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Corticotropin-Releasing Factor Facilitates Epileptiform–activity In The Entorhinal Cortex Via CRF2 Signaling Mechanisms

Lalitha Kurada

CORTICOTROPIN-RELEASING FACTOR FACILITATES EPILEPTIFORM–ACTIVITY IN THE ENTORHINAL CORTEX via CRF_2 SIGNALING MECHANISMS

by

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This dissertation, submitted by Lalitha Kurada in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Date

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Title: Corticotropin-Releasing Factor Facilitates Epileptiform Activity in the Entorhinal Cortex *via* CRF_2 Signaling Mechanisms

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Lalitha Kurada
May 07, 2015

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ABSTRACT

Temporal lobe epilepsy (TLE) is characterized by hyperexcitability of limbic structures. The entorhinal cortex (EC) is involved in the initiation and maintenance of TLE. Layers II and III of the EC in particular are hyperexcitable and are more susceptible to epileptogenesis. TLE is influenced in a complex manner by the stress-released epileptogenic neuropeptide, corticotropin-releasing factor or hormone (CRF/CRH). Nevertheless, the action site and underlying mechanisms of CRF in epilepsy are not fully understood. Here we found that the EC expresses high levels of CRF and CRF_2 receptors without the expression of CRF_1 receptors. CRF increased the frequency of picrotoxin (PTX)-induced epileptiform activity via CRF_2 receptors and requires cyclic AMP (cAMP). However, application of selective protein kinase A (PKA) inhibitors reduced, not completely blocked CRF-induced enhancement of epileptiform activity suggesting that PKA is only partially required. Furthermore, endogenously released CRF is also involved in the epileptogenesis.

Among various ionic conductances maintaining neuronal excitability, the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and the conducting current I_h has strongly been implicated in TLE. Whereas, layer III of the EC shows preferential neuronal loss in TLE, layer II is spared and becomes hyperexcitable. Since the stellate neurons of layer II express high levels of HCN channels, we investigated the role of HCN channels in CRF- mediated facilitation of epileptiform activity. In the

presence of HCN-channel blocker-ZD7288, CRF failed to increase the frequency of epileptiform activity but still augmented the numbers of synchronizing events within an epileptiform activity and the duration of epileptiform activity. This suggests that part of the effects of CRF on epilepsy is mediated via HCN channels. Furthermore, using perforated patch clamp recordings we found that CRF increased I_h recorded from layer II stellate neurons via activation of CRF_2 receptors. cAMP, not PKA was responsible for CRF-mediated facilitation of I_h. At the cellular level, CRF depolarized the membrane potential resulting in increase in neuronal excitability and action potential firing. These mechanisms facilitate an increase in epileptiform activity mediated by CRF, in the EC. Our results provide a novel cellular and molecular mechanism whereby CRF modulates epilepsy.

CHAPTER I

INTRODUCTION

Epilepsy

Epilepsy is defined as "a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition" (International League Against Epilepsy (ILAE) (Fisher et al., 2005b). Seizures are complex manifestation of transient, abnormal and synchronous enhancement of neuronal excitability in response to adverse internal and external variables with different and distinct effects (Fisher et al., 2005a, Engel et al., 2006). Epilepsy is a chronic neurological disorder characterized by two or more unprovoked seizures (Duncan et al., 2006). Epileptogenesis is the sequence of events in which a normal brain transforms into a hyperexcitable epileptic neuronal circuit. Epilepsy is the third most common, multifarious and devastating neurological disorder affecting 50 million people worldwide (Hauser and Kurland, 1975, Hirtz et al., 2007, Kobau et al., 2008) and more than two million people. Epilepsy usually presents in childhood and people over 65 years age, but may occur for the first time at any age (Hauser et al., 1993, Olafsson et al., 2005). Five percent of the population suffer a single seizure at some time in their life.

Etiology of Epilepsy

Etiology is an important determinant of treatment, prognosis, and clinical course. Based on the etiology, the ILAE Commission divided epilepsies into three distinct categories (Berg et al., 2010). a. Genetic epilepsies in which seizures are the core symptom of a disorder due to a known or presumed genetic defect(s) (Berg et al., 2010). b. Structural or metabolic epilepsies which are the result of structural or metabolic condition, including acquired disorders and genetic conditions, in which there is a separate condition between the genetic defect and the epilepsy (Berg et al., 2010). c. Epilepsy of an unknown etiology, also referred to as idiopathic epilepsy, accounts for 70% of epilepsies where the cause is not currently known, but may be of genetic origin or the result of a separate, unrecognized disorder (Hauser et al., 1991, Duncan et al., 2006, Berg et al., 2010).

Seizure Classification

Based on the seizure semiology and electroencephalography (EEG), ILAE (Gastaut, 1969, Epilepsy, 1981, 1989, Luders et al., 1993, Engel, 2001) classified epileptic seizures into: 1. Partial or focal seizures, in which the abnormal electrical discharge originates from a localised epileptic focus. Partial seizures can be further subdivided into: a. simple partial seizures are the most common type of epilepsy and do not affect consciousness. b. Complex partial seizures affect consciousness. In adults, partial seizures are most the most common form accounting for 60 – 70 % of all seizures (Hauser et al., 1993, Forsgren et al., 1996, Oun et al., 2003).2. Generalized seizures involve many parts of the brain. Generalized seizures can be further subdivided into:

a. Absence seizures (petit mal) which typically occur in childhood. These seizures are distinguishable by brief lapses of consciousness lasting less than 30 seconds such as staring, blinking, rolling of the eyes, or arm movements, followed by full awareness. b. Myoclonic seizures which are rapid, shock-like jerks of a muscle or group of muscles. c. Clonic seizures which occur when several myoclonic seizures occur in succession. d. Tonic seizures which cause muscle stiffening, usually in back, legs and arms. e. Tonic-clonic seizures (grand mal) which are characterized by a stiffening of body (tonic phase) and jerking movements (clonic phase). A person sometimes loses consciousness during a tonic-clonic seizure and may also have shallow breathing and a loss of bowel/bladder control. They are the most common form of generalized seizures. f. Atonic seizures which cause an abrupt loss of normal muscle tone for seconds resulting in head nods, jaw drops or even falls. Partial seizures may spread to other parts of the brain and transform to generalized seizures. 3. Unknown seizures such as epileptic spasms and the events that are not clearly diagnosed into one of the categories above. Generalized and partial seizures occasionally may lead to continuous or recurrent seizures lasting longer than 30 minutes without full recovery of consciousness. Continuous generalized seizures, known as status epilepticus is a life-threatening condition and requires an immediate pharmacological treatment. Continuous partial seizure activity, epilepsia partialis continua, though less life threatening, if left untreated for prolonged conditions results in focal neuronal damage and generalize into status epilepticus.

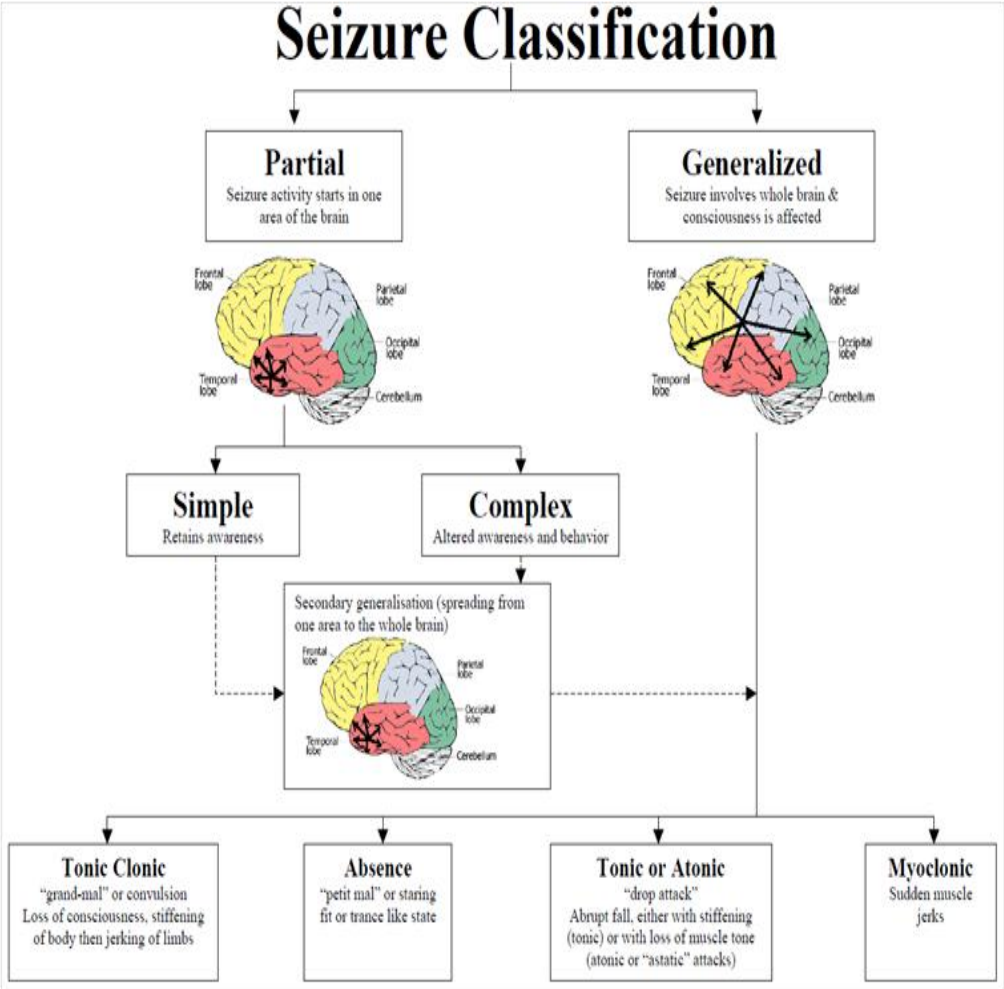


Figure 1. ILAE classification of seizures. (Adopted from: Epilepsy.org.au)

Epileptic and Non-Epileptic Events

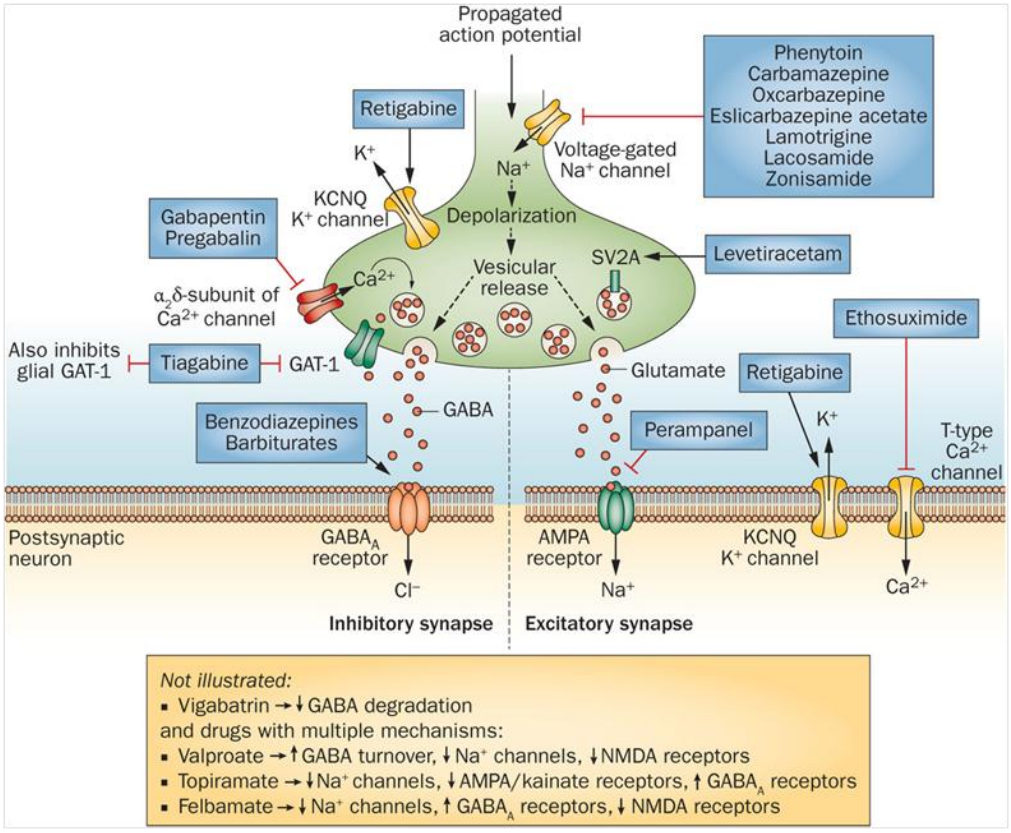
Electroencephalography (EEG) recordings in epileptic patients show sharp wave, spike, spike-and-slow wave complex and the multiple spike-and-slow-wave complex patterns which are referred to as epileptiform activity. Subclinical or interictal discharges are often observed between seizures which also exhibit the patterns of epileptiform activity. However, it must be noted that not all seizures are epileptic. For example, medical conditions such as narcolepsy, heat stroke, cardiac arrhythmia and low blood

sugar have symptoms similar to epileptic seizures, but show no abnormal electrical activity (Hauser et al., 1996). Non-epileptic events can occur in both epileptic and non-epileptic patients.

Treatment Strategies

Current treatment strategies for seizures focus exclusively on prophylaxis or seizure suppression, thus providing only a symptomatic treatment (Rogawski and Loscher, 2004) without clear influence on the cause of disease and produce various side effects (Pitkanen and Sutula, 2002). Anti-epileptic drugs (AEDs) are the most commonly used treatment strategy for epilepsy. AEDs are designed to restore the normal balance between excitation and inhibition of the neuronal network (Avoli, 1983, Mody et al., 1992). AEDs target a number of mechanisms such as: increase the inhibitory neurotransmission via gamma-aminobutyric acid (GABA), decrease the excitatory glutamatergic transmission, and reduce neuronal excitability by modulation of sodium or calcium channels (Figure 2). However, a substantial proportion of patients (~30%) do not respond to AEDs (Perucca et al., 2007) and continue to have seizures despite carefully optimized drug treatment (Regesta and Tanganelli, 1999). In patients with intractable epilepsy, other treatments may be needed. In some cases, a ketogenic diet rich in fatty acids and free from carbohydrates or a vagus nerve stimulator has been proven to be helpful. However, the effects have not always been promising (Danielsson et al., 2008). Removal of epileptic tissue can be a cure for a select population with clear epileptogenic focus. In some cases of febrile seizures, corticosteroid treatment is often preferred.

However, there is no easy cure or prophylactic regimen. Intractability to currently available anticonvulsants may indicate multiple mechanisms of seizure generation.Hence, even when one mechanism is targeted, the others still can exacerbate the disease.



Adopted from (Bialer and White, 2010, Loscher and Schmidt, 2012)

Figure 2. Clinically approved AEDs with a wide spectrum of mechanisms of action. Effects on both inhibitory (left-hand side) and excitatory (right-hand side) nerve terminals.
(AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; GABA, γ-aminobutyric acid; GAT-1, sodium- and chloride-dependent GABA transporter 1; SV2A, synaptic vesicle glycoprotein 2A).

Therefore, advances in the knowledge of the underlying mechanisms of epilepsies would allow for more rational therapeutic approaches to this challenging neurological disorder

(Perucca et al., 2007). Temporal lobe epilepsy (TLE) is one of the most prevalent intractable epilepsies in adults, and the cellular and molecular basis for the pharmacoresistance of the epilepsy has so far remained elusive (Engel, 1996).

TLE

TLE manifests as a partial seizure (Kwan and Brodie, 2000). There are many causes of TLE such as brain injuries, brain tumors, vascular malformations and developmental abnormalities. TLE can be mesial TLE (MTLE) or lateral TLE. The MTLE or limbic epilepsy is the most common form of TLE (Wiebe, 2000) and involves the interaction among neuronal networks in limbic structures such as the hippocampus, amygdala and the entorhinal cortex (EC) (Engel, 1993). The LTLE is the less common form of TLE and arises in the neocortex. In about 60% of TLE patients, seizures spread from temporal lobe to the adjacent occipital, frontal, parietal as well as to the temporal lobe on the contralateral side of the brain. This process is called secondary generalization. The result is a convulsive (grand mal) seizure. Whereas some of the TLE patients develop abnormal tissue damage such as the mesial temporal sclerosis, which is amenable to resection, the number of options is limited for those without such lesions. Therefore, unraveling additional mechanisms would allow new targets for future therapy developments not only for patients with non-surgical intractable epilepsy, but also for other types of epilepsies.

TLE and the EC

In patients with intractable seizures, seizure onset is frequently detected in the hippocampus. However, human and animal studies provide increasing evidence that other

structures of the limbic system, such as the amygdala, parts of neocortex, and the EC also play important roles. In line with these studies, the EC has been implicated in the development and propagation of limbic seizures in TLE patients (Rutecki et al., 1989, Spencer and Spencer, 1994, Bartolomei et al., 2001) and in animal models mimicking this disorder (Dreier and Heinemann, 1991, Bragdon et al., 1992, Jones et al., 1992, Heinemann et al., 1993, Avoli et al., 1996). However, the processes leading to spontaneous seizures involving the EC have not been fully determined.

Structure of the EC

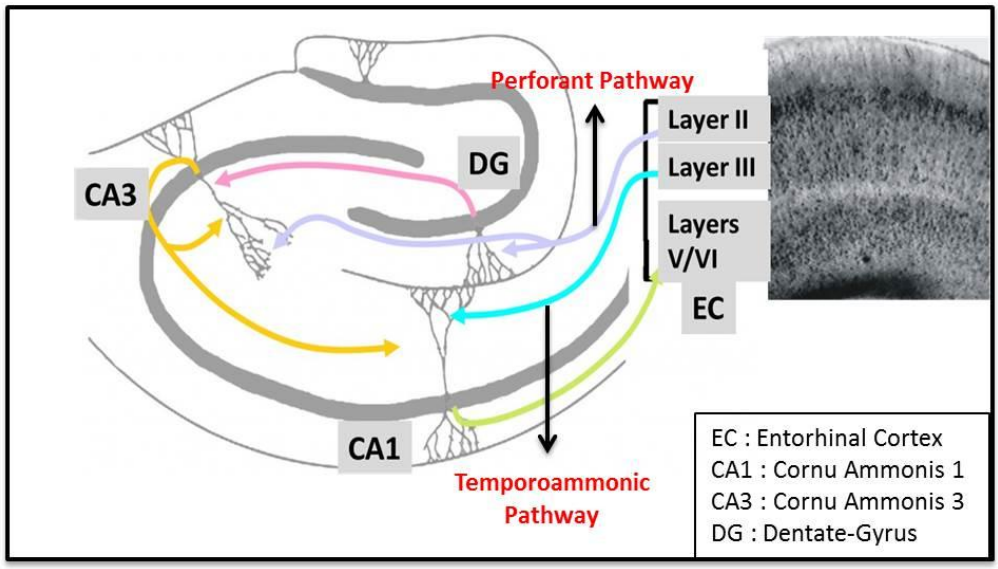
Anatomically, the EC is a six-layered pivotal structure (layers I-VI, Figure 2) (Mulders et al., 1997) integrating information from the parahippocampus, prefrontal cortex, and the frontal cortex (Apergis-Schoute et al., 2006). Hence the EC is considered the gateway mediating the majority of connections between the hippocampus and other cortical areas (Witter et al., 1989, Witter et al., 2000a, Witter et al., 2000b). Layer I is the molecular layer, which has a scarcity of cells, whereas layer IV is the cell-sparse, fiber-rich narrow layer that constitutes the lamina dissecans. Layer II is mainly made up of densely packed, large and medium sized pyramidal and stellate cells. The most abundant cell type throughout layer II in medial EC (mEC) is the stellate cells, located within the superficial and middle layer II. The soma of these cells is quite variable but their spiny dendritic tree is their defining characteristic. Layer III consists of a high density of pyramidal neurons (Dickson et al., 1997, Gloveli et al., 1997). Sensory inputs from olfactory structures, parasubiculum, perirhinal cortex, claustrum, amygdala and neurons in the deep layers of the EC (layers V–VI) converge onto the superficial layers (layer

II/III) of the EC (Witter et al., 1989, Burwell, 2000) which give rise to dense projections to the hippocampus. The axons of the stellate neurons in layer II of the EC form the perforant path that innervates the dentate gyrus and Cornu Ammonis (Steward and Scoville, 1976), whereas those of the pyramidal neurons in layer III form the temporoammonic pathway that synapses onto the distal dendrites of pyramidal neurons in CA1 and the subiculum (Steward and Scoville, 1976, Witter et al., 2000a, Witter et al., 2000b). Furthermore, neurons in the deep layers of the EC (layers V–VI) relay a large portion of hippocampal output projections back to the superficial layers of the EC (Kohler, 1986, Dolorfo and Amaral, 1998a, b, van Haeften et al., 2003) and to other cortical areas (Witter et al., 1989). Accordingly, the EC is not only involved in the induction and maintenance of TLE (Spencer and Spencer, 1994, Avoli et al., 2002), but also closely involved in the consolidation and recall of memories (Haist et al., 2001, Squire et al., 2004, Dolcos et al., 2005, Steffenach et al., 2005), Alzheimer's disease (Hyman et al., 1984, Kotzbauer et al., 2001), and schizophrenia (Falkai et al., 1988, Arnold et al., 1991, Joyal et al., 2002, Prasad et al., 2004).

Role of the EC in TLE

Clinical and animal studies show that the EC is a site of seizure initiation and maintenance. The EC is also involved in acutely induced epileptiform discharges (Walther et al., 1986, Wilson et al., 1988, Jones and Lambert, 1990, Pare et al., 1992, Stringer and Lothman, 1992, Bear and Lothman, 1993, Rafiq et al., 1993). Preferential loss of layer III neurons of the EC is seen in both human TLE (Kim et al., 1990, Du et al., 1993) as well as animal models of epilepsy (Du and Schwarcz, 1992, Du et al., 1995).

With the onset of epilepsy, CA3 neurons are lost and the Schaffer collateral (SC) pathway connecting CA3 to the CA1 pyramidal neurons is disrupted (Ben-Ari and Cossart, 2000). Under these conditions, the TA pathway has been suggested as a major



(Adopted and modified from © 2012 Neural Circuits and Memory Lab).

Figure 3. EC forms the gateway to the hippocampus and other cortical areas. Superficial layers (II, III) send sensory inputs to the hippocampus. Stellate neurons in layer II form Perforant path with DG cells and CA3 Pyramidal neurons in layer III form Temporoammonic pathway connecting CA1 and subiculum
CA1 sends it output to layers V/VI of the EC which in turn relay back to the superficial layers.

excitatory drive to the principal neurons of the CA1 region (Barbarosie et al., 2000, Avoli et al., 2002, Wu and Leung, 2003). The neurons of layer II are well spared in TLE. However, they become hyperexcitable (Bear et al., 1996) due in part to a reduction in inhibitory input (Kobayashi and Buckmaster, 2003), and proexcitatory alterations in sodium channel gating parameters (Hargus et al., 2011). Additionally, removal of the

parahippocampal gyrus along with the EC, showed better seizure management in patients with refractory epilepsy (Siegel et al., 1990, Engel, 1993).

Auras and Seizure Precipitation Factors

In some cases, epileptic seizure can be predicted. Auras are symptoms that occur in some epileptic patients before the onset of a seizure. These include a strange taste or odor or sounds, feeling of numbness or tingling, anxiety and nausea. Whereas in some epileptic patients, seizures are triggered (Berg et al., 1995). Triggering factors can be environmental or endogenous in origin and cause a transient lowering of the seizure threshold, or chemical or physiological stimulation capable of precipitating an ictal event (Aird, 1983, Nakken et al., 2005). In most cases, multiple factors are involved in setting off seizures and are rarely predictable. However, in some cases it is possible to determine what triggers the onset of seizures. In addition to determining the underlying causes of a seizure disorder, identifying and managing factors that precipitate seizures can aid in developing behavioral or lifestyle changes that can improve seizure control and the patient's need for medication. Some known potential precipitants that often encourage seizures include: alcohol consumption, alcohol withdrawal, various medical conditions such as multiple sclerosis, fever, photosensitivity, drugs, sleep deprivation, anxiety and stress.

Stress and Epilepsy

Stress is the more frequently self-reported precipitant of seizures in patients with refractory epilepsy (Frucht et al., 2000, Spector et al., 2000, Nakken et al., 2005, Haut et al., 2007, Sperling et al., 2008). Stress can be internal or environmental signals perceived

as a potential threat (Behan et al., 1995b, Cortright et al., 1995, Hsu and Hsueh, 2001, Calabrese et al., 2007). Although stress does not cause epilepsy, it can make a person more prone to seizures. Additionally, getting anxious about having seizures can add to stress and thus forms a vicious cycle of seizures and stress. In support of these clinical studies, stress pathways have been shown to promote neural activity in a variety of ways, suggesting its direct contribution to hyperexcitability resulting in spontaneous seizures. The mammalian brain is equipped with numerous sensing devices to identify stress, as well as mechanisms to respond to the stress signals (Behan et al., 1995b, McEwen, 2011). Mild or acute stress is often adaptive as it enhances functions of the hippocampus and other brain regions by augmenting synaptic plasticity, to cope with the situation if it ever occurs again (Behan et al., 1995b, Cortright et al., 1995, Joels et al., 2011). However, the same mechanisms when chronically activated render the brain more susceptible to numerous detrimental effects (Cortright et al., 1995, Joels et al., 2007, Joels et al., 2011), including seizure precipitation.

Stress Mechanisms Activate the Excitatory Neuropeptide CRF

How does the brain detect and reacts to a stressful stimulus and trigger seizures? When an individual appraises a situation as a threat, stress responses get activated (Lazarus and Folkman, 1984). Two major components are principally engaged to adopt to stress challenges; i) the sympathetic-adrenomedullary system is activated for rapid- and short-lived responses and results in increased systemic levels of norepinephrine and epinephrine, and increased levels of norepinephrine in the brain. ii) The hypothalamic-pituitary-adrenal (HPA) system is activated for delayed, sustained responses and involves

various hormones and neuropeptides (Behan et al., 1995b, Cortright et al., 1995, De Kloet et al., 1998, Calabrese et al., 2007) (for a detailed review, see (Turnbull and Rivier, 1997)). Activation of the HPA axis involves the limbic system in the brain, which is composed of the amygdala, prefrontal cortex and the hippocampus (Figure 3). The amygdala creates an emotional response in reaction to stress, while the hippocampus creates a memory of the threat to deal with it in a better way in future. Stressful or threatening stimuli pass through the amygdala and reach the paraventricular nucleus (PVN) of the hypothalamus via neurochemical pathways. The PVN then releases corticotropin releasing factor (CRF) into the adenohypophyseal portal circulation (Whitnall, 1993). CRF activates CRF receptors on anterior pituitary corticotrophs resulting in the release of adrenocorticotrophin hormone (ACTH) (Turnbull and Rivier, 1997). ACTH then signals the pituitary gland to stimulate adrenal glands to release cortisol (corticosterone in rodents) (Vale et al., 1981) Cortisol and other glucocorticoids released from the adrenal cortex, help to mobilize resources for sustained actions. These include changes such as increased blood glucose and lipid levels, increased heart rate, blood pressure as well as immune responses involving circulating T cells, B cells and lymphocytes. In the short term, these resources are useful in helping the body to deal with stressors. However, chronic activation of these mechanisms may contribute more to the development of various diseases.

Overview of the CRF System

CRF is known as the most potent epileptogenic neuropeptide. Therefore, appreciation of the circuitry affected by the CRF-system is particularly germane to

understand the effects of CRF on seizure threshold, seizure susceptibility and epileptogenesis. CRF plays a fundamental role in responding to stressors exposed to the human body (Hemley et al., 2007) and mediates both short and long term responses to stress.

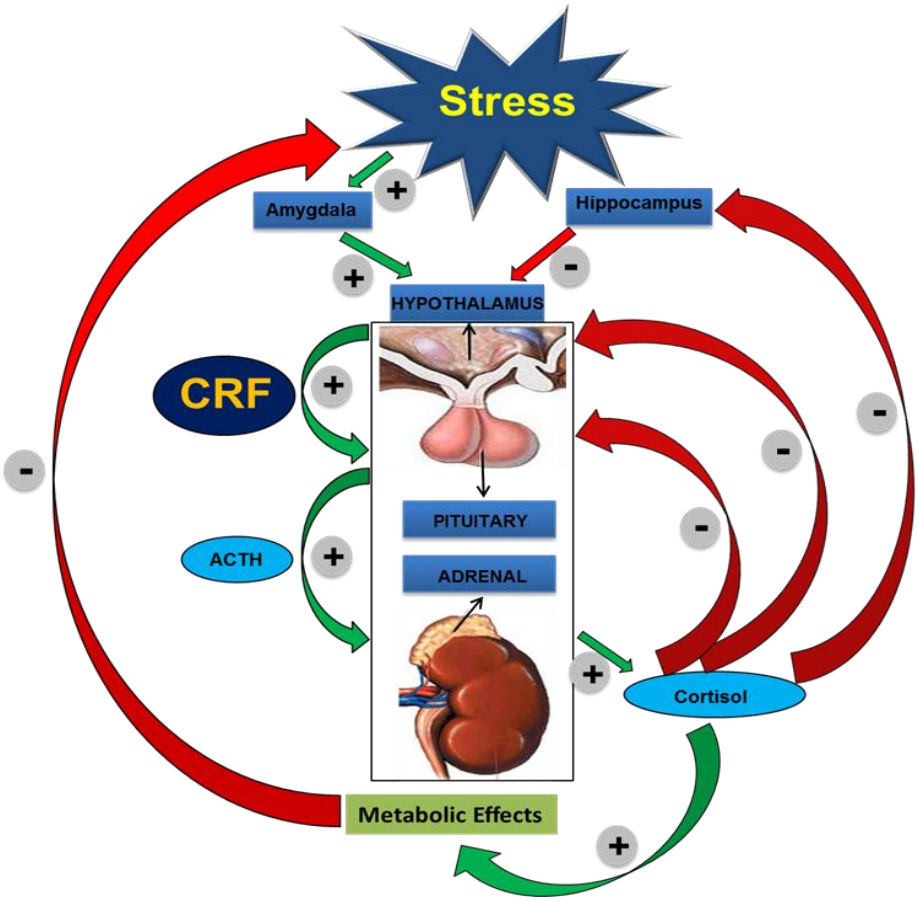


Figure 4. Schematic illustration of the HPA axis signaling.
Stress signals stimulate CRF neurosecretory cells in PVN of the hypothalamus to release CRF.
CRF then activates ACTH secretion from corticotrophs of anterior pituitary gland.
ACTH travels to adrenal glands of kidneys and stimulates release of cortisol.
To protect against prolonged activity as well as help regain homeostasis, the HPA system is carefully modulated through negative feedback exerted by cortisol at anterior pituitary, PVN and the hippocampus.
- : Inhibition, + : Activation.

Dysregulation of the CRF–system has been implicated in a myriad of "stress-related" disorders, including epilepsy (Baram and Hatalski, 1998, Hauger et al., 2006).

CRF is known to change neuronal function in a rapid and reversible manner. CRF is an excitatory neuromodulator (Dunn and Berridge, 1990). There are numerous CRF pathways outside the HPA axis (Lymangrover and Brodish, 1973) where CRF acts as central neuromodulator (Swanson et al., 1983). The CRF signaling system comprises of 4 CRF family ligands, a binding protein, and two receptors (Vale et al., 1981, Behan et al., 1995a, Steckler and Holsboer, 1999, Ryabinin et al., 2002, Fekete and Zorrilla, 2007).

CRF family ligands. CRF, urocortins (Ucn_1, Ucn_2 or stresscopin-related peptide, and Ucn_3 or stresscopin), urotensins (UTn_1, UTn_2, and UTn_3), and sauvagine (Svg) are the four ligands that belong to the CRF family.

CRF gene location and homology. CRF is found in all vertebrates (Batten et al., 1990, Bhargava and Rao, 1993) and mammals (Paull et al., 1982, Sakanaka et al., 1986, Stolp et al., 1987) including primates (Millan et al., 1986) and humans (Chan et al., 1982, Binder and Nemeroff, 2009). The CRF gene contains two exons separated by an intron that is around ~600–800 base-pair long (Furutani et al., 1983, Shibahara et al., 1983, Jingami et al., 1985, Roche et al., 1988, Morley et al., 1991, Stenzel-Poore et al., 1992b, Keegan et al., 1994, Mimmack et al., 1998) and is located on chromosome 8q13. The first exon contains mostly untranslated mRNA, while the second exon encodes the entire translated region of CRF precursor. Although CRF mRNAs differ in length in all these species, the CRF gene is highly conserved and the same gene sequence is found in almost all animals (De Souza et al. 2000). Human CRF is identical to that of rat and differs from

ovine by only seven amino acids. This homology of the CRF gene has been exploited to learn more about human CRF as well as to develop antagonists, using CRF from other species (Hemley et al., 2007).

CRF structure. CRF is a 41 amino acid polypeptide (Vale et al., 1981), cleaved from the pro-peptide at dibasic amino acids (lysine or arginine residues) (Shibahara et al., 1983, Roche et al., 1988, Morley et al., 1991, Stenzel-Poore et al., 1992b). The glycine residue in the C-terminal, Gly-Lys serves as a template for amidation. The 3D- structure of the peptide is not fully discovered. However, it is believed that CRF is made up of a defined alpha helix with an unstructured terminal end at each side, which may also form a helix when bound to a receptor. This double helical confirmation might possibly be the biologically active form of CRF (Grigoriadis et al. 2001). CRF is released at synaptic terminals upon depolarization (Smith et al. 1986) and is characterized by saturable, reversible, specific binding to its receptors (De souza et al, 1985).

CRF Distribution. CRF is ubiquitous in the mammalian central nervous system (CNS) (Swanson et al., 1983, Dautzenberg and Hauger, 2002). Apart from the PVN and lateral area of the hypothalamus, CRF immunoreactivity has been detected in various extra-hypothalamic regions such as cerebellar cortex (Cha and Foote, 1988, Arzt and Holsboer, 2006), locus coeruleus (Cha and Foote, 1988), olfactory bulb (Bassett et al., 1992) and limbic structures including the EC (Bassett et al., 1992, Park et al., 2003), amygdala (Bassett and Foote, 1992) and the hippocampus (Yan et al., 1998, Park et al., 2003). The distribution of CRF neurons in the brain is consistent with its role in endocrine, physiological and behavioral responses to stress. The identity of the CRF-

containing neurons can be either GABAergic (Primus et al., 1997, Yan et al., 1998) or glutamatergic (Cain et al., 1991, Valentino et al., 2001).

Regulation of CRF Expression. Stress adaptation involves either down-or up-regulation of CRF expression, depending on the brain regions. CRF gene transcription site involves the promoter and 5′-upstream region containing glucocorticoid response

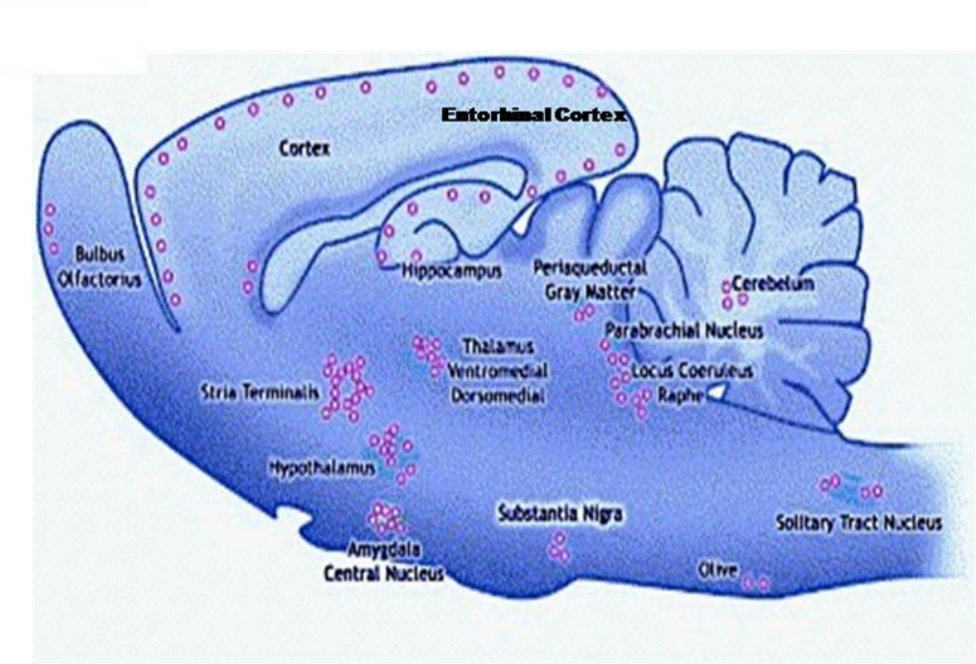


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Figure 5. Distribution of CRF mRNA.
CRF-like immunoreactivity is seen in regions including the hypothalamus, amygdala, BNST and cortex.

element (GRE) and cyclic AMP (cAMP) response elements (CRE), and Pit-1, Oct-1, Oct-2 and *Caenorhabditis elegans* Unc86(POU) transcription factor binding sites (Roche et al., 1988, Xu et al., 2001, Parham et al., 2004). In rodents, exogenous central CRF administration generates a large increase in CRF mRNA expression in the PVN (Parkes

et al., 1993). This finding is consistent with studies in which acute or chronic stress significantly increases endogenous CRF mRNA expression in hypothalamic PVN and the central nucleus of amygdala (Ma et al., 1999, Makino et al., 1999, Figueiredo et al., 2003, Shepard et al., 2005). Additionally, increases in CRF mRNA induced by acute stress are mediated via CRE-binding protein (CREB) phosphorylation by protein kinase A (PKA) (Itoi et al., 1996, Kovacs and Sawchenko, 1996). While acute stress initially triggers a rapid increase in CRF mRNA in rat PVN, chronic stress results in an increase in inducible cAMP early repressor (ICER) mRNA expression, thus preventing cAMP-dependent CRF gene transcription (Shepard et al., 2005). Failure of this regulatory mechanism could therefore be one of the major contributors for CRF hypersecretion.

Regulation of CRF release. In addition to the gene transcription mediated control, CRF release is regulated by a number of other factors such as angiotensin, vasopressin, neuropeptide-Y, substance P, atrial natriuretic peptide, activin, melanin concentrating hormone, β-endorphin, and possibly CRF itself. Whereas neurotransmitters such as acetylcholine, norepinephrine, histamine and serotonin promote CRF release, GABA inhibits the release of CRF. In addition, cytokines such as interleukin-1β, tumor necrosis factor, eicosanoids, and platelet-activating factor have been shown to activate the HPA-axis by increasing hypothalamic CRF expression.

CRF Receptors. CRF transduces neuronal and endocrine signals by binding to two G-protein coupled receptor types, CRF receptor 1 (CRF_1; also known as CRFR1and CRHR1) and CRF receptor 2 (CRF_2; also known as CRFR2 and CRHR2) (Perrin et al., 1993, Lovenberg et al., 1995b, Dautzenberg and Hauger, 2002). CRF receptors are

component of seven transmembrane α-helical proteins that belong to class II or B receptor superfamily.. Human CRF_1 and CRF_2 receptor genes are mapped to chromosomes 17q21 and 7p14, respectively (Polymeropoulos et al., 1995, Meyer et al., 1997). CRF-mediated signal transduction is triggered when CRF-type ligand binds to the interior of the helical protein core (Perrin and Vale, 1999, Hillhouse and Grammatopoulos, 2006) (De Souza, 1995, Dautzenberg and Hauger, 2002, Chatzaki et al., 2006, Fekete and Zorrilla, 2007). Distinct genes encode for CRF_1 and CRF_2 receptors, while retaining a 70% peptide sequence homology in all species. Post translational splicing results in isoforms with variants having altered intercellular or transmembrane domains (Lovenberg et al., 1995a, Zmijewski and Slominski, 2010). CRF receptors are highly conserved and are present in a wide variety of vertebrates, including but not limited to mouse (Vita et al., 1993, Kishimoto et al., 1995, Perrin et al., 1995a, Stenzel et al., 1995), rat (Chang et al., 1993, Perrin et al., 1993, Lovenberg et al., 1995a) and humans (Chen et al., 1993, Vita et al., 1993). CRF_1 and CRF_2 display distinct pharmacological profiles (De Souza, 1995, Dautzenberg and Hauger, 2002, Chatzaki et al., 2006, Fekete and Zorrilla, 2007) and are widely distributed in the extrahypothalamic circuits; with some splice variants located in specific peripheral tissues (Potter et al., 1994, Chalmers et al., 1995, Hiroi et al., 2001). Recently, a third CRF receptor subtype termed CRF_3 was cloned from catfish, which is highly homologous to CRF_1 receptor. However, no such subtype has been reported to date in humans (Arai et al., 2001).

CRF_1 receptors. CRF_1 was cloned from several species, including human, mouse, rat (Chen et al., 1993, Dieterich et al., 1997) and has no known functional genetic

polymorphism. CRF_1 is the primary endocrine transduction pathway of CRF, and is found at high densities in the anterior pituitary. In the brain, CRF_1 are expressed mainly in the medial septum, pituitary, cerebral cortex, cerebellum, hippocampus, amygdala, raphe nuclei, hindbrain, and olfactory bulb (De Souza et al., 1985, Chang et al., 1993, Primus et al., 1997, Rominger et al., 1998, Hauger et al., 2006). Peripheral CRF_1 receptors are located in adrenal gland, ovaries, testes and skin. The mRNA distribution for CRF_1 correlates well with the known distribution of CRF binding sites (Bittencourt and Sawchenko, 2000b). When expressed in cells, CRF_1 exhibits an identical *in vitro* pharmacological profile similar to that in brain and pituitary. Based on these observations, several novel CRF receptor-antagonist molecules were developed to probe the physiological significance of this receptor (McCarthy et al., 1999). CRF binds with high affinity to CRF_1 (Perrin and Vale, 1999, Hauger et al., 2003). Urocortins (Ucn), urotensins, and sauvagine also bind to CRF receptors. Among the urocortins, Ucn1 binds to CRF_1 receptors with affinity similar to CRF. Both CRF and Ucn1 can therefore be considered as endogenous ligands for CRF_1 (Hauger et al., 2003).

CRF_2 receptors. CRF_2 receptors are known to exist in 4 isoforms: $CRF_2\alpha$, $CRFR_2\alpha$-tr, $CRF_2\beta$, and $CRF_2\delta$. $CRF_2\alpha$ is a 411 amino acid protein and is about 71% identical to CRF_1 (Lovenberg et al., 1995b). $CRF_2\alpha$ is localized to subcortical regions, including lateral septum, paraventricular, ventromedial nuclei of the hypothalamus and the EC (Lovenberg et al., 1995a). $CRF_2\alpha$-tr is a novel short variant of 2α-isoform cloned from the rat amygdala (Miyata et al., 1999). $CRF_2\alpha$-tr exhibits differential brain expression and is pharmacologically related to $CRF_2\alpha$. $CRF_2\beta$ is a 431 amino acid

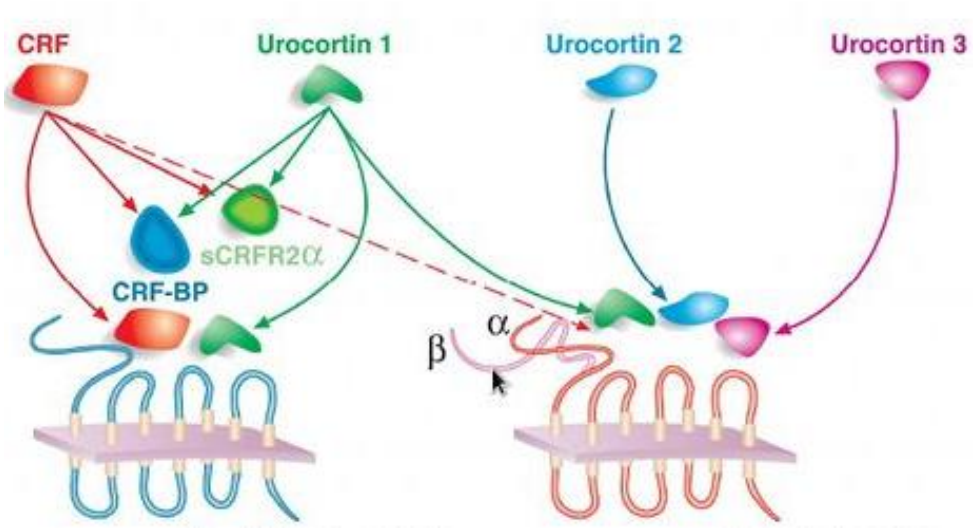
protein and differs from $CRF_2\alpha$ in that the first 34 amino acids in the N-terminal extracellular domain are replaced by 54 different amino acids (Perrin et al., 1995b). $CRF_2\beta$ is localized primarily to heart, skeletal muscle, cerebral arterioles and choroid plexus (Chalmers et al., 1995). $CRF_2\delta$ in human brain (Kostich et al., 1998) is expressed in amygdala and the hippocampus, whereas no such subtype exists in rat. The binding affinity of CRF to CRF_2 is 15 times lower than that of CRF_1 (Perrin and Vale, 1999, Hauger et al., 2003). CRF-like peptides UTn1, sauvagine, generally binds to CRF_2. More importantly, Ucn2 and Ucn3 have nearly 100 times greater affinity to CRF_2 than CRF (Hauger et al., 2003), and show little or no effect on CRF_1. Thus Ucn is hypothesized as the endogenous ligand for CRF_2 (Vaughan et al., 1995).

CRF-binding protein. CRF levels are maintained by a soluble 322 amino acid, 37 kDa glycoprotein, known as CRF-binding protein (CRF-BP). CRF-BP buffers the amount of free CRF in extracellular compartment (Jahn et al., 2005), both in periphery and in the brain. CRF-BP was first inferred from studies in pregnant humans and was thought to block the effects of CRF, to attenuate activation of the HPA axis. Later, studies established that CRF-BP is highly expressed in brain, plasma, heart, lungs, