Affective Pain Hypersensitivity in the Amygdala Kindling Model of Temporal Lobe Epilepsy

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ABSTRACT

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To examine comorbid pain sensitivity, temporal lobe epilepsy was modeled with a 42-stimulation amygdala kindling paradigm using rats. Sham and kindled rats' mechanical allodynia was not different before the formalin conditioned place aversion (FCPA) test. FCPA behaviour was not different, but twenty-four hours later kindled rats showed mechanical allodynia. Thermal sensitivity 48 hours after FCPA was not different. A second experiment revealed no difference in pre- and interictal mechanical and thermal sensitivity. Kindled rats did display a higher frequency of pain behaviours in the formalin nociceptive test, and greater early growth response 1 expression in the anterior cingulate cortex (ACC). The final experiment examined FCPA behaviour of sham and kindled rats given an ACC infusion of control (EGFP), or inhibitory designer receptor exclusively activated by designer drug (hM4Di). Kindled-EGFP rats did not spend a different amount of time in either compartment but Kindled-hM4Di rats spent more time in the formalin-paired compartment.

Keywords: amygdala, anterior cingulate cortex, amygdala kindling, affective pain, physical pain, formalin, von Frey, Hargreaves, formalin conditioned place aversion, formalin nociceptive test

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Figure 8. Sham (N = 5) and Kindled rats (N = 4) did not show a significant difference in paw withdrawal at baseline or 1 day and 1 week after the formalin test when the force (in grams) necessary to elicit withdrawal of the hindpaw in the von Frey test (A) or the latency to withdraw from the thermal stimulus in the Hargreaves test (B) was measured. Data is represented as the Mean ± SEM.

Figure 9. (A) Kindled rats (N = 4) displayed a significant difference in the frequency of behaviour in pain categories 1-3 and (B) total pain intensity score than Shams (N = 5). Data is represented as the Mean ± SEM.

Figure 10. A greater total number of EGR1+ cells per mm2 were found in the ACC of Kindled (N = 4) than Sham rats (N = 5) (A). Comparison of the number of EGR1+ Cells per mm2 in the rostral and caudal ACC showed greater expression in the caudal ACC than rostral ACC of kindled rats but no difference between kindled and sham rats (B). Data is represented as the Mean ± SEM.

Figure 11. All rats underwent electrode placement surgery in preparation for amygdala kindling and infusion of the ACC with EGFP or hM4Di. After a recovery period, rats received either 42 kindling or sham stimulations over 14 days. Rats then started the formalin conditioned place aversion test 24 hours after the last stimulation which occurred between day 15 to 21.

Figure 12. Locomotor activity was not significantly different between Sham-EGFP (n = 6), Kindled-EGFP (N = 6), and Kindled-hM4Di (N = 4) rats as no difference was observed in total distance traveled in the FCPA apparatus (A). The time spent in the formalin-paired and non-paired compartments of the FCPA test reflected that sham-EGFP spent significantly more time in the non-paired compartment compared to the formalin-paired compartment. The opposite was shown for Kindled-hM4Di rats which spent more time in the formalin-paired compartment but kindled-EGFP rats showed no difference in choice of compartment (B). A conditioned preference score was calculated reflecting preference for the aversive context, surprisingly when compared to the other rats Kindled-hM4Di rats spent more time in the formalin-paired compartment after conditioning than the same compartment during habituation (C). No significant difference was found between Sham- or Kindled-EGFP rats CPA scores. Data is represented as the Mean ± SEM.

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ACC	anterior cingulate cortex
AD	after discharge
BLA	basolateral amygdala
cACC	caudal anterior cingulate cortex
CAMKII	calcium/calmodulin regulated kinase II
CA1	cornu ammonis 1
CeA	central amygdala
CNO	clozapine-N-oxide dihydrochloride
CPA	conditioned place aversion
DAB	3,3'-diaminobenzidine
DREADD	designer receptor exclusively activated by designer drug
EGR1	early growth response 1
FCPA	formalin conditioned place aversion
fMRI	functional magnetic resonance imaging
FNT	formalin nociceptive test
GABA	γ-amino butyric acid
Glu	glutamate
IEGs	immediate early genes
IQ	intelligence quotients
MOR	mu-opioid receptors
NMDA	*N*-methyl-D-aspartate
PWT	paw withdrawal threshold

PB	parabrachial nucleus
PBS	phosphate buffered saline
p-ERK	phosphorylated-extracellular signal regulated kinase
PKCδ	protein kinase C-delta
rACC	rostral anterior cingulate cortex
rCBF	regional cerebral blood flow
RSFC	resting state functional connectivity
SFG	superior frontal gyrus
Som	somatostatin
S1	primary somatosensory
TLE	temporal lobe epilepsy
VF	von Frey
Wistar/RAJ	Wistar albino Glaxo/Rijswijk

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CHAPTER 1

General Introduction

Mesial temporal lobe epilepsy (TLE) results from repeated seizure discharges that arise from the hippocampus and amygdala, structures that are involved in learning, memory, and emotion (Maillard et al., 2004; Rajmohan & Mohandas, 2007; Sah et al., 2003). Individuals with TLE often report comorbid cognitive and emotional impairments in addition to seizure activity (Gilmour et al., 2016; Ottman et al., 2011). These comorbid impairments can produce a significant impact on the person's quality of life that can even exceed the seizures themselves (Johnson et al., 2004). Comorbid pain with epilepsy has been investigated using insurance claims which revealed a higher prevalence of all pain related symptoms and conditions as well as opioid prescriptions for individuals with epilepsy than without (Wilner et al., 2016). Despite the prevalence of comorbid pain across health conditions and the involvement of limbic brain regions in mediating emotional (affective) pain responses, studies have only begun to address the relationship between pain and TLE (Cragg et al., 2018; Gilmour et al., 2016; Ottman et al., 2011; Rajmohan & Mohandas, 2007). As seizures localized to the amygdala have been shown to produce anxiety-like behaviour using the amygdala kindling model of TLE it is possible that another emotionality change occurs in which negative affective pain-like behaviour is heightened (Fournier et al., 2020; Kalynchuk et al., 1997; Maillard et al., 2004; Melzack & Casey 1968; Price, 2000).

In this thesis, I will examine the development of enhanced physical and affective pain-related behaviours after amygdala kindling in rats. In the following sections, I will first review the major nociceptive pathways of the brain with emphasis placed on discussing the neural circuits important for encoding and evaluation of physical and emotional pain in humans and

animals. Next, I will broadly present the clinical neurobiology of TLE and associated behavioural comorbidities and discuss the relationship between seizures and pain. I will present evidence of the shared overlap between neural circuits impacted by kindling that contribute to enhanced emotionality and those involved with pain processing. Finally, I will conclude this section with a discussion of the Objectives of my thesis research and the specific hypotheses.

1. Neurobiology of Somatosensory and Affective Pain

As defined by the International Association for the Study of Pain, pain is considered an "unpleasant sensory and emotional experience associated with actual or impending tissue damage or described in terms of such damage" (Raja et al., 2020). Pain is an inevitable and inescapable part of the human experience. As a clinical syndrome, pain can arise from a variety of sources in human populations, from trauma to cancer to illnesses such as diabetes. Given the numerous distinct pathologies and mechanisms that can result in the emergence of diverse pain conditions within the human population, it is not surprising that chronic pain is major cause of human suffering with significant social and economic costs (Anderson et al., 2019; Moulin et al., 2002).

When a noxious stimulus is encountered, multiple pathways are activated that simultaneously produce an autonomic response, sensory response which results in a motor response to remove oneself from the experience and emotional response that provides motivation to avoid the stimulus and negative emotions (Gauriau & Bernard, 2002). The neural circuitry involved in the processing of pain information has been well characterized (Gauriau & Bernard, 2002; Lu et al., 2016; Melzack & Casey, 1968; Ong et al., 2019; Price, 2000; Singh et al., 2020). In general, noxious stimulation causes activation of peripheral Aδ- and C-fibers that enter the spinal cord via the dorsal roots which terminates mainly on large projection neurons within the

superficial (Rexed layers I and II) and deep (Rexed layer V) laminae of the dorsal horn. From here, the experience of pain is mediated by two distinct pathways. The first pathway, which is critical for encoding and detecting the physical features of a painful stimulus, such as its location, intensity, and quality, is related to the Aδ system and involves the crossed fibers of the ascending lateral spinothalamic tract which terminates in the ventral posterior thalamus. From the thalamus, information is sent to the primary somatosensory (S1) cortex to drive the sensory-discriminative aspects of pain. The other pathway is associated with the C fiber system and provides the basis for the affective and visceral components of pain. From the spinal cord, this pathway projects rostrally and bilaterally to diffusely innervate the posterior and intralaminar thalamic nuclei, which engages the anterior cingulate cortex, brain stem nuclei, and limbic areas hence contributing to the emotional and aversive experience of pain.

2. The Role of the Amygdala in Pain Modulation and Pain-Related Behaviours

Pain includes a strong emotional component. The affective dimension of pain includes the motivation to reduce the experience of pain (Melzack & Casey, 1968; Price, 2000). However, persistent periods of pain can be maladaptive and have been shown to be significantly associated with depression and anxiety disorders. Indeed, depressed individuals – who are naturally shifted towards experiencing and exhibiting greater negative emotions – are twice as likely to develop chronic pain compared with nondepressed people. Although much progress has been made in uncovering neural mechanisms that underlie the sensory detection of noxious stimuli and the processing of nociceptive information at the spinal level, there is still uncertainty regarding how supraspinal circuits transform this sensory information into the emotional experience of pain.

The amygdala is an almond-shaped brain area located deep within the mesial temporal lobes. It consists of a set of anatomically and functionally distinct nuclei involved in the assignment

of emotional significance (either positive or negative) to environmental information (Janak & Tye, 2015). A large body of literature in animals and humans have highlighted a pivotal role of the amygdala in emotional learning and memory as well as in regulating autonomic and stress-related functions (Herman et al., 2005; LeDoux, 1994; Maren, 2001; McGaugh et al., 2002, Phelps, 2004; Phelps & LeDoux, 2005; Roozendaal et al., 2009). Moreover, increasing evidence has further linked amygdalar dysfunction in the etiology of various neuropsychiatric conditions, including anxiety, depression, and addiction (Kilts, 2001; Rauch et al., 2003; Whalen, 2002).

Given the importance of the amygdala as a key neural substrate in mediating negative affective states, it should not be surprising that it also plays a prominent role in modulating the affective-motivational and cognitive dimensions of pain (Phelps & LeDoux, 2005). In fact, many studies have shown that amygdaloid neurons are preferentially activated by various types of somatic, chemical, and visceral noxious stimuli (Veinante, Yalcin, & Barrot, 2013). Decreasing amygdala activity through either lesion or pharmacological inactivation inhibits pain-related behaviours in several different animal models (Han & Neugebauer, 2005; Pedersen et al., 2007; Fu et al., 2008; Herbert et al., 1999). Importantly, increasing amygdala activity exacerbates ongoing pain as well as generate novel pain responses in the absence of any tissue injury or pathology (Carrasquillo & Gereau, 2007; Da Silva et al., 2019; Han et al., 2010; Myers & Greenwood-Van Meerveld, 2010, Ji et al., 2013; Lico et al., 1974). Additionally, enhanced functional connectivity between the amygdala and sensorimotor cortical regions was shown to predict greater experiences of pain unpleasantness during an evoked-negative emotional state in human participants (Gandhi et al., 2020). This suggests that intrinsically higher than normal levels of amygdala activity, particularly in response to negative affective stimuli, could be a significant risk factor for the development of maladaptive emotional responses to pain. Indeed, it has been

well documented that amygdala hyperactivity occurs across multiple chronic pain conditions in humans, including irritable bowel syndrome, arthritis, and mononeuropathy raising the possibility that early amygdalar dysfunction might contribute to the transition from acute to chronic pain-related symptoms and behaviours after injury or trauma (Bonaz et al., 2002; Kulkarni et al., 2007; Petrovic et al., 1999; Qi et al., 2016; Simons et al., 2014).

2.1 Organization of the Amygdala

The amygdala is a functionally heterogenous structure comprised of dozens of nuclei that include the lateral, basolateral, and central nuclei (Pitkänen et al., 2000). While many studies support that the amygdala is a critical junction for the integration of emotional and nociceptive information, the contribution of specific amygdaloid nuclei to the emotional-affective dimension of pain is still being elucidated. Figure 1 illustrates a non-exhaustive summary of the transmission of information through the major nuclei of the amygdala including interactions it has with structures in the physical (black) and emotional (red) pain pathway.

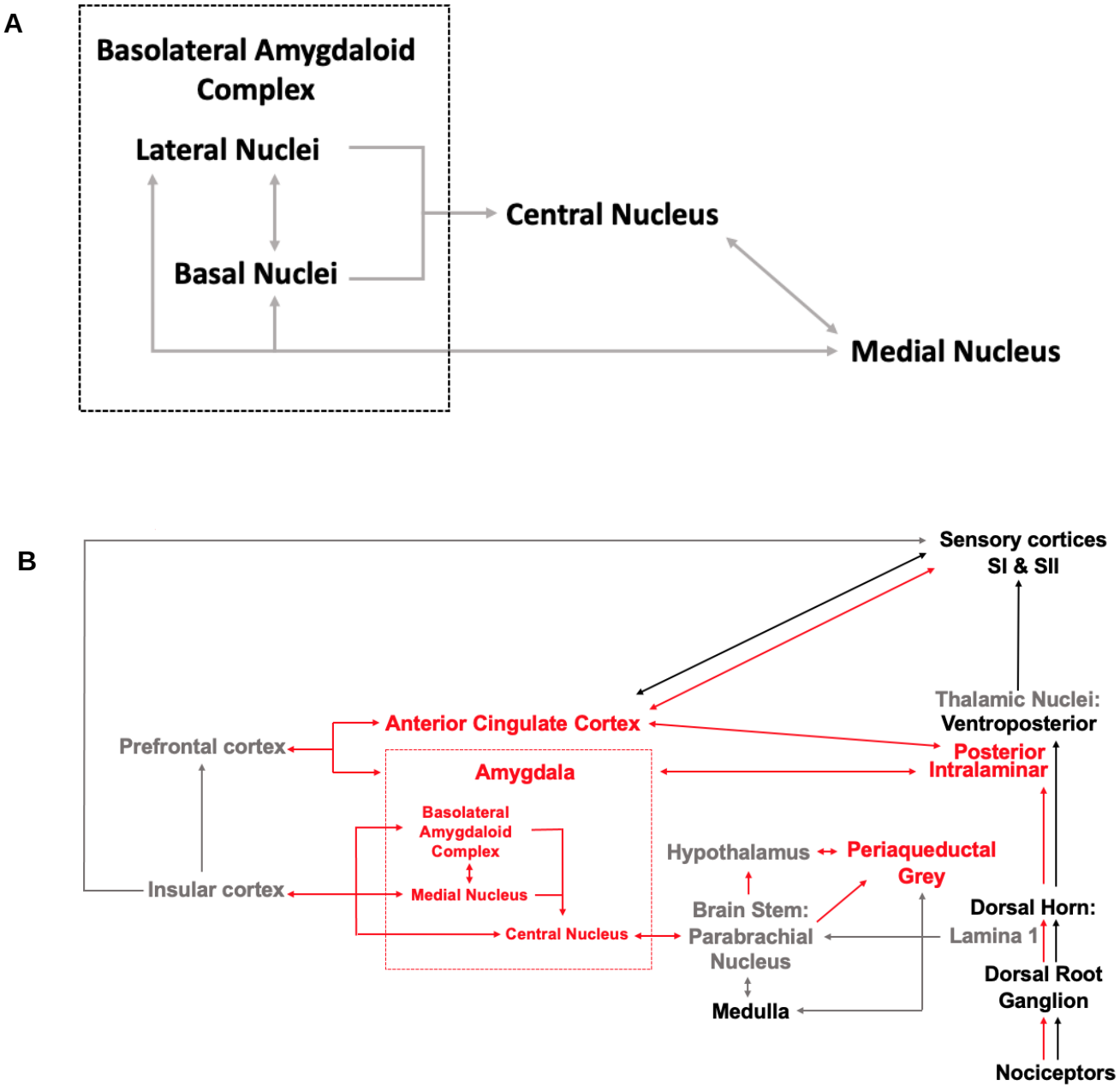

Figure *1*. A non-exhaustive (A) summary of the transmission of information through the major nuclei of the amygdala and (B) interactions of the amygdala with structures in the physical (black) and emotional (red) pain pathway. In the pain pathway diagram, structures involved in either pain pathway are grey.

The lateral and basolateral nuclei, collectively form the basolateral amygdala complex (BLA). The BLA complex is considered the main entry point of the amygdala and receives polymodal sensory and nociceptive information from various thalamic and cortical areas, such as the insular and sensory cortices, anterior cingulate cortex, and medial prefrontal areas. Projections of the BLA to the medial prefrontal cortex and anterior cingulate cortex are thought to be important in providing emotion- and value-based information which can help to guide behaviours associated with decision-making and behavioural control in response to pain (Holland & Gallagher, 2004; Laviolette & Grace, 2006). In rats, bilateral lesions of the BLA block the development of chronic neuropathic pain after spare nerve ligation as well as abolish formalin-induced place aversion learning (Li et al., 2013; Tanimoto et al., 2003). Furthermore, in an arthritic rodent model, chronic arthritic pain causes an enhancement of glutamatergic synaptic transmission in the BLA (Ren & Neugebauer, 2010). Additionally, neuroimaging studies in humans have found similar evidence of BLA hyperactivity and altered functional connectivity within the BLA that is associated with the onset of chronic pain (Cai et al., 2018; Simons et al., 2014). In addition to this, accumulating evidence also suggests that BLA neurons are important for encoding the negative affective qualities as well as the perceived aversiveness of painful stimuli. For example, Corder and colleagues (2019) recently showed that chemogenetic inhibition of BLA neurons reduced both attending and escape-related behaviours to mechanical and thermal noxious stimuli but had no effect on behaviours associated with sensory detection and withdrawal. Interestingly, similar findings have been observed in humans. For instance, patients with Urbach-Wiethe disease (a rare congenital lipoid proteinosis disorder characterized by bilateral amygdala calcification) that show selective BLA damage demonstrate intact detection and discrimination of both noxious and non-noxious stimuli, but report diminished experiences of unpleasantness and occurrences of protective

behaviours to noxious stimuli (Koen et al., 2016). These findings suggest that BLA neurons may be important in shaping pain experiences by providing an evaluation of nociceptive information that in turn triggers the occurrence of avoidant and protective responses associated with pain.

It is believed that associative processing within BLA networks attaches emotional significance to incoming sensory inputs (LeDoux, 2007; Paré, 2003). This highly processed affective and cognitive polymodal information from the BLA is then sent to the central amygdala (CeA), the main output nucleus of the amygdala, which in turn regulates behavioural responses through interactions with various autonomic and modulatory centers of the hypothalamus and the brainstem (Bourgeais et al., 2001). A second pathway (i.e., the spino-parabrachio-amygdaloid pathway), sends direct and raw nociceptive information from the spinal cord to the pontine parabrachial nucleus (PB) and from there directly to the CeA (Bernard et al., 1990; Bernard & Besson, 1990). Based on the input and output projections of the CeA, this brain region has received increasing attention as a "nociceptive center" that is ideally positioned to link experience and emotional states with behavioural responses to painful stimuli in both normal and pathological states (Davis & Whalen, 2001; Neugebauer et al., 2004; Veinante et al., 2013). Consistent with the critical function of the CeA in pain modulation, recent studies have shown pain-related plasticity in this brain region promotes hypersensitivity in pathological states (Dworsky-Fried et al., 2022; Liang et al., 2020; Louwies et al., 2021; Min et al., 2011; Wilson et al., 2019; Xie et al., 2017). PB efferents to the CeA involved in the transmission of nociceptive pain have also been implicated in learning and memory of pain inducing stimuli including changes in defense behaviour (Han et al., 2015). Taken together, these findings suggest that maladaptive changes in the CeA can induce persistent hypersensitivity as well as alterations in affective behaviours, which are all commonly comorbid in chronic pain conditions in both humans and rodents.

3. The Role of the ACC in Pain Modulation and Pain-Related Behaviours

Accumulating evidence suggests that the anterior cingulate cortex (ACC) is a critical brain region in the modulation of pain states (Fuchs et al., 2014). Early reports indicated that surgical ablation of the ACC and surrounding tissue decreased pain-related unpleasantness without affecting the subject's ability to discriminate the intensity or localization of a painful stimulus. Indeed, cingulotomy has been performed with success for the treatment of several intractable pain conditions, including cancer pain, lower back pain, and neuropathic pain (Ballintine et al., 1967; Boccard et al., 2014; Sherman, 1973). More recently, neuroimaging studies further implicate the ACC in pain processing (Hutchinson et al., 1999; Lenz et al., 1998; Vogt et al., 1996; Yuan et al., 2013). In one study, Tolle and colleagues (1999) measured ratings of unpleasantness and regional cerebral blood flow (rCBF) following a noxious thermal stimulus. They found that ratings of pain unpleasantness were positively correlated with increased rCBF in the left posterior ACC. Interestingly, the use of hypnotic suggestion to selectively decrease pain affect and unpleasantness prior to and during application of a noxious stimulation was found to change rCBF in the ACC but not in the somatosensory cortex (Rainville, 1997). Finally, training participants to decrease activity of the ACC using real-time functional magnetic resonance imaging (fMRI) feedback resulted in lower pain ratings in both healthy controls and chronic pain patients (Chapin et al., 2012). These findings suggest that the ACC is involved in the processing of pain-related affect but not sensory processing or localization of the noxious stimulus.

Behavioural studies in experimental animals further support a role for the ACC in mediating pain responses in several different pain models. The formalin test is one such model that results in a progressive and long-lasting hyperalgesia that is thought to closely mimic clinical pain (Coderre & Melzack, 1992; Dubuisson & Dennis, 1977). In this model, subcutaneous injection of

diluted formalin (1%-10%) to glabrous areas of the hind paw generates behavioural responses, such as paw favoring, paw elevation, and licking/biting/shaking of the paw (Abbott et al., 1995; Cao et al. 2009; Gaumond et al., 2002; Lei et al., 2004; Wheeler-Aceto et al., 1990). The occurrence of formalin-induced nociceptive behaviours occurs over two distinct phases: early (first) and late (second) phases. The early phase lasts approximately 5 to 10 minutes after injection and is associated with marked nociceptive responses. This phase is then followed by a quiescent interphase interval of 5-10 minutes, in which nociceptive behaviours are attenuated. Following this interphase, a late phase of responding can be observed for up to 60 to 90 minutes after injection and is associated with a gradual elevation in nociceptive behaviours (Coderre & Melzack, 1992). This biphasic response to formalin is prominent in both rats and mice and likely reflects an initial direct activation of nociceptive sensory afferents by formalin, which is then followed by afferent activation produced by inflammatory mediators following tissue injury and mechanisms of central sensitization (Coderre & Melzack, 1992). Electrophysiological recordings show that ACC neurons are active in response to noxious stimuli, including formalin (Yamamura et al., 1996). Lesions of the ACC have been shown to spare the formalin evoked nociceptive behaviours but disrupt the occurrence of pain-related aversive learning in rats (Gao et al., 2004; Johansen et al., 2001).

The formalin conditioned place aversion (FCPA) test uses an aversive conditioning paradigm in which rats receive an injection of formalin prior to confinement in one compartment and no injection or saline prior to confinement in another compartment (Johansen et al., 2001). Then on the final day of the test the time spent in the formalin paired and saline or non-paired compartments are recorded. A magnitude of conditioned place aversion (CPA) may also calculated by taking the difference between the time spent in the formalin paired compartment on test day and the same compartment during habituation. Therefore, avoidance behaviour in the FCPA test

reflects greater affective pain when rats exhibit a larger magnitude of CPA or more time spent in the safe compartment. Johansen and colleagues (2001) used the FCPA test to examine the role of the ACC in affective pain following lesions of the rostral ACC (rACC) and caudal ACC (cACC). They found that formalin evoked nociceptive behaviour was preserved when either region of the ACC was lesioned. However, only rACC lesioned rats showed reduced avoidance of the formalin paired compartment. Diminished pain-related affect but not nociceptive behaviour in the FCPA test following lesions of the ACC was also observed by Gao and colleagues (2004). These studies overwhelmingly suggest that the ACC may be manipulated to examine avoidance behaviour without impacting the physical pain response. Other studies have gone on to show that distinct neural populations, protein expression, and sex differences also influence affective pain measured by the FCPA test (Cao et al., 2014; Jarrin et al., 2020; Lei et al., 2004).

Across all studies avoidance behaviour in the FCPA test and activation of the downstream signaling pathways important for formalin-induced plasticity suggest the ACC is a vital structure in the affective pain response (Cao et al., 2014; Gao et al., 2004; Jarrin et al., 2020; Johansen et al., 2001; Lei et al., 2004). Combined with the evidence from Gao and colleagues (2004) suggesting affective pain is also influenced by the amygdala, this suggests that the relationship between the ACC and amygdala may be important for the affective pain response of individuals with TLE. Our discussion will turn to the structural changes associated with the development of pain sensitization which may explain the risk of developing comorbid pain with TLE.

4. Neuroplasticity of Pain and Pain Sensitization

It is now recognized that pain can be divided into three major types: nociceptive, neuropathic, or nociplastic pain. Nociceptive pain involves direct stimulation of peripheral nociceptors and is associated with actual or impending tissue injury or damage that causes pain.

It is generally acute in nature and resolves once the tissue heals or the noxious stimulus has ceased. Neuropathic pain can be caused by disease or injury to the peripheral nerves and can become chronic in nature. Nociplastic pain, is mechanically distinct from nociceptive pain, and does not involve obvious evidence of direct activation of peripheral nociceptors or clear evidence of disease or lesion of the somatosensory system causing the pain (International Association for the Study of Pain, 2017). The presence of nociplastic pain implies altered or augmented functions of pain-related pathways that give rise to pain hypersensitivity.

A number of neuroplastic changes in the spinothalamic tract have been suggested to promote nociplastic pain in both humans and animal models (Boadas-Vaello, et al., 2017; Latremoliere & Woolf, 2009; Melzack et al., 2006; Nijs et al., 2014; Petersen-Felix & Curatolo, 2002; Seifert & Maihöfner, 2011; Willis Jr, 2001). Studies using rats have shown that central sensitization results in increased activation of spinothalamic structures due to functional changes of neurons and microglial including increased receptor and signalling molecule activity (Ferrari et al., 2013; Ferrari et al., 2014; Ferrari et al., 2015; Hathway et al., 2009; Kawasaki et al., 2004; Woolf & Thompson, 1991; Wu et al., 2017; Zhou et al., 2019). However, hyperalgesia has also been shown to be promoted by inhibition of mu-opioid receptors involved in pain relief (Araldi et al., 2015). Moreover, inhibiting the neuroplastic changes associated with hyperalgesic priming such as nerve growth factor and protein kinase C expression inhibits hyperalgesia (Zhou et al., 2019). These neuroplastic changes underlying the development of nociceptive pain and hyperalgesia, resemble the pathological hyperexcitability shown during the development of epilepsy (Ong, 2020). To understand how pain sensitization may occur in individuals with epilepsy, we will review the anatomical and behavioural changes associated with a focus on temporal lobe epilepsy.

5. Temporal Lobe Epilepsy: Diagnosis and Characteristics

Epilepsy is a chronic neurological condition characterized by spontaneous seizures. It is one of the most frequent neurological disorders affecting about 50 million people worldwide (World Health Organization, 2019). It is estimated that over 260,000 Canadians have epilepsy (Mapping Connections: An Understanding of Neurological Conditions in Canada, 2018). In Ontario, epilepsy affects over 95,000 people with 30% living with uncontrolled seizures (https://epilepsyontario.org/). Diagnoses for epilepsy are based mainly on the localization of the seizure discharge and the type of seizure that occurs (Scheffer et al., 2017). Focal onset seizures are the most common type of seizures and refer to situations in which the abnormal electrical activity is normally localized to a discrete brain area within only one hemisphere affected. However, focal seizures can ultimately spread or generalize to involve both cerebral hemispheres, and in these situations, we often refer to the seizure events as focal seizures with secondary generalization. If awareness is impaired or affected at any time during the seizure, then it is called a focal impaired awareness seizure (originally complex partial seizure). Symptoms of focal seizures will depend on the area of the brain where the abnormal discharges are localized too (Scheffer et al., 2017).

Temporal lobe epilepsy is one of the most frequent types of adult-onset epilepsy and is characterized clinically by the presence of pathology as well as the development of spontaneous recurrent seizures originating from temporal lobe or limbic foci. TLE is commonly divided into *limbic* and *neocortical* forms (Ojemann, 2000). In neocortical TLE, the seizure focus is localized to the lateral temporal lobes, whereas in limbic or mesial TLE, the seizure focus is localized to either the hippocampus, amygdala, or both (Maillard et al., 2004).

TLE is also often associated by a unique pattern of neuropathological changes primarily involving the hippocampus and surrounding structures (often referred to as hippocampal sclerosis), in which gliosis and the loss of select hippocampal neurons and aberrant sprouting of axonal connections is believed to promote hyperexcitability and epileptogenesis (Houser, 1999; Isokawa et al., 1993; Mathern et al., 1994; Mathern et al., 1997). People who have TLE often show unusual changes in behaviour during ictal (period of seizure activity) or interictal (time between subsequent seizure) periods. Recurrent seizure discharges within the mesial temporal lobes are often accompanied by significant impairments in learning and memory as well as emotional regulation (Bragatti et al., 2010; de Oliveira et al., 2010; Helmstaeder et al., 2003; Johnson et al., 2004; Maillard et al., 2004; Ottman et al., 2011; Rajmohan & Mohandas, 2007; Tavakoli et al., 2011; Tellez-Zenteno et al., 2007; Weatherburn et al., 2017; Xu et al., 2018). The nature of these behavioural deficits suggests involvement of the hippocampus and the amygdala. The amygdala is most well recognized for its central role in emotional behaviour, as well as in the modulation of cognitive functions (Davis, 1994; LeDoux, 1992; McGaugh et al., 1996) In TLE, in addition to hippocampal damage, extensive neuropathology is also present in the amygdala in a significant subpopulation of patients. The most common pathology of the amygdala in TLE is atrophy (reduced volume associated with neuronal cell loss) (Coan et al., 2013b; Peedicail et al., 2020). Interestingly, the severity of amygdala atrophy does not appear to correlate with the frequency of seizures, the age of the patient, or the age of onset of epilepsy, but it appears to be associated with the chronicity of epilepsy (Bernasoconi et al., 2005; Guerreio et al., 1999; Kalviainen et al., 1997; Salmenpera et al., 2001). Histopathological examination from post-mortem or resected samples of patients with TLE have found that the regions of the amygdala that present with the most severe damage are the lateral and basal nuclei (Pitkänen et al., 1998). Similarly, in non-human primates

and rodent models of TLE, the basolateral portions of the amygdala are the most susceptible to seizure-induced damage (Pitkänen et al., 1998; Tuunanen et al., 1996). Since the amygdala modulates cognitive functions and plays a central role in emotional behaviour and affective disorders, then it should not be surprising that amygdala dysfunction in TLE is important not only for its role in the generation of seizures, but also for its role in the emotional and cognitive impairments that often accompany epilepsy.

6. The Amygdala Kindling Model of Temporal Lobe Epilepsy

Animal models of TLE have been used to study the morphological, physiological, and behavioural changes associated with seizure activity in the limbic system (Coulter et al., 2002). The development of animal models has greatly accelerated our understanding of the pathophysiology of TLE. Animal models have allowed us to systematically examine factors that contribute to the development and maintenance of a chronically epileptic state and to advance intervening methods that can alleviate the aversive consequences of seizure activity (Coulter et al., 2002). The primary goal of any animal model of epilepsy is to encompass the wide spectrum of clinical observations that represent the particular epileptic condition in humans. Considering the vast heterogeneity in clinical presentations of TLE, it is not surprising that several different experimental models have been advanced each with their own strengths and weaknesses in modeling the neurobiological complexity of TLE. For the sake of brevity, I will only concentrate my discussion to the electrical kindling model of epilepsy.

Kindling refers to the process whereby daily repetitive electrical stimulation of certain brain regions results in the gradual progression and intensification of motor seizures (Goddard et al., 1969). At first, the electrical stimulation produces little change in ongoing behaviour of the animal, but concurrent electrographic recordings reveal the presence of high amplitude, low

frequency epileptiform discharges occurring at the site of stimulation. This brief focal seizure event or after discharge (AD) shows little propagation to other brain sites. However, with repeated stimulation, the focal AD event increases in both duration and complexity and begins to spread throughout the expanding epileptogenic network culminating in the occurrence of a fully generalized motor convulsive seizure. The kindling model has been valuable for helping researchers investigate the cellular and molecular mechanisms that contribute to the development of epilepsy (e.g., epileptogenesis) as well as understand how seizures can affect cognitive and behavioural processes.

Studies in rodents have suggested that the amygdala may be one of the more epileptically prone regions of the brain (Goddard et al., 1969). Kindling proceeds much faster by repeated electrical stimulations of the amygdala than other limbic regions such as the hippocampus (McIntyre & Racine, 1986). In addition, kindling-induced interictal discharges tend to be first initiated within the amygdala and piriform cortex regardless of the kindling site (Racine et al., 1988). Furthermore, in studies involving exposure to the potent epileptogenic nerve agent soman, exposure to toxic levels of this nerve agent caused a rapid increase in levels of extracellular glutamate first in the amygdala before other limbic and cortical structures suggesting a critical involvement of the amygdala in the early development of soman-induced seizures (Lallement et al., 1991). Finally, with respect to the 10 functionally distinct nuclei that compose the amygdala, the basolateral nucleus appears to play the most important role in the initiation and spread of seizures. For example, prolonged electrical stimulation of the BLA appears to trigger status epilepticus and recurrent seizures more readily than application to other amygdaloid nuclei, such as the medial and central nuclei of the amygdala (Mohapel et al., 1996). Thus, both clinical findings and animal studies appear to support the idea that the amygdala plays a prominent role in the

symptoms and pathogenesis of epilepsy with the basolateral amygdala appearing to be most suspectable to generation of seizures.

