclonic seizures are brief, sudden jerking movements and contractions that affect one muscle or groups of related muscles without any change in awareness. Absence seizures (formerly petit mal) generally include a sudden period of behavioural arrest and unresponsiveness (“blanking out”) and may be accompanied by eye blinking or head nodding. GTC

seizures (formerly grand mal) involve cycles of bilateral stiffening (tonic) and jerking (clonic) of all limbs with impaired consciousness. Atonic seizures involve the sudden and complete loss of body tone resulting in a fall ("drop attack") or drooping of the head.

The temporal lobe is the most frequent origin site of focal seizures, specifically the limbic type, termed mesial temporal lobe epilepsy (TLE) which involve the internal structures of the temporal lobes (Blair, 2012). This type of focal epilepsy arises from the hyperexcitable neurons in limbic brain regions involving the hippocampus and amygdala, or from structures of the parahippocampal gyrus, such as the entorhinal cortex and perirhinal cortices (Bernasconi et al., 2003). The most common radiological and histopathological finding of TLE (seen in approximately 56-70% of medically refractory TLE cases; Malmgren & Thom, 2012) involves the presence of hippocampal sclerosis, which is defined by a stereotyped pattern of neuronal cell loss and reactive fibrillary gliosis primarily affecting the CA1, CA3 and CA4 (hilus) regions of the hippocampus (Blümcke et al., 2013). The dentate gyrus granule cell layer can be relatively preserved. However, 40 to 50% of cases may show dispersion or looser packing of the neurons in the granule cell layer.

It is increasingly realized that the neuropathology of TLE can extend beyond the hippocampus to affect nearby structures, including the amygdala, parahippocampal gyrus, lateral temporal cortex, and parts of the thalamus. These widespread pathological changes may be relevant not only in the establishment of networks involved with seizure development and susceptibility but also with emergence of co-morbid behaviours and experiential symptoms frequently associated with TLE.

1.2.2 Behavioural and Physical Comorbidities of Epilepsy

As discussed, people with TLE often report a host of psychiatric, cognitive, and physical symptoms during the period around a seizure event or ictus. These peri-ictal symptoms can include the presence of symptoms or clusters of symptoms that precede the seizure (pre-ictal), occur as an expression of the seizure activity (ictal), or immediately follow the seizure (post-ictal). The presence of pre-ictal symptoms, such as irritability or dysphoria, are especially common and manifest a few minutes or hours to even days before the ictus. In contrast, symptoms at the time of the seizure are usually brief, often lasting less than 30 s, and may initially present as simple partial seizure which can evolve into a complex partial seizure or a secondarily generalized tonic-clonic seizure. The most common ictal symptoms associated with TLE involve fear, depression, or psychosis.

Symptoms can also begin immediately after a seizure (post-ictal) or during the seizure-free period between episodes (interictal). Post-ictal refers to the period of transition from the end of the ictal phase to the individual's normal (pre-seizure) level of awareness and function to the time between the end of the seizure (ictus). During this period, individuals may experience various symptoms, such as confusion and unresponsiveness, aphasia, motor weakness, sensory changes, and amnesia. The duration and severity of these symptoms can vary, lasting anywhere from minutes to hours, or even days. Finally, when the individual experiences recurrent seizures, the return to the baseline period between consecutive seizures is referred to as the interictal period. The interictal state represents, by definition, a period of seizure freedom. However, it is essential to recognize that the interictal state continues to manifest abnormalities in

network and circuit function, highlighting the chronic disruptive effects that seizure activity can have on the brain.

Table 1. Definitions of Epilepsy Terminology

Term	Definition
Focal seizures	Localized to a single origin point in one cerebral hemisphe
focal to bilateral tonic-clonic seizures	Focal seizures that propagate from one hemisphere to both hemispheres.
Generalized seizures	Localized to multiple origin points across both cerebral hemispheres.
Generalized tonic-clonic seizures	Characterized by a cycle of tonic movements, stiffening of limbs, and clonic movements, jerking or twitching of
Myoclonic seizures	Characterized by quick, sudden movements affecting one more more muscles. Can be focal or generalized in
Atonic seizures	Characterized by a complete loss of control in the body, resulting in a fall or drooping of the head.
Peri-ictal symptoms	Symptoms that occur in during some form of neuronal seiz activity.
Pre-ictal symptoms	Symptoms that occur immediately prior to seizure onset.
Ictal symptoms	Symptoms that occur during the seizure period.
Post-ictal symptoms	Symptoms that occur immediately following seizure completion.
Interictal symptoms	Symptoms that occur with no active neuronal seizure activ

1.2.2.1 Interictal Comorbidities

While seizures are the hallmark symptom of epilepsy, they are not the only symptom. For many PWE and their families, the burden of the disease is largely caused by presence of comorbid conditions, including behavioural, cognitive, psychiatric and physical disorders. These associated comorbidities are now recognized to be an integral part of the pathophysiology of epilepsy and arise from a complex myriad of genetic, molecular, and physiological changes in the brain.

These interictal symptoms can include impairments in cognitive functioning and emotional regulation, with memory deficits and mood disorders occurring at a prevalence of up to 30% in people with epilepsy (Kanner, 2016; McCagh et al., 2009). In addition to deficits in memory, cognitive symptoms including poor concentration, disorientation, impairments in language, visuoperception, learning, and executive functioning are also commonly reported alongside epilepsy (Lin et al., 2012; McCagh et al., 2009). With earlier onset or prolonged duration of seizure-related neural activity, these cognitive symptoms can exhibit increased severity (Lin et al., 2012). However, while reduction or reversal of cognitive deficits in a majority of seizure types is possible, it depends upon the effective control of seizure activity (McCagh et al., 2009).

Concurrently, the impacts of cognitive challenges are compounded by affective disorders, such as mood disorders, psychosis, and mood instability (Kanner et al., 2004). In some studies, these mood disorders, such as major depression and anxiety, are reported at nearly twice the prevalence in individuals with epilepsy in comparison to the general population (Keezer et al., 2016). In addition, similar brain structures are implicated in the

physiopathology of these disorders, such as the amygdala (Lorenzetti et al., 2010; van Eijndhoven et al., 2009). Moreover, the changes in the volume of the amygdala and plasticity of BLA neurons have been shown to contribute to the progression of depressive mood-related models (Monkul et al., 2007; Eiland et al., 2012; Vyas et al., 2006). Overall, these mood disorders, in addition to dementia and migraines are amongst the most common psychiatric and neurological predictors of worse QOL in PWE (Kanner et al., 2016; Vinti et al., 2021). Hence, understanding these additional comorbidities is crucial in PWE.

Alongside reports of mental comorbidities, increasing reports of other physical conditions have come to light in people with epilepsy, such as cardiovascular, respiratory, and chronic pain conditions (Keezer et al., 2016). In fact, pain-related comorbidities are reported with higher prevalence within PWE than in the general population, with over 70% reporting post-ictal headaches, up to 52% experiencing migraines in their lifetime, up to 25% diagnosed with chronic pain disorders, and up to 18% reporting fibromyalgia (Cragg et al., 2018; Gray, 2022; Téllez-Zenteno et al., 2005; Rasker et al., 2021).

In spite of the efforts to become seizure free, many people with epilepsy have an undiagnosed and untreated comorbid impairment (Gaitatzis et al., 2012). The presence of the comorbid impairment alone can also influence the effectiveness of seizure-related treatment, or exacerbate negative affects of such treatment. Therefore, underscoring the importance of understanding the various cognitive, affective, and physical comorbidities experienced by people with epilepsy.

1.2.2.2 Pain symptomology in epilepsy

Although reports of comorbid pain conditions are greater in PWE than the general population, seizure-related changes in pain sensitivity are not extensively studied. For example, in some focal seizures with impaired awareness, hot objects or surfaces are held with no memory or sensation of its heat, or loss of consciousness occurs near a source of extreme heat resulting in falling into the heat results in severe injuries, even leading to death (DeToledo & Lowe, 2004; Zia Ziabari et al., 2022; Wang et al., 2024; Rao et al., 2020). In a survey conducted on 51 individuals with TLE, 51% reported decreased nociception in the ictal or post-ictal period (Szűcs et al., 2015). Of which, 12 patients (23%) reported experiencing one or more seizure-related burns, where 5 patients (42%) lost awareness and fell onto a hot surface or fire during a generalized seizure, and 7 patients (58%) held onto a hot object or reached into boiling fluid with no experience of or response to the pain during a complex partial seizure. One explanation for the extended period of insensitivity to the damaged tissue required for the severity of burns could be ictal and post-ictal analgesia (Szűcs et al., 2015). As reports of post-ictal analgesia have increased in the past decade, studies have begun to investigate the mechanisms involved (Samineni et al., 2011; Coimbra et al., 2001; de Freitas et al., 2013; de Oliveira et al., 2006).

In chronic pain, a repeated and prolonged noxious stimuli, such as an injury, causes changes in neurons involved in pain stimulus previously not involved in the injury (Latremoliere & Woolf, 2009; Bourne et al., 2014; Meacham et al., 2017). Similarly, this plastic change in the sensitization of pain-related neurons can provide insight into the

mechanisms that underlie seizure-related changes in pain sensitivity. One study suggests that endogenous opioid system mechanisms contribute to the immediate analgesia post-ictally, while additional mechanisms relying on serotonergic or acetylcholine may contribute to prolonged post-ictal analgesia (Coimbra et al., 2001). Evidently in pain studies, the application of naloxone, a wide-ranged opioid antagonist, has also been shown to be effective in blocking post-convulsive analgesia (Urca et al., 1981; Navratilova et al., 2015; Coimbra et al., 2001). Conversely, it is also possible that initial post-ictal analgesia involves widespread inhibition of neuronal activity immediately following the end of a seizure (Mareš et al., 1982). A key mediator of both the generation and completion of seizures involve GABA, an inhibitory neurotransmitter widely distributed within the central nervous system (CNS) (Shore et al., 2020; Briggs & Galanopoulou, 2011). In summary, post-ictal inhibition-related analgesia could be mediated by inhibition by GABA-release (Cheng spinal; Yam 2018), which may also interact with opioid peptides in descending pain pathways (Szűcs et al., 2015, Bourbia & Pertovaara, 2018).

Despite the occurrence of ictal and post-ictal analgesia resulting in injury, some rat studies have reported an increase in post-ictal sensitivity (Velioglu et al., 2017, 2018). Even so, while interictal and post-ictal analgesia has been seen in both humans and rat models, the exact temporal pattern of changes in the pain sensitivity of the amygdala kindling model have yet to be elucidated. While alternative animal models of epilepsy have begun to investigate how pain sensitivity can change during the interictal period,

investigation into the interictal changes in pain sensitivity that can be seen in the amygdala kindling model remains lacking.

1.3 Animal Models of Epilepsy

Animal models have played a fundamental and critical role in advancing our understanding of the pathophysiology of epilepsy and in discovering novel antiepileptic and disease-modifying treatments. Several animal models have been developed that employ different methods to study seizures and epilepsy. However, these models can be broadly divided into genetic or induced models. Genetic models result in spontaneous seizures and involve animals that carry spontaneous or naturally arising mutations or have had mutations introduced through the application of transgenic technologies, such as gene knockout or knock-in techniques (Knowles et al., 2022) . Induced models typically involve chemical or electrical approaches to evoke acute or chronic seizures (Coulter et al., 2002).

Acute models (e.g., maximal electroshock, pentylenetetrazole, local application of insulin, or flurothyl administration) are valuable for identifying neurobiological processes involved in generating or terminating seizures or to screen potential anti-ictogenic drugs that suppress seizures acutely (Schwartzkroin & Engel, 2006). Chronic models of epilepsy can include post-status epilepticus models, where epilepsy develops following a period of status epilepticus produced by acute administration of a chemoconvulsant agent, such as pilocarpine or kainic acid, or kindling. Kindling involves repeated induction of seizure activity through the application of initially subconvulsant treatments,

which result in a gradual evolution and development of motor convulsive seizures of increasing severity and duration. Kindling can be induced through daily electrical stimulation to forebrain structures, such as the amygdala, or by repeated administration of sub-convulsant drugs, such as pentylenetetrazole. It is believed the kindling process mirrors the unfolding of molecular, cellular and molecular events that lead to persistent epileptogenic abnormalities, especially those present between seizures or interictally (Farrell et al., 2017; Morimoto et al., 2004).

1.3.1 Kindling as a model of plasticity and sensitization

Beginning with Graham Goddard's (1967, 1969) serendipitous discovery that daily brief electrical stimulation to the amygdala initially had little effect on behaviour, but after repeated application could cause a progressive evolution in the duration and severity of motor convulsive seizures that persists even after a period of absence of stimulation, the kindling model has since become one of the most important methods for studying potential neural mechanism underlying the epilepsy development.

The kindling process begins with the elicitation of a brief focal seizure or after-discharge (AD) event at the site of stimulation. Electrographically the AD is characterized by a low frequency, high amplitude discharge, accompanied by minimal behavioural changes (Goddard et al., 1969). Several studies (Goddard et al., 1969; Racine, 1972) have shown that the kindling process critically depends on the generation of the AD as stimulation intensities below the current required to elicit an AD does not produce kindling. Initial ADs begin restricted to the site of stimulation, propagating minimally to surrounding brain regions and may produce brief focal seizure symptoms. Over time, the

necessary afterdischarge threshold (ADT) reduces progressively, leading to increased AD duration, complexity, and expanded propagation, ultimately producing a fully generalized motor convulsive seizure. Seizures produced from kindling are classified according to the extended eight stage Racine scale (Pinel & Rovner, 1978) which describe a gradual progression of seizure-related behaviour beginning from arrest in mobility, to head and facial automatisms, eventually leading to forelimb clonic-tonic movement, rearing, and loss of equilibrium. Reaching a fully kindled state requires three consecutive stage 5 (bilateral forelimb clonus and rearing leading to loss of equilibrium) or greater convulsions.

Characteristics of kindling such as the degree of convulsive seizures and rate of stimulations required to reach a to kindled state vary depending on the region chosen for stimulation (Goddard et al., 1969; McNamara et al., 1980). In some brain regions, such as the neocortex and thalamus, stimulations for upwards of 200 days are unable to produce seizure-related behaviours (Goddard et al., 1969). Even specific nuclei and subregions within brain structures vary in their sensitivity to kindling, such as the caudate-putamen which ranged from 30 days to inability to produce seizure behaviours. Some brain regions require many stimulations in order to produce seizure behaviours, such as the septal area (55), hippocampus (50-77), and preoptic area (63 stimulations), while others kindle with fewer stimulations, such as the olfactory area (29), piriform cortex (24), and amygdala (15 stimulations). Early observations described a pattern associating fewer stimulations required with fewer anatomical connections to the amygdaloid complex (Goddard et al., 1969, Racine et al., 1988). This and other investigations lead to the

theory that the pathway responsible for lowering the ADT importantly involves the amygdala (Löscher & Ebert, 1996; Racine et al., 1988).

