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Corticotropin releasing factor receptor type 1 signaling in epilepsy and traumatic brain injury

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Abstract

Stress increases the frequency by which epileptic seizures occur. Corticotropin-releasing factor (CRF) coordinates neuroendocrine, autonomic and behavioral response to stress. This thesis sought to study the cellular and molecular mechanisms by which CRF regulates the activity of neural circuits in the piriform cortex (PC) in normal and epileptic states. The PC is richly innervated by CRF and 5-HT containing axons arising from the central amygdala and raphe nucleus. $CRFR_1$ and $5-HT_{2A/C}Rs$ have been shown to interact in a manner where CRFR activation subsequently potentiates the activity of $5-HT_{2A/C}Rs$. The first purpose of this thesis was to determine how the activation of $CRFR_1$ and/or $5-HT_2Rs$ modulates PC activity at both the circuit and cellular level. Voltage-sensitive dye imaging showed that CRF acting through $CRFR_1$ dampened activation of layer II in the PC and interneurons of the endopiriform nucleus. Application of the selective $5-HT_{2A/C}R$ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) following $CRFR_1$ activation potentiated this effect. Blocking the interaction between $CRFR_1$ and $5-HT_2R$ with a Tat-$CRFR_1$-CT peptide abolished this potentiation. Application of forskolin did not mimic $CRFR_1$ activity but instead blocked it, while a protein kinase A antagonist had no effect. However, activation and antagonism of protein kinase C (PKC) either mimicked or blocked CRF modulation respectively. DOI had no effect when applied alone indicating that the prior activation of $CRFR_1$ receptors was critical for the DOI activity. This data shows that CRF and 5-HT, acting through $5-HT_{2A/C}Rs$, reduce the activation of the PC. This modulation may be an important blunting mechanism of stressor behaviors mediated through the olfactory cortex.

Anxiety and stress conditions induce neurons arising from the central amygdala and local interneurons to release CRF in PC, where it normally dampens excitability. The second aim

of this thesis was to determine the role of CRF in stress associated epilepsy. We showed that CRF increased the excitability of PC in rats subjected to kindling, a model of temporal lobe epilepsy. In non-kindled rats, CRF activates its receptor, a G protein-coupled receptor (GPCR) and signals through a $G\alpha_{q/11}$ mediated pathway as identified in the first aim of this thesis. After seizure induction, CRF signaling occurred through a pathway involving $G\alpha_s$. This change in signaling was associated with reduced abundance of regulator of G-protein signaling protein-2 (RGS2), which promotes the switch in $CRFR_1$ signaling cascade to a $G\alpha_s$ dependent mechanism. RGS2 knockout mice responded to CRF in a similar manner as epileptic rats. These observations indicate that seizures produce changes in neuronal signaling that can increase seizure occurrence by converting a beneficial stress response into an epileptic trigger.

People with traumatic brain injury often develop epileptic seizures. The mechanisms underlying this are poorly understood. Considerable evidence suggests that association of stressful life experiences in brain injured patients lead them to develop post-traumatic stress disorder. CRF release in brain regions that are implicated in epileptogenesis make these situations worse. The third aim of this thesis was to understand the role of CRF in inducing excitability in PC after brain injury. We found that CRF has variable effects on the interneurons of ipsilateral and contralateral PC. Altogether, its actions lead to increased excitability of PC compared to healthy rat PC. The extent of excitability produced by CRF and the signaling mechanism of $CRFR_1$ after brain injury were similar to CRF actions and $CRFR_1$ signaling mechanism in kindling induced epilepsy.

Overall, this thesis study provides the basic mechanisms by which certain forms of epilepsy, both stress and injury induced develops. It also points out the important discovery of this project that is, the capability of GPCRs to switch signaling cascades depending on the pathological condition of the brain.

Co-authorship

Chapter 2 was previously published as "Suppression of piriform cortex activity in the rat by corticotropin-releasing factor 1and serotonin 2A/C receptors" in *Frontiers in cellular neuroscience*, 2014, 9.200 (Co-authored by C. Narla, H. A. Dunn, S. S. G. Ferguson, M.O. Poulter). Western blotting was done by H. A. Dunn. All other work including data analysis and writing were performed by C. Narla. Reprinted with permission from *"Frontiers in Cellular Neuroscience"*.

Chapter 3 was previously published as "A switch in G protein coupling for type 1 corticotropin-releasing factor receptors promotes excitability in epileptic brains" in *Science Signaling, 2016,* 9: 432 ra60 (Co-authored by C. Narla, T. Scidmore, J. Jeong, M. Everest, P. Chidiac and M.O. Poulter). Immunohistochemistry and QPCR were performed by T. Scidmore, J. Jeong and M. Everest. All other work including data analysis and writing was performed by C. Narla. Reprinted with permission from AAAS.

Chapter 4 entitled "CRFR$_1$ signaling in traumatic brain injury" was co-authored by C. Narla, M. Everest, and M. O. Poulter. QPCR and immunohistochemistry were performed by M. Everest. All other work including data analysis and writing was performed by C. Narla.

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Abbreviations

AHF	Adapting high frequency
ALF	Adapting low frequency
aPC	Anterior piriform cortex
CNS	Central nervous system
CRF	Corticotropin releasing factor
$CRFR_1$	Corticotropin releasing factor receptor type 1
$CRFR_2$	Corticotropin releasing factor receptor type 2
GABAA	Gamma-amino butyric acid type A receptor
GAT-1	Gamma-amino butyric acid (GABA) transporter 1
IR	Immunoreactivity
ISI	Interspike interval
LOT	lateral olfactory tract
mm	Millimeter
mM	Millimolar
mV	Millivolt
NAvHF	Non-adapting very high frequency
NMDA	N-methyl-D-aspartate
PC	piriform cortex
pPC	Posterior piriform cortex
PTSD	Post-traumatic stress disorder
QPCR	Quantitative real time polymerase chain reaction
RMP	Resting membrane potential
RGS2	Regulator of G-Protein signalling protein 2
RGS 17	Regulator of G-Protein signalling protein 17
sALF	Strongly adapting low frequency
SAP97	Synapse associated protein 97
TBI	Traumatic brain injury
VSDI	Voltage sensitive dye imaging
wALF	Weakly adapting low frequency

Chapter 1. General introduction and thesis overview

1.1 Introduction:

The human brain is a highly complex and well-organized organ that controls sophisticated behaviors such as learning, memory, emotion, sexual functions and heart rate. A typical human brain is estimated to have 80-120 billion nerve cells, the smallest functioning units and signaling elements of the nervous system (Azevedo et al., 2009;Herculano-Houzel, 2009). Anatomically distinct regions of the brain have been described to function similarly to a network of linked computers which work together to process information. Unlike computers, the output of the brain depends on the precise timing patterns that occur between brain regions. There are several studies that argue that the synchrony between neural networks is important for most cognitive functions such as working memory, emotions and decision making (Singer, 1999;Schnitzler and Gross, 2005;Varela et al., 2001).

Perhaps owing to its complexity, the human brain is susceptible to many different kinds of diseases and disorders. One neurological disorder that is manifested by aberrant neuronal network synchrony is epilepsy.

1.2 Epilepsy:

Epilepsy is a chronic neurological disorder causing misery in the lives of millions of victims around the globe and has been a cause of mortality and morbidity (Banerjee et al., 2009;Blume et al., 2001). It is one of the oldest and most known conditions to humankind and that there are 50 million people suffering from it as per World Health Organisation report 2001a & 2001b. There is

no certain age group that is most affected or particularly at risk. According to international league against epilepsy (ILAE), epilepsy is characterized by at least two unprovoked seizures occurring less than 24 hours apart or one unprovoked seizure and a probability of further seizures similar to the general recurrence risk after two unprovoked seizures, occurring over the next 10 years or the diagnosis of an epilepsy syndrome. The epileptic seizure is defined as a paroxysmal and excessive electrical neuronal discharge in the brain that results from too much excitation or too little inhibition in the area in which the abnormal discharge starts (Chang and Lowenstein, 2003). This desynchronization between excitatory and inhibitory responses can lead to neuronal hyperactivity over the time and develop seizures that are able to generalize to other regions of the brain from the foci of initiation. It manifests as a wide range of behavioral abnormalities including uncontrolled muscle movements loss of consciousness and sensory auras (Fisher et al., 2005). Its impact on health care system is huge and onerous. The heterogeneous etiology of the condition and deficiency in the biomarkers for its diagnosis and treatment make it an important condition to address (Stern, 2011).

There are several factors that can trigger epileptic seizures in an individual that include simple day to day activities such as lack of sleep, skipping a meal, flashing light etc. Complex psychiatric disorders, which include anxiety disorder, pathophysiology associated with stress and major depressive disorder can exacerbate the occurrence of more frequent seizures (Kanner et al., 2012)**.** Other conditions that can also affect brain physiology such as degenerative diseases, brain infections, brain hemorrhages and ischemia could lead to the development of epileptic seizures (Vinters et al., 1993). In several patients, genetic factors, mutations in the brain development, neurodegeneration and defective physiology of ion channels contribute to seizures (Sander, 1996).

Many types of seizures exist that have been broadly classified depending on whether they have a local onset or a more distributed origin. Specifically, localized seizures (partial or focal onset seizures) are confined to a region and may or may not spread to the other parts of the brain. Partial seizures are further divided based on their ability to affect consciousness. If consciousness is affected it is a complex partial seizure, if not, it is a simple partial seizure. The distributed seizure involves large parts of the brain or the origin of the seizure cannot be determined (Chabolla, 2002). "Generalized seizures" is the term often used interchangeably with distributed seizures. They can further be classified based on their effect on the body into absence seizures, myoclonic, tonic-clonic, clonic, tonic and atonic seizures. Losing consciousness is also a characteristic feature of generalized seizures (Shorvon, 2004). In both types, there is a disruption of normal brain activity in both the hemispheres.

Epileptic seizures are further classified into syndromes that are a collection of different types of seizures or combinations of seizures. Complex partial seizures are the most common among epileptic syndromes and affect 40-50% of patients with epilepsy. As 75-80% of complex partial seizures arise in the temporal lobe structures, this type of epilepsy was termed temporal lobe epilepsy (TLE) (Loscher and Schmidt, 1994).

1.2.1 Temporal lobe epilepsy (TLE):

Temporal lobe has been the most epileptogenic region of the human brain and TLE is the most common and occurring epilepsy type in humans. Mesial temporal regions that include the amygdala, hippocampus, and parahippocampal regions are the most affected regions in TLE (Engel et al., 1997). Hippocampal sclerosis has been the most common cause for TLE and mesial TLE is the best-studied epilepsy type of all epilepsies (Tatum, 2012). Mesial temporal lobe

epilepsy (MTLE) is used interchangeably for lateral temporal lobe epilepsy (LTLE) due to the involvement of mesial temporal lobe structures. There is an estimate that MTLE contribute to 40% of all epilepsies in adults.

Hippocampal sclerosis (HS) is a form of MTLE and is accounted for 70% of MTLE cases (Bruton, 1988;Wolf et al., 1993;Babb, 1999). The rest of the MTLE lesions are not associated with HS (Thom, 2004;Panayiotopoulos, 2005). HS is a combination of atrophy and astrogliosis of mesial structures: amygdala, hippocampus, uncus, parahippocampal gyrus and the entorhinal cortex (Tatum, 2012). Different regions of the hippocampus are vulnerable to damage in various degrees. CA1 and CA3-CA4 regions of the hippocampus are the most vulnerable to damage whereas granule cells of the dentate gyrus and pyramidal cells of CA2 region are resistant to seizure generation (Loscher and Brandt, 2010). Several studies reported a link between MTLE associate HS and cognitive deficits including impaired verbal and visual memory performance (Hermann et al., 1997;Gleissner et al., 1998;Helmstaedter, 2002;Wieser, 2004). A reorganization of excitatory network and neurogenesis have also been reported besides the neuronal death (Sutula et al., 1989;Babb et al., 1991;Isokawa et al., 1993;Isokawa, 1997;Blumcke et al., 2001;Crespel et al., 2005). This reorganization in excitatory drive and synaptic connections made by neuronal cells formed as a result of neurogenesis contribute to the imbalance between excitatory and inhibitory drive in the hippocampal formation and limbic system (Chang and Lowenstein, 2003). Observation of hippocampal specimens revealed that granule cell dispersion may be associated with early seizure onset or status epilepticus(Blumcke et al., 1999a). Changes in glutamatergic and GABAergic synaptic transmission in hippocampus further alleviate/contribute to the imbalance. Altered expression of NMDA receptors (Isokawa and Levesque, 1991;Mathern et al., 1997;Mathern et al., 1998b;de Lanerolle et al., 1998), AMPA receptors (Babb et al., 1996) and

kainate receptors (Mathern et al., 1998a) in the epileptic hippocampus can alter the physiological properties of these channels and contribute to the neuronal hyperexcitability in hippocampus. Altered inhibitory neurotransmission as a result of changes in $GABA_A$ receptor subunit has also been reported in MTLE patients (Loup et al., 2000). Increased zinc sensitivity as a result of subunit reorganization in $GABA_A$ receptors and subsequent occlusion of receptor channel has been reported (Sharonova et al., 2000). This change in receptor sensitivity leads to impairment in inhibitory neurotransmission in the hippocampus (Shumate et al., 1998;Brooks-Kayal et al., 1999). In addition, MTLE can co-exist with second temporal lobe epileptogenic pathology such as cortical dyslamination; focal cortical dysplasia type I or gliomal tumors. (Blumcke et al., 2002;Blumcke et al., 1999b). Anatomic data confirm the importance of medial thalamus to MTLE. Cell loss and thus a reduction in volume in thalamus, caudate and amygdala contribute to MTLE (Spencer, 2002).

Neocortical temporal lobe epilepsy (NTLE) is another form of temporal lobe epilepsy and is very rare compared to the occurrence of MTLE (Tatum, 2012) (Bercovici et al., 2012). The precipitation factors for MTLE; febrile seizures, brain infections, head injury or perinatal complications rarely contribute to NTLE (Gil-Nagel and Risinger, 1997). Auditory hallucination, psychic experiences, visual impulses, cephalic sensation and vertiginous symptoms account for triggering 60% of NTLE seizures (Kennedy and Schuele, 2012;Maillard et al., 2004). Unresponsiveness and staring are the clinical signs to identify NTLE. These symptoms are followed by secondary generalization and clonic movements (O'Brien et al., 1996). Another important manifestation of NTLE is seizure occur often less than a minute compared to MTLE where the seizure occurs for more than a minute.

1.3 Psychiatric co-morbidities of Epilepsy:

1.3.1 Anxiety:

Anxiety and affective disorders, stress and depression are major complications for the precipitation of epileptic seizures (Kalinin et al., 2010). The presence of these conditions further exacerbates the condition of having seizure disorder in patients with epilepsy and ultimately the quality of life is compromised. Ample evidence based on research argue that patients having a psychiatric complication such as anxiety, depression or suicide in association with epilepsy are at more risk in comparison to general population (Jacoby et al., 1996;McCagh et al., 2009;Mensah et al., 2007;Pompili et al., 2005). These psychological illnesses were highly underrated and undertreated despite their impact on the health care system and the misery they create in the lives of patients (de Boer et al., 2008). Of all the psychiatric complications mentioned, anxiety is the most prevalent and most reported among patients with epilepsy (Collings, 1990;Jacoby et al., 1996). A clinical study reported in 193 patients with epilepsy revealed that the occurrence of anxiety disorders in association with major depressive disorder or sub-syndromic depression episodes greatly diminished the quality of life compared to the patients with anxiety disorders alone (Kanner et al., 2010). Epidemiological studies revealed that the prevalence of anxiety disorders is twice compared to the healthy general population (Verrotti et al., 2014). A study conducted in Canadian population reported that the prevalence of anxiety disorders are second to suicide ideation in patients with epilepsy and its lifetime prevalence was 22.8% (Tellez-Zenteno et al., 2007). The presence of seizure phobia (unpredictability of seizures in public places) exacerbates the symptoms of anxiety. This also results in the development of agoraphobia and

social phobia in patients with epilepsy. Epilepsy-related factors that are associated with anxiety include early age of onset, poor seizure control and increased seizure severity (Mensah et al., 2007;Jacoby et al., 1996). Very severe anxiety has been reported in patients with chronic refractory disorders (Beyenburg et al., 2005). Several studies attempted to clearly unravel the relationship between anxiety and epileptic seizures but their reports largely remained as speculations. There is a great body of evidence that suggests that $GABA_A$ receptor dysfunction or disorders that cause interneuron degeneration have great relevance in the development of anxiety and epileptic seizures. However, little is known about the neurotransmitter system that is implicated in anxiety and stress; Corticotropin-releasing factor (CRF) involvement in the generation of epilepsy.

1.3.2 Stress:

Stress is another psychological illness that is associated with epilepsy (Verrotti et al., 2014) and is reported to be a most frequent precipitant of seizures (Frucht et al., 2000;Haut et al., 2003;Nakken et al., 2005;Neugebauer et al., 1994). In medical or biological terms stress is a physical, mental or biological factor that results in a complex reaction from neurologic or endocrine systems (Verrotti et al., 2014). Autonomic nervous system and Hypothalamo-pituitary-adrenal axis mediate the body's physiological response to stress. Activation of these systems releases catecholamines and adrenocortical steroids (Verrotti et al., 2014). During acute stress (stress exposure for a shorter period of time) conditions brain responds by releasing certain hormones that alter specific function but the levels are restored back to basal levels after the stress exposure is subsided. In chronic stress (Stress exposure for longer periods) the physiological response persists for longer periods and cause irreversible changes in brain processes which may precipitate chronic neurological disorders such as epilepsy. This change in expression levels of

stress hormones and thus a change in neuronal excitability may partially explain the association of stress with epilepsy. A study reported in epileptic patients assessed the order of most precipitant factors for epilepsy. Most patients in the study have cited stress as the most precipitant factor in the order of frequency (Frucht et al., 2000). Stress, when exposed during early life or chronically, increase the risk of advancement of epileptic seizures (van Campen et al., 2014). Several studies reported in humans that exposure to stress during early life had long lasting effects up to adulthood (Gutteling et al., 2005;Lyons-Ruth et al., 2000;O'Connor et al., 2005). Such a long exposure can predispose an individual to develop epilepsy (Koe et al., 2009).

1.3.3 Depression:

Depression and suicide are other major psychiatric co-morbidities pathogenically associated with epilepsy. A study reported that the prevalence of depression and suicide is 4-5 times more in patients with epilepsy compared to healthy subjects (Strine et al., 2005). A meta-analysis and systematic review published in the journal *Neurology* revealed that the prevalence of occurrence of lifetime depression in epilepsy patients is 20% (Fiest et al., 2013). But some other studies have reported the symptoms of depression may be seen in 40-60% of patients with epilepsy (Grabowska-Grzyb et al., 2006). The difference in the number is attributed to the study design and the assessment factors considered diagnosing depression. Grabowski-Grzyb et al. have reported that depression was more common in patients with TLE with a focus in dominant hemisphere (Grabowska-Grzyb et al., 2006). In a recent study published in 2012 substantiated reports made by Grabowski-Grzyb et al. and reported the pathogenetic association of depression with TLE (Garcia, 2012). Kanner et al proposed that pathogenic association of depression and epilepsy may be bidirectional and thus epilepsy may increase the onset of depression and depression acting as a

risk factor for epilepsy (Kanner, 2011). One explanation how epilepsy supports the onset of depression is through stress. Unpredictability about the occurrence of seizures may cause the patient to undergo stress, sadness, despair, low self-esteem and thus the onset of depression (Verrotti et al., 2014). Conversely depression may support seizure onset. It has been reported that there was decreased noradrenergic and serotonergic neurotransmission in depression and epilepsy (Jobe et al., 1999). Upregulation of HPA axis associated with disturbances in glutamate and GABA have been reported in the development of depression in epilepsy patients (Kanner, 2011). Zobel et al found that HPA hyperactivity in epilepsy and depression may be associated with developmental abnormalities in the hippocampus and frontal lobes (Zobel et al., 2004). At this point, it is imperative to note that any change in the HPA axis result in the dysregulation of CRF system and thus relate depression associated epilepsy with CRF. Moreover, increased CRF immunoreactivity has been reported in the frontal cortical brain regions of depressed suicide individuals (Merali et al., 2004).

1.4 Corticotropin-releasing factor (CRF):

CRF is a 40 amino acid containing polypeptide that plays a pivotal role in the coordination of neuroendocrine, autonomic and behavioral responses to stress (Owens and Nemeroff, 1991;Vale et al., 1981). The amino acid sequence has been highly conserved throughout evolution process that CRF-like compounds have been used by organisms for millions of years to adapt to stress responses (Owens and Nemeroff, 1991). Following immunohistochemistry and radioimmunoassay studies CRF and CRF receptor immunoreactivity has been found in several brain regions; hippocampus, lateral hypothalamus, cortex, piriform cortex, amygdala, BNST, central gray area, dorsal tegmentum, locus ceruleous, parabrachial nucleus, dorsal vagal complex of rats (Rodaros

et al., 2007;Van et al., 2000;Potter et al., 1994;Swanson et al., 1983). There are several reports that CRF is anxiogenic in rodents (Buwalda et al., 1997;Magalhaes et al., 2010). CRF cell bodies have been widely distributed throughout neocortex, prefrontal cortex, cingulate and insular cortical areas (Swanson et al., 1983). Gray et al. have described the projection pathways of CRF neurons from central amygdala to parvocellular region of paraventricular nucleus (PVN) and parabrachial nuclei in the brain stem (Gray et al., 1989;Moga and Gray, 1985). CRF neurons from BNST project to parabrachial nuclei and to the dorsal vagal complex where they control autonomic responses (Gray and Magnuson, 1987). CRF-immunoreactive fibers from Edinger- Westphal nucleus, sympathetic and sensory ganglia terminate in layers of the spinal cord (Chung et al., 1987;Krukoff, 1986;Skofitsch et al., 1985). CRF *mRNA* is identified in several brain regions including PVN (Young et al., 1986a;Lightman and Young 1987), supraoptic nucleus of hypothalamus (Lightman and Young, 1987), inferior olive (Young et al., 1986b) and olfactory bulb (Imaki et al., 1989) using *in situ* hybridization. Northern blot analysis confirmed the presence of CRF *mRNA* in the cerebral cortex (Thompson et al., 1987;Suda et al., 1988). Thompson et al. reported that CRF *mRNA* is present in every major brain structure except cerebellum (Thompson et al., 1987). This widespread distribution of CRF in the nervous system implicates CRF peptide and its role as a neurotransmitter. Strategical distribution of CRF, high concentrations in certain brain regions whereas low concentrations in others implicate that its functional role would most likely vary among these areas.

1.5 CRF receptors:

CRF binds to two different types of receptors namely corticotropin-releasing factor receptor 1 (CRFR$_1$/ CRF$_1$ receptor) and corticotropin-releasing factor receptor 2 (CRFR$_2$/CRF$_2$

receptor) (Stengel and Tache, 2010). CRF binds to $CRFR_1$ with very high affinity compared to $CRFR_2$ (Perrin et al., 1993;Lovenberg et al., 1995). These G-protein coupled receptors have been cloned from two distinct genes that share 70% homology in amino acid composition (Bale and Vale, 2004a;Grammatopoulos and Chrousos, 2002). They signal by coupling to G-proteins resulting in the stimulation of adenylate cyclase in most cases of signaling (Grammatopoulos and Chrousos, 2002). Various isoforms of $CRFR_1$ and $CRFR_2$ exist as a result of alternative splicing (Grammatopoulos and Chrousos, 2002;Pisarchik and Slominski, 2001;Wu et al., 2007). $CRFR_1\alpha$ is found to bind with adenylate cyclase whereas the other isoforms do not have the ligand binding or signaling domains (Grammatopoulos and Chrousos, 2002;Pisarchik and Slominski, 2001;Pisarchik and Slominski, 2004). Various isoforms of $CRFR_2$ are known in humans; 2α, 2β, 2γ and in rodents; 2α and 2β (Hauger et al., 2003). Both $CRFR_1$ and $CRFR_2$ exhibit distinct pharmacological binding properties. $CRFR_1$ have high affinity for CRF, Urocortin 1 (Ucn 1) but no affinity to Ucn 2 and Ucn 3 whereas $CRFR_2$ have low affinity for CRF but high affinity for Ucn 1, Ucn 2 and Ucn 3 (Smart et al., 1999;Vaughan et al., 1995;Reyes et al., 2001;Dautzenberg et al., 2004;Dautzenberg and Hauger, 2002;Hauger et al., 2003). The dense distribution of $CRFR_1$ is found in the forebrain, subcortical limbic structures such as the amygdala. Although the expression of $CRFR_1$ is low in the hypothalamus during basal conditions, the elevated expression is reported during stress (Imaki et al., 2001;Bonaz and Rivest, 1998;Konishi et al., 2003). Highest concentrations of these receptors are distributed in olfactory bulb followed by cerebellum which is followed by cortical and limbic structures (Owens and Nemeroff, 1991). $CRFR_2$ are expressed in the hindbrain structures; dorsal raphe nucleus, choroid plexus, area posterma and nucleus of the solitary tract (Bittencourt and Sawchenko, 2000).

1.6 Corticotropin-releasing factor receptor 1 signaling:

As other members of G-protein coupled receptor (GPCR) family members, CRF receptors have the intrinsic ability to bind to heterotrimeric G-proteins. Studies in animal tissues and cultured cells revealed that CRF receptors are highly promiscuous in selecting and activating Gα subunits including Gαs, Gαo, Gαq/11, Gαi1/2 and Gαz thus linked to several second messenger pathways (Blank et al., 2003;Grammatopoulos et al., 1999;Grammatopoulos et al., 2001). Initially, when CRFR$_1$ was characterized, it was reported that CRFR$_1$ signal through activation of Gs and adenylate cyclase in the brain. The resultant increase in cellular levels of cAMP (Cyclic adenosine monophosphate) represents the second messenger associated with CRF receptor activation (Labrie et al., 1983;Labrie et al., 1982a;Labrie et al., 1982b;Giguere et al., 1982;Wynn et al., 1984;Litvin et al., 1984;Hoffman et al., 1985;Sobel, 1985;Millan et al., 1987). CRFR$_1$ activated cAMP/PKA pathway further activate several downstream signaling molecules such as ERK$_{1/2}$, membrane guanylyl cyclase, the NF- κB transcription factor, glycogen synthase kinase-3 and tyrosine hydroxylase to produce end response (Haug and Storm, 2000;Aggelidou et al., 2002;Kovalovsky et al., 2002;Zhao and Karalis, 2002;Facci et al., 2003;Nemoto et al., 2005). In the brain, CRFR$_1$ activated cAMP/PKA-mediated MAPK/ERK$_{1/2}$ phosphorylation has been reported in behavioral and memory adaptation to stress responses and neuroprotection in the hippocampus (Sananbenesi et al., 2003;Elliott-Hunt et al., 2002). Battaglia et al. reported that CRFR$_1$ may be coupled to second messengers other than cAMP (Battaglia et al., 1987). Punn et al. showed that Gαq/PKC pathway and Gβγ dependent receptor tyrosine kinase transactivation mechanisms were involved in ERK$_{1/2}$ stimulation by CRFR$_1$ overexpressed in Human embryonic kidney 293 cells (HEK-293)

(Punn et al., 2006). Gαi and Gαo proteins, PI3-K, MAPK kinase 1, Raf-1 kinase have also been implicated in CRFR$_1$ induced ERK1/2 activation (Punn et al., 2006;Brar et al., 2004). Owing to the multiplicity of CRF receptor subtypes and their promiscuity in activating several G-proteins and second messenger pathways responsible for distinct end responses, CRFR$_1$ are very important candidates in the pathological manifestation of plethora of brain disorders directly or indirectly by activating the other neurotransmitter systems (Cronin et al., 1986). Further characterization and elucidation of signaling mechanisms of these receptors are essential to explore their potential as novel therapeutic targets in human neurological and cognitive disorders.

1.7 CRF and its receptors in anxiety, stress, depression, and epilepsy:

CRF and its receptors have long been implicated in the manifestation of anxiety, stress, and depression. Although the effect of CRF on anxiety and depression is unambiguous, several studies reported the use of CRFR$_1$ antagonists as potential candidates for the treatment of psychopathologies (Paez-Pereda et al., 2011;Kehne and Cain, 2010). The increased expression of CRFR$_1$ in depressed individual brains (Nemeroff et al., 1984;Hartline et al., 1996) reveal that antagonism of these receptors may treat depression. However, there have been reports that the antagonism of CRFR$_1$ had no significant therapeutic value in depression patients (Binneman et al., 2008). In another study by Held et al. showed that treatment with R121919, a CRFR$_1$ antagonist produced effects similar to classical antidepressants such as tricyclic antidepressants, monoamine oxidase inhibitors and noradrenaline reuptake inhibitors (Held et al., 2004). Some results suggest that chronic administration of antidepressants reduced the sensitivity of CRF neurons to stress (Stout et al., 2002). Recently Fernandez Macedo et al. reported that CRFR$_1$ expression increased in the hippocampus in a learned helplessness model of depression (Fernandez Macedo et al., 2013).

Several studies have reported that antidepressants suppress CRF gene expression in rodent brain (Brady et al., 1992;Brady et al., 1991;Reul et al., 1993) and that CRF activity downregulation is a common pathway of antidepressant therapeutic effect (Licinio et al., 2004).

CRF has been widely implicated in the regulation of stress responses since its discovery decades ago (Binder and Nemeroff, 2010). CRF neurons co-express CRF and vasopressin in the hypothalamus (Aubry et al., 1999;Whitnall et al., 1987) which upon release act synergistically on the secretion of adrenocorticotropic hormone (ACTH) from the pituitary (Antoni, 1986). This mechanism is believed to cause HPA plasticity and thus precipitate intensified and long-lasting stress responses (Aubry, 2013). CRF neurons also project to locus coeruleus where they control the noradrenergic component of the stress response. The release of CRF from limbic regions induces anxiety in response to stress (Koob and Thatcher-Britton, 1985). $CRFR_1$ are essential for the activation of the endocrine response to stress. Studies in $CRFR_1$ knockout mouse (KO) showed reduced anxiety responses and dysregulation of HPA axis (Smith et al., 1998;Timpl et al., 1998). In comparison to $CRFR_1$ expression, $CRFR_2$ is more limited to specific brain regions. Studies using $CRFR_2$ KO mice revealed that mice showed increased anxiety in contrast to $CRFR_1$ KO mice with reduced anxiety (Bale and Vale, 2004b). Kasahara et al. reported that overexpression of CRF in mouse brain showed anxiety (Kasahara et al., 2007). The amygdala that is involved in the emotional and autonomic response to stress expresses CRF receptors, CRF perikarya, and terminals (Chalmers et al., 1995). Increased CRF immunoreactivity has been identified after stress in basolateral nuclei (Becker et al., 2007). CRF system also controls the release of brain dopamine. It was recently shown in mice that CRF increased dopamine release in nucleus accumbens through combined activation of $CRFR_1$ and $CRFR_2$. And this effect was completely dissipated after severe stress exposure (Lemos et al., 2012). CRF binding protein (CRFBP) has also been implicated in

stress. It was shown that CRFBP can block ACTH secretion mediated by CRF (Potter et al., 1991). Upregulation of CRFBP gene in pituitary and amygdala has been reported in rats subjected to acute stress. This increase in the CRFBP is reported to contribute to the feedback mechanism of the HPA axis (Herringa et al., 2004).

These observations in CRF and its receptor pathophysiology in distinct brain regions place CRF in the intersection of Venn diagram of anxiety, stress, and depression. The relation between CRF and epilepsy still needs to be well established. Failing in adaptation to stress and novel ambiances may cause neuronal network excitability that impacts/initiates seizures in rats (Midzyanovskaya et al., 2005). This observation is substantiated by studies involving epileptic human subjects. Temkin and Davis reported that patients with epilepsy reported stress as the precipitant factor for seizure generation in their study (Temkin and Davis, 1984). Stress caused by acoustic stimulus was reported to cause audiogenic seizures in C57BL/6 mice (Iturrian and Fink, 1969;Schreiber and Graham, Jr., 1976;Henry and Bowman, 1968;Fuller and Collins, 1968). Evidence from the literature shows that the stress modulatory neuropeptide, CRF increases seizure activity during postnatal development (Baram and Hatalski, 1998). Higher doses of CRF administered intracerebroventricularly induce electrographic and behavioural signs of seizure similar to the seizure activity observed following electrical kindling of amygdala (Weiss et al., 1986). Stress exposure has been shown to upregulate CRF gene in several limbic regions and increased the excitability of CRF-expressing pyramidal neurons in the hippocampus (Baram et al., 1997). In fact, CRF receptors are highly expressed in amygdala and hippocampus; and *in vivo* activation of these receptors induce severe age-dependent seizures. CRFBP immunoreactivity is highly expressed in entorhinal cortex after seizures may contribute to feedback inhibitory regulation of the pro-convulsant activity of endogenous CRF systems (An et al., 2003;Park et al.,

2003). This evidence indicates that CRF and CRFR$_1$ activation contribute to the development of triggered seizures.

The current understanding of epilepsy associated with stress pathology is more focused in hippocampus and amygdala. However, there are other seizure prone regions in the brain that have reciprocal connections with hippocampus and amygdala such as Piriform cortex (PC). PC has been implicated in seizure genesis and is strongly epileptogenic like hippocampus (Loscher and Ebert, 1996). Autoradiographic studies and in situ hybridization studies revealed that neurons that release CRF and express CRFR are present in the layers of PC (Van et al., 2000;De Souza et al., 1985). However, little is known about the functionality of CRF and its receptors in modulating PC during seizures.

1.8 Piriform cortex

Piriform cortex (PC) is a phylogenetically old part of the brain (paleocortex) and receives direct synaptic input from the olfactory bulb. It is the largest subdivision of olfactory cortex and all the olfactory information reaches here first. In rat brain, it extends over the lateral and ventral surface of the forebrain. The term piriform has been coined owing to its *pear*-shaped structure in the brains of certain animals. In humans, the structure that is similar to PC lies within the uncus on the ventral and medial surface of the cortex and is relatively small. The primary olfactory cortex represents the areas of cerebral cortex receive information from the olfactory bulb. The olfactory input is carried through the lateral olfactory tract and is delivered to the anterior olfactory nucleus, the anterior cortical nucleus of the amygdala, peri amygdaloid complex, entorhinal cortex, and PC. Besides olfactory processing, PC has also been implicated in the study of memory processing, the spread of excitatory waves and in epileptogenesis (Loscher and Ebert, 1996). It has

been known for many years that the PC is one of the most seizurogenic regions of the brain (McIntyre and Plant, 1989;McIntyre and Kelly, 1990).

1.8.1 Functional anatomy and structure of PC:

PC consists of a relatively simple three-layered structure compared to six layers of the neocortex. However, The cellular morphology and physiology and the circuit connections are similar between three layered PC and six-layered neocortex (Haberly and Sutula, 1992a). In fact, the PC may serve as a simple model for the most complex neocortex (Loscher and Ebert, 1996). The three layers of PC are divided into Layer I, II and III. Layer I is divided into layer Ia which contains the lateral olfactory tract (axons of mitral and tufted cells). Layer Ib receives connections from other parts of PC and other olfactory cortical areas (Price, 1973). Layer II is a dense layer consisting of perikaryon. As Layer I, it is also divided into a superficial layer IIa which contains semilunar cells (S cells) and a deep and dense layer IIb which consists of cell somata of superficial pyramidal cells (SP cells) (Haberly and Price, 1978b). Layer III shows structural variation one examines the deeper regions (Cajal, 1955;Valverede, 1965;Haberly, 1983). A moderately high density of deep pyramidal cells and lower density of large multipolar cells are the characteristic features of a superficial layer. The density of nonpyramidal cells gradually increases with the increase in depth. Associational axons that form synapses on pyramidal cell dendrites can be seen in layer III as with layer Ib. Endopiriform nucleus (DEn) which can also be regarded as Layer IV of the piriform cortex consists of spiny multipolar neurons which are also seen in small numbers in the deeper region of Layer III (Behan and Haberly, 1999;Valverede, 1965;Tseng and Haberly, 1989). Although it is divided into anterior and posterior PC, the cell morphology and cell layers are similar throughout its structure. The borderline between anterior PC (aPC) and posterior PC

(pPC) is the disappearance of lateral olfactory tract (LOT) and concomitant increase in the size of interneuronal layer (layer III) and adjacent DEn (Loscher et al., 1995;Honack et al., 1991;Haberly and Sutula, 1992b;Ketchum and Haberly, 1993;Federico et al., 1994).

1.8.2 Connections to the PC:

The major source of input to PC is from mitral cells of olfactory bulb via lateral olfactory tract that runs along the lateral surface of the anterior PC. These axons form LOT spread all over piriform cortex make synapses on apical dendrites of pyramidal cells in layer Ia. These synapses are excitatory in nature and use glutamate as a neurotransmitter (Collins and Howlett, 1988). In addition to the input from olfactory bulb, the PC also receives projections from basal forebrain, the thalamus, the hypothalamus and the brain stem. PC also receives intrinsic connections from the other layers within PC. They include excitatory connections from axon collaterals of PC pyramidal cells. These collaterals are unmyelinated and make asymmetric synapses on either multipolar cells close to their origin (Haberly and Presto, 1986) or run into layer III for some distance and ascend to layer Ib where they contact apical dendrites of other pyramidal cells (Haberly and Behan, 1983). The multipolar cells which received asymmetric connections from pyramidal cells, in turn, make symmetric connections back onto pyramidal cells in layer II. These connections are thought to be important for the inhibitory feedback mechanism (Haberly and Feig, 1983). Horizontal cells in layer I and multipolar cells in layer III provide inhibitory intrinsic input to PC (Haberly and Feig, 1983). Innervation from subcortical regions which comprise cholinergic and monoaminergic fibers modulate pyramidal cells and interneurons of PC. The cholinergic modulation is thought to contribute to memory processing in PC (Haberly and Bower, 1989). Monoaminergic input arising from median and dorsal raphae nucleus excites GABAergic

interneurons through activation of 5HT2A receptors (Sheldon and Aghajanian, 1990;Sheldon and Aghajanian, 1991;Marek and Aghajanian, 1994) thus exerting a control over pyramidal cell firing (Gellman and Aghajanian, 1993). In addition, pyramidal cell layer of PC also receives direct excitatory serotonergic innervation. Dense noradrenergic fibers from locus coeruleus and dopaminergic fibers from the ventral tegmental area innervate PC (Haberly and Price, 1978a) where they thought to excite GABAergic interneurons (Gellman and Aghajanian, 1993). Histaminergic projections from the hypothalamus to PC have also been reported (Haberly and Price, 1978a). These distinct connections from various nuclei of the brain implicate PC in the pathophysiology of disorders associated with those neurotransmitters.

1.8.3 Connections from PC:

Pyramidal cells of PC receive major synaptic input from the olfactory bulb. At the same time, strong ipsilateral projections from pyramidal cells run back and innervate olfactory bulb (Luskin and Price, 1983). Commissural connections from anterior PC project to contralateral PC(Haberly and Price, 1978b). Connections to the nuclei outside olfactory system include insular cortex, various nuclei of amygdala (Veening, 1978;Wakefield, 1980;Carlsen et al., 1982;Ottersen, 1982;Russchen, 1982), entorhinal cortex, subiculum (Krettek and Price, 1977b;Krettek and Price, 1978a), mediodorsal thalamus (Krettek and Price, 1977b;Price and Slotnick, 1983), hypothalamus and ventral putamen (Krettek and Price, 1978a;Krettek and Price, 1978b). Hence, PC has reciprocal connections with many nuclei of the ipsilateral olfactory system and insular neocortex, limbic system and hypothalamus implicating that any malfunction in the PC may associate it pathologically in disease states involving these regions.

1.8.4 PC involvement in epilepsy:

The functional anatomy of the PC has the properties that subject it to epileptic seizures. Its involvement in seizure generation is best-studied using amygdala kindling model of epilepsy. During the electrical stimulation of limbic brain regions, PC displayed large after discharges indicating its early activation in kindling process. The reciprocal interconnections with the amygdala, hippocampus, and entorhinal cortex make PC an important candidate in epilepsy. Although the connections to amygdala from PC are direct, the connections from basolateral amygdala (BLA) to PC are indirect and are via dorsal endopiriform cortex that has immense connections with layers of PC (Haberly and Price, 1978b;Krettek and Price, 1977a;Luskin and Price, 1983). Entorhinal cortex, an input structure and ventral subiculum, an output structure to the hippocampus respectively receive connections from deep cells of the PC. The connections form lateral entorhinal cortex to the posterior PC and dorsal endopiriform nuclei (Luskin and Price, 1983) provides the second pathway for the spread of the limbic seizure activity in addition to PC/BLA circuit.

1.9 Kindling model of epilepsy:

Kindling is a model of complex partial seizures with secondary generalization parallel to TLE in humans and has been a good model to study the pathophysiology of TLE, focal epileptic seizures and in the antiepileptic drug development. Periodical electrical stimulation of amygdala or hippocampus or the regions of the limbic system via stereotactically implanted depth electrodes lead to the development of complex partial seizures (Sato et al., 1990). One of the main criteria of kindling model is the distribution of the seizure activity to other regions and recruit them into the discharge leading to increased sensitivity to the focal electrical stimulation. Thus the repeated

electrical stimulation will increase the seizure severity while the threshold for the seizures is reduced (Racine, 1972a). This gradual progression in seizure severity in kindling is slower compared to the post status induced epileptogenesis and is comparable to various syndromes of human TLE (Sutula and Ockuly, 2006). The severity of seizures evoked by the kindling of amygdala or hippocampus is usually rated using Racine scale (Racine, 1972b). Stage 1 involves freezing, eye closure, unusual sniffing and mouth twitches. Stage 2 correspond to facial clonus and head nodding behavior. Stage 3, unilateral forelimb clonus usually contralateral to the stimulation electrode. Stage 4, rearing and bilateral clonus. Stage 5, rearing and falling associated with generalized clonic seizures. As the animal reaches stage 5 it is considered to be fully kindled. The increased sensitivity to focal stimulation persists for longer periods and thus reflective of persistent changes in the brain pathophysiology. Spontaneous seizures induced by kindling have also been observed in other species such as baboons, cats, and dogs. (Loscher and Schmidt, 1988).

PC has been proposed to require the smallest number of stimulations in limbic system to kindle while the hippocampus needs the largest number of stimulations compared to other limbic brain regions (McIntyre and Plant, 1989) making PC the most pro-epileptogenic region in the brain. There is accumulating evidence that PC play a critical role in the generation and propagation of the epileptic discharge induced after the electrical stimulation of other brain regions such as amygdala in addition to serving as the primary focus. Consequently, PC can act as a nodal point as it has efferent and afferent connections with other limbic and extra-limbic regions of the brain. McIntyre et al and Milgram et al reported that continuous limbic brain stimulation resulted in damage to PC even though the stimulation was applied to the amygdala (McIntyre et al., 1982;Milgram et al., 1985). The resultant structural damage in PC in association with

up/downregulated levels of amino acid neurotransmitters and loss of interneurons render PC more epileptogenic region in the brain.

Lesional studies revealed that animals with complete loss of PC were unable to develop secondarily generalized seizures after hippocampus kindling suggesting that discharge from hippocampus may need to access PC before it can be generalized (McIntyre and Kelly, 1990). Also, lesions in the posterior PC hindered seizure progression in rats after chemical induced kindling in the amygdala (Wahnschaffe et al., 1993). Mechanically induced lesions in the transition zone between anterior PC and posterior PC reduced effect in amygdala kindling rates (Racine et al., 1988). Overall, these lesional studies indicate that PC plays a prominent role in facilitating the development and progression of kindling.

Kindling of the amygdala may serve as a model of anxiety or stress. It has been shown that Wistar rats showed increased anxiety measured in the elevated plus maze one-week post amygdala kindling (Adamec, 1990a). Partial kindling of amygdala or ventral hippocampus in cats showed permanent fearful responses to the threat which may serve as a model for human anxiety (Adamec, 1990a;Adamec, 1990b). These findings provide preliminary observations which suggest that experimental limbic epilepsy increases anxiety interictally and alter responses to stress. The effects of kindling on stress and anxiety were shown to mediate through CRF receptors as α-helical CRF, a CRFR antagonist blocked these stress responses (Adamec and McKay, 1993). It has also been suggested that kindling may increase the release of CRF from amygdala in response to stress (Adamec and McKay, 1993) which indicates that kindling model provides an important basis to study epilepsy stress and anxiety associated with CRF pathology.

1.10 Traumatic brain injury:

Traumatic brain injury is a major risk factor for developing pharmacoresistant epilepsy. Posttraumatic epilepsy accounts for 8-33% of civilian head injuries and about 50% in military populations (Lowenstein et al., 1992;Lowenstein, 2009). TBI can be subdivided into primary insults where there is a physical damage from a blunt force; and secondary insult which include increased glutamate release, inflammation as a result of injury, oxidative stress, altered neuronal connectivity and metabolic dysregulation (d'Avila et al., 2012;Khan et al., 2011;Andriessen et al., 2010;Readnower et al., 2011;Ward et al., 2011). There is an extended period lasting for months or even years in most of the patients following the insult before the occurrence of a first seizure. There is a strong association between head trauma and the development of a seizure focus in the temporal lobe (Jennett, 1973). Multiple molecular, cellular and network level events can occur during this latent period, which underlie epileptogenesis. To date, there is no cogent explanation by which head traumas can lead to the development of focal seizure. Unraveling these events require intense research and may reveal important therapeutic targets.

The experience of stressful life events in brain-injured patients exacerbates the occurrence of seizures. Post-traumatic stress disorder (PTSD) and traumatic brain injury often occur with each other. PTSD is a disorder that develops in response to a traumatically stressful life event. This is associated with fluctuations in HPA axis and thus implicate CRF in its pathology (Charney et al., 1993). High basal cerebrospinal fluid CRF concentrations have been reported by different studies in PTSD patients (Bremner et al., 1997;Baker et al., 1999). To date there are no reports of PC circuitry is affected after moderate to severe traumatic brain injury, if so whether modulations in stress system have any deleterious effects in PC functionality. Thus, the considerable evidence

about the involvement of CRF in post-traumatic epilepsy in various brain regions from the literature suggests that extensive investigation is required to understand its effects in PC following brain injury.

1.10.1 Controlled cortical impact model of TBI:

TBI has been effectively modeled in rodents using controlled cortical impact (CCI) (Lighthall et al., 1989;Mannix et al., 2011;Walker et al., 2012). There were several reports that this model of TBI reproduces spontaneous, behavioral and electrographic seizures that resemble human PTE in 9-36% of animals (Hunt et al., 2009;Statler et al., 2009;Bolkvadze and Pitkanen, 2012). The cortical damage as a result of CCI often extends into underlying hippocampus (Mtchedlishvili et al., 2010;Zhou et al., 2012). The extent of molecular or cellular events that occur in the brain following CCI depends on the severity of the injury. We have chosen moderate to severe injury parameters to impact sensorimotor cortex which lies dorsally to the PC in rat brain.

1.11 Thesis overview:

Despite much work done to unravel the mechanisms by how anxiety and stress lead to the development of epileptic seizures, there is still much needed to be done to have a better understanding of this aspect of neurological disorders. Moreover, the involvement of PC in stress/anxiety and brain injury induced epilepsy has not been studied.

My hypothesis for this thesis is that $CRFR_1$ and $5HT_{2a/c}$ functionally interact and this interaction may increase the activity of PC to a moderate level in healthy non-kindled animals. After kindling induced epilepsy, activation of $CRFR_1$ may increase the activity of PC to a level

that further supports epileptic spread in its layers. After the brain injury, PC ipsilateral to the injury is more epileptogenic compared to contralateral PC. My thesis is outlined

1. To study the functioning and signaling of CRF receptors and their interaction with $5HT_{2a/c}$ in a non-kindled rat PC.

2. To understand the role of $CRFR_1$ activation and signaling pathways that it follows in PC after kindling induced epilepsy.

3. To study the functioning and signaling of $CRFR_1$ in PC after traumatic brain injury.

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Chapter 2. Suppression of piriform cortex activity in rat by corticotropin-releasing factor

2.1 Introduction

In the central nervous system, corticotropin-releasing factor (CRF) binds with high affinity to $CRFR_1$ but with only moderate affinity to $CRFR_2$ (Zorrilla et al., 2014). CRF within CNS originates from cell bodies in the central amygdala as well as interneurons in the cortex (Anisman et al., 2007). $CRFR_1$ receptor activation has been shown to have a number of differing effects on cellular behavior that varies with brain region. In the prefrontal cortex, it prolongs the Serotonin (5-HT) induced increase in GABAergic synaptic activity (Tan et al., 2004). While in CA1 pyramidal neurons it modulates potassium currents (A and delayed rectifier types) (Kratzer et al., 2013). It may also dampen excitability by inhibiting NMDA-induced currents in cultured hippocampal neurons (Sheng et al., 2008). In the amygdala, CRF activates a Kv3 type potassium channels that contribute to action potential repolarization, therefore, slowing firing frequency (Fu and Neugebauer, 2008). Thus the effects of CRF seem to be highly diverse. This varied functionality may arise from the heterogeneity of G proteins that couple to CRF receptors. For example, in above-mentioned studies these actions were mediated by either protein kinase A (PKA); (Fu and Neugebauer, 2008) or protein kinase C (PKC); (Tan et al., 2004) signaling pathways.

A similar situation is apparent for the neurotransmitter 5-HT which arise from cells located in the Raphae nucleus [Sheldon and Aghajanian, 1990]. Its activity throughout the brain is highly diverse. For example, Aghajanian and co-workers found that serotonin is excitatory on interneurons and inhibitory on pyramidal cells of piriform cortex (PC) (Marek and Aghajanian,

1996;Sheldon and Aghajanian, 1990;Sheldon and Aghajanian, 1991). However, unlike CRF, this diversity in the activity of 5-HT seems to be due to the wide range of 5-HT receptor subtypes that are heterogeneously expressed throughout the brain.

The heterogeneous functions of both these neurotransmitters are thought to be important in mediating stressor or anxiety responses (Eison, 1990;Hayley et al., 2005). For example, it has been well documented that DOI, a $5\text{-HT}_{2A/C}R$ agonist produces very strong anxiety responses, and increases in extrahypothalamic CRF has been associated with anxiety disorders (Magalhaes et al., 2010;Anisman et al., 2008). $CRFR_1$ activation can enhance $5\text{-HT}_{2A/C}$ R-mediated signaling via a mechanism involving the recruitment of endosomal $5\text{-HT}_{2A/C}R$ to the plasma membrane (Magalhaes et al., 2010). Furthermore, the same study showed that prior dosing of mice with a $CRFR_1$ agonist enhanced DOI-induced anxiety. However, an understanding of how these neurotransmitters affect the neural circuit behavior is incomplete and more research is needed to understand the impact of their activation on local circuit behavior.

One region of the brain that highly expresses both $CRFR_1$ and 5-HT_2Rs is the PC (Van et al., 2000;Pazos et al., 1985). The PC is of interest in epilepsy because its circuitry easily supports seizures (Ekstrand et al., 2001). We have recently shown that piriform cortex excitability is under the control of a feed forward disinhibitory circuit that potentiates excitatory input from the lateral olfactory tract (LOT); (Birjandian et al., 2013). How CRF and/or 5-HT may modulate this circuit is not known. Therefore, the purpose of this study was to understand how $CRFR_1$ activation and their possible interaction of $5\text{-HT}_{2A/C}R$ modulate the activity of the PC.

2.2. Materials and Methods:

All the procedures performed for this project were in accordance with the guidelines of Canadian council of animal care and approved by The University of Western Ontario council on animal care.

2.2.1 Slice preparation and dye loading:

Adult male Sprague-Dawley rats weighing 150-180g were used in all experiments. They were housed individually with free access to food and water under a continuous 12-hour light/dark cycle. Animals were anaesthetized with ketamine-medetomidine hydrochloride combination and then perfused through heart with an ice-cold artificial cerebrospinal fluid (ACSF) in which sodium ions was replaced by choline ions. The composition of this ACSF used is choline chloride, 110 mM; KCl, 2.5 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 0.5 mM; $MgCl_2$, 7mM; Sodium Pyruvate, 2.4 mM; Ascorbate, 1.3 mM; Dextrose, 20 mM (McIntyre et al., 2002). The brains were perfused to flush any blood out of the vessels to prevent the iron in the blood from oxidizing and causing damage to neuronal cells. The brain was rapidly removed following perfusion and the region containing anterior piriform cortex was carefully cut into a block to facilitate slicing by a Vibratome (slices were 400 µM thick). The slices were incubated at room 37°C for 30 min and subsequently moved to room temperature (22°C) bath for 45 min. The perfusion, slicing and incubation procedures were carried out in choline-ACSF with continuous supply of carbogen (95% O_2 and 5% CO_2 mixture). These slices were used for both voltage-sensitive dye imaging (VSDI) and patch clamp recording. For the VSDI slices were incubated in the voltage sensitive dye Di-4-ANEPPS (D-199, Invitrogen Molecular probes Inc., OR, USA) for 35 min. The stock solution of the dye was dissolved in ethanol (22mg/ml). On the day of experiment, the dye incubation was

prepared by mixing 60µl of dye stock with 500µl of fetal bovine serum (FBS), 500µl of ACSF and 310µl of 10% cremophore-EL solution. The concentration of dye in the final solution was 0.1mg/ml. After incubation slices were washed for 8-10 min with ACSF and transferred to recording chamber. The temperature of the bath was maintained at 32°C during recordings and continuously supplied with carbogen bubbled ACSF having a composition of NaCl, 110 mM; KCl, 2.5 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.0 mM; $MgCl_2$, 2.0 mM; dextrose, 20 mM . The pH and osmolarity of the solutions were adjusted to 7.3-7.4 and 297-305mOsm respectively.

2.2.2 Stimulation protocol

A platinum/iridium electrode (Microprobes, Inc., MD, USA) with a tip diameter of 200-300 µM was used to stimulate lateral olfactory tract (LOT) of PC. The stimulation of each slice was in the range of 160-200 µA, each square pulse was 2.0 ms in length. The electrode was connected to a stimulator (S88X dual output square pulse stimulator, Grass Technologies, Astro-Med, Inc., QC, Canada), which controlled the pulse frequency and train duration (Birjandian et al., 2013).

2.2.3 Voltage-sensitive dye imaging

Each optical recording was about 10s in length and consisted of two phases. The first contained recording of background activity for 2s followed by the application of stimulus for 1s with a train of frequencies ranging from 5-80Hz. The acquisition rate was set at 5ms/frame. The camera saturation was set around 50% for each recording. Optical signals were recorded by a CMOS camera (Micam Ultima Brain vision, Inc., Tokyo, Japan) mounted on top of an upright microscope (Fixed stage upright microscope, BX51WI, Olympus). The light from a 100W halogen lamp source (HLX 64625, Microlites Scientific, Corp.) passed through an excitation filter ($\lambda = 530$

± 10 nm). A long pass emission filter (λ > 590 nm) collected the fluorescent signals. A long working distance objective was used in the experiments (XLFluor 4X N.A.0.28, Olympus). The movies were analyzed using Brain Vision Analyzer (Tokyo, Japan) software. A detailed explanation of the technique is described elsewhere (Birjandian et al., 2013).

2.2.4 Patch clamp electrophysiology

Patch clamp recordings were done as previously described in (McIntyre et al., 2002;Gavrilovici et al., 2012). Patch electrodes were pulled from borosilicate glass capillaries and filled with K^+-gluconate solution having a composition (in mM) of: potassium gluconate 147, KCl 1, $CaCl_2$ 2, HEPES 10, EGTA 10, Glucose 10, MgATP 2, GTP 0.3 (300 mOsm, pH 7.3-7.4). Whole-cell patch clamp recordings were made with an Axon Instruments Patch 700B amplifier (Molecular Devices, CA, USA) from neurons in layers II & III of anterior PC. Series resistance compensation was performed in all recordings. The initial access resistance was < 20 MΩ and compensated by 50-70%. Bridge balance was set to auto to correct the voltage drop across the membrane. All experiments were performed at 32°C. Stimulation of the lateral olfactory tract for these recordings was done in an identical manner as that used for VSDI. Excitability of the cells was assayed by current-clamp protocols (Axon instruments; Clampex 10.3; 500 ms pulses in 50 pA increments). For recordings done in Layer III we have previously identified by the cluster analysis 5 differing functional phenotypes (Gavrilovici et al., 2012). These clusters were named according to the terminology described by (Kroner et al., 2007)) and (Ascoli et al., 2008)). To decide whether interneuron significantly slowed action potential generation (adapted) an interspike interval ratio (II_R) was calculated. The II_R is the ratio between the last spike interval at the end of the 500 ms current pulse divided by the first spike interval. An II_R <1.25 were termed as non-adapting cells while neurons with II_R >1.25 were classified as adapting cells. As well cells were classified based

on their firing frequency (FF) at 2X current threshold (I_{2t}). Cells that had an average FF > 50 Hz were classified as high frequency (HF) while those FF < 50 Hz were termed as low frequency (LF). Cells with a FF of > 100 Hz were classified as very high frequency (vHF).

2.2.5 Tat peptide co-immunoprecipitation:

Human embryonic kidney (HEK293) cells were maintained in Eagle's minimal essential medium with 10% fetal bovine serum. HEK293 cells were seeded onto 10cm dishes 24 hours before transfection to attain 70-80% confluency. Transient transfection was performed using a modified calcium phosphate protocol described by Ferguson and Caron (2004). Cells were transfected with 1μg of YFP-SAP97 and 1μg of either FLAG-5-HT$_{2A}$R or HA-CRFR$_1$. These constructs have been described previously (Dunn et al., 2014;Dunn et al., 2013). 18 hours post-transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin containing 0.25% EDTA. 24 hours after the removal of transfection reagents, cells were treated with Tat-Scrambled or Tat- CRFR$_1$-CT for 2 hours at the described concentrations (Tao et al., 2008). Cells were subsequently lysed in lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, and 1% Triton X-100) containing protease inhibitors (1mM AEBSF, 10μg/ml leupeptin, and 5μg/ml aprotinin) for 20 min on a rocking platform at 4 °C. Samples were collected into 1.5-ml Eppendorf tubes and centrifuged at 15,000 x g for 15 min at 4 °C to pellet insoluble material. A Bronsted-Lowry protein assay was performed, and 400μg of protein from each condition was incubated for 1–2 h at 4 °C with either FLAG-immunoprecipitation beads from Sigma-Aldrich or protein G-Sepharose and mouse anti-HA antibody (1:50). After incubation, beads were washed three times with cold lysis buffer and incubated overnight at room temperature in 3x SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and

immunoblotted to identify co-immunoprecipitated YFP-SAP97 (rabbit anti-GFP, 1:1000) with either FLAG-5-HT$_{2A}$ R or HA- CRFR$_1$ in the presence or absence of Tat- CRFR$_1$-CT.

2.2.6 Reagents

DOI, H-89, Forskolin and BIS were obtained from Sigma-Aldrich Co. St. Louis, MO, USA. CRF, antisauvagine-30 and antalarmin were obtained from Tocris Biosciences, Ellisville, MO, USA. Tat- CRFR$_1$-CT peptide was obtained from CanPeptide Inc, Point Claire, PQ, Canada. The Tat-CRFR$_1$-CT sequence used was YGRKKRRQRR-PTRVSFHSIKQSTAV. This peptide has been shown previously to prevent the functional synergism between CRFR$_1$ and 5HT$_2$Rs (Magalhaes et al., 2010). Di-4-ANEPPS stock solution was prepared in alcohol and cremophore-EL solution which can be stored at 4 degree C for 2 months. Fetal bovine serum and ACSF were added on the day of experiment. CRF stock was dissolved in HBSS and milli-Q water mixture. DOI was prepared in alcohol and Milli-Q water. Forskolin, H-89, BIS, Antalarmin, antisauvagine-30 and PMA stock solutions were prepared in Dimethyl sulfoxide (DMSO). All the stock solutions were made 1000 times more concentrated than working concentrations.

2.2.7 Statistical analysis

Comparison of all measured values was done using two- way ANOVA or repeated measures ANOVA as appropriate. Post-Hoc comparisons were carried out using a Fisher Exact Test. A Mann-Whitney test (non-parametric) was used to evaluate the action potential number data taken from pyramidal cell recordings. The significance for all tests was set at $p < 0.05$. All statistical evaluations were done using Statview Software.

2.3 Results

2.3.1 Distribution of response in layers of PC after stimulation with 80 Hz pulse

We have recently demonstrated that the activation of the PC through the stimulation of the lateral olfactory tract (LOT) induces a novel feed-forward disinhibitory loop that potentiates activation of the Layer II pyramidal cell layer (see (Birjandian et al., 2013)) for a complete description). Responses were followed by voltage-sensitive dye imaging of the PC brain slices (sampling rate is 200 frames/second) that include all three layers along with the underlying dorsal endopiriform nucleus (DEn). When the (LOT) was stimulated with a bipolar electrode with frequencies ranging from 20-80 Hz for 1-second layer II was first activated. This initial response was followed about 40-50 ms later by the activation of the DEn and then (after about 200 ms) the deactivation of layer III. An example of a response is shown in Figure 2.1.
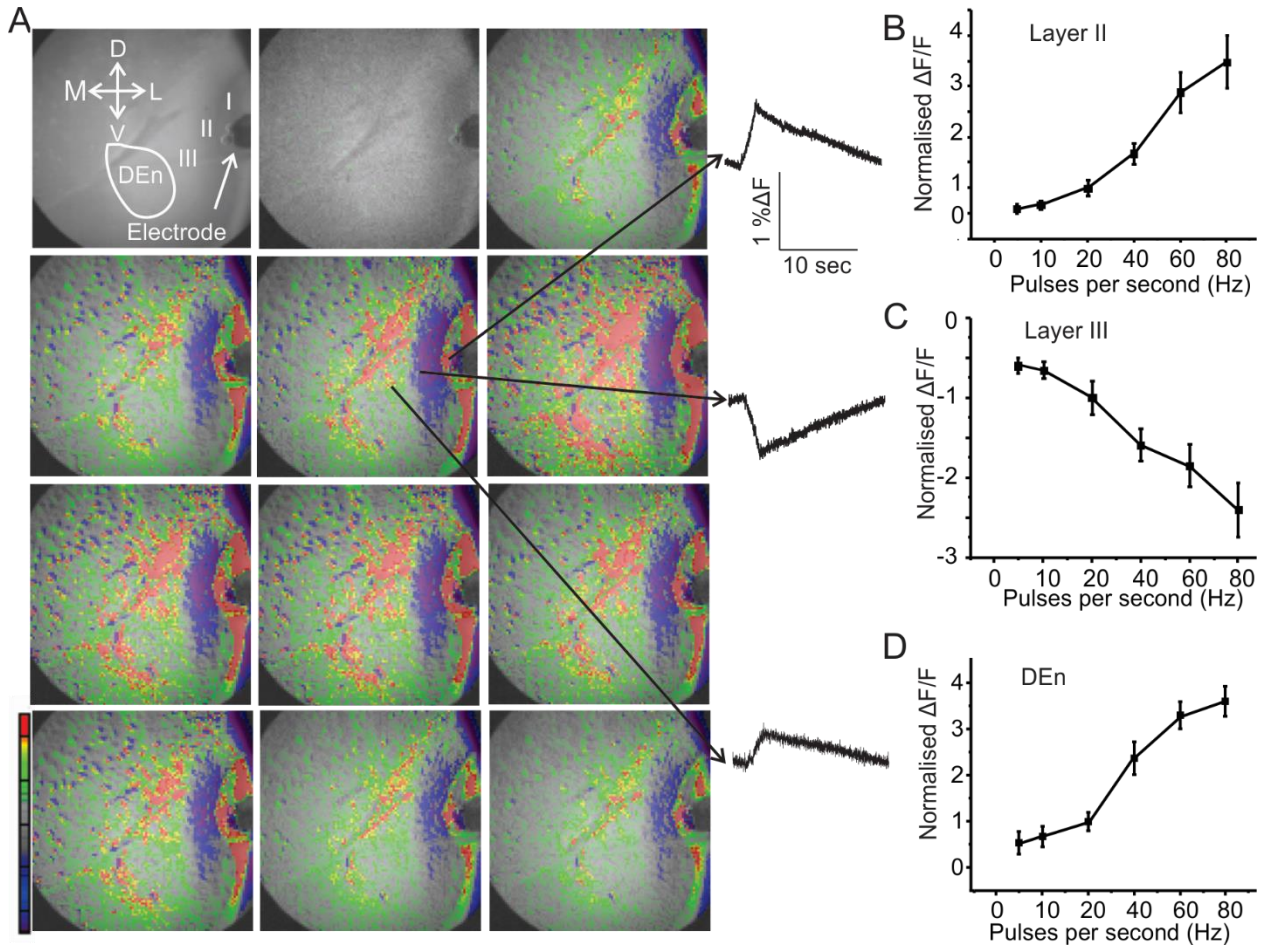
Figure 2.1 Example recording showing the distribution of the signal in the layers of PC. Activation of piriform cortex involves three kinds of responses originating in different anatomical regions. The panel in (A) show a 20s long recording where an 80Hz stimulation was used (red, green, yellow represents depolarization: blue, magenta, violet represent hyperpolarization). Waves on the right side indicated by arrows represent depolarization responses from layer II and Dorsal endopiriform nucleus (DEn) and a hyperpolarizing response from layer III (arrows in the first panel represent the orientation of the slice. D: dorsal: V: ventral; M: medial; L: lateral). (B,C,D) Graphs of normalized change in ΔF/F vs. stimulus intensity from 9 recordings in the layer II, layer III, and

Den respectively. Activation of each response increased over the range of stimulation of 20-80Hz (γ-range).

The first frame, before the stimulation, shows of the PC layers I, II, III and endopiriform nucleus (DEn). Each subsequent frame in the picture has a time difference of 2s from the preceding frame. The excitatory responses originating from layer II and DEn are shown in Figure 1B and 1D. The inhibitory response originating from layer III is shown in Figure 1C. The change in fluorescence in these recordings is color coded by the software so that excitation is shown by green, yellow and red while decreased activity is shown by blue and magenta colors. Our interpretation of this sequence of events is that the initial activation of the layer II activates inhibitory neurons in the DEn that then, in turn, inhibit layer III neurons. This disinhibition of layer III potentiates the activation of layer II neurons (again see (Birjandian et al., 2013) for complete characterization of this response). In Figure 1B-D we show the relationship between the frequency of stimulation and change in the magnitude of the response in layers II, III and DEn respectively showing that the (de)activation occurs over the gamma frequency band of stimulation trains.
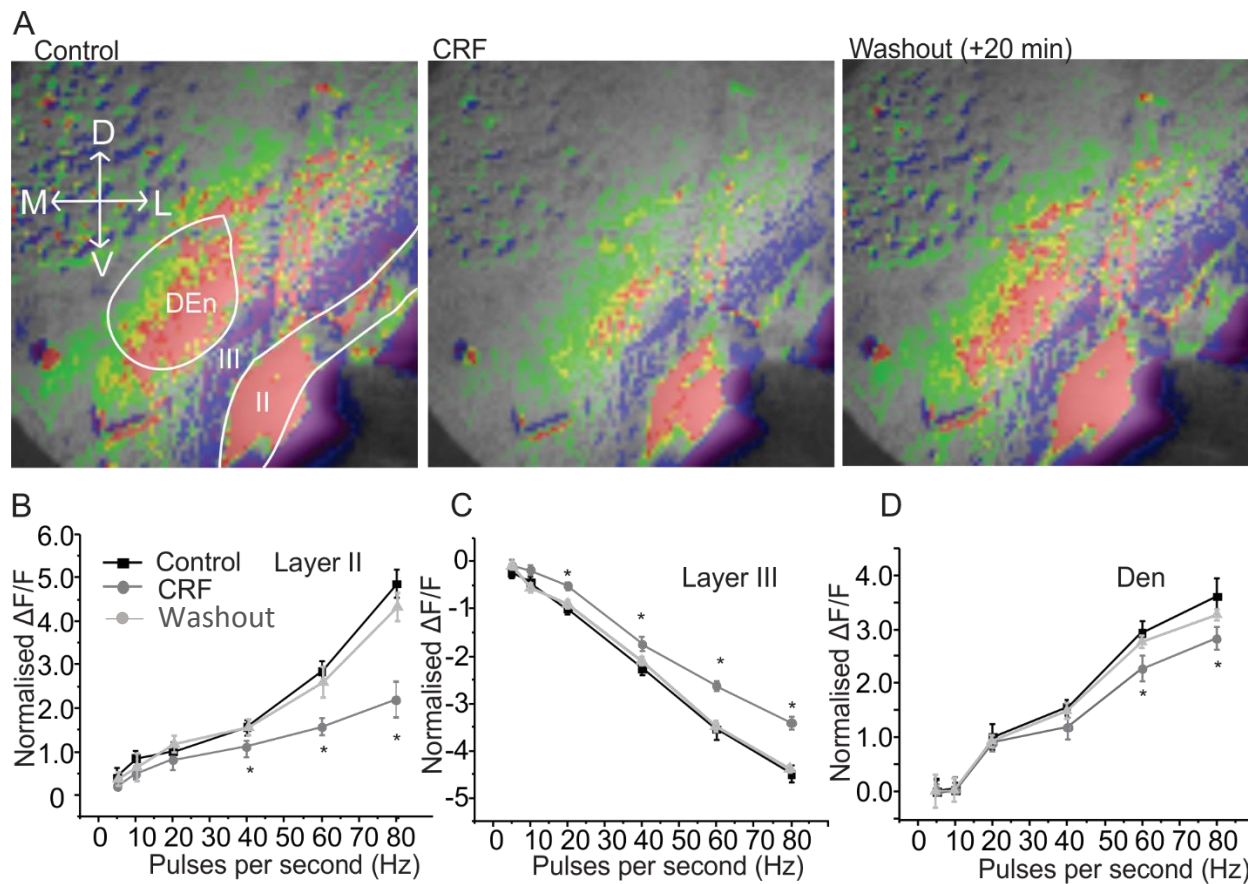
Figure 2.2 Distribution of signal after CRF (100 nM) application and washout in PC. Activation of CRFR$_1$ in PC reduced the activation of pyramidal cell layer (layer II) and dorsal endopiriform nucleus (DEn) but attenuated the deactivation of the interneuronal layer (layer III). Representative images taken 1 second after the end of stimulation are shown in A before (left) and after the application of 100 nM CRF (middle 15 min) and after 20 min wash (right). In panels B, C and D we show quantification of CRFR$_1$ effects over the range of stimulation frequencies used to activate the circuit. CRF was most effective on the 60 and 80 Hz stimulations * $p < 0.05$.

2.3.2 CRF reduces the activity of the PC

We wanted to investigate how CRF might modulate this response. This was done by comparing control input/output relationship (as shown in Figure 1B-D) to those obtained after ACSF containing 100 nM CRF was perfused over the slice. In Figure 2.2 A, the left panel shows the control response (2 seconds after the stimulation) to an 80 Hz train. The middle panel shows the equivalent response after 15-20 minutes of 100 nM CRF application and the rightmost panel shows the response after CRF washout. CRF reduced the activity of pyramidal cell layer as evidenced by decreased signal (less red) in layer II ($p < 0.001$, n=9). Similarly, the activity of neurons in DEn was also reduced which can be seen by decreased green/yellow/red scaling of the fluorescence signal. The decreased blue/violet color in layer III represents decreased disinhibitory drive arising from the DEn neurons. In Figure 2.2 B,C, and D, we show the effect of CRF on the entire input/output relationships in the three areas tested. CRF reduced the activity of layer II pyramidal cells and the cells in DEn as the curves shifted downwards (Figure 2.2 B and D). Application of CRF also reduced the inhibition of layer III, thus shifting the curve upwards. This CRF effect was reversible within 10 minutes (Figure 2.2 C). We also found that the effects of CRF were most robust within γ-frequency stimulation range (40-80Hz). There was no significant difference in fluorescence signals within θ to high β frequency range (5-20Hz).
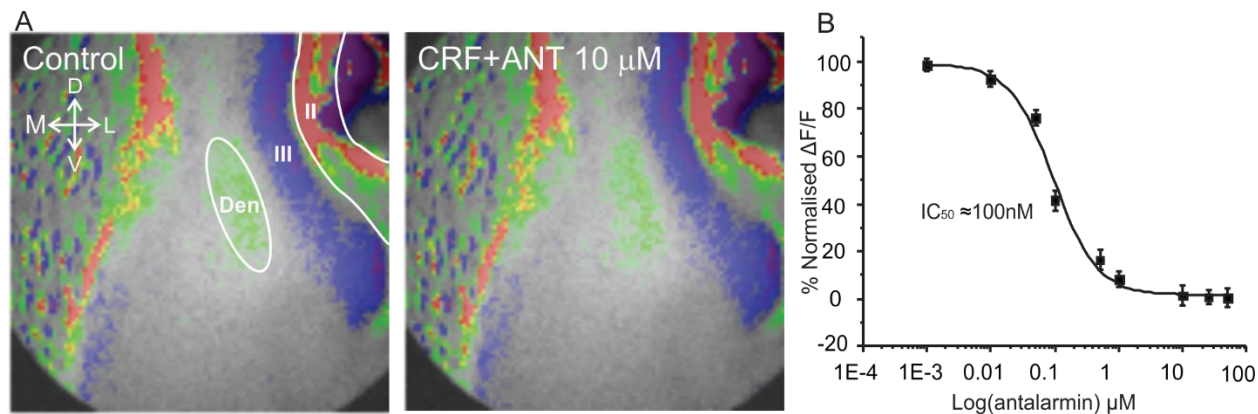
Figure 2.3 CRFR$_1$ antagonism in PC. The effects of CRF are blocked by the CRFR$_1$ antagonist antalarmin. In A, we show a representative image of the control recording (on the left). The image on the right shows that in presence of 10 µM antalarmin CRF was without effect on the activation of the PC circuitry. In B we show dose inhibition curve for CRFR$_1$ receptor blockade in layer II of PC over the range of antalarmin concentrations used. The IC$_{50}$ for antalarmin was found to be 100 nM.

2.3.3 CRFR$_1$ mediate the actions of CRF in PC

CRF exerts its actions through two major kinds of receptor subtypes named CRFR$_1$ and CRFR$_2$ (Zorrilla et al., 2014). These two receptors are widely distributed in the rat central nervous system (Primus et al., 1997;De Souza et al., 1985;Van et al., 2000). Since these receptors bind to different G proteins, activate various signaling cascades and produce different effects it is essential to understand which CRFR subtype mediate the actions of CRF in this context. We employed antalarmin, a CRFR$_1$ antagonist to see if it blocks the actions of CRF. We found that antalarmin at a concentration of 10 µM abolished the activity of CRF (100 nM, $p < 0.001$, $n = 10$). In Figure 2.3 A, we show that there is no difference in control (left panel) and CRF and antalarmin treated slice (right panel). In Figure 2.3 B, we show the dose/inhibition curve of antalarmin in the presence of 100 nM CRF. We found that the IC$_{50}$ of antalarmin was 100 nM. We also tested antisuvagine-30, a CRFR$_2$ antagonist to determine whether CRFR$_2$ has any role in the actions of CRF. Antisauvagine-30 was not able to block the actions of CRF (data not shown). These data indicate that CRF reduces the excitability of the PC through the activation of CRFR$_1$.
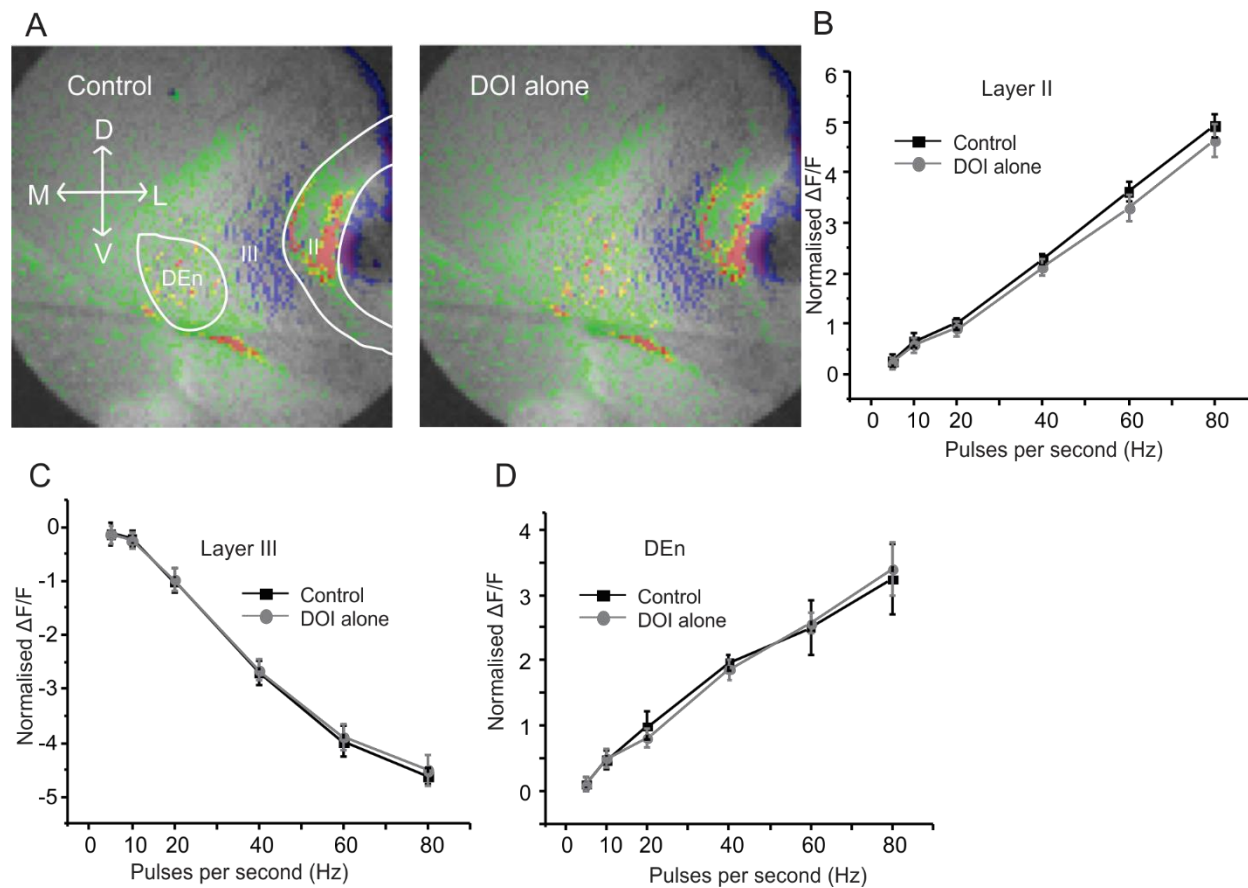
Figure 2.4 5-HT$_{2A/C}$ R activation in PC. Activation of serotonin receptors alone had no effect on piriform cortex circuitry. Representative images in A were taken before and after application of DOI, a 5-HT$_{2A/C}$R agonist. In B, C & D we show that DOI was without effect in each region followed over the entire range of stimulation frequencies used to activate the circuit.

2.3.4 DOI alone failed to activate the layers of PC

It has been previously reported that $CRFR_1$ potentiates $5\text{-}HT_2R$ mediated signaling *in vitro* (Magalhaes et al., 2010). Therefore, we wished to see if these receptors could alter PC activation and perhaps act in similar synergistic manner. We used the relatively selective $5\text{-}HT_{2A/C}$ receptor agonist DOI. Surprisingly, DOI (10 µM) had no effect on the PC circuit activation (n = 5). Figure 2.4 A shows images of the signal distribution (1 second after the end of 1 second 60 Hz stimulus) before and after the application of DOI. The quantification of these results shows that no frequency of stimulation altered circuit activity in all three layers of PC (Figure 2.4 B, C, and D).
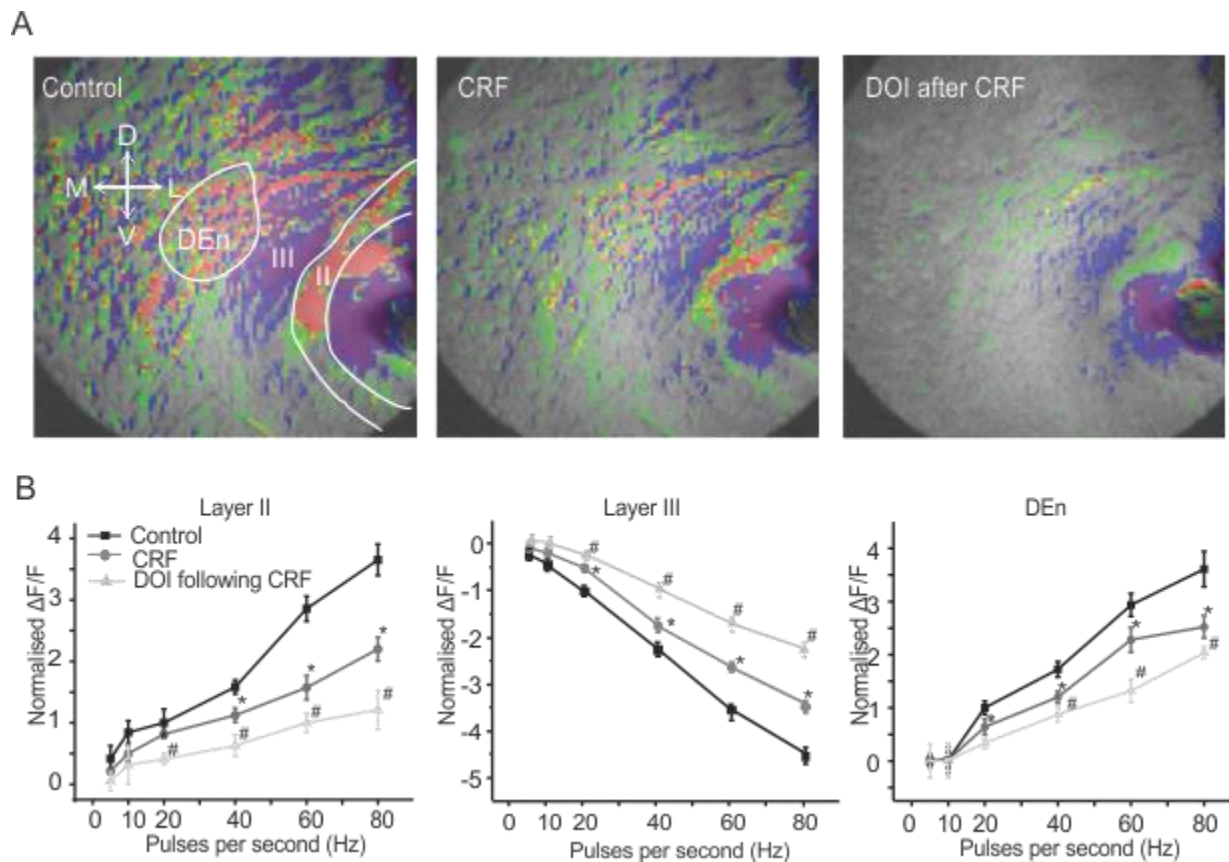
Figure 2.5 Activation of 5-HT$_{2A/C}$ receptors following the activation of CRFR$_1$. Activation of CRFR$_1$ before the application of DOI potentiated the CRF effects. Representative images in A show control (left), after the application of CRF (middle) and the subsequent application of DOI following CRF (right). The image on right was taken after 15 min of perfusion with CRF. In B, C & D we show quantitation of CRFR$_1$ and 5-HT$_{2A/c}$R activation over the range of stimulation frequencies used to activate the circuit. # p < 0.01. =CRF effect is significantly different compared to control. #= DOI following CRF effect is significantly different from CRF. DOI was immediately applied after the CRF application was stopped. However, there was at least 15-17 min time lapse between when the DOI application was started and the slice was stimulated for the recording. The perfusion bath emptying rate was maintained at 1ml per min. When DOI was added in the absence of CRF

2.3.5 Prior activation of CRFR$_1$ is required for DOI to activate the layers of PC

To test if prior CRFR$_1$ activation could induce a DOI response we first applied CRF and then DOI. Under these conditions, DOI produced similar responses to those produced by CRF. In Figure 2.5 we show images of responses taken before and after the application of these agonists. In Figure 2.5 A, the control response is in the left panel while in the middle panel we show the activity of CRF and in the right panel, we show the effect of DOI. It is evident that DOI further reduced the effects of the activation of this circuit (n = 9). In Figure 2.5 B we show the quantitation of the effects of CRF and DOI after CRF in all three layer of PC. These data show that 5-HT$_{2A/C}$R activity is dependent on the prior activation of CRFR$_1$. Unlike CRF, the effects of DOI were not reversible within 30 minutes of "washouts" that were attempted in these experiments.
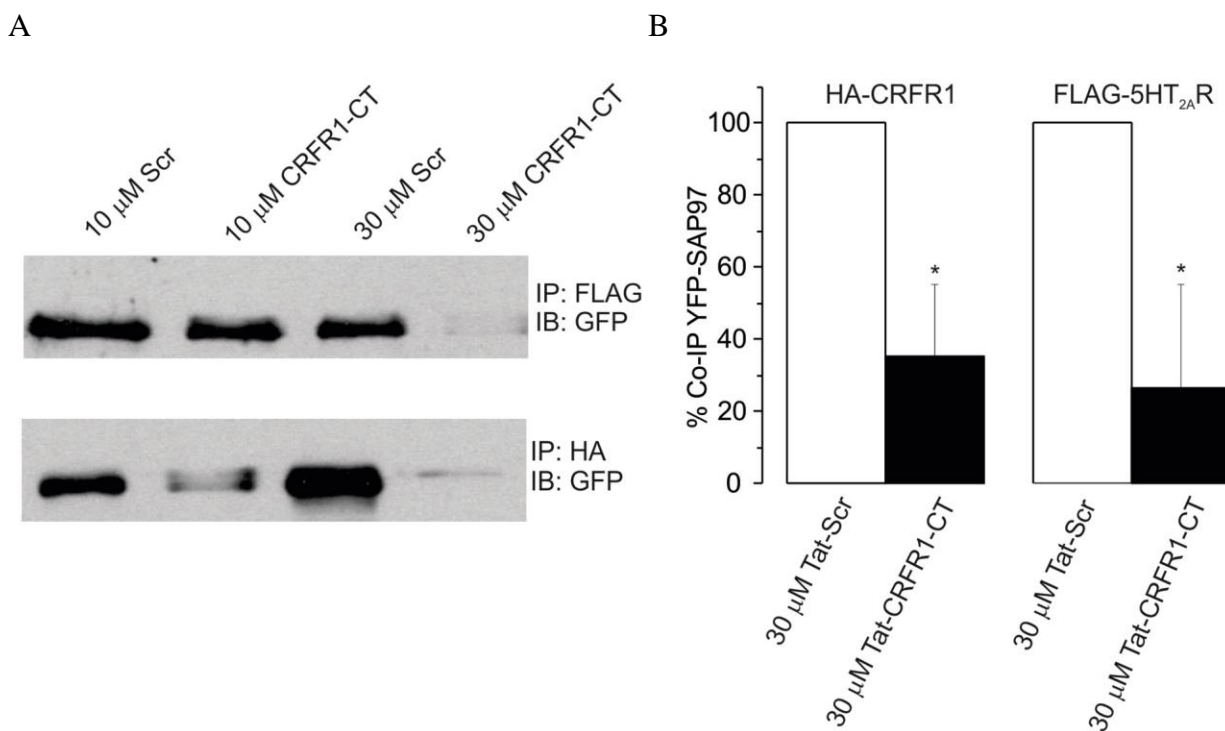
Figure 2.6 Co-immunoprecipitation of synapse associated protein 97(SAP97) with $CRFR_1$ and $5HT_{2A}Rs$ is blocked by the $Tat-CRFR_1-CT$ peptide. (A). HEK293 cells transiently transfected with YFP-SAP97 and either FLAG-5-$HT_{2A}R$ or HA-$CRFR_1$. Cells were transfected with control or $CRFR_1$-CT tat-tagged peptide. YFP-SAP97 co-immunoprecipitation with either FLAG-5-$HT_{2A}R$ or HA-$CRFR_1$ is significantly inhibited following $CRFR_1$-CT treatment. (B) Quantitative densitometric analysis of co-immunoprecipitated YFP-SAP97 western blots, $*p < 0.05$.

2.3.6 The activity of DOI is dependent on CRF and 5HT$_2$ receptor complex formation

It has been previously reported that the activation and subsequent desensitization of CRFR$_1$ lead to a complex formation by CRFR$_1$ with 5-HT$_2$R on endosomes involved in trafficking to the cell membrane (Magalhaes et al., 2010). Therefore, we wanted to determine if the DOI effect after CRFR$_1$ stimulation may occur through the crosstalk of CRFR$_1$ and 5-HT$_{2A/C}$R, which has previously been demonstrated to involve PDZ interactions (Magalhaes et al., 2010). To do this, we utilized a Tat-tagged peptide corresponding to the final 15 amino acids of the CRFR$_1$ carboxyl terminus (Tat-CRFR$_1$-CT) which includes a class I PDZ-binding motif similar to that found in 5-HT$_{2A/C}$R. This peptide was previously demonstrated to abolish the functional crosstalk between CRFR$_1$ and 5-HT$_{2A/C}$R (Magalhaes et al., 2010). Therefore, we investigated whether this peptide could sequester PDZ domain-containing proteins capable of interacting with class I PDZ-binding motifs, thereby preventing their interaction with CRFR$_1$ and 5-HT$_{2A/C}$Rs. Figure 2.6 shows that 30 μM of Tat-CRFR$_1$-CT was sufficient to block the interaction of HA-CRFR$_1$ with PDZ domain-containing protein YFP-SAP97 in HEK293 cells. Additionally, the interaction of FLAG-5-HT$_{2A}$R with PDZ domain-containing proteins was inhibited at 30 μM of Tat-CRFR$_1$-CT, as evidenced by a lack of interaction between FLAG-5-HT$_{2A}$R and YFP-SAP97 (Figure 2.6 A and B). Therefore, Tat-CRFR$_1$-CT is capable of blocking the interaction of both CRFR$_1$ and 5-HT$_{2A}$Rs with PDZ domain-containing proteins, thereby providing a potential mechanism for the inhibition of receptor crosstalk.
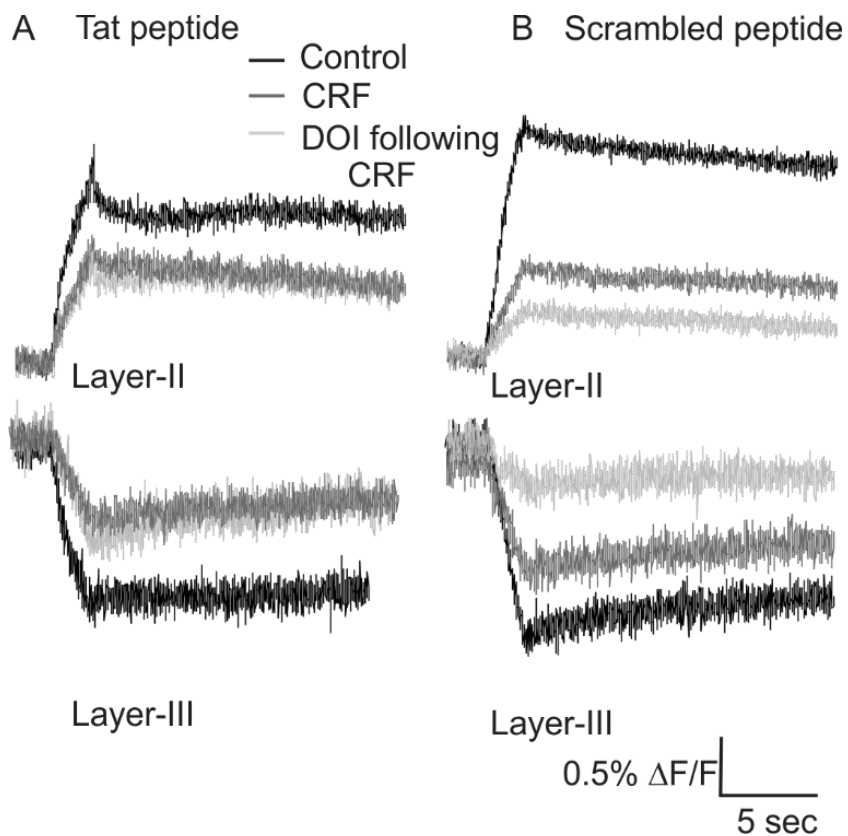
Figure 2.7 Tat-CRFR$_1$-CT peptide abolished the activity of DOI but not CRF. Representative traces A of layer II (top panel) and layer III (bottom panel) shows that after the incubation of slices with Tat-CRFR$_1$-CT peptide CRF was able to show its usual effects but DOI even applied after CRF failed to show its effect. (B) Incubation of slices in scrambled peptide did not prevent the interaction of CRFR$_1$ and 5-HT$_{2A/C}$Rs.

2.3.7 CRFR$_1$ and 5HT$_{2A/C}$Rs functionally interact with each other

Next, we determined if the same peptide could prevent the activity of DOI after CRF in brain slices. As a control, we also used a scrambled Tat-tagged sequence. The Tat-CRFR$_1$-CT peptide was incubated with the slices for 40 minutes before the application of CRF and DOI following CRF. CRF produced effects comparable to the effects that it produced in the absence of Tat-CRFR$_1$-CT while the DOI activity after CRF receptor activation was abolished (Figure 2.7). The scrambled peptide was without effect and DOI was able to further potentiate the activity of CRF. These observations are consistent with the interpretation that in order for DOI to have an effect CRFR$_1$ must interact with 5-HT$_2$Rs through a complex involving PDZ domain-containing proteins.
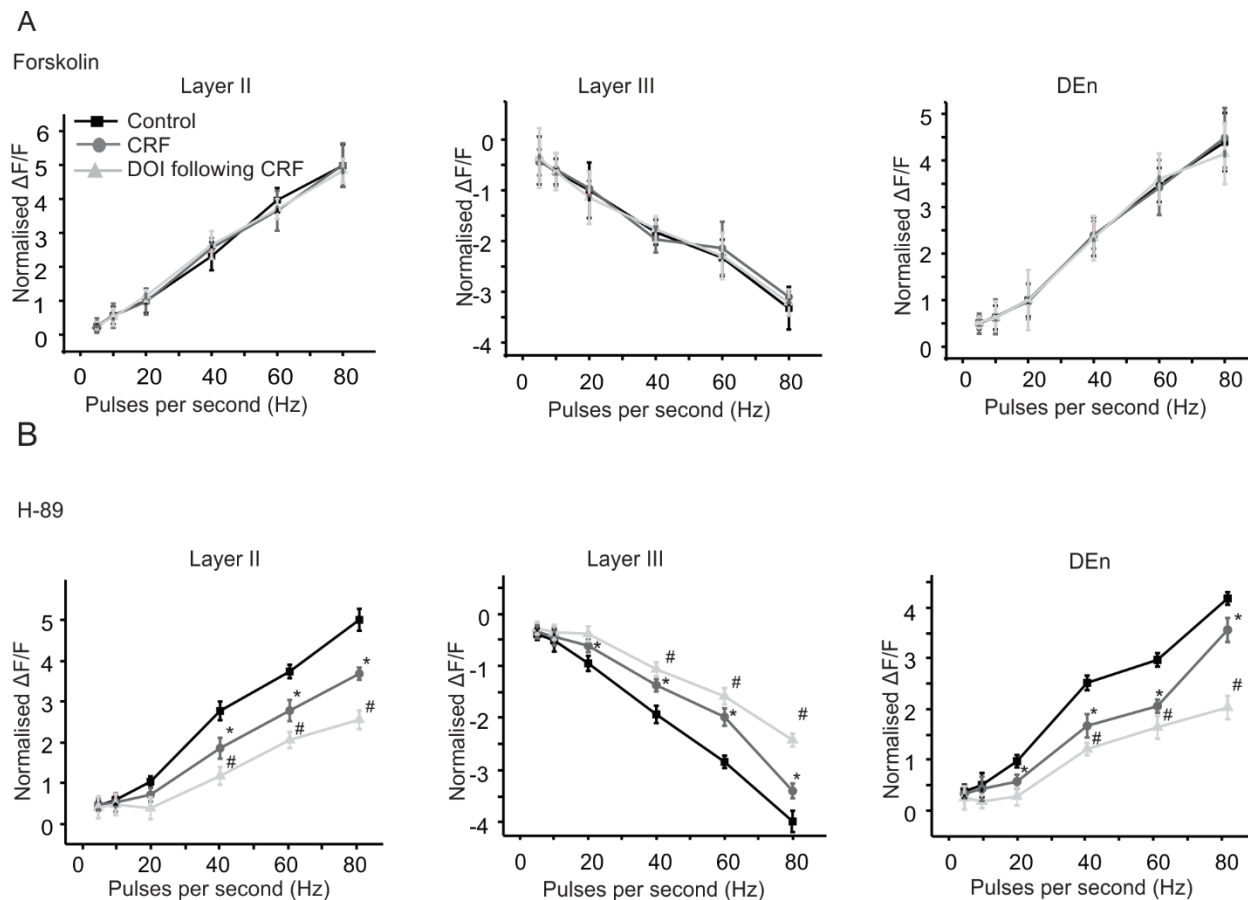
Figure 2.8 Activation of adenylate cyclase abolished CRF/DOI activity while antagonism of PKA has no effect. In A we show the quantification of the effects of adenyl cyclase activation which blocked the effects of CRF/DOI in the layer II, III and DEn. B shows that the inhibition PKA by H-89 (a PKA antagonist) has no effect on the CRF/DOI responses in all regions. * CRF effect is significantly different compared to control. # DOI following CRF effect is significantly different from CRF *# p < 0.05

2.3.8 The effects of CRF are mediated through protein kinase C activation

We found that $CRFR_1$ mediates the actions of CRF as antalarmin blocked the actions of CRF. Next, we determined which signaling cascade is activated by $CRFR_1$ stimulation. Although the $CRFR_1$ is usually classified as GPCR that is coupled to $G\alpha_s$ (activation of adenyl cyclase), there are several reports that $CRFR_1$ signaling can activate $G\alpha_{q/11}$ cascades as well (Blank et al., 2003;Elliott-Hunt et al., 2002;Wietfeld et al., 2004). To see if a $G\alpha_s$ mediated pathway may be activated by $CRFR_1$ simulation we first used the adenylyl cyclase activator forskolin (20μM, for 15-20 min) to see if it would mimic the effects of CRF in all layers of PC. It did not produce effects like CRF. (not shown). However, the subsequent application of CRF and/or DOI also had no effect and so activation of adenyl cyclase occludes the effects of CRF/DOI (Figure 2.8 A). Next, we used the PKA antagonist H-89 (10μM) to see if it could affect the CRF/DOI responses. It had no activity on either the CRF or the DOI response (Figure 2.8 B). The responses of CRF and DOI after CRF were still significant in the presence of H-89 ($p < 0.05$, n = 6)). Thus antagonism of PKA activity does not affect the CRF/DOI responses
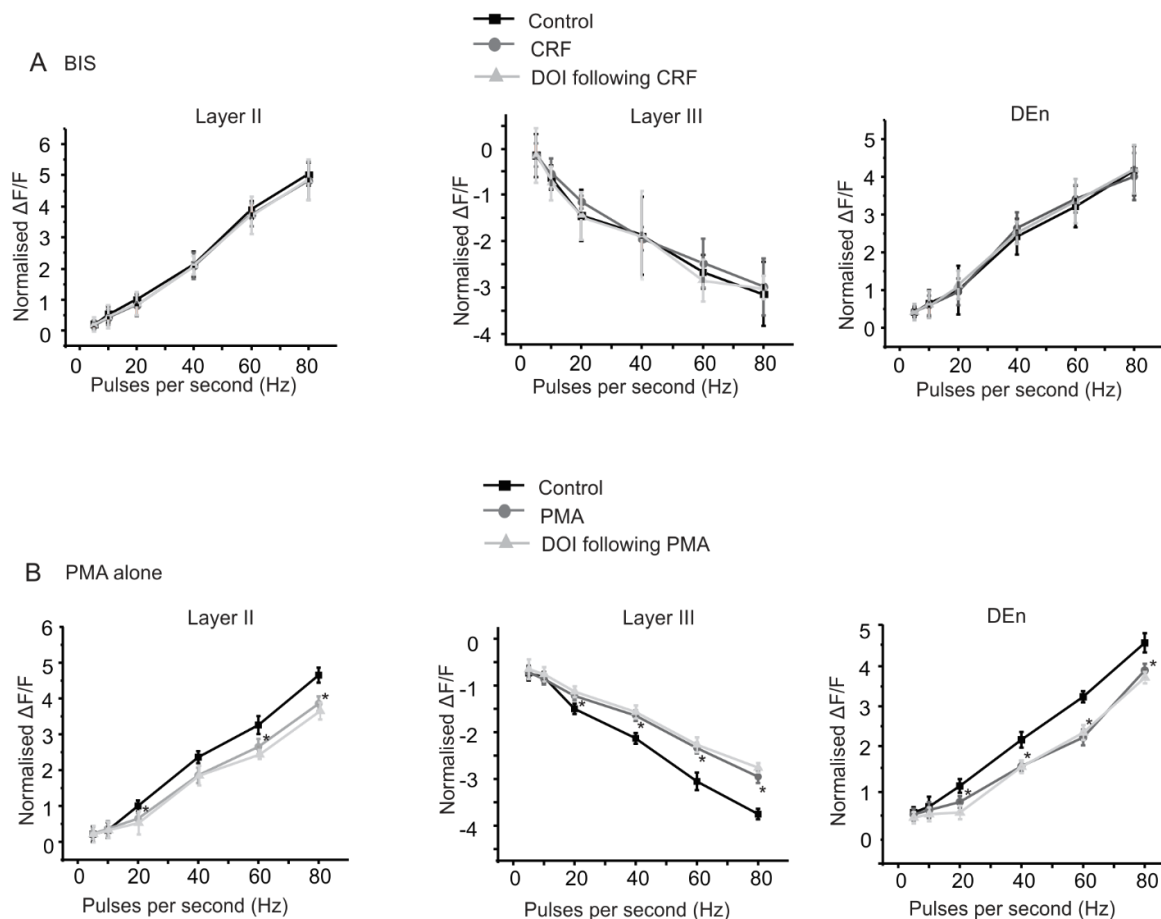
Figure 2.9 CRFR$_1$ activity is mediated through PKC activation. Application of BIS, a PKC antagonist prevented the effects of CRF and DOI following CRF. In A, we show quantitation of PKC antagonism in the presence of CRF and DOI over the range of stimulation frequencies used to activate the circuit. Application of PMA, a PKC agonist alone mimicked the CRF effects. However, application of DOI following PMA failed to produce a further change in the circuit activity. In B we show quantitation of PKC agonism in the presence of both CRF and DOI over the range of frequencies used to activate the circuit. $*p < 0.05$. PMA effect is significantly different compared to control.

Thus antagonism of PKA activity does not affect the CRF/DOI responses. Next, we applied Bisindolylmaleimide-I (BIS) (100nM), a PKC antagonist to the slices before the application of CRF and DOI following CRF. We found that BIS blocked the effects of CRF and DOI following CRF (Figure 2.9 A; n = 6). In the next step, we applied PMA alone, an activator of PKC to the see if it could mimic the effects of CRF. We observed that PMA (100nM) produced effects like CRF. However, DOI applied after PMA failed to produce a further change in the PC activity (Figure 2.9 B; n = 6).
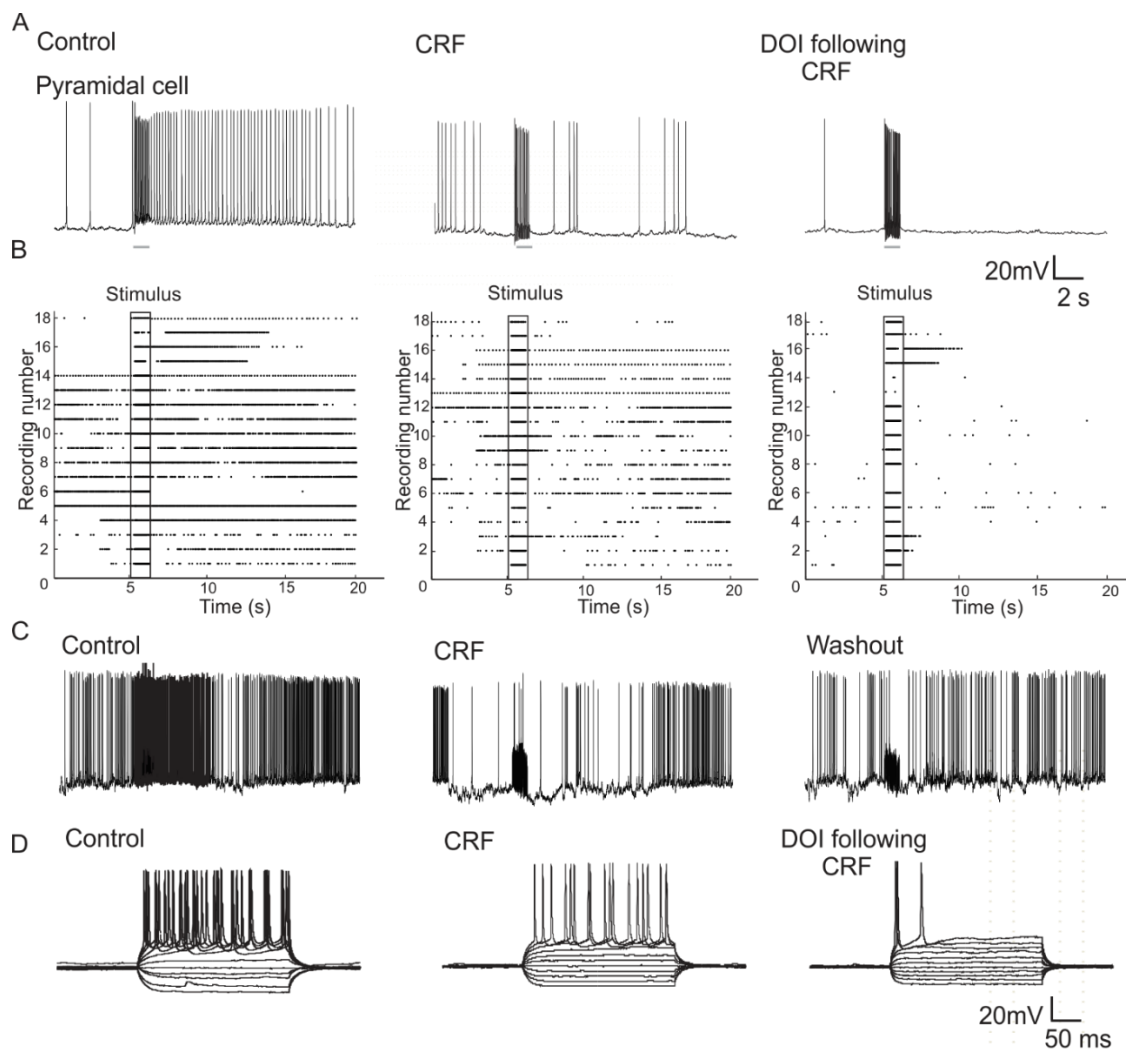
Figure 2.10 Patch clamp recordings from layer II pyramidal cells show that CRF and DOI reduced action potential firing. In A, a current clamp recording from a pyramidal cell we show that CRF and subsequently DOI attenuated the excitation produced by a 1 sec 80 Hz pulse. In B a raster plot of 18 recordings from layer II cells shows a summary of these data. In C like in the VSD recordings, the effects of CRF were slowly reversible in pyramidal cells after washout. In D a current clamp recording from a typical pyramidal cell shows that the number of action potentials produced by current injection over a range of current steps was reduced by CRF and DOI. This effect was primarily due to increased accommodation with no prominent change in firing frequency (see text for a complete description)

2.3.9 Single cell activity correlates to VSDI

In order to understand how CRF/DOI may affect the activity of individual cells, we conducted whole-cell patch clamp recordings in the layers II and III of PC. To examine the responses in the Layer II we stimulated LOT using 80 Hz trains which were identical to stimuli used for VSD imaging. The response to this train was reduced by CRF (Figure 2.10 A) even though each stimulus generated an action potential during the train (not shown). Thus CRF does not seem to have any effect on the efficacy of the stimulus only the response to them. The addition of DOI after CRF further reduced the efficacy of these stimuli. In Figure 2.10 B we show a raster plot of 18 recordings done from pyramidal cells showing that both CRF and DOI reduced the activity induced by LOT stimulation (n =10 recordings). The effects of CRF were reversible after about 15-20 minutes of washing (Figure 2.10 C), but we saw no reversibility within the same time frame for DOI after CRF applications. Finally, we also found that CRF and DOI reduced the excitability of pyramidal cells as equivalent current pulses were unable to generate same activity during a 500 ms pulse of current (Figure 2.10 D). In control recordings at 2 times the threshold current required to induce an action potential (I_{2t}) the average interspike frequency of the train (60 Hz) was 44.8 ± 1.1 Hz while in CRF and DOI this was 39.0 ± 2.0 Hz ($p < 0.01$) and 42.9 ± 1.3 Hz respectively ($p > 0.05$ compared to control). Thus the average frequency of trains generated was not largely changed. However, the number of action potentials generated by this current injection was greatly reduced (Control 11 ± 1, CRF 6 ± 1 and DOI 2 ± 1 $p < 0.001$ for all comparisons). Thus the pyramidal cells generated action potentials at similar or slightly reduced frequencies but the cells accommodated to the stimulus generating fewer action potentials.
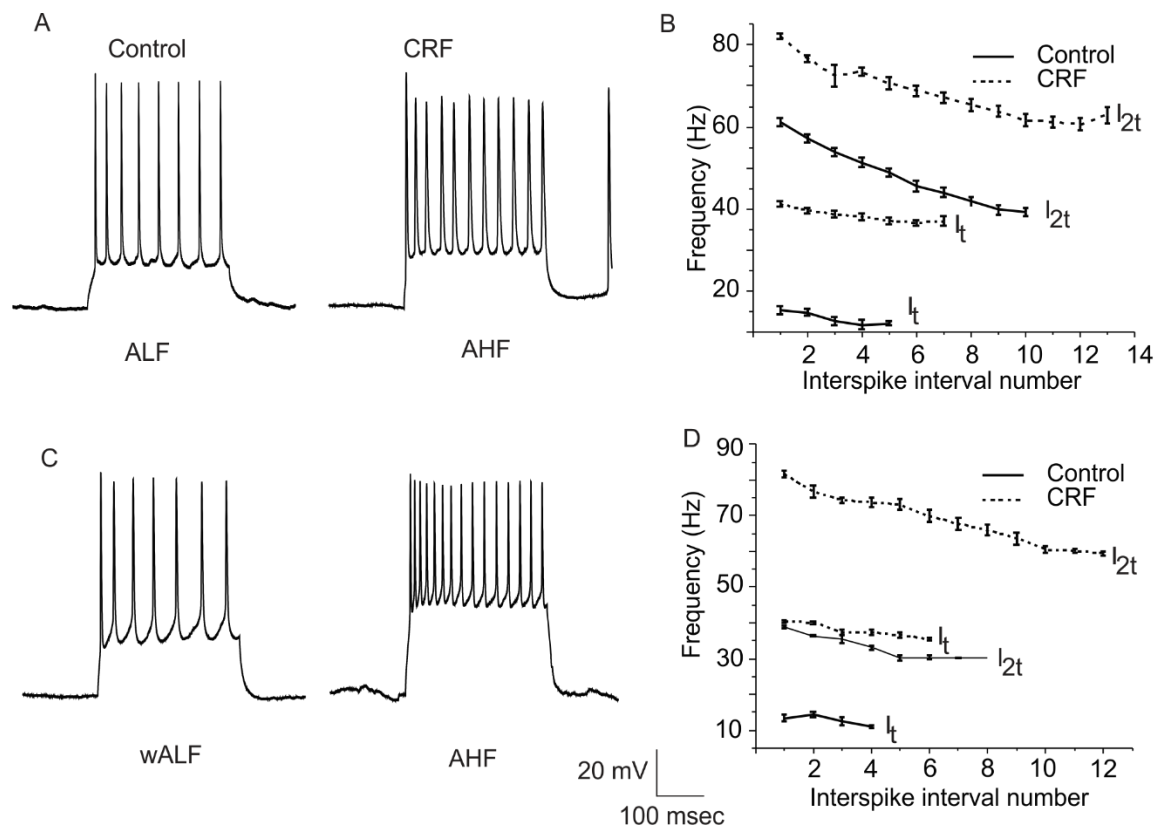
Figure 2.11 Class of interneurons exhibited increased firing frequency after the application of CRF. A subpopulation of interneurons from layer III increased their firing frequency after the application of CRF. In A, we show an example of ALF (Adaptive low Frequency) interneuron that converted to AHF (Adaptive high frequency) firing pattern after the application of CRF. The graphs in panel B show the average of firing frequency against interspike interval number from 14 ALF at one times threshold and 2 times threshold (I_t and I_{2t} respectively). Panel C shows an example of a wALF (Weakly adapting low frequency) interneuron that converted to AHF type after the application of CRF. A plot of the average of firing frequency against interspike interval number from the 5 ALF interneurons that converted to AHF after the application of CRF is shown in D.

2.3. 10 Interneurons exhibit varied spiking patterns after CRF application

CRF had variable and highly complex effects on the excitability of cells located in layer III which are almost exclusively GABAergic interneurons. In contrast to pyramidal cells, DOI had no effect on any recordings done in this cell layer. Our lab alumni previously showed that interneurons in Layer III fire with 5 well-defined and differing spike patterns (Gavrilovici et al., 2012). The naming of these firing patterns was adopted from the nomenclature defined in Ascoli et al. (2008). Here we found that CRF altered the spiking patterns of some, but not all these subtypes. There were two types of outcomes on these interneurons. One response converted low frequency firing patterns to high-frequency ones. While the second type of response showed a conversion from a high-frequency spiking patterns to low. In some recordings, there was no apparent effect of CRF.

In Figure 2.11 A, we show an example of the conversion of an adapting low-frequency interneuron (ALF; < 50 Hz average firing frequency at 2 times threshold (I_{2t}); also see methods for the description of classifications) to an adapting high-frequency phenotype (AHF; > 50 Hz at I_{2t}). All interneurons having this initial phenotype responded in this manner (Control: 44.0 ± 0.1; CRF: 62.3 ± 0.6 Hz, $p < 0.001$, $n = 14$). The graph in Figure 2.11 B shows the averaged interspike frequency versus spike interval relationship for all 14 recordings. One line shows the relationship at the threshold (I_t) where a train of action potentials is elicited, while the other shows the relation at I_{2t}. It is evident that CRF produced a dramatic change in the input/output responses of these cells. Similarly, those cells that had the weakly adapting low frequency phenotype (wALF) all converted to an AHF pattern (Figure 2.11 C; Control: $29.1 0 \pm 0.6$; CRF: 69.1 ± 0.8 Hz, $p < 0.001$, $n = 6$). The average change in the input/output for this population is shown in Figure 2.11 D.
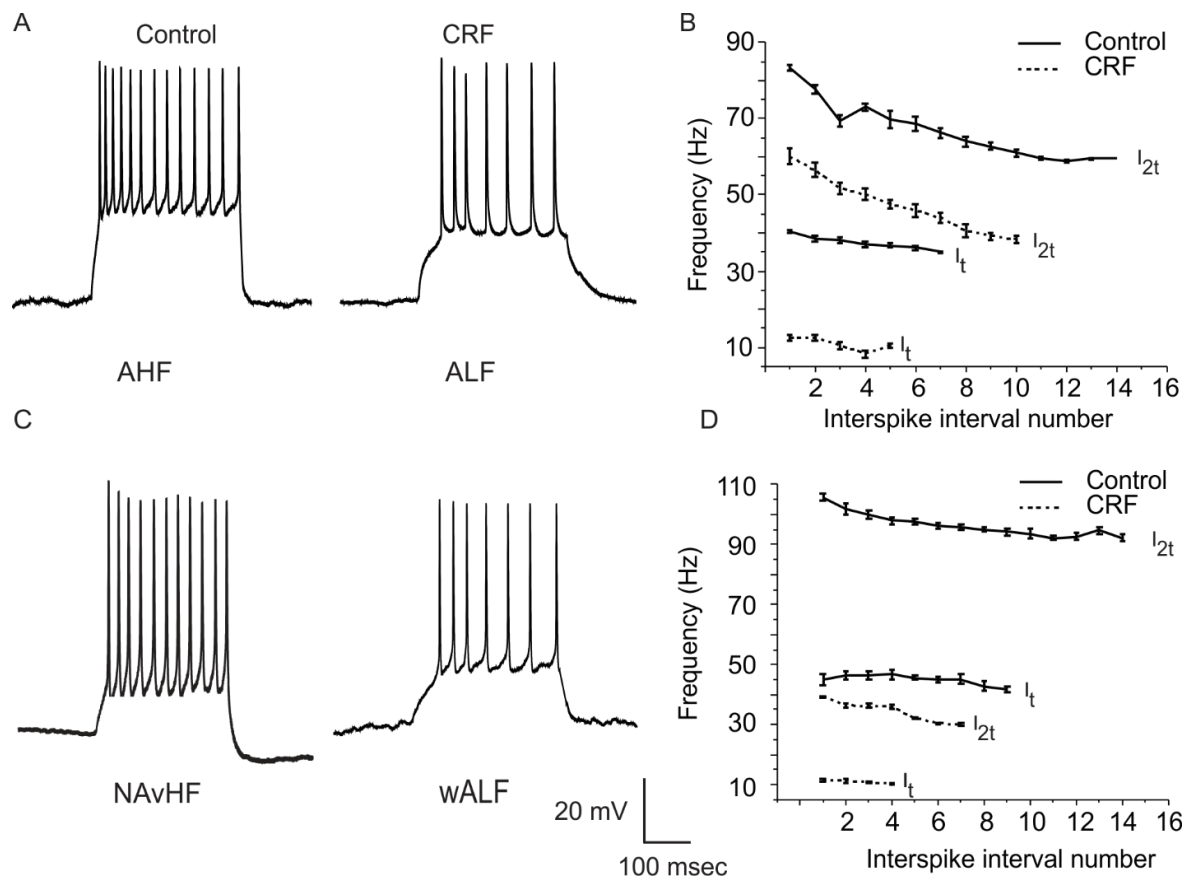
Figure 2.12 Class of interneurons exhibited reduced firing frequency after the application of CRF

Another subpopulation of interneurons from layer III reduced their firing frequency after the application of CRF. In an example of AHF interneuron that converted to ALF phenotype after the application of CRF is shown. The plots in panel B show how the average firing frequency versus interspike interval number was changed in the 6 interneurons where this occurred at one-time threshold and 2 times threshold (I_t and I_{2t} respectively). Similarly, in C we show an example of an NAvHF (Non-adapting very high frequency) interneuron that converted to the wALF phenotype after CRF application. Panel D shows the quantification of the firing frequency against interspike interval number for 5 NAvHF interneurons.

The second type of responses was more variable. Interneurons that initially fired with an AHF phenotype sometimes converted to ALF pattern (Figure 2.12 A & B; Control: 62.9 ± 0.9; CRF: 43.9 ± 1.0 Hz, p < 0.001, n = 6) but another cohort did not change (n =7; not shown). Non adapting very high frequency interneurons (NAvHF; >100 Hz) similarly had variable responses; some did not change (n = 4) while another group converted to the weakly adapting low frequency interneuron phenotype (wALF; Figure 2.12 C & D, Control: 101.3 ± 1.0; CRF: 28.1 ± 0.9 Hz, p < 0.001, n = 5). The fifth pattern that we identified (Gavrilovici et al., 2012), strongly adapting low frequency, which occurred in less than 5% of the 205 recordings, was not seen in 38 recordings we did here and so we are unsure as to the effects of CRF on these cells.

2.4. Discussion

Disturbances in homeostasis due to stressors are thought to mitigate the onset of depression or anxiety (de Kloet et al., 2005). The physiological mechanisms following stressful stimuli involve various brain regions, such as the amygdala, hippocampus and nucleus accumbens. These areas interact and integrate their responses by releasing neuromodulators that regulate stressor reactions (Mora et al., 2012). The importance of these brain regions during stress and anxiety has been extensively studied (McEwen, 2007). The PC, a part of the limbic region that is interconnected with the amygdala, hippocampus and prefrontal cortex (Loscher and Ebert, 1996), has not been extensively studied to determine how it may respond to stressors. Odor perception can induce stressor responses which are highly context dependent (Kadohisa, 2013;Krusemark et al., 2013) and therefore it is reasonable to hypothesize the PC could be modulated by neurochemical processes depending on the nature of the stressor. In this context, we have shown here that CRF and 5-HT receptors, which are implicated in stress, anxiety and depression in many regions (Kahn et al., 1988;Eison, 1990;Davis, 1992;Aghajanian and Davis, 1975;Merali et al., 2004;Muller et al., 2003;Risbrough and Stein, 2006;Arborelius et al., 1999;Naughton et al., 2000) have profound effects on the PC. What is surprising is that although CRFR and 5-HT$_2$R activation are known to be anxiogenic, they dampened the overall activity of the PC. Thus deactivation of PC would seem to be the usual response to these ligands in this particular brain region. As the primary function of the PC is olfaction this may, in fact, be a reflexive response to dampen output to the amygdala from the PC which is presumably already being, through prefrontal or other pathways, activated by the perceived threat. Our data also indicate that serotonergic neurotransmission on 5-HT$_2$R receptors, in the stimulation paradigm used here, when CRF is

released (presumably from the central amygdala or local CRF-containing neurons). Thus, it would seem that amygdala activation is required for this CRF/DOI interaction to occur.

The effects of DOI were dependent on prior $CRFR_1$ activation requiring the formation - $CRFR_1$ - $5\text{-}HT_2R$ complex. However, the interaction of $CRFR_1$ and $5\text{-}HT_{2A/C}$ Rs appears to only occur on pyramidal cells as the interneurons of layer III were unaffected by DOI application. As pyramidal cells express both $5\text{-}HT_{2A/C}$ Rs we are unsure what subtype(s) are being trafficked. Although the application of PMA mimicked CRF, activation of PKC did not induce DOI responses (like CRF). This outcome fits with the interpretation that the DOI activity is dependent on $CRFR_1$ activation and presumably $CRFR_1$ endocytosis. So although PKC activation dampened excitability, DOI could not add to this effect as $CRFR_1$ activation had been bypassed.

CRF activity seems to vary with brain region and cell type. Another study using VSDI demonstrated that CRF-enhanced hippocampal excitation (von et al., 2011) and several patch clamp studies reported that CRF was able to facilitate action potential firing in the hippocampus (Aldenhoff et al., 1983;Blank et al., 2003;Hollrigel et al., 1998). CRF has also been shown to depress excitatory neurotransmission in the hippocampal formation (Sheng et al., 2008), but we found no evidence of this here as LOT stimulation was unaffected by CRF. It has also been shown that in the prefrontal cortex CRF decreased the excitability of pyramidal cells by enhancing GABA release (Tan et al., 2004). Although we did not measure GABA release here, this outcome seems unlikely. The NAvHF interneurons whose excitability was reduced densely innervates layer II pyramidal cells and therefore a reduction in GABA release would be predicted. The reason for these highly variable outcomes throughout the brain is unclear. However, these outcomes cannot be explained by CRF interacting with differing CRFRs. It is more likely that divergent actions of

CRF are, at least, in part due to divergent signaling pathways as other studies have shown that CRFR$_1$ can activate either Gα_s or G$\alpha_{q/11}$ signaling. Elliot-Hunt and co-workers reported that CRF activated PKA and MAPK signaling pathways in the hippocampus (Elliott-Hunt et al., 2002). In another study, Sheng et al. (2008) observed, in cultured hippocampal neurons, inhibition of PKC abolished CRF regulation of NMDA currents. (Tan et al., 2004) showed that forskolin did not mimic the actions of CRF but PKC activation did; observations that are identical to ours. Thus it seems that CRF signally is promiscuous varying from cell to cell and region to region. Interestingly we found that activation of adenyl cyclase by forskolin- occluded the effects of CRF. The mechanism by which this occurs is unclear but may involve heterologous desensitization of the CRFR$_1$ itself as a consequence of forskolin-mediated activation of PKA. As we have found no evidence CRF signals through differing cascades here (although it cannot be ruled out), it seems more likely that the CRFR$_1$ activation is linked to divergent effector cascades that alter neuron function.

The most profound effect of the CRFR$_1$/5HT$_{2A/C}$R interaction is the dampening of the layer II excitability. These likely accounts for the reduced activation the circuit with the highly variable effects of CRF on interneurons in layer III modulating the overall response. Although, it would also seem reasonable to conclude that the reduced excitability of the majority of interneurons would also enhance the dampening of circuit activation. Nevertheless, the fact that the activity of CRF in layer III varied changing from one functionally distinct interneuron subtype to another argues for more subtle modulatory roles for CRF within this circuit. Given that an important function of interneurons is to control network synchrony this suggests that CRF in layer III modulates the firing patterns of the PC and may also be important for governing the impact of circuit activation. This variability in CRF effects has been observed before; when administered

intracerebroventricularly it produced both inhibitory and excitatory effects on dorsal raphae nucleus neuron firing rate. Both effects were mediated through $CRFR_1$ as antalarmin attenuated CRF's activity (Kirby et al., 2000). So in summary, our data suggest that the most salient outcome of CRF/DOI activity is to reduce the layer II activity which then attenuates the disinhibition.

5-HTR activation also has been shown elsewhere to produce heterogeneous effects in many brain regions. Here we show DOI reduced PC pyramidal cell activity but had no effect on the interneurons in Layer III. By contrast activation of $5\text{-}HT_{1C}R$ (subsequently renamed $5\text{-}HT_{2C}R$) receptors in PC produced increased pyramidal cell activity (Sheldon and Aghajanian, 1990). In same study activation of $5\text{-}HT_2Rs$ in PC increased the occurrence of spontaneous inhibitory postsynaptic potentials (IPSP's) on pyramidal cells. This was shown to be due to increased firing of interneurons located on the border between layers II and III. Initially which subtype of $5\text{-}HT_2R$ was activated was not known as the serotonin was used as the agonist and a nonselective $5\text{-}HT_2R$ antagonist was used to block this effect. Later it was shown that this effect was mediated by the $5\text{-}HT_{2A}R$ and not $5\text{-}HT_{2C}R$ (Marek and Aghajanian, 1994). We found that activation of the $5\text{-}HT_{2A/C}Rs$ on pyramidal cells decreased excitability although this required previous $CRFR_1$ activation. This argues that the effects are mediated by $5\text{-}HT_{2A/C}Rs$, consistent with the findings of Magalhaes et al. (2010). We also found that DOI had no effect on interneuron spiking patterns. These results are surprising as $5\text{-}HT_{2A/C}Rs$ are expressed in this layer and one might expect activation to alter the activity of cells in this layer. Therefore, it would seem that the increased interneuron activity observed by Aghajanian and coworkers is not due to changes in their intrinsic excitability and likely represents increased excitatory drive onto these interneurons or alteration in the neurotransmitter release mechanisms.

In summary, this study shows how neuronal activity through PC layers is altered by the activation of $CRFR_1$ and $5\text{-}HT_{2A/C}Rs$. To emphasize it is surprising that two neurotransmitters that heighten anxiety would reduce excitability in a circuit which has strong connections to the hippocampus, amygdala, and prefrontal cortex. This may represent a behavioral feedback mechanism to blunt odor-induced stressor responses. What is also striking is the highly complex and varied nature of the $CRFR_1$ effects on the differing cell populations. The use of the selective Tat- $CRFR_1$-CT peptide indicates these effects of DOI are mediated through an interaction between $CRFR_1$/$5\text{-}HT_{2A/C}Rs$ with one or multiple PDZ domain-containing proteins (like SAP97), but it doesn't rule out other similar interactions may occur with other receptor subtypes. Finally, it seems $CRFR_1$ may signal through differing G protein signaling cascades and so it may be possible under differing conditions, epilepsy, chronic stress or perhaps head injury, that these pathways may be plastic and modulation of this network and others may be dynamic.

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A switch in G protein coupling for CRFR$_1$ promotes excitability in epileptic brains

3.1 Introduction

Aversive emotional experiences increase both the frequency and severity of seizures in humans and in animal models (Frucht et al., 2000;de Boer et al., 2008). Epileptic patients are also more vulnerable to suicide, anxiety attacks, and depression than healthy individuals (Hettema et al., 2006;Pompili et al., 2005). Negative emotions such as worry and fear resulting from stress and depression affect neuronal activity within many brain regions, including limbic structures, such as the amygdala, hippocampus, and piriform cortex; these brain regions also support epileptiform activity. The epileptic brain could be more susceptible to otherwise normal stress responses or alternatively, the brain state could change the nature of the stress response so that the brain state now promotes seizures. Several hormones and neurotransmitters are increased by anxiety and stress conditions and might, therefore, be involved in stress-dependent seizurogenesis. Basal concentrations of stress hormones are increased in epileptic patients compared to healthy individuals. For instance, increased seizure frequency is observed with increased concentrations of cortisol in epilepsy patients (Galimberti et al., 2005). The polypeptide hormone corticotropin-releasing factor (CRF), which also acts as a neurotransmitter, might also serve to increase seizure frequency and has been linked with various psychological disorders (Arborelius et al., 1999). The presence of CRF-immunoreactive cell bodies in paraventricular nucleus links CRF to the endocrine stress axis and the dense distribution of CRF immunoreactivity in the dorsal raphae nucleus and locus coeruleus implicate CRF in the modulation of monoaminergic pathways. Individuals with major depressive disorder may have hyperactive hypothalamic-pituitary-adrenal

axis (HPA) functioning, including CRF hypersecretion and increased *CRF* mRNA in PVN (Raadsheer et al., 1994).

In other portions of the CNS, CRF is released from axons arising from cells of the central amygdala and interneurons (von et al., 2011;Anisman et al., 2007;Zieba et al., 2008). It acts on type-1 corticotropin releasing factor receptors (CRFR$_1$) with high affinity and with low affinity on CRFR$_2$, both of which are G-protein coupled receptors (GPCRs). We have shown that in piriform cortex, CRF acting through CRFR$_1$ dampens excitation of the piriform cortex circuitry *(*Narla et al.*, 2015)*, whereas it usually increases excitability in other brain regions (von et al., 2011). This activity is likely mediated through activation of G$_{q/11}$ signaling path since PKC activation and antagonism either mimic or block the activity of CRF respectively.

Because epileptic seizures are exacerbated by anxiety and stress, we decided to test the hypothesis that the effect of CRF could depend on the underlying condition of the brain or its disease state. We used the kindling model of epilepsy which mimics many aspects of temporal lobe epilepsy in humans (McIntyre and Gilby, 2009). In contrast to the normal brain state, we found that CRF augmented the principal cell excitation in kindled piriform cortex. This alteration in function appears to be due to a change in the signaling pathway activated by the CRFR$_1$, which occurs through the downregulation of a regulator of G protein signaling protein type 2 (RGS2).

3.2 Materials and Methods:

Procedures were performed in accordance with the guidelines of Canadian Council of Animal Care and approved by The University of Western Ontario Council on Animal Care.

3.2.1 Electrode implantation

Adult male Sprague-Dawley rats weighing 150-180g were anesthetized with Ketamine & medetomidine hydrochloride combination and implanted with two bipolar stimulating ∕ recording electrodes bilaterally in the basolateral amygdala with the following coordinates: 2.6 mm posterior to Bregma, 4.5 mm lateral to midline and 8.0 mm ventral (Paxinos & Watson, 1986). The electrodes were constructed of two twisted strands of 0.127-mm diameter Diamel-insulated Nichrome wire and were attached to male Amphenol pins. The electrodes were implanted and secured to the skull with jeweler's screws. The electrode assembly was fixed to the skull by dental acrylic cement (McIntyre & Molino, 1972).

3.2.2 Kindling procedure

Kindling is a phenomenon in which the delivery of daily electrical impulses to the limbic region, such as the amygdala, lead to the development of seizures and epilepsy. The rats were allocated to two groups. A control group (sham) of rats was implanted but never kindled and an experimental group was kindled 1 week after the surgery. The afterdischarge threshold (ADT) was determined in each amygdala by delivering a 2-s 60-Hz sine wave stimulus of progressively increasing intensity (15, 25, 35, 50, 75, 100, 150, 200, 250, 300 and 350 lA) until an ADT was triggered (McIntyre & Plant, 1993). The rats were stimulated daily until six generalized stage 5 convulsions were elicited. Seizure severity and duration were recorded daily during the kindling acquisition. At 5 days after the last stage 5 seizure the ADT has determined again (post-kindling ADT) using the same protocol as for pre-kindling ADT. Brain slices were isolated from the sham rats or from those which had been fully kindled and allowed to recover for a minimum of 2 weeks

after the last seizure. Rats were deeply anaesthetized with sodium pentobarbital (60 mg ∕ kg, i.p.) and then perfused through the heart with an ice-cold Ringer's solution in which sodium was replaced by choline [containing (in mm): choline Cl, 110; KCl, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 0.5; $MgCl_2$, 7; Na pyruvate, 2.4; ascorbate, 1.3; dextrose, 20] (as described in McIntyre et al., 2002b). After perfusion, the brain was rapidly removed and the temporal lobe area was excised as a block. The block was sliced coronally with a Vibratome (300-lm-thick sections). The slices were obtained from 1.5 to 0.3 mm relative to bregma. The slices were incubated at 37 °C for 30 min and subsequently moved to a room-temperature (22 °C) bath for at least 45 min. Slicing, incubation, and storage were all performed in the choline solution. The Ringer's solution used during electrical recordings was similar to the choline solution except that pyruvate and ascorbate were removed, equimolar NaCl replaced the choline Cl and $MgCl_2$ were used at a concentration of 2 mm. In order to slow the miniature inhibitory postsynaptic current (mIPSC) frequency, $CaCl_2$ was present at 0.5 mm. All solutions were maintained at pH 7.4 and bubbled with 5% $CO_2 ∕ 95\%$ O_2 (carbogen).

3.2.3 Slice preparation and dye loading

Adult male Sprague-Dawley rats weighing 150-180g or C57BL/6 mice weighing 20-25gms were used in our experiments. They were housed individually with free access to food and water under a continuous 12-hour light and dark cycle. Animals were anesthetized with a ketamine-medetomidine hydrochloride combination and then perfused through the heart with an ice-cold artificial cerebrospinal fluid (ACSF) in which sodium ions were replaced by choline ions(Gavrilovici et al., 2006). The composition of ACSF used comprised of choline chloride, 110 mM; Potassium chloride, 2.5 mM; Sodium dihydrogen phosphate, 1.2 mM; Sodium bicarbonate, 25 mM; Calcium chloride, 0.5 mM; Magnesium chloride, 7mM; Sodium pyruvate, 2.4 mM; Ascorbate, 1.3 mM; Dextrose , 20 mM (McIntyre et al., 2002). The brains were perfused to flush

any blood out of the vessels and thereby prevent the iron in the blood from oxidizing and causing damage to neuronal cells. The brain was rapidly removed following perfusion and the region containing anterior piriform cortex was carefully cut into a block to facilitate slicing by a Vibratome (slices were 400 µM thick). The slices were incubated at $37^{\circ}C$ for 30 min and subsequently moved to a room temperature ($22^{\circ}C$) bath for 45 min. The perfusion, slicing, and incubation procedures were carried out in choline-ACSF with a continuous supply of carbogen (95% O_2 and 5% CO_2 mixture). These slices were used for both voltage-sensitive dye imaging (VSDI) and patch clamp recording. For VSDI, slices were incubated in the voltage sensitive dye Di-4-ANEPPS (D-199, Invitrogen Molecular probes Inc., OR, USA) for 35 min. The stock solution of the dye was dissolved in ethanol (22mg per ml). On the day of the experiment, the dye incubation was prepared by mixing 60 µl of dye stock with 500 µl of fetal bovine serum (FBS), 500 µl of ACSF and 310 µl of 10% cremophore-EL solution. The concentration of dye in the final solution was 0.1 mg per ml. After incubation slices were washed for 8-10 min with ACSF and transferred to a recording chamber. The temperature of the bath was maintained at $32^{\circ}C$ during recordings and continuously supplied with carbogen bubbled ACSF having a composition of NaCl, 110 mM; KCl, 2.5 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.0 mM; $MgCl_2$, 2.0 mM; dextrose, 20 mM. The pH and osmolarity of the solutions were adjusted to 7.3-7.4 and 297-305mOsm, respectively.

A platinum-iridium electrode (Microprobes, Inc., MD, USA) with a tip diameter of 200-300 micrometers was used to stimulate the lateral olfactory tract of the piriform cortex. The stimulation of each slice was in the range of 160-200 µA, each square pulse was 2.0 ms in length. The electrode was connected to a stimulator (S88X dual output square pulse stimulator, Grass

Technologies, Astro-Med, Inc., QC, Canada), which controlled the pulse frequency and train duration (Birjandian et al., 2013).

3.2.4 Voltage-sensitive dye imaging

Each optical recording was about 10 s in length and consisted of two phases. The first contained a recording of background activity for 2 s followed by the application of stimulus for 1 s with a train of frequencies ranging from 5-80 Hz. The acquisition rate was set at 5 ms/frame. The camera saturation was set at approximately 50% for each recording. Optical signals were recorded by a CMOS camera (Micam Ultima Brain vision, Inc., Tokyo, Japan) mounted on top of an upright microscope (Fixed stage upright microscope, BX51WI, Olympus), sections were illuminated using the light from a 100W halogen lamp source (HLX 64625, Microlites Scientific, Corp.) passed through an excitation filter ($\lambda = 530 \pm 10$ nm). A long pass emission filter ($\lambda > 590$ nm) collected the fluorescent signals. A long working distance objective was used in the experiments (XLFluor 4X N.A.0.28, Olympus). The movies were analyzed using Brain Vision Analyzer (Tokyo, Japan) software. A detailed explanation of the technique is described elsewhere (Birjandian et al., 2013).

3.2.5 QPCR analyses

Brain tissue from microdissected piriform cortex was placed in Trizol (Invitrogen, Catalog #75649) and homogenized. Extraction of RNA from the homogenate was performed following the manufacturer's protocol. To assess the purity of extracted RNA, a NanoDrop 1000 spectrometer was used. RNA (2 μg) samples with appropriate purity ($\geq$260/280 levels) were reverse transcribed using the Invitrogen Superscript II with oligo(dT) primers. For the PCR reaction, 2 μl (40 ng) of cDNA were placed in duplicates in flat cap PCR strips. A master mix containing SYBR Green Fast Mix for IQ, along with milli-Q water and the primers for the protein of interest were prepared according to the manufacturer's instructions (Quanta Biosciences). 23 μl of master mix was then

added to each PCR tube to make the total volume 25 µl. The strips were then placed in a Bio-Rad

MyIQ thermocycler for subsequent PCR reaction and fluorescence detection. Primers were

designed and purchased (IDT) for the mRNAs encoding G alpha protein stimulatory (GNAS), G

protein q polypeptide (GNAQ), a regulator of G protein 2 (RGS2) and Regulator of G protein 17

(RGS17). The primer sequences were as follows: *GNAS*: F 5' -CTG CCT CGG CAA CAS TAA

GAC-, R 5' GCA GCT GCT TCT CGA TCT TTT - , GNAQ: F 5' -ATG CTA CGA TAG ACG

GCG-, R 5' –ATA GGA AGG GTC AGC CAC AC-, *RGS2*: F 5' –CGG GAG AAA ATG AAG

CGG AC- , R 5' -AGT TTT GGG CTT CCC AGG AG-, *RGS17*: F 5' –CTA AGA TGG CAG

CCA GTC GG-, R 5' CCT GCG TGC CTT CAT TTT GT-. Primer efficiency was quantified using

a five-point 10X dilution series of rat brain cDNA. PCR primer sets were evaluated for efficiency

at 55 °C annealing temperature. Cyclophilin A (*PPIA*) (Sigma-Aldrich, Catalog #1475-017&

#1475-018) was the reference gene to normalize expression (F 5'-CCG CTG TCT CTT TTC GCC

GC-, R 5' − CGA ACT TTT GTC TGC AAA CAG GCT CG-). Furthermore, 84 genes (Fig. S3)

that encode proteins involved in the rat dopamine and serotonin pathways were evaluated using

RT^2 Profiler™ rat PCR arrays (PARN 158ZA-12, Qiagen). Equal amounts of pooled cDNA from

non-kindled and kindled rat brains (5 each) were used as a template for their own respective plates.

1 plate was run for the non-kindled and kindled conditions, each well on the plate contained pooled

genetic material of 5 individual rats. Each plate was run twice on separate occasions. The plate

also contained 5 housekeeping genes, whose cycle thresholds were averaged and used for

normalization of the data. Differences in the amount of mRNA present were evaluated through a

comparison of the Δ cycle thresholds (Ct) between non-kindled and kindled brain samples. ΔCt is

the Ct (reference gene) − Ct (gene of interest). Mean ΔCt between kindled and non-kindled rats

were statistically analyzed using a Student's t-test. Data are expressed as a mean ± standard error

of the ΔCt values.

3.2.6 Phospho-site protein kinase screen

Samples of 250 mg of rat brain piriform cortex were washed three times with ice-cold PBS then homogenized in lysis buffer as described in Kinexus Bioinformatics protocol http://www.kinexus.ca/. The tissue was sonicated 4 times for 10 sec in an ice bath at 20% power with a Branson Sonicator. The homogenate was then centrifuged at 20,000 g for 30 min at 4C in an Eppendorf Centrifuge 5810R. The supernatant was assayed for protein concentration using the Bradford Assay (Bio-Rad). At a concentration of 1mg/ml in SDS-PAGE sample buffer, the samples were boiled for 4 min then shipped on wet ice for the Phospho-site protein kinase screen (cat# KPSS 11.0) at Kinetworks^{TM} Screening Services (Kinexus Bioinformatics Corporation, Vancouver, B.C). This assay consists of the analysis of 54 proteins that have a phosphorylation-dependent function in intracellular signaling. The complete list of the proteins assayed and the epitopes analyzed are provided in Table 3.2. and further information can be found at http://www.kinexus.ca/. The assay quantitatively shows whether a protein has increased or decreased target phosphorylation.

3.2.7 Tissue Preparation and Fixation for immunohistochemistry

The following methodology has been described in detail elsewhere (Gavrilovici *et al*., 2012). The rats were anesthetized and perfused with 0.1 M phosphate-buffered saline (PBS), followed by Lana's fixative (4% paraformaldehyde and 20% picric acid in PBS). The brain was removed from the skull, and stored in Lana's fixative for 24 h, and then placed in phosphate buffer solution with 30% sucrose for 48 h at 4°C. The brains were then flash frozen at −80°C and sectioned coronally (40 μm) using cryostat at −15°C. The sections were stored in cryoprotectant solution at −20°C.

3.2.8 Immunostaining

Sections were washed with PBS with 0.2% Triton X-100 three times for 5 min and blocked with 10% donkey serum and 10% goat serum in PBS with 0.025% Triton X-100 and 1% bovine serum albumin (BSA) for 1 h. The primary antibodies were diluted in PBS with 1% BSA and 0.025% Triton X-100. The sections were labeled with a single primary antibody and incubated for 24 h at 4°C. The primary antibodies used were 1:250 anti-rabbit CRF antibody (Abcam, ab11133), 1:250 anti-rabbit $CRFR_1$ antibody (Abcam, ab150561), and 1:1000 anti-rabbit $CRFR_2$ antibody (Abcam, ab75168). Sections were then washed two times with PBS with 0.2% Triton X-100 for 5 min and incubated with a secondary antibody for 1 h at room temperature. The secondary antibody used was Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, A-11034) diluted in PBS with 1% BSA and 0.025% Triton X-100 and was diluted 1:1000 for CRF and $CRFR_2$ and 1:2000 for $CRFR_1$. Sections were then washed three times with PBS with 0.2% Triton X-100 for 10 min and incubated in 1% Sudan Black B (Sigma-Aldrich, S2380) in 70% ethanol for 5 min. Sections were rinsed twice with 70% ethanol for 1 min, followed by two 5 min washes with PBS. Sections were mounted on glass slides with glass coverslips using Prolong Gold Antifade Reagent with DAPI mounting medium (Molecular Probes, P36935). Primary antibodies used were 1:500 rabbit anti-G protein alpha S antibody (Abcam, ab83735) and 1:250 rabbit anti-GNAQ antibody (Abcam, ab75823). The secondary antibody used was Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, A-11034) diluted in PBS with 1% BSA and 0.02% Triton X-100 at a ratio of 1:1000. Primary antibody anti-RGS2 was used at 1:500 (Sigma-Aldrich, GW22245F) with secondary antibody Cy3 anti-chicken IgY at 1:500 (Cedarlane, 703-166-155).

3.2.9 Image Acquisition and Analysis

Confocal images were obtained using an Olympus IX51 inverted microscope using either a 60× oil immersion objective (numerical aperture (N.A.) = 1.4) or for large field of viewing a 20X objective (N.A. 0.8) on a PerkinElmer UltraVIEW spinning disc confocal unit. The microscope was equipped with a Hamamatsu Orca ER CCD camera (1344 × 1024 pixels). All images were acquired using Volocity software (version 4.2.1, Improvision). A stack of 10 images with 0.2 μm spacing in the z-plane was taken. RGS2 images (Fig. 4) were acquired using a Zeiss LSM 510 Meta confocal microscope with a 40X (1.2 N.A.) objective. For Figure 4 the intensity of immunoreactivity is indicated by adding a channel (colored in white) that contains only pixels 3X above background. For the semiquantitative analysis in Fig 4C, we averaged the total amount of pixels in the field above this background value for comparison.

3.2.10 Statistical analysis

All statistical evaluations were done using Statview Software. Two-way ANOVAs were conducted to compare the neuronal responses after the application of BIS, PMA, H89 and Forskolin in the piriform cortex layers in response to LOT stimulation. For CRF responses in the presence of BIS, PMA, Forskolin and H89 a repeated measures ANOVA was used in which responses of CRF in the absence of drugs served as the within group measure. The pixel/voxel counts were analyzed using t-test. Follow-up comparisons were conducted using Fisher's protected least significance different test to maintain p at 0.05.

3.3 Results:

A Nonkindled Rat
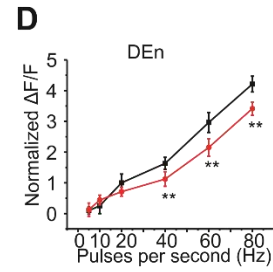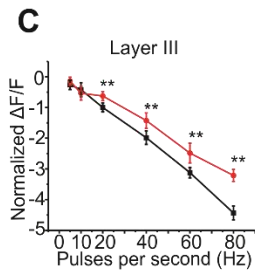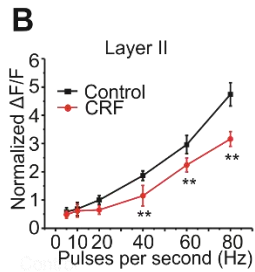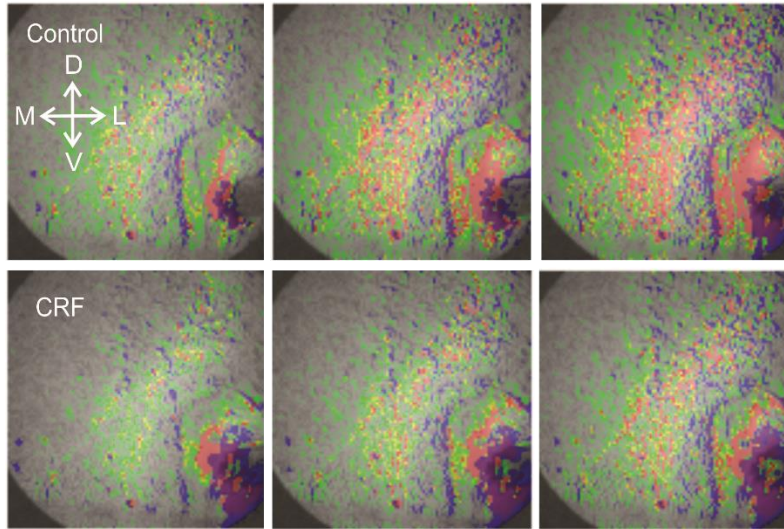
E Kindled Rat
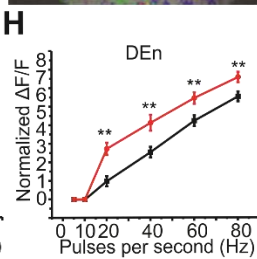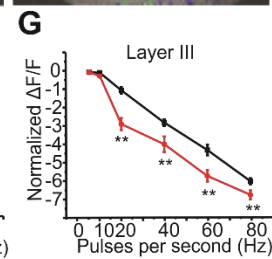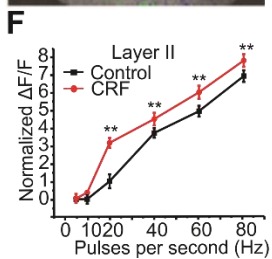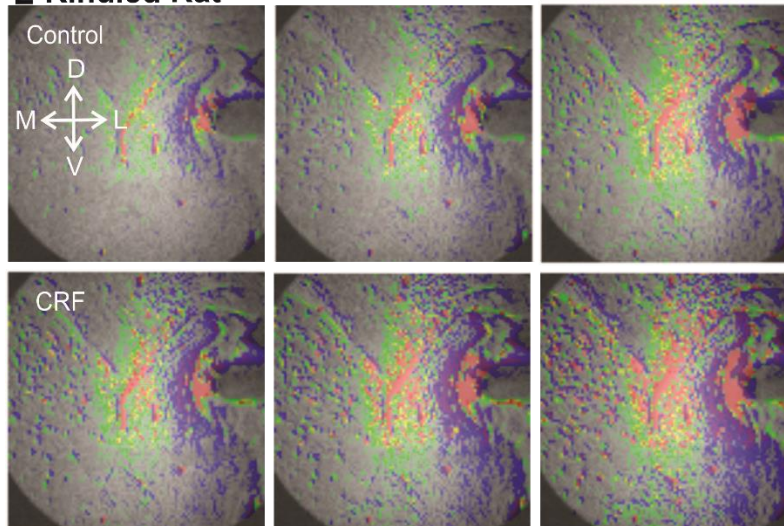
Figure 3.1 (A) Representative images showing activation of piriform cortex from non-kindled rats, without (top) or with CRF perfusion before recording (bottom). Each image in the bottom row was taken at the same time interval as that of its corresponding image in the top row. (B to D) Quantification of $CRFR_1$ activation shows that the activity of layer II pyramidal cells and interneurons of DEn are decreased while the activity of layer III interneurons is increased in non-kindled animals over the range of stimulation frequencies used to activate the circuit. ** $p < 0.01$. $n = 7$ slices from 6 rats. (E) Representative images showing activation of layers of piriform cortical slices from kindled rats, without (top) or with CRF perfusion before recording (bottom). (F to H) Quantification of $CRFR_1$ activation shows that the activity of layer II pyramidal cells and interneurons of DEn are increased while the activity of layer III interneurons is decreased over the range of stimulation frequencies used in piriform cortical slices from kindled rats to activate the circuit. ** $p < 0.01$. Kindled $n = 13$ slices from 9 rats. Arrows in indicate the orientation of slice. D, dorsal; V, ventral; M, medial; L, lateral. Red indicates highest $\Delta F/F$; orange, yellow and green indicates medium $\Delta F/F$; blue a reduction in $\Delta F/F$.

3.3.1 Activation of CRFR$_1$ activated the layers of PC

Upon activation of the piriform cortex by stimulation of the lateral olfactory tract in non-kindled rats, CRF reduced the excitation of Layer II Dorsal Endopiriform nucleus (DEn) neurons and the inhibition of layer III inhibitory interneurons, resulting in reduced disinhibition of layer II neurons (Fig. 3.1 A to D), as indicated by voltage-sensitive dye imaging. By contrast, in kindled rats, CRF increased Layer II and DEn activity, whereas in Layer III it further decreased the neuronal activity, enhancing disinhibition (Figure 3.1 E to H). Thus after kindling CRF-enhanced circuit activation, an effect opposite to that in sham-operated control rats. We hypothesized that this switch in activity could be due to changes in the relative abundance of CRFRs (Type 1 compared 2) or in CRF abundance (an increase so that endogenous release might activate CRFR$_2$).
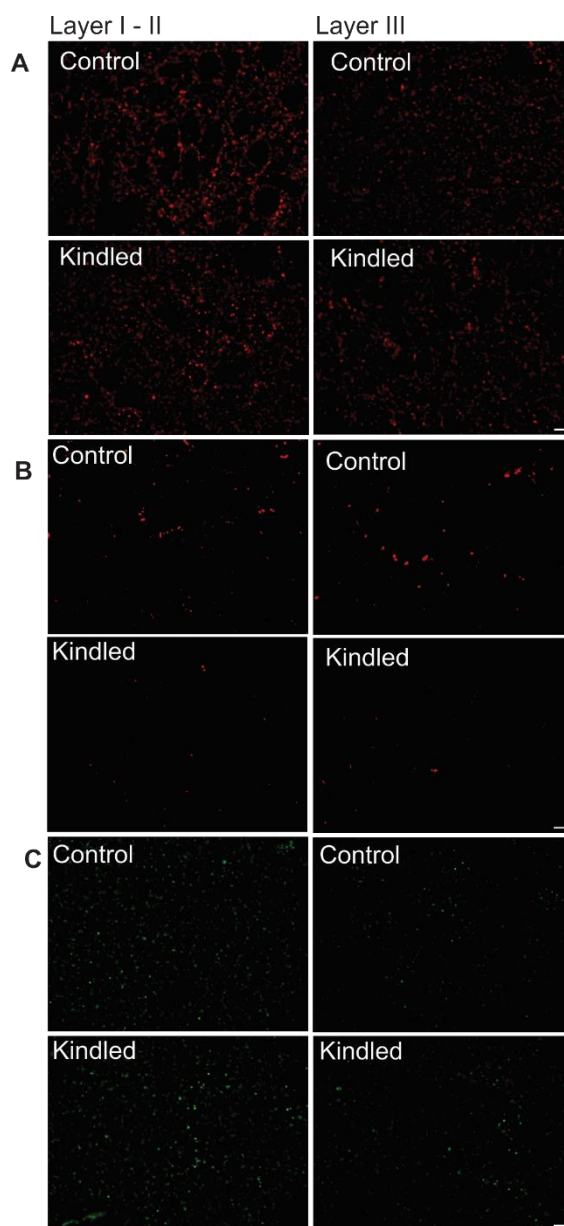
Figure 3.2 No change in CRFR$_1$-ir, CRFR$_2$-ir or CRF-ir after kindling. (A) Representative deconvolved confocal images of CRFR$_1$ immunoreactivity before and after kindling in layer I - II and layer III of the PC. Scale bar = 5 µM. (B) Representative deconvolved confocal images of CRFR$_2$ immunoreactivity before and after kindling in layer I - II and layer III of the PC. Scale bar = 5 µM. (C) Representative deconvolved confocal images for CRF immunoreactivity before and after kindling in layer I - II and layer III of the PC. Scale bar = 5 µM. Images are representative of 5 rats per treatment each case.

3.3.2 No changes in CRFR$_1$, CRFR$_2$, or CRF immunoreactivity in the layers of the piriform cortex after kindling

Immunohistochemical analyses revealed that CRFR$_1$ protein abundance was unaltered in all of the layers of the piriform cortex (Fig 3.2 **A**) and that CRFR$_2$ abundance was low and unchanged as well (Fig 3.2 **B**). The distribution and abundance of the CRF peptide were also unchanged (Fig 3.2 **C**).
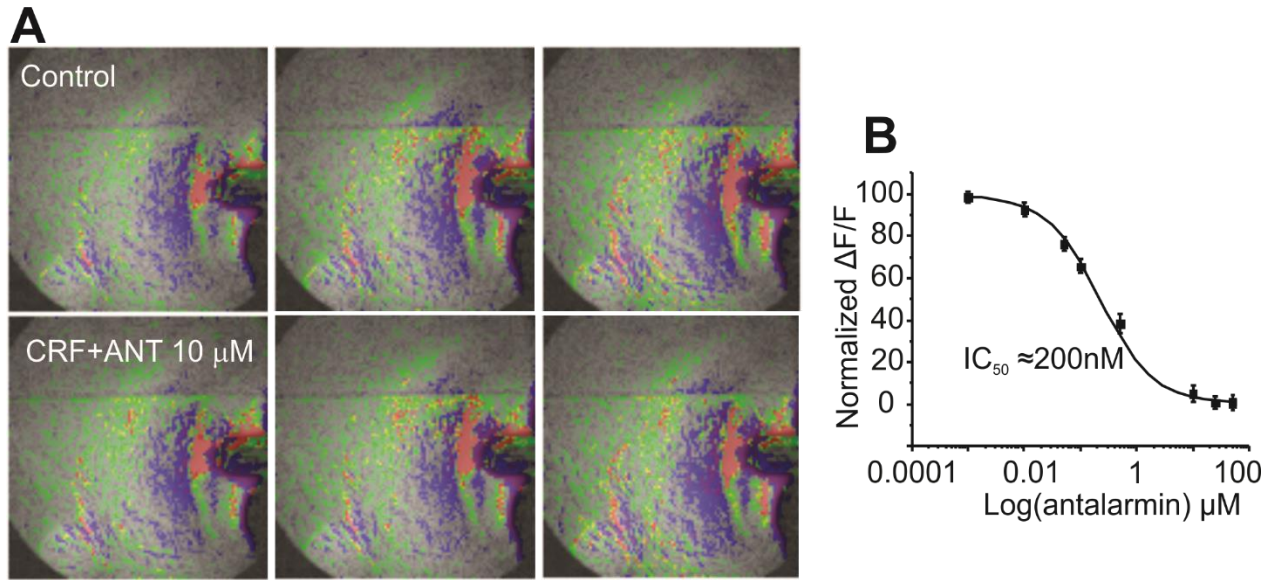
Figure 3.3 CRFR$_1$ antagonism in PC after kindling. (A) Representative images of the activation of the piriform cortex layers in slices at differing time points before (top) and after the application of CRF in the presence of the CRFR$_1$ antagonist antalarmin. The responses are similar to control responses in the presence of CRF and CRFR$_1$ antagonist. (B) Dose-inhibition curve for CRFR$_1$ activity in layer II over the range of concentrations of antalarmin used. n =9 slices from 7 rats.

3.3.3 CRF signaled through activation of CRFR$_1$

It was essential to investigate the type of CRFR mediating the responses of CRF in kindled animals. These experiments to antagonize both the receptor subtypes of CRFR may provide crucial information about the switch in polarity of responses from normal animals or kindled animals. As in normal controls, CRF acted through the activation of CRFR$_1$ because of antalarmin, a CRFR$_1$ antagonist, blocked the effects of CRF in kindled rats (Fig. 3.3). The IC$_{50}$ for antalarmin increased to about 200 nM which is in contrast to the 100 nM that has been previously reported by our group in non-kindled rats (Narla *et al.,* 2015).
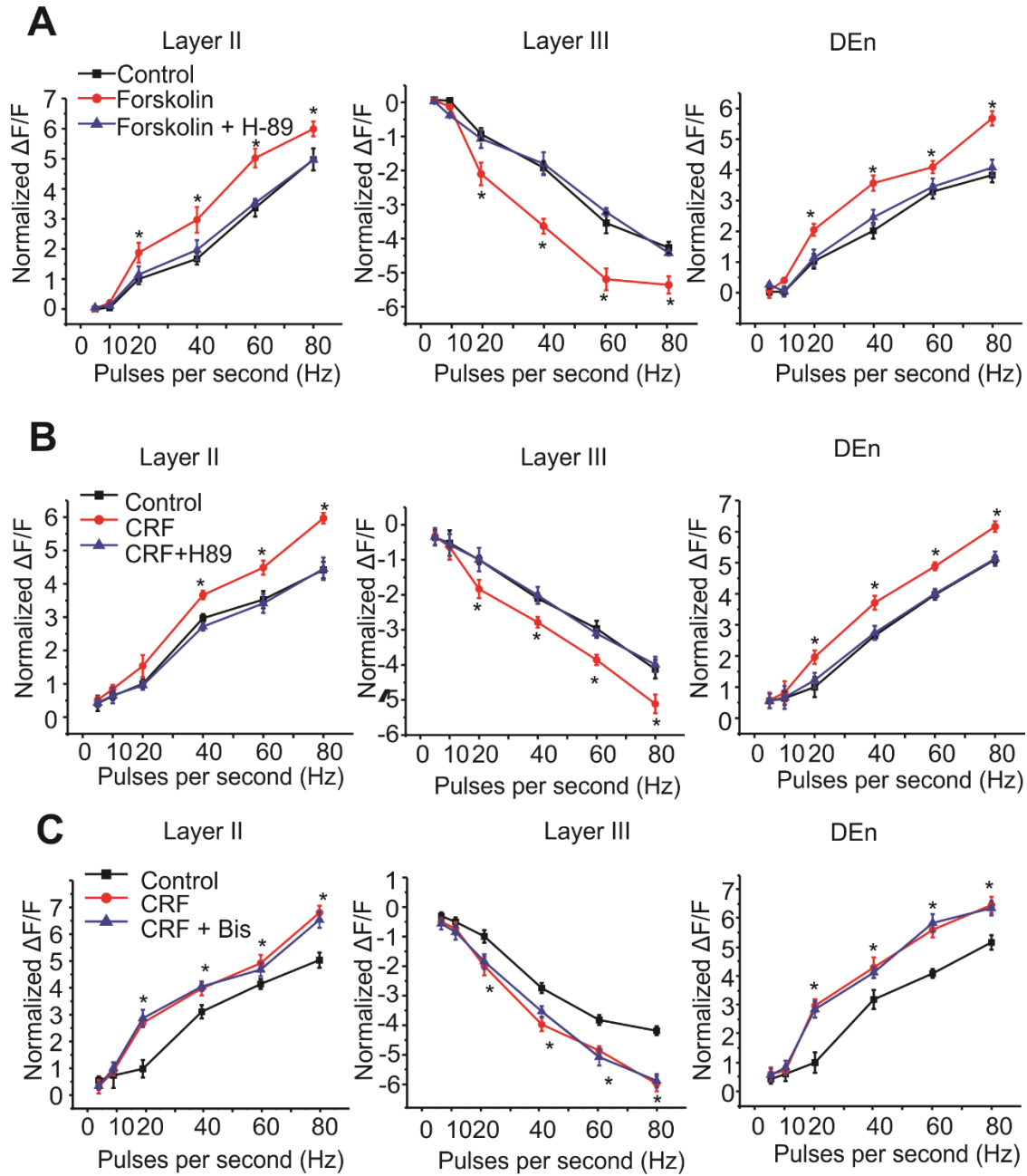
Figure 3.4 PKA and PKC antagonism in kindled PC in the presence of CRF. (A) Quantification of the effect of forskolin (an adenylyl cyclase activator) with or without subsequent administration of H-89, on $CRFR_1$ activation in piriform cortical slices from kindled animals * $p < 0.05$, n =11 slices from 9 rats/group. (B) Quantification of the effect of the PKA inhibitor H-89 on CRF-mediated activation of $CRFR_1$ in piriform cortical slices from kindled animals. * $p < 0.05$, n=9 slices from 8 rats/group. (C) Quantification of the effect of the PKC antagonist Bis on CRF-mediated activation of $CRFR_1$ in piriform cortical slices from kindled animals. *$p < 0.05$, n = 9 slices from 7 rats/group.

3.3.4 CRFR$_1$ signaled by coupling with Gα_s in epileptic animals

Because there was no change in CRFR or CRF peptide abundance and the effects were mediated through the same receptors, we next investigated the signaling pathways involved in CRF responses. In my earlier study, it was observed that in non-kindled animals, CRFR$_1$ signals through activation of G$\alpha_{q/11}$ and thus linked to PKC-mediated signaling (Figure 2.8). G$\alpha_{q/11}$ activated phospholipase C (PLC) hydrolyzes phosphatidyl 4,5-bisphosphate (PIP$_2$) to diacylglycerol (DG) and inositol triphosphate (IP$_3$). DG, a second messenger activates PKC and facilitates further signaling cascades. But in kindled animals CRFR$_1$ signaled through the activation of a Gα_s mediated pathway. It was first found that forskolin, an activator of adenylyl cyclase that mimics the effect of activated Gα_s, mimicked CRF responses and this was antagonized by the protein kinase A inhibitor H-89 (Figure 3.4 A). H-89 blocked the effects of CRF (Figure 3.4 B). The application of the PKC inhibitor Bisindolylmaleimide (BIS) did not block the effects of CRF (Figure 3.3 C), contrasting with non-kindled animals that application of BIS blocks the effects of CRF in non-kindled animals (Figure 2.9)
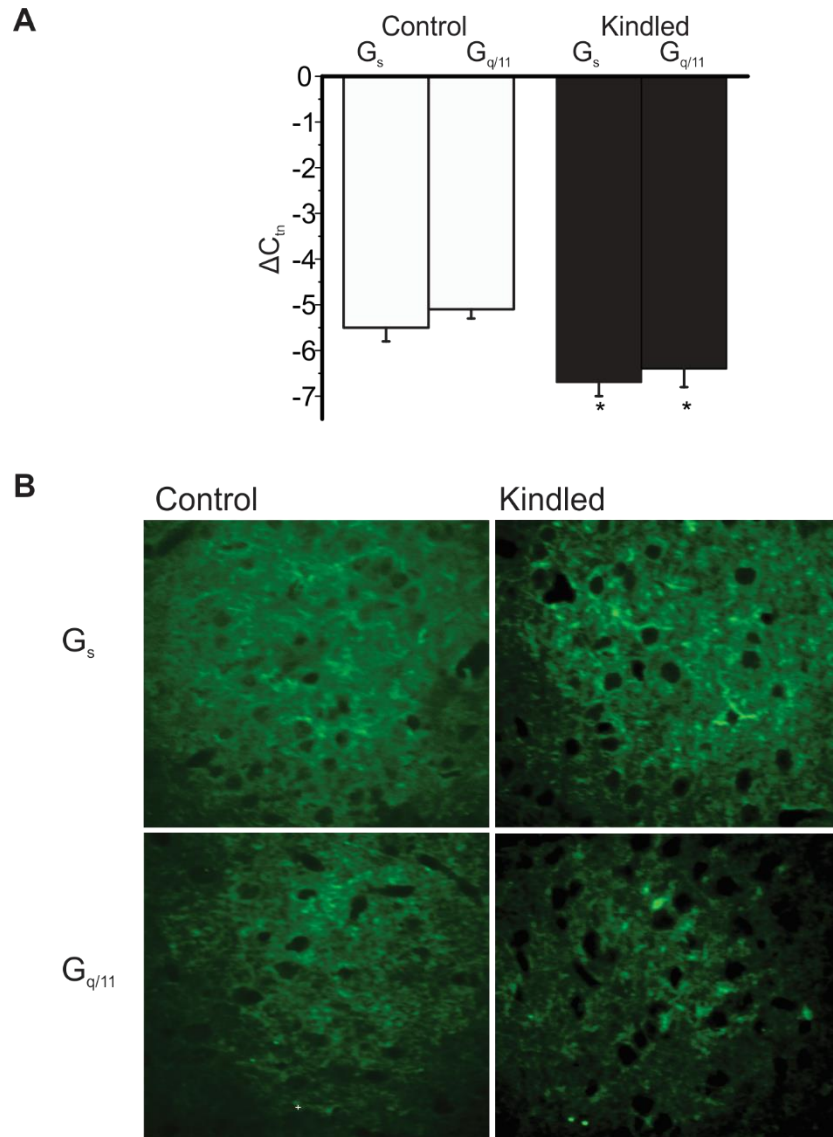
Figure 3.5 The expression of $G\alpha_s$ and $G\alpha_{q/11}$ is unchanged after kindling. (A) QPCR analysis of $G\alpha_s$ and $G\alpha_{q/11}$ mRNA. N=5 rats per treatment (B) Representative deconvolved confocal images of $G\alpha_s$ and $G\alpha_{q/11}$ immunoreactivity before and after kindling in layer II of PC. Images are representative of 5 rats per treatment. * $p < 0.05$.

3.3.5 The expression of Gα_s and G$\alpha_{q/11}$ is unchanged after kindling

We first hypothesized that the change in signaling pathways after kindling could be due to changes in the relative abundance of Gα_s and *G$\alpha_{q/11}$* mRNA and protein. For example, an increase in Gα_s abundance or a decrease in G$\alpha_{q/11}$ abundance could favor the coupling of CRFR$_1$ to Gα_s. However, *Gα_s* mRNA expression decreased and protein abundance was unchanged. The abundance of *G$\alpha_{q/11}$* mRNA and protein was also unchanged (Figure 3.5). These observations, therefore, do not provide an explanation as to why the signaling was altered.

Table 3.1 List of 22 relevant *mRNA* targets analyzed in the commercial qPCR array

Number	QPCR target list
1.	Adenylate cyclase 1
2.	Adenylate cyclase 2
3.	Adenylate cyclase 3
4.	Adenylate cyclase 5
5.	Caspase 3
6.	Cyclin-dependent kinase 5
7.	cAMP responsive element binding protein 1
8.	Mitogen-activated protein kinase 1
9.	Phosphodiesterase 10A
10.	Phosphodiesterase 4, cAMP-specific (Phosphodiesterase E2 dunce homolog, drosophila)
11.	Phosphodiesterase 4B, cAMP-specific
12.	Phosphodiesterase 4C, cAMP-specific
13.	Phosphodiesterase 4D, cAMP-specific (Phosphodiesterase E2 dunce homolog, drosophila)
14.	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
15.	Phosphoinositide-3-kinas, catalytic, gamma polypeptide
16.	Phospholipase A2, group 1
17.	Phospholipase C, beta 1
18.	Phospholipase C, beta 2
19.	Phospholipase C, beta 3 (phosphatidyl inositol-specific)
20.	Protein phosphatase 1, regulatory(inhibitor) subunit 1B
21.	Protein kinase, cAMP-dependent, catalytic, alpha
22.	Synuclein, alpha interacting protein

Table 3.2 List of *mRNA* transcripts changed after kindling

Gene Name	Abbreviation	Control ΔCtn	Kinlded ΔCtn	Difference
Cytochrome P450, family 2, subfamily d, polypeptide 4	Cyp2d4	-7.9	-9.4	-1.5
Glial cell derived neurotrophic factor	Gdnf	-12.4	-10.6	1.8
5-hydroxytryptamine (serotonin) receptor 1A	Htr1d	-8.8	-11.2	-2.4
5-hydroxytryptamine (serotonin) receptor 3a	Htr3b	-14.7	-13.2	1.5
Phospholipase C, beta 2	Plcb2	-9.7	-8.5	1.2
Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	Slc6a4	-12.8	-11.8	1.0
Tryptophan 2,3-dioxygenase	Tdo2	-13.6	-11.4	2.2
Tyrosine hydroxylase	Th	-7.8	-8.8	-0.9
Tryptophan hydroxylase 1	Tph1	-12.3	-10.7	1.6
Tryptophan hydroxylase 2	Tph2	-10.2	-12.1	-1.9

This analysis did not reveal changes in transcript abundance that would predict altered preference for $G\alpha_s$ versus $G\alpha_{q/11}$ for activation. Green indicates increased mRNA abundance and red indicates decreased abundance. N= 4 biological replicates.

3.3.6 No alteration in mRNAs encoding proteins involved in GPCR signaling

We next considered the possibility that another mRNA(s) coding for protein(s) involved in intracellular signaling was altered. To assay for this, we employed commercially available QPCR arrays that permit the quantitation of mRNAs encoding 84 differing proteins involved in second messenger signal transduction, out of which only 22 transcripts encoded proteins that were potentially downstream of GPCRs (Table 3.1). We found that the expression of 6 transcripts was increased and 4 were decreased (Table 3.2). However, none of the proteins encoded by these mRNAs were considered to be potentially involved in any kind of process that would cause the shift in the preferred signaling cascade. For example, we found transcript abundance for PKC isoforms was increased, a result that might predict increased, rather than decreased, PKC signaling.

Protein	Abreviation	Epitope	CPM Control	CPM Kindled	% change
Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)	Erk1	T202+Y204	1513	791	-48%
Focal adhesion protein-tyrosine kinase	FAK	S910	1342	1061	-21%
Focal adhesion protein-tyrosine kinase [Y397]	FAK	Y397	301	91	-70%
G protein-coupled receptor-serine kinase 2	GRK2 (BARK1)	S670	493	790	60%
G protein-coupled receptor-serine kinase 2 [S670] (82)	GRK2 (BARK1)	S670	2049	1329	-35%
Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK))	JNK	T183+Y185	2140	1357	-37%
Protein-serine kinase C alpha	PKCa	S657	8631	6048	-30%
Protein-serine kinase C beta 1/2	PKCb1/2	T500	7781	9147	18%
Protein-serine kinase C beta 2	PKCb2	T641	7662	11764	54%
Protein-serine kinase C delta	PKCd	S664	6992	11477	64%
Protein-serine kinase C gamma	PKCg	T514	7420	4403	-41%
Protein-serine kinase C gamma	PKCg	T674	4070	6810	67%

Figure 3.6 Changes in the phosphorylation of signaling proteins with kindling. (A) Western blots of control and kindled brain samples. (B) Quantification of 10 proteins with altered phosphorylation state. These proteins are not known to be involved in changing the signaling preferences of GPCRs. Green indicates increased phosphorylation and red indicates decreased phosphorylation in control, compared to kindled. N= 5 biological replicates. (C). The total list of proteins that were tested for the change in the phosphorylation state.

Table 3.3: Total list of proteins that were tested for the change in the phosphorylation state

| Number	Genes and proteins
1.	cAMP-dependent protein-serine kinase catalytic subunit alpha/beta [Thr^{198}]
2.	cAMP-dependent protein-serine kinase catalytic subunit beta [Ser^{339}]
3.	Double-stranded RNA-dependent protein-serine kinase [Thr^{451}] (65)
4.	Double-stranded RNA-dependent protein-serine kinase [Thr^{451}] (73)
5.	Epidermal growth factor receptor-tyrosine kinase [Tyr^{1138}]
6.	ErbB2 (Neu) receptor-tyrosine kinase [Tyr^{1248}]
7.	Extracellular regulated protein-serine kinase 1 (p44 MAP kinase) [Thr^{202}+Tyr^{204}]
8.	Extracellular regulated protein-serine kinase 2 (p42 MAP kinase) [Thr^{185}+Tyr^{187}]
9.	Extracellular regulated protein-serine kinase 5 (Big MAP kinase 1 (BMK1)) [Thr^{218}+Tyr^{220}]
10.	Focal adhesion protein-tyrosine kinase [Ser^{722}]
11.	Focal adhesion protein-tyrosine kinase [Ser^{843}]
12.	Focal adhesion protein-tyrosine kinase [Ser^{910}]
13.	Focal adhesion protein-tyrosine kinase [Thr^{397}]
14.	G protein-coupled receptor-serine kinase 2 [Ser^{670}] (70)
15.	G protein-coupled receptor-serine kinase 2 [Ser^{670}] (82)
16.	Insulin receptor [Tyr999]
17.	Insulin receptor/Insulin-like growth factor 1 receptor [Tyr^{1189}/Tyr^{1190}]
18.	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [Thr^{183}+Tyr^{185}] (37)
19.	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [Thr^{183}+Tyr^{185}] (38)
20.	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [Thr^{183}+Tyr^{185}] (44)
21.	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) [Thr^{183}+Tyr^{18}5] (46)
22.	Kit/Steel factor receptor-tyrosine kinase [Tyr^{703}]
23.	Mitogen-activated protein-serine kinase p38 alpha [Thr^{180}+Tyr^{182}] (36)
24.	Mitogen-activated protein-serine kinase p38 alpha [Thr^{180}+Tyr^{182}] (40)
25.	p21-activated kinase 1/2/3 (serine/threonine-protein kinase PAK 1/2/3) [Ser^{144}/Ser^{141}/Ser^{154}]
26.	Protein kinase C-related protein-serine kinase 1 [Thr^{774}]
27.	Protein kinase C-related protein-serine kinase 2 [Thr^{816}]
28.	Protein-serine kinase C alpha [Ser^{657}]
29.	Protein-serine kinase C alpha/beta 2 [Thr^{638}/Thr^{641}]
30.	Protein-serine kinase C beta 1/2 [Thr^{500}]
31.	Protein-serine kinase C beta 2 [Thr^{641}]
32.	Protein-serine kinase C delta [Ser^{664}] (75)
33.	Protein-serine kinase C delta [Ser^{664}] (79)
34.	Protein-serine kinase C delta [Tyr^{313}] (75)
35.	Protein-serine kinase C delta [Tyr^{313}] (79)
36.	Protein-serine kinase C epsilon [Ser^{729}]
37.	Protein-serine kinase C eta [Ser^{674}]

38.	Protein-serine kinase C gamma [Thr^{514}]
39.	Protein-serine kinase C gamma [Thr^{655}]
40.	Protein-serine kinase C gamma [Thr^{674}]
41.	Protein-serine kinase C mu (Protein Kinase D) [Ser^{738}+Ser^{742}]
42.	Protein-serine kinase C mu (Protein kinase D) [Ser^{910}]
43.	Protein-serine kinase C zeta/lambda [Thr^{410}/Thr^{412}]
44.	Protein-tyrosine kinase 2 [Tyr^{579}]
45.	Yes-related protein-tyrosine kinase [Tyr^{507}] (44)
46.	Yes-related protein-tyrosine kinase [Tyr^{507}] (46)
47.	Yes-related protein-tyrosine kinase [Tyr^{507}] (48)

3.3.7 Changes in the phosphorylation of signaling proteins with kindling does not account for switch in CRFR₁ signaling

 We also considered the possibility that the phosphorylation states of various proteins involved in GPCR cascades could account for the change. To this end, we used a commercially available service that provides a relative assessment of the phosphorylation of 45 proteins involved in signal transduction (Total list in Table 3.3). Again, while we found that various proteins showed changes in their phosphorylation state after kindling, none would account for a switch in signaling efficacy or preference (Figure 3.6).
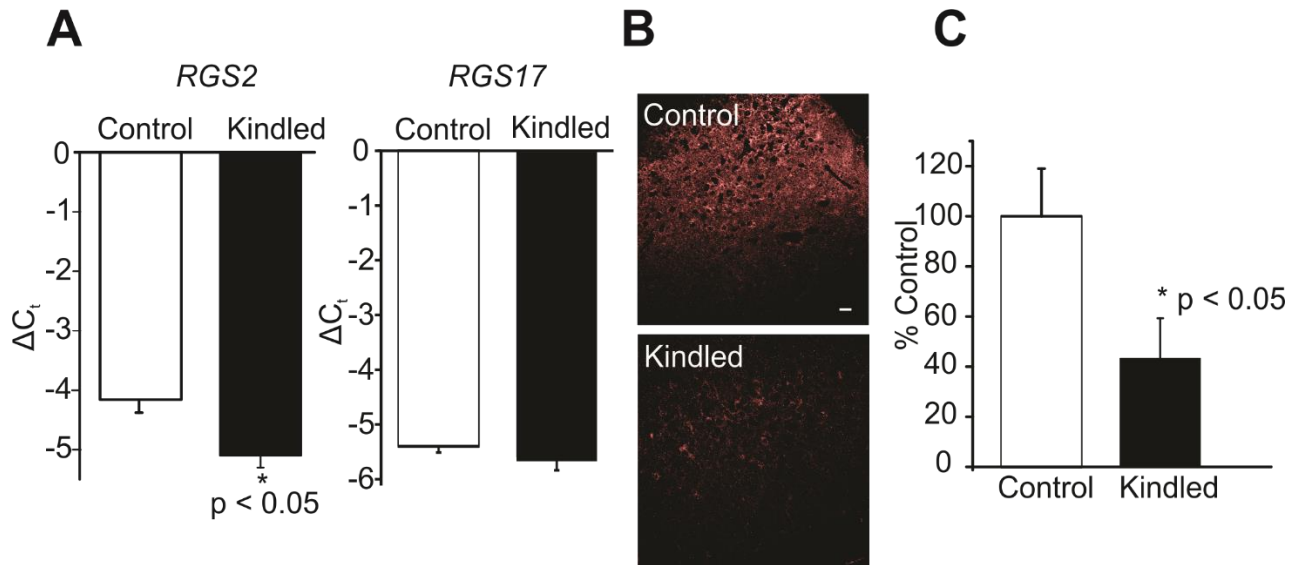
Figure 3.7. Alteration in the abundance of RGS2. Δ cycle threshold (ΔCt) was compared between kindled (n=11) and non-kindled (n=9) samples. *$p < 0.05$. ΔCt is the Ct(reference gene) - Ct(gene of interest). The analysis shows that *RGS2* mRNA transcripts are significantly decreased in kindled piriform cortex in comparison to control. No significant difference is observed in *RGS17* mRNA expression between non-kindled and kindled animals. (B) Representative confocal images indicating reduced RGS2 abundance in layer II after kindling. The brightest pixels three times above background have been marked in white to indicate differences in expression (scale bar is 20mm). (C) The relative percentage of pixels that were above this cutoff (n =5 slices from 5 Control rats; n = 4 slices from 4 kindled rats *$p < 0.05$).

3.3.8 Alteration in the abundance of RGS2 accounts for the switch in CRFR$_1$ signaling

Finally, we hypothesized that the abundance of regulators of G protein signaling proteins (RGS) may be altered. These proteins inhibit or enhance the activity of heterotrimeric G proteins in vitro (Grafstein-Dunn et al., 2001;Heximer et al., 1999;Ingi et al., 1998a). Two RGS proteins, namely RGS2 and RGS17, are abundant in the pyramidal cell layer of the piriform cortex (Grafstein-Dunn et al., 2001). QPCR analysis showed that in kindled rat brain, expression of *RGS2*, but not that of *RGF17*, was decreased (Figure 3.7 A)**.** In addition, RGS2 protein abundance in the Layer II of the piriform cortex was reduced (Figure 3.7 B). There have been reports that RGS2 interact with several adenylate cyclase isoform and thus decreases cAMP production thereby limit G$_{\alpha s}$ mediated signaling (Roy et al., 2006a;Sinnarajah et al., 2001).
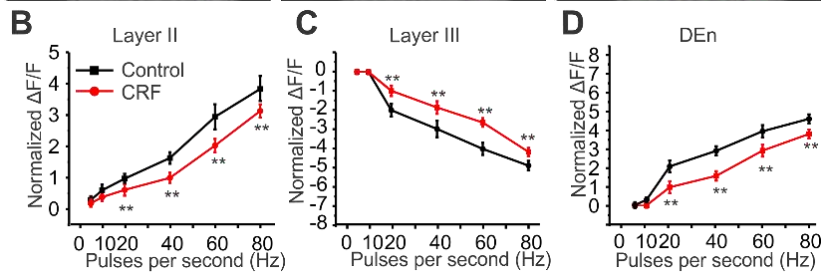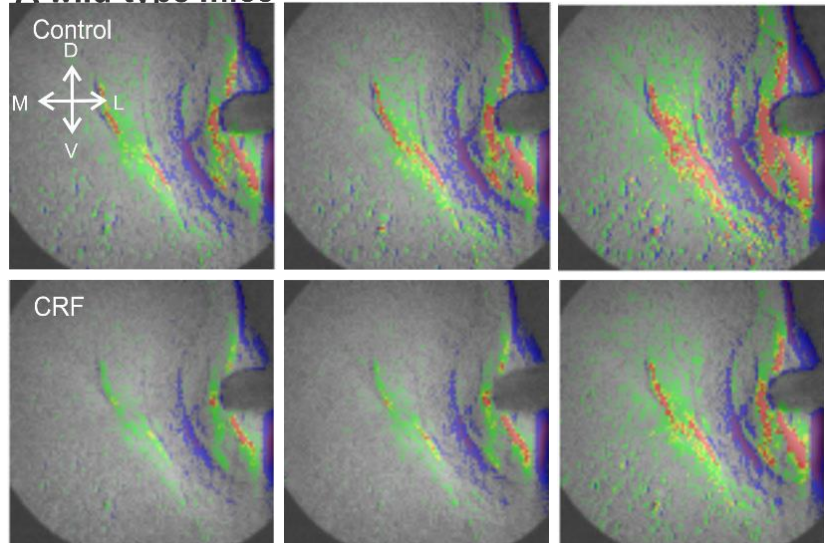
A wild-type mice
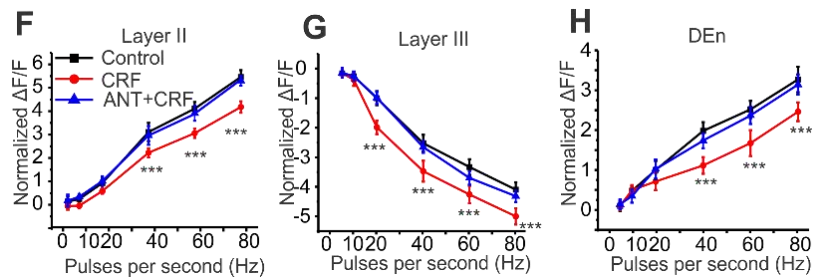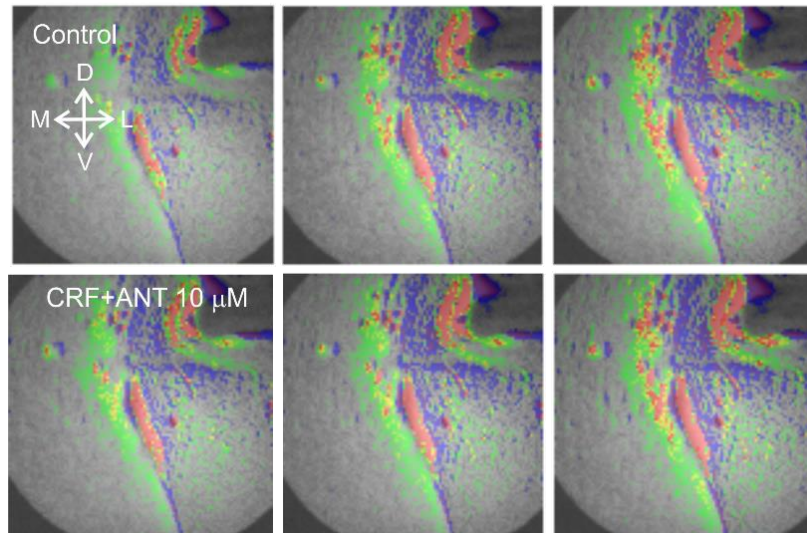
Figure 3.8 Activation and antagonism of $CRFR_1$ in mouse PC. (A) Representative images of activation of layers of piriform cortex from WT mice, without (top) or with CRF perfusion before recording (bottom). (B to D) Quantification of $CRFR_1$ activation show decreased excitation in layer II and DEn and increased disinhibition of layer III interneurons in piriform cortical slices from WT mice over the range of stimulation frequencies used to activate the circuit. ** $p < 0.01$. n=8 slices from 6 mice per treatment. (E) Representative images of activation of layers of piriform cortex from non-kindled WT mice, without (top) or with perfusion of CRF and antalarmin before recording (bottom). (F to H) Quantification of $CRFR_1$ activation over the range of stimulation frequencies used in piriform cortical slices from wild-type mice to activate the circuit. *** $p < 0.005$, n = 6 slices from 5 mice per treatment. Each image in the bottom row of (A) and (E) was taken over the same time interval as that of its corresponding image in the top row.

3.3.9 CRF responses in wild-type mouse PC are similar to CRF responses in non-kindled rat PC

These observations suggest that downregulation of RGS2 may increase $G_{\alpha s}$ signaling and thus may account for the switch in the $CRFR_1$ responses after kindling. To test this hypothesis, we assessed whether CRF responses in RGS2 knockout mice were similar to those observed in kindled rat brain slices. First, we established that CRF activity in wild-type mice was similar to that in non-kindled rats (Narla et al., 2015). In wild-type mouse brain slices CRF responses were similar to those seen in the rat (Figure 3.8 A-D). Because in mice $CRFR_1$ and $CRFR_2$ are equally abundant (unlike in rat, where $CRFR_1$ predominates) we verified that this response was mediated by $CRFR_1$ by showing it was blocked by the $CRFR_1$ antagonist antalarmin (Figure 3.8 E-H).
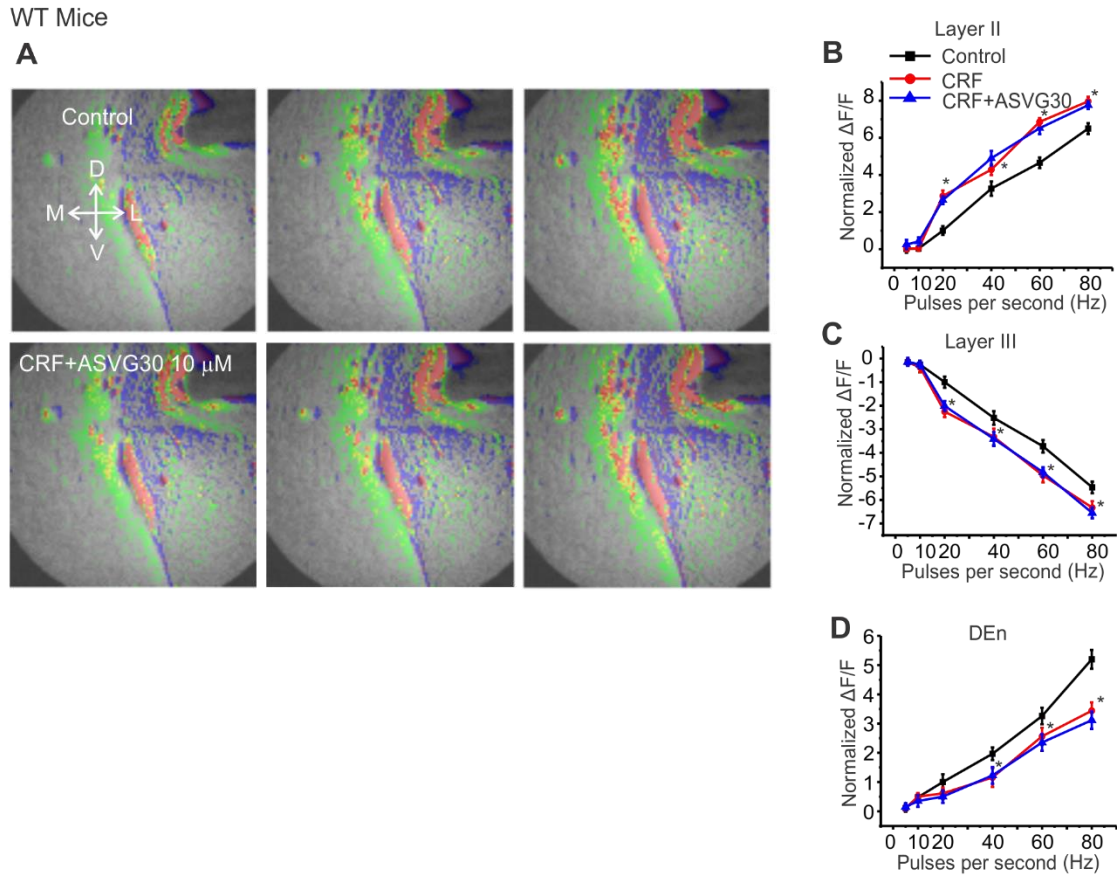
Figure 3.9 Antagonism of CRFR$_2$ in mouse PC. A. Representative images of activation of layers of piriform cortex from non-kindled WT mice, without (top) or with perfusion of CRF and anti sauvagine 30 before recording (bottom). (B to D) Quantification of CRFR$_2$ antagonism over the range of stimulation frequencies used in piriform cortical slices from wild-type mice to activate the circuit. ** $p < 0.005$, n = 5 slices from 5 mice per treatment. Each image in the bottom row of (A) was taken over the same time interval as that of its corresponding image in the top row.

3.3.10 CRFR$_2$ antagonism did not block the effects of CRF

Mouse PC express CRFR$_2$ with equal abundance as CRFR$_1$. So simply considering that CRFR$_1$ are mediating the effects of CRF is not ideal. So we tested if CRFR$_2$ is involved in observed CRF effects by using CRFR$_2$ selective antagonist Antisauvagine-30. Application of the CRFR$_2$ antagonist antisauvagine-30 did not block the effects of CRF. N=6 rats (Figure 3.9).

A RGS2 KO mouse
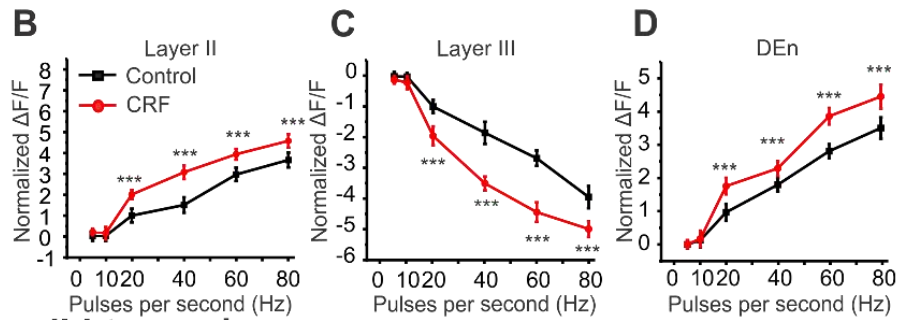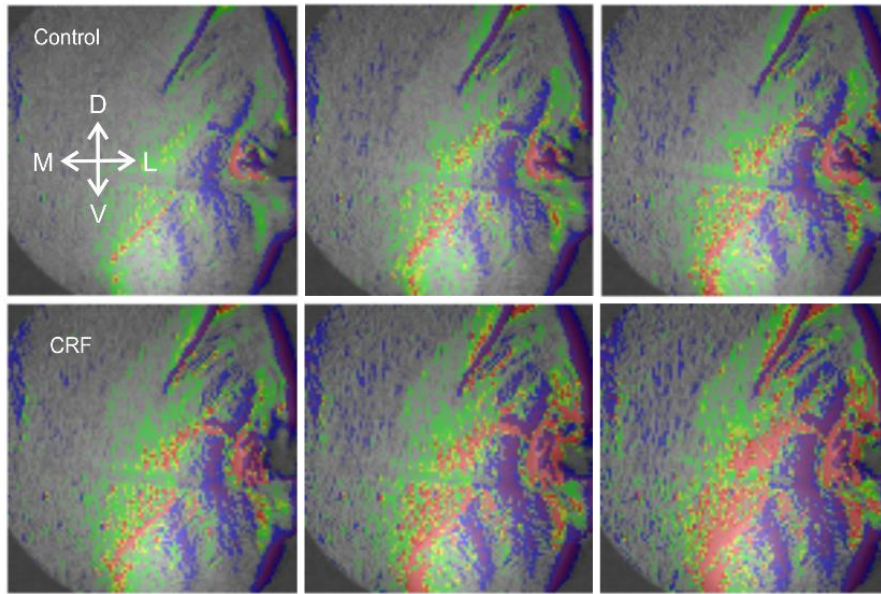
wild-type mice
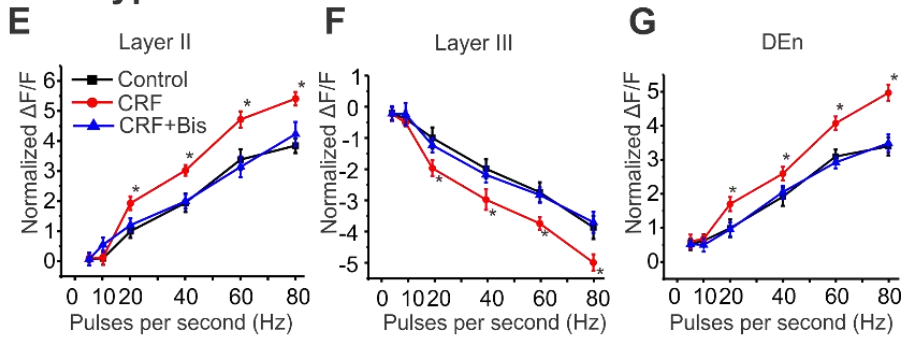
RGS2 KO mice
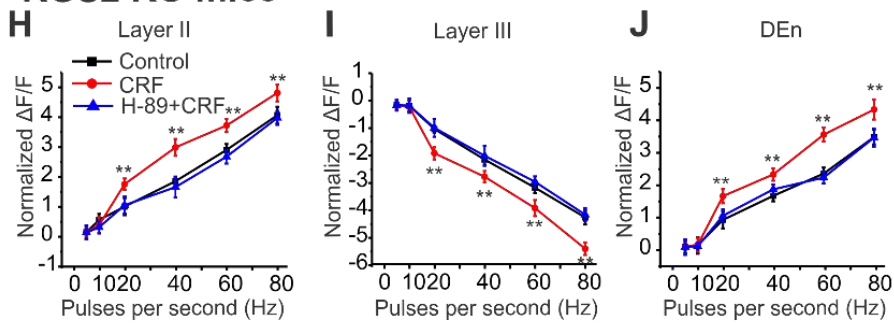
Figure 3.10 $CRFR_1$ activation and PKA antagonism in RGS2 KO mouse and PKC antagonism in WT mouse. (A) Representative images of activation of layers of piriform cortex from non-kindled RGS2 knockout mice, without (top) or with CRF perfusion before recording (bottom). Each image in the bottom row was taken at the same time interval as that of its corresponding image in the top row. (B to D) Quantification of $CRFR_1$ activation in RGS2 KO mice shows increased excitation in layer II and DEn whereas increased disinhibition in layer III of piriform cortical slices from non-kindled RGS2 knockout mice over the range of stimulation frequencies used to activate the circuit. *** $p < 0.005$, n = 6 slices from 5 mice/group. (E to G) Quantification of the effect of the PKC inhibitor BIS on CRF-mediated activation of $CRFR_1$ over the range of stimulation frequencies used in piriform cortical slices from non-kindled wild-type mice to activate the circuit. ** $p < 0.01$, n = 8 slices from 6 mice/group. (H to J). Quantification of the effect of H-89 on CRF-mediated activation of $CRFR_1$ over the range of stimulation frequencies used in piriform cortical slices from non-kindled RGS2 knockout mice to activate the circuit. ** $p < 0.01$, n = 6 slices from 5 mice per group.

3.3.11 CRF responses in RGS2 KO mouse PC are similar to CRF responses from kindled rat PC

With the confirmation that CRF responses in wild-type mice were similar as those in non-kindled rats, we next examined CRF responses in RGS2 deficient mice. Like in kindled rats (Fig. 3.1 E), CRF increased the excitability of the piriform cortex in RGS2 KO mice (Figure 3.10 A to D). In wild-type mice, we found that the PKC antagonist BIS blocked the effects of CRF, indicating that $CRFR_1$ likely signaled by a $G_{q/11}$ dependent pathway (Figure 3.10 E, F, and G). These observations are similar to our observations in the non-kindled rats. By contrast, in RGS2 KO mice, the PKA inhibitor H-89 blocked the effects of CRF, revealing that PKA activation was essential for signaling of $CRFR_1$ (Figure 3.10 H, I and J).
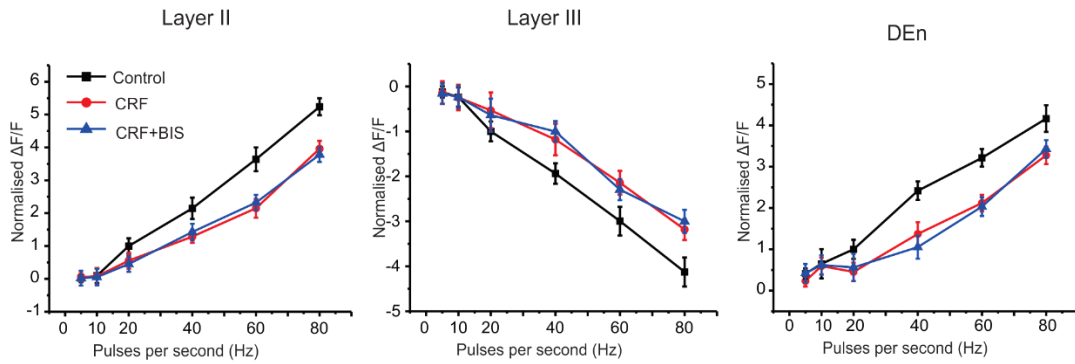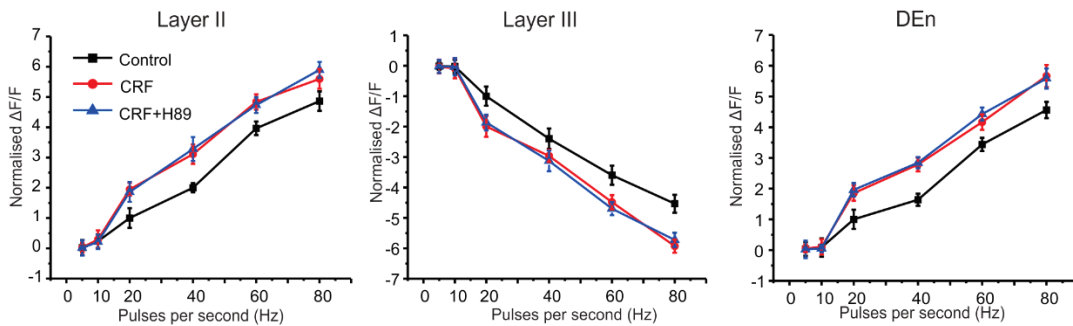
Figure 3.11 PKC antagonism in RGS2 KO mouse and PKA antagonism in WT mouse. (A) Quantification of the effect of BIS (PKC blocker) on $CRFR_1$ activation in piriform cortical slices from RGS2 KO mouse * $p < 0.05$, n =7 slices from 4 mice/group. (B) Quantification of the effect of the PKA inhibitor H-89 on CRF-mediated activation of $CRFR_1$ in piriform cortical slices from WT mouse. * $p < 0.05$, n=5 slices from 5 mice/group.

3.3.12 PKC or PKA antagonism had no effect on CRFR$_1$ activation in WT and RGS 2 KO mouse respectively

We next investigated if there was any change in the responses of CRF in the presence of PKC blockers in RGS2 KO mouse and PKA blocker in WT mouse. We found that PKC antagonism using BIS in RGS2 KO mouse and PKA antagonism in WT mouse using H89 did not affect CRFR$_1$ signaling (Figure 3.11).

3.4 Discussion

In an animal model that resembles human temporal lobe epilepsy, we showed that the stress-related neurotransmitter CRF became excitatory and potentially more seizurogenic, reversing the polarity of its activity from inhibitory to excitatory in the piriform cortex. Our observations indicate that the epileptic state was accompanied by greater susceptibility to stressor-induced excitability in a brain region that supports seizures and seizure genesis. 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 neurochemical alterations support seizure onset. This observation is opposite to the one where the stressor responses are otherwise "normal' (cell signaling and polarity of the response is constant) but the epileptic state makes the brain more susceptible to an otherwise physiological response. The extent to which this alteration in brain state may account for other co-morbidities or susceptibilities seen with those with epilepsy (depression for example) is not known. In addition to epilepsy, it also possible that similar alterations may occur in individuals who have suffered a traumatic brain injury and who can experience increased frequency of anxiety- and stress-induced seizures in brain regions that have not been directly injured. Our observations, therefore, provide an increased impetus to consider "bystander" outcomes that may exacerbate pathophysiological neurological outcomes.

The change in the polarity of the $CRFR_1$ signaling occurs through the switch in the signaling cascade by which CRF acts. Although $CRFR_1$ was initially classified as a GPCR that activated adenylyl cyclase (Hauger et al., 2003), CRFR activation can activate different G protein cascades (Blank et al., 2003;Wanat et al., 2008;Wietfeld et al., 2004). For example, the neuroprotective effects of CRF in the hippocampus are mediated by the activation of PKA and MAPK signaling pathways (Elliott-Hunt et al., 2002). Moreover, $CRFR_1$ couples to $G_{q/11}$ in

BALB/c mice hippocampal neurons, whereas it couples to $G_{\alpha s}$, and G_i in C57BL/6N mice hippocampal neurons (Blank et al., 2003). In cultured hippocampal neurons, CRF regulation of NMDA currents is mediated by PKC, which correlates with increased phospholipase C–β3 abundances (Sheng et al., 2008). In prefrontal cortex pyramidal neurons, CRF activates CRFR$_1$ signaled through activation of PKC to enhance stressor responses (Tan et al., 2004). As previously mentioned, we have shown that activated CRFR$_1$ suppresses piriform cortex activity through a PKC-dependent mechanism (Narla et al., 2015). Thus, it seems clear that depending on the region and pathological condition of the brain, CRFR$_1$ can activate distinct G proteins resulting in activation of multiple signaling pathways. The present findings show that RGS2 protein may be an important mediator of this variation in CRFR$_1$ signaling.

GPCR signaling involves a series of complex events that lead to a conformational change of the receptor upon ligand binding and then activation of G-protein by promoting the exchange of GTP and GDP associated with Gα. Many factors influence these events, including perceiving the extracellular signals through transducing them to heterotrimeric G-proteins and to downstream effectors. RGS proteins interact with Gα subunits to attenuate GPCR mediated signaling by accelerating Gα-GTPase activity (Grafstein-Dunn et al., 2001) and/or by inhibiting G-protein/effector interaction (Cunningham et al., 2001;Abramow-Newerly et al., 2006;Zhao et al., 2013). RGS2 can inhibit $G_{\alpha s}$ -stimulated adenylyl cyclase activity even though it does not promote the GTPase activity of $G_{\alpha s}$ (Ingi et al., 1998a;Roy et al., 2006b;Roy et al., 2003). RGS2 may bind directly to $G_{\alpha s}$ (Roy et al., 2003;Ko et al., 2001) or to adenylyl cyclase (Salim et al., 2003;Sinnarajah et al., 2001;Roy et al., 2006a), and thus RGS2 could produce its inhibitory effect on cAMP production by interfering with $G_{\alpha s}$ -adenylyl cyclase coupling. Overall, these reports corroborate our findings that the presence of RGS2 in control rats decreased the production of

cAMP and thus suppressed the CRFR$_1$-stimulated activity of G$_{\alpha s}$. However various RGS proteins (including RGS2) possess GTPase-activating protein (GAP) activity for G$_{q/11}$ and also function as potent inhibitor of G$_{q/11}$ signaling compared to other types of RGS proteins (Ingi et al., 1998a;Heximer et al., 1999;Hepler, 1999;Kehrl, 1998;Sunahara et al., 1997). Thus, it is still not clear how RGS2 is working before and after kindling; namely, how changes in RGS2 abundance might selectively favor either G$_{q/11}$- or G$_{\alpha s}$ -mediated effects of CRFR$_1$ activation. Our findings indicated that in the absence of RGS2 (in the KO mice) or when RGS2 abundance is low, as occurs in the kindled rat, CRFR$_1$ activation seemed to "prefer" to signal through G$_{\alpha s.}$ While it is easy to rationalize how a decrease in RGS2 could increase G$_{\alpha s}$ -mediated signaling, a concomitant decrease in G$_{q/11}$-mediated signaling would not be expected. This finding recalls the decreased G$_{12/13}$ signaling effect that occurs upon a decrease in RGS5 in vascular smooth muscle (Arnold et al., 2014), although in that case the G protein would not be considered a potential target of the RGS protein. Although RGS2 can inhibit CRFR$_1$-stimulated G$_{q/11}$ signaling, the targeting of RGS proteins to particular pathways in vivo may be governed by additional factors such as scaffolding proteins (Zhao et al., 2013), and thus a decrease in RGS abundance would not necessarily result in increased activation of all of its potential G protein partners.

RGS2 is present in brain regions that are implicated in the pathophysiology of anxiety and depression, such as hippocampus, amygdala, cerebral cortex, hypothalamus and raphe nucleus of rats and mice (Ingi and Aoki, 2002;Neubig and Siderovski, 2002;Leygraf et al., 2006a;Ingi et al., 1998b). These regions have reciprocal connections with the piriform cortex and pathological changes in these regions may negatively affect the physiology of piriform cortex and perhaps seizurogenesis. Hippocampal slices from RGS2 KO mice have increased excitation in CA1 pyramidal cells and these mice are more anxious compared to their wild-type counterparts

(Oliveira-Dos-Santos et al., 2000). Moreover, a mutation in the RGS2 gene causes anxiety in mice (Yalcin et al., 2004) and decreased RGS2 abundance is associated with depression-like behavior in mutant mice (Lifschytz et al., 2012). Thus, the relationship between RGS2 abundance and behavior may depend on whether anxiety or depression is also present, which may limit the usefulness of drugs targeting RGS2.

Several studies have reported the clinical implications of RGS2 and complications associated with its polymorphisms. RGS2 has been proposed to be an important drug target in anxiety-related disorders, depression and is associated with panic disorders in humans (Amstadter et al., 2009a;Otowa et al., 2011). Several studies have reported the pathogenetic association of single nucleotide polymorphisms (SNPs) especially rs4606 in the RGS2 gene and neuropsychiatric disorders. RGS2 gene polymorphism has been associated with the development of panic disorder associated with agoraphobia in 173 subjects of German descent (Leygraf et al., 2006b). Furthermore, abnormalities in serotonin receptor function due to RGS2 gene polymorphisms have been found in Japanese suicide victims (Cui et al., 2008). Examination of postmortem brains revealed significant differences in allele frequencies of two SNPs (SNP 2&3) in suicide victims compared to controls. Moreover, RGS2 immunoreactivity is greatest in the amygdala and BA9 region of the prefrontal cortex of suicide subjects compared to controls suggesting that the increased RGS2 abundance alter GPCR intracellular signaling (Cui et al., 2008). In 2004 Florida hurricane victims, the C allele of rs4606 SNP of RGS2 has been correlated with increased chances of developing generalized anxiety disorder, posttraumatic stress disorder and ideation to commit suicide (Amstadter et al., 2009a;Amstadter et al., 2009a;Amstadter et al., 2009b). Behavioral studies and functional magnetic resonance imaging have identified the association of the G allele of rs4606 SNP in anxiety-related phenotype and increased activation of the insular cortex, a part

of the limbic system, in human subjects (Smoller et al., 2008). Genetic variation such as rs4606 SNP that lead to reduced RGS2 abundance is associated with antipsychotic-induced Parkinsonism (Greenbaum et al., 2007;Greenbaum et al., 2009). Together, polymorphisms in *RGS2* in different brain regions are associated with various neurological disorders. More research is needed to understand the physiology and pathophysiology of RGS2 proteins and thus their clinical implications in human neurological disorders.

In summary, our observations provide a mechanism by which anxiety and stressors may exacerbate the occurrence of seizures. The epileptic state alters the function of a stress neurotransmitter that increases the excitability of a brain region that supports seizurogenesis, although it is uncertain how general this effect may be. Other brain pathophysiological states (such as stroke, repeated concussion, or traumatic brain injury) may also produce similar alterations in signaling in many GPCR responses. Thus, we will need to consider how mental disorders may be mitigated by the altered pathophysiological state of the brain.

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Chapter 4. Altered CRFR$_1$ signaling in rat piriform cortex: a mechanism of development of stress associated post-traumatic epilepsy

4.1 Introduction:

Traumatic brain injury (TBI) refers to injury to the brain as a result of many insults including concussions, impacts to the head, blast injuries as well as others. (Maas et al., 2008a). It affects a wide array of individuals in the population regardless of age, background and health status. It is a critical public health problem throughout the world causing deaths and lifelong disabilities in the young adults (Maas et al., 2008b). By 2005-2006, it was estimated that 5.3 million people in the United States of America (Langlois and Sattin, 2005) and 7.7 million people in the European Union (Tagliaferri et al., 2006) were living with a disability related to TBI. As per the brain injury association of Canada, the annual incidence of acquired brain injury is 44 times more common than spinal cord injuries, 30 times more common than breast cancer. Brain injury accounts for 11,000 deaths per year out of 165,000 individuals affected in Canada. TBI is a leading cause of acquired epilepsy (Campbell et al., 2014). The incidence of TBI is also increasing in the middle income and low-income countries (Maas et al., 2008b). Neurocognitive deficits such as impaired attention and psychological health issues such as depression (30-70% of people with TBI develop depression) are common in TBI affected individuals (Roozenbeek et al., 2013). A cascade of molecular, electrophysiological and cellular changes as a result of TBI initiate several comorbidities include seizure precipitation that may be recurrent and causes the development of post-traumatic epilepsy (PTE) (Lucke-Wold et al., 2015;Pitkanen et al., 2014). The underlying pathophysiology is poorly understood and thus making it uncertain for health care individuals to diagnose and treat.

Mild traumatic brain injury (mTBI) has been shown to predispose people to psychiatric disorders such as anxiety, depression, mood disorders and disruptive behaviors (Emery et al., 2016). For example, social anxiety was increased in a group of people as a result of TBI (Curvis et al., 2016). There is also evidence that people with mTBI are more likely to experience anxiety episodes, a precipitating factor for seizuregenesis (Luis and Mittenberg, 2002;Vanderploeg et al., 2007).

Corticotropin-releasing factor (CRF), a 40 amino acid containing neuropeptide is well known to be implicated in stress and anxiety disorders. It is released in various brain regions during stress and mediates endocrine/behavioral responses to stress. Activation of $CRFR_1$ through intra-amygdala injections of CRF showed anxiety-like behavior in mice (Cipriano et al., 2016). In another study activated $CRFR_1$ augmented serotonergic neurotransmission in the prefrontal cortex and this lead to the development of anxiety in mice (Magalhaes et al., 2010). There are numerous case studies linking TBI to post-traumatic stress disorder (PTSD) (Hoge et al., 2008;Levin et al., 2001;Greenspan et al., 2006;Gaylord et al., 2008). PTSD is a disorder associated with alterations in hypothalamic-pituitary-adrenal (HPA) axis resulting in increased CRF concentration in the CSF (Yehuda, 2001). PTSD has been a major complication in Iraq and Afghanistan war veterans who were subjected to concussions while executing their duties (Seal et al., 2016). People with PTSD are at high risk to experience psychogenic nonepileptic seizures (PNESs) (Diprose et al., 2016) and they are 50% more likely to experience epileptic seizures (ESs) compared to healthy subjects. A recent study reported that Wister rats exhibited higher levels of CRF released in ventromedial prefrontal cortex post-traumatic stress resulting in stress-induced avoidance of predator odor-paired chamber and this effect was blocked by $CRFR_1$ antagonists (Schreiber et al., 2016). Persistent elevations of central CRF release have also been reported to contribute to panic disorders

(Gold et al., 1988;Roy-Byrne et al., 1986). Patients with panic disorders exhibit anxiety scores identical to anxious epileptic patients (Adamec, 1990). All of these clinical findings do not provide an insight of whether increased central CRF release and abnormalities in CRF functioning as a result of TBI may lead to epilepsy.

We recently reported that CRF exacerbates seizures in kindling model of epilepsy through the activation of corticotropin-releasing factor receptor type 1 ($CRFR_1$) in piriform cortex (PC), one of the most seizuregenic regions of the brain (Narla et al., 2016). These effects of $CRFR_1$ are mediated through $G\alpha s$ activation. Normally CRF dampened the excitability of PC in healthy/sham rats (Narla et al., 2015). This effect is also mediated by $CRFR_1$ but through the activation of $G\alpha_{q/11}$. We have also shown that CRF augments serotonin receptor function through the activation of $CRFR_1$ and this lead to further suppression of PC. Based on these observations it is very difficult to predict the functionality of CRF or the associations it may form with other neurotransmitter systems in the brain. Given the complexity of CRF pathology and CRFR signaling ambiguity, it is unclear what is the role of CRF on TBI-induced changes in the neuronal circuitry of rat PC. The aim of the current study is to answer two questions. 1) How does CRF activity change after brain injury and whether it suppresses or supports epileptogenesis in PC? 2) What signaling pathway does $CRFR_1$ follow? Does it vary from a non-injured brain?

4.2 Materials and Methods:

Procedures were performed in accordance with the guidelines of Canadian Council of Animal Care and approved by The University of Western Ontario Council on Animal Care.

4.2.1 Traumatic brain injury procedure- Controlled cortical impact

Adult male Sprague-Dawley rats weighing 150-180g were used in our experiments. They were anesthetized with ketamine-medetomidine hydrochloride combination and placed in a stereotaxic frame for positioning under a pneumatic impactor (Precision science instruments). The animals were placed under 2% isoflurane to maintain the anesthesia throughout the surgery. Scalps were shaved and rats were placed in a stereotactic apparatus. Eye ointment (LacriLube) was applied throughout the procedure to prevent irritation and drying. The scalp was scrubbed with povidone-iodine, a bacteriostat, and 70% ethanol respectively with fresh, sterile cotton swabs. A 6-8 mm longitudinal incision was made in the scalp and sterile tissue clamps were used to held the incision open to expose the skull. Fascia was scored and gently removed with a scalpel. Sterile saline was used to stop any bleeding and to assist in viewing bregma clear. Stereotactic coordinates were determined and a craniotomy was performed at 2.0 mm anterior to Bregma, 4.8 mm lateral to the midline. Animals received a unilateral injury to the surface of the brain at above mentioned coordinates. The injury parameters consisted of 2.5 mm cortical compression at a speed of 3.5 m/s for 500 ms. Sham animals received a craniotomy but did not receive an impact to the brain. Following the impact, the skull cap was placed back in place using Vetbond tissue glue. The scalp incision was closed using surgical grade suture. Animals were then removed from isoflurane anesthesia and placed in a clean cage. The temperature was maintained at 37 °C with the use of a heating pad.

4.2.2 Slice preparation and dye loading

Animals were housed individually with free access to food and water under a continuous 12hour light and dark cycle. Animals were anesthetized with a ketamine-medetomidine hydrochloride combination and then perfused through the heart with an ice-cold artificial cerebrospinal fluid (ACSF) in which sodium ions were replaced by choline ions(Gavrilovici et al., 2006). The composition of ACSF used comprised of choline chloride, 110 mM; Potassium chloride, 2.5 mM; Sodium dihydrogen phosphate, 1.2 mM; Sodium bicarbonate, 25 mM; Calcium chloride, 0.5 mM; Magnesium chloride, 7mM; Sodium pyruvate, 2.4 mM; Ascorbate, 1.3 mM; Dextrose, 20 mM (McIntyre et al., 2002). The brains were perfused to flush any blood out of the vessels and thereby prevent the iron in the blood from oxidizing and causing damage to neuronal cells. The brain was rapidly removed following perfusion and the region containing anterior piriform cortex was carefully cut into a block to facilitate slicing by a Vibratome (slices were 400 µM thick). The slices were incubated at $37^{\circ}C$ for 30 min and subsequently moved to a room temperature ($22^{\circ}C$) bath for 45 min. The perfusion, slicing, and incubation procedures were carried out in choline-ACSF with a continuous supply of carbogen (95% O_2 and 5% CO_2 mixture). These slices were used for both voltage-sensitive dye imaging (VSDI) and patch clamp recording. For VSDI, slices were incubated in the voltage sensitive dye Di-4-ANEPPS (D-199, Invitrogen Molecular probes Inc., OR, USA) for 35 min. The stock solution of the dye was dissolved in ethanol (22mg per ml). On the day of the experiment, the dye incubation was prepared by mixing 60 µl of dye stock with 500 µl of fetal bovine serum (FBS), 500 µl of ACSF and 310 µl of 10% cremophore-EL solution. The concentration of dye in the final solution was 0.1 mg per ml. After incubation slices were washed for 8-10 min with ACSF and transferred to a recording chamber.

The temperature of the bath was maintained at 32°C during recordings and continuously supplied with carbogen bubbled ACSF having a composition of NaCl, 110 mM; KCl, 2.5 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.0 mM; $MgCl_2$, 2.0 mM; dextrose, 20 mM. The pH and osmolarity of the solutions were adjusted to 7.3-7.4 and 297-305mOsm, respectively.

A platinum-iridium electrode (Microprobes, Inc., MD, USA) with a tip diameter of 200-300 micrometers was used to stimulate the lateral olfactory tract of the piriform cortex. The stimulation of each slice was in the range of 160-200 µA, each square pulse was 2.0 ms in length. The electrode was connected to a stimulator (S88X dual output square pulse stimulator, Grass Technologies, Astro-Med, Inc., QC, Canada), which controlled the pulse frequency and train duration (Birjandian et al., 2013).

4.2.3 Voltage-sensitive dye imaging

Each optical recording was about 10 s in length and consisted of two phases. The first contained a recording of background activity for 2 s followed by the application of stimulus for 1 s with a train of frequencies ranging from 5-80 Hz. The acquisition rate was set at 5 ms/frame. The camera saturation was set at approximately 50% for each recording. Optical signals were recorded by a CMOS camera (Micam Ultima Brain vision, Inc., Tokyo, Japan) mounted on top of an upright microscope (Fixed stage upright microscope, BX51WI, Olympus), sections were illuminated using the light from a 100W halogen lamp source (HLX 64625, Microlites Scientific, Corp.) passed through an excitation filter ($\lambda = 530 \pm 10$ nm). A long pass emission filter ($\lambda > 590$ nm) collected the fluorescent signals. A long working distance objective was used in the experiments (XLFluor 4X N.A.0.28, Olympus). The movies were analyzed using Brain Vision Analyzer (Tokyo, Japan) software. A detailed explanation of the technique is described elsewhere (Birjandian et al., 2013).

4.2.4 Tissue Preparation and Fixation for immunohistochemistry

The following methodology has been described in detail elsewhere (Gavrilovici *et al*., 2012). The rats were anesthetized and perfused with 0.1 M phosphate-buffered saline (PBS), followed by Lana's fixative (4% paraformaldehyde and 20% picric acid in PBS). The brain was removed from the skull, and stored in Lana's fixative for 24 h, and then placed in phosphate buffer solution with 30% sucrose for 48 h at 4°C. The brains were then flash frozen at −80°C and sectioned coronally (40 μm) using cryostat at −15°C. The sections were stored in cryoprotectant solution at −20°C.

4.2.5 Immunostaining

Sections were washed with PBS with 0.2% Triton X-100 three times for 5 min and blocked with 10% donkey serum and 10% goat serum in PBS with 0.025% Triton X-100 and 1% bovine serum albumin (BSA) for 1 h. The primary antibodies were diluted in PBS with 1% BSA and 0.025% Triton X-100. The sections were labeled with a single primary antibody and incubated for 24 h at 4°C. The primary antibodies used were 1:250 anti-rabbit CRF antibody (Abcam, ab11133), 1:250 anti-rabbit $CRFR_1$ antibody (Abcam, ab150561), and 1:1000 anti-rabbit $CRFR_2$ antibody (Abcam, ab75168). Sections were then washed two times with PBS with 0.2% Triton X-100 for 5 min and incubated with a secondary antibody for 1 h at room temperature. The secondary antibody used was Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, A-11034) diluted in PBS with 1% BSA and 0.025% Triton X-100 and was diluted 1:1000 for CRF and $CRFR_2$ and 1:2000 for $CRFR_1$. Sections were then washed three times with PBS with 0.2% Triton X-100 for 10 min and incubated in 1% Sudan Black B (Sigma-Aldrich, S2380) in 70% ethanol for 5 min.

Sections were rinsed twice with 70% ethanol for 1 min, followed by two 5 min washes with PBS. Sections were mounted on glass slides with glass coverslips using Prolong Gold Antifade Reagent with DAPI mounting medium (Molecular Probes, P36935). Primary antibodies used were 1:500 rabbit anti-G protein alpha S antibody (Abcam, ab83735) and 1:250 rabbit anti-GNAQ antibody (Abcam, ab75823). The secondary antibody used was Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, A-11034) diluted in PBS with 1% BSA and 0.02% Triton X-100 at a ratio of 1:1000. Primary antibody anti-RGS2 was used at 1:500 (Sigma-Aldrich, GW22245F) with secondary antibody Cy3 anti-chicken IgY at 1:500 (Cedarlane, 703-166-155).

4.2.6 Image Acquisition and Analysis

Confocal images were obtained using an Olympus IX51 inverted microscope using either a $60\times$ oil immersion objective (numerical aperture (N.A.) = 1.4) or for large field of viewing a 20X objective (N.A. 0.8) on a PerkinElmer UltraVIEW spinning disc confocal unit. The microscope was equipped with a Hamamatsu Orca ER CCD camera (1344×1024 pixels). All images were acquired using Volocity software (version 4.2.1, Improvision). A stack of 10 images with 0.2 μm spacing in the z-plane was taken.

4.2.7 Reagents

H-89, Forskolin, and BIS were obtained from Sigma-Aldrich Co. St. Louis, MO, USA. CRF, antisauvagine-30, and antalarmin were obtained from Tocris Biosciences, Ellisville, MO, USA. Tat- $CRFR_1$-CT peptide was obtained from CanPeptide Inc, Point Claire, PQ, Canada. Di-4-ANEPPS stock solution was prepared in alcohol and cremophore-EL solution which can be stored at 4 °C for 2 months. Fetal bovine serum and ACSF were added on the day of the experiment. CRF stock was dissolved in HBSS and milli-Q water mixture. Forskolin, H-89, BIS, Antalarmin, antisauvagine-30 and PMA stock solutions were prepared in Dimethyl sulfoxide

(DMSO). All the stock solutions were made 1000 times more concentrated than working concentrations.

4.2.8 Statistical analysis

All statistical evaluations were done using Statview Software. Two-way ANOVAs were conducted to compare the neuronal responses after the application of BIS, PMA, H89 and Forskolin in the piriform cortex layers in response to LOT stimulation. For CRF responses in the presence of BIS, PMA, Forskolin and H89 a repeated measures ANOVA was used in which responses of CRF in the absence of drugs served as the within group measure. The pixel/voxel counts were analyzed using t-test. Follow-up comparisons were conducted using Fisher's protected least significance different test to maintain p at 0.05.

4.3 Results:

A Non-brain injured rat

B Layer II

C Layer III

D Den
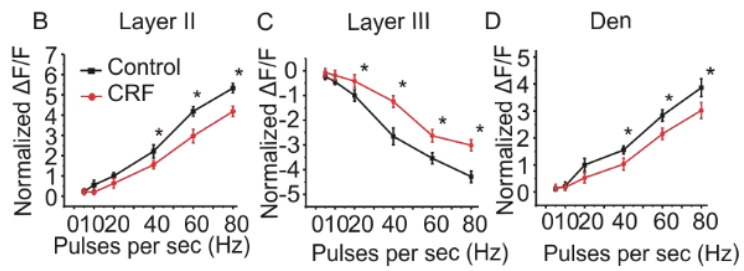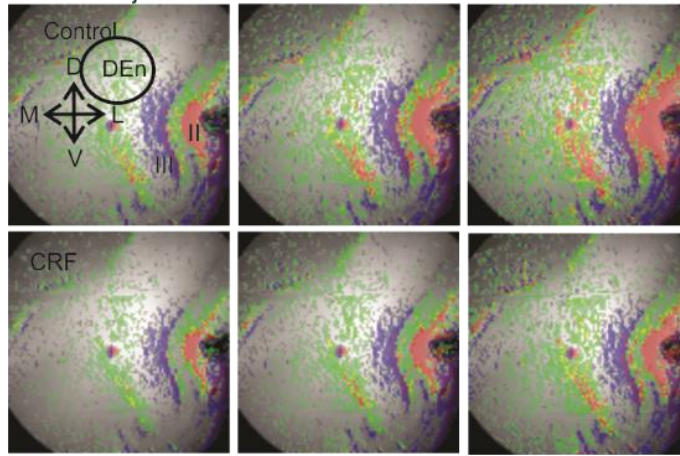
E Traumatic brain injured rat (ipsilateral to injury)

F Layer II

G Layer III
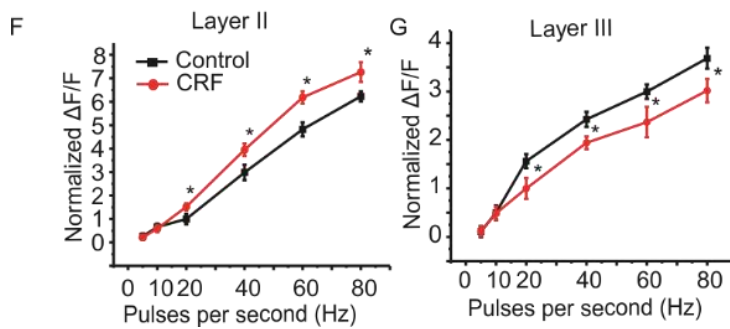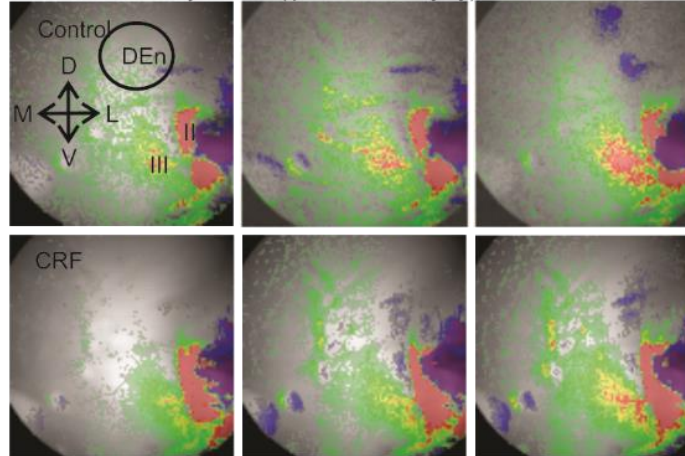
Figure 4.1 CRF application increased ipsilateral PC activity (A) Representative images showing activation of piriform cortex from non-kindled rats, without (top) or with CRF perfusion before recording (bottom). Each image in the bottom row was taken at the same time interval as that of its corresponding image in the top row. (B to D) Quantification of $CRFR_1$ activation shows that the activity of layer II pyramidal cells and interneurons of DEn are decreased while the activity of layer III interneurons is increased in non-brain injured animals over the range of stimulation frequencies used to activate the circuit. * $p < 0.05$. n = 5 slices from 4 rats. (E) Representative images showing activation of layers of ipsilateral piriform cortex from brain injured rats, without (top) or with CRF perfusion before recording (bottom). (F & G) Quantification of $CRFR_1$ activation shows that the activity of layer II pyramidal cells is increased while the activity of layer III interneurons is decreased in over the range of stimulation frequencies used in piriform cortical slices from kindled rats to activate the circuit. * $p < 0.05$. Kindled n = 9 slices from 7 rats. No response is observed from interneurons of DEn. Arrows in indicate the orientation of slice. D, dorsal; V, ventral; M, medial; L, lateral. Red indicates highest ΔF/F; orange, yellow and green indicates medium ΔF/F; blue a reduction in ΔF/F.

4.3.1 Application of CRF increased the excitability of ipsilateral PC of injured brain

Activation of piriform cortex of non-brain injured rat is characterized by three phases of response in its layers II, III and dorsal endopiriform nucleus (DEn) (Fig.1 A top row) as indicated by voltage-sensitive dye imaging (VSDI). Stimulation of lateral olfactory tract (LOT) with different frequencies ranging from 5-80 Hz elicited a frequency dependent increase in these responses (Fig.4.1 B-D). Corticotropin-releasing factor reduced the excitability of layer II and DEn while reduced the inhibition of layer III, contributing to diminished disinhibition of layer II pyramidal cells. (Fig 4.1 A bottom row and B-D). By contrast, in brain injured rats, the conventional responses from PC were affected in such a way that there was very little or no DEn responses observed and the inhibitory response from layer III was now increased in PC ipsilateral to the injury (Fig 4.1 E top row and F-H). CRF application further reduced the excitation of layer III but increased the excitation of layer II pyramidal cells (Fig 4.1 E bottom row and F-H). Thus after brain injury, CRF increased circuit activation, an effect opposite to non-brain injured rats. We hypothesized that this change in neuronal response is attributed to neuronal degeneration in DEn following brain injury or the damage to axons that innervate DEn from layer II. This hypothesis stems from literature that showed interneuronal degeneration following brain injury. Immunohistochemical analyses revealed that there was no axonal damage or neuronal loss in DEn implicating that other cellular mechanisms would underlie the observed changes in PC after traumatic brain injury.

A Traumatic brain injured rat (contralateral to injury)

B Layer II
C Layer III
D Den
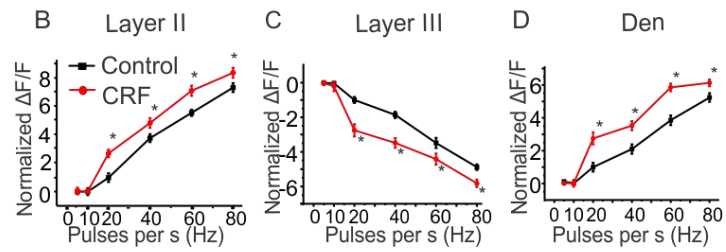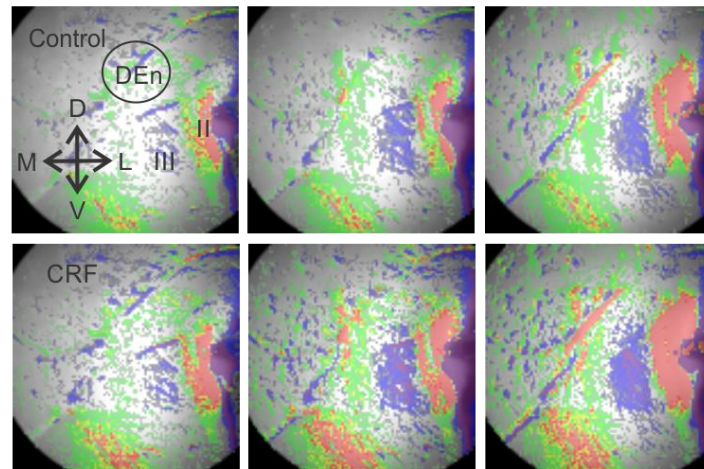
E CRFR1 antagonism (ipsilateral to injury)

F Layer II
G Layer III
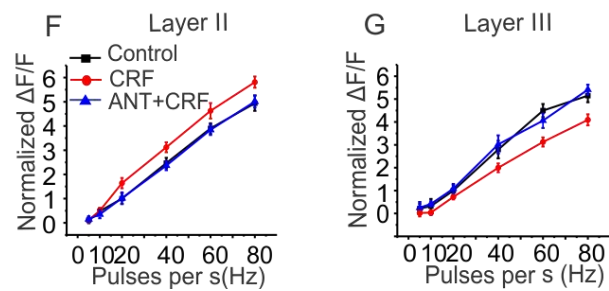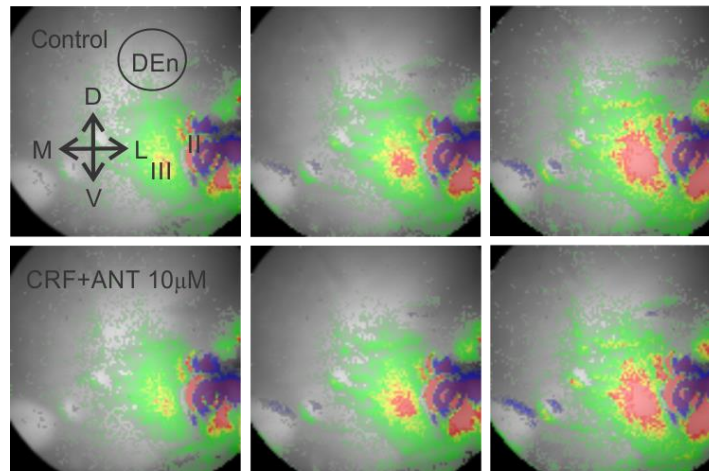
Figure 4.2 Application of CRF increased contralateral PC activity in brain-injured rats. CRFR$_1$ mediated these effects (A) Representative images showing activation of layers of contralateral piriform cortical slices from brain injured rats, without (top) or with CRF perfusion before recording (bottom). (B to D) Quantification of CRFR$_1$ activation shows that the activity of layer II pyramidal cells and interneurons of DEn are increased while the activity of layer III interneurons is decreased over the range of stimulation frequencies used in contralateral piriform cortical slices from brain injured rats to activate the circuit. ** $p < 0.01$. n = 8 slices from 6 rats. (E) Representative images of the activation of the piriform cortex layers in slices at differing time points before (top) and after the application of CRF in the presence of the CRFR$_1$ antagonist antalarmin. The responses are similar to control responses in the presence of CRF and CRFR$_1$ antagonist. (F & G) Quantification of CRFR$_1$ activation in the presence of antalarmin in layer II and layer III of ipsilateral PC. n =6 slices from 5 rats.

4.3.2 Application of CRF differentially activate layers of contralateral PC of injured brain compared to ipsilateral PC

We next investigated if the effects of brain damage would reach the contralateral PC and if there was any difference in the neuronal responses compared to the ipsilateral PC or non-injured PC. We found out that there were three phases of responses in the contralateral PC as observed with a non-injured brain (Fig 4.2 A top row and B-D). However, CRF became excitatory as opposed to dampening the PC circuitry in non-injured PC (Fig 4.2 A bottom row and B-D). These observations (three phases of activity) are very similar to our previous report where we showed that CRF is excitatory after kindling induced epilepsy (Narla et al., 2016). Despite the absence of three phases of activity in ipsilateral PC, it is similar to contralateral PC in a manner there was increased excitatory activity after the application of CRF.

4.3.3 CRFR$_1$ mediate the effects of CRF in injured rat brain

We next examined what type of receptors mediate these effects as we already know that in non-injured and kindled animals CRF signaled through activation of CRFR$_1$ in PC (Narla et al., 2015;Narla et al., 2016). We found that antalarmin antagonized the effects of CRF in both ipsilateral and contralateral PC confirming that CRF signaled through activation of its abundantly available receptor subtype CRFR$_1$ in PC (Fig 4.2 E-G).

Ipsilateral PC to injury
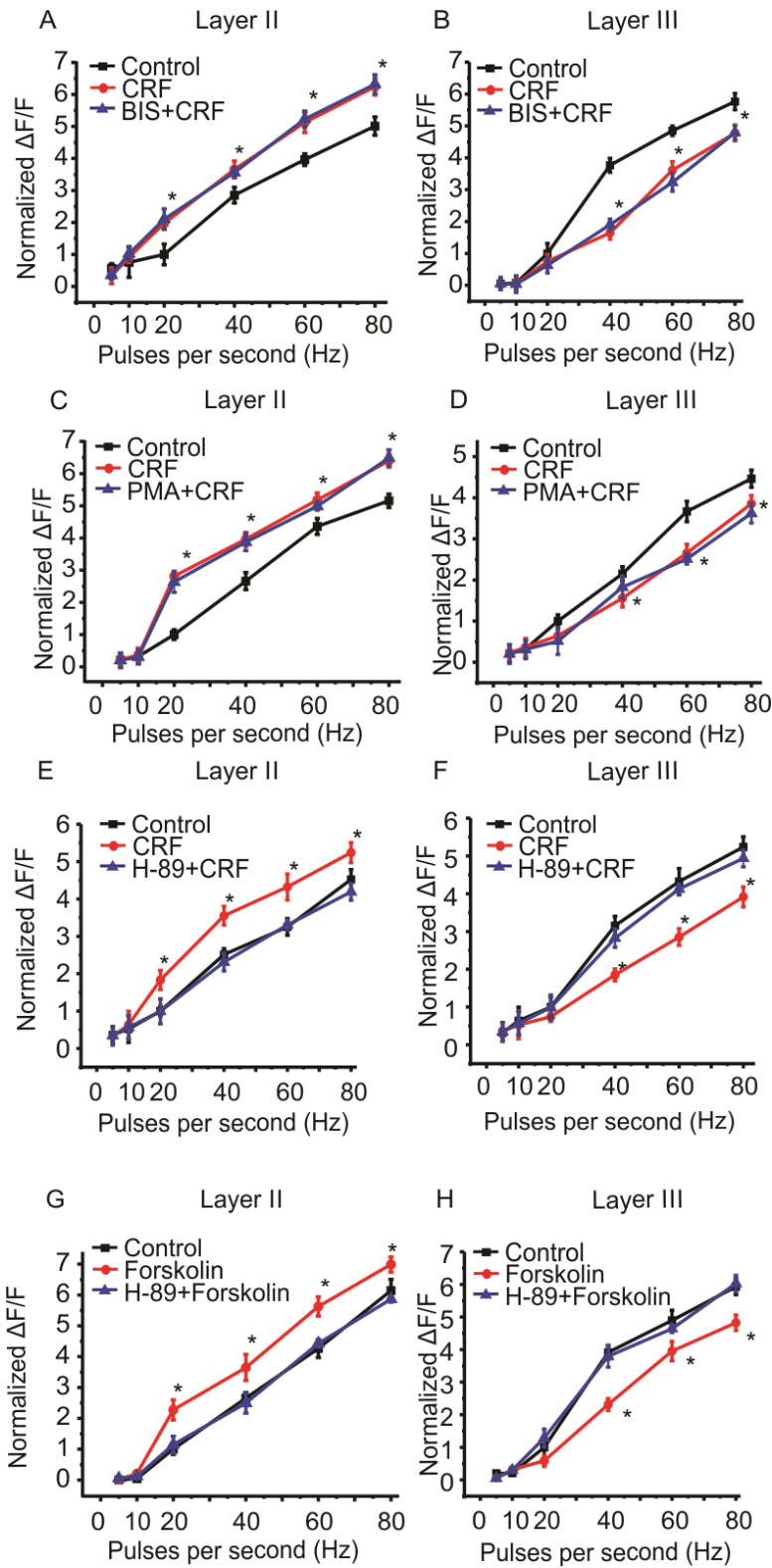
Figure 4.3 PKA and PKC antagonism in ipsilateral PC in the presence of CRF. (A & B) Quantification of the effect of BIS on $CRFR_1$ activation in ipsilateral piriform cortical slices from brain-injured animals * $p < 0.05$, n =8 slices from 8 rats/group. (C & D) Quantification of the effect of the PKC inhibitor, PMA on CRF-mediated activation of $CRFR_1$ in ipsilateral piriform cortical slices from brain-injured animals. * $p < 0.05$, n=9 slices from 8 rats/group. (E & F) Quantification of the effect of the PKA antagonist H-89 on CRF-mediated activation of $CRFR_1$ in ipsilateral piriform cortical slices from brain-injured animals. * $p < 0.05$, n = 9 slices from 7 rats/group. (G & H) Quantification of effect of forskolin, an adenylate cyclase activator with or without subsequent administration of H-89 on ipsilateral piriform cortical slices from brain-injured animals * $p < 0.05$, n = 8 slices from 6 rats/group

4.3.4 CRFR$_1$ signaled by coupling with Gα$_s$ in ipsilateral PC of brain-injured animals

CRFR1s have been known to couple to both Gα$_{q/11}$ and Gα$_s$ proteins to produce end responses (Narla et al., 2015;Narla et al., 2016;Blank et al., 2003). In our previous reports, we showed that CRFR$_1$ couples with Gα$_{q/11}$ and thus activates PKC in non-kindled animals whereas it couples with Gα$_s$ in kindled animals and activates PKA (Narla et al., 2015;Narla et al., 2016). Therefore, we next tested which signaling pathway CRFR$_1$ adopted in the current study. We found out that inhibition of PKC by bisindolylmaleimide (BIS) or activation by PMA did not block nor mimic respectively the effects of CRF (Fig 4.3 A-D). H-89, a PKA antagonist blocked the effects of CRF in both layers of ipsilateral PC (Fig 4.3 E-F). Forskolin, an activator of adenylyl cyclase that mimics the effect of activated Gα$_s$, produced a response like CRF. This was antagonized by the protein kinase A (PKA) inhibitor H-89 (Fig. 4.3 G-H).
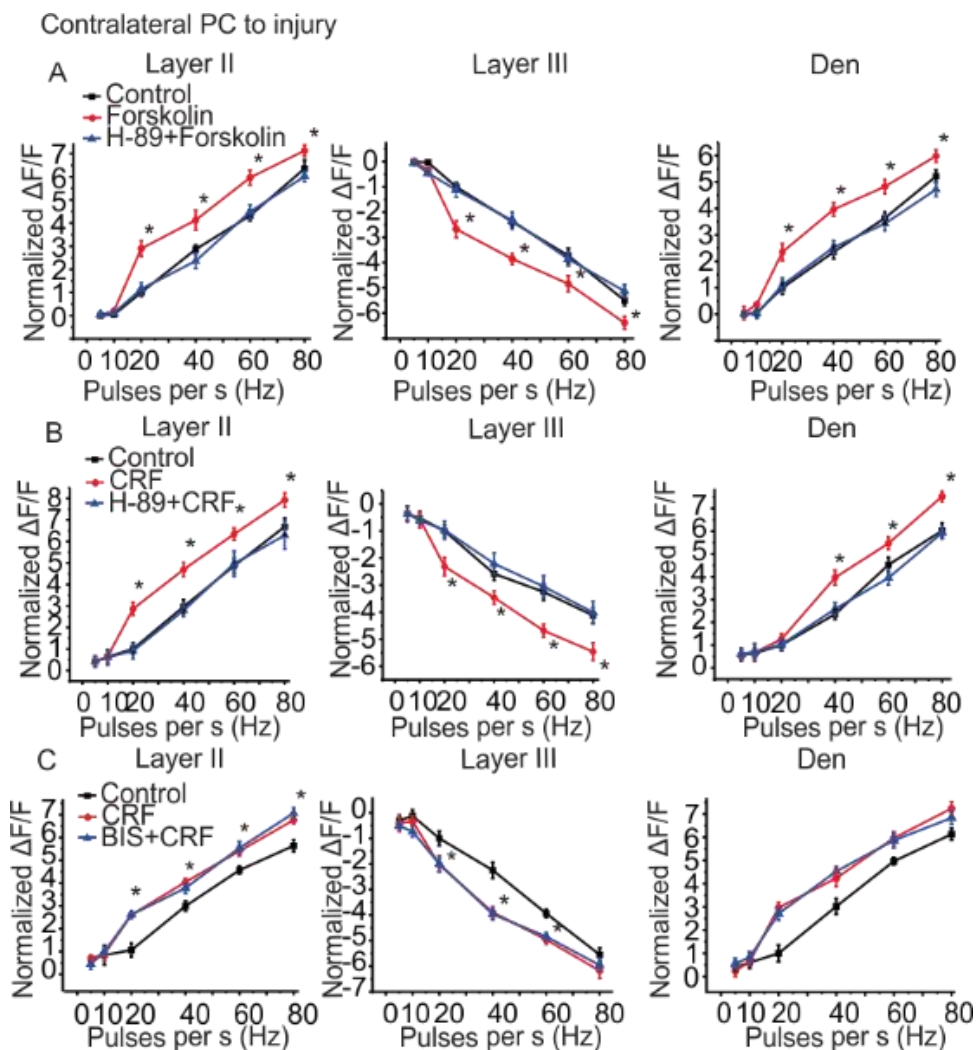
Figure 4.4 PKA and PKC antagonism in contralateral PC in the presence of CRF. (A) Quantification of the effect of forskolin (an adenylyl cyclase activator) with or without subsequent administration of H-89, on $CRFR_1$ activation in contralateral piriform cortical slices from brain-injured animals * $p < 0.05$, n =7 slices from 6 rats/group. (B) Quantification of the effect of the PKA inhibitor H-89 on CRF-mediated activation of $CRFR_1$ in contralateral piriform cortical slices from brain-injured animals. * $p < 0.05$, n=6 slices from 6 rats/group. (C) Quantification of the effect of the PKC antagonist Bis on CRF-mediated activation of $CRFR_1$ in contralateral piriform cortical slices from brain-injured animals. *$p < 0.05$, n = 6 slices from 6 rats/group.

4.3.5 CRFR$_1$ signaled by coupling with Gα_s in contralateral PC of brain-injured animals

Similar observations were made in the contralateral PC where the blockade of PKC had no effect on CRF responses but blockade of PKA removed CRF effects (Fig. 4.4). Thus it is evident that CRFR$_1$ signaled through activation of a Gα_s mediated pathway in both ipsilateral and contralateral PC following the injury similar to kindling induced epilepsy.
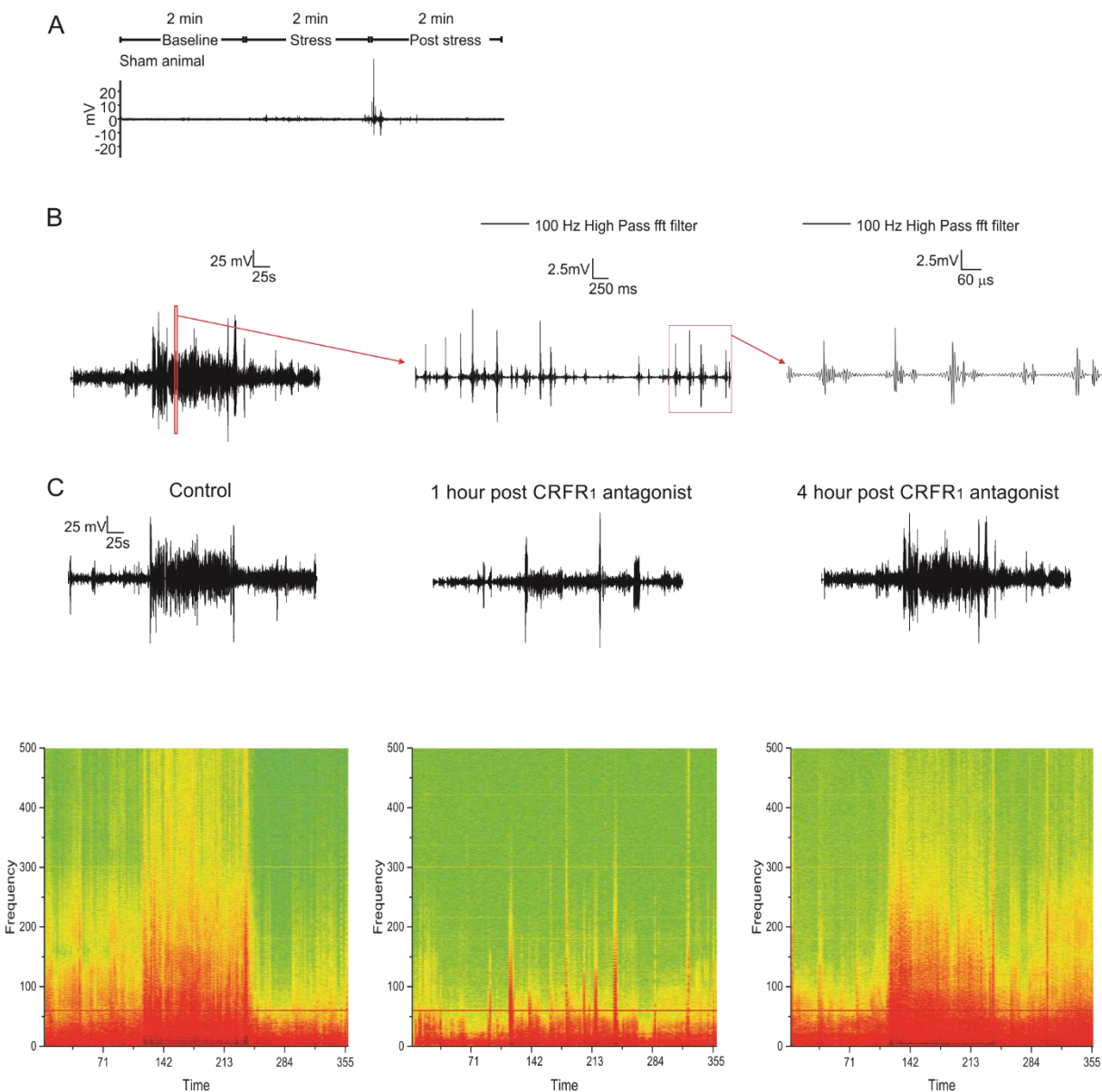
Figure 4.5 CRFR₁ antagonism *in vivo* in brain-injured animals. (A) Trace showing the tail pinch protocol as a model of stress in the healthy non-brain injured the animal. Two minutes of baseline recording followed by two mins of stress which is followed by two minutes of recording in the absence of stress. (B) Traces from the ipsilateral amygdala to the injury of a brain injured animal at different time scales. (C) Traces and power spectrums before, 1-hour and 4-hour post application of CRFR₁ antagonist, CP154526.

4.3.6 Antagonism of CRFR₁ *in vivo* showed reduced electrical discharge from the amygdala of brain injured rats

We next hypothesized that antagonizing CRFR$_1$ block the neuronal hyperexcitability. We tested this hypothesis in brain-injured animals using CP-154526, a CRFR$_1$ antagonist. These animals have bilaterally implanted electrodes in their amygdalae which were used implanted during the time of controlled cortical impact injury. This procedure for implanting electrodes in amygdalae was adopted from kindling model of epilepsy that has been well established in our lab. We designed a model of stress in which the animals were subjected to a tail pinch stressor. The protocol was designed such a way that there was a baseline recording for 2 min in the absence of stressor followed by two min recording in the presence of a stressor. This is further followed by a two min recording in the absence of stressor to see if the brain discharge would come back to baseline levels. The total recording in one animal lasted for 6 min. A representative recording from a sham animal was shown in Figure 4.5 A where it was evident that there was a little discharge from amygdala even during stressor. But in brain-injured animals, there was great discharge from the amygdala during stressor (Fig 4.5 B left a trace). We show the same response from brain injured animal as in the left trace but at a different time scale in the middle trace and last trace of Fig 5 B to show high-frequency oscillations. Highlighted inset in red on the traces denote the region from which the following trace was drawn. In Fig 4.5 C we show the pharmacology of CP154526, a CRFR$_1$ antagonist along with spectral analysis in a brain injured animal. The animal displayed reduced brain activity during stressor compared to control trace 1-hour post injection of CP154526 in the dosage of 30mg/kg (IC$_{100}$) (Fig 4.5 C left and middle traces). After 4 hours post CP154526, the brain activity seemed to come back to normal and was comparable to control recording (Fig 4.5 C last trace). Spectral analysis showed variation in power density comparable to trace above

the power spectrum. A power spectrum describes the power or amplitude of the signal as a function

of frequency.
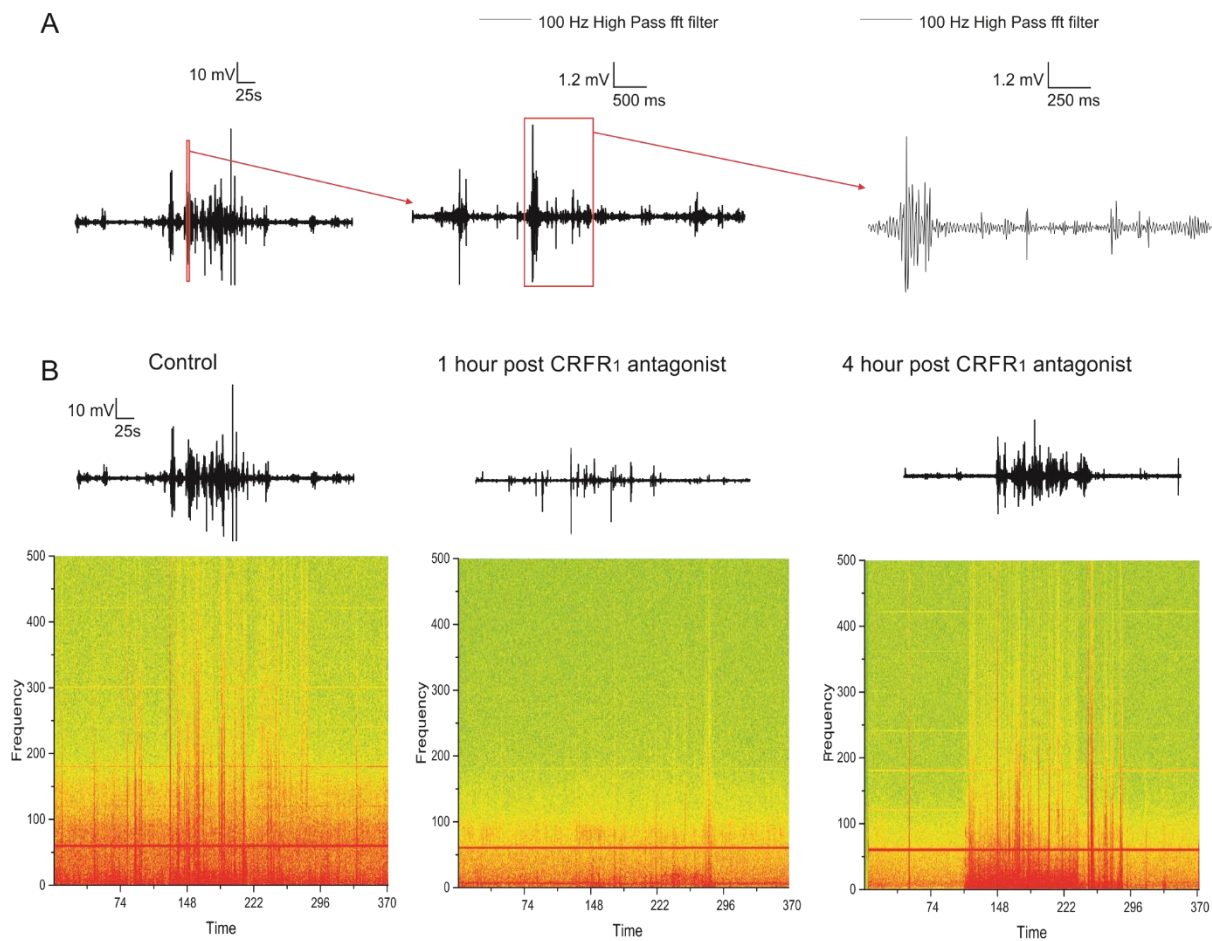
Figure 4.6 CRFR$_1$ antagonism *in vivo* in kindled animals. (A) Traces from the amygdala-kindled animal at different time scales. (B) Traces and power spectrums before, 1-hour and 4-hour post application of CRFR$_1$ antagonist, CP154526.

4.3.7 Antagonism of $CRFR_1$ *in vivo* showed reduced electrical discharge from the amygdala of kindled rats

With a confirmation that $CRFR_1$ antagonism prevents discharges from amygdala following the brain injury, we hypothesized that injection of CP154526 may produce comparable results in kindled animals. Since IC_{100} of CP154526 prevented discharges from amygdalae of brain-injured animals, we decided to test IC_{50} in kindled animals. Surprisingly, IC_{50} was able to prevent the discharges from amygdala of kindled animals. The similar protocol as conducted in the case of brain-injured animals was followed for kindled animals. In Fig 4.6 A left trace, we show the response from a kindled animal in the absence of $CRFR_1$ antagonist. Same response but with a different time scale was shown in the middle and last traces of Fig 4.6 A. In 4.6 B, CP154526 pharmacology has been shown along with spectral analysis.

Discussion:

Traumatic brain injury and its consequences have been a burden on the health care system around the globe, especially in the western world. TBI is a major risk factor in the development of pharmacoresistant epilepsy. In a model of controlled cortical impact, a model of traumatic brain injury to the sensorimotor cortex of the rodent brain we showed that the circuitry of the piriform cortex is affected in a manner that it easily facilitates the occurrence of epileptic seizures. CRF, a stress neurotransmitter exhibited excitatory effects following TBI and became seizuregenic which is otherwise inhibitory physiologically in PC (Narla et al., 2015). Our observations indicate that pathological mechanisms that accompany brain injury make PC susceptible to stressor-induced excitability and thus supports epileptogenesis. These findings provide an explanation for how war veterans or hockey players who suffered head traumas or concussions can develop posttraumatic epilepsy. It is also evident that mechanisms underlying brain injury induced excitability share similarities with kindling induced alterations in the PC of the rodent brain.

The effect of TBI is twofold in rat brain. In PC ipsilateral to the injury interneurons of layer III became excitable while the interneurons of endopiriform nucleus were unresponsive. There should be two reasons to cause this either cell loss or degeneration of axons of layer II pyramidal cells that innervate DEn.

Immunohistochemical analysis of PC revealed no cell death or axonal loss implying that other mechanisms rendered the interneurons of DEn unresponsive. Sloviter (1987) described disruption of functional inhibition, in the presence of viable interneurons and functionally active $GABA_A$ receptors in a dentate area of the hippocampus (Sloviter, 1987;Sloviter, 1991). Later the same author reported similar effect in the CA1 region of the hippocampus (Sloviter, 1991). Another study lead by Bekenstein and Lothman (1993), reported a loss of GABA-mediated

inhibition of hippocampal principal cells in temporal lobe epilepsy. However, immunohistochemical analysis revealed no GABAergic interneuron loss in these areas (Bekenstein and Lothman, 1993) (Lothman et al., 1996). This intriguing paradox has been described as "dormancy of interneurons" where the inhibitory basket cells became quiescent and disconnected rather than dysfunctional. Sloviter described this phenomenon is due to paired pulse inhibition. Similar observations were reported by Jefferys and Traub (1998) in tetanus toxin model of chronic epilepsy where they provided evidence for the existence of functionally dormant interneurons and strongly supported the aforementioned studies (Jefferys and Traub, 1998). These observations provide an explanation for the quiescence of interneurons of DEn ipsilateral PC following brain injury in our study.

Another study demonstrated a long-term synaptic depression (LTD) in hippocampal interneurons which was thought to be responsible for the strengthening of excitatory neurotransmission seen during epileptiform activity (McMahon and Kauer, 1997;Kauer and McMahon, 1997). Brief high-frequency stimulation of pyramidal cells of the stratum radiatum resulted in depression (LTD) of synapses on interneurons that innervated by pyramidal cells. The same synapses on those particular interneurons displayed LTP when the associated pyramidal cells were stimulated with low frequency stimulations. A similar mechanism could be implicated in the ipsilateral PC where high-frequency afferent activity during epileptiform bursting from pyramidal cell layer to DEn resulting in LTD at the synapses of interneurons of DEn and thus contribute to dormant interneuron hypothesis observed in DEn. Further, these findings are supported by other two studies that show in the absence of postsynaptic inhibitory control in the hippocampus, long-lasting potentiation was observed in CA1 pyramidal cells (Wigstrom and Gustafsson, 1983) and

latent synaptic pathways become functional in CA3 pyramidal neurons (Miles and Wong, 1987) after tetanic stimulation.

It has been reported by our group in 2013 that the involvement of interneurons of DEn plays a vital role in controlling the excitability of PC in healthy rats (Birjandian et al., 2013). The disappearance of DEn activity in the current study contributed to flip in the polarity of the interneuronal response of layer III from inhibitory to excitatory. These pathological observations resemble our previous findings reported in 2013 where severing the connections between layer II and deeper layers of PC caused DEn to become unresponsive and layer III responses became excitatory from inhibitory (Birjandian et al., 2013). These circumstances would make one expect that the increased incoming inhibitory drive from layer III (as a result of dysfunctional control of layer III interneurons by DEn interneurons) induce lesser excitability in layer II pyramidal cells compared to non-brain injured rats. Interestingly, the layer II pyramidal cells are still excitable to an extent that is still greater than that of responses from layer II of healthy rats (Fig 4.1). This finding is supported by evidence from the literature that many biochemical, molecular and physiological alterations contribute to network reorganization and increased glutamate signaling following injury(Nilsson et al., 1994;Folkersma et al., 2011). Cantu et al (2015) showed that TBI increased glutamate network activity in the cortex by compromising GABAergic networks (Cantu et al., 2015). This finding by Cantu et al may also provide an explanation to an extent for the incapability of DEn to control layer III. Plastic reorganization of hippocampal and neocortical circuitry has been reported following TBI in immature rats (Card et al., 2005). The pathological network change in our study that the inhibitory interneurons became excitatory in layer III and the dormancy of interneurons of DEn explains that TBI reorganizes the circuitry in a manner that it may support epilepsy.

Active immune surveillance and inflammatory responses can take place in the brain following injury resulting in the release of pro-inflammatory mediators such as cytokines and nitric oxide (NO) (Hemmer et al., 2002). In addition, glial cells actively take part in the immune response in the CNS and contribute to synaptic plasticity (Bains and Oliet, 2007;Volterra and Meldolesi, 2005). This abnormal expression of inflammatory mediators during CNS inflammation result in the impairment of synaptic plasticity and in the destabilization of neuronal networks (Di et al., 2008). The neuroinflammation following TBI may provide another explanation for the increased principal cell activity in layer II.

In PC contralateral to the injury, responses were normal where there was activity from DEn and the interneurons of layer III were inhibited as opposed to ipsilateral PC. These responses in contralateral PC following TBI are very similar to the responses we have reported after kindling (Narla et al., 2016). Important to note that the responses in layer II of PC contralateral to the injury were stronger compared to its counterpart (Fig.4.2). This could be explained by reduced GABAergic drive from layer III to layer II and increased glutamatergic network activity within layer II as a result of TBI. The decrease in glutamate transporter may also contribute to increasing in hyperexcitability as observed by Sullivan et al (1998) immediately following the injury (Sullivan et al., 1998). The vulnerability of GABAergic interneurons to excitotoxic damage has been reported in hippocampal CA1 neurons after induced epilepsy (Dudek and Shao, 2003;Dinocourt et al., 2003). The increased glutamate signaling as a result of brain injury may cause excitotoxic damage to interneurons of PC and thus may make it susceptible to seizure generation. These observations reveal that the network reorganization resulting from TBI is pathologically significant and provide an explanation of why people subjected to concussions are vulnerable to develop epilepsy. Although PC in both hemispheres is affected dissimilarly, they

collectively would make the brain susceptible to develop epilepsy. It can also be inferred that the molecular changes in the brain induced by traumatic injury are confined only to the certain perimeter around the damage and may or may not involve other regions depending on the intensity of the injury.

The responses following Corticotropin-releasing factor application are interesting. In ipsilateral PC, CRF reduced excitation of layer III interneurons thus it contributed to hyperexcitability of principal cells in layer II. In contralateral PC, CRF increased the excitation of DEn interneurons while it increased the inhibition of interneurons of layer III both of which contributed to increased excitation of layer II pyramidal cells. Some studies reported that exogenously applied CRF potentiated the excitatory potential of glutamate in the cerebellum (Bishop and King, 1992) and hippocampus (Aldenhoff et al., 1983). Thus CRF may potentiate the effects of excitotoxic damage already caused by the injury to interneurons and further enhance the deleterious effects of TBI. These effects of CRF are mediated through its G-protein coupled receptor type 1 ($CRFR_1$) as antalarmin, a $CRFR_1$ antagonist blocked these effects. $CRFR_1$ signaled through the activation of $G\alpha s$ after brain injury opposing to $G\alpha q$ in healthy rats. This observation is similar to that of our previous observations in kindled model of epilepsy (Narla et al., 2016) implicating that $CRFR_1$ follow similar molecular changes after brain injury as it would undergo after kindling induced epilepsy. This surprising similarity between TBI and kindling implies that the signaling pathways that $CRFR_1$ would follow depend on the pathological condition of the brain.

Corticotropin-releasing factor has been implicated in several brain disorders such as anxiety, stress, depression and neuropathic pain. Clinical studies revealed that increased cerebrospinal fluid concentrations of CRF are associated with increased neural release of CRF in PTSD (Post et al., 1982). Several studies have found altered HPA axis and CRF function in PTSD

(Smith et al., 1989;Mason et al., 1986;Pitman and Orr, 1990;Yehuda et al., 1993). Consistent with these reports preclinical studies reported a chronic increase in CRF release following stress exposure (Dallman and Jones, 1973). PTSD and traumatic brain injury often comorbid as brain injuries are often associated with traumatic experiences (Hoge et al., 2008;Bryant, 2011;Ohry et al., 1996;Hickling et al., 1998;Gaylord et al., 2008;Greenspan et al., 2006;Levin et al., 2001). To date, few mechanisms have been proposed such as upregulated glutamate neurotransmission, axonal damage and compromised interneuronal circuits causing the development of epilepsy. Even though these mechanisms are true they do not explain how the presence of PTSD, a common aftermath of brain injuries further exacerbates seizures. Characterization of PTE in PC is a unique finding of this study. With our findings, we propose that increased CRF levels in the CSF (Bremner et al., 1997;Yehuda, 2001) and several brain regions as a result of brain injury further exacerbate the occurrence of epilepsy by many folds.

Intrigued by the pathophysiological involvement of CRF and its receptor, CRFR$_1$ in exacerbation of epilepsies in our past (Narla et al., 2016) and current findings we propose that CRFR$_1$ antagonists may have profound clinical importance as antiepileptic drugs. Consistent with this idea, CP-154526, a CRFR$_1$ antagonist reduced the discharge from amygdala of kindled (epileptic) rats following stress exposure in our *in vivo* experiments. CRF receptor antagonists have long been under investigation for treating stress-related disorders such as anxiety and depression though they were less promising (Valdez, 2009;Zorrilla and Koob, 2004). No clinical study till date has investigated or argued about the use of CRFR$_1$ antagonists in epilepsy. Exogenously applied CRF-induced excitotoxicity that resulted in degeneration of interneurons (Bishop and King, 1992;Aldenhoff et al., 1983). There were reports that CRFR antagonists are effective in providing neuroprotection in the hippocampus following seizure (Maecker et al., 1997)

and after cerebral ischemia (Lyons et al., 1991). Selective $CRFR_1$ antagonists may offer neuroprotection against interneuronal loss and help in controlling seizures following brain injury.

In summary, our findings provide a mechanism by which individuals affected by traumatic brain injury develop stress associated epilepsy. Molecular mechanisms following brain injury alter the function of stress hormone/neurotransmitter that increases the excitability of piriform cortex, a region that supports seizures. Also, this is the first report that described interneuronal dormancy in brain injury and in a brain region other than those previously reported. These observations collectively support the idea that CRF1 receptor antagonists have the potential to become antiepileptic medication and thus require clinical investigation.

4.5 Reference List

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Chapter 5. General Discussion

The piriform cortex, a part of the temporal lobe, is the largest subdivision of olfactory cortex; the first region of the olfactory cortex to which the olfactory information is sent. The olfactory cortex receives monosynaptic input from the olfactory bulb. Of all the regions of olfactory cortex, the PC is the largest and is also referred to as the primary olfactory cortex. It is a phylogenetically ancient cortical structure and contains only three layers as opposed to the six layers of the neocortex (Neville and Haberly, 2003). It's simple three-layered structure along with an increased understanding of its connections with other regions makes it a good candidate as a model of study in cortical sensory processing (Loscher and Ebert, 1996). The piriform cortex is also highly epileptogenic. It is interconnected with other epileptogenic regions in the limbic system and other cortical areas such as the amygdala, hippocampal formations, and the rhinal cortex. PC acts as a junction between these regions and distributes seizure activity throughout the limbic system. The functional anatomy of these regions involve oscillatory network activity and can also lead to pathological hypersynchronization resulting in seizure generation. Closely associated with the PC is endopiriform nucleus (DEn), a large region of multipolar cells which has been implicated in generation and propagation of seizures (Piredda and Gale, 1985;Piredda and Gale, 1986;Demir et al., 1998;Demir et al., 1999;Demir et al., 2001;Vaughan and Jackson, 2014). All of the functions of DEn are unknown but may have a role in the integration of olfactory sensory information (Sugai et al., 2012). Owing to its simple structure and association with other epileptic regions of the brain, PC constitutes an excellent model for studying network dynamics.

Anxiety, stress, and depression are the most commonly agreed stressors that are associated with epilepsy (Adamec, 1990a). Several psychological disturbances have been known as stressors

which precede the onset of anxiety and depression in non-epileptic individuals. Human studies indicate that limbic epilepsy predisposes patients with epilepsy to respond to psychological stressors with anxiety and depression (Adamec, 1990b). Studies in animal models of epilepsy support this observation This is an awkward starting amygdala of right hemisphere increased stress-induced ulcerations in Wistar rats (Henke and Sullivan, 1985) and increased anxiety as measured in elevated plus maze a week post kindling (Adamec, 1990a). Fearful responses in felines are a model of anxiety states in humans. Partial kindling of the amygdala and hippocampus in felines showed a permanent increase in fearful responses to species of characteristic threat, a sign of stress and anxiety (Adamec and Stark-Adamec, 1983;Adamec, 1991).

Corticotropin-releasing factor is a 41 amino acid peptide which is widely distributed in the brain and is known to mediate many behavioral responses to stress (Vale et al., 1981;Chappell et al., 1986;Palkovits et al., 1985;Binder and Nemeroff, 2010). CRF neurons in hypothalamus coexpress CRF and vasopressin which synergistically work on adrenocorticotropic hormone release from the pituitary. It results in hypothalamic-pituitary-adrenal (HPA) axis plasticity to sustain a response to long-lasting and intense stress (Antoni, 1986). Centrally, the release of CRF in response to stress in limbic regions induces fear and anxiety responses (Koob and Thatcher-Britton, 1985). CRF has also been implicated in major depressive disorder and in bipolar mood disorders. Increased levels of CRF concentration in the cerebrospinal fluid has been reported in the depressed individuals (Banki et al., 1987;Hartline et al., 1996;Nemeroff et al., 1984). However, little is known about the involvement of CRF in epileptogenesis and thus how it may exacerbate the frequency of seizures in patients with stress, anxiety, and depression.

We have selected electrical kindling of amygdala as a model to study epilepsy associated with stress and anxiety in our experiments. Several lines of evidence support that this model best represents human temporal lobe epilepsy. Kindling of amygdala and hippocampus in felines showed a permanent increase in fearful responses to species of characteristic threat, a sign of stress and anxiety and this is a model to study anxiety states in humans (Adamec and Stark-Adamec, 1983;Adamec, 1991). Also, that amygdala kindling in Wister rats has been described as a model of stress (Henke and Sullivan, 1985). Based on these observations amygdala kindling model of epilepsy best represent human temporal lobe epilepsy along with the states of anxiety and stress.

Traumatic brain injury brain injury is a consequence of concussions, head blast injuries and alike resulting in cellular and molecular changes in the brain that pathologically affect the brain function and may predispose an individual to other disease states. People who are subjected to traumatic brain injury are more likely to develop post-traumatic stress disorders and stress associated seizurogenesis. We believed that CRF could be a candidate to explore in this situations as it is well known to modulate hypothalamic-pituitary-adrenal axis in response to stress. To understand the function of CRF in epilepsy and traumatic brain injury it was important to elucidate the role of CRF in the healthy brain first. Therefore, the present thesis addressed following questions:

1. What is the role of CRF in healthy rat piriform cortex? What kind of CRF receptors mediate these effects? Does CRF system influence other neurotransmitter systems such as serotonin?

2. How does the functionality of CRF change after kindling? Do the same receptors mediate these effects as in healthy rats? If yes, is there any change in the signaling cascades?

3. What is the role of CRF in traumatic brain injury? Do the CRF receptors follow same signaling pathway as in healthy animals?

This section focuses on the main findings of these studies and analyzes functions of CRF and its receptor signaling pathways in kindling induced epilepsy and traumatic brain injury.

5.1 Corticotropin-releasing factor actions in healthy rat piriform cortex:

Fluctuations in homeostasis due to stressors are responsible for the onset of anxiety episodes and depression (de Kloet et al., 2005). Various brain regions, such as the amygdala, hippocampus and nucleus accumbens mediate stress responses by releasing various stress neurotransmitters. These neurotransmitters/neuromodulators mediate stress responses (Mora et al., 2012). Piriform cortex, a brain region that has extensive connections with these stress mediating brain regions has been poorly studied. PC as a part of olfactory cortex processes odor stimuli and can induce stressor responses (Kadohisa, 2013;Krusemark et al., 2013). Stress neurotransmitters such as CRF is released from interneurons of PC and serotonin is released from efferent fibers arising from dorsal raphae nucleus to PC. It is hypothesized that CRF and serotonin modulate neuronal activity of PC in response to stress. CRF binds to its receptor subtypes $CRFR_1$ and $CRFR_2$ whereas serotonin binds to a variety of its receptors and their subtypes ranging from 5HT1 to 5HT2. $CRFR_1$ are more predominant and various 5HT1 and 5HT2 subtypes are expressed in PC. Activation of these receptors are implicated in stress, anxiety, and depression in many regions and are known to be anxiogenic (Kahn et al., 1988;Eison, 1990;Davis, 1992;Aghajanian and Davis,

1975;Merali et al., 2004;Muller et al., 2003;Risbrough and Stein, 2006;Arborelius et al., 1999;Naughton et al., 2000). Surprisingly, activation of these receptors turned out to be anxiolytic in PC and thus dampened the overall circuit activity. This observation is an unusual response to these ligands. Although it is unusual, it appears that deactivation of PC is a reflexive response to reduce the output of amygdala which is activated by perceived threat and thus seems usual in this particular brain region. Activation of the serotonergic system alone by the application of DOI did not alter the neuronal circuit behavior of PC. However, the activation of serotonergic neurotransmission following the activation of CRF system further dampens the excitability of PC conveying that the interaction or prior activation of $CRFR_1$ is necessary to prepare the animal for the stress response resulting from perceived threat.

Corticotropin-releasing factor displayed varied functionality depending on the cell type and region of the brain. Hippocampal excitation and action potential facilitation by CRF has been reported (von et al., 2011;Aldenhoff et al., 1983;Hollrigel et al., 1998;Blank et al., 2003). Depression of excitatory neurotransmission in hippocampal formation by CRF has also been reported (Sheng et al., 2008). In the prefrontal cortex, CRF decreased the pyramidal cell firing by increasing GABA release via prolonged serotonergic neurotransmission (Tan et al., 2004). The reason for this highly variable behavior of CRF is unclear. We hypothesized that CRF signal through different G-proteins depending on the region of the brain. Supporting for this hypothesis comes from several observations that $CRFR_1$ signaled through either PKA or PKC in various brain regions (Blank et al., 2003;Elliott-Hunt et al., 2002a;Tan et al., 2004). We found that $CRFR_1$ coupled to $G\alpha_{q/11}$ through activation of PKC in PC and this coupling lead to a dampening of the circuit activity. The most profound effect is observed after $5HT_{2A/2C}$ receptor activation following $CRFR_1$ where there was a further reduction in the activity of PC. This implies that $CRFR_1/5HT_{2A/2C}$

interaction accounts and is necessary for further reduction in excitability of PC. Most important observation is that CRF showed variable effects on interneurons in layer III and DEn where it reduced inhibition (positive magnitude) of interneurons of layer III but decreased the excitation (negative magnitude) of interneurons of DEn. Overall this contributes to dampening the layer II activity.

In summary, our observations show how neuronal activity in the PC is altered following the activation of $CRFR_1$ and 5HT2A/C Rs. It is a surprising finding that how two neurotransmitters that heighten anxiety would dampen the excitability of circuit that has strong interconnections with other regions such as the hippocampus, amygdala and prefrontal cortex which are also implicated in anxiety. Another important finding of the study is the highly complex and varied nature of $CRFR_1$ effects in different cell populations and capability to activate different signaling cascades involving multiple G-proteins. This capacity of $CRFR_1$ to couple with multiple G-proteins may become plastic under different conditions such as epilepsy and brain injury.

5.2 Corticotropin-releasing factor signaling in kindling induced epilepsy:

Intrigued by actions and the functionality of $CRFR_1$ in healthy non-kindled animals we pursued to characterize CRF and $CRFR_1$ functionality in epileptic animals. Thus, the goal of this study is to first understand the functioning of CRF in kindling induced epileptic animals followed by elucidation of $CRFR_1$ signaling mechanisms. And finally to correlate these findings with non-epileptic animal data. In kindling model of epilepsy, a model of epilepsy that resemble human temporal lobe epilepsy, we found that CRF became excitatory and potentially more seizuregenic reversing its polarity from inhibitory to excitatory. This observation is quite opposite to our finding in non-kindled animals. This indicates that stressor induces excitability to accompany epileptic

state in a brain region that supports epileptogenesis suggesting that the underlying brain pathology may be an important factor in worsening epilepsy associated with heightened anxiety states. The resulting neurochemical alterations following the stressor-induced excitability support epilepsy onset.

The actions of CRF in the epileptic brain are mediated through activation of $CRFR_1$ as observed with a non-epileptic brain. This is confirmed by the blockade of CRF responses by antalarmin, a $CRFR_1$ antagonist. This is an interesting finding that CRF binding to same receptor subtype produces opposite effects in kindled animals compared to healthy animals. It indicates a change in the signaling mechanism that $CRFR_1$ adopted in epileptic animals compared to healthy animals. We tested this hypothesis and found that the change in the polarity of the $CRFR_1$ signaling, indeed, occurs through the switch in the signaling cascade by which CRF acts. $CRFR_1$ has been shown to activate different G-protein cascades (Blank et al., 2003;Wanat et al., 2008;Wietfeld et al., 2004). For example, the neuroprotective effects of CRF in the hippocampus are mediated by the activation of PKA and MAPK signaling pathways (Elliott-Hunt et al., 2002b). Moreover, $CRFR_1$ couples to $G_{q/11}$ in BALB/c mice hippocampal neurons, whereas it couples to $G\alpha_s$, and $G\alpha_i$ in C57BL/6N mice hippocampal neurons (Blank et al., 2003). In cultured hippocampal neurons, CRF regulation of NMDA currents is mediated by PKC, which correlates with increased $PLC\beta3$ abundance (Sheng et al., 2008). In prefrontal cortex pyramidal neurons, CRF activates $CRFR_1$ signaled through activation of PKC to enhance stressor responses (Tan et al., 2004). Thus, it seems clear that depending on the region and pathological condition of the brain, $CRFR_1$ can activate distinct G proteins resulting in activation of multiple signaling pathways.

Our interest to understand the root cause of the switch in the $CRFR_1$ signaling mechanism lead to test a myriad of hypotheses which include changes in the relative abundance of $G\alpha_s$ and $G\alpha_{q/11}$ mRNA and protein. Thus, we hypothesized that an increase in $G\alpha_s$ or a decrease in $G\alpha_{q/11}$ abundance favors the coupling of $CRFR_1$ to $G\alpha_s$. This was not the case as $G\alpha_s$ mRNA was decreased and the protein abundance was not changed. $G\alpha_{q/11}$ mRNA was also not changed. These observations did not provide an explanation as to why the switch in the signaling of $CRFR_1$ occurred. We next tested the hypothesis that mRNAs encoding other proteins involved in intracellular signaling may be altered. Although there were changes in the expression of some transcripts, the proteins coded by these transcripts were not involved in any kind of process that would cause the shift in the preferred signaling cascade. We next considered the possibility that the phosphorylation states of various proteins involved in GPCR cascaded could account for the change only to learn that none would account for a switch in the signaling efficacy or preference. We next identified RGS proteins as candidates of interest and their abundance may be altered. There have been several reports that RGS proteins inhibit or enhance the activity of heterotrimeric G-proteins in vitro (Grafstein-Dunn et al., 2001;Heximer et al., 1999;Ingi et al., 1998a). Two RGS proteins RGS2 and RGS17 are widely expressed in piriform cortex (Grafstein-Dunn et al., 2001). qPCR analysis revealed that expression of RGS2 protein abundance decreased but not RGS 17. There have been reports that RGS2 interact with several adenylate cyclase isoform and thus decreases cAMP production thereby limit $G\alpha_s$ mediated signaling (Roy et al., 2006;Sinnarajah et al., 2001). These observations suggested that downregulation of RGS2 may increase $G\alpha_s$ signaling and thus may contribute to switching in $CRFR_1$ signaling after kindling. Supporting experiments conducted in RGS2 knockout mice (KO) revealed that CRF responses were similar to kindled rat responses and $CRFR_1$ mediated these effects. Overall, these reports corroborate our findings that

the presence of RGS2 in healthy animals decreased the production of cAMP and thus suppressed the CRFR$_1$ stimulated the activity of Gα_s. The absence of RGS2 in the case of RGS2 KO mice and when RGS2 abundance is low as in the case of epileptic animals (kindled), CRFR$_1$ activation seemed to prefer to signal through Gα_s.

RGS2 is expressed in brain regions that are implicated in the pathophysiology of anxiety, stress and depression, such as hippocampus, amygdala, cerebral cortex, hypothalamus and raphae nucleus of rodents (Ingi and Aoki, 2002;Neubig and Siderovski, 2002;Leygraf et al., 2006;Ingi et al., 1998b). These regions are interconnected with PC and any pathological changes in these regions would negatively affect the neuronal activity of PC and may lead to seizurogenesis. Animal models involving RGS2 KO or mutation of *RGS2* gene showed increased anxiety and depression-like behaviors (Yalcin et al., 2004;Lifschytz et al., 2012). These observations indicate that any changes in the RGS2 abundance may precipitate anxiety and depression states and thus exacerbate epileptogenic potential of the brain.

In summary, our findings provided a novel mechanism by which anxiety and stressors may heighten the incidence of seizures. The presence of epileptic state alters the functionality of stress neurotransmitter that increases the excitability of brain which supports seizurogenesis. Other pathophysiological brain states such as concussions, stroke, and traumatic brain injury may also lead to similar alterations, such as those observed in this study, in many GPCR responses.

5.3 Corticotropin-releasing factor receptor function in traumatic brain injury:

Traumatic brain injuries are very common nowadays and people are subjected to head injuries in a variety of situations such as playing sports, slips, and falls or while executing their duties at work as in the case of war veterans. It has been a burden on the health care system around

the globe, especially in the western world. Brain injured patients are more at risk of developing pharmacoresistant epilepsy. The presence of stress or anxiety adds complexity to the situation. The underlying mechanisms are not fully understood and thus demands intense research over the years. Based on our findings in an animal model of epilepsy, we predicted that CRF might play a crucial role in network reorganization of PC and thus involve in precipitation of stress associated epilepsy in brain injured patients. In a controlled cortical impact model of traumatic brain injury, we showed that the network activity of PC is pathologically affected in a manner that it may support epilepsy. CRF, a stress neurohormone showed excitatory effects in PC following traumatic brain injury and became potentially seizuregenic. These findings provide an explanation how individuals with brain injuries develop posttraumatic epilepsy.

Traumatic brain injury to the sensorimotor cortex of rat brain has a variable effect on the circuitry of PC ipsilateral and contralateral to the injury. In ipsilateral PC, layer III became excitatory while DEn was unresponsive following the stimulation of LOT. Immunohistochemical analysis revealed no cell loss or degeneration of pyramidal cell axons that convey excitatory input to DEn from layer II. An intriguing paradox, named, "Dormancy of interneurons" has been described in hippocampus and dentate gyrus, a phenomenon in which there is disruption of functional inhibition, in the presence of viable interneurons and functionally active $GABA_A$ receptors (Sloviter, 1987;Sloviter, 1991;Bekenstein and Lothman, 1993;Lothman et al., 1996). In this phenomenon, the inhibitory basket cells become quiescent and disconnected rather than dysfunctional and are due to paired pulse inhibition. These observations provide an explanation for the disappearance of activity from DEn. The excitability of layer III interneurons, as opposed to inhibition in healthy animals, is explained by the lack of GABAergic inhibitory drive from DEn as a result of dormancy of interneurons. Several other mechanisms such as long-term depression

of glutamatergic synapses on DEn interneurons as a result of high-frequency epileptiform bursting from pyramidal cell layer further contribute to interneuronal dormancy. This high-frequency epileptiform bursting of layer II pyramidal cells may be due to destabilizing neuronal networks and impaired synaptic plasticity as a result of the inflammatory response following the injury (Bains and Oliet, 2007;Volterra and Meldolesi, 2005;Di et al., 2008).

In PC contralateral to the injury, responses were normal where there was activity from DEn and the interneurons of layer III were inhibited as opposed to ipsilateral PC. These responses in contralateral PC following TBI are very similar to the responses we have observed after kindling induced epilepsy. There were stronger responses in layer II of PC contralateral to the injury compared to its counterpart. This is explained by reduced GABAergic drive from layer III and increased glutamatergic activity within layer II as a result of TBI. Also downregulation of glutamatergic transporters and increased excitotoxic damage to interneurons contribute to seizure susceptibility of PC. These observations indicate that network changes and hyperexcitability following TBI are pathologically significant and provide an explanation for why people subjected to concussions are vulnerable to develop epilepsy.

The responses following CRF application are interesting. CRF reduced the excitability of layer III of ipsilateral PC thus contributed to increased excitability of already hyperexcitable pyramidal cell layer. In contralateral PC, CRF increased the excitation of layer II by reducing inhibition of layer III and excitability in DEn. These effects of CRF are mediated through its G-protein coupled receptor type 1 ($CRFR_1$) as antalarmin, a $CRFR_1$ antagonist blocked these effects. $CRFR_1$ signaled through the activation of $G\alpha_s$ after brain injury opposing to $G\alpha_{q/11}$ in healthy rats. This observation is similar to that of our previous observations in kindled model of epilepsy

implicating that the presence of pathological brain states induces $CRFR_1$ to adopt a signaling mechanism different from a normal brain.

Intrigued by the pathophysiological involvement of CRF and its receptor, $CRFR_1$ in exacerbation of epilepsies in our past and current findings we propose that $CRFR_1$ antagonists may have profound clinical importance as antiepileptic drugs. Consistent with this idea, CP-154526, a $CRFR_1$ antagonist reduced the discharge from amygdala of kindled (epileptic) rats following stress exposure in our *in vivo* experiments. CRF receptor antagonists have long been under investigation for treating stress-related disorders such as anxiety and depression though they were less promising (Valdez, 2009;Zorrilla and Koob, 2004). No clinical study till date has investigated or argued about the use of $CRFR_1$ antagonists in epilepsy. Exogenously applied CRF-induced excitotoxicity that resulted in degeneration of interneurons (Bishop and King, 1992;Aldenhoff et al., 1983). There were reports that CRFR antagonists are effective in providing neuroprotection in the hippocampus following seizure (Maecker et al., 1997) and after cerebral ischemia (Lyons et al., 1991). Selective $CRFR_1$ antagonists may offer neuroprotection against interneuronal loss and help in controlling seizures following brain injury.

In summary, our findings provide a mechanism by which individuals affected by traumatic brain injury develop stress associated epilepsy. Molecular mechanisms following brain injury alter the function of stress hormone/neurotransmitter that increases the excitability of piriform cortex, a region that supports seizures. Also, this is the first report that described interneuronal dormancy in brain injury and in a brain region other than previously reported. These observations collectively support the idea that CRF1 receptor antagonists may become potential antiepileptic drugs and require clinical investigation.

5.4. What is the importance of these findings and what do they implicate?

Corticotropin-releasing factor plays a critical role in anxiety, depression and stress responses. Its actions are divergent in various brain regions. Some studies argue that CRF is excitatory and contributes to anxiety and epilepsy whereas others suggest that it is inhibitory. Various conditions such as a region of interest in the brain, different cell types, abundance and predominance of its receptor subpopulations, the concentration of CRF, an abundance of RGS proteins, interaction with other neurotransmitter systems, differences in species and/or strains and the presence of disease states influence the actions of CRF. It has been shown that CRF is excitatory in the hippocampal formation (von et al., 2011) whereas we found that it is inhibitory in piriform cortex of healthy rats. This reveals that CRF shows a high degree of specificity in its actions depending on the brain region. Whole cell patch clamp recordings in our experiments demonstrated that CRF reduced the excitability of layer II pyramidal cells in PC whereas it increased the excitability (disinhibition) of layer III interneurons. This shows that CRF has the capacity to differentially modulate the neuronal populations within the same brain region. Despite being interneurons, the interneurons of endopiriform nucleus displayed decreased firing patterns upon CRF application whereas layer III interneurons exhibited increased firing patterns. This demonstrates that CRF goes further extremes within the same brain region to differentially activate a similar class of neuronal populations. Altogether, this denotes that CRF alters the network dynamics of PC circuitry in a manner to suppress its activity. The flip in CRF responses following epilepsy and brain injury

The concentration of endogenously released CRF would influence its effects on its receptors. High concentrations of CRF has been shown to activate $CRFR_2$ whereas low concentrations activate $CRFR_1$ (Kirby et al., 2008). Discrete distribution of these receptors in

various brain regions imply diverse physiological functions and thus implicate variation in CRF actions (Van et al., 2000). Kirby et al (2008) showed that CRF modulates 5-HT DRN neurons in a direct as well as indirect manner on a GABAergic synaptic activity via its both receptor subtypes. This is one of many classic examples of CRF modulation of other neurotransmitter systems. Thus the actions of CRF may not directly exert desired or undesired effects but indirectly via its interactions with other neurotransmitter systems such as GABA, serotonin and alike. Differences in the distribution and abundance of CRF receptors also contribute to CRF's diversified effects. Mice express $CRFR_2$ with equal abundance as $CRFR_1$ whereas in rats $CRFR_1$ predominates $CRFR_2$ (Chen et al., 2005;Van et al., 2000). Blank et al (2003) reported that $CRFR_1$ signaled through activation of different G-proteins in two different mouse strains which lead to increased conditioned fear in one strain but not the other suggesting that CRF actions are dependent on the strain investigated (Blank et al., 2003). Thus, myriad factors influence CRF actions in the brain and hence may arise a question how effectively these findings can be extrapolated to alleviate human suffering? The findings of my thesis that $CRFR_1$ switches signaling from a healthy rat PC to epileptic and traumatically injured brain are novel. Lack or limited availability of healthy human brain tissue to test the applicability of these findings is a limitation of this study. Conducting clinical trials to assess the candidacy of $CRFR_1$ antagonists may provide more clinical applicability of novel findings of this thesis.

5.5 Future studies:

Performing multiple current clamp and voltage clamp recordings at the same time in different layers of PC could provide further information about the temporal dynamics of neuronal excitability. Also employing advanced techniques such as optogenetics and microelectrode array provide a great wealth of information that otherwise could not be attained. Our work indicates that

CRFR$_1$ antagonists would make better antiepileptic drugs to counter stress induced epilepsy. Further clinical studies are required to evaluate their potency to treat epilepsy in human subjects and to discover more potent and clinically relevant CRFR$_1$ antagonists.

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APPENDIX A: ETHICS APPROVAL (Rat and Mouse)

AUP Number: 2010-017

PI Name: Poulter, Michael

AUP Title: Electrophysiological and Genetic Studies of the Kindled Central Nervous System

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-017 has been approved.

1. This AUP number must be indicated when ordering animals for this project.

2. Animals for other projects may not be ordered under this AUP number.

3. Purchases of animals other than through this system must be cleared through the ACVS office.

Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible for ensuring that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Thompson, Sharla H

on behalf of the Animal Use Subcommittee

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- **Chakravarthi Narla**, Tanner Scidmore, Jaymin Jeong, Michelle Everest, Peter Chidiac and Michael O Poulter. "A switch in G protein coupling for type 1 corticotropin-releasing factor receptors promotes excitability in epileptic brains" in *Science Signaling, 2016,* 9: 432 ra60.
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- **Chakravarthi Narla**, VSP Pavan Kumar, Ranjith Babu Vegicharla, Ramana Gangireddy "Pro-Convulsant activity of Progesterone like compounds in Kainic acid induced convulsions; implications for the possible mechanisms". January – March 2011 RJPBCS Vol 2 Issue 1 Page No. 874