6.1 Histopathological Changes Associated with Amygdala Kindling

Studies of the structural and functional changes involved in epileptogenesis during amygdala kindling have examined changes in volume, neuronal morphology, density, and neurotransmission (Aroniadou-Anderjaska et al., 2008; Löscher & Schwark, 1987; Morimoto et al., 1987; Morimoto, 1989; Rainnie et al., 1992; Tuunanen & Pitkänen, 2000; von Bohlen und Halbach et al., 2004). Depending on the number of stimulations and method used to quantify amygdalar histology, studies of the number of amygdalar neurons following amygdala kindling report mixed results (Tuunanen & Pitkänen, 2000; von Bohlen und Halbach et al., 2004). However, when morphology of the amygdala was examined, greater degeneration of axons was observed ipsilaterally in all regions except the medial amygdala for kindled rats compared to controls (von Bohlen und Halbach et al., 2004). From these studies it appears that structural changes observed in neuron density and axon morphology may occur with a greater number of motor seizures as 15 motor seizures induced pathology in the amygdala whereas 5 motor seizures did not (Tuunanen & Pitkänen, 2000; von Bohlen und Halbach et al., 2004).

Despite the degeneration of axons observed in the amygdala, studies of synaptic potentiation concentrations of neurotransmitters such as γ-amino butyric acid (GABA) and glutamate (Glu) in the amygdala support hyperexcitability following kindling (Aroniadou-Anderjaska et al., 2008; Löscher & Schwark, 1987; Morimoto et al., 1987; Morimoto, 1989; Rainnie et al., 1992). Concentrations of GABA and related molecules (i.e., GAD and [^{3}H] GABA binding protein) conducive to excitation of inhibitory neurons through $GABA_A$ receptors have been found to be lower in amygdala kindled rats than shams (Löscher & Schwark, 1987).

The result is a lack of excitation of GABAergic neurons leading to disinhibition of glutamatergic neurons in the BLA producing pathological excitatory transmission in the amygdaloid complex (Löscher & Schwark, 1987; Morimoto, 1989; Sah et al., 2003). Moreover, the glutamatergic response also supports excitability through *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors after kindling (Rainnie et al., 1992). In amygdala kindled rats, this hyperexcitability not only progresses seizure activity exemplified by evolution to motor seizures but also promotes comorbid conditions of TLE (Botterill et al., 2014; Botterill et al., 2015; Fournier et al., 2020; Goddard, 1969; Hannesson et al., 2008; Kalynchuk et al., 1997; Löscher & Schwark, 1987; Pinel & Rovner, 1978a; Racine, 1972).

7. Comorbidities in Epilepsy

In addition to experiencing seizures, a significant proportion of people diagnosed with epilepsy will also present with additional comorbid health conditions (Boro & Haut, 2003). The interictal period that occurs between seizure events is marked with an absence of seizure activity. However, for many individuals with epilepsy, the occurrence of these comorbidities can reduce their quality of life often to a greater extent than the seizure factors (i.e., frequency, duration) themselves (Johnson et al., 2004; Pulsipher et al., 2006).

The comorbidities of epilepsy encompass somatic, neurobehavioural, and psychiatric conditions, with over 50% of patients with active epilepsy presenting with at least one comorbidity within these three domains. Several larger population-based studies report that the prevalence of some of them may be up to eight times higher in people with epilepsy than in the general population (Seidenberg et al., 2009). Cognitive impairments and behavioural disturbances, such as memory loss, anxiety, and depression, are considered the most common

and devastating co-morbidities associated with epilepsy. Table 1 shows comorbidities of humans with TLE mapped to relevant behaviours in the amygdala kindling model.

Table 1

Comorbidities of Humans with Temporal Lobe Epilepsy (TLE) mapped to relevant behaviours in the amygdala kindling model

Comorbidity	Humans with TLE	Amygdala Kindling Model
Cognition		
	Visuospatial function	Learning (DFC)
	Executive function	Memory (DFC, TFC)
	Naming	
	Memory	
	Abstraction	
	Intelligence quotients (verbal, performance, full-scale)	
Emotionality		
	Depression	Increased or decreased locomotor activity (OF, EPM)
	Anxiety	Reduced Exploration of novel environments (OF)
	Mood Disorder	Aggression or defensive behaviour (RTC, SIT)
Medical		
Physiological	Cardiovascular	Cardiovascular
	Respiratory	Respiratory
Pain		
	Chronic pain	Post-ictal hypoalgesia
	Chronic headache disorders	
	Migraines	
	Neuropathic pain Fibromyalgia	
	Post-ictal Mechanical Threshold increase	

Note. DFC = Delay fear conditioning, TFC = Trace fear conditioning, OF = Open field, EPM = Elevated plus maze, RTC = Resistance to capture, SIT = Social interaction test

7.1 Cognition

Patients with mesial TLE have shown impairments in visuospatial and executive function, naming, abstraction, memory as well as reduced mean verbal, performance, and full-scale intelligence quotients (IQ) (Tavakoli et al., 2011; Xu et al., 2018). The ability to alleviate cognitive impairments using surgical and pharmacological treatment was found to be dependent on elimination of seizure activity (Helmstaeder et al., 2003). Helmstaeder and colleagues (2003) found that that while chronic mesial TLE is associated with an overall worsening of memory impairment, the progression of memory decline could be stopped or even reversed if seizures could be effectively controlled. Indeed, patients that continued to experience seizures despite surgical or medical treatment exhibited ongoing impaired cognitive performance and accelerated memory decline that was worsened if surgical intervention was performed on the left side (Helmstaeder et al., 2003).

A number of studies have been conducted using the amygdala kindling model of TLE to better understand how limbic pathology influences learning and memory. Spatial memory or learning deficits following amygdala kindling have been shown to occur in a variety of tasks with increasing numbers of seizures (Becker et al., 1992; Hannesson et al., 2008; Letty et al., 1995; Nieminen et al., 1992; Sherafat et al., 2013). Impairments in learning and memory across fear conditioning paradigms have also been shown (Botterill et al., 2014; Botterill et al., 2015; Fournier et al., 2013). The impairment in fear learning and memory is unsurprising due to the comorbid emotional changes observed in the amygdala kindling model and humans with TLE (Fournier et al., 2020; Kalynchuk et al., 1997; Kalynchuk et al., 1998; Kalynchuk et al., 1999; Tellez-Zenteno et al., 2007; Weatherburn et al., 2017).

7.2 Emotion

Individuals with epilepsy report a higher lifetime prevalence of comorbid major depression, mood disorder, and anxiety than the general population (Tellez-Zenteno et al., 2007; Weatherburn et al., 2017). The prevalence of psychiatric diagnoses is also specifically higher for people with TLE than the general population (Bragatti et al., 2010; de Oliveira et al., 2010). Approximately 25% of individuals with TLE report having depression and of the ~ 43% of individuals that reported a mood disorder just over half (57%) had concurrent depression (Bragatti et al., 2010). Mood disorder was also reported by approximately half of another sample of individuals with TLE (de Oliveira et al., 2010). Neuroimaging studies used to examine the structural and functional changes in individuals with TLE and comorbid depression, anxiety, affective aggression, or psychosis, showed changes in amygdalar volume and metabolic processes compared to those without psychiatric comorbidity or controls (Chen et al., 2012; Richardson et al., 2007; Tebartz van Elst, 2000; Yilmazer-Hanke, 2016).

The long-term amygdala kindling model has become a well-established model of comorbid anxiety and fear behaviour in TLE (Kalynchuk, 2000). Amygdala kindled rats display anxiety-like behaviour such as a lack of exploratory behaviour in open spaces during the open field test compared to controls (Fournier et al., 2020; Helfer et al., 1996; Kalynchuk et al., 1997; Kalynchuk et al., 1998). Despite both anxiolytic and anxiogenic behaviour reported in the elevated plus maze, amygdala kindled rats behaviour has been suggested by Fournier and colleagues (2020) to be reflective of greater negative affect shown by the increased locomotor activity contributing to more interaction with the open arms (Helfer et al., 1996; Kalynchuk et al., 1997; Kalynchuk et al., 1998). Heightened negative affect in amygdala kindled rats has also been shown during social situations between rats and during retrieval by researchers (Fournier et

al., 2020; Kalynchuk et al., 1997; Kalynchuk et al., 1998; Kalynchuk et al., 1999). In these situations, amygdala kindled rats respond with increased aggression or defensive behaviour (Fournier et al., 2020; Kalynchuk et al., 1999). The heightened fear and anxiety-like behaviour of amygdala kindled rats compared to controls was still present two months later, suggesting that changes in emotionality are present in the interictal period (Kalynchuk et al., 1998).

7.3 Medical Conditions

It has become increasingly recognized that people with epilepsy can also suffer from several other conditions, such as a higher prevalence of cardiovascular, respiratory, and chronic pain disorders than people without epilepsy (Boro & Haut, 2003; Elliott et al., 2009; Pulsipher et al., 2006). Indeed, patients with epilepsy report higher rates of chronic headache disorders, migraines, neuropathic pain, and fibromyalgia at a much higher frequency than the general population (Ottman et al., 2011; Pulsipher et al., 2006). Unfortunately, the number of medical comorbidities reported by individuals with TLE was found to increase the longer an individual has epilepsy, and as the number increases self-reported quality of life decreases (Pulsipher et al., 2006). Only a small number of studies have examined the autonomic response following amygdala kindling with most focusing on impairments produced in cardiovascular and respiratory system function (Pansani et al., 2021; Ruiz-Salinas, 2016; Totola et al., 2019).

Whereas comorbid impairments in cognitive, emotional, and autonomic functions are known to occur with epilepsy, they are frequently underdiagnosed and undertreated. The presence of comorbidities can influence treatment decision as often the choice of antiepileptic medication can either positively or negatively affect the comorbid condition. Although seizure freedom remains the primary goal of the treatment of epilepsy, comorbidities represent an important predictor for reduced quality-of-life. Thus, a better understanding of the neurobiological changes

that give rise to epilepsy-associated comorbidities will greatly improve our ability to successfully treat these conditions.

7.4 Post-ictal Pain and Nociceptive Sensitivity

Despite people with epilepsy reporting a greater number of pain conditions than people without epilepsy, changes in pain sensitivity as a comorbidity of TLE has only been examined in one study (Guieu et al., 1992; Ottman et al., 2011; Pulsipher et al., 2006). When measured at least 2 days after a seizure, individuals with TLE displayed an increased threshold for painful stimuli compared to controls (Guieu et al., 1992). The reflex response of the femoral biceps measured by electromyography following a brief electrical shock paradigm were initiated slower in both legs of individuals with TLE than controls (Guieu et al., 1992). Interestingly, this effect was not found for individuals with generalized tonic clonic epilepsy relative to controls (Guieu et al., 1992). Guieu and colleagues (1992) suggest that the higher nociceptive threshold for individuals with TLE is unlikely to be influenced by post-ictal hypoalgesia since the range in measurement included instances where it had been more than 7 days since a seizure event. Similarly, another study found that approximately half of people with epilepsy report the absence of feeling or physically responding to pain following the experience of a noxious stimulus in the ictal or post-ictal period. This was also true for people who experienced burn injuries in the ictal and post-ictal period, of whom 58% had TLE (Szűcs et al., 2015).

Similar to individuals with TLE, hypoalgesia is exhibited in the post-ictal period by amygdala kindled rats allowing the model to be used to understand seizure-induced changes in the pain response (Frenk & Yitzhaky, 1981). No studies have examined pain sensitivity of amygdala kindled rats during the interictal period, yet other models of epilepsy suggest that interictal pain sensitivity is enhanced compared to controls (Velioglu et al., 2017; Velioglu, et al., 2018). While

the studies employing alternative epilepsy models support the notion that pain and epilepsy can co-occur, the majority of work in amygdala kindled rats have not adequately addressed whether changes in pain sensitivity occur in the interictal period. Given the comorbid pain conditions reported by individuals with epilepsy and the similarities in neuroplasticity underlying kindling and pain, we aimed to study whether amygdala kindled rats' experience pain sensitivity during the interictal period.

8. Kindling As A Model of Pain Hypersensitivity

Many of the neurobiological processes involved in the development of chronic pain appear to share similarity to a kindling-like mechanism. First, continuous and repetitive nociceptive stimulation following specific injuries (e.g., spinal cord injuries, diabetic neuropathy, etc.) produce chronic pathophysiological changes in pain processing that result in symptoms worsening over time. During kindling, initially sub-optimal electrical stimulation also when applied in a repeated manner results in a progressive increase in seizure frequency and worsening of seizure-related cognitive and affective symptoms (Botterill et al., 2014; Fournier et al., 2013; Goddard, 1969; Kalynchuk et al., 1997; Pinel & Rovner, 1978a; Racine, 1972). Second, the development of chronic pain reflects the transition from an initial loss of neuronal activity due to a primary lesion (e.g., nerve or spinal cord injury) and gradual development of a state of hyperexcitability with ectopic discharges that arise from the disrupted neuronal network (Latremoliere & Woolf, 2009). These neuroplastic changes reduce pain threshold and sensitize pain pathways that contribute to the emergence of hyperalgesia and allodynia (Baliki et al., 2014). In a similar manner, kindling is also based upon a change and sensitization of the intrinsic properties of affected neurons, so that these neurons become more excitable and readily respond to both normal and sub-optimal synaptic inputs (Ong, 2020). Third, there is substantial overlap in the brain regions that are engaged by

ongoing pain and those that contribute to the behavioural effects associated with amygdala kindling. And finally, many different anticonvulsant medications also show efficacy for the treatment of pain-related disorders.

Evidence for using the amygdala kindling model to study pain sensitivity comes from the comorbid emotional changes induced by amygdala kindling, including impairments in fear conditioning and heightened fear and anxiety-like behaviours as a result of hyperexcitability in the hippocampus and amygdala (Botterill et al., 2014; Botterill et al., 2015; Kalynchuk et al., 1997; Kalynchuk et al., 1998; Kalynchuk et al., 1999; Ong, 2020). The structural and functional changes associated with this increase in fear-related emotions may enhance the pain response due to the involvement of the hippocampus and amygdala in pain pathways (Apkarian et al., 2016; Neugebauer et al., 2004; Ong, 2020; Price, 2000). Specifically, the hyperexcitability induced by amygdala kindling may increase motivational-affective behaviours relating to the emotional aspect of pain (Melzack & Casey, 1968; Price, 2000; Rome et al., 2001). The similarity between the negative emotional changes in kindling and pain hypersensitivity made amygdala kindling the ideal choice for studying the relationship between affective pain sensitivity and TLE.

9. Objectives

Affective pain has long been associated with the ACC which reciprocally connects to another structure implicated in emotionality, the amygdala (Melzack & Casey, 1968; Price, 2000; Rajmohan & Mohandas, 2007; Sah et al., 2003; Singh et al., 2020). Since a seizure focus may develop in the amygdala of some individuals with TLE, there is potential for epileptogenesis to influence negative affect in the ACC (Maillard et al., 2004; Melzack & Casey, 1968; Rajmohan & Mohandas, 2007; Sah et al., 2003). The amygdala kindling model allows for epileptogenesis and comorbidities of TLE to be studied using rats by establishing a seizure focus

in the amygdala via electrical stimulation and produces cognitive impairments, anxiety-like behaviour, and fear sensitization (Botterill et al., 2014; Botterill et al., 2015; Fournier et al., 2020; Goddard et al., 1969; Kalynchuk et al., 1997). This thesis uses the amygdala kindling model to investigate whether a comorbid increase in affective pain occurs in TLE.

The experiment in Chapter 2 examined whether amygdala-kindled rats display hypersensitization of the affective pain response in the FCPA test compared to non-kindled controls. It was hypothesized that interictal affective pain sensitization would result from amygdala kindling such that greater avoidance would be displayed by kindled rats compared to controls in the FCPA test shown by less time spent in the formalin-paired compartment. It was also hypothesized that amygdala kindled rats would also display mechanical and thermal pain sensitivity compared to controls in the von Frey and Hargreaves' tests respectively.

Another experiment in Chapter 3 addressed differences in pain sensitization between controls and amygdala kindled rats prior to kindling and in the interictal period. To assess changes in the pain response across time mechanical and thermal pain thresholds were measured prior to kindling, then again 24 hours and one week after the last stimulation. It was hypothesized that when mechanical and thermal thresholds were measured prior to kindling no differences in paw withdrawal threshold (PWT) or latency would occur between control and amygdala kindled rats but that kindled rats would withdraw sooner than controls when measured again 24 hours and a week after kindling.

Differences in activation of the ACC and affective behaviours during the formalin nociceptive test (FNT) were also addressed. It was hypothesized that during the FNT amygdala-kindled rats would display more elevation, licking or biting, and shaking or flinching of the injected paw than controls when injected with the inflammatory stimulus formalin.

Immunohistochemical analysis of early growth response 1 (EGR1) expression in the ACC was conducted as a relative indicator of neuronal activation which was hypothesized to be greater 90 minutes after FNT in amygdala kindled rats than controls (Imbe & Kimura, 2017; Ko et al., 2005).

The final experiment in Chapter 3 used a designer receptor exclusively activated by designer drug (DREADD) to inhibit the ACC and examine whether avoidance behaviour of amygdala kindled rats was eliminated during the FCPA test. It was hypothesized that kindled rats with an intra-ACC injection of EGFP would display greater avoidance of the formalin-paired compartment than Sham-EGFP, which would show more avoidance than kindled rats with an intra-ACC injection of inhibitory DREADD.

CHAPTER 2

Amygdala Kindling Impairs Formalin Conditioned Place Aversion Learning

1. Introduction

Support for pain as a multidimensional experience comes from multiple models that suggest pain to be the combination of physical and affective responses to stimuli (Melzack & Casey, 1968; Price, 2000; Price & Harkins, 1992). The physical response consists of reflexive pain-like behaviours including withdrawal from stimuli whereas the affective response consists of the emotional experience (Melzack & Casey 1968; Price, 2000). Price and Harkins' (1992) two-stage model of the affective-motivation dimension of pain describes pain modulation as the integration of both immediate and latent affect with sensory and cognitive processes. Sensitization of the negative affective emotions (i.e., unpleasantness, anxiety, fear) associated with pain may occur during TLE due to hyperexcitability of the amygdala and amygdalar networks as well as the high prevalence of comorbid emotionality impairments among people with TLE (Aroniadou-Anderjaska et al., 2008; Bragatti et al., 2010; de Oliveira et al., 2010; Löscher & Schwark, 1987; Maillard et al., 2004; Morimoto et al., 1987; Morimoto, 1989; Price & Harkins, 1992; Rainnie et al., 1992).

The relationship between pain and TLE has not been extensively studied but is supported by one indirect assessment of comorbid pain conditions reported by individuals with epilepsy (Ottman et al., 2011). The prevalence of chronic and neuropathic pain, fibromyalgia, and migraine headache was found to be 1.33 to 1.96 times higher for individuals with epilepsy than without epilepsy (Ottman et al., 2011). Recent works have also examined whether pain behaviours are altered in animal models of epilepsy (Coimbra et al. 2001; Frenk & Yitzhaky, 1981; Samineni et al., 2011; Velioglu et al., 2018). For example, audiogenic-evoked seizures or

acute PTZ-induced convulsions were found to elevate thermal pain thresholds during the post-ictal period, a finding that is consistent with the evidence of post-ictal analgesia (Coimbra et al., 2001; Samineni et al., 2011). Consistent with these findings, CeA kindled rats exhibit elevated thermal pain latencies during the tail-flick test during the post-ictal period after evocation of seizure (Frenk & Yitzhaky, 1981). These findings suggest that unlike in human clinical epilepsy, pain sensitivity may be elevated postictally in animals.

The post-ictal pain response has been the focus of studies investigating the relationship between epilepsy and pain, whereas limited attention has been given to pain in the interictal period. Although evidence suggests that post-ictal pain sensitivity may be influenced by the type of seizure experienced or the time since the seizure occurred (Coimbra et al., 2001; Frenk & Yitzhaky, 1981; Samineni et al., 2011). Indeed, reports of elevated hypersensitivity to thermal pain have been reported during ictal and interictal periods for WAG/Rij rats, a genetic model of generalized absence epilepsy (Velioglu et al., 2018).

The amygdala kindling model was used to assess the relationship between TLE and pain. Amygdala-kindled rats have been shown to exhibit heightened anxiety and fear responses in fear-conditioning and anxiety-like behaviour measures such as the open field test (Fournier et al., 2020; Hannesson et al., 2008; Kalynchuk et al., 1997). Kindling induced hyperexcitability of the amygdala that contributes to the changes in emotionality may lead to sensitization of the pain response due to the amygdala's connections with the parabrachial nucleus and ACC (Aroniadou-Anderjaska et al., 2008; Bernard et al., 1990; Bernard & Besson, 1990; Kalynchuk et al. 2000; Rajmohan & Mohandas, 2007; Sah et al., 2003; Singh et al., 2020) This is true for post-ictal thermal pain sensitization which has been shown in CeA kindled rats, but no studies have examined kindling induced pain sensitization in the interictal period (Frenk & Yitzhaky, 1981;

Yitzhaky et al., 1982). This experiment will measure mechanical and thermal thresholds as well as FCPA to characterize the influence of kindling on interictal pain sensitization.

2. Method

2.1 Animals

Sixteen male Long-Evans rats (Charles Rivers Laboratories; Montreal, Quebec, Canada), weighing approximately 190 – 230 grams at the beginning of experiment, were used in this study. All animals were paired housed until surgery after which they were single housed. All animals received ad *libitum* access to food and water throughout the duration of the study. The housing condition was at 20°C on a 12:12 hour light schedule with lights off at 1900 h. Figure 2 shows the general design and timeline for this study. All animals were treated in accordance with the Canadian Council for Animal Care and the Trent University Animal Care Committee.

2.2 Electrode Placement Surgery

All animals underwent stereotaxic surgery for implantation of a single bipolar electrode into the left basolateral amygdala using previously published methods (See Fournier et al. 2013, for a full discussion of the surgical procedures). Briefly each rat was anesthetized with isoflurane (5% induction, 2.5% maintenance) and an incision was made down the scalp so that overlying skin and fascia could be extracted. A single bipolar electrode was stereotaxically aimed at the left basolateral amygdala (-2.88 mm posterior to bregma, +5.0 mm medial/lateral to bregma, and -8.5 mm ventral from the surface of the skull, Paxinos & Watson, 2007) with the incisor bar set in the flat skull position. The electrode assembly was secured to the skull surface with four stainless steel screws and dental acrylic. After surgery, rats received an injection of saline (5 cc, s.c.), enrofloxacin (Baytril; 0.03 – 0.05 ml; 50 mg/kg, I.P.) and carprofen (5 mg/kg) to provide hydration, analgesia, and reduce the risk of infection and/or inflammation. Rats were monitored

in a warmed recovery cage for the display of full mobility before being returned to home cages. A topical antibiotic ointment (polymyxin B ointment, Polysporin ® Extra Strength) was applied to the incision site to promote healing. Rats received daily postoperative treatment of enrofloxacin and carprofen for 6 days before the onset of the experiment.

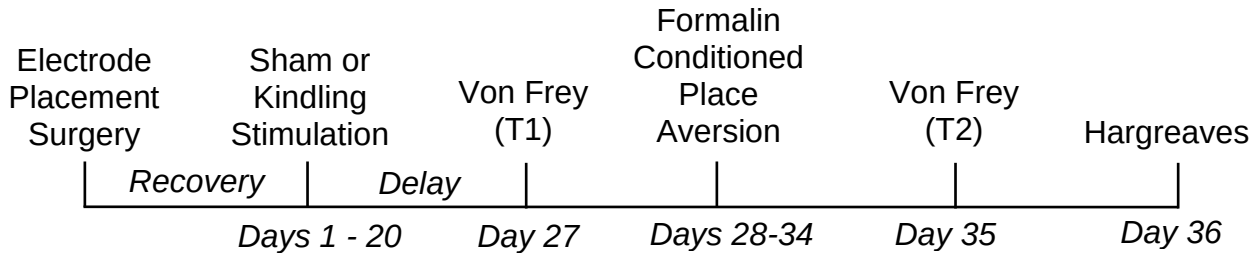

Figure 2. Rats received electrode placement surgery and were able to recover before the start of the amygdala kindling treatment. Twenty days of amygdala kindling consisting of sham or kindling stimulations were delivered until rats received 42 stimulations total. One week later, rats began testing with the Von Frey test. Twenty-four hours later rats began the 7-day formalin conditioned place aversion test consisting of two days of habituation, four days of conditioning with an aversive and safe stimulus in paired contexts, then a testing day. The next day, rats completed the von Frey test again. Finally, rats completed the Hargreaves test.

2.3 Amygdala Kindling Procedure

The rats were allowed at least 1 week to recover after surgery and were then randomly assigned into kindled (n=7) and sham (n=7) groups. All rats received a total of 42 stimulations. Three stimulations were delivered each day (5 days per week) with a minimum of 3-4 hours between consecutive stimulations. Prior to stimulation, the rats were placed into a glass container (51 cm X 25.7 cm X 27.5 cm) with commercial bedding, the wire lead attached, and the stimulation delivered. The electrical stimulation comprised of a 1 s, 60 Hz square-wave pulse with a peak-to-peak amplitude of 800 uA. For sham stimulations, rats were handled in the same manner and were connected to the wire lead, but no current was delivered.

The behavioural convulsion elicited after each stimulation was scored according to a modified criteria outlined by Pinel and Rovner (1978a). Stage 0 convulsions were classified as an arrest in behavioural mobility, Stage 1 convulsions were characterized by orofacial automatisms, Stage 2 convulsions were defined as orofacial automatisms with repeated head nodding, Stage 3 convulsions consisted of forelimb clonus and mastication and salivation, Stage 4 convulsions were associated with generalized convulsions consisting of rearing and unilateral tonic extension of the forelimb, Stage 5 convulsions involved rearing, bilateral clonus followed by loss of equilibrium (e.g., falling), and Stage 6 were associated with multiple bouts of Stage 5. To be considered kindled, rats must have had at least three consecutive stage 5 seizures over the course of the 42 stimulations. Kindled rats that did not reach this threshold were removed from analysis.

2.4 Behavioural Assessments

2.4.1 Mechanical allodynia (von Frey test)

The mechanical response threshold to a nociceptive stimulus was measured using an ascending, calibrated series of von Frey filaments, starting with an intermediate filament with a bending force of 4.31 g (filament size 10) for 6 seconds. This test was performed using the SUDO method of Bonin et al. (2014) with the range of filaments with bending forces from lowest to highest of: 3.84 g (filament size 7), 4.08 g (filament size 8), 4.17 g (filament size 9), 4.31 g (filament size 10), 4.56 g (filament size 11), 4.74 g (filament size 12), 4.93 g (filament size 13), and 5.07 g (filament size 14). Inter-paw (i.e., left-right) stimulation occurred immediately after one another. Intra-paw (i.e., left - left) stimulation occurred two minutes apart. Rats were placed in a clear cube with lid (approximately 20 cm x 20 cm x 14 cm) for a 30-minute habituation period prior to the first stimulation. At least one grey wall was inserted to separate the rat from the view of rats in adjacent cubes. A mesh stand (90 cm x 38 cm x 40 cm) served as the floor of the apparatus which was elevated approximately 55.90 cm on poles from the base to allow for application of filaments (Touch Test Sensory Evaluator; Exacta ™ Precision & Performance; North Coast Medical, Inc., Morgan Hill, Canada) to the dorsal surface of the hind paw. After the first 15 minutes of the habituation period elapsed, the experimenters entered the room to allow the rats to habituate to their presence before testing began. The apparatus was cleaned with Oxivir Five 16 concentrate solution (Johnson Diversey) before testing the next rat.

The mean force (in grams) applied by filaments that elicited withdrawal was calculated by taking a mean of the mean the forces (in grams) applied by filaments that elicited withdrawal in the left and right hind paw.

2.4.2 Thermal Hyperalgesia (Hargreaves' test)

Thermal pain sensitivity was measured during the Plantar test in which the plantar hindpaw is stimulated with radiant heat (Hargreaves Apparatus, Ugo Basile, Type 7370). Rats were placed into individual plastic cages with glass floors for 5 min before the experiment. A thermal stimulus was focused through the glass using an infrared light source onto the plantar surface of the rat's hindpaw until the subject lifted the paw away from the source. The paw withdrawal latency was automatically measured to the nearest 0.1 s. A cut-off latency of 20 s was used to avoid tissue damage. The time between intra-paw (i.e., left-left) stimulation was ten minutes whereas the time between inter-paw (i.e., left-right) stimulation was five minutes. A total of three trials were recorded for each foot. The apparatus was cleaned with Oxivir Five 16 concentrate solution (Johnson Diversey) before testing the next rat.

2.5 Formalin Conditioned Place Aversion Test

2.5.1 Habituation

An image of the apparatus is included in Figure 3. During day one and two rats were placed in the middle compartment (19 cm x 26 cm x 33 cm) of the apparatus that was white with a white solid plastic floor and the doors were closed for the first two minutes of testing. After two minutes, the doors were removed, and the rat was allowed to explore all three compartments of the apparatus for the remaining 13 minutes of the habituation session. One compartment (34 cm x 26 cm x 33 cm) had vertical striped walls, a wire square grid floor, and smelt of cinnamon scent (~30 ul of 100% pure cinnamon cassia (*Cinnamomum cassia*) essential oil per 1 L of deionized H_2O, Now Essential Oils; Now Foods, Bloomingdale, IL) whereas the other compartment (34 cm x 26 cm x 33 cm) had solid grey walls, a wire bar floor, and smelt of 1% acetic acid (99.7% diluted in deionized H_2O; ACP, Quebec, Canada). All compartments were

cleaned with 70% ethanol and new scents were placed in the bottom of the apparatus between each rat for all habituation, conditioning, and testing sessions.

To determine which compartment was preferred by the rats across the two habituation sessions, an experimenter examined the time spent in the striped or grey compartment and assigned the compartment with the greatest amount of time spent (i.e., preferred) across the two sessions to be paired with an injection of formalin during conditioning.

2.5.2 Conditioning

The conditioning paradigm occurred over four days during which rats received a formalin injection on an alternating schedule (ex., days one and three no injection administered, days two and four formalin injection administered). On the formalin-paired days, rats received a 5% formalin (37% Formaldehyde diluted in 0.9% Saline; 50 ul; 30-gauge needle) intraplantar hind paw injection then were immediately placed into the preferred compartment. On the second formalin-paired conditioning session, injections were given in the opposite hind paw than the first formalin-paired conditioning session. No injections were administered on days when rats were placed in the non-preferred compartment. Rats remained in the context for 60 minutes on all conditioning days.

2.5.3 Test

On the final day rats were returned to the middle compartment of the apparatus with the doors removed to allow them to move between compartments during the 15 minutes of the testing session. Rats were perfused on a later date, and brains were collected and stored in phosphate buffered saline (PBS) with 0.1% sodium azide at 4°C.

A webcam mounted on the ceiling in conjunction with AnyMaze (Version 6.0; Stoelting, Wood Dale, IL) was used to capture the movement of the animal in the apparatus and the time

spent in each compartment for each rat during the habituation, conditioning, and testing sessions was computed.

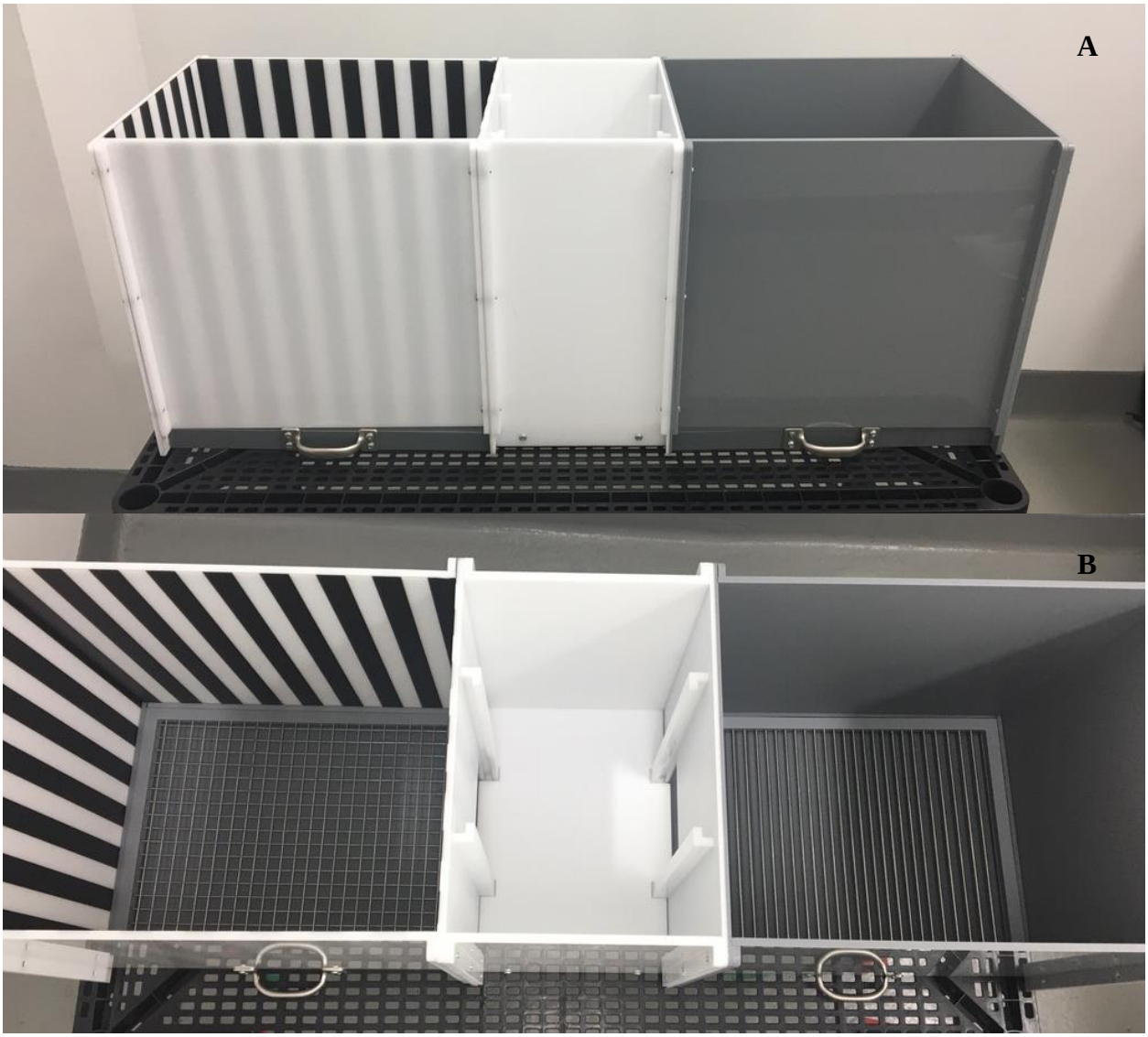

Figure 3. A frontal (A) and aerial (B) view of the formalin conditioned place aversion apparatus. The compartment with vertical stripes on the walls included a wire mesh grid floor and was cinnamon scented whereas the compartment with grey walls included a wire bar floor and was 1% acetic acid scented. Between each trail the entire apparatus was cleaned using 70% ethanol. Two removable sliding doors with surfaces matching the respective compartments were used to block access to adjacent compartments are not pictured.

2.6 Statistical Analysis

Behavioural data was analyzed using R-Studio. The mean number of stimulations required to evoke first Stage 5 or higher event as well as the total number of Stage 5 or higher events recorded during kindling was analyzed using Student t-tests. A two-way mixed measures analysis of variance (ANOVA) with test session (Time 1 and Time 2) as the within subject factor and group (sham vs. kindling) was used to analyze the change in mean force (in grams) applied to elicit withdrawal reflex at each time point of the von Frey test. For the thermal (Hargreaves) plantar test, the latency to withdraw the paw was recorded to the nearest second and was analyzed using a two-tailed Student t-test. A series of two-tailed paired t-tests and a one-way between subjects ANOVA was used to examine differences in time spent in the formalin-paired and non-paired compartments during the FCPA test. Finally, a CPA score was calculated for each animal by taking the difference of the time spent in the formalin-paired compartment during the test session with the time spent in this compartment on the second habituation day during the preconditioning stage. A negative value represents a greater aversion to the formalin-paired chamber. A two-tailed Student t-test was used to examine if there was a difference in CPA score. The criteria for statistical significance was set as $P < .05$. The data is presented as the mean and standard error of the mean (SEM) in all Figures.

3. Results

3.1 Kindling Characteristics

Two rats died due to complications during surgery and one rat from the kindled group was removed due to never developing stage 5 seizures over the 42 stimulations. Thus, the final number of rats in each group was: Sham (N=7) and Kindled (N=6). The mean number of stimulations required to evoke the first stage 5 seizure was 9.86 ($\pm$ 3.02) and the total number of stage 5 or higher events was 10.29 ($\pm$ 2.62).

3.2 Kindled Rats Showed A Delayed Enhancement of Mechanical But Not Thermal Pain Sensitivity

To evaluate whether kindling altered mechanical thresholds, we employed the von Frey test, in which a series of filaments of different forces were applied to the rat hind paws. The mechanical withdrawal threshold of the hind limbs was recorded and averaged across both hind paws. These tests (Time 1 and Time 2) were conducted 7 and 15 days after kindling was completed. The results of a two-way repeated measures ANOVA are presented in Figure 4a. There was a significant group by time interaction effect [$F(1,12)=4.46$, $P<.05$]. However, there was no significant main effect for time [$F(1,12)=3.97$, $P=.069$] or group [$F(1, 12)=.166$, $P=.691$]. The major source of the interaction was the decrease in mechanical thresholds at Time 2 compared to Time 1 for the kindled rats [$t(6)=2.57$, $P<.041$], but not for the non-kindled controls [$t(6)=-.09$, $P=.925$] suggesting a delayed development of mechanical allodynia after kindling.

Heat sensitivity was then examined in the Hargreaves' test by measuring the time it took for rats to withdrawal from radiant heat stimulation on the plantar-hind paw. In contrast to mechanical pain hypersensitivity, heat withdraw latency was not significantly different between the kindled and sham control rats ($t(12)=-.411$, $P=.822$, Figure 4b).

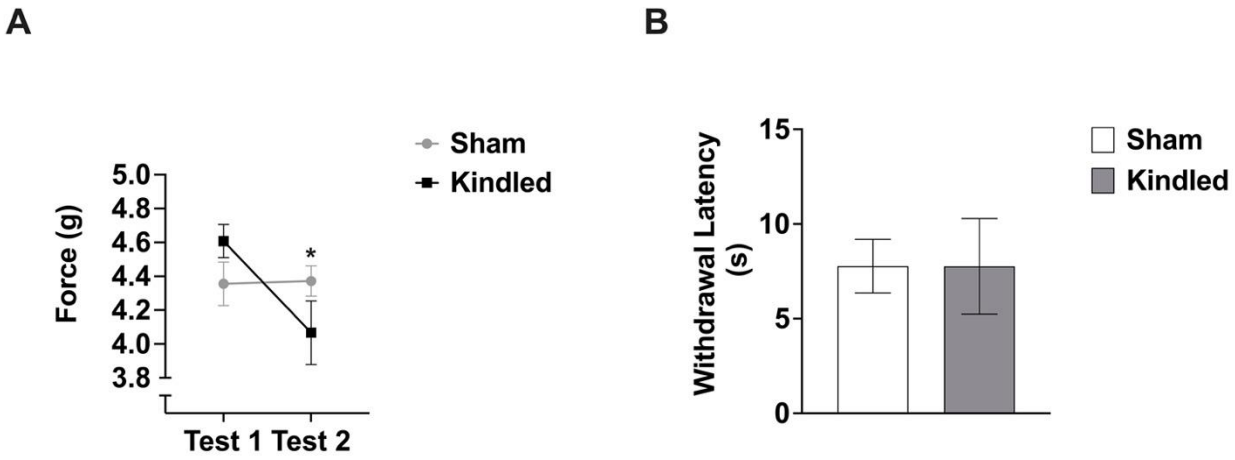

Figure 4. In the von Frey test of mechanical allodynia, Sham- (N = 6) and Kindled-rats (N = 7) showed a tendency toward a significant difference in the force (in grams) required to elicit the withdrawal response using the SUDO method (A). Sham- (N = 6) and Kindled- rats (N = 7) did not show a significant difference in withdrawal latency from a U.V. beam during Hargreaves' test of thermal hyperalgesia (B). Data is represented as the Mean ± SEM.

3.3 Kindled Rats Show Impaired Pain-related Aversion Behaviour

Amygdala kindling is known to enhance fear and anxiogenic responses in rats (Fournier et al., 2020; Kalynchuk et al., 1997). Given that fear and pain sensitization are mediated by in part by overlapping neural circuits, we set out to examine if kindling could affect pain-related aversion using the FCPA task (Al-Chalabi et al., 2019; Fournier et al., 2020; Gauriau & Bernard., 2002; Kalynchuk et al., 1997; Singh et al., 2020; Wilson et al., 2019). As shown in Figure 5, when formalin injection was paired with a particular compartment in the place conditioning apparatus, non-kindled rats spent significantly less time in the formalin (pain)-paired compartment compared to the non-paired compartment [paired t-test, $t(5)=4.36$, $P<.005$]. This difference was not found for the kindled group, which spent a similar amount of time in either compartment [paired t-test, $t(5)=1.95$, $P=.098$]. Next, CPA scores (time spent in the formalin-paired compartment on the post-conditioning day – the time spent in the formalin-paired compartment on the preconditioning day) were also computed to further examine the degree of aversive response to the formalin-conditioned compartment. While CPA scores of the non-kindled group were smaller than that of the kindled group, this difference was not statistically significant [$t(12)=-.812$, $P=.433$, Figure 6]. Taken together, these results could suggest that kindled rats exhibit some difficulty in learning to associate formalin-evoked pain with a particular context associated with noxious stimulation.

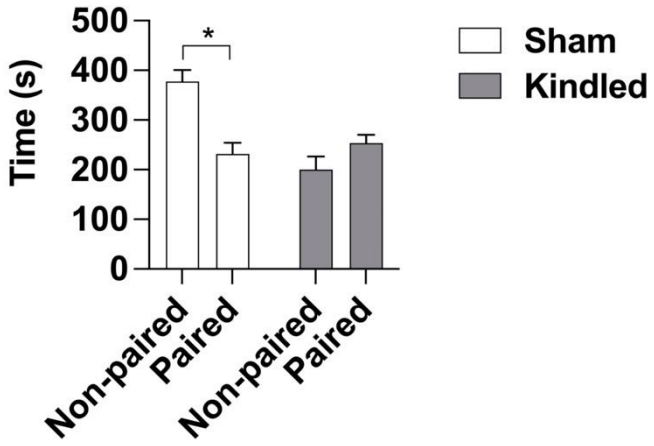

Figure 5. Sham- (N = 6) and Kindled- rats (N = 7) did not show a significant difference in time spent in the safe or aversive compartment of the formalin conditioned place aversion apparatus. Data is represented as the Mean ± SEM.

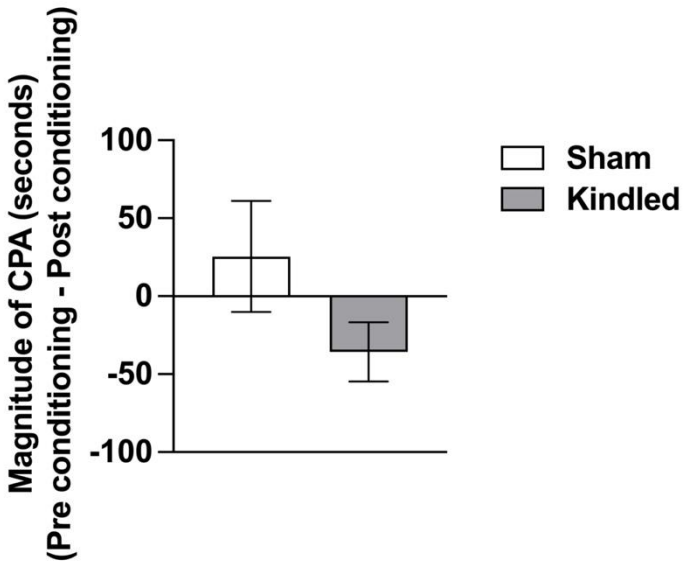

Figure 6. Sham- (N = 6) and Kindled- rats (N = 7) did not show a significant difference in the magnitude of conditioned place aversion following the formalin conditioned place aversion test. Data is represented as the Mean $\pm$ SEM.

4. Discussion

To examine the relationship between pain and TLE, pain sensitivity of amygdala kindled rats was measured in the interictal period. A 42-stimulation amygdala kindling paradigm localized to the ipsilateral left basolateral nucleus of the amygdaloid complex was used to produce the model of TLE. It was predicted that heightened mechanical and thermal pain as well as affective pain would be displayed by kindled rats compared to controls in the von Frey, Hargreaves, and FCPA tests, respectively.

During the first session of von Frey (i.e., Test 1) there was no significant difference in the applied force that sham and kindled rats withdrew from on average suggesting no difference in sensitivity to a mechanical stimulus. When re-tested twenty-four hours after the completion of FCPA (i.e., Test 2) kindled rats withdrew their hind-paws at a significantly smaller force applied than sham rats. The delay in hypersensitivity of the mechanical pain response in basolateral amygdala kindled rats may have been due to seizure-induced changes, as such other measurements throughout the interictal period are needed to determine the onset and/or persistence of hypersensitivity. Kindled rats' mechanical pain sensitivity may also be due to silent c-fibers, a nerve type that only become activated following an inflammatory stimulus (Dubin & Patapoutian, 2010). Isolating the contribution of silent c-fibers during the von Frey test may be challenging due to activation of these fibers by both mechanical and inflammatory stimuli but it could address their role in kindled rats' heightened mechanical sensitivity following inflammatory stimulation during the FCPA test.

Hargreaves was the last test to be conducted which showed no difference in sham and kindled rats' latency to withdrawal from a thermal stimulus in the interictal period. This is consistent with Frenk and Yitzhaky (1981), who examined the thermal threshold of CeA kindled

rats prior to experiencing a Stage 3 seizure event using a modified version of the tail-flick test. Thermal thresholds of kindled rats pre- and post-AD reflected an increase in pain tolerance or analgesia as the withdrawal latency increased compared to controls. Analgesia was also found to increase in magnitude as the number of stimulations (i.e., days 2 - 6) and magnitude of AD increased (Frenk & Yitzhaky, 1981). It is possible that the thermal analgesia observed in the post-ictal period does not persist into the interictal period, but future studies will need to assess this at different times points relative to the last stimulation. The lack of sensitivity to a thermal stimulus in the current study was surprising as mechanical sensitivity was observed twenty-four hours prior but may also support that the time course of seizure-induced sensitivity in the interictal period is different for thermal and mechanical pain.

On the test day of the FCPA test sham rats spent more time in the safe-paired (i.e., no injection) than the formalin-paired compartment but there was no difference in the amount of time spent in either compartment by kindled rats. The magnitude of CPA score likewise did not reveal any differences between kindled and sham rats. The limited difference in aversion of the formalin-paired compartment before and after conditioning could indicate a learning impairment in the kindled rats. In another aversive conditioning paradigm using the delay fear conditioning task, short- and long-term (i.e., 30 vs 99 stimulation) amygdala kindled rats displayed learning and memory impairments compared to controls (Botterill et al., 2014). Memory impairment in long-term kindled rats have also been observed in the trace fear conditioning task when compared to controls (Botterill et al., 2014; Botterill et al., 2015). This may suggest that kindled rats learning and/or memory of context-dependent aversion is impaired in the FCPA test such that the lack of aversion reflects inability to acquire or retrieve the formalin-paired context.

As the first study to examine pain sensitization of amygdala kindled rats, this experiment allowed us to gain knowledge of mechanical and thermal pain thresholds, and negative affective avoidance behaviour in a model of TLE. Pain thresholds and affective pain-like behaviours could have been influenced by the timeline of administration of the pain assays relative to the last seizure event. Affective pain measurement could also have been influenced by a learning impairment produced by the 42-stimulation amygdala kindling paradigm. Future studies measuring mechanical pain may choose to assess the threshold in the days surrounding the 15th day since the last stimulation in order to further characterize the pain response of amygdala kindled rats.

CHAPTER 3

Role of the Anterior Cingulate In Modulating Affective Pain After Amygdaloid Kindling

1. Introduction

The affective pain response is an important aspect of pain accounting for emotions experienced because of a noxious stimulus (Melzack & Casey, 1968; Price, 2000; Price & Harkins, 1992). Animal models of pain-like behaviour have allowed for affective pain to be studied using the formalin conditioned place aversion (FCPA) test which establishes aversion using association of a context with exposure to a noxious inflammatory stimulus (Gao et al., 2004; Johansen et al., 2001; Johansen & Fields, 2004). One structure that is proposed to influence the affective pain response is the anterior cingulate cortex (ACC), which has been shown to be activated following exposure to a noxious stimulus in humans (Hutchinson et al., 1999; Lenz et al., 1998; Vogt et al., 1996). Evidence for the modulation of affective pain by the ACC has also been shown in rat models in a variety of studies including those that used cell signaling manipulation, optogenetics, and lesioning techniques (Cao et al., 2014; Gao et al., 2004; Lou et al., 2015; Jarrin et al., 2020; Johansen et al., 2001; Tochiki et al., 2015).

Rat models have shown that the rostral and caudal divisions of the ACC play distinct roles in pain affect (Johansen et al., 2001; Malin et al., 2007). The rACC is implicated in emotional processes more so than the cACC which is implicated in cognition (Devinsky et al., 1995). Studies investigating affective pain after lesioning the entire ACC or the rACC found that rats displayed a reduction in avoidance behaviour in the FCPA test but no difference in formalin nociceptive behaviours compared to controls (Gao et al., 2004; Johansen et al., 2001). The rACC's connection to the BLA also plays an important role in retention of aversion conditioning as impairments were produced when either structure was lesioned while the other was excited (Malin et al., 2007). Therefore, activation of the ACC especially the rACC plays a role in

affective pain and contributes to the aversive learning paradigm (Johansen et al., 2001; Malin et al., 2007). Further analyses that isolated the effect of glutamatergic neurons in the ACC using optogenetics found the same trend in affective pain behaviour. Bilateral inhibition of Glu neurons in the ACC was shown to abolish avoidance behaviour in the FCPA test compared to controls whereas formalin nociceptive behaviours remained intact but presented differently between male and female rats at specific time points (Jarrin et al., 2020). Activation of Glu neurons in ACC have also been shown to be activated by NMDA activity during affective pain (Lei et al., 2004). Inhibition of NMDA eliminated avoidance of the formalin-paired compartment of the FCPA test and Fos expression in the ACC but not pain-like behaviours in response to a formalin injection (Lei et al., 2004). Given the pattern of pain-modulation by the ACC in Glu neurons and activation of NMDA receptors, the affective pain response of individuals with TLE may be influenced by seizure-induced changes in the limbic system and ACC.

Support for seizure-induced changes in the ACC that could influence pain-related affect comes from studies of individuals with TLE as well as rat models. Resting state functional connectivity (RSFC) of the ventral ACC bilaterally and dorsal ACC contralaterally in individuals with TLE showed a reduction compared to the same structures in controls (Jo et al., 2019). RSFC reduction was also found in several divisions of the cingulate cortex to the superior frontal gyrus (SFG) of individuals with right TLE, but an increase was observed in the hippocampus in the left cornu ammonis 1 (CA1) region (Zhang et al., 2020). Interestingly, the amygdala kindling model of TLE has been shown to produce a post-ictal increase in c-fos expression which decreases during the interictal period to levels comparable to controls (Dragunow et al., 1988). Functional changes in the ACC following amygdala kindling are still being assessed but support for

affective pain hypersensitivity comes from reciprocal connections with the amygdala, a structure shown to have increased NMDA activity and underly co-morbid fear and anxiety-like behaviours (Fournier et al., 2020; Hannesson et al., 2008; Kalynchuk et al., 1997; Lei et al., 2004; Rainnie et al., 1992).

In this section, I will investigate the influence of the ACC in pain-related behaviour and negative affect. First, I will examine the impact of kindling on nociceptive behaviours in the FNT and determine whether kindling affects expression of Egr1, an immediate early gene, in the ACC during noxious stimulation with formalin. Next, I will employ a chemogenetic approach to selectively inhibit ACC neurons to determine if ACC hyperactivity mediates affective pain of kindled rats.

2. Method

2.1 Animals

A total of 34 male Long Evans (Charles Rivers Laboratories; Montreal, Quebec, Canada) weighing approximately 250 – 300 g were used as subjects. Upon arrival, the rats were paired housed in conventional rectangular polypropylene cages with standard laboratory bedding until surgery. The housing room was kept on a 12:12 light:dark cycle with light on at 0700 h local time. Ambient temperature was held at 20°C (±3°C). Food and water were available *ad libitum* throughout the experiments. The design of this study is shown in Figure 7. All animals were treated in accordance with the Canadian Council for Animal Care and the Trent University Animal Care Committee.

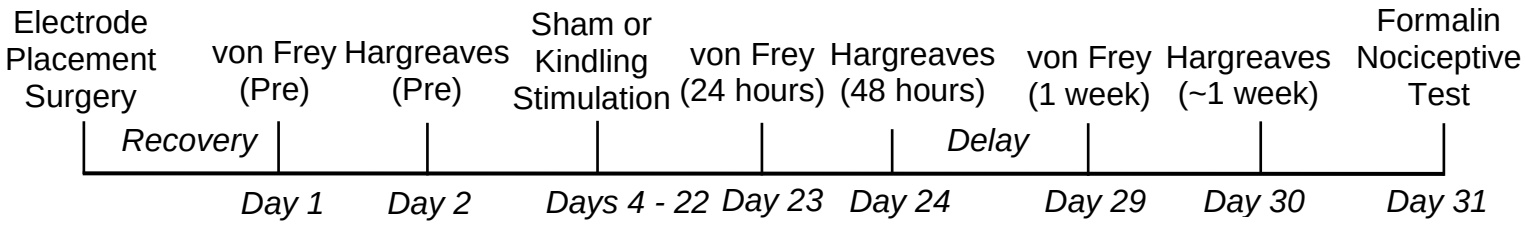

Figure 7. Following electrode placement surgery and recovery, rats completed the von Frey and Hargreaves test twenty-four hours apart. Rats then underwent amygdala kindling for a total of 42 stimulations or sham stimulations across 20 days. Twenty-four hours later von Frey was remeasured, then twenty-four hours later Hargreaves was remeasured. After a one-week delay, von Frey and Hargreaves were measured on the same schedule. Twenty-four hours after the last Hargreaves test, the formalin nociceptive test was conducted. Rats were sacrificed 90 minutes after the hind-paw injection with formalin and brain tissue was collected and stored for immunohistochemistry.

2.2 Electrode Placement Surgery

All animals underwent stereotaxic surgery for implantation of a single bipolar electrode into the left basolateral amygdala using the same methodology as outlined in Chapter 2.

2.3 Intracranial Viral Injections

For chemogenetic DREADD experiments, rats were anesthetized with isoflurane and head-fixed into a stereotaxic frame and then were bilaterally injected with either a AAV8-CaMKIIa-hM4D(Gi)-mCherry or AAV8-CamKIIa-EGFP viral construct into the rostral ACC at the following coordinates: AP +/- 2.52 mm, ML +/- 0.5 mm, and DV – 2.2 mm (Paxinos and Watson, 2007). For viral injections, a 32-gauge Neuros syringe needle (1.0 μl, Model 65458-02, Point Style 4, Hamilton) was attached to the stereotaxic frame and lowered from the skull surface to the target site. Each site was injected with 200 nl of virus at a rate 50 nl/min. The needle remained in place for an additional 5 minutes after each injection to facilitate for diffusion of the virus and then it was slowly retracted. Following viral injection, a single bipolar electrode was then implanted into the left basolateral amygdala using methods as described in Chapter 2. After implantation of the electrode, the surgical site was closed and antibiotic ointment was applied and hydrating fluids were administered (saline, 5 cc, s.c.). The rat was transferred from the stereotaxic apparatus to a warmed recovery cage until fully mobile. Post-surgical analgesics (carprofen, 5 mg/kg, s.c.) and antibiotics (enrofloxacin, 5 mg/kg, s.c.) was administered for five days as per institutional Standard Operating Procedures.

2.4 Drugs

Clozapine-N-oxide dihydrochloride (CNO, 10 mg/kg, HB 6149, Hello Bio) was dissolved in 0.9% (w/v) saline and freshly prepared at the time of use. The formalin solution

was prepared using formaldehyde (37%, Sigma Aldrich) and preservative free saline (sodium chloride injection BP 0.9%).

2.5 Kindling Procedure

Rats were allowed to recover for a minimal of three weeks and were then randomly assigned into kindled and sham groups. All rats received a total of 42 stimulations. Three stimulations were delivered each day (5 days per week) with a minimal of 3-4 hours between consecutive stimulations. Prior to stimulation, the rats were placed into a glass container (51 cm X 25.7 cm X 27.5 cm) with commercial bedding, the wire lead attached, and the stimulation delivered. The electrical stimulation was comprised of a 1 s, 60 Hz square-wave pulse with a peak-to-peak amplitude of 800 μA. For sham stimulations, rats were handled in the same manner and were connected to the wire lead, but no current was delivered.

The behavioural convulsion elicited after each stimulation was scored according to a modified criteria outlined by Pinel and Rovner (1978a). Stage 0 convulsions were classified as an arrest in behavioural mobility, Stage 1 convulsions were characterized by orofacial automatisms, Stage 2 convulsions were defined as orofacial automatisms with repeated head nodding, Stage 3 convulsions consisted of forelimb clonus and mastication and salivation, Stage 4 convulsions were associated with generalized convulsions consisting of rearing and unilateral tonic extension of the forelimb, Stage 5 convulsions involved rearing, bilateral clonic followed by loss of equilibrium (e.g., falling), and Stage 6 were associated with multiple bouts of Stage 5. To be considered kindled, rats must have had at least three consecutive stage 5 seizures over the course of the 42 stimulations. Kindled rats that did not reach this threshold were removed from analysis.

2.6 Behavioural Tests

2.6.1 Von Frey Filament Test

Paw withdrawal thresholds were measured using von Frey hairs at multiple times (1 day, 7 days post-kindling). The procedure for von Frey testing was identical to those described in Chapter 2 except rats were placed in the testing room for 2 hours before a 1-hour habituation to the testing apparatus (see Mechanical Allodynia test).

2.6.2 Thermal Pain

Thermal hyperalgesia was evaluated by measuring the paw-withdrawal latency in response to a radiant heat stimulus applied to the core of the plantar surface of the rat's hind paw. The procedures for this test were identical to those described in Chapter 2 except rats were placed in the testing room for 2 hours before a 30-minute habituation to the testing apparatus (Hargreaves' test).

2.6.3 Formalin Test

For the formalin test, rats were individually placed into Plexiglas observation chambers and were allowed to acclimate to the chambers and test stand for 30 minutes before testing commenced. Immediately following acclimation rats received an intraplantar injection of 30 μl of 2.5% (v/v) formalin and were placed back into the observation chamber for a 60 min test session. A video camera was positioned below the glass floor as well as on either side of the observation chamber to record pain responses. Formalin-evoked behaviours were scored by trained observations blind to the experimental conditions of each animal. The behaviours were divided into four categories: (0) the injected paw is not favoured; (1) the injected paw has little or no weight on it; (2) the injected paw is elevated and is not in contact with any other surface; (3) the injected paw is licked, bitten, or shaken. Each pain behaviour category was scored for each animal for the

first ten seconds of every minute of the test. However, due to technical issues which resulted in partial loss of video streams at random points across the 60-minute recording, we had to restrict our analysis to the period between 20 and 40 minutes after formalin injection as this period was common for all subjects. A weighted pain intensity score was calculated by multiplying the frequency an animal engaged in each category by it assigned weigh, summing these products, and then dividing by the total number of observations. After testing was complete, the floor and observation chambers were cleaned with Oxivir Five 16 concentration (1:16 dilution).

2.6.4 Formalin Conditioned Place Aversion

Rats were trained in a three-compartment shuttle box using the same procedures outlined in Chapter 2. Two of the compartments were used for "conditioning" (i.e., formalin was paired with one or the other) and each contained distinct visual, textural, and olfactory cues. A neutral middle zone permitted shuttling between the two conditioning compartments. The experimental procedures were video recorded and analyzed using AnyMaze software. For the conditioning trials, rats were treated with CNO (10 mg/kg, i.p.) thirty minutes before intraplantar formalin injection and confinement into one of the two conditioning compartments. The compartments were cleaned with Oxivir Five 16 concentration (1:16 dilution) after each rat underwent habituation, conditioning, or post-conditioning testing.

2.7 Tissue Preparation, Immunostaining, and Quantification

Rats were deeply anesthetized 90 minutes after formalin testing with sodium pentobarbital (340 mg/ml, Euthansol) and underwent transcardiac perfusion with room temperature saline followed by ice-cold 4% (w/v) formaldehyde fixative (pH=7.4) that was freshly prepared from depolymerized paraformaldehyde. The brains were extracted and post fixed in the same fixative for up to 72 h at 4°C before being placed into PBS containing 0.1% (w/v) sodium azide. Brains

were embedded in 3% agarose with 0.3% gelatin (Gelatin Type A, Fisher Scientific, New Jersey USA) and sectioned on a vibrating microtome (VT1000, Leica Canada). Brain tissue was sectioned in a 1 in 6 interval at 50 μm in the coronal plane. The sections were stored in PBS with 0.1% sodium azide until immunohistochemical analysis.

Brain sections were processed for Egr1 immunoreactivity according to previously published methods (Kalinina et al., 2022). Briefly, sections were incubated with a rabbit anti-Egr1 antibody (1:1000, Santa Crus Biotechnology USA) diluted in a solution containing 5% (v/v/) normal goat sera, 1% (w/v) bovine serum albumin, and 0.3% (v/v) Triton X-100 dissolved in PBS. Following this, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (1:500, room temperature, Vector Labs) and then incubated with avidin-biotin peroxidase complex (1:500, 1 h, Vectastain ABC Elite). Immunolabeling was visualized using 2.5 (w/v) nickel sulfate, 0.02% (w/v) 3,3'-diaminobenzidine (DAB), and 0.000083% (v/v) H2O2 to produce a blue and black product. Sections were mounted on charged slides (Superfrost Plus, Fisher Scientific), air dried, and then briefly rinsed in water before undergoing dehydration through an ascending series of 70%, 90%, 95%, and 100% ethanol and clearing with xylene before cover slipping with Entellan mounting medium (EM Microscopy Sciences).

Quantification of Egr1+ cells was conducted on digital images collected from a Nikon TI2-E inverted microscope at 4x magnification for identification of the region and 20X magnification for quantification (Nikon Instruments, USA). All images were converted into TIFF format and processed in ImageJ (http://rsbweb.nih.gov/ij/; National Institute of Health, USA). Images were converted to (8-bit) grayscale and the number of Egr1+ cells in the ACC were counted using the Threshold feature and Particle Analyzer tool in ImageJ with parameters for threshold, size, and

circularity held constant for all images. The boundaries of the ACC were outlined according to standardized atlas plates of Paxinos and Watson (2007).

2.8 Statistical Analysis

All statistical analyses were performed using either R-Studio or Statistical Package for Social Sciences (SPSS v. 28). Behavioural data was analyzed using either a mixed model ANOVA design with the between subject factor of group and the within subject factor of phase of testing or by using two-tailed Student t-tests and paired t-tests, where appropriate. For analysis of kindling stages, group differences in kindling Stage 5 events were compared or two-tailed Student t-test conducted. For Egr1 immunoreactivity, Egr1 labelling (neurons/mm^2) was averaged across 3-5 sections comprising the rostral and caudal ACC for each animal and the group means calculated. Analyses was carried out using a two-tailed Student t-test and paired t-test. Finally, Pearson correlations were used to examine Egr1 labelling and pain intensity scores. For all analyses, statistical significance was set at $P < .05$. Data are presented as mean and standard error of the mean (SEM).

3. Results

3.1 Experiment 1: Effect of Kindling on Formalin-Induced Nociceptive Behaviours

3.1.1 Kindling Characteristics

Rats were assigned to receive either 42 electrical (kindled, N=5) or sham stimulations (N=5). Kindling of the amygdala produced the typical progression of behavioural seizures in most rats. The initial stimulations elicited a short period of behavioural arrest that was typically followed by orofacial automatisms. With subsequent stimulations, these episodes progressed to Stage 5 seizures characterized by bilateral forelimb clonus, rearing, and loss of equilibrium. Kindled rats reached the first Stage 5 event after approximately 6.0 ± 2.04 stimulations. The total number of

Stage 5+ events was 20 ± 4.49 events. One rat never developed kindled seizures beyond Stage 1 (orofacial automatism) and was subsequently removed from all analyses. Sham control rats never exhibited seizures during kindling. The final group composition was kindled (N=4) and sham (N=5).

3.1.2 Kindling Does Not Affect Mechanical Or Thermal Pain Sensitivity

After completion of kindling, all rats underwent tests for mechanical (von Frey) and thermal (Hargreaves) pain sensitivity 24 hrs and 48 hrs after receiving their last stimulation. These tests were also repeated again one week later. Importantly, baseline mechanical allodynia [$t(7)=1.17$, $P=.279$] and thermal hyperalgesia [$t(7)=1.54$, $P=.176$] measurements prior to kindling or sham stimulation were not significantly different between groups. The results of a two-way repeated measures ANOVA found no significant interaction effect for group (sham vs. kindling) and phase of testing (1 or 2 days vs. 1 week) [$F(2,14)=0.127$, $P=.882$] or main effect of group [$F(2,14)=.441$, $P=.65$] for mechanical thresholds to elicit a withdrawal reflex (Figure 8a). Similarly, there was no significant interaction between group and time [$F(2,14)=2.74$, $P=.099$] or main effect of group [$F(2,14)=1.00$, $P=.39$] for heat withdrawal latency during the Hargreaves test (Figure 8b).

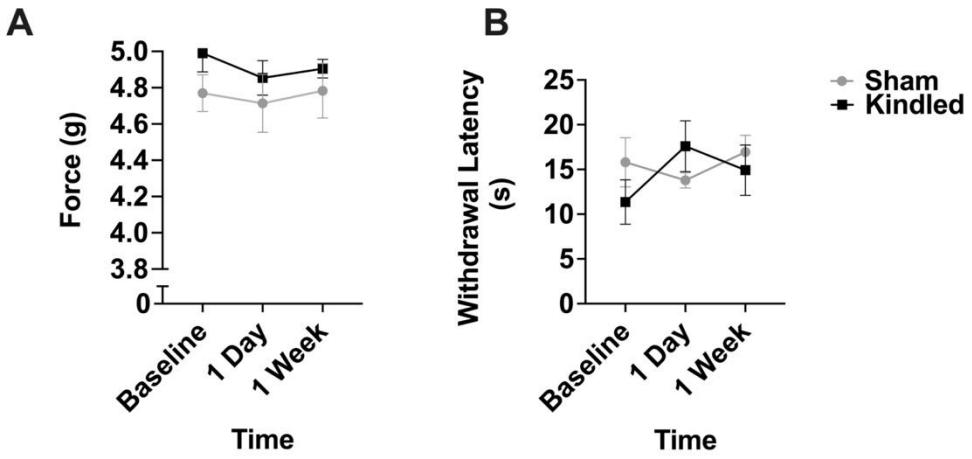

Figure 8. Sham (N = 5) and Kindled rats (N = 4) did not show a significant difference in paw withdrawal at baseline or 1 day and 1 week after the formalin test when the force (in grams) necessary to elicit withdrawal of the hindpaw in the von Frey test (A) or the latency to withdraw from the thermal stimulus in the Hargreaves test (B) was measured. Data is represented as the Mean $\pm$ SEM.

3.1.3 Formalin Evoked More Affective Responses From Kindled Rats

Rats underwent a formalin test 13 days after receiving their last kindling or sham stimulation. A single intraplantar injection of 30 µl of 2.5% formalin was delivered into the plantar surface of hindpaw. Formalin injections evoked typical nocifensive responses in all animals. As shown in Figure 9a, kindled rats engaged in more frequent paw lifting [$t(7)=4.52$, $P<.001$] and licking, biting, or shaking of the injected paw [$t(7)=4.95$, $P<.001$] compared to sham controls. Further analysis of the pain intensity scores showed that kindled rats had significantly higher pain scores than sham controls [$t(7)=4.45$, $P<.003$, Figure 9b].

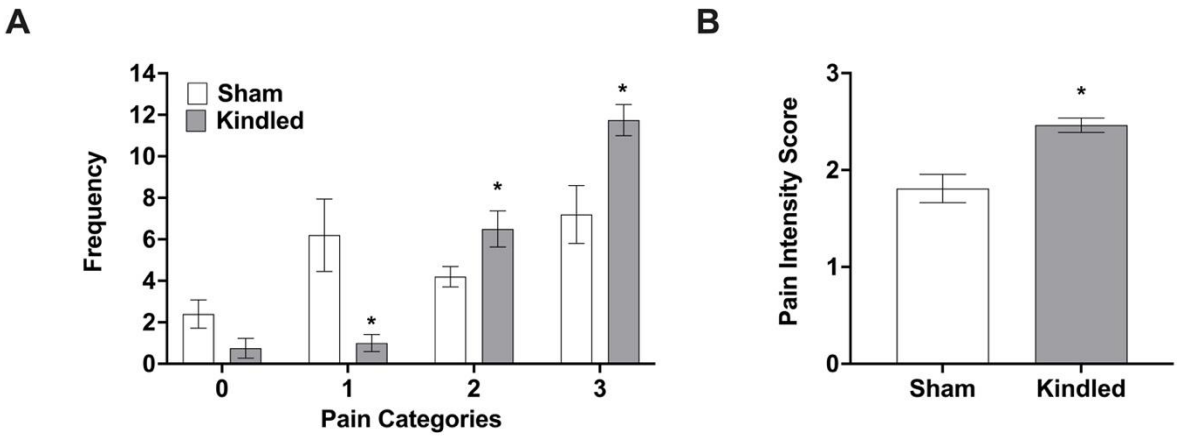

Figure 9. (A) Kindled rats (N = 4) displayed a significant difference in the frequency of behaviour in pain categories 1-3 and (B) total pain intensity score than Shams (N = 5). Data is represented as the Mean ± SEM.

3.1.4 Kindled Rats Show Greater Activation of the Anterior Cingulate Cortex after Inflammatory Pain

To examine brain areas that could mediate the elevated pain sensitivity to formalin stimulation after kindling, we perfused rats 90 minutes after the formalin test was completed and conducted immunostaining probing for the neuronal activity-dependent marker Egr1. We restricted our analysis to the ACC given past work showing that ACC is activated by noxious stimuli and plays a critical role in affective pain perception (Gao et al., 2004; Jarrin et al., 2020; Johansen et al., 2001; Johansen & Fields, 2004; Lei et al., 2004). In agreement with past work, we found a moderately strong positive correlation between pain intensity and the number of Egr1+ cells in the ACC [$r(9)=.68$, $P<.042$]. Further analysis showed that total Egr1 labeling was higher for kindled rats than sham controls after formalin injection [$t(7)=7.68$, $P<.001$, Figure 10a], which was consistent with the above findings that kindled rats exhibited greater aversive and emotional responses to formalin. This increase in the number of Egr1+ cells was observed across both rostral ($P=.040$) and caudal ($P=.001$) segments of the ACC for kindled rats. However, the increase in Egr1 immunoreactivity for kindled rats appeared to be highest for the caudal ACC segments than the rostral ACC segments [paired t-tests, $t(3)=-2.49$, $P<.045$, Figure 10b].

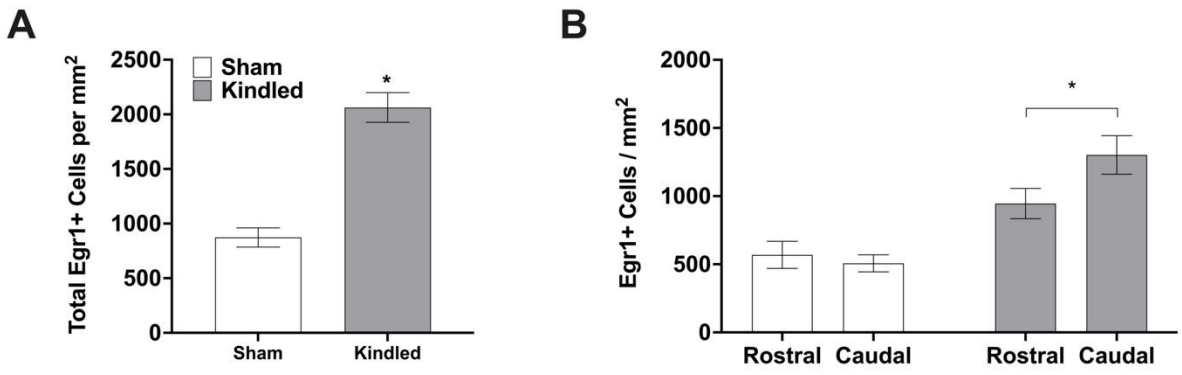

Figure 10. A greater total number of EGR1+ cells per mm2 were found in the ACC of Kindled (N = 4) than Sham rats (N = 5) (A). Comparison of the number of EGR1+ Cells per mm2 in the rostral and caudal ACC showed greater expression in the caudal ACC than rostral ACC of kindled rats but no difference between kindled and sham rats (B). Data is represented as the Mean $\pm$ SEM.

3.2 Experiment 2: Effect of Chemogenetic Inhibition of Rostral Anterior Cingulate Cortex on Pain-Aversive Learning after Kindling

Given that we observed increased activation of the ACC in kindled rats compared to controls in response to noxious stimulation and the evidence that ACC activity is critical for the expression of affective pain, we decided to test whether the elevated activity of ACC neurons mediated the heightened pain-related emotional behaviour seen after kindling. To do this, we employed a chemogenetic based strategy that would permit us to selectively inactivate excitatory neurons in the ACC of sham and kindled rats and investigate pain-related avoidant and preference behaviours using the FCPA task. The outline for this experiment is shown in Figure 11.

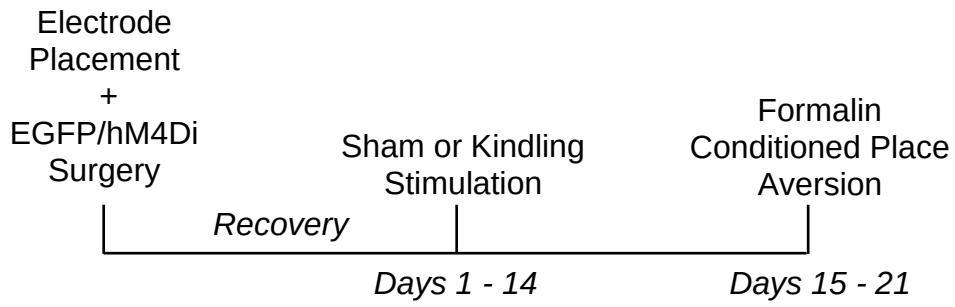

Figure 11. All rats underwent electrode placement surgery in preparation for amygdala kindling and infusion of the ACC with EGFP or hM4Di. After a recovery period, rats received either 42 kindling or sham stimulations over 14 days. Rats then started the formalin conditioned place aversion test 24 hours after the last stimulation which occurred between day 15 to 21.

3.2.1 Targeting Inhibitory DREADDs to Anterior Cingulate Cortex

To achieve chemogenetic modulation of ACC activity, we bilaterally injected rats with an AAV8-CamKIIa-hM4D(Gi)-mCherry virus (herein referred to as hM4Di) or with an AAV8-CamKIIa-EGFP control virus. Preliminary analysis confirmed that our injections successfully targeted the rACC with all subjects showing at least moderate levels of expression of mCherry or EGFP.

To assess any influence of CNO treatment on mechanical sensitivity rats were administered an intraperitoneal injection of CNO (N=3) or saline (N=4) 30 minutes before VF testing. A mixed model ANOVA did not reveal an effect of group [$F(1,5)= .337$, $P=.587$] or interaction between group (i.e., CNO vs. Saline) and time of assessment (i.e., baseline, 30 minutes, or 60 minutes) suggesting mechanical sensitivity was not significantly influenced by CNO treatment [$F(2,10) = .579$, $P=.578$, Supplementary Figure 1].

3.2.2 Kindling Characteristics

Three rats were removed from the study for not reaching Stage 5 seizures during kindling. Therefore, the final group composition was: Sham-EGFP (N=6), Kindled-EGFP (N=6), and Kindled-hM4Di (N=4). There was no difference between Kindled-EGFP and -hM4Di rats for the frequency of Stage 5+ seizures [$t(8)=.21$, $P=.71$] or for the number of stimulations required to the first Stage 5+ seizure event [$t(8)=.10$, $P=.92$] indicating that kindling progression was similar for both groups.

3.2.3 Effect of Kindling on Formalin Conditioned Place Aversion

The FCPA task combines the formalin test of tonic, persistent pain with the place-conditioning paradigm to assess for pain-related aversion learning. The test is run over 7 days: Days 1-2: pre-conditioning; Day 3-6: conditioning; and Day 7: post conditioning test.

For preconditioning sessions, rats were placed into a 3-chamber shuttle-box for two separate days for 15 minutes. Exploratory activity as defined as the total distance travelled in the apparatus each session was examined using a two-way repeated measures ANOVA with preconditioning session (day 1 and day 2) as the within subject factor and group (Sham-EGFP vs. Kindled-EGFP vs. Kindled-hM4Di) as the between subject factor. Although there was no significant interaction between group and preconditioning session [$F(2,13)=.251$, $P=.782$] or main effect of preconditioning session [$F(1,13)=4.13$, $P=.063$], there was a significant main effect of group [$F(2,13)=18.01$, $P<.001$]. Subsequent analysis revealed that both kindled groups (Kindled-EGFP, $P<.003$; Kindled-hM4Di, $P<.001$) were significantly more active and travelled further distance than the sham group infused with the control (EGFP) virus (Figure 12a).

To examine the effect of kindling on pain-related aversion learning, rats underwent place avoidance conditioning (Days 3 through 6). CNO was administered to all rats 30 minutes before intraplantar injections of formalin on the conditioning days (e.g., Days 4 and 6). After conditioning was completed, a postconditioning test was performed (Day 7) and the time the rat spent in each compartment (e.g., formalin-paired and non-paired compartments) was measured. As shown in Figure 12b, Sham-EGFP rats spent significantly more time in the non-paired compartment compared to the formalin-paired compartment [$t(5)=2.905$, $P<.034$] indicating that controls acquired the test during conditioning. By contrast, Kindled-EGFP rats showed no difference in time spent in the formalin-paired and non-paired compartments [$t(5)=1.41$, $P=.218$] suggesting that kindling impaired the expression of pain-aversive learning as reported in Chapter 2. However, to our surprise, for hM4Di-infused kindled rats treated with CNO, chemogenetic inactivation of the rACC during conditioning appeared to reverse the effect of kindling with these rats now

spending more time in the compartment paired with formalin than the non-paired compartment during the post-conditioning test [$t(3)=7.33$, $P<.005$].

To provide a more quantitative estimation for the above findings, we computed a conditioned preference score to measure the compartment preference after conditioning. This score was computed by taking the time spent in the formalin-paired chamber and dividing this value by the total time spent in both formalin-paired and unpaired chambers. Thus, a higher score indicates a greater preference towards the formalin (aversive) compartment. The results of a one-way ANOVA revealed a significant group effect for conditioned preference scores [$F(2,16)=8.74$, $P=.003$]. Post hoc analyses showed that conditioned preference scores were significantly higher for hM4Di-infused kindled rats given CNO compared to all other groups (All Ps < .017, Figure 12c).

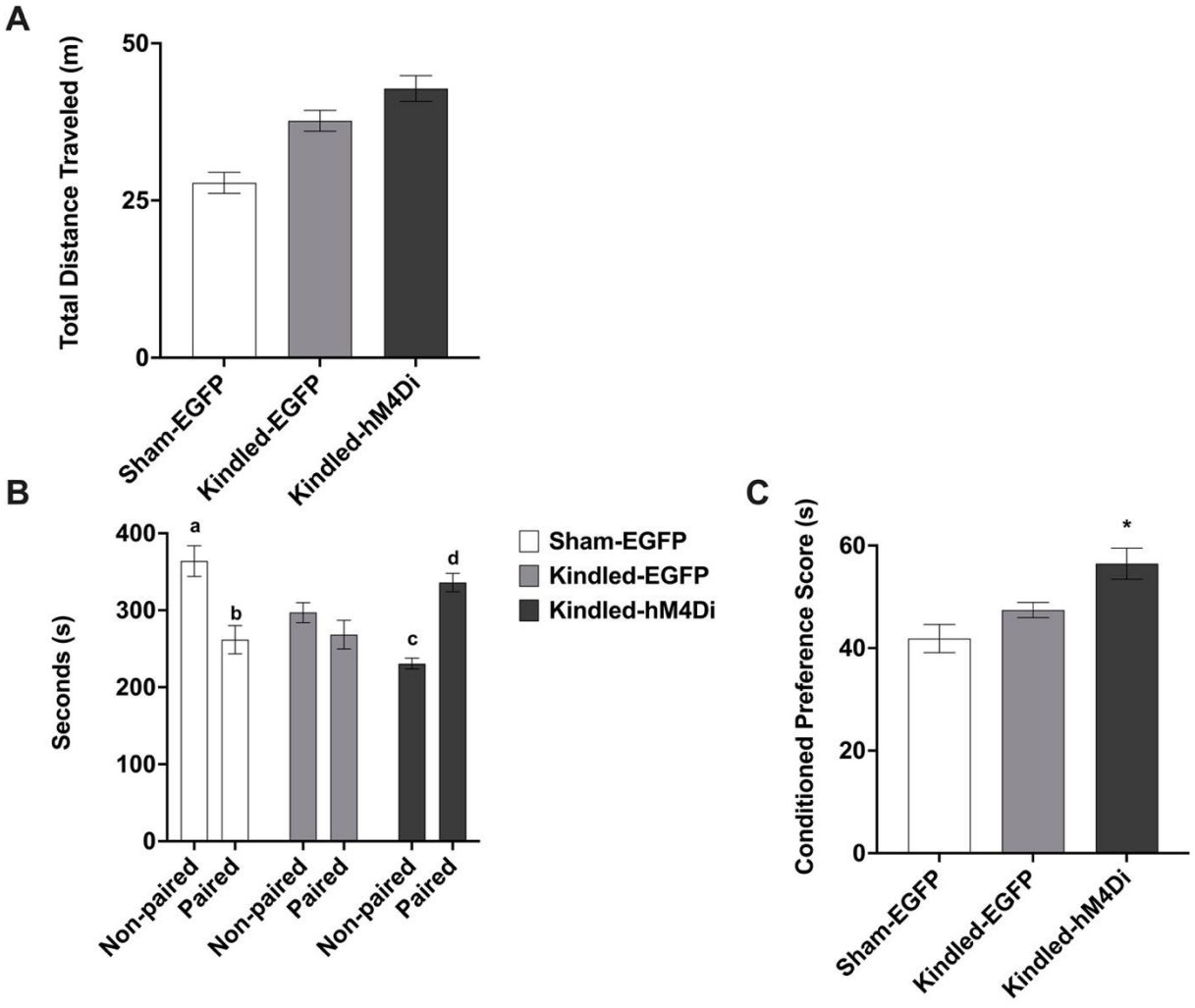

Figure 12. Locomotor activity was not significantly different between Sham-EGFP (n = 6), Kindled-EGFP (N = 6), and Kindled-hM4Di (N = 4) rats as no difference was observed in total distance traveled in the FCPA apparatus (A). The time spent in the formalin-paired and non-paired compartments of the FCPA test reflected that sham-EGFP spent significantly more time in the non-paired compartment compared to the formalin-paired compartment. The opposite was shown for Kindled-hM4Di rats which spent more time in the formalin-paired compartment but kindled-EGFP rats showed no difference in choice of compartment (B). A conditioned preference score was calculated reflecting preference for the aversive context, surprisingly when compared to the other rats Kindled-hM4Di rats spent more time in the formalin-paired compartment after conditioning than the same compartment during habituation (C). No significant difference was found between Sham- or Kindled-EGFP rats CPA scores. Data is represented as the Mean $\pm$ SEM.

4. Discussion

The amygdala kindling model of TLE was used in two studies to examine differences in interictal pain modulation by the ACC during the FNT and FCPA tests, respectively. In the first study, kindled rats were predicted to exhibit a higher frequency of formalin-induced pain behaviours compared to controls in the FNT. A chemogenetic approach was then used in the second study to investigate the role of the ACC in avoidance behaviour after an inhibitory DREADD was activated in the bilateral rACC during formalin-conditioning. It was predicted that the greatest amount of time spent avoiding the formalin-paired compartment would be observed in Kindled-EGFP rats, then Sham-EGFP rats, followed by Kindled-hM4Di rats.

Kindled rats did not show a difference in mechanical or thermal pain sensitivity compared to controls during repeated assessment across the interictal period. However, kindled rats did exhibit a greater frequency of pain behaviours and overall pain intensity score compared to controls in the FNT. This supports kindling-induced hypersensitivity to the noxious inflammatory stimulus formalin but not thermal and mechanical modalities. Activation of the ACC assessed 90 minutes after administration of formalin in the FNT supports that pain-like behaviours induced by formalin were correlated with Egr1 expression and adds to the literature suggesting activation of the ACC during the FNT And FCPA test (Gao et al., 2004; Jarrin et al., 2020; Johansen et al., 2001; Johansen & Fields, 2004; Lei et al., 2004). This is the first study to show that amygdala kindling increases EGR1 expression compared to controls and that a greater increase is observed in the cACC than rACC. Despite this increase in cellular activation of the ACC after an injection of formalin in experiment 1, Kindled rats did not show an increase in avoidance behaviour during the FCPA test relative to controls in Chapter 2 or the next study.

The final study examined the effect of kindling on affective pain by comparing avoidance in the FCPA test between controls (Sham-EGFP) and kindled rats with (Kindled-hM4Di) or without (Kindled- EGFP) chemogenetic inhibition. An initial analysis of kindling characteristics between Kindled-EGFP and -hM4Di rats revealed no difference in progression of seizure events. Avoidance behaviour of Sham-EGFP rats was consistent with the FCPA literature in that post-conditioning avoidance of the formalin-paired compartment was greater than the non-paired compartment (Gao et al., 2004; Johansen et al., 2001). Importantly and contrary to our predictions, Kindled-hM4Di rats spent more time in the formalin-paired than the non-paired compartment on test day. Kindled-hM4Di rats' conditioned preference score was also significantly higher than Sham- or Kindled-EGFP rats, which reflects significantly more time spent in the formalin-paired compartment on test day than during preconditioning. The lack of compartment avoidance observed in Kindled-EGFP rats in this study and Chapter 2 may be explained by the role of the rACC in aversive conditioning and the interictal opioid response.

The effect of mu-opioid receptors (MOR) on Glu neurons in the ACC may be important due to the initial increase in opioids induced by amygdala kindling followed by interictal decrease in the ACC and amygdala except for the central region (Rocha et al., 1993). A full review of pain mediation via MOR in Glu neurons is provided by Sánchez-Blázquez and colleagues (2013). Briefly, calcium/calmodulin regulated kinase II (CAMKII) typically promotes negative feedback inhibition of MOR that regulates stimulation of Glu receptors to reduce tolerance from prolonged activation. Due to the repeated release of opioids following Stage 5 seizures during amygdala kindling, tolerance may occur (Rocha et al., 1994). If kindled rats in this study and Chapter 2 were experiencing opioid tolerance as a result of amygdala kindling, it would be expected that Glu neurons would be less active in the rACC during formalin-

conditioning and avoidance would be reduced. Introducing a CAMKII inhibitor would restrict the negative feedback mechanism of MOR activity promoting greater Glu activity, but it may still not be enough to overcome kindling induced tolerance. Moreover, levels of opioids are found to decrease during the interictal period suggesting an even greater likelihood that the effect of formalin on Glu neurons in the rACC is being supressed.

The dual ability of kindling to reduce avoidance in the FCPA test but hypersensitivity in the FNT test may be understood through careful analysis of the rACC in the affective pain pathway. Inactivation of rACC using lesioning, optogenetics, and antagonists all produced a pattern of reduced CPA but intact pain-like behaviours in response to formalin (Gao et al., 2004; Johansen et al., 2001). Johansen and Fields (2004) revealed a learning impairment to be the loss of avoidance behaviour in the FCPA test. When glutamatergic neurons of the rACC are inhibited during formalin conditioning rats do not acquire the FCPA test but pain-like behaviours to formalin are preserved. Moreover, avoidance behaviour was observed if inhibition was induced after conditioning. This supports that activation of the rACC mediates aversive learning and works in unison with the pain-like response to formalin during the affective pain response. Thus, accounting for the increase in pain-like behaviours after formalin administration in the FNT but lack of FCPA by kindled rats observed in our studies.

As the first experiment investigating the ACCs role in the affective pain response using a model of TLE, an aversive learning impairment appeared to occur in kindled rats which may be induced by MOR receptor activity. Second, sensitivity of pain-like behaviours in response to an acute inflammatory stimulus (i.e., formalin) was supported following amygdala kindling (Wheeler-Aceto et al., 1990). However, given that Kindled-hM4Di rats spend more time in the formalin-paired compartment and higher preference score it is likely that kindled rats are able to

learn but kindling is influencing their aversive behaviour. Whereas additional studies will be needed to examine the role of pain-modulation by the ACC induced by seizure activity across the interictal period, this study provides insight on the ACC's function in the negative affective pain pathway of individuals with TLE (Al-Chalabi et al., 2018; Gauriau & Bernard, 2002; Price, 2000; Wei et al., 2008).

CHAPTER 4

General Discussion

Individuals with TLE not only experience seizure activity arising from temporal lobe foci, but also comorbid impairments in cognition, memory, and emotion associated with limbic system dysfunction (Bragatti et al., 2010; de Oliveira et al., 2010; Maillard et al., 2004; Tavakoli et al., 2011; Tellez-Zenteno et al., 2007; Weatherburn et al., 2017; Xu et al., 2018). Studies examining interictal emotionality changes using rat models of TLE have shown sensitization of anxiety and fear-like responses with seizure-induced amygdala pathology (Fournier et al., 2020; Helfer et al., 1996; Kalynchuk et al., 1997; Kalynchuk et al., 1998; Kalynchuk et al., 1999). As such, it is unsurprising that the amygdala would be recruited in the emotional experience of pain, known as affective pain (Melzack & Casey 1968; Price, 2000). To investigate a potential relationship between TLE and comorbid affective pain, interictal pain responses were measured using the amygdala kindling model of TLE.

1. Mechanical Pain Occurs After An Inflammatory Stimulus

Our experiments revealed that mechanical and thermal pain responses were not significantly different between sham and kindled rats when measured using the von Frey and Hargreaves tests, respectively. Confirmation that kindled rats did not exhibit predisposed physical pain sensitivity in the interictal period was of importance to limit confounding factors during formalin conditioning in the FCPA test. It is not currently known why sham and kindled rats were inconsistent with Fu and colleagues (2001) who found that up to 3 days after formalin administration non-kindled rats displayed thermal hypoalgesia but mechanical hyperalgesia. More consistent with Fu and colleagues (2001), 48 hours after the second formalin-paired conditioning trial of the FCPA test in the first experiment kindled rats tended toward greater

mechanical sensitivity than sham rats. Interestingly, this suggests that exposure to a noxious inflammatory stimulus may have promoted a lasting influence on mechanical pain responses but not thermal pain responses during the interictal period.

The mechanical sensitization shown by kindled rats' may be due to changes in the CeA as Sugimoto and colleagues (2021) showed that 13 days after administration of an orofacial formalin injection mechanical hyperalgesia occurred during hind-paw stimulation and that chemogenetic inhibition of the bilateral or right CeA before testing increased rats' paw withdrawal threshold. Kindling the basal nucleus of the amygdala has been shown to reduce the number of somatostatin neurons that typically inhibit the pain response (Tuunanen et al., 1997; Wilson et al., 2019). To determine whether this is a mechanism underlying kindled rats' mechanical sensitivity after noxious stimulation, a future study may examine mechanical sensitivity and the number or activity of Som-CeA neurons of basolateral kindled rats before and after an injection of formalin.

2. Increased Pain-Related Nociceptive Behaviour and EGR1 Expression in Amygdala Kindled Rats

When interictal pain sensitivity was measured in response to formalin during the FNT, kindled rats displayed a greater overall pain intensity score and higher frequency of elevation, and licking, biting, or shaking of the injected paw than sham rats. The FNT was chosen to assess differences in pain-like behaviours after formalin independent of context association which is a necessary component for avoidance behaviour in the FCPA test (Abbott et al., 1995). Multiple studies have found that formalin induced pain-like behaviours persist when either the entire ACC or distinct regions of the ACC are functionally inhibited by lesions, optogenetics, or antagonists (Gao et al., 2004; Jarrin et al., 2020; Johansen et al., 2001; Lei et al., 2004). Seizure-induced

changes may be driving the increase in pain-like behaviours, but more studies will need to examine whether pain-like behaviours in the FNT are sensitized after amygdala kindling. To assess physiological changes in the ACC, activation was investigated 90 minutes after the FNT to determine if more expression of EGR1 occurred in kindled than sham rats.

EGR1 expression was shown to be increased in kindled rats compared to sham rats, reflecting greater cellular activation of the ACC. The cACC was shown to have a greater amount of EGR1 expression than the rACC in kindled than sham rats suggesting greater recruitment of the cACC in the physical pain response to formalin. Studies of phosphorylated extracellular signal regulated kinase (p-ERK) activation have shown increased expression in the ACC as a response to formalin (Luo et al., 2015; Tochiki et al., 2015). As such, it may be possible that upregulation of other IEGs coincide with the increase in pain-like behaviours after kindling. Identification of IEGs upregulated in the ACC by formalin should be investigated in future studies given our study has shown that increased EGR1 expression in the ACC is produced by 2.5% formalin and kindling in the FNT.

Despite the increase in pain-like behaviour in the FNT and EGR1 expression in the ACC, the interictal affective pain response measured by avoidance of the formalin-paired compartment in the FCPA test was not found to be significantly different between sham and kindled rats. Calculation of the magnitude of CPA produced during the FCPA test suggested no significant difference in avoidance of the formalin-paired context on test day than the same context on the second habituation day when compared to shams. A similar result was found for Kindled-EGFP but not Sham-EGFP or Kindled-hM4Di rats in our second FCPA experiment which used chemogenetic inhibition to examine avoidance behaviour in the absence of glutamatergic neuron activity in the ACC.

3. Kindle-EGFP Rats Exhibit an Impairment in FCPA

Kindled-EGFP rats did not show a preference for the formalin or safe paired compartments in the FCPA test which supports a kindling induced impairment in this study and Chapter 2. Although unlikely, a few limitations in the design and apparatus of the FCPA test in our experiments could have reduced the ability to distinguish between compartments on test day such as our choice of apparatus and number of habituation days. Future experiments may use more distinct conditioning compartments and a single habituation day to improve rats' ability to distinguish between the compartments. We suspect this impairment is more likely due to kindling promoting seizure-induced changes in the amygdala which influence the ACC due to reciprocal connections between the structures (Rainnie et al., 1992; Rajmohan & Mohandas, 2007; Sah et al., 2003; Singh et al., 2020). Under normal conditions the amygdala moderates the pain response via the lateral and capsular nuclei of CeA which are composed of protein kinase C-delta (PKCδ) or somatostatin (Som) receptor expressing neurons, with efferent transmission of the CeA primarily controlled by Som neurons during resting state (Adke et al., 2021; Pitkänen et al., 1997; Sah et al., 2003). The mechanical and thermal pain responses have been shown to be reduced by Som and increased by PCK-δ CeA neuron activation in a rat model of chronic pain produced by spare nerve injury (Wilson et al., 2019). Amygdala kindling may disrupt moderation of pain by CeA neurons as Tuunanen and colleagues (1997) have shown that the number of Som-CeA neurons are reduced after kindling of the basal amygdala nuclei. In this case, increased pain sensitivity would occur as PKC- δ neurons would be uninhibited in the absence of Som neurons. To understand how kindling may influence the pathway between CeA and ACC during the interictal period, an examination of how Som-CeA neurons may influence hypersensitivity in the

FNT and negative affect in the FCPA test should be conducted. Interestingly, inhibition of the ACC in the kindled-hM4Di rats appears to improve acquisition of the FCPA test.

4. Kindled Rats with Chemogenetic Inhibition of the ACC Exhibit Intact FCPA

The tendency of Kindled-hM4Di rats to spend more time in the formalin compartment and larger conditioned preference score than Sham- or Kindled-EGFP rats is interesting as this suggests less affective pain. The avoidance behaviour of Kindled-hM4Di rats not only opposes the pattern of reduced avoidance behaviour of rats with functional inhibition of the ACC using other methods but also appears to discredit a learning impairment in Kindled-EGFP rats (Cao et al., 2014; Gao et al., 2004; Jarrin et al., 2020; Johansen et al., 2001; Lei et al., 2004). It was surprising that in this study inhibition of the rACC during formalin conditioning reduced avoidance as Johansen and Fields (2004) have shown that rACC inhibited rats are unable to acquire the FCPA test. The behaviour of Kindled-hM4Di rats is likely influenced by kindling in the absence of the rACC but more research is needed to identify any changes in the pathway between the CeA and ACC that could lead to less affective pain while the rACC is inhibited. One way that kindling could be influencing FCPA behaviour is through pain relief of a chronic pain state. Relief of chronic constriction injury pain by chemogenetic inhibition of glutamatergic neurons in the ACC, nucleus accumbens, or ventral tegmental area during conditioning has been shown to induced preference for that compartment (Gao et al., 2020). Future studies should examine if kindling induces a chronic pain state in the interictal period that is relieved through chemogenetic inhibition of the rACC.

5. Considerations and Conclusions

These experiments support the use of the amygdala kindling model to study comorbid negative affect in TLE. The FCPA test has shown that amygdala kindling produces an

impairment in aversive behaviour. The ACC was implicated in the negative affective processes of TLE underlying the impairment which was supported by avoidance behaviour of rats in the FCPA test that received chemogenic inhibition and kindling treatment. We recognize that kindled rats' impairment in the FCPA test could be due to a lack of distinct cues in the apparatus and two habituation days. To increase the odds of successfully distinguishing between the two compartments future studies are encouraged to use distinct visual, olfactory, and tactile cues as well as one habituation day.

Lastly, kindling induced hypersensitization was shown in response to formalin without assessing avoidance in the FNT, further suggesting that the reduction in avoidance during the FCPA test is due to an impairment in kindled rats. The increase in pain-like behaviour for kindled rats in the FNT coincided with an increase in EGR1 expression suggesting that activation of the ACC is involved in formalin-induced pain behaviour and other IEGs may be influencing the pain response in kindling models or individuals with TLE. Using larger samples sizes may strengthen the ability to draw conclusions on pain behaviour in future studies but overall, these experiments suggest TLE may produce a comorbid emotional pain impairment as a result of the seizure-induced changes in the amygdala through a relationship with the ACC which is known to moderate negative affect (Al-Chalabi et al., 2019; Gauriau & Bernard, 2002; Price, 2000; Tuunanen et al., 1997; Wei et al., 2008; Wilson et al., 2019).

Other studies may attribute the mechanical allodynia and increased negative affect accompanied by elevated EGR1 expression in the ACC to formalin-induced neuroinflammation or excitotoxicity (Ji et al., 2018; Hoffman et al., 2022). As increased EGR1 expression suggests greater cellular activation of the ACC in kindled rats after formalin, analyses of specific cell-types such as glial cells could determine the influence of neuroinflammation on EGR1

expression in future studies (Ji et al., 2018). To examine the role of neuroinflammation in kindled rats' formalin related pain behaviours, studies could quantify expression markers such as GFAP in the ACC after the FNT (Ji et al., 2018). Recently, formalin induced excitotoxicity has been proposed as an alternative mechanism underlying behaviour in the FNT (Hoffman et al., 2022). At this time, evidence for a reduction in formalin-induced excitation of C or Aδ-fibers due to excitotoxicity in skin tissue of mice and rats does not discredit formalin's ability to sensitize and produce inflammatory responses in the spinothalamic tract and structures involved in pain (Hoffman et al., 2022). Formalin-induced silent c-fiber excitation may be involved in kindled rats mechanical hyperalgesia after the FCPA test as both mechanical and inflammatory stimuli activate this fiber type (Dubin & Patapoutian, 2010). As Hoffman and colleagues (2021) did not distinguish silent c-fibers from other c-fibers, it may be useful to determine if excitotoxicity occurs in fibers that respond to inflammatory stimuli after formalin and any resulting changes in mechanical pain sensitivity. We believe it is possible that kindling induced neural sensitization occurs alongside neuroinflammatory and excitotoxic processes throughout the nervous system which will need to be examined in additional studies.

Overall, these experiments suggest the ACC is an important structure in formalin-induced pain sensitivity after amygdala kindling. Not only has formalin-induced pain been shown to be associated with activation of the rACC and to a greater extent the cACC, but chemogenetic inhibition has been shown to reduce aversion in the FCPA test likely due to dysmodulation of the connections between the rACC and BLA (Malin et al., 2007). These experiments are the first to use the basolateral amygdala kindling model to study interictal pain and demonstrate that it is useful for understanding the neurological changes associated with comorbid pain in TLE (Ottman et al., 2011; Pulsipher et al., 2006).

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Supplementary Figures

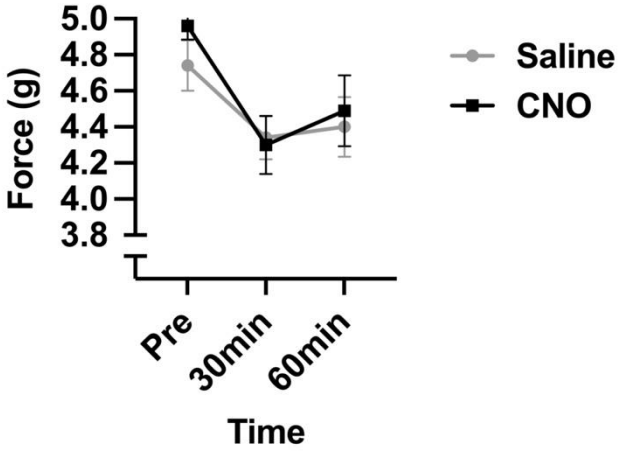

Figure 1. The force necessary to elicit paw withdrawal in the von Frey test was not significantly different between rats when measured prior to or 30 minutes and 60 minutes after injection with Saline (n = 4) or CNO (n = 3).