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High Frequency Oscillations are Phase-Amplitude Coupled in Stress Induced Seizures Following Traumatic Brain Injury

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Abstract

Traumatic Brain Injury (TBI) often leads to the development of epilepsy, especially with the occurrence of stressful events. Stressors increase the levels of corticotropin-releasing factor (CRF) in the amygdala, which can be damaged by the secondary effects of TBI. It is hypothesized that the activity of CRF receptor type 1 (CRFR₁) in the amygdala is altered post-TBI and supports the generation of epileptiform waves, namely high-frequency oscillations (HFOs). Sprague-Dawley rats were given a moderate TBI and *in vivo* recordings of the amygdala were taken during the administration of an acute tail pinch stressor. The stressor increased broadband activity which included the occurrence of HFOs. Moreover, HFO amplitudes were found to be coupled to the phase of a simultaneous theta wave (4 – 8Hz). Furthermore, application of a CRFR₁ antagonist disrupted the generation of HFOs and their phase-amplitude coupling with theta, and these effects were reverted after washout of the antagonist.

Keywords

epilepsy, traumatic brain injury, stress, corticotropin-releasing factor, high frequency oscillations, phase-amplitude coupling

Summary for Lay Audience

Traumatic brain injury (TBI) causes lasting changes which make the brain susceptible to developing epilepsy. Stressful events that occur after TBI often trigger the occurrence of seizures and increase the likelihood that the brain will become epileptic. The exact interaction between the effects of TBI and stress that leads to epilepsy is unclear and needs investigation. It is known that the amygdala, a small region of the brain that plays a key role in processing stress, is often damaged due to the effects of TBI. Therefore, this phenomenon can be studied by measuring the activity of the amygdala that has been affected by TBI and stress. We can simulate these effects and measure the outcome using an animal model. For the experiments in this thesis, Sprague-Dawley rats were given a moderate TBI via a surgical process and a recording electrode was implanted in the amygdala of each rat. The brain-injured rats were stressed for short periods of time by applying a stressor called the tail pinch. Recordings of the amygdala during stress revealed the occurrence of high-frequency oscillations (HFOs), which are brainwaves that are implicated in the development of epilepsy. Furthermore, these HFOs were found to be interacting with another simultaneously occurring brainwave called the theta oscillation, which seemed to be acting like a pacemaker for the HFOs. This peculiarity, called phase-amplitude coupling, may be a contributing mechanism to the interaction between TBI and stress that leads to epilepsy.

Co-Authorship Statement

All data presented in this thesis was analyzed by the thesis author, under the guidance of supervisors and advisory committee. Part of the electrophysiological data presented were gathered by Chakrivarthi Narla, a PhD alumnus of the M.O. Poulter lab. The data presented in Figures 4 – 7 were published in *eNeuro* in April 2019 and are reproduced here with the permission of the authors.

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Table of Contents

Abstract	ii
Keywords	iii
Summary for Lay Audience	iv
Co-Authorship Statement.....	v
Acknowledgements.....	vi
Table of Contents.....	vii
List of Abbreviations	x
List of Figures	xi
Chapter 1	1
Chapter 1: Introduction	1
1.1 Epilepsy	1
1.1.1 History of Epilepsy	2
1.1.2 Seizures	3
1.2 Traumatic Brain Injury (TBI) and Post-Traumatic Epilepsy (PTE).....	4
1.3 Stress.....	5
1.3.1 Amygdala.....	6
1.3.2 Corticotropin-Releasing Factor (CRF)	7
1.4 Electrophysiology	8
1.4.1 <i>In vivo</i> Extracellular Recording	8
1.4.3 High Frequency Oscillations (HFOs)	10
1.5 Cross-Frequency Coupling (CFC) and Phase-Amplitude Coupling (PAC).....	11
1.6 Rationale	14
1.6.1 Hypothesis.....	14

Chapter 2.....	15
Chapter 2: Methods.....	15
2.1 Animals.....	15
2.2 Stereotaxic Surgery.....	16
2.2.1 Bilateral Electrode Implantation.....	16
2.2.3 Post-Operative Procedure.....	17
2.3 <i>In vivo</i> Recording Protocol.....	19
2.4 Data Analysis.....	20
2.4.1 Filtering for LFP Oscillations and HFOs.....	21
2.4.2 Wavelet Spectral Analysis.....	21
2.4.3 Modulation Index.....	23
2.4.4 Surrogate Analysis.....	25
Chapter 3.....	26
Chapter 3: Results.....	26
3.1 Stress in post-TBI amygdala produces increased electrographic responses, namely HFOs.....	26
3.2 Theta and HFOs are phase-amplitude coupled during stress in post-TBI amygdala.....	28
3.3 CRFR ₁ antagonist (CP 154526) disrupts theta-HFO coupling.....	30
3.4 TBI rats under stress show higher excitability than sham rats in 4kHz amygdala recordings.....	32
3.5 CRFR ₁ antagonism <i>in vivo</i> in post-TBI amygdala reduced electrographic response and HFOs during stress in 4kHz recordings.....	36
3.6 CRFR ₁ activity mediates fast ripples and PAC between theta and fast ripples during stressor in post-TBI amygdala in 4kHz recordings.....	38
3.7 Oscillation-triggered comodulogram shows underlying theta activity in fast ripple ranges.....	40

Chapter 4.....	42
4. Discussion	42
4.1 Stress Exacerbates Post-Traumatic Epileptogenesis	42
4.2 High-Frequency Oscillations are Increased and Phase-Amplitude Coupled during Stress	43
4.3 Altered Interneurons May Lead to Epileptiform Activity	44
4.4 Limitations.....	45
4.5 CRFR ₁ Antagonists as an Anti-Epileptic Drug	46
4.7 Conclusion.....	46
References.....	48
Curriculum Vitae	58

List of Abbreviations

HFO: High frequency oscillation

TBI: Traumatic brain injury

TLE: Temporal lobe epilepsy

PTE: Post-traumatic epilepsy

CRF or CRH: Corticotropin-releasing factor; also known as corticotropin-releasing hormone

CRFR₁: Corticotropin-releasing factor receptor type 1

CRFR₂: Corticotropin-releasing factor receptor type 2

LFP: Local field potential

CFC: Cross-frequency coupling

PAC: Phase-amplitude coupling

MI: Modulation Index

List of Figures

Figure 1: Electrographic recordings of sham and TBI amygdala during stress.....	27
Figure 2: Comodulogram of 10s segment during stress in TBI amygdala	29
Figure 3: PAC in TBI rat amygdala during stress in non-drug, CRFR ₁ antagonist, and washout	31
Figure 4: Stress induces a larger electrographic response in the amygdala of TBI than in sham rats.....	34
Figure 5: CRFR ₁ antagonist reduces HFO activity in TBI amygdala during stress.....	37
Figure 6: Comodulograms depict theta-HFO PAC in non-drug and washout conditions, but not after administration of CRFR ₁ antagonist.....	39
Figure 7: OTC shows coupling between theta and fast ripples that is attenuated by CRFR ₁ antagonist	41

Chapter 1

Chapter 1: Introduction

In brief, the goal of this thesis is to study the effect of acute stress on the excitability of the amygdala following a traumatic brain injury by measuring high-frequency oscillations. To understand the context behind this investigation, this thesis will start broadly with the definition of epilepsy, how traumatic brain injury leads to epilepsy, and how stress precipitates its development. Then, it will explore the methods in which this phenomenon can be studied, including the basics of electrophysiological extracellular recording, the definition of high-frequency oscillations, and the concept of cross-frequency coupling.

1.1 Epilepsy

Epilepsy is one of the most common chronic neurological disorders, affecting between 0.5-1% of the Canadian population (Tellez-Zenteno et al., 2004; Reid et al, 2012). It has a substantial impact on the quality of life of affected individuals and has an estimated cost of approximately \$13 billion each year (Begley and Durgin, 2015). Epilepsy is defined as a neurological condition in which the brain is abnormally susceptible to generating seizures. Seizures are transient occurrences of aberrantly excessive or synchronous neuronal activity that create abnormal behaviours including but not limited to jerking muscle movement, cognitive deficiencies, and loss of consciousness (Scharfman, 2007). The clinical definition of epilepsy is defined as a brain state that generates two or more unprovoked seizures that occurs more than 24 hours apart (Fisher et al. 2014). Unprovoked in this sense means that they were not evoked to occur by means of stimulation and are idiopathic. The process by which a healthy brain becomes epileptic

is complex and not fully understood. There is no ‘one cause’ that leads to epilepsy; indeed, many different mechanisms may be involved in producing many different epileptic brains. Different forms of epilepsy have different manifestations and implications for treatment and management. This speaks to the significant undertaking we have in understanding the disorder and to the humble paucity of understanding that we currently have of this condition and to the workings of the brain.

1.1.1 History of Epilepsy

Epilepsy has been witnessed and studied for millennia and had been the subject of much mysticism and wonder. Early shamans and healers who observed people spontaneously convulsing thought them to be possessed by evil spirits. In fact, the word “seizure” originates from the purported seizing of a person’s mind and body by the gods as a form of punishment for immoral acts or simply for their wanton will. Those afflicted by epilepsy were perceived as unholy or morally defective and were treated as lesser citizens (Magiorkinis et al, 2010).

One of the first recorded cases that referred to a biological origin to epilepsy was made by Hippocrates in 400BC (Temkin, 1933). He described epilepsy as the result of an imbalance of humours, a prevailing theory of physiology at the time, rather than due to godly intervention or a failure of moral character. Moreover, he astutely linked epilepsy to a malfunction in the brain, a monumentally innovative insight in a time when the brain was believed to be merely a large vessel for cooling the blood, and the seat of consciousness was thought to reside in the heart (Laskaris, 2002). He wrote that we “ought to know that from nothing else but the brain come joys, delights, laughter and sports, and sorrows, griefs, despondency, and lamentations”, and that

“by the same organ we become mad and delirious”, which had a prevailing impact for understanding epilepsy and the brain in general (Hippocrates, 400 BC).

Our modern understanding of epilepsy begins in the 19th century with the seminal work of Bouchet and Cazauvieilh (1825) examining cadavers affected by epilepsy, specifically observing profound structural changes in the brain. Jackson (1899) provided descriptive clinical details of seizures and suggested possible mechanisms that became foundational to the study of epilepsy. Technological advances in the 20th century, notably the invention of the electroencephalogram (EEG), allowed for further study of brains and seizures (Jasper et al, 1951). Parallel to these breakthroughs, the fundamentals of nerve function were elucidated by Hodgkin and Huxley (1952) advancing knowledge about the brain and epilepsy. Present day research in epilepsy builds upon the technological and anatomical foundations presented by these seminal works.