1.3.2 Behavioural effects of kindling

Kindling can produce various changes in behaviour depending on kindling location (Goddard et al., 1969). To start, perirhinal and hippocampal kindling tends to produce deficits in memory, while producing less robust changes in negative affective behaviour (Barnes et al., 2005). Likewise, though kindling of the striatum can produce seizures, no changes in affective behaviour are observed (Teskey et al., 2006). In contrast, kindling of the amygdala has shown significant increases in anxiogenic (increased anxiety-related) and defensive fear behaviours in both rats and cats (Kalynchuk et al., 1997, 1998, 1999; Fournier et al., 2020; Hiyoshi et al., 1990). Highlighting the suitability of amygdala kindling in investigating affective behaviour in TLE.

While amygdala kindling produces seizure-related convulsions at a low number of stimulations, continual stimulations for an increased duration of time produces additional changes in behaviour. A fear conditioning paradigm found that while short-term (30 stims) amygdala kindled rats displayed no difficulty in learning the fear-associated memory compared with controls, long-term (99 stims) amygdala kindled rats displayed difficulty with the association (Botterill et al., 2014). However, when learning the association become more complex, both short and long-term kindled rats showed difficulty in learning the fear-associated memory, implicating long-term amygdala kindling in producing greater impairments in fear learning and memory than short-term kindling. Indeed, long-term kindling of the amygdala also exhibits heightened anxiogenic

behaviour and vigilance to novel environmental factors (Kalynchuk et al., 1997, 1998, 1999; Fournier et al., 2020). In particular, extreme escape behaviours have been observed in long-term amygdala kindling, displaying heightened negative affect and indifference to the test context (Kalynchuk et al., 1997; Fournier et al., 2020). This increase in fear and threat-reactive behaviour in long-term amygdala kindled rats has also been found to persist for months, with no return to control levels of affect (Kalynchuk et al., 1998). On top of these behavioural changes, long-term amygdala kindling produces the affective and cognitive changes parallel to TLE in humans without producing spontaneous seizures. The gradual development of affective and cognitive symptoms following progressive increases in seizure-behaviour can be attributed to the propagation of AD that sensitizes and persistently increases the excitability of neurons (Cheng et al., 2020). This, coupled with the gradual loss of GABAergic neurons and overall inhibition of neuronal networks, then produces changes in how regions in the brain process external stimuli and produce behaviour. Thus, kindling models, particularly long-term amygdala kindling, provide an effective framework for exploring sensitization in epilepsy and investigating associated affective behaviours.

1.4 Neurobiology of Pain Sensitization

Pain is defined by the International Association on the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Raja et al., 2020). While everyone has experienced pain at certain points in their lives, this more commonly entails a clearly

defined cause and successful resolution to the pain. Yet, chronic pain disorders occur at a prevalence of 20-30% worldwide (DeRidder et al., 2021). These disorders can occur due to any trauma or injury that becomes long-lasting (Moshourab et al., 2015). These can include traumatic brain injury, spinal cord injury, nerve damage, and more. Additionally, clear differences in the prevalence of chronic pain disorders and anxiety disorders exist between women and men. More than half of chronic pain conditions are reported at a higher rate in women than men, and despite this difference most pain-related animal research have been conducted in male animals (Osborne & Davis, 2022; Bartley & Fillingim, 2013). Similarly, women are reported with a higher lifetime prevalence of an anxiety disorder, and nearly twice as likely to experience panic disorders (McLean et al., 2011; Burani & Nelson, 2020). Therefore, investigating the neural mechanisms that potentiate or sensitize pain, along with the specific brain regions involved in these pain behaviours, is crucial for understanding and addressing the complexities of pain processing.

1.4.1 Circuitry of pain sensitization

Pain signalling triggered by a noxious stimuli are first detected by mechanical, thermal, and chemical nociceptors in the cutaneous skin and internal organs which are then transmitted from peripheral primary afferent nerve fibres to the spinal cord of the CNS (Das, 2015). These nerve fibres that are myelinated, Aδ-fibres, or unmyelinated, C-fibres, then travel through the dorsal root ganglia in the spinal cord to the superficial and out of the deeper laminae to the brain (Willis et al., 2004). Neurons then branch into two separate pathways, each with a distinct purpose. The first pathway, the spinothalamic

tract, begins with the primary afferent neurons passing through Rexed laminae layers I of the superficial to the deep laminae and through secondary afferent neurons decussating in the spinal cord, which then terminate in the ventral posterior or ventral medial nuclei of the thalamus. Thalamic neurons then communicate with the primary somatosensory (S1) cortex for the localization and differentiation of the noxious stimuli. The second pathway, the spinoreticular tract, is involved in the activation of endogenous analgesia systems, and coordinates motivational-affective responses (Willis & Westlund, 1997). In this tract, the nerve fibres first decussate in the superficial Rexed laminae layer II to the deeper laminae layers IV-VIII. The second-order neurons then ascend the spinal cord to connect in various brain regions. The neurons of the spinoreticular tract in particular synapse in the mesencephalic reticular formation, the peri-aquaductal gray (PAG), and the intralaminar nuclei in the thalamus. Nuclei in the thalamus then project to various limbic regions, such as the anterior cingulate cortex, the hypothalamus, and the amygdala (Willis & Westlund, 1997). These pathways thus map how the sensory and affective components of pain are localized.

1.4.2 Pain Sensitization

Generally, pain can be categorized into three types, nociceptive, neuropathic, and neuroplastic. Nociceptive pain involves the activation of pain through the activation of nociceptors in the skin and internal organs in the absence of sensitization, and can be triggered by mechanical, thermal or chemical noxious stimuli (Das, 2015). Neuropathic pain is defined by the IASP as “initiated or caused by a primary lesion or dysfunction in the nervous system” (Merskey, 1994). It is described as abnormal sensory allodynia in

response to non-noxious (normally absent of pain) or hyperalgesia to noxious stimuli (increased pain when pain was previously already present) (Das, 2015). Neuroplastic pain refers to hypersensitivity that occurs beyond damage to tissue, where cellular and molecular mechanisms change the plasticity of synapses involved in pain encoding. When these changes occur in the peripheral nervous system, it is referred to as "peripheral sensitization", and encompasses changes at the receptor and transmitter level (Das, 2015). On the other hand, "central sensitization" refers to functional changes in neuronal network activation within the spinal cord and pain-related brain regions (May, 2008).

The formalin test is an inflammatory model of chronic and tonic pain developed to enable observation of long-lasting nociceptive pain (Dubuisson & Dennis, 1978). In this model, noxious pain develops from the injection of diluted formalin (0.02-15%) into the subcutaneous glabrous of the hindpaw which produces well-described spontaneous pain-related behaviours (Dubuisson & Dennis, 1978; Abbot et al., 1995; Fischer et al., 2014; Lee et al., 2002; Hunskaar et al., 1987). These behaviours include paw flinching, elevation, favouring, licking, biting, and shaking. These nociceptive responses occur over an extended period and tend to occur in a biphasic pattern (Abbot et al., 1995). The acute phase (phase I) consists of a short response, lasting approximately 5-10 following injection, and is thought to be associated with peripheral primary afferent C-fiber nociceptors (Coderre et al., 1993; Dickenson and Sullivan, 1987; Shibata et al., 1989). Next, a quiescent interphase characterized by a period of 5-10 minutes, where any remaining peripheral neurons are inactivated by unprocessed formalin, producing an

attenuation of pain behaviours (Fischer et al., 2014). After this, a period of extended tonic nociceptive responses occurs (phase II). During this phase, initial afferent C-fiber inputs is thought to contribute to central sensitization of spinal dorsal horn neurons resulting in the prolonged pain-related spontaneous paw behaviours (Coderre et al., 1993; Dickenson and Sullivan, 1987; Shibata et al., 1989; Willis & Westlund, 1997). Consequently, variations in pain sensitivity can be understood through pain sensitization mechanisms and investigated using the formalin test.

1.4.3 Neural circuits in Affective Pain

When examining affective pain modulation, special focus can be given to understanding neural pathways that play crucial roles in this process. Distinct structures within the medial pre-frontal cortex (mPFC), specifically the anterior cingulate cortex (ACC) and the insular cortex, have been well-documented with important roles in the modulation of pain and pain-related affective behaviour, such as unpleasantness and aversion (Vogt, 2005; Shackman et al., 2011; Bushnell, 2013; Johansen et al., 2001; Devinsky et al., 1995; Bush et al., 2000). In rats, aversion was observed to be produced in the absence of any noxious stimuli when ACC excitatory glutamate receptors were activated, and when antagonists were applied to the same receptors, expected aversion to noxious stimuli was absent (Johansen & Fields, 2004). In modelling neuropathic pain, the dorsal ACC was found to be active in response to peripheral noxious stimuli, however once neuropathic pain was induced by nerve injury, the neurons of the dorsal ACC remained active regardless of presence of noxious stimuli (Zhao et al., 2018).

The effect of the mPFC in affective pain can be studied in the formalin-induced conditioned place aversion (F-CPA) test which produces a learned aversion by developing an association between an injection of formalin prior to seclusion in a distinct compartment, while no treatment or saline is received prior to seclusion in another distinct compartment (Johansen et al., 2001). The time spent on the first and last day, pre- and post-conditioning, in the formalin-paired and no injection or saline-paired compartment is recorded for analysis, and the magnitude of conditioned place aversion (CPA) was indicated by less time spent in the paired chamber on post-conditioning day. While studies have implicated the mPFC as an important player in the development of aversion in the CPA model, lesioning the ACC found an intact aversion response, indication that more investigation into other structures involved in pain learning, such as the BLA, is necessary (Johansen et al., 2001; Marek et al., 2013; McGarry et al., 2017). Furthermore, lesions to the amygdala have been shown to interfere with the affective components of pain without affecting the nociceptive sensations (Calvino et al., 1982; Tershner and Helmstetter, 2000; Gao et al., 2004). In addition, activation of connections from the mPFC to the BLA has been shown to diminish aggressive behaviour and atypical plasticity within the BLA (Wei et al., 2017). This clearly outlines the importance of connections between structures such as the mPFC and BLA in understanding the multi-modal aspects of pain.

1.4.4 Role of the amygdala in pain sensitization

The experience of pain is accompanied by a strong negative affective component, so much so that a bi-directional relationship exists between patients with depression and

anxiety experience pain more intensely (Haythornthwaite et al., 1991; Wilson et al., 2001; Rhudy and Meagher, 2003). In addition, many recent papers have begun to investigate the overlapping neural circuits between affective depressive and fear behaviours in chronic pain conditions (Becker et al., 2023; Zhou et al., 2019; Chen et al., 2022). Thus, understanding the amygdala, a structure frequently investigated within these disorders, and its role in pain can clarify how epilepsy and pain conditions sensitize.

The amygdala is an almond-shaped limbic structure found in the medial temporal lobe, adjacent to the hippocampus. It has long been acknowledged that the amygdala is a crucial structure in the integration of negative affective input, however the role that specific amygdaloid nuclei play in fear and nociceptive information within epilepsy has yet to be revealed (Aggleton, 1993; Dicks et al., 1969; Calvino et al., 1982; Tershner and Helmstetter, 2000; Gao et al., 2004). The amygdala is made up of multiple groups of nuclei (Price et al., 1987; Pitkänen et al., 2006). First, the basolateral group (BLA) consists of the lateral, basal, and basomedial nuclei, and maintains connections with the neocortex. The superficial group consists of multiple nuclei that connect with the olfactory and accessory olfactory system. The third group includes the central nucleus (CeA) and contains neurons that synapse in the autonomic regions of the lateral hypothalamus and other brain stem structures.

The BLA is a combination of nuclei consisting of excitatory glutamate neurons and inhibitory interneurons (Janak & Tye, 2015; Pitkänen et al., 1997). Nuclei subsets within the BLA uniquely code for the magnitude and degree of noxious stimuli, and are involved in attributing motivational affect to chosen protective behaviours in threatening

situations (Corder et al., 2019). More specifically, the lateral nuclei of the BLA receives sensory information from the hippocampus, thalamus, association regions, and the prefrontal cortex structures. Information from the lateral nuclei is then projected to the basal and basomedial nuclei, as well as to the CeA, which modulate how BLA output produces behaviour. In a F-CPA study, the amygdala was lesioned in rats with an excitotoxin and found to interfere with the magnitude of CPA produced (Gao et al., 2004). In addition, increased excitatory glutamatergic receptor activity from the mPFC, more specifically the prelimbic cortex, to the BLA increased vigilance in nerve injury mice (Gao et al., 2023). Hence, not only does the BLA play a key role in negative affective behaviour, but its ability to modulate pain-related information is of equal significance.

The CeA is a main output structure of the BLA, and consists of the lateral (CeL) and medial (CeM) subdivisions (Janak & Tye, 2015; Pitkänen et al., 1997). The neurons of the CeA are primarily inhibitory GABA neurons projecting from the CeL to the CeM, with a unique subset of neurons that appear to play a crucial role in mediating pain and fear-related behaviours (Gilpin et al., 2016; Wilson et al., 2019). CeA activity has been recognized as a key player in processing nociceptive information, now termed the "nociceptive amygdala" due to the many nociceptive inputs it receives through the CeL (Neugebauer et al., 2004). These nociceptive sources include spinal cord and brainstem input through the spino-parabrachio-amygdaloid pain pathway (Gauriau & Bernard, 2002). The CeA then communicates with a large portion of the forebrain and brainstem regions in modulating affective behaviour (Bourgeais et al., 2001; Price et al., 2003).

Pharmacological and optogenetic inhibition of input from the CeL to the CeM, and the general inhibition of the CeM produces anxiogenic behaviours in mice (Ciocchi et al., 2010). In addition, a fear conditioning paradigm found that CeM neuron activation and deactivation correlated with increased and decreased freezing during testing, respectively, indicating the role of CeM in learned conditioned fear responses (Duvarci et al., 2011). Ultimately, noxious information from the BLA are processed by the CeL, where potentiation of noxious stimuli results in the production of anxiogenic behaviours through CeM outgoing projections.

1.5 Objectives and Purpose

Literature on affective pain and pain-related behavioural outcomes have accumulated evidence relating connections within the BLA and pain-associated structures, which has historically been implicated in fear and anxiogenic behaviour (Chen et al., 2022; Zhuo, 2016). As discussed, the overlap between brain structures contributing to affective anxiogenic behaviour and pain-related responses have begun to be unveiled. Furthermore, pain-related and affective disorders occur at a disproportionately higher rate in PWE than in the general population (Keezer et al., 2015; Cragg et al., 2018; Gray, 2022; Téllez-Zenteno et al., 2005; Rasker et al., 2021). Yet, the role of BLA and connecting structures in the sensitization of pain requires further investigation. The amygdala kindling model in rats produces seizure activity as an epileptogenesis model that relies on electrical stimulation to able to produce anxiety-related and fear behaviour which parallels comorbidities seen in TLE (Kalynchuk et al., 1997, 1998, 1999). Thus, in

this thesis, the long-term amygdala kindling model will be used to examine affective and nociceptive pain in TLE. Further, differences in male and female pain within TLE will be observed.

First, the formalin-induced conditioned place aversion (F-CPA) task will investigate whether long-term amygdala kindled rats display an increased affective pain response when compared to non-kindled controls. It was hypothesized that amygdala kindling would sensitize the interictal affective component of pain and would produce a greater aversion in kindled rats when compared to control rats. This would be indicated by less time spent in the compartment paired to the noxious stimulus, formalin.

Next, affective pain processing behaviours were assessed using the formalin pain assay task. It was hypothesized that during the formalin task, amygdala kindling rats would display a greater frequency and duration of paw attending behaviours, such as elevation, licking, biting, shaking, or flinching of the injected paw when compared to controls, where the inflammatory stimulus was formalin. Immunohistochemical analysis of the expression of the immediate early gene c-Fos in regions of interest along the pain pathway including the mPFC, BLA, CeA, and PAG. It was hypothesized that neuronal activation would be greater in the mPFC, BLA and CeA (Morland et al., 2016; Seno et al., 2018) 90 minutes after the formalin test in amygdala kindled rats, and would be lower in the PAG (Liu et al., 2017) in amygdala kindled rats when compared to controls.

Lastly, immunofluorescence of the CeA and PVN were analyzed using double fluorescent staining with novel protein kinase C δ (PKCδ) and early growth response 1 (Egr1), another immediate early gene. It was hypothesized that more activation of PKCδ

and Egr1 would be seen in the CeA 90 minutes after the formalin test in amygdala kindled rats, while less activation of PKCδ and Egr1 would be seen in the PVN 90 minutes after the formalin test in amygdala kindled rats when compared with control rats (Li et al., 2023).

CHAPTER 2

METHODS

2.1. Animals

Forty male (n=20) and female (n=20) Long-Evans rats weighing between 190 and 240 g at the time of arrival (Charles Rivers Laboratories; Montreal, Quebec, Canada) were used as subjects. All rats were paired housed with the same sex in conventional rectangular polypropylene cages with standard laboratory bedding and with food and water provided ad libitium. The housing room was maintained at a constant temperature of 22 ± 1 C and kept on a 12:12 hr light:dark cycle with lights on at 0700 h local time. All procedures were conducted during the light phase between 0800 and 1800 h. Rats were given at least one week of daily handling before starting any procedures. All procedures were approved by Trent University's Animal Care Committee and were in compliance with the standards set by the Canadian Council on Animal Care. Efforts were made to minimize the number of animals used in this study. A timeline of the experiment is provided in **Figure 1**.

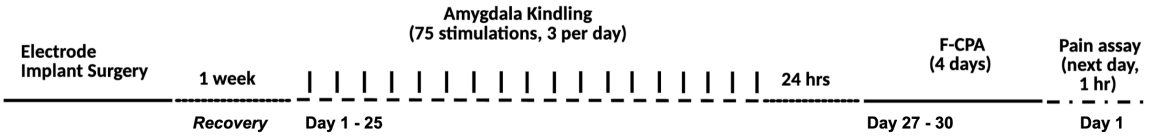

Figure 1. Electrode placement surgery was conducted on all rats for the purpose of amygdala kindling. Following a minimum one week recovery period, rats received either 75 kindling or sham stimulations over 25 days. After one rest day, rats were then placed in the formalin-induced conditioned place aversion test between days 27 and 30. On day 31, rats underwent the formalin pain assay test, and were euthanized.

2.2 Electrode Placement Surgery

A total of 37 rats underwent biphasic electrode stereotaxic surgery. All 40 rats underwent biphasic electrode stereotaxic surgery, ideally divided across group and sex, resulting in 10 rats for each male or female, kindled or sham groups.

Rats weighed an average of 277 ± 55g at the time of surgery. Rats were individually anesthetized with 2.5-5% isoflurane and treated with an analgesic (carpofen, 10 mg/kg, i.p.). The rat was secured in a stereotaxic frame and an incision was made along the scalp in a rostral-caudal direction. The skin was retracted and the overlying fascia was removed to expose the cranial surface. A single bipolar electrode (MS-303-2-, Plastics One, Roanoke VA) was implanted into the left basolateral amygdala (-2.8 mm posterior from bregma, -5.0 mm medial/lateral, 8.0 mm ventral from the cranial surface/dura) using stereotaxic coordinates (Paxinos & Watson, 2007). The electrode assembly was secured to the skull using jeweler screws and dental acrylic. Rats received an injection of 0.9% (w/v) saline (5 ccs) to replenish lost fluids. An injection of an antibiotic (Bayril, ~5 mg/kg, i.p.) was given and topical cream (Polysporin) applied to the incision site to reduce the risk of infection. Rats were allowed to recover in a cage with a heated pad until full mobility before being returned to their home cages. After surgery, rats were housed singly with enrichment items. Rats were monitored for seven days post-surgery for a minimum and received daily postoperative treatment of carprofen and Baytril.

2.3. Amygdala Kindling Procedure

A total of 37 rats successfully underwent electrode placement. Male and female rats were then randomly assigned to either sham (male: n=10; female: n=11) or kindled (male: n=7; female: n=9) groups. The kindling procedure followed our standard published methods (Fournier et al., 2020). Briefly, rats received three daily electrical stimulations to the left basolateral amygdala using an isolated pulse stimulator (Model 2100, A-M Systems, Sequim, WA, USA) with the following parameters: 1 msec biphasic square wave pulses, 60 pulses per second for 1 s, with a current amplitude of 800 uA (peak-to-peak). These parameters have been shown to successfully evoke afterdischarges in the amygdala after each stimulation and produce kindling (Racine, 1972). Electrical stimulations were delivered three times per day with a minimum of 3 hrs between consecutive stimulations. Stimulations were delivered each day, for 25 days, until animals received a total of 75 stimulations. Sham rats underwent the same handling procedure as the kindled groups, except no current was delivered.

Kindling seizures were classified according to the eight stage modified Racine scale (Pinel and Rovner, 1978). Stage 0 convulsions were defined by behavioural arrests in mobility, Stage 1 convulsions were classified as orofacial automatisms, stage 2 convulsions included orofacial automatisms and head nodding, stage 3 convulsions consisted of forelimb clonus and mastication and salivation, stage 4 convulsions were indicated by rearing and unilateral tonic extension of the forelimb, stage 5 convulsions included bilateral clonic forelimb movement and rearing leading to loss of equilibrium (i.e., falling), stage 6 refers to generalized convulsions consisting of multiple bouts of

stage 5 convulsions. In order to be considered kindled, rats are required to have had at least three consecutive stage 5 or greater seizures. Kindled rats that did not exhibit this threshold were removed from the study.

2.4. Behavioural Testing

2.4.1 Formalin conditioned place avoidance

Place conditioning apparatus.

Formalin conditioned place avoidance (F-CPA) was conducted in a three-compartment chamber consisting of two rectangular compartments (34 cm X 26 cm X 33 cm) and a smaller middle white box (19 cm X 26 cm X 33 cm). The two larger compartments had distinct visual, tactile and olfactory cues (see **Figure 2**). One compartment consisted of gray walls with a metal grid (mesh) floor and dilute acetic acid (1% v/v) as a scent. The other compartment consisted of vertically stripped walls with a metal rod floor and a dilute cinnamon extract (0.00003% v/v). The middle (starting) box had white walls and a smooth floor and was in the middle of the apparatus towards the centre point of each compartment. On the preconditioning and postconditioning test days, the start box door was opened, enabling each rat to travel freely across the three compartments. The compartments were cleaned with 70% (v/v) ethanol and new scents were reapplied to the bottom of the compartments after each animal. A digital camera was mounted to the ceiling, recorded each session, and allowed for tracking and quantifying of the animal's activity in each compartment using AnyMaze software.

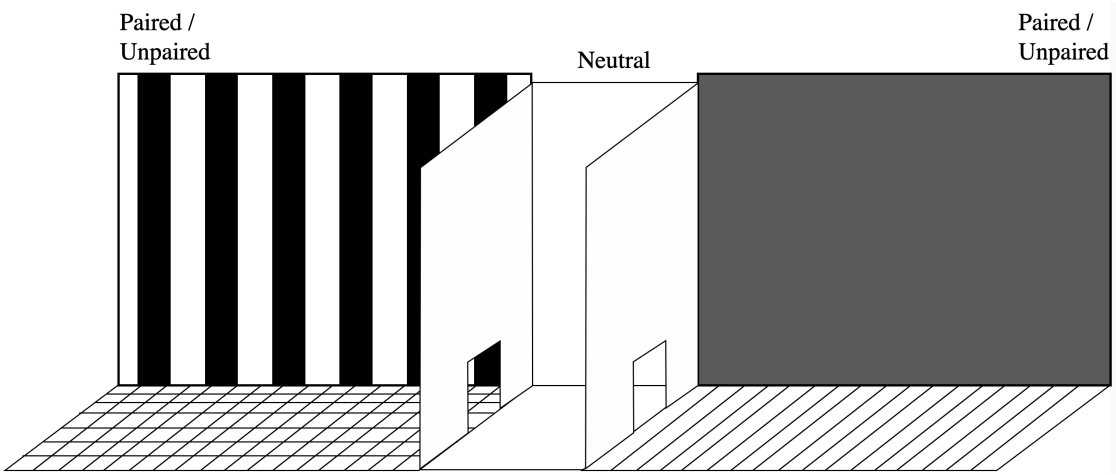

Figure 2. A representative diagram of the formalin-induced conditioned place aversion apparatus. The gray compartment contained a metal parallel bar floor and was 1% acetic acid scented, while the vertical stripped compartment contained a grid metal wire floor and was cinnamon scented. The apparatus was cleaned between each animal session with 70% ethanol. Passage between the three compartments was controlled by two removable sliding doors with colouring matching their respective compartments and was not pictured.

CPA Procedure

The F-CPA behavioural test was conducted over four days (see Figure 3) and consisted of a preconditioning session (Day 1), two conditioning sessions (Days 2 and 3) and one post-conditioning test (Day 4). On Day 1 (preconditioning day), rats were placed in the middle (start) box for 2 minutes, with entry to each adjacent compartment closed off. The doors were then removed, and the rat was given 23 minutes to freely explore all three compartments. Time spent in the chambers was quantified, and the preferred and non-preferred compartments were identified. A preferred chamber was used for conditioning (i.e. formalin / pain pairing) and was considered if the animal spent more than 30% of time in the chamber during preconditioning. On Day 2 (conditioning unpaired), the rat was placed into their non-preferred chamber for 40 minutes with the door closed. On Day 3 (conditioning paired), the rats were given an intraplantar injection of 5.5% (v/v) formalin (37% Formaldehyde diluted in 0.9% Saline) at a volume of 50 µl into either the left or right hindpaw (counter-balanced across groups) and then confined in their preferred chamber for 40 minutes with the door closed. On Day 4 (postconditioning), the procedure was the same as in Day 1 with rat allowed 23 minutes to freely explore all compartments of the apparatus.

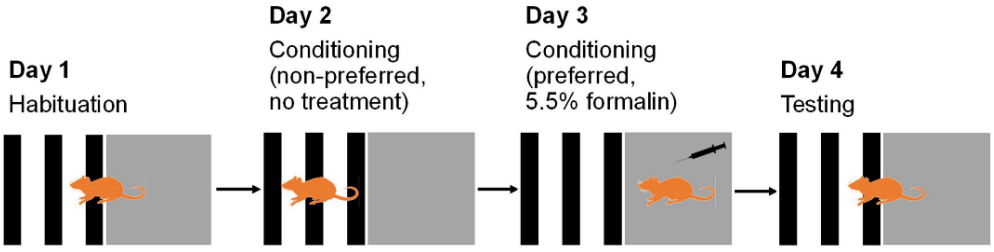

Figure 3. A visual representation of the formalin-induced conditioned place aversion task. Rats habituated to the apparatus on day 1 for 25 minutes and roamed freely. On day 2 rats were placed in the non-preferred and non-paired compartment for 40 minutes with no treatment. On day 3, rats received a 5.5% formalin injection (i.p.) into their hind paw and placed in the preferred and paired compartment for 40 minutes. Rats spent day 4 freely roaming the apparatus for 25 minutes, and time spent and behavioural measures were recorded using ANY-maze.

2.4.2 Nociceptive Pain Assay

Twenty-four hours after the F-CPA was completed, nociceptive and nocifensive behaviours was examined using the formalin test. During this procedure, rats were placed individually into Plexiglas observation chambers and allowed to habituate 30 minutes to acclimate to the apparatus. A dilute 5.5% v/v formalin solution was injected subcutaneously in the plantar surface of the hind paw not injected during the F-CPA procedure. After injection, the rat was immediately returned to the observation chambers and then monitored for 60 minutes. The observation procedure was video recorded with cameras positioned above, below and adjacent to the observation boxes. Formalin-evoked behaviours were quantified using AnyMaze software by an experimenter blind to the treatment conditions of the animals. The behaviours were divided into four categories: 0 – the injected paw does not show favouring and has normal contact with the floor, 1 – the injected paw is in contact with the surface but has reduced weight placed on it, 2 – the injected paw is elevated and is not in contact with any surface, 3 – the injected paw is licked, bitten, or shaken (Faramarzi et al., 2016; Abbot, 1995). Each category of formalin-evoked behaviour was recorded during the 60-minute test. A weighted pain intensity score was computed by multiplying the frequency of each category by its assigned weight and then dividing the sum of these products by the total frequency of each category. In addition to the above measures, the frequency of paw flinching was also recorded during the test session (Yin et al., 2016).

2.5 Tissue Preparation

2.5.1 Perfusions and Sectioning

Rats were euthanized 90 minutes after completion of the formalin test using sodium pentobarbital (Euthansol, Dormatol) and then perfused transcardially using 0.1 M phosphate-buffered saline (pH = 7.2-7.4) followed by 4% paraformaldehyde. The brain was extracted and placed in the same fixative for 48 hrs before being transferred into phosphase buffered saline (PBS, 1X, pH=7.4) containing 0.1% w/v sodium azide. The brains were then cryoprotected (until they sunk) in an ascending series of sucrose (10, 20, 30% w/v) diluted in PBS. Finally, brains were stored in 30% sucrose containing 0.1% sodium azide diluted in PBS until sectioned.

Brains were embedded in cryomolds containing Optimal cutting temperature (OCT) and then flash frozen in 2-methylbutane cooled in liquid nitrogen. Once frozen brains were sectioned on a cryostat at a thickness of 40 μm, they were stored in 0.1% sodium azide in 1X PBS until processed.

2.5.2 Immunohistochemistry and Quantification

Sections were stained for c-Fos, a marker of neuronal activity, using previously published methods (Fournier et al., 2020). All washes and incubations were carried under gentle agitation at room temperature unless otherwise noted. Free-floating sections were first washed several times in PBS and then incubated in 1% (v/v) hydrogen peroxide for 30 minutes to reduced endogenous peroxidase staining. After several rinses in PBS, the sections were incubated in a blocking solution for 1 hr containing 5% normal horse

serum, 1% bovine serum albumin, and 0.3% Triton X-100 diluted in PBS and then placed into blocking solution now containing rabbit anti-c fos antibody (1:10,000, Millipore Canada) overnight at 4°C. The following day, sections were rinsed several times in PBS and then incubated with a biotinylated horse anti-rabbit antibody (1:500, Vector labrs) diluted in 0.3% v/v Triton X-100 in PBS at room temperature. After this, sections were washed again in PBS and then incubated for 1 h in avidin-biotin-peroxidase complex (1:500, Vectastain ABC Elite) in 0.3% (v/v) Triton X-100 in PBS. Fos immunoreactivity was visualized with 2.5 (w/v) nickel sulphate, 0.02% (w/v) 3,3'-diaminobenzidine (DAB), and 0.000083% (v/v) hydrogen peroxide dissolved in 0.175 M sodium acetate. The sections were then mounted onto Superfrost Plus charged slides (Fisher Scientific), air dried, then briefly rinsed in deionized H_2O before placing them through increasing concentrations of 50%, 70%, 95%, and 100% ethanol and clearing with Xylene before coverslipping with Entellan mounting medium (EM Microscopy Sciences).

A Nikon Eclipse Ti2 Elements inverted microscope was used to take images of each section (Nikon Instruments, USA) The dimension of each image was identified using the 2X magnification and constructed from images taken at 10X magnification. All images were converted into the TIFF format and processed using Fiji (Schindelin et al., 2012). The images were converted to grayscale (8-bit) and cropped down to the left and right hemispheres for each section.

A semi-automated cell counting procedure, as described by Bourgeois et al., 2021 was used to estimate the number of Fos+ cells across four brain regions of interest (ROIs). The brain ROIs included the mPFC, CeA, BLA, and PAG. To quantify Fos

signal, landmarks were manually placed on each brain image to indicate their corresponding coronal area on the brain atlas (Paxinos and Waton, 2006), such as corpus callosum, hippocampus, and piriform cortex. These landmarks were then used to align the brain image before applying homographic transformation using the *WARP Image* plugin (BigWarp, Bogovic et al., 2016). Precoded ROI from the brain atlas were then overlaid onto the warped images and Fos positive cells were counted using a custom-made batch macro. The custom macro included a background subtraction using the roller ball plug-in (radius = 50 pixels), followed by thresholding. Threshold images were then processed using the *Analysis Particle* tool with the number of Fos positive cells set between 0 (minimal) and 12 (maximum) pixels. All parameters were held constant across all images to help reduce biases when applying thresholding to the brain sections. The number of Fos+ cells was normalized by dividing these numbers by by the area of the region to yield the number of Fos positives per unit area (Fos per mm^2).

2.5.3 Immunofluorescence and Quantification

In addition, sections were also double stained with novel protein kinase C delta (PKCδ), a protein found to be expressed in neurons involved in pain sensation (Han, 2015; Singh, 2022) and early growth response 1 (Egr1), another immediate early gene used as an indicator for the activation of neurons. All sections were washed and incubated under gentle agitation at room temperature unless stated otherwise. Free-floating sections were first rinsed several times with PBS and then incubated in an antigen retrieval solution (10 mM sodium citrate, diluted in deionized H20, pH = 9.5-9.0) for 30 minutes at 80°C. After several rinses in PBS, the sections were incubated in a blocking solution

for 1 hr containing 5% (v/v) normal goat sera, 1% (w/v) bovine serum albumin, and 0.3% (v/v) Triton X-100 diluted in PBS then incubated in a blocking solution additionally containing mouse anti-PKCδ antibody (1:2000, BD Biosciences) and rabbit anti-Egr-1 (1:1000, Cell Signalling) for 72 hours at 4°C. The following staining day, sections were rinsed several times with PBS and then incubated in Alexa Fluor 488-conjugated goat anti-mouse (1:500, Invitrogen, A11001) and Alexa Fluor 546-conjugated goat anti-rabbit (1:500, Invitrogen, A11035) diluted in 0.3% (v/v) Triton X-100 in PBS for 2 hrs at room temperature under minimal lighting. The sections were then mounted onto charged slides (Superfrost Plus, Fisher Scientific), air dried for a minimum of 2 minutes and coverslipped immediately with 100 µl of VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories, H-1800, Newark, California).

The Nikon Eclipse Ti2 Elements inverted microscope was used to take images at 20X magnification, using 2X and 10X magnification to identify regions of interest in order to quantify PKCδ+ and Egr1+ cells (Nikon Instruments, USA). The ND Acquisition Wizard and Capture Z-series plugins were used to capture images with the wavelengths set to 488 (PKCδ) and 546 (Egr1), and stop distances between each Z-stack image set to a minimum of 1 µm and a maximum of 3 µm. All images were saved as NDS files and manually counted using the Cell Counter plugin in Fiji (Schindelin et al., 2012). Files were imported using the Bio-formats importer plugin (Linkert, 2010) and the CeA and PaV boundaries were determined according to Paxinos and Watson's atlas plates (2007).

2.6 Statistical Analyses

All statistical calculations were run using R-studio. Kindling stages and weight differences were compared using the two-tailed Student t-test. The F-CPA and Formalin Tests were analyzed using a mixed model ANOVA design consisting of the between-subject factors of kindling status and sex and the within-subject factor of the phase of testing and/or compartment. When applicable and proper, two-tailed Student t-tests and paired t-tests will also be used. For each animal, the *c-Fos* labelling (neurons/mm^2) was averaged across 3-4 sections, and group means were calculated. For PKCδ/Egr1 immunofluorescence, the positive PKCδ/Egr1 counts for each animal were summed across 3-4 sections, and group means were calculated. Two-tailed Student t-tests and paired t-tests were run on *c-Fos* immunoreactivity and PKCδ/Egr1 immunofluorescence. Statistical significance was set at $P < .05$ for all analyses run. Data in figures and text are presented as the mean and standard error of the mean (SEM).

CHAPTER 3
RESULTS

3.1. Introduction

Pain-related and psychiatric conditions are reported at an increased prevalence in people with epilepsy. Coupled with growing research linking structures involved in anxiety, such as the basolateral amygdala, with pain, this leads us to investigate these structures in the context of epilepsy. More specifically, the amygdala kindling model of epilepsy has been shown to produce anxiety and fear-related behavioural changes that parallels comorbid symptoms in TLE (Kalynchuk et al., 1999). In summary, the long-term amygdala kindling model will be used to investigate sensitization of affective and nociceptive pain processing in TLE. Furthermore, kindling-related affective changes will be assessed with the F-CPA task. In addition, nociceptive behaviours in kindled and non-kindled rats will be assessed with the formalin test, and subsequent will be assessed for neurobiological changes using c-Fos and PKCδ/Egr1 immunohistological staining.

3.2 Animals

Only rats with confirmed electrode placements that correctly targeted the left basolateral amygdala were included in the study. During stereotaxic surgery, 3 rats (2 males; 1 female) died due to complications. In addition, two male rats failed to exhibit consecutive Stage 5 or higher motor convulsions during electrical kindling and were subsequently removed from the study. The final group values were 7 male/sham, 9

female/sham, 8 male/kindled, and 11 female kindled, summing up to a total of 35 rats consisting of 16 sham and 19 kindled rats.

3.2.1 Kindling Characteristics

Male and female rats that received 75 electrical stimulations to the left basolateral amygdala showed the typical evolution of evoked limbic motor convulsions during the experiment. Figure 4A presents the mean number of each Racine seizure score. A series of independent t-tests did not find differences between male and female kindled rats in the mean number of Stage 1 (p = .518), Stage 2 (p = .676), Stage 3 (p = .837), Stage 4 (p = .229), or Stage 5 seizures (p = .462). The kindling rates were similar for male and female kindled rats (Figure 4B). The male kindled group required 17.75 ± 1.3 stimulations to reach the first generalized motor seizure (Stage 5 or higher) event, whereas the female kindled group required 14.36 ± 0.7 stimulations. The mean difference between the two groups did not reach statistical significance [t(11.9)=-0.788, p=0.445]. There was also no difference in the number of stimulations required to evoke partial seizures (Stage 2). As with past work (Galic et al., 2008), neither male nor female kindled rats displayed spontaneous seizures during regular observation or handling over the duration of the study.

Changes in body weight are a common comorbidity of epilepsy. Previous work has shown that men and women with epilepsy tend to have a higher body mass compared to the general population (Steinhoff et al., 1996), with women potentially being at greater risk for obesity. In rats, amygdala kindling has also been associated with significant weight gain (Fournier et al., 2009; Innes et al., 1977; Loscher et al., 2003; Hum et al.,

2009). To examine the effect of kindling on weight gain for male and female rats, we examined the percentage change in body weight at sacrifice (Day 31) relative to the body weight measured on the first day of kindling (Figure 4C). Analysis of the percentage change in body weight revealed a significant effect of the group [$F(1,31)=33.27$, $p < .0001$], with the kindled group gaining approximately 74% more weight compared to the sham group. There was no main effect of sex [$F(1,31)=1.77$, $P=.193$], and the interaction between group and sex was also not significant [$F<1.00$, $P=.818$]. However, analysis of the absolute body weight at sacrifice revealed significant effects of group [$F(1,31)=56.80$, $p < .0001$] and sex [$F(1,31)=110.41$, $P<.001$], but no significant interaction between group and sex [$F(1,31)=2.09$, $P=.158$]. As shown in Figure 4C, the kindled group weighed significantly more than the sham group at the end of the study.

These data suggest that male and female amygdala-kindled rats display similar kindling rates and expressions of Racine convulsive events. Additionally, both male and female kindled rats experienced equivocal weight gain during kindling. Based on these similarities in kindling profiles, we combined the male and female subjects for each group condition to increase statistical power and simplify analyses of the behavioural and histological data.

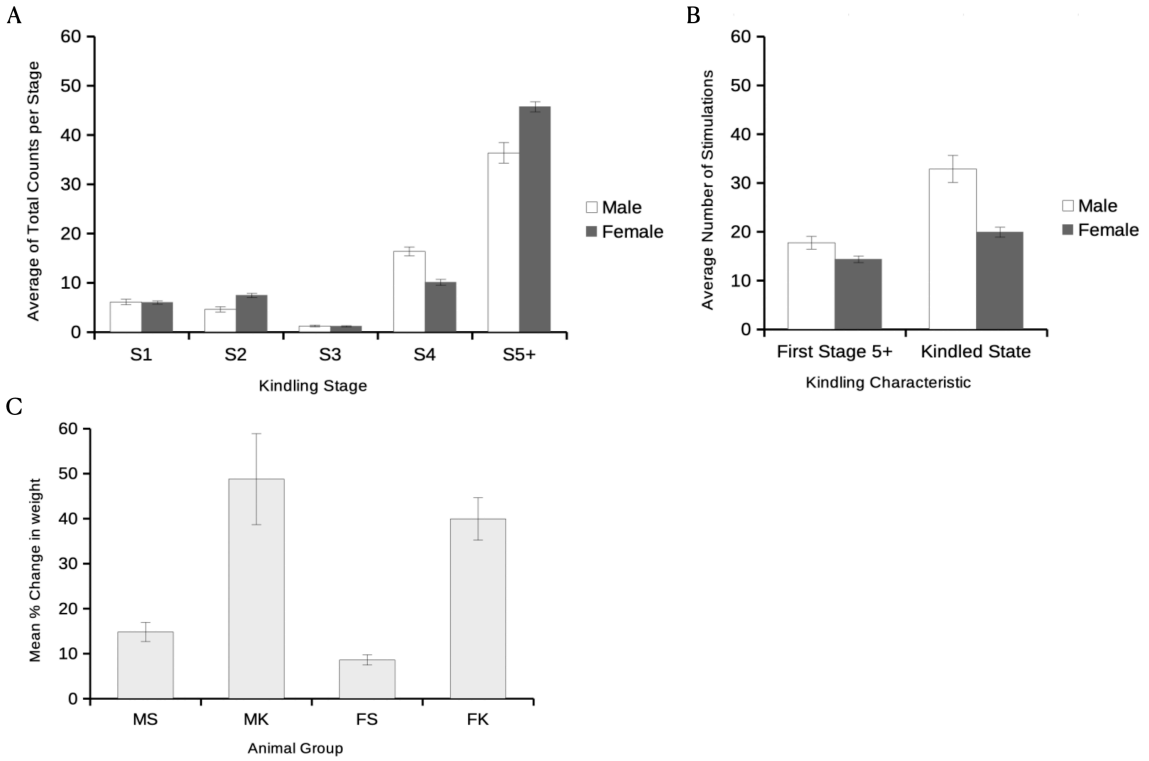

Figure 4. Animal weight and kindling characteristics. Male (n=8) and female kindled (n=11) rats did not show a significant difference between total counts of Stage 1-5+ convulsions elicited (A), or the number of stimulations required to reach the first Stage 5+ or a fully kindled state (B). Kindled male (MK, n=8) and female (FK, n=11) rats (n=19, Kindled) gained significantly more weight than sham male (MS, n=7) and female (FS, n=9) rats (n=16, Sham) during kindling (C). Data is presented as the Mean ± SEM.

3.3 Effect of long-term kindling on formalin-induced conditioned place avoidance

Because of the well-documented relationship between anxiety and chronic pain disorder (Ferguson & Ahles, 1988; Asmundson & Katz, 2009; Chen et al., 2022), we assessed pain-associated learning by using a conditioned place avoidance (CPA) task—an established model to investigate the affective dimension of pain. In this procedure, aversion induced by a noxious stimulus, such as intraplantar injection of the formalin, produces pain responses that have temporal proximity with the conditioning session of the CPA procedure. This results in the animal displaying avoidance (i.e. spending less time) of a compartment previously paired with formalin during a treatment-free post-test session. Several brain regions, including the basolateral amygdala, have been previously reported to be critical in acquiring pain associated CPA (Tanimoto et al., 2003). Thus, we sought to determine whether long-term amygdala kindling might enhance pain-associated learning on this task.

Prior to our main experiment, a pilot study was run to establish the threshold for dose of formalin required to produce nociceptive behavioural responses. This pilot consisted of three treatment doses given 1%, 2.5%, and 5.5% formalin diluted in saline, and a control group (n=5, each). A statistically significant CPA response was found in control [$t(4)=-10.29$, $p<.001$] and the 5.5% treatment group [$t(4)=-5.85$, $p=.004$] but no other groups ($p>.05$), and therefore 5.5% will be the formalin concentration used for the main experiment (see Appendix, Supplementary Figure S1).

Activity levels during the 25-minute preconditioning (Day 1) session were examined to determine each subject's preferred chamber and identify potential group differences in exploratory behaviour and motor activity. There were no significant group differences in either total distance travelled [$F(1,33)=0.281$, $p=.599$, Figure 5A] or mean movement velocity [$F(1,33)=0.044$, $p=.835$, Figure 5B] during this session. Furthermore, a chi-square analysis [22 striped vs. 13 gray subjects; $\chi^2(1)=2.31$, $p=.128$] revealed no significant bias for the assigned formalin-paired chamber.

For the conditioning days (Day 2 and Day 3), the animal's preferred chamber (stripped vs. gray) on the preconditioning day was assigned as the formalin-paired (pain) chamber. The animal's conditioned place aversion (CPA) to the pain chamber was then calculated by subtracting the animal's time spent in the pain chamber on preconditioning day from that of the post-conditioning day. There were no significant differences in CPA across conditioning days between the groups [$F(1,33)=0.021$, $p=.884$, Figure 5C]. Further analysis on the individual group learned aversion showed that neither sham (one-sample t-test, $p=.144$, one-tailed) nor kindled (one-sample t-test, $p=.082$, one-tailed) rats successfully learned the formalin-induced aversion.

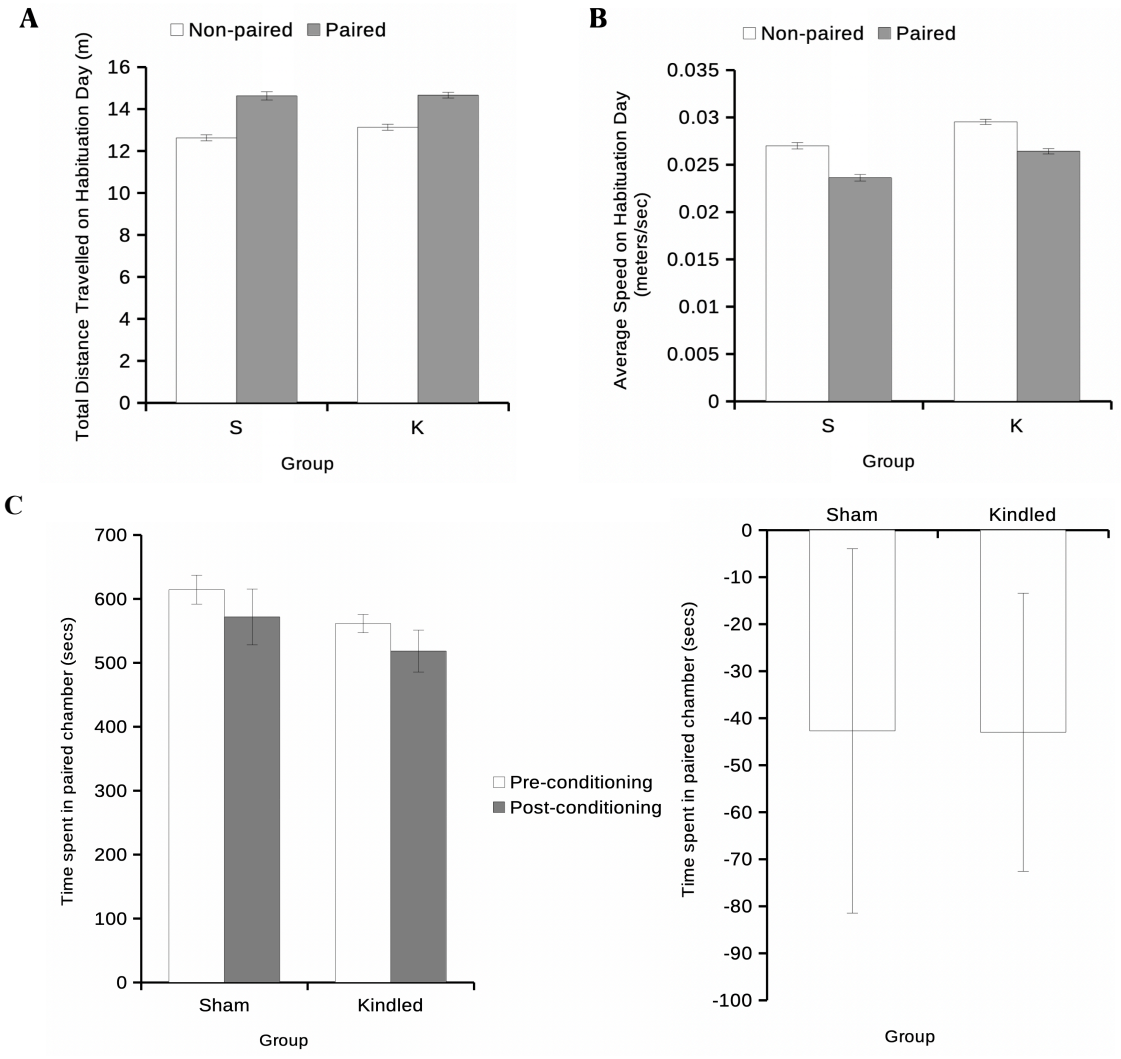

Figure 5. In the formalin-induced conditioned place aversion test, both (A) average speed and (B) distance travelled were not significantly different between sham (n=16, S) and kindled (n=19, K) rats. (C) The time spent in the paired chamber on pre-conditioning and post-conditioning day (left), with the difference in time spent (right) shown between sham and kindled rats. Data is shown in mean ± SEM.

3.3.1 Reconsidering Pre-Conditioning Preference

Following no significant CPA found in our animal groups, we re-evaluated the preferences established on pre-conditioning day. We found that a small but significant proportion of animals (4 out 35, $\chi^2(1)=20.83$, $p<.001$] tended to show a preference for the neutral (middle) chamber over the other two chambers. This bias was not specific to either group (data not shown), and were excluded for this analysis, but included in further tests. Figure 6 shows the mean difference scores for time spent in the formalin-paired chambers on the post-test and the pre-conditioning session. While sham controls spent a similar amount of time in the formalin-paired chamber during the post-test as they did on the pre-conditioning session (one-sample t-test, $p=.277$, one-tailed), kindled rats spent significantly less time in this compartment on the post-conditioning test day (one-sample t-test, $p=.032$, one-tailed). These results suggest that kindled rats learned the aversion to formalin pain, whereas sham rats exhibited difficulty making this association.

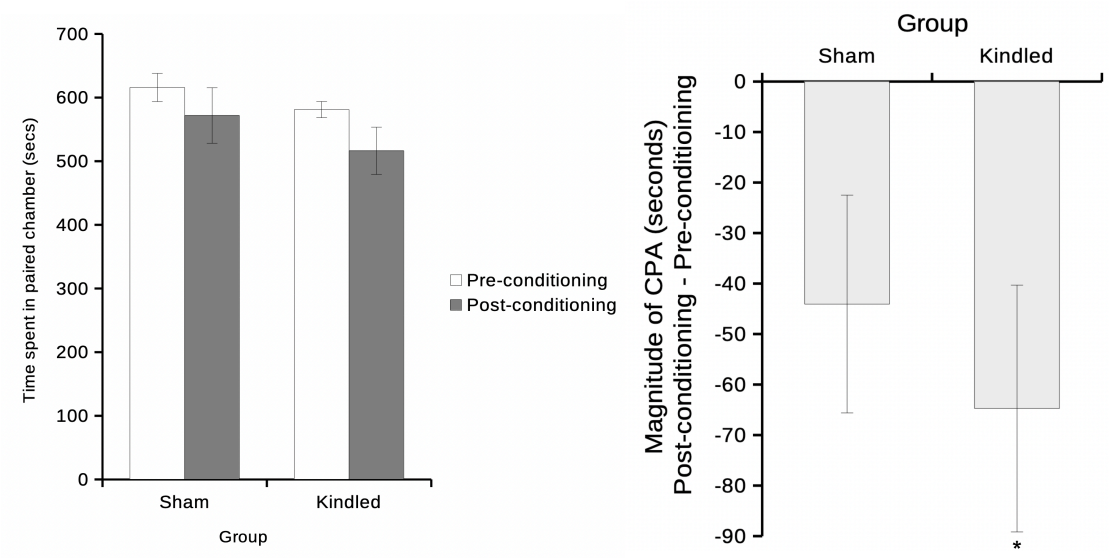

Figure 6. The magnitude of CPA (in seconds) calculated from the difference between the time spent in the paired compartment on post-conditioning day from the pre-conditioning day is shown for sham and kindled rats (right), with the time spent on each pre- and post-conditioning day shown as well (left). A significant aversion developed in kindled (n=15, p=.032), but not sham (n=16, p=.277) rats. Data is shown in mean ± SEM. *P<.05.

3.4 Effect of long-term kindling on formalin-evoked nociceptive behaviour

To examine if kindling might sensitize neural circuits critical in processing of pain, we characterized evoked nociceptive responses of kindled rats on the formalin pain assay test. Each rat received an intraplantar injection of a 5.5% formalin solution, and their pain behaviour was monitored per 5 min intervals for 60 min. Consistent with previous studies (Dubuisson & Dennis, 1978; Abbot et al., 1995), formalin injection evoked a classic biphasic response with a short-lasting acute phase (0-15 min), followed by an interphase (10-15 min), in which spontaneous nociceptive behaviours (i.e. elevation, flinching, licking or biting of the injected paw) was attenuated. This was followed by a second tonic phase beginning approximately 15 min after formalin injection and persisting until 60 mins.

A series of t-tests were used to examine for group differences in the total frequency of each nociceptive behaviour (0-3) during the 60 min time period, but found no significant differences between kindled and sham rats in the mean number of category 0 ($p = .89$), 1 ($p = .79$), 2 ($p = .13$), or 3 ($p = .46$) behaviours (Figure 7A). In addition, the total composite weighted pain score was not significantly different between kindled and sham rats (two-sample t-test, $p=.21$, two-tailed, Figure 7B). To further examine nociceptive responses across the different phases after formalin injection, we computed weighted composite scores during every 5 min bin across each of the different phases (Figure 7C). While the weighted pain scores were found to be different between each acute, inter-, and tonic phases [$F(1, 35)=23.92$, $p<.0001$], no group differences were found between each phase [$F(1,35)=0.054$, $p=.817$]. Further t-tests were run on the

weighted pain scores for each bin and found that sham rats showed a greater mean than kindled rats for the 10 minute bin [$t(34.9)=2.926$, $p=.006$].

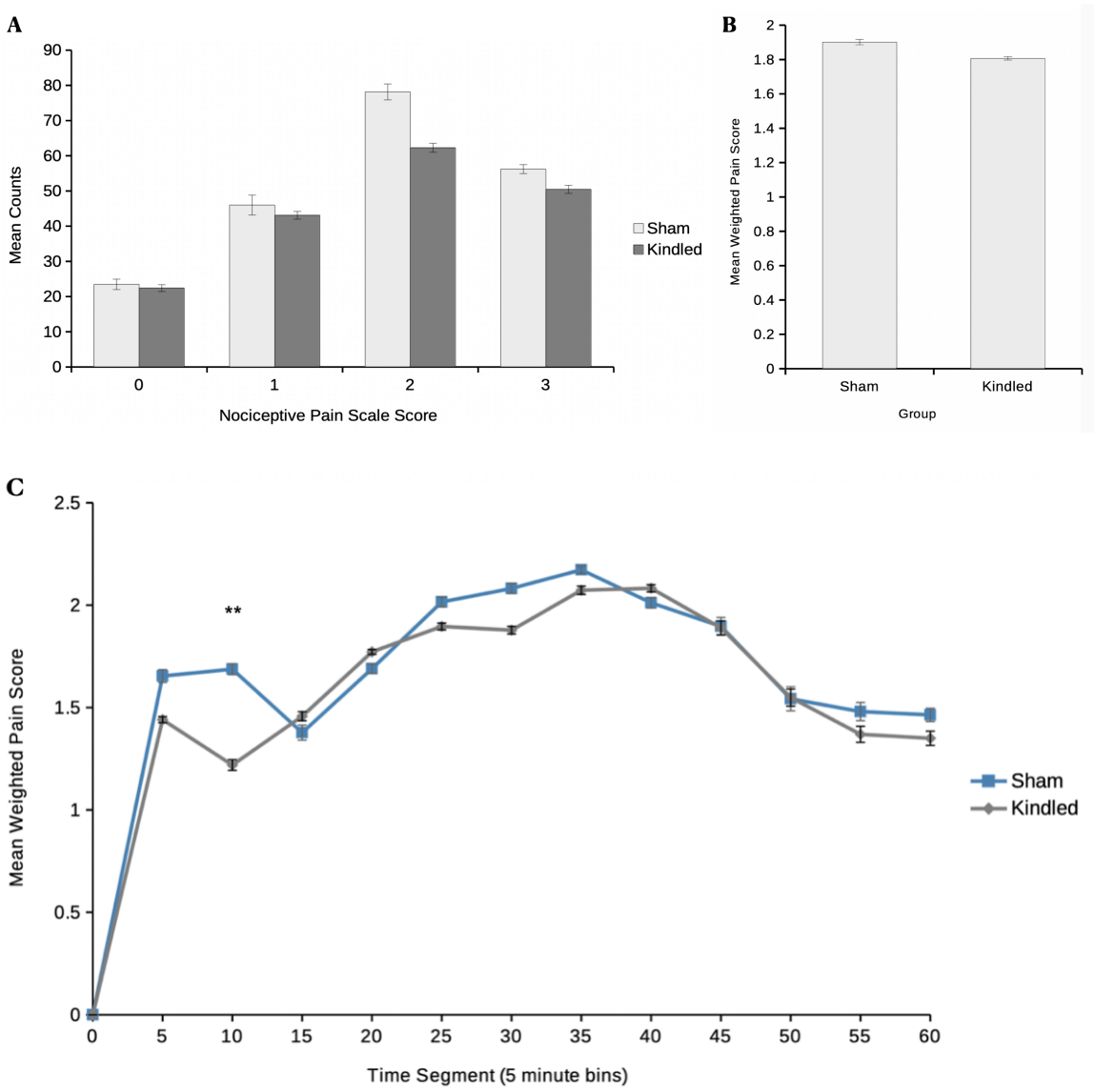

Figure 7. Formalin pain assay test scores. (A) Mean counts per pain score and (B) the cumulative weighted pain scores are shown for each group across the full hour. Weighted pain scores for each five minute bin are shown for each group (C), with significance found during the 10 minute bin between kindled (n=19) and sham (n=16) rats (p=.006). Data is shown in mean ± SEM. **P<0.01.

In order to investigate the potential changes in plasticity that seizures can have on nociceptive behaviour, the relationship between the frequency of generalized motor seizures and cumulative weighted pain scores were examined. The mean counts of each nociceptive behaviour and their weighted pain score for phase I and II are shown in Figure 8A and 8B, respectively. The total Stage 5 and 6 seizures were compared with the overall acute phase I or tonic phase II weighted pain scores for each kindled animal using Pearson correlations (Figure 8C and D). A very weak decrease in pain scores correlates with increasing Stage 5 (r=-.14) or 6 (r=-.04) seizure frequency in phase I. In contrast, a moderate increase in Stage 5 seizures correlates with increased pain (r=-.35), and a moderate decrease in pain correlates with increasing frequency of Stage 6 seizures in phase II (r=.40). Further t-tests indicate that Stage 5 (p=.56) or 6 (p=.87) seizures during phase I did not significantly correlate with the weighted pain scores. Pain scores during phase II increased insignificantly with greater Stage 5 seizures (p=.14) and trended towards decreasing with greater Stage 6 seizure frequency (p=.09). These findings suggest that frequency of Stage 5+ seizures have no effect on phase I of the formalin test, while a moderate increase in Stage 5 seizure positively correlates with pain scores in phase II, and a moderate decrease in pain scores is seen as Stage 6 seizure frequency decreases.

In summary, kindled rats showed lower pain scores during acute phase I of the formalin test, but this effect did not persist into phase II. Additional investigation into the effect of seizure frequency on pain scores found that seizure frequency had no effect on

phase I, while more Stage 5 seizures trended towards increased pain scores and more Stage 6 seizures trended towards decreased pain scores in phase II.

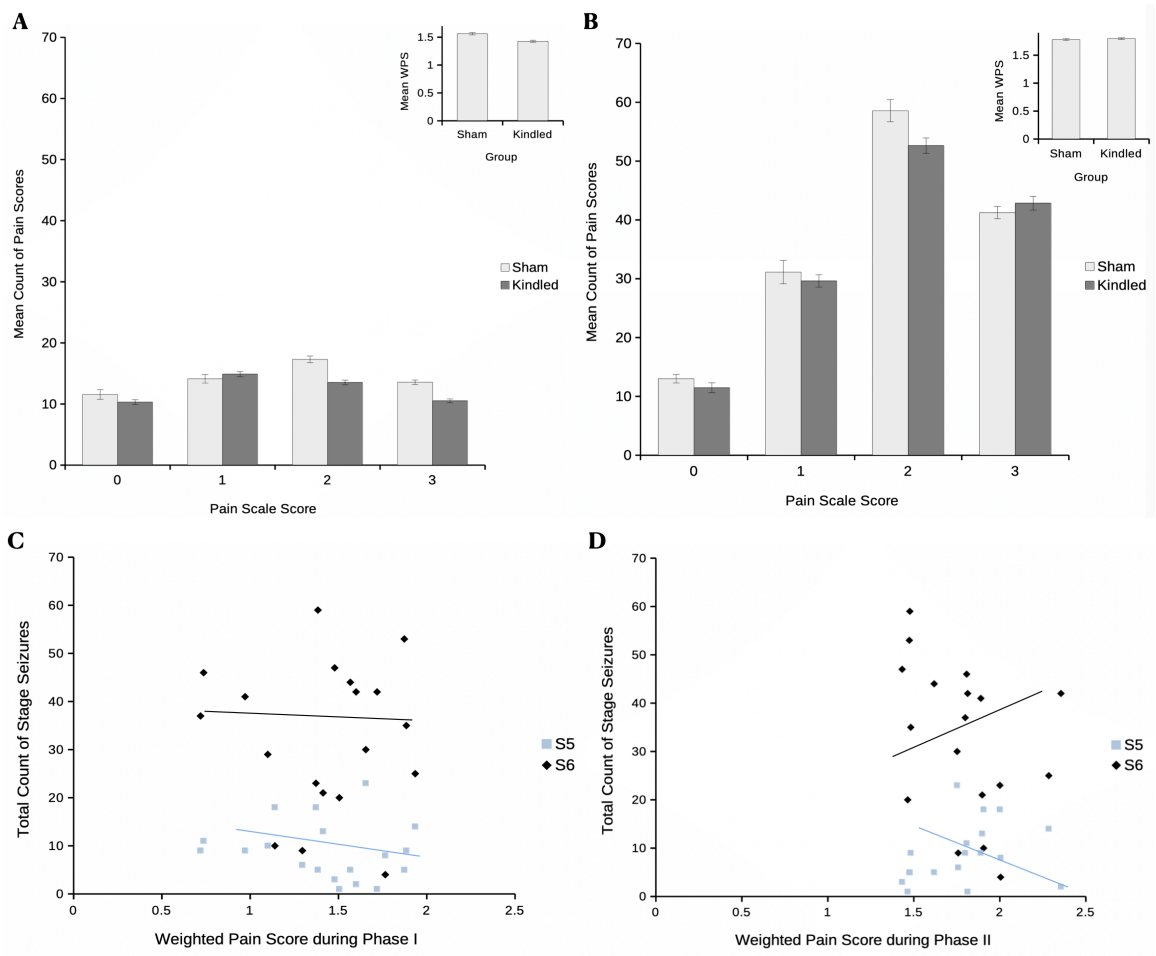

Figure 8. The mean pain scores by count for sham (n=16) and kindled (n=19) rats are shown with their composite weighted pain scores for phase I (A), and phase II (B). A scatterplot of the relationship between the weighted pain score for each kindled rat and their total number of Stage 5 (S5) or 6 (S6) seizures during phase I (C) and phase II (D) with their respective correlation lines are shown (phase I, S5 r=-.14, S6 r=-.04; phase II, S5 r=-.35, S6 r=.40), where no significance was found in each correlation. Where relevant, data is shown in mean ± SEM.

3.5 Immunohistology

3.5.1 Formalin-induced Fos expression in Brain Regions Involved in Emotion and Pain

Using the immediate early gene product c-Fos as a marker of neuronal activity (Bullitt, 1990), we examined the impact of kindling on brain regions known to be important in the processing of nociceptive and emotional information. Fos protein expression was examined in rats euthanized 90 to 120 minutes after formalin injection in the following brain regions: basolateral and central amygdala, medial prefrontal cortex, and periaqueductal gray.

The number of Fos-immunoreactive cells within BLA, CeA, mPFC, and PAG was compared using a series of independent t-tests. Examples of Fos-immunoreactivity for each brain region and group are shown in Figure 9. No differences between kindled and sham rats were found in the BLA [$t(32.9)=0.02$, $p=.99$], CeA [$t(28.4)=1.38$, $p=.18$], or mPFC [$t(21.6)=0.152$, $p=.88$]. Interestingly, sham rats showed a greater c-Fos expression than kindled rats did PAG [$t(14.1)=3.49$, $p=.003$].

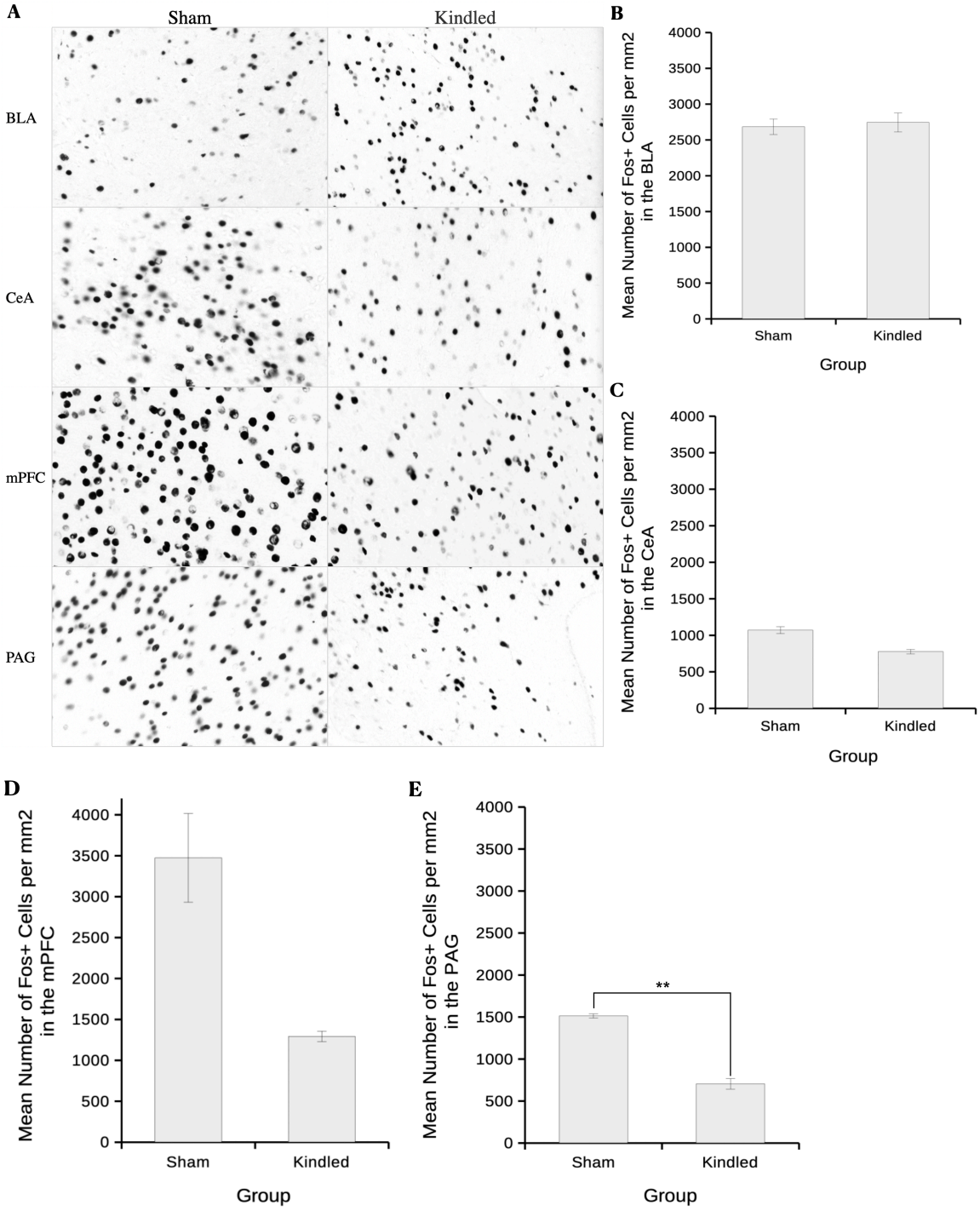

Figure 9. A photomicrograph of c-Fos expression for the BLA, CeA, mPFC, and PAG, and their respective positive mean number of c-Fos cell counts per mm^2 (BLA, B; CeA, C; mPFC, D; PAG, E) is shown for sham (n=16) and kindled (n=19) rats, where sham rats showed greater PAG expression than kindled rats (p=.003). Data is shown in mean ± SEM. **P<.01.

3.5.2 Formalin-induced expression of PKCδ positive Brain Regions in Emotion and Pain

Sections were stained with protein kinase C δ (PKCδ) due to its expression during in nociceptive and affective behaviour. The immediate early gene, early growth response 1 (Egr1), was also used as a marker of neuronal activity. Particular interest was placed in the activation of PKCδ positive neurons within the central amygdala (CeA) and paraventricular hypothalamus (PVH) due to their roles in anxiogenic and pain modulation (Botta, 2015; Wilson et al., 2019; Li et al., 2023; Ji et al., 2024a,b).

The percentage of cells counted with PKCδ positive, Egr1 positive, and both PKCδ/Egr1 (i.e., co-localized) positive expression within the CeA and PVH were examined by a few mixed ANOVAs. The immunofluorescence for each group and brain region are pictured in Figure 10. Kindled rats were not found to have different expression from sham rats between each positive cell type within the CeA [$F(4,124)=2.192$, $p=.074$] and the PVH [$F(1,27)=0.026$, $p=.082$]. The highest percent of cells were found to be the PKCδ positive cells, followed by Egr1 positive cells, and co-localized cells in both the CeA [$F(4,124)=163.359$, $p<.00001$] and PVH [$F(4,108)=9.269$, $p<.00001$]. In summary, no group comparisons between cell types were significant between CeA and PVH.

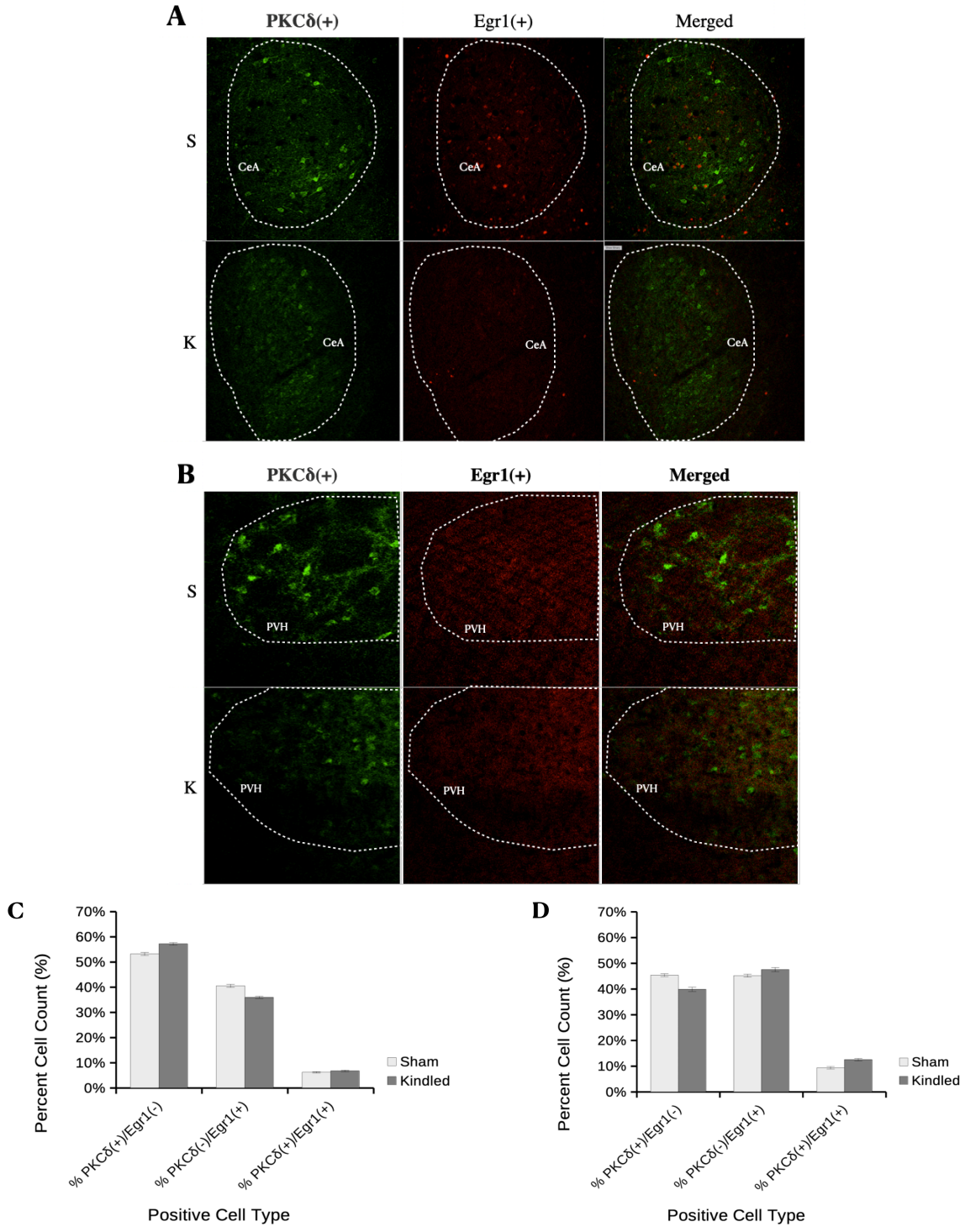

Figure 10. Photomicrographs for Positive PKCδ and Egr1 Expression in the CeA (A) and PaV (C) between sham (n=16) and kindled (n=19) rats. Percent positive PKCδ and Egr1 Expression in the CeA (B) and PaV (D).

CHAPTER 4

4.1 General Discussion

Studies have long implicated connections within the BLA and limbic system in fear and anxiogenic behaviour (Cendes et al., 1994; Aggleton, 1993; Dicks et al., 1968). In addition, recent evidence has begun to unveil the relationship between affective anxiety-related and pain-related symptoms and disorders (Chen et al., 2022; Zhuo, 2016). Despite this, the exact mechanisms behind the changes in pain sensitization in TLE have yet to be elucidated. The anxiogenic and fear-related behaviour induced from seizure activity within the amygdala kindling epileptogenic model which relies on electrical stimulation parallels the affective comorbidities seen in TLE (Kalynchuk et al., 1997, 1998). Hence, this thesis focuses on investigating the potential overlap between TLE and comorbid affective pain, where interictal pain changes were observed in the long-term amygdala kindling model. Further, the affective component of pain was examined through the F-CPA task, nociceptive pain behaviours were examined in the formalin test, and the neurobiological changes that occur following formalin-induced pain in amygdala kindled rats were investigated using c-Fos and PKCδ/Egr1 immunohistological staining.

4.2 Formalin-induced Conditioned Place Aversion

In our experiment, the F-CPA test was used to assess the effect of long-term amygdala kindling on affective and nociceptive pain by comparing learned avoidance between sham and kindled rats. Kindling characteristics were considered prior to analysis of F-CPA test results and found no significant difference in the progression of seizure

events between male and female rats, excluding sex from further analysis and combining across groups. The initial insignificant aversions found led to the discovery of animals with middle compartment preferences, which introduces the issue of our formalin-pairing not occurring in their true preferred compartment. Following the exclusion of these animals, a significant conditioned place aversion to the paired chamber was seen in kindled rats, however sham rats failed to develop the CPA. As mentioned previously, increasing impairments in memory and learning are reported with increasing stimulations in amygdala kindling (Botterill et al., 2014), it would not be unexpected to find the long-term kindled rats unable to learn the F-CPA avoidance. Moreover, long-term amygdala kindling may induce structural and functional changes that influence affective learned avoidance (Pikanen et al., 1998). And thus, difficulty learning the associated aversion could be understood in our kindled rats, however absence of learned aversion in sham rats is unseen in the literature, which indicates that F-CPA avoidance of the paired compartment should occur in the sham rats (Johansen et al., 2001; Gao et al., 2004).

These results clearly indicate some difficulty in the learning of aversion to the formalin-paired chamber. Interestingly, however, kindled rats were able to successfully learn this aversion which suggests that long-term amygdala kindling may potentiate the affective processing of the formalin stimuli enough to produce a clearer aversion in our F-CPA task than in our sham rats. This would support our hypothesis that amygdala kindling may potentiate overlapping pathways to produce an enhanced affective pain experience. Few studies have been conducted analyzing the potential for epileptic neuroplastic changes to induce increases in pain sensitivity, however one study did find a

similar pattern. Velioglu and colleagues (2017) found an interictal increase in sensitivity to thermal discomfort in a genetic model of absence epilepsy. In addition, kindled rats displaying greater avoidance or sensitivity towards negative affect is reflexive of the increased fear and defensive behaviour previously recorded (Kalynchuk et al., 1997, 1998, 1999; Fournier et al., 2020).

Despite the fascinating suggestion of our F-CPA results from the comparison of kindled rats learning CPA and sham rats being unable to, it is still important to discuss the lack of learned aversion in sham rats, and also by extension, the possible causes for the difficulty in learning the CPA in our task. To start, concerns may be brought to the dose of formalin, however the dosage of 5.5% formalin was chosen due to a significant aversion seen in non-kindled pilot rats, and thus the dosage is an unlikely cause (see Appendix, Figure S1). Next, other studies that utilize the CPA paradigm assign more days to establish a strong association between the stimulus and the paired compartment (Prus et al., 2009). Even so, the successful CPA produced during the pilot study was conducted with the same protocol as the current study, ruling the number of conditioning days out as a possible reason behind unsuccessful CPA. After any apparent issues in the protocol have been addressed, attention turns to potential confounds within the apparatus.

Discussion within the paradigm that associates an aversive (CPA) or preferable (CPP) stimulus with a distinct compartment, an important consideration is whether the animal is required to make a “forced choice” between the stimulus or non-stimulus associated chamber, or whether they may make an “unforced choice” where more than one compartment has no association with the stimulus (Prus et al., 2009). Our apparatus

consists of two non-stimulus related compartments, the non-paired distinct compartment and the neutral middle chamber. As animals have been removed from statistical analysis due to showing preference to the middle chamber, this highlights the issue of the middle chamber in affecting the development of the learned CPA. While significant aversion was captured during pilot experiments, the contribution of the middle chamber in learned aversion still warrants further investigation.

Taken together, the ability of kindled rats to learn the aversion to the noxious formalin-related compartment in contrast to sham rats may indicate a potentiation of affective pain due to long-term amygdala kindling. Despite this, further investigation into the sensitivities of the F-CPA task is necessary for future usage of this paradigm in affective pain-mediated changes in amygdala kindled rats.

4.3 Formalin nociceptive pain response

Two days following the completion of the F-CPA test, the formalin test was conducted to observe the changes in nociceptive attending and nocifensive pain-related responses to a formalin plantar injection. An overall biphasic progression of nociceptive pain was observed, with phase one occurring between 0 and 15 minutes, an interphase between 15 and 20 minutes, and phase two occurring from 20 minutes to the end of the test. This biphasic progression is well-documented in literature (Abbot et al., 1995; Dubuisson & Dennis, 1978).

During phase one of the formalin test, kindled rats showed lower nociceptive pain scores than sham rats did, which contrasts the increased affect observed within the F-CPA

task. According to the literature, the beginning phase of the formalin test engages with the peripheral nociceptors (Coderre et al., 1993; Dickenson and Sullivan, 1987; Shibata et al., 1989). This suggests that long-term amygdala kindling produced a decrease in the pain threshold of peripheral neurons that persisted into the interictal period, as the nociceptive pain behaviours were observed on the sixth day following the last stimulation.

The CeA is a main output structure of the amygdaloid nuclei, recieving a majority of its information from the BLA (Janak & Tye, 2015). CeA activity has been recognized as a key player in processing nociceptive information, now termed the "nociceptive amygdala" due to the many nociceptive inputs it receives through the CeL (Neugebauer et al., 2004). These nociceptive sources include nociceptive spinal cord and brainstem information through the spino-parabrachio-amygdaloid pain pathway (Gauriau & Bernard, 2002). Several studies targeting the CeA have identified it as a key player in formalin-induced nociceptive behavioural changes, more so than the BLA (Manning & Mayer, 1995; Torres-Rodriguez et al., 2024). Hence, amygdala kindling may have potentiated CeA connections and led to decreased nociception, which we further investigated with c-Fos and PKCδ+ staining. Another possible explanation involves potentiation of peripheral neuronal and receptor activation changes, which has been noted to occur post-operatively, and may involve changes in nociceptors due to kindling-mediated sensitization. Although more targeted drug-mediated antagonist based studies would be required to further elucidate the exact receptor mediating peripheral nociceptor changes.

The decrease in nociception could also have been due to previous exposures to pain, such as the previous formalin injection received during the F-CPA task, or their electrode implantation surgery. The increased pain threshold on the formalin test day may have been influenced by the formalin-paired conditioning that occurred during the F-CPA test. While we are unsure whether signs of inflammation such as redness, edema, or enlargement of the paw were observed in the F-CPA injected paw on the formalin test day, some studies suggest that a formalin injection of 5% can induce a hyperalgesia that increases in the 1 to 3 days following injection and can persist for up to 4 weeks (Fu et al., 2001; Zhang et al., 2007). This could make it difficult to distinguish between the effect of kindling, the first formalin injection, and the second formalin injection on the nociceptive behaviours observed in the formalin test. However, effects of kindling were likely still captured as both sham and kindling received the same pattern of injections. Therefore, while the effect of consecutive formalin injections within a short period of time should be considered, it is not a likely confound within this experiment.

Previous studies have also found that surgeries are able to produce post-operative pain that persisted for between 20 to 40 days (Xu & Brennan, 2011). In addition, studies that implemented conditioned place preference or avoidance paradigms which utilized surgery for cannula placement or tissue removal with a recovery period less than the minimum 20 days (Der-Avakian et al., 2005; Sakoori & Murphy, 2004). This indicates that testing may have occurred while pain sensitization due to surgery was still present, and were nonetheless able to produce their conditioned response. It is possible that the formalin nociceptive assay, which occurred 38 days post-surgery, was able to capture a

neuroplastic change in kindled rats. More specifically, that the amygdala kindling produced changes in neuronal activation that interacted with post-operative pain sensitization which produced a decreased nociception, or rather, a greater tolerance to pain in kindled rats than in sham rats. In order to confirm this theory, further testing on specific hyperalgesic or allodynic sensitivities would need to be conducted across several timepoints.

In summary, kindled rats showed less peripheral pain during the acute phase than sham rats, indicating a potential change in peripheral nociceptor activation due to kindling. However, these effects did not persist beyond phase I.

4.4 c-Fos activity

Ninety minutes following the injection of formalin for the formalin test of nociceptive behaviours, and 30 minutes following the end of observations for pain-related behaviours, rats were euthanized, perfused, and brains were collected for further immunohistology analysis. Brain sections were analyzed for the effect of kindling on c-Fos expression during formalin-induced nociceptive behaviours. Overall, no significant difference in positive c-Fos expression was found between kindled and sham rats in the BLA or the mPFC in response to the noxious stimuli. Interestingly, the formalin-induced nociceptive behaviours induced greater PAG c-Fos expression in sham than in kindled rats.

No significant differences in c-Fos activation were found in the BLA, CeA, or mPFC between all animal groups during the formalin test. These results are in complete

contrast to findings in literature, which indicate that an increase in BLA and CeA activation and decreased mPFC activation in negative affective behaviours (Ong et al., 2019; Jarrin et al., 2020; Gao et al., 2004; Xiao & Zhang, 2018; Kiritoshi & Neugebauer, 2018; Jhang et al., 2018). In accordance with the nociceptive pain scores recorded during the formalin test, as increased activation in the pathway between the mPFC and the BLA has been shown to produce increased pain (Gao et al., 2023), this indicates that a decrease in activity in the mPFC and BLA should be seen in kindled rats. In addition, as discussed previously, CeA activation has been implicated in changes of nociception seen during the formalin test, where increased activation results in increased pain and anxiety-related behaviour (Chou et al., 2022). Although no significant comparisons were found, this may have been due to the unique populations of inhibitory neurons that make up the CeA, such as the PKCδ+ and SOM+ expressing neurons. This suggests that changes seen in nociceptive pain behaviours during the formalin assay were not a result of these structures, and that the amygdala kindling may have produced neuroplastic changes beyond the initial site of stimulation.

The periaqueductal gray (PAG) is a key modulator in the sensation of pain and expression of threat-related defensive behaviours (Benarroch, 2008; Ho et al., 2018). It consists of inhibitory projections through the rostral ventromedial medulla and efferently to modulate peripheral nociceptors. It receives information relating to noxious stimuli through the spinothalamic pathway, while also exhibiting bi-directional relationships with higher cortical structures through the spinoreticular and other pathways, such as the mPFC and BLA (Benarroch, 2008). This is where the long-term amygdala kindling may

produce changes in c-Fos activation within the PAG, resulting in changes in pain experience. Interestingly, a lower positive c-Fos activation was found in the PAG due to the noxious stimuli in kindled than in sham rats. Current literature would suggest that a lower PAG activation results in decreased engagement in the pain modulation process, resulting in a increased unmodulated peripheral pain response (Gao et al., 2023; Mendes-Gomes et al., 2011). However, our kindled rats instead displayed less pain than the sham rats did during the formalin test, a counterintuitive result. This result could be better understood if our c-Fos activation was further divided into its ventrolateral (vlPAG) and dorsolateral (dlPAG) subregions, each activated during modulation of pain and defensive behaviours, respectively (Bourbia & Pertovaara, 2018; Lovick, 1993). With decreased ventrolateral activation increasing pain and decreased dorsolateral activation decreasing fear or defensive behaviour. If majority of the kindled activation is due to vlPAG activation, producing the decreased pain, and sham rat activation consists of increased dlPAG activation with minimal vlPAG activation, this could explain the role of the PAG in the resulting difference of formalin assay pain behaviour.

In sum, the c-Fos expression patterns found indicate that decreased PAG activation was associated with decreased phase I nociceptive responses observed in the kindled rats when compared to sham rats, but were insufficient to produce changes in phase II. However, long-term amygdala kindling did not produce changes in the CeA, BLA, or mPFC in response to behavioural responses to noxious stimuli. Conversely, the interpretability of these results may be confounded by the reliability of c-Fos as an IEG (Aparicio et al., 2022). It is possible that the differences, or lack thereof, may be muddled

by the inability to ensure that all positive staining is not due to the additional marking of glial cells. In addition, the inability to stain inhibited neurons and information about the network of connections that the activated neurons participate in further complicate understanding neuronal activation. Crucially to the formalin test, is the inability to place c-Fos neuronal activation in temporal relevance to the exact biphasic pain response progression. Despite this, c-Fos allows enough specificity and activation to a wide range of behaviours that is beneficial in these initial investigations into the roles of these regions of interest in pain and epilepsy, directing further research. Within this experiment, confounding factors that could produce differences in c-Fos activation such as the use of anesthesia were kept the same between all animals. Even so, the patterns of c-Fos activity will require further analysis through markers of greater specificity and the chemogenetic or optogenetic investigation of neuron activity involved in order to fully elucidate the connectivity that contributes to these patterns.

4.5 PKC activity

Next, the effects of kindling on the activation of PKCδ, a protein involved in pain processing, and Egr1, an indicator of neuronal activity, during nociceptive behaviours in the formalin test were analyzed. More specifically, the CeA and PVH were analyzed due to their roles in anxiety-related behaviours and pain modulation.

First, no significant comparisons were found in PKCδ and Egr1 activation between kindled and sham rats due to formalin-induced nociception in the CeA. However, this may be in line with literature. The central amygdala consists of the lateral

(CeL) and medial (CeM) subdivisions (Janak & Tye, 2015; Pitkänen et al., 1997), which consists primarily of inhibitory GABA neurons projecting from the CeL to the CeM (Gilpin et al., 2016). The CeL is made up of two distinct populations of inhibitory GABAergic neurons, somatostatin (SOM) expressing and protein kinase C δ (PKCδ) expressing neurons (Wilson et al., 2019). The activation of SOM+ neurons has been shown to contribute to reduced nociception and becomes inactive in nerve injury models. In contrast, the activation of PKCδ+ neurons contributes to increased nociception and becomes sensitized in nerve injury models (Wilson et al., 2019). With this in mind, our lack of difference in PKCδ+ activation within the CeA may be in line with the decreased nociceptive behaviours in kindled rats. Considering the bidirectional connection between PKCδ+ expressing and SOM+ expressing neurons and their activation that works hand-in-hand to result in changes in nociception, further staining with SOM+ expression co-localized with an activity indicator, such as EGR1, would be required to obtain a complete picture of the CeA's role in nociceptive changes within the formalin assay.

In addition, no significant group comparisons were found in the percent of PKCδ positive (PKCδ+), Egr1 positive (Egr1+), and co-localized cells within the PVH. Studies show that different neuron populations within the PVH play unique roles in modulating the experience of pain (Li et al., 2023; Ji et al., 2024a; Ji et al., 2024b). Pain sensations could be attenuated if inhibitory GABAergic neurons are deactivated or if oxytocinergic neurons are activated, while pain could be magnified if PVH excitatory glutamatergic neurons are activated (Ji et al., 2024a; Ji et al., 2024b). Despite the possibility for these plastic changes in pain sensitization within kindling, our results did not reveal an effect.

In summary, patterns of PKCδ+ expression in the CeA may reflect the decrease in nociception seen in the formalin assay, however further investigation into the activation of SOM+ is necessary to obtain a complete picture. Contrastly, no significant differences were found within the PVH, suggesting that the PVH was not actively playing a role in pain attenuation during the formalin assay. It is also important to note that Egr1 was used as the IEG marker instead of c-Fos due to issues encountered during procurement. Initially, while it will be important to verify the cross-reliability of c-Fos and Egr1, the decision to use Egr1 in the fluorescence double-labelling brought minimal concerns, due to literature supporting comparable neuronal staining between the two options (Gallo et al., 2018).

4.6 Trends in male and female formalin-induced nociception

While this study was not statistically able to analyze difference in sex within kindling, differences between sex may still have influenced the results of this experiment. Indeed, some patterns did emerge between male and female rats within their groups. While no differences in sex were observed during the F-CPA test, female rats showed a pattern of greater pain than male rats persisting from the beginning (20 min) to the mid-tonic phase (30 min; see Appendix, Figure S2). Additionally, immunohistology showed greater c-Fos activation within the CeA in female rats (see Appendix, Figure S3), and lower PKCδ activation within the PVH in female kindled rats during the formalin test (see Appendix, Figure S4).

Several studies report a similar increase in the pain response of female rats in phase II of the formalin test as well as in models of fear conditioning, and visceral deep tissue pain (You et al., 2006; Traub & Ji, 2013; Archer, 1975), although studies more specific to kindling found significant difference between male and female pain (Harton et al., 2017; Wintink et al., 2003). Literature on the tonic phase II of the formalin test as engaging in central sensitization of the CNS neurons (Coderre et al., 1993; Dickenson and Sullivan, 1987). Compounding this are the increased CeA c-Fos activity and decreased PKCδ within the PVH, which corresponds to literature noting an increase in the CeA and a decrease within the PVH contributing to increased nociception and decreased pain modulation, respectively (Botta, 2015; Li et al., 2023). These trends suggest that differing neurophysiological changes in sensitization to pain can occur within male and female rats, however a more robust sample size will be necessary in order to fully elucidate true statistical differences.

4.7 Conclusion and Considerations

In this experiment, the long-term amygdala kindling model of epileptogenesis was used to investigate the changes in pain sensitivity by examining the changes in the negative affective and nociceptive responses to noxious stimuli. While formalin-induced conditioned place aversion was difficult to establish, amygdala kindling was able to potentiate the noxious-stimuli induced aversion enough to produce a significant learned aversion. Counterintuitively, during the formalin assay kindled rats showed lower nociception than sham rats. Further immunohistochemistry showed lower c-Fos

activation in the PAG, which was again counterintuitive to the respective lower nociception. Additional PKCδ+ activation found no significant differences suggesting an absence or decrease of pain potentiation, but necessitates further SOM+ activation to complete the picture.

Overall, while F-CPA results indicate that amygdala kindling potentiates pain sensitivity, our formalin assay in contrast indicates that amygdala kindling attenuates pain sensitivity. This suggests that amygdala kindling sensitizes affective and nociceptive components of pain pathways differently. Immunohistochemical data further complicates interpretations of our results as c-Fos activation within the PAG was found to be lower in kindled rats, usually indicating less pain modulation and more pain, than in sham rats. However, further investigations into the subregions may elucidate what the PAG activation indicates for amygdala kindling and nociception. Lastly, despite no differences in PKCδ+ expression found within the CeA and PVH, due to the unique neuronal populations within these structures, these patterns may be in line with current literature. In addition, further staining of PKCδ's counterpart, somatostatin, within the CeA can further support this theory.

Despite extensive research within the specific management of pain conditions and epilepsy conditions, less research has explored the unique overlap and extensive impact of these comorbid conditions. More specifically, little is understood about the neurophysiology behind the comorbidity of pain conditions within TLE and other epilepsy conditions. In addition, in this thesis amygdala kindling produced different effects on affective and nociceptive behavioural responses, reflected within the neuronal

activation data found. Similar to the increased rate of affective disorders, we were also able to show that the long-term amygdala kindling model is capable in producing changes in affective avoidance behaviour, without the detriments with extended kindling. Specifically, identifying it as a useful mediator between the too subtle changes in models with fewer stimulations, and the disruptive nature of spontaneous seizures and accompanying brain damage in greater stimulations. In addition, the long-term amygdala kindling model may also be capable of producing sensitization of peripheral nociceptors, reducing pain, highlighting a potential avenue for investigating epilepsy-related pain analgesia.

Importantly, this thesis was able to demonstrate the suitability of the long-term amygdala kindling model of epilepsy in investigating seizure-induced pain changes. We demonstrated that kindling can influence sensitization along affective and nociceptive pain pathways in ways that produce different pain experiences. Ultimately, our investigations have begun to shed light on the complexity of neurophysiological changes behind the pain experiences in people with epilepsy, opening doors for more targeted treatments and highlighting the importance of understanding this unique phenomenon.

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Appendix

Supplementary figures

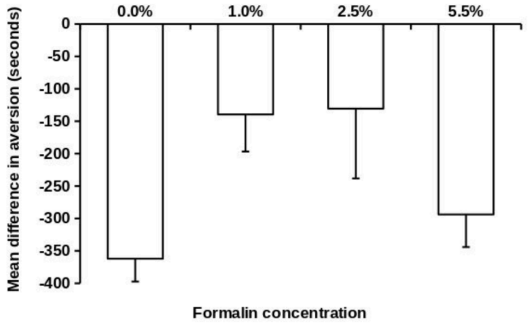

Figure S1. Pilot of varying doses of formalin in the formalin-induced conditioned place aversion test (n=5, per dose). Mean differences in aversion (seconds) are shown for each concentration by subtracting time spent in paired compartment on post-conditioning from pre-conditioning day. Saline doses (p<.001) and 5.5% produced aversion (p=.004), while other doses did not (p>.05). Data is shown in mean ± SEM.

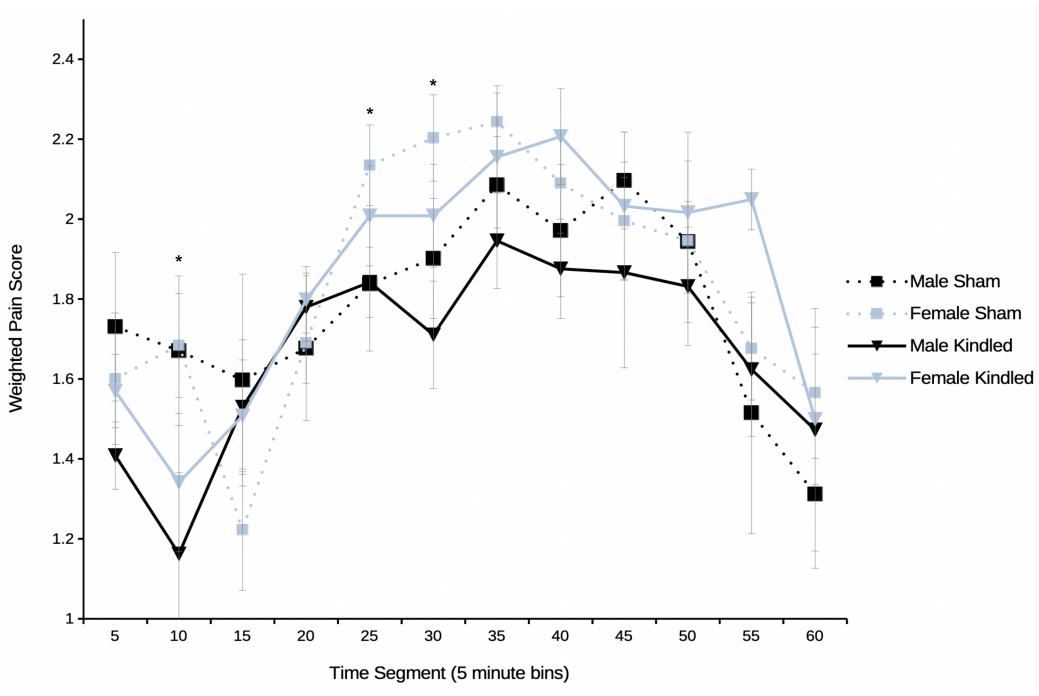

Figure S2. Formalin weighted pain assay test scores for each five minute segment shown across group and sex. Kindled rats (n=19) showed less pain than sham rats (n=16) at the 10 minute bin (p=.011), and male rats (n=16) showed less pain than female rats (n=19) during the 25 minute (p=.034) and 30 minute bins (p=.033). Data is shown in mean ± SEM. *P<0.05.

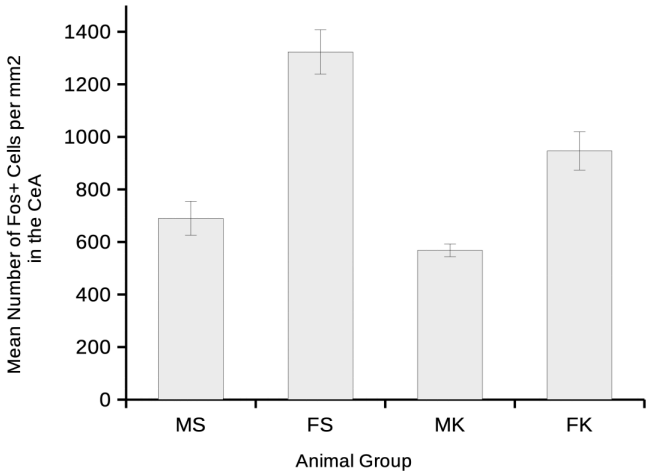

Figure S3. Positive c-Fos expression counts per mm² in the CeA across group and sex (Male sham, n=7, MS; Female sham, n=9, FS; Male kindled, n=8, MK; Female kindled, n=11, FK). Female rats (n=20) showed a greater expression than male rats (n=15) [F(1,31)=5.451, *p*=.026]. Data is shown in mean ± SEM.

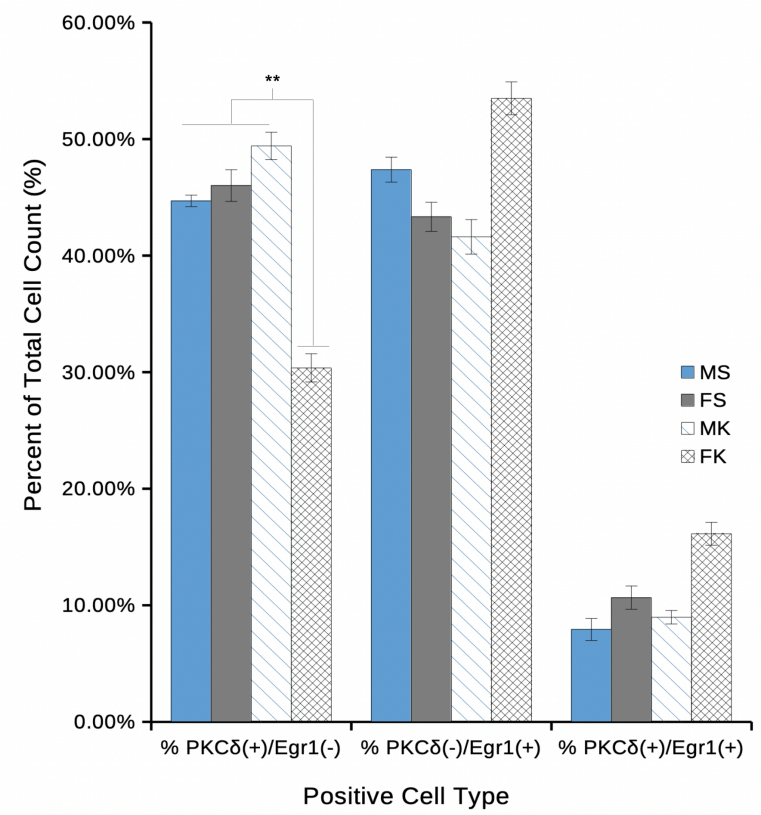

Figure S4. Percent positive PKCδ, Egr1, and Co-Localized Expression in the and PVH. Female kindled rats (n=9, FK) displayed a significantly lower %PKCδ(+)/Egr1(-) cells than male kindled (n=8, *p*=.0012, MK), male sham (n=7, *p*=.0226, MS) and female sham rats (n=9, *p*=.0086, FS). Data is shown in mean ± SEM. ***P*<.01.