1.1.2 Seizures

Seizures are sudden and transient occurrences that last from 30 seconds to minutes at a time that are the result of uncontrolled bursts of neuronal activity in the brain. This activity has been found to be the result of abnormal synchronized action potential firing in a group of neurons (Bromfield et al, 2010). The excessive excitatory signalling stems from a perturbation in the normal balance between excitation and inhibition of neuronal firing (Scharfman et al, 2007). This imbalance can be caused by an over-excitation of excitatory neurons or by a failure of the inhibitory interneurons that normally attenuate excessive firing. The epileptic brain is thus characterized by networks of neurons that are configured in a hyperexcitable manner.

The activation of these hyperexcitable networks results in seizures, which are categorized into two main groups: generalized seizures and focal seizures (also called partial seizures). In generalized seizures, neurons in multiple regions of the brain fire excessively together and result in convulsions and loss of consciousness. Focal seizures occur when the abnormal firing is localized in a unilateral region in the brain and can cause hallucinations or fugue states (Scheffer et al., 2017). The region in which the focal seizure is localized is called the epileptic focus. Temporal lobe epilepsy (TLE) is the most common type of epilepsy involving focal seizures (Télliez-Zenteno and Hernández-Ronquillo, 2011).

1.2 Traumatic Brain Injury (TBI) and Post-Traumatic Epilepsy (PTE)

One of the leading causes of the development of temporal lobe epilepsy is traumatic brain injury (TBI; Yeh et al, 2013; Xu et al., 2017). TBI refers to an external force that injures the brain and the subsequent changes that occur. Compared to general population, individuals affected by a TBI have on average a 3-fold higher risk of developing TLE (Martin et al., 2014). Epilepsy that occurs following TBI is referred to as post-traumatic epilepsy (PTE) and accounts for 20% of cases of acquired epilepsy (Agrawal et al., 2006). The exact reason for the development of PTE remains unclear, but the link between TBI and the development of epilepsy is well documented (Rehman et al., 2015; Chen et al., 2017). It is known that acutely, TBI causes tissue deformation leading to damage to neurons, glia, and blood vessels which may result in a seizure immediately following injury (Mendelow and Crawford, 1997; Laurer et al., 2000). There are many mechanisms that could explain why this acute seizure occurs (Hunt et al., 2013). It is commonly thought that a primary driver of acute seizures is an immediate excessive release of glutamate in response to the injury followed by a significant increase in extracellular calcium concentration

that causes excitotoxicity and neuronal cell death (DeLorenzo et al., 2005). However, not all cases of TBI lead to an acute seizure, and the occurrence of an initial seizure is not predictive of the development of PTE or its severity (Aroniadou-Anderjaska et al., 2008). Following the TBI, there is a latent period that is seizure-free that lasts from two weeks to months or even years. During this period, neurons and glia undergo a cascade of molecular, cellular, and conformational changes, including inflammatory responses, perturbed cellular calcium homeostasis, formation of scar tissue called cicatrix, and structural alterations to neural circuitry such as axonal sprouting and neurogenesis (Mendelow and Crawford, 1997; Hunt et al., 2013). This secondary damage is thought to lead to a disturbance in the brain's ability to maintain the normal balance of excitatory and inhibitory neurotransmission, and is called epileptogenesis, or the gradual process by which a brain becomes epileptic (Herman, 2002). After this period, seizures may occur repeatedly and these late seizures are understood to be due to a lasting change in the brain's structure (Hunt et al., 2013). Further investigation of the mechanisms of epileptogenesis following TBI are required as they are poorly understood in current literature.

1.3 Stress

Stress is a complicated response that involves a cascade of hormonal and behavioural responses that ready an organism to adapt to perceived or actual dangers (Paykel, 1979). Notably, a heightened level of stress is a precipitating factor for seizures after TBI (Reddy and Rogawski, 2002) with stress being the most commonly reported trigger for seizures in epileptic patients (Joëls, 2009). Numerous case studies have shown that patients comorbid with TBI and a stress disorder have a notably high risk of developing epilepsy (Arborelius et al., 1999; Pompili et al., 2005; Hetteema et al., 2006). Specifically, war veterans with TBI and PTSD are a subpopulation

that has one of the highest odds of developing PTE (Salazar et al., 1995). Neural processes that generate and regulate responses to stress are poorly understood and the mechanism by which stress exacerbates epileptogenesis in PTE is the subject of much investigation.

1.3.1 Amygdala

The amygdala is a small almond-shaped subcortical structure that resides in the temporal lobe and exhibits increased activity in response to stress. Stress affects neuronal activity in many brain regions, including the amygdala which is well recognized for its central role in the expression and modulation of emotion (Davis and Shi; 1999). The amygdala is integral in processing emotional responses and providing an affective context to sensory processes and is thus a canonical site of study for responses to stress such as conditioned fear and anxiety (Benini and Avoli, 2005; Aroniadou-Anderjaska et al., 2007).

The amygdala is often implicated in epilepsy as an “epileptic focus”, or a region from which epileptic neuronal activity originates and spreads to other structures (Lv et al., 2014). Extensive damage is present in the amygdala in a significant subpopulation of epileptic patients, especially in those with a TBI (Aroniadou-Anderjaska et al., 2007). Epileptic patients with amygdalar damage often report an “aura” that occurs before the onset of seizures, which are premonitory changes in emotion or sensation that alert the patient that a seizure is about to occur such as sudden sensations of terror and anger (van Elst et al., 2009). These clinical presentations of stress processed by the amygdala are also supported by evidence from animal models, which has shown that the amygdala is hyperactivated in stressed states (Roosendaal et al., 1997; Pitman et al., 2012). Thus, the amygdala provides a site of study that facilitates the investigation of the effects of both stress and TBI on epileptogenesis.

1.3.2 Corticotropin-Releasing Factor (CRF)

Corticotropin-releasing factor (CRF) is a 40 amino acid neuropeptide that is implicated in the response to stress, including in several psychiatric disorders such as anxiety, panic disorder, PTSD, and depression (Arborelius et al. 1999; Boyer 2000). It is released ubiquitously in the central nervous system, notably by neurons of the central amygdala. Intracerebroventricular administration of CRF results in seizures and epileptiform activity in the amygdala in rats (Weiss et al. 1986). Moreover, repeated bouts of CRF-induced seizures were found to make the amygdala more and more susceptible to producing a seizure via electrical stimulation (Arborelius et al., 1999). CRF acts with high affinity on type 1 CRF receptors (CRFR₁), a G-protein-coupled receptor, and with low affinity on type 2 CRF receptors (CRFR₂), both of which are highly expressed in the basolateral amygdala (Dedic et al., 2018). The action of CRFR₁ neurons is predominately involved in the control of anxiety-like behaviour (Merali et al., 2004). Previous work has shown that in epileptic piriform cortex, a structure that is highly interconnected with the amygdala, activation of CRFR₁ augments principal cell excitation. This alteration in function appears to be due to a change in the signalling pathway activated by the CRFR₁ following epileptogenesis, which occurs through attenuated expression of a regulator of G-protein signalling protein type 2 (Narla et al., 2015). These receptors are structurally and functionally similar to those found in the amygdala, and an analogous alteration may take place in epileptic amygdala (Fu & Neugebauer 2008). In summary, CRFR₁ activation in the amygdala is associated with the response to stress and is implicated in epileptogenesis.

1.4 Electrophysiology

Galvani (1791) discovered that neuronal functioning could be assessed based on its electrical activity. By using metal, glass, or silicon electrodes to measure the transmembrane voltage changes that occur with the flux of ions between neurons and their extracellular environment, the workings of neurons could be measured with a high signal-to-noise ratio. This method is called electrophysiology and has been regarded as the “gold standard” for studying neurons for the past century (Scanziani and Häusser, 2009). Since Galvani, the experimental techniques and equipment have been improved and refined, but the fundamentals remain the same.

The current most common method of observing large-scale neuronal signalling in humans is through the electroencephalogram (EEG), which uses an array of macro-electrodes placed on the scalp to pick up summed electrical signals that are conducted from the cortex and subcortical structures through the skull and to the scalp (Varela et al., 2001; Steriade, 2006). While this method is the least invasive type of electrophysiology, it is also less specific temporally and spatially in assessing neuronal activity than more precise methods such as *in vivo* extracellular recording.

1.4.1 *In vivo* Extracellular Recording

There are always trade-offs in choosing a method of recording electrical signals from the brain. Typically, as signals get more exact temporally or spatially, they also become more invasive and costly. This trade-off can be worth the improved signal, often justifying the more invasive *in vivo* recording, or implanting electrodes surgically in live brain tissue. This is a method that can gather information about neuronal workings at a higher signal-to-noise ratio and with more

specificity than EEG recordings. These electrodes lie in the extracellular space between neurons and it is thus called an extracellular recording (Heinricher, 2004).

The flow of ions that occurs during neural functioning results in an electric field that is conducted through the extracellular space. An extracellular recording captures the voltage changes of this electric field, and results in oscillations that exist within a frequency range called the local field potential (LFP; Buzsaki and Draguhn, 2004). The LFP is a complex phenomenon that gives a window into the mechanisms of neuronal communication (Engel, 2001; Canolty and Knight, 2010). It is the summed electrical activity that is comprised of the rhythmic discharge of groups of neurons and can influence and coordinate the timing of neuronal firing (Buzsaki and Watson, 2012). Within the LFP, there are several discrete oscillations that are delineated into small bands of frequency ranges. These oscillations are highly conserved in mammals, birds, and fish and have been shown to have distinct roles in normal functioning (Buzsaki and Draguhn, 2004; Fries, 2009). For example, the theta oscillation (4 – 8 Hz) has been implicated in important and complex brain functions such as memory consolidation and navigation in the hippocampus and prefrontal cortex (Canolty et al., 2006; Colgin, 2013). While the exact function and mechanism of theta and other rhythms are currently unknown, it is undeniable that these LFP oscillations are pervasive throughout the brain and play a key role in higher-level functions.

1.4.3 High Frequency Oscillations (HFOs)

High frequency oscillations (HFOs) are oscillations in the broadband signal that have a much faster frequency (>120 Hz) than those that are found in the normal range of an LFP (Jacobs et al., 2012). They are transient and local phenomena that are thought to be the manifestations of the bursts of neuronal activity that characterizes seizures. These bursts are the result of hypersynchronous firing of action potentials of excitatory pyramidal cells which are ubiquitous in cortex and in limbic structures such as the hippocampus and amygdala (Bragin et al., 2004; Haufler and Pare, 2014). HFOs occur on a much smaller spatial and temporal scale than LFP oscillations and can be specific to 1mm^3 of brain tissue and a millisecond timescale (Staba et al., 2017). There is an abundance of evidence that supports the presence of these HFOs before, during, and after seizures in epileptic animal models as well as in epileptic human patients (Ponomarenko et al, 2003; Bragin et al., 2004; Jefferys et al., 2012). The presence of HFOs in a specific area of brain tissue is considered a biomarker for epileptic tissue. Resection of brain tissues that display HFOs in human patients has been shown to ameliorate seizure outcomes in cases of epilepsy refractory to medication (Rosenow and Lüders, 2001). Notably, resection of epileptic amygdala has been more successful than resections of epileptic hippocampus in decreasing epileptic activity (Boling, 2018).

HFOs are generally categorized into two types: ripples (120 - 250Hz) and fast ripples (250 - 500Hz; Jiruska et al., 2017). The exact mechanism of HFO production is under investigation, but both types are present in epileptic tissue and reflect hyper-excitabile network dynamics. However, ripples can be present during normal processes such as in REM sleep and consummatory behaviour, while fast ripples are more often exclusive to epileptic states (Jefferys et al., 2012).

Ripples are caused by the rhythmic and synchronous firing of action potentials in an ensemble of pyramidal cells. However, physiological limits, such as the absolute refractory period during repolarization after an action potential, prevent firing of individual neurons at fast ripple frequencies (Ibarz et al., 2010). Therefore, the occurrence of fast ripples by the firing of one synchronous group of neurons is unlikely. Indeed, fast ripples are believed to arise from alterations in the network dynamics of these pyramidal cells (Bikson et al., 2003; Jiruska et al., 2010). For example, loss or damage to pyramidal cells in a cluster of neurons could prevent the normal synchronization of these cells through gap junctions, leading to two disparate synchronously firing groups that are offset from each other that results in an oscillation at fast ripple frequencies (Roopun et al., 2010). As well, two offset firing groups could result from a change in the morphology of axons that normally provide an input into the cluster of pyramidal cells due to axonal sprouting or damage (Ibarz et al., 2010). Finally, frequencies at fast ripples could occur by the rapid firing of a group of neurons that lack the normal level of synchrony, resulting in a chaotic firing pattern. While it is unclear which of these proposed mechanisms is predominant in PTE, it is evident that fast ripples are a marker for a hyperexcitable epileptic group of neurons (Thomschewski et al., 2019).

1.5 Cross-Frequency Coupling (CFC) and Phase-Amplitude Coupling (PAC)

Cross-frequency coupling (CFC) is a phenomenon that is thought to contribute to complex functions such as memory and decision making (Canolty and Knight, 2010). CFC is the coupling of a higher frequency signal to a lower frequency signal of a different origin. The idea is that these two waves work together; the slower and broader low frequency wave that exists across a

large population of neurons coordinates the faster and more locally occurring high frequency wave (Tort et al., 2008; Aru et al., 2015). If the two waves that are coupled together arise from different biophysical origins, the coupling is not likely to have spurious correlations based on volume conduction or synchronized noise (Dvorak and Fenton, 2014). Phase-amplitude coupling (PAC) is a type of CFC in which the phase of a low frequency wave drives the amplitude of a higher frequency wave. For example, bursts of high frequency wave activity could have a rhythmic pattern of occurring on the troughs of a low frequency wave (Ibrahim et al., 2014). PAC has been studied in rodents, non-human primates, and in humans and plays a key role in integrating information within networks during complex functions such as memory encoding and selective attention (Canolty and Knight, 2010).

Tort et al. (2010) developed a method which quantifies the intensity of PAC using a measure called the Modulation Index (MI). This index is able to detect coupling between two frequency ranges of interest. It measures the magnitude of nesting of the amplitude of one frequency, called the “amplitude-modulated frequency”, within the phase of another frequency, called the “phase-modulating frequency”. The measure compares the distribution of the amplitude of the amplitude-modulated frequency among discrete and uniform bins of the phase of the phase-modulating frequency. This distribution is compared to a null distribution using a premetric called the Kullback-Leibler distance, which is a widely used statistical tool to calculate the difference between two probability distributions. The null distribution used in the calculation of the MI is flat, where amplitude is spread out perfectly evenly over each phase bin, representing an absence of any coupling. The MI value ranges from 0 to 1, in which a value of 0 indicates that the distribution is perfectly similar to the flat distribution, while a value of 1 indicates that the amplitude oscillation exists only in one phase bin, both of which are physiologically unlikely

extremes. The MI value must be interpreted against a surrogate value that is generated by random shuffling of frequency pairings for the specific experiment in order to determine the significance of that value.

1.6 Rationale

TBI often leads to the development of refractory epilepsy. The severity and occurrence of seizures in PTE is exacerbated by events of stress. The interaction between TBI and stress in epileptogenesis is poorly understood and can be investigated using *in vivo* extracellular recordings in the amygdala. This thesis investigates the generation of HFOs by CRFR₁ neurons and possible PAC of these HFOs to LFP oscillations during stress in the amygdala following TBI.

1.6.1 Hypothesis

In an altered amygdala affected by the secondary effects of TBI, the occurrence of a stressor is hypothesized to cause increased excitability through activation of CRFR₁, resulting in the production of pathophysiological HFOs. Furthermore, it is hypothesized that an underlying LFP oscillation supports the generation of these HFOs through PAC.

Chapter 2

Chapter 2: Methods

2.1 Animals

Adult male Sprague-Dawley rats weighing 150 – 180 g were used in all experiments. They were housed individually with free access to food and water under a continuous 12/12 h light/dark cycle. Ketamine medetomidine hydrochloride (0.1mL/100g) was given intraperitoneally as anaesthetic. The rat is placed in an anaesthetic chamber and gas isoflurane (at 5% concentration) is administered in a stream of oxygen at 2-4L/min until the rat is recumbent and unresponsive to stimuli. The rat is then given an injection of buprenorphine (0.05Mg/kg) subcutaneously as a further anaesthetic and taken out of the chamber to have its site of surgery shaved with a hair clipper. The appropriate plane of anaesthesia is verified by testing the withdrawal reflex (toe-pinching) and palpebral reflex (eyelid closing in response to stimulus). Breathing is constantly visually monitored. Temperature is maintained at 37°C using a heating pad and monitored with a rectal thermometer. The front incisors of the rat are clamped onto the bite bar of the stereotaxic apparatus, and ear bars covered with xylocaine gel (topical analgesic) are placed in the ear canals to stabilize the head and ensure the top of the head is level. Eye ointment (LacriLube) was applied throughout the procedure to prevent irritation and drying of the eyes. Throughout surgery, gas isoflurane is titrated to keep the rat at the surgical plane of anaesthesia (1.0 to 2.0% isoflurane concentration).

2.2 Stereotaxic Surgery

The shaved surgical area is cleaned with povidone-iodine, 80% ethanol, and bacteriostat. A vertical scalpel incision is made from between the eyes to the interaural line, about a 6 to 8mm longitudinal cut along the midline. Blood and tissues on the skull are wiped away using cotton swabs and gauze. The surgical site is held open using sterile tissue clamps that clip onto and peel back the subcutaneous membrane. The fascia is scored and gently removed with a scalpel. Bregma is located and used as the origin coordinate for the stereotaxic system.

A small hole about 1.0 mm in diameter is drilled 2.0 mm anterior to bregma and 4.8 mm lateral to the midline and carefully cut out of the skull. The piece of the skull from the craniotomy is kept moist in a saline solution for later reattachment. The pneumatic impactor (Precision Science Instruments) is placed over the craniotomy site and the parameters of the machine are set to simulate moderate TBI. The injury parameters consisted of 2.5-mm cortical compression at a speed of 3.5 m/s for 500 ms (Dixon et al., 1991). The skull piece is then reattached over the area using VetBond tissue glue. Sham animals received a craniotomy in the same fashion but did not receive an impact to the brain. The vitals and plane of anaesthesia of the rat are carefully monitored throughout the process.

2.2.1 Bilateral Electrode Implantation

The site of electrode implantation in the amygdala, 2.6 mm posterior to bregma and 4.8 mm lateral to the midline, is found and marked using a pencil (Paxinos and Watson, 1986). 2 mm burr holes are made in these spots, as well as in five other spaced out spots on the skull (one in

each quadrant separated by the coronal suture and the sagittal suture, and one below lambda). The grounding jeweller's screw is placed in the top-left burr hole, and the other five holes are filled with regular jeweller's screws. The electrodes were made of two twisted strands of 0.127 mm diameter diamel-insulated nichrome wire and were attached to male amphenol pins. The electrode is affixed to the crossbar on the stereotax and lowered slowly into the burr hole at one of the electrode sites until a depth of 8.0 mm (Paxinos and Watson, 1986), and then slightly withdrawn to relieve stress in the tissue at the tip of the electrode. Dental acrylic is then used to keep the electrode in place.

The implanted electrode and grounding wire are inserted into a plastic headplug, and then the skull and jeweller's screws are covered with dental acrylic, so that only the top of the headplug is left uncovered. The headplug allows for contact with the electrodes by the recording device during experiments while keeping them safe and out of reach at other times. The acrylic is allowed to dry and then the remaining skin gap below the site is sutured. The rat is removed from the ear bars and bite bar of the stereotax. Topical application of bupivacaine gel is applied on the margins where the acrylic meets the head of the rat. The rat is given 1 mL/kg of saline subcutaneously after surgery for hydration and allowed to regain consciousness under a heating pad in a clean cage. The procedure lasts approximately 60 - 90 minutes.

2.2.3 Post-Operative Procedure

Rats are kept in clean recovery cages with Bed-o-Cob bedding for the first 24 hours after surgery. Water and food pellets are readily provided. Meloxicam (1.5Mg/kg) is given subcutaneously once a day for two days following surgery as analgesic. As well, buprenorphine

(0.05Mg/kg) is given every 8 hours for up to two days after surgery. The rats are examined everyday for one week post-surgery for normal grooming, behaviour, weight gain, and adaptation to their headcap. These details are recorded on the post-op sheet. After one week, the weight and condition of the rat is recorded once a week. If discomfort is noticed, buprenorphine (0.05Mg/kg) is administered once every eight hours as needed to relieve pain.

2.3 *In vivo* Recording Protocol

Both TBI and sham surgery rats underwent the same recording protocol at least one week after surgery. During this protocol, a rat was transferred to a recording chamber and connected to the head-cap of the recording system. Following 5 min of acclimatization to the chamber environment, baseline measurement of the brain activity was taken via the implanted electrodes for 2 min (after amplification using a Grass instruments Model 55 preamplifier; a Molecular Devices Digidata 1550 A/D converter; sampling rate = 4 kHz). Following this 2 minute segment, a paper clip of moderate strength was placed on the middle of the tail of the animal to act as an acute stressor. The tail was covered with a paper towel or a clean cotton rag before the clip was applied to prevent pain and local damage of the tissue. This stressor was applied for 2 min, and at the end of this time, the clip was removed. The recording continued for a further 2 min in the absence of stress. This procedure is not considered to be painful.

6-minute electrophysiological recordings were taken every two days after recovery from surgery. These recordings consisted of 2 minutes of baseline, followed by 2 minutes of stress, then 2 minutes of post-stress. Stress was delivered by applying a tail pinch using a binder clip affixed to the midpoint of the tail. After three weeks, rats were given CP 154526 via subcutaneous injection (50mg/kg) before recording, to observe the effects of the CRFR₁ antagonist.

2.4 Data Analysis

Recordings were analyzed using MATLAB 2018a (MathWorks). To visualize the spectral responses that occurred during the stressor, the raw signal was filtered using FIR filters or convolved with complex Morlet wavelets to extract frequency-specific power and to transform the signal into a time-frequency representation. The Morlet wavelets were constructed with seven wavelet oscillations (Tallon-Baudry et al., 1997):

$$w(t, f_0) = A \exp(-t^2/2\sigma_t^2) \exp(2i\pi f_0 t),$$

where w is the morlet wavelet, t is time, and σ describing the Gaussian shape around f_0 , the central frequency, with

$$\sigma_f = 1/2\pi\sigma_t$$

$$f_0/\sigma_f = 7$$

and the normalization factor A equal to

$$(\sigma_t \sqrt{\pi})^{-1/2}$$

The recordings were also visually checked for spikes that when filtered may produce artifacts resembling fast ripples (Béнар et al., 2010).

2.4.1 Filtering for LFP Oscillations and HFOs

To examine the average responses of the two groups across the entire HFO range, the recordings were high pass filtered at 120 Hz using a FIR filter. Power spectra were then generated for the baseline and stress response. The two spectra were then subtracted and normalized. For the five rats analyzed in each group, sham and TBI, these subtracted and normalized spectra were then averaged to generate an aggregate response for these two groups.

2.4.2 Wavelet Spectral Analysis

Analyses were done using MATLAB 2018a (MathWorks). For the results shown in Figures 1 – 3, the raw signal was filtered into LFP oscillations, ripple, and fast ripple ranges using band-pass FIR filters. For Figures 4 -7, raw signals were filtered into oscillations using convolution with complex Morlet wavelets. To extract low frequency phase, the real component of the result of convolution of the raw signal with wavelets (centered at 1 – 21 Hz in steps of 2 Hz) was taken. The amplitude envelope of higher frequencies was extracted by squaring the result of convolution of the raw signal and wavelets (centered at 20 – 500 Hz in steps of 10 Hz). Peaks of high power activity during the stress epoch of the recording within band-specific frequencies were found. Peaks of high power were defined as areas in the spectrogram space that had amplitudes greater than >95% of the whole 6 minute recording. One-second windows centered on each high power peak were analyzed for their strength of phase-amplitude coupling using the modulation index (MI; Tort et al., 2010). A window length of 1 s was chosen because this length matched the duration of HFO bursts during stress. The HFO amplitudes within each window were normalized and then binned into co-occurring theta phases spaced into 20° segments. The normalized mean high frequency amplitude value at each low frequency phase bin, known as the

p_j value in the calculation of the MI (Tort et al., 2010), for each high-power window for all rats in each drug condition were pooled. Pooling these 1 s windows found over the 2 min stress epoch gives a more robust estimation of coupling than simply calculating the MI over a longer time window, especially during the drug condition, in which the signal-to-noise ratio is much lower due to reduction in occurrence and amplitude of HFO events. The pooled normalized values were used to calculate the MI at each frequency pairing during each drug condition and are represented in comodulograms showing preferred frequency of coupling in Figure 6.

Oscillation-triggered comodulograms (OTC) were created by summing 1s of raw recordings centered on the high power peaks to reveal underlying rhythmic low-frequency activity (Dvorak and Fenton, 2014; Samiee and Baillet, 2017) as shown in Figure 8. The OTC is a measure of PAC that requires fewer assumptions on the data and reveals if there is a strong underlying slow wave in the activity of another frequency of interest and is used here to augment the evidence provided by the MI analysis.

2.4.3 Modulation Index

The following steps were performed to calculate the MI value, as adapted from Tort et al. (2010).

1. First, the raw signal, $x_{raw}(t)$, is filtered at the 2 frequency ranges under analysis (f_p and f_A). The filtered signals are denoted as $x_{fp}(t)$ and $x_{fA}(t)$.
2. The time series of the phases of $x_{fp}(t)$ [denoted as $f_{p(t)}$] is obtained taking the real component of the result of convolution with a 7-oscillation Morlet wavelet. The square of the result of convolution was taken as the time series of the amplitude envelope of $x_{fA}(t)$ [denoted as $A_{fA(t)}$] The composite time series [$f_{p(t)}$, $A_{fA(t)}$] is then constructed, which informs the amplitude of the f_A oscillation at each phase of the f_p rhythm.
3. The phases $f_{p(t)}$ are binned into eighteen 20° intervals (0° to 360°), and the mean of A_{fA} over each phase bin is calculated. We denote as $A_{fAfp(j)}$ the mean A_{fA} value at the phase bin j .

4. We then apply the entropy measure H , defined by:

$$H = - \sum_{j=1}^N p_j \log p_j,$$

where $N = 18$ (i.e., the number of bins) and p_j is given by

$$p_j = \frac{\langle A_{f_A} \rangle \phi_{f_p}(j)}{\sum_{j=1}^N \langle A_{f_A} \rangle \phi_{f_p}(j)}.$$

5. The MI is finally obtained by normalizing H by the maximum possible entropy value

(H_{max}), which is obtained for the uniform distribution $p_j = 1/N$ (and hence

$$H_{max} = \log N):$$

$$MI = \frac{H_{max} - H}{H_{max}}.$$

Comodulograms are then obtained by systematically matching each $f_{p(t)}$ to each $A_{f_A(t)}$, creating all possible composite pairings and then representing the MI value in a pseudocolour plot.

2.4.4 Surrogate Analysis

Surrogate analyses were performed via shuffling the phase of one window with the amplitude of another window ($n = 10,000$) in the frequency pairing of interest and then averaging the calculated MI in each of those shuffled outcomes. Custom MATLAB scripts were written for these analyses and are available upon request.

Chapter 3

Chapter 3: Results

In contrast to mice, rats in this TBI model rarely develop spontaneous seizures (Bolkvadze and Pitkänen, 2012). Nevertheless, as reported in the past (Pitkänen et al., 2009, 2014), injured rats do have epileptiform activity. We wanted to determine whether TBI rats would respond to an acute stressor differently than sham rats and whether electrophysiological responses from the stressor responsive nuclei, the amygdala, may be affected. The acute tail pinch was used to deliver a stressor to rats while recording from the amygdala (Gibb et al., 2008).

3.1 Stress in post-TBI amygdala produces increased electrographic responses, namely HFOs

Extracellular electrophysiological recordings were taken in the amygdala in both TBI rats ($n = 6$) and sham surgery rats ($n = 4$). As shown in Figure 1, the recording protocol for each trial was 6 minutes in length: 2 minutes of baseline activity, followed by 2 minutes under stress using an acute tail pinch, and then 2 minutes of the response in the absence of the stressor. The tail pinch was used because it is a simple and effective way to deliver an acute, easily terminable stressor that is not considered to be painful. Sham and TBI rats were equally agitated by the stressor and attempted to remove the clip from their tail. In sham surgery animals, the stressor resulted in a mild increase in activity, likely reflecting processing by amygdala nuclei involved in stress (Fig 1A). Comparatively, in the TBI animal (Fig 1B), the same stressor resulted in a larger increase in

activity. HFOs were a prominent of this increase in activity during stress, as shown when the raw signal is filtered by FIR filter into HFO ranges (>120 Hz).

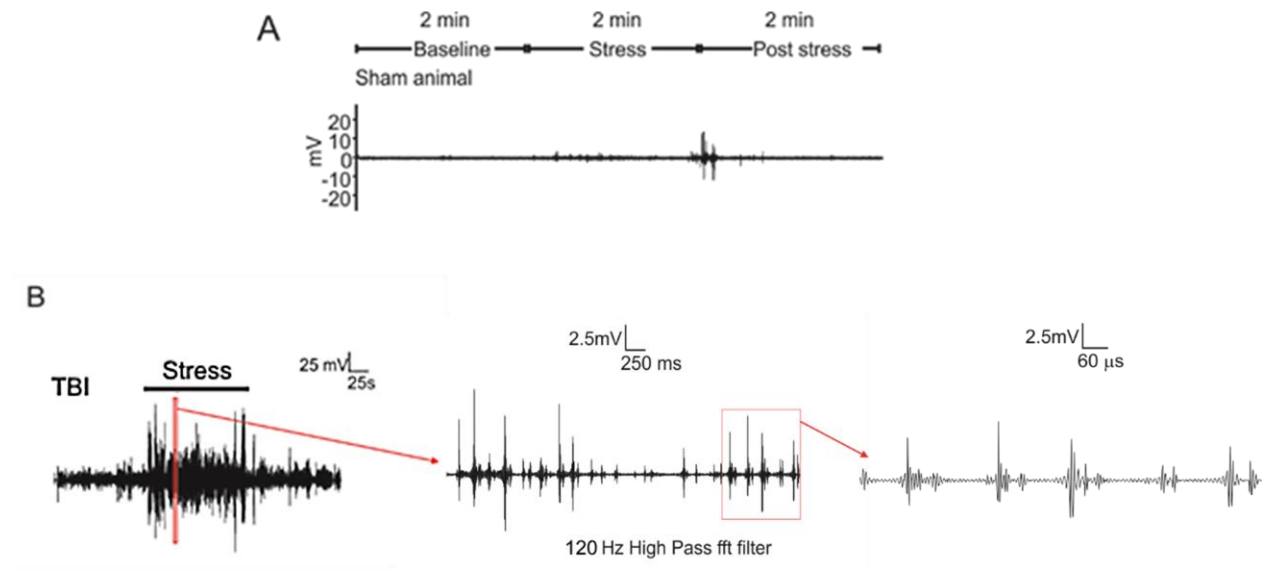


Figure 1: Electrophysiological recordings of sham and TBI amygdala during stress. **A**, Sham rats show mild increases in electrophysiological activity under acute stress. **B**, Comparatively, TBI animals show a larger increase in activity during application of the stressor. The area delineated by the red box is enlarged and high pass filtered at 120Hz and shown at the arrowhead. It is further enlarged again to depict the HFOs that are present in the electrical response to the stressor. Scales are provided to depict the magnitude of time (milliseconds) in the x-axis and of voltage change (mV) in the y-axis.

3.2 Theta and HFOs are phase-amplitude coupled during stress in post-TBI amygdala

In Figure 2, 10s segments of high HFO occurrence during stress in TBI rats were visually chosen for analysis after filtering the raw signal into HFO frequencies (>120 Hz). Then, a comodulogram was generated for each segment (Fig 2A) by systematically analyzing the coupling between each possible pairing between phase-modulating frequencies in the LFP [delta (0.5 – 4 Hz), theta (4 – 8 Hz), alpha (8 – 12 Hz), beta (12 – 16 Hz), low gamma (30 – 80 Hz), high gamma (80 – 120 Hz)] with amplitude-modulated frequencies [gamma (30 – 120Hz), ripple (120 – 250 Hz), fast ripple (250 – 500 Hz)]. The frequency ranges here were extracted by filtering the raw signal with bandpass FIR filters. These revealed theta as the dominant phase-modulated rhythm, and HFOs in both the ripple and fast ripple ranges as the amplitude-modulated rhythm.

The recordings shown in Figures 1 - 3 were taken at a sampling rate of 1000 Hz as part of a preliminary exploration into this phenomenon. While the Nyquist frequency of the initial sampling rate is 500 Hz and is theoretically able to detect frequencies up to the upper limit of fast ripple frequencies (250 – 500 Hz), a sampling rate of at least four times the highest frequency of interest is recommended to decrease aliasing that occurs in filtering. Thus, in further analyses presented in Figures 4 - 7, the sampling rate was increased to 4000 Hz. This increased resolution aided detection of fast ripples and differentiation between ripple and fast ripple activity.

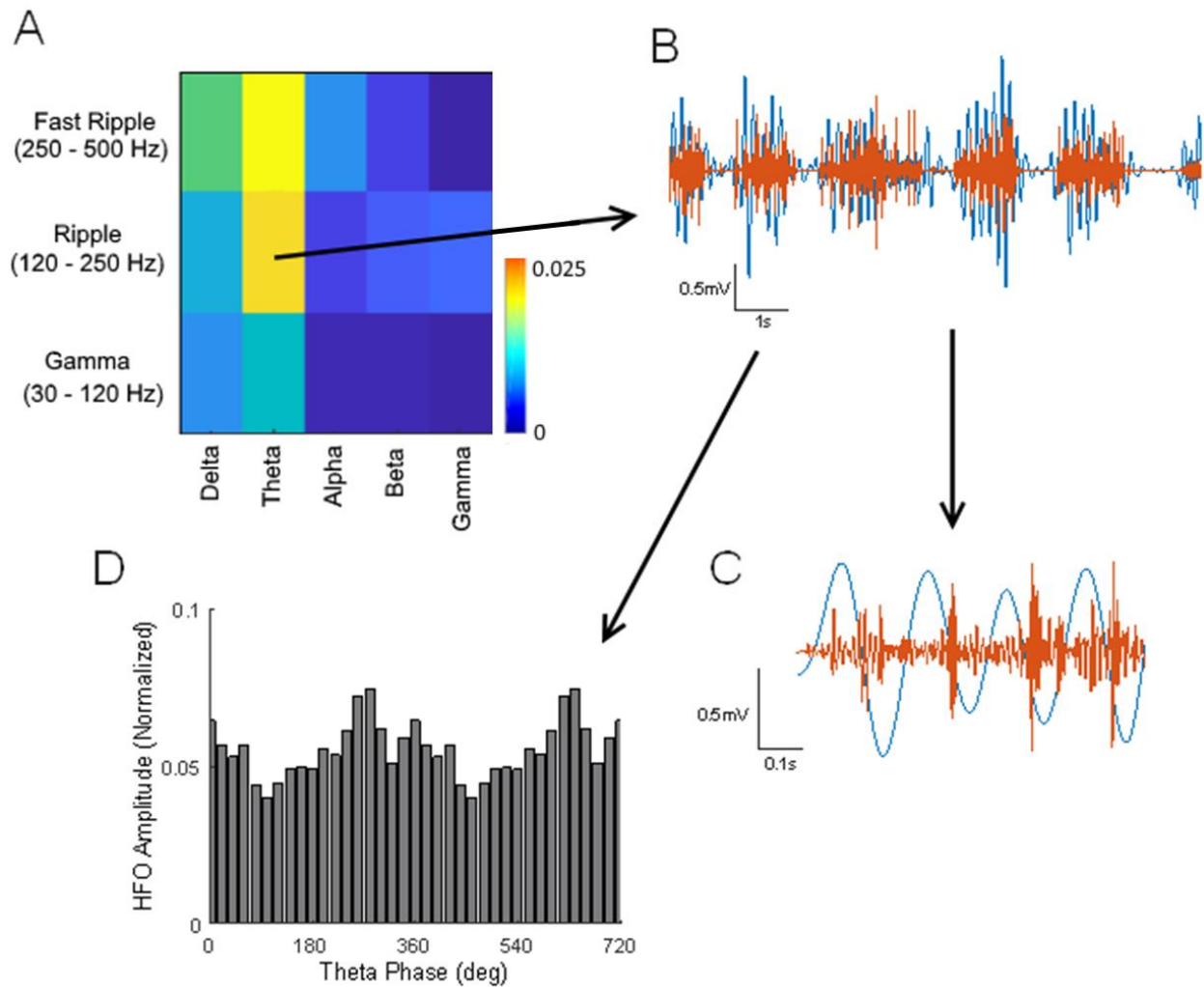


Figure 2: Comodulogram of 10s segment during stress in TBI amygdala. (A) Pseudocolour plot of MI between low frequency phase-modulating rhythm (y-axis) and high frequency amplitude-modulated rhythm (x-axis) during the 10s segment. Warmer colours represent higher magnitude of coupling. Highest coupling in this example was between theta (4 - 8 Hz) and ripple (120 - 250 Hz) range, followed by coupling between theta and fast ripple (250 – 500Hz). **(B)** The 10s segment of recording during stress filtered into theta (blue) and fast ripple (orange) waves that were used to generate the MI value represented in the square of the comodulogram indicated by the arrow. **(C)** Zoomed-in window of a 1s segment of B, showing that bursts of fast ripple amplitude occur preferentially on the falling edge of the theta phase. **(D)** Phase-amplitude plot of 10s segment in B, which shows that HFO amplitude mildly prefers the 280 – 300° phase bin.

3.3 CRFR₁ antagonist (CP 154526) disrupts theta-HFO coupling

In order to investigate the effect of the acute stressor on CRFR₁ activation, a protocol of recording with and without CP 154526, a CRFR₁ antagonist, was established, as shown in Figure 3. A recording of TBI rats with the same recording protocol was taken before administration of the drug, then 3h after intraperitoneal injection (50 mg/kg) of the drug, then post-washout 24h after drug administration. In these recordings shown in Figure 3 A-C, the signal was filtered into theta (4 – 8 Hz; blue) and non-specific HFO (120 – 500 Hz; orange) ranges, chosen due to their high level of coupling. The MI was calculated in these 10s segments during stress and averaged per drug condition. In non-drug recordings, as expected, high amplitudes of HFOs were demonstrably phase-locked to theta ($MI = 1.82 \times 10^{-2} \pm 0.006$). After the drug was applied, the phase-amplitude coupling between theta and HFOs were markedly decreased ($MI = 3.20 \times 10^{-3} \pm 1 \times 10^{-3}$). After washout, the coupling returned ($MI = 1.91 \times 10^{-2} \pm 8 \times 10^{-3}$). The magnitude of coupling measured by shuffling phases and amplitudes from all conditions was similar to and slightly higher than the magnitude found during the drug condition (surrogate $MI = 4.33 \times 10^{-3} \pm 1.4 \times 10^{-2}$). Here, PAC exhibited a “fall-max” pattern during non-drug and post-washout conditions, in which the maximum amplitude of HFOs were coupled to the falling edge of the theta phase, which has been reported in other studies of phase-amplitude coupling (Zhang et al., 2017).

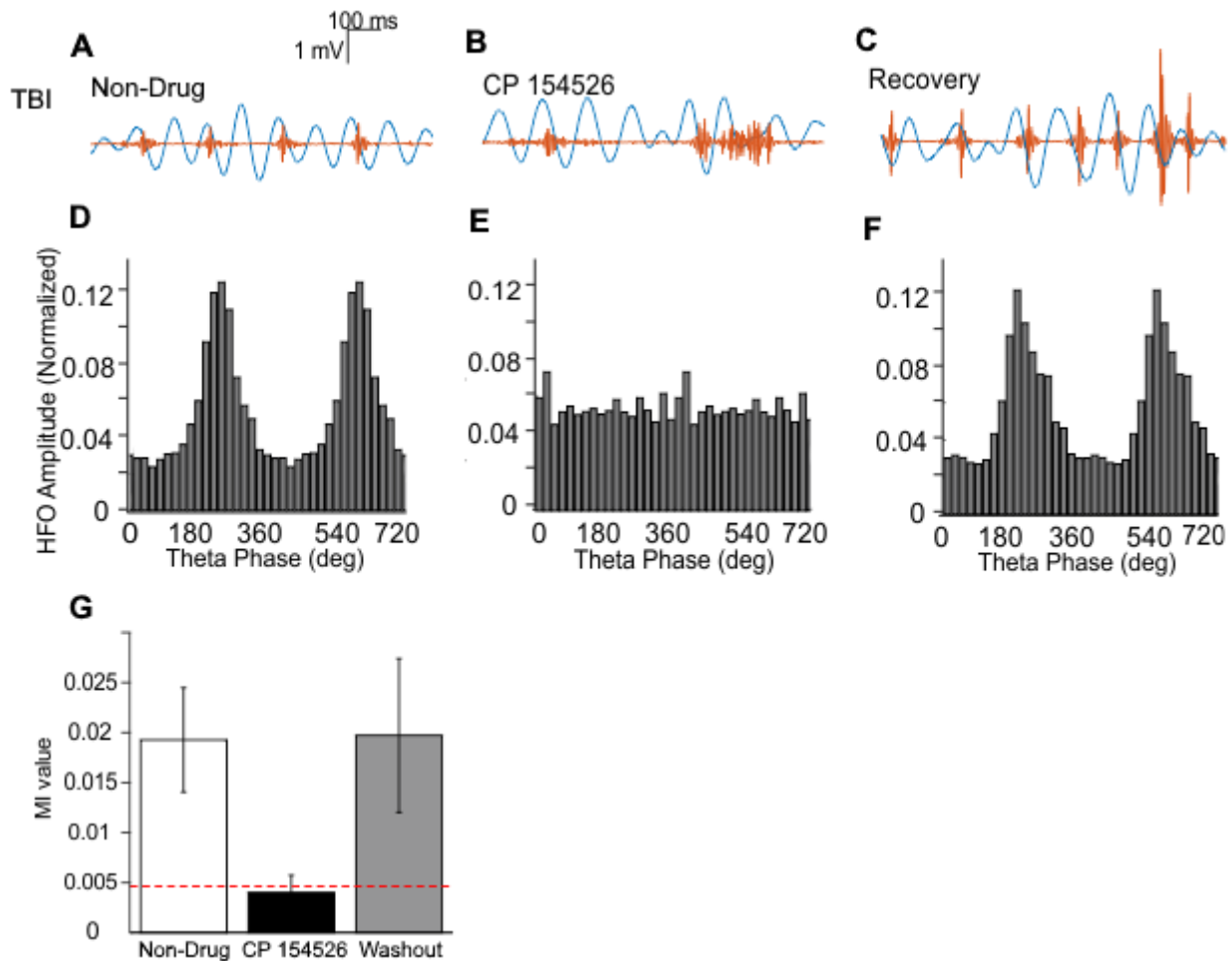


Figure 3: PAC in TBI rat amygdala during stress in non-drug, CRFR₁ antagonist, and washout. 10s segments recorded at 1kHz sampling rate from one rat are shown here. (A) High amplitude HFO activity (orange) repeatedly occurs on the falling edge of the theta rhythm. (B) This phase-amplitude coupling is disrupted after intraperitoneal injection of CP 154526, a CRFR₁ antagonist. (C) Coupling recovers after washout. (D to F) Phase-amplitude coupling plots depict average amplitude of HFOs at theta phases binned in 20° segments during stress in non-drug, drug, and post-washout conditions. (G) The Modulation Index (MI), a measure of the magnitude of coupling, was averaged in the segments collected from all TBI rats during the different drug conditions. The mean difference between non-drug and drug, and between drug and post-washout conditions were statistically significant (ANOVA; $p < 0.05$). The red dotted line denotes MI from shuffled surrogates ($n = 10^6$).

3.4 TBI rats under stress show higher excitability than sham rats in 4kHz amygdala recordings

Of interest was whether ripples (120 – 250 Hz) and fast ripples (250 – 500Hz) became more prevalent as the increased occurrence of the latter wave forms is more closely associated with epileptic activity (Jefferys et al., 2012). A new cohort of rats were operated on in the same procedure with sham surgery (n = 5) and TBI rats (n = 5) in preparation of use with the recording setup with an increased sampling rate of 4000Hz. Again, in sham rats the responses to this stressor were mild (Fig. 4A) and less high frequency content was present as seen in the spectrogram (Fig. 4E), although the rats were equally agitated and attempted to remove the clip from their tail in response to the stressor. In Figure 4B, a typical electrical response from a TBI rat recorded from the amygdala during a tail pinch stressor is shown. This electrophysiological response in the TBI rats was accompanied by stage 2–3 seizures (Racine scale; mean = 2.5, 0.4 SD; n = 5; Racine et al., 1988). Sham rats did not demonstrate any visual seizure behaviors. In Figure 5C, we show a subsample of the response in Figure 5B (150 ms). The unfiltered sample is shown in black, while the band-passed 120 – 250 Hz is in red, and high pass filtered above 250 Hz is in blue, showing both ripples and fast ripples, respectively. In Figure 5D, we show the averaged power spectra of the high-passed filtered recordings (>120 Hz) from five sham TBI and five TBI rats. This was done by subtracting the power spectra of the baseline from the spectra during stressor responses. The baseline corrected spectra were then normalized and averaged. We found little or no increase in power at high frequencies in the sham rats, but in TBI rats, the power increased by 2.5-fold, particularly >250 Hz (p = 0.001, Kolmogorov–Smirnov distribution test). A spectral analysis of the entire response using a Morlet wavelet transform revealed a small

increase in power at frequencies up to 100 Hz in the sham rats but HFOs (ripples and fast ripples) were rare, although some were observed (Fig. 5E). This was very different in TBI rats (Fig. 4F), where both kinds of ripples occurred during the application of the stressor. Figure 4F, inset, shows the wavelet analysis over a 500-ms window of the spectrogram, demonstrating occurrence of HFO bursts.

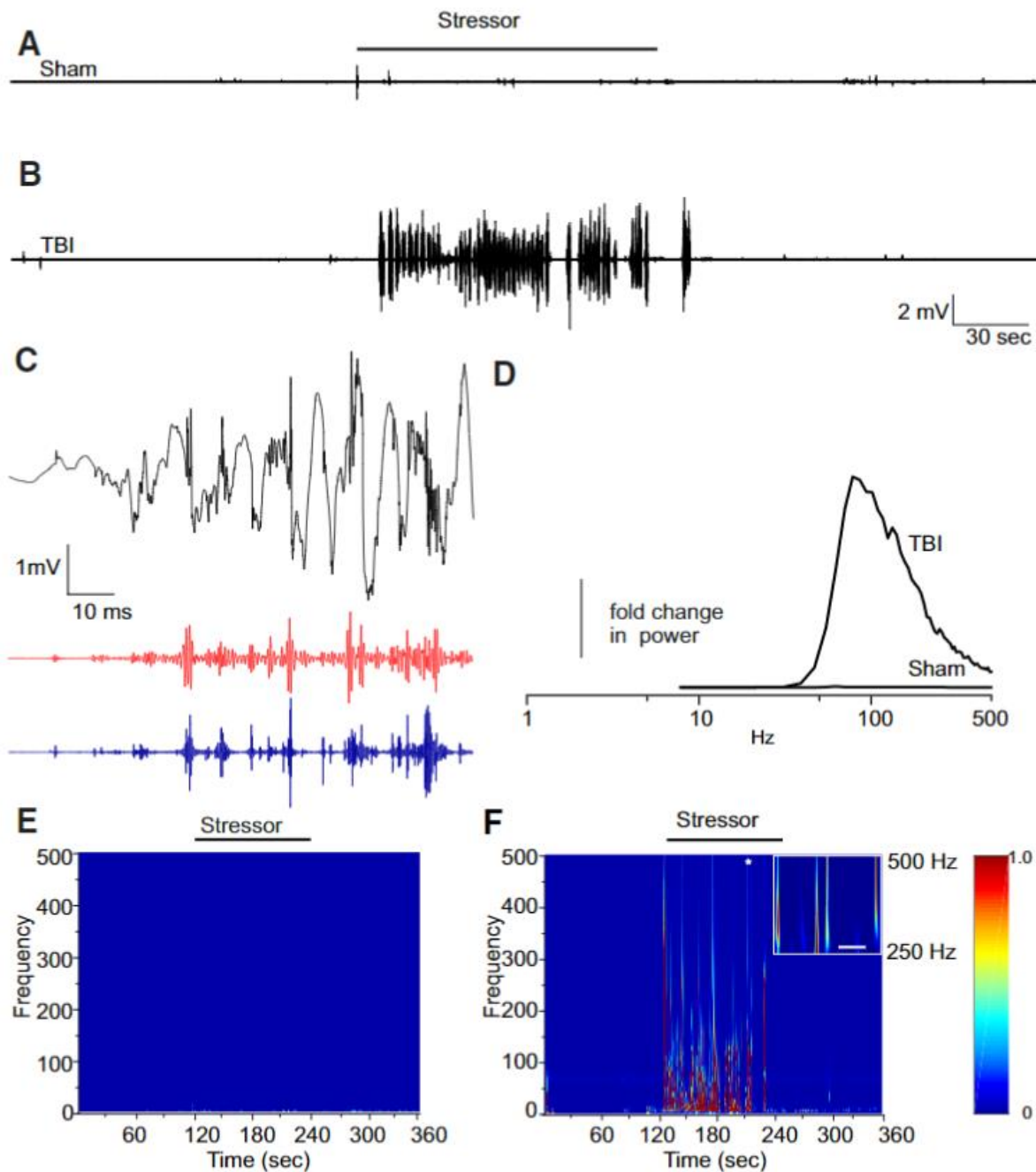


Figure 4: Stress induces a larger electrographic response in the amygdala of TBI than in sham rats. **A**, Recording of TBI rat amygdala response from a sham-operated rat showed a mild electrographic response to a tail pinch stress, while in **B**, TBI-injured rats showed a large increase in electrical activity. **C**, A 100-ms sample of a response where ripples (red; 120 - 250 Hz) and fast ripples (blue; 250 – 500 Hz) are clearly visible. **D**, Mean normalized power spectra

of sham (n = 5) and TBI rats (n = 5). HFOs compared to lower frequencies showed a 2.5-fold change in power in TBI rats, but little change in sham rats. **E,F**, A comparison of frequency content of sham rats' response using Morlet wavelet analysis shows that sham rats have only small response to the stressor, while in TBI rats, large increases in electrical activity during stressor. The inset smaller window shows well-resolved fast ripple activity (the white * marks approximate time from which the window was obtained; white scale bar is 100 ms).

3.5 CRFR₁ antagonism *in vivo* in post-TBI amygdala reduced electrographic response and HFOs during stress in 4kHz recordings

Figure 5 shows a typical electrophysiological response from a TBI rat and its attenuation by the CRFR₁ antagonist CP 154526 (1.5h after an intraperitoneal injection of 50 mg/kg). All rats did not exhibit seizure behaviour during the drug condition. The accompanying Morlet wavelet analyses showed a marked attenuation of activity in both high and low frequencies 3h after the injection, which was reversed in the recording 24h later.

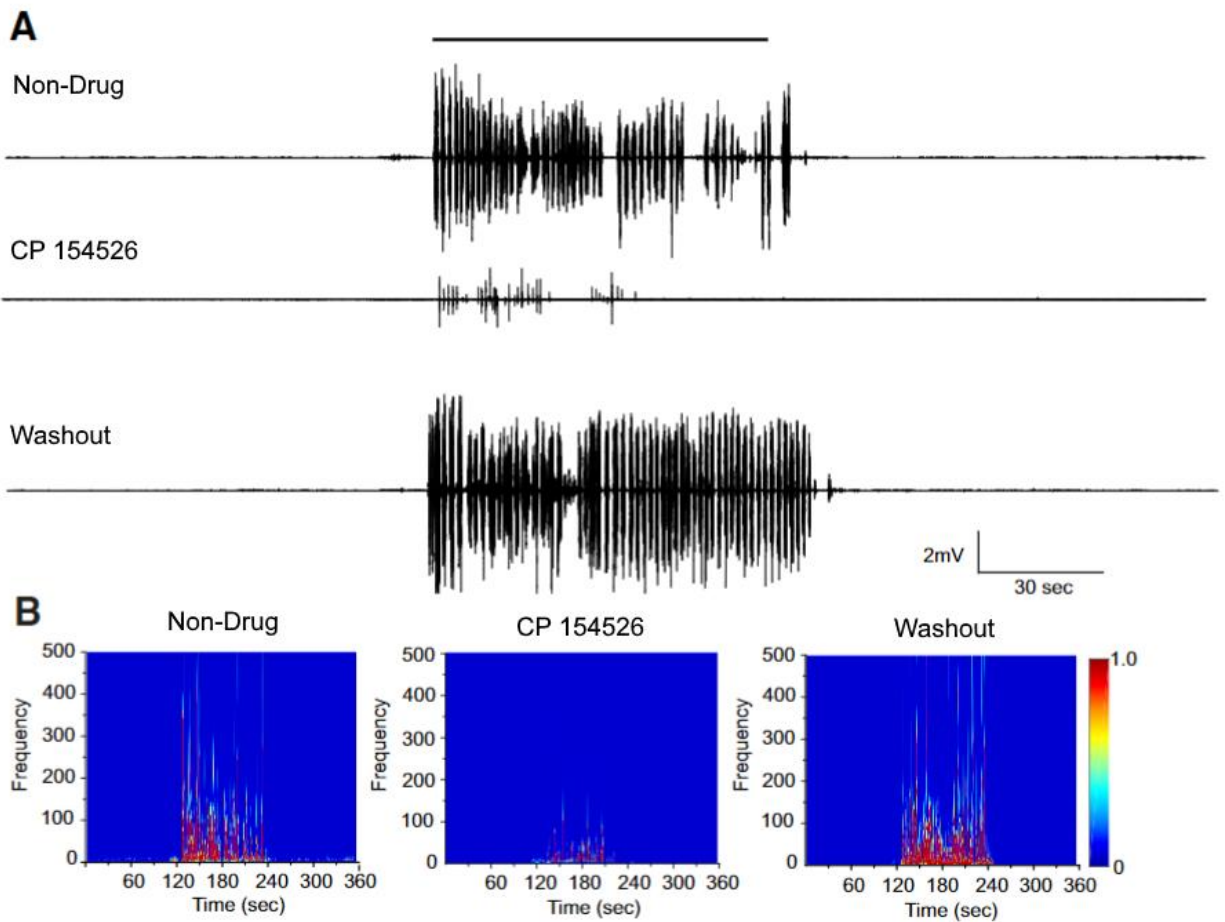


Figure 5: CRFR1 antagonist reduces HFO activity in TBI amygdala during stress. **A**, Tail pinch response from a TBI rat followed by the response after the application of CP 154526, a CRFR₁ antagonist, which is followed by a recovery trace after 24 h of drug washout. Duration of stress is indicated by the bar placed above the control response which was for 2 min. **B**, Morlet spectrograms which correspond to the traces shown in A.

3.6 CRFR₁ activity mediates fast ripples and PAC between theta and fast ripples during stressor in post-TBI amygdala in 4kHz recordings

We next examined whether the high-frequency activity was cross-frequency coupled to any particular frequencies. Figure 6 shows a summary of the PAC (Tort et al., 2010) between low-frequency activity (binned on x-axis) versus high frequencies (binned on the y-axis) in the signals before, during, and after CRFR₁ antagonist treatment. The averaged MI values from all five rats are shown in this analysis and are color-coded as a heat map. The non-drug responses showed that again in theta (4 – 8 Hz), there was a high magnitude of PAC in both the ripple and fast ripple bands. This was particularly prominent between the fast ripple whose MIs were the largest (MI = 2.05×10^{-3} vs surrogate MI = 5.47×10^{-5}). In the CP 154526-treated rats, the coupling between theta and high-frequency bands was again attenuated. These MIs calculated in the presence of antagonist were below chance, as compared to surrogate analyses of shuffling (MI = 1.28×10^{-3} vs surrogate MI = 4.11×10^{-3}). After recovery, the coupling between theta and the ripple and fast ripple bands were again present (MI = 1.56×10^{-3} vs surrogate MI = 2.77×10^{-5}). These recordings did not exhibit the same fall-max pattern in coupling.

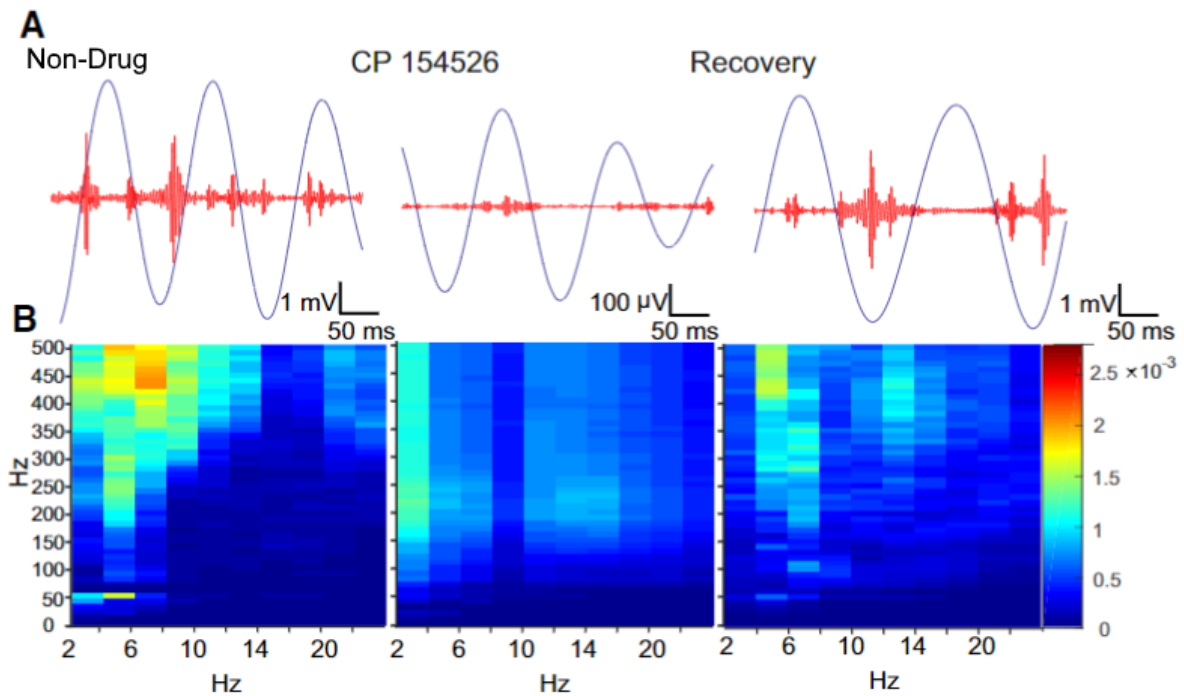


Figure 6: Comodulograms depict theta-HFO PAC in non-drug and washout conditions, but not after administration of CRFR₁ antagonist. A, upper traces: Examples of fast ripple activity and theta in non-drug, CP 154526, and after 24h drug washout from TBI rats under stress filtered into (4 – 8 Hz, blue) and fast ripples (250 – 500 Hz, orange) bands. B, Comparison of MIs between low-frequency phase on x-axis and higher frequency amplitude between 250 and 500 Hz on y-axis. Fast ripples are significantly and highly coupled over the frequency bins corresponding to theta rhythm. Ripple activity is also coupled to a lesser extent in the same range. This relationship is abolished by CRFR₁ antagonism. Very low-frequency activity (2–4 Hz) is apparently coupled over a wide range of high-frequency activity. The MI values corresponding to this coupling during antagonist are below chance. After 24h, the PAC returned.

3.7 Oscillation-triggered comodulogram shows underlying theta activity in fast ripple ranges

Finally, to confirm the PAC analysis, an oscillation-triggered comodulogram was also calculated (Dvorak and Fenton, 2014). This calculation was done for the pooled data before, during, and after CRFR₁ antagonism. The crests of fast ripple activity aligned at a slow oscillation of 0.2 s between crests, or an underlying wave that resonated at 5 Hz (theta). This analysis supports the observations using the MI described by Tort et al. (2010) by providing another measure of coupling that uses the raw data and does not rely on assumptions of linearity or harmonic artifacts that can occur after filtering.

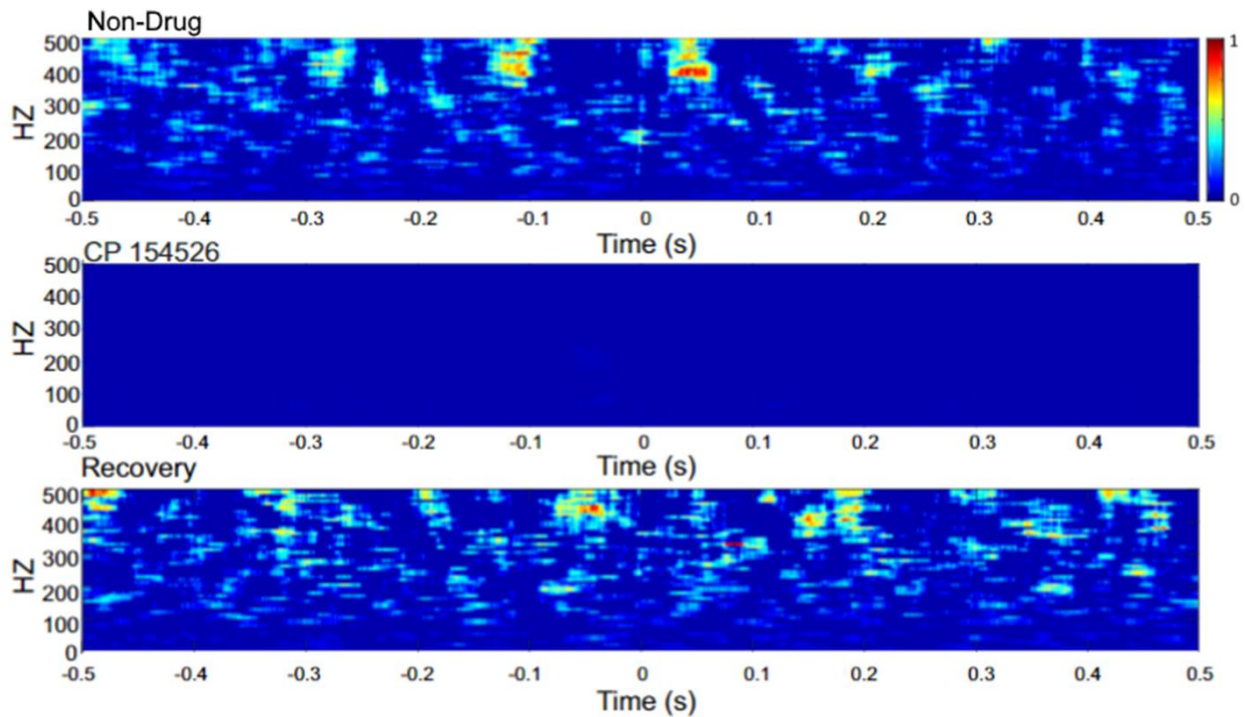


Figure 7: OTC shows coupling between theta and fast ripples that is attenuated by CRFR₁ antagonist. Another method of showing CFC from data recorded during a stressor in TBI rats. The oscillation-triggered comodulogram (OTC) shows coupling between fast ripples and an underlying 5 Hz theta oscillation without antagonist and after washout of antagonist. During antagonist, destructive interference of the pooled LFPs shows no significant coupling.

Chapter 4

4. Discussion

4.1 Stress Exacerbates Post-Traumatic Epileptogenesis

TBI is a major risk factor in the development of epilepsy (Xu et al., 2017). PTSD and TBI are often comorbid as brain injuries are often associated with traumatic experiences (Ohry et al., 1996; Hickling et al., 1998; Levin et al., 2001; Greenspan et al., 2006; Gaylord et al., 2008; Hoge et al., 2008; Bryant, 2011). To date, a few mechanisms have been proposed to account for the high association of PTSD and the development of epilepsy such as up-regulated glutamate neurotransmission, axonal damage, and compromised interneuronal circuits (Guerriero et al., 2015). CRF has been implicated in several brain disorders such as anxiety, stress, depression, and neuropathic pain. Clinical studies have shown that increased concentrations of CRF in the CSF are associated with PTSD (Post et al., 1982) and several studies have found altered hypothalamic pituitary adrenal (HPA) axis and CRF function in PTSD (Mason et al., 1986; Smith et al., 1989; Pitman and Orr, 1990; Yehuda et al., 1993). Consistent with these reports, studies in humans showed a chronic increase in CRF release following stress exposure (Dallman and Jones, 1973). In TBI rats, CRF exhibited excitatory effects in the PCtx, a region that has strong interconnections with the amygdala. Therefore, these changes in CRF activity have a wide impact that either originate in the PCtx or involve changes that associate other limbic regions to produce the electrophysiological responses we observed in the amygdala.

Importantly, the results indicate that pathologic mechanisms that accompany brain injury make the brain susceptible to stressor-induced increases in the excitability of the amygdala. In

particular, the phase coupling between pHFOs and LFP oscillations seem important as this coupling may aid in the spread of the high-frequency activity contributing to epileptogenesis. Indeed, it has been noted in previous studies that HFOs have a surprising ability to spread and exhibit high coherence across much larger volumes than one might expect (Haufler and Pare, 2014). These findings also provide insight into why those who have suffered TBI and PTSD have a 3-fold increase in the incidence to develop epilepsy, which is often pharmaco-resistant (Hitiris et al., 2007).

4.2 High-Frequency Oscillations are Increased and Phase-Amplitude Coupled during Stress

Recently, HFOs have been the subject of great interest in epileptogenesis. Here, both ripples (120 – 250 Hz) and fast ripples (250 - 500 Hz) have been investigated. Ripples are generally thought to be physiologic, whereas fast ripples are generally considered to be pathologic, supporting the generation of epileptic seizures (Bragin et al., 1999; Jefferys et al., 2012). This view is by no means settled and other phenomena may also need to be considered as contributing to the occurrence of seizures. These include whether they are bursting, like observed here, or steady state HFOs, phase locked to a behavioural or electrical event, and their location (Jefferys et al., 2012). Also, it has been reported that ripples are associated with epileptic behaviour in animal models (Shiri et al., 2016) as well as in humans (Weiss et al., 2016). The other issue is whether pHFOs are an epiphenomena or causative of the epileptic state. Evidence that the latter may be true comes from work showing that during the transition period from the latent to the chronic phase in the pilocarpine model of mesial temporal lobe epilepsy, there is an increased incidence of HFO activity in the EC and CA3 region of the hippocampus (Salami et al., 2014)

and the blocking of their occurrence with the anti-epileptic drug levetiracetam impeded the generation of seizures. Here, we have recorded HFOs from the amygdala, a region of the brain that readily supports the development of seizures (McIntyre et al., 2002). Previously, HFOs have been isolated in the amygdala and associated limbic regions (DEn, central amygdala, EC, and PCtx; Ponomarenko et al., 2003; Haufler and Pare, 2014; Salami et al., 2014). In general, these studies have found that HFOs occur regionally in a highly synchronous manner so that HFOs occurring in one region are highly correlated with occurrences in other regions. In fact, it was noted that an apparently unusual property of HFOs relative to LFP oscillations was their high coherence (Haufler and Pare, 2014). LFP coherence usually decreases with distance, and this reduction is quite steep for their fast components such as gamma (Steriade et al., 1996; Collins et al., 2001). In addition, the main difference between what was observed in past reports and the findings here is that the fast ripples were highly phasic locked to theta (4 – 8 Hz). A recent study has shown that high-power low-frequency oscillations coupled to HFOs could be readily identified in a brain region of patients with pharmaco-resistant epilepsy and when this region was subsequently removed, better clinical outcomes of seizures were observed (Cotic et al., 2016). This supports the idea that the cross-frequency coupling of HFOs to LFP oscillations seems to be highly epileptogenic.

4.3 Altered Interneurons May Lead to Epileptiform Activity

While it is clear that stress generates HFOs, the mechanism by which this occurs is unclear. It seems likely that activation of CRFR₁ is influencing the activity of the local circuit neurons in the amygdala to produce these network effects, as evidenced by the decrease in HFO amplitude and occurrence following administration of the CRFR₁ antagonist. Changes in network activity

often implicates changes in interneuron function, and in some cases, CRF application converted slow-firing interneurons to fast-firing ones, and conversely converted fast-firing interneurons into slow-firing ones, disrupting normal networking (Narla et al., 2015). Moreover, interneurons may become functionally silent after an insult. Evidence for this interpretation comes from a study where the perforant path model of temporal lobe epilepsy was used. Sloviter (1987) described disruption of functional inhibition in the presence of viable interneurons and functionally active GABA_A receptors in the dentate gyrus and CA1 areas of hippocampus (Sloviter, 1987, 1991). Similar observations were also observed by two other studies showing no loss of GABAergic interneurons (Bekenstein and Lothman, 1993; Lothman et al., 1996) but a reduction in inhibitory activity. Similar observations were reported in the tetanus toxin model of epilepsy where evidence for the existence of functionally dormant interneurons was shown (Jefferys and Traub, 1998). Further studies will be required to provide insight into how CRF affects the rewiring of amygdala neurons following TBI.

4.4 Limitations

One of the main limitations of animal models of PTE is that only a relatively small proportion of animals develop PTE. Non-traumatic animal models of epilepsy have a relatively high incidence of post-intervention epilepsy with reliable induction of multiple seizures per day. By contrast, experimental PTE is associated with a much lower seizure frequency and duration. In one study of PTE using continuous intracranial monitoring for 11 months after TBI, the average rate of seizures was one every 1–2 weeks (Kharatishvili et al., 2006). In another study of PTE using the lateral fluid percussion injury model, 94% of experimental animals showed electroencephalogram (EEG) evidence of non-convulsive seizures, but these generally lasted less

than ten seconds (Campbell et al., 2014). Unlike many other experimental models of epilepsy, experimental PTE often leads to generalized epilepsy that is relatively easy to evaluate using video monitoring to establish frequency and severity of seizure activity (Racine, 1972).

4.5 CRFR₁ Antagonists as an Anti-Epileptic Drug

Our current findings suggest that CRFR₁ antagonists may have a clinical importance as antiepileptic drugs. CRFR₁ antagonists have long been under investigation for treating stress related disorders such as anxiety and depression, although the results have not been promising (Zorrilla and Koob, 2004; Valdez, 2009). No clinical study has investigated or argued for the use of CRFR₁ antagonists to attenuate epileptogenesis following TBI. Also, it has been observed that exogenously applied CRF induced excitotoxicity of interneurons (Aldenhoff et al., 1983; Bishop and King, 1992), which suggests that CRFR₁ antagonists may be effective in providing neuroprotection in the hippocampus following seizure (Maecker et al., 1997) and after cerebral ischemia (Lyons et al., 1991).

4.7 Conclusion

In summary, our findings provide a mechanism by which individuals affected by TBI develop stress-associated PTE. CRFR₁ activation in the amygdala is associated with changes in excitability in the amygdala following brain injury. In particular, the generation of fast ripples during the response and their PAC to LFP oscillations could be particularly epileptogenic. Our observations indicate that the epileptic state was accompanied by greater susceptibility to stressor-induced excitability in a brain region that supports seizures. These findings suggest that the underlying brain pathology may be an important determinant in the exacerbation of epilepsy

in response to heightened anxiety. In effect, the neuronal network alterations support hyperexcitability and seizure onset, and the epileptic state makes the brain more susceptible to an otherwise physiological response to stress.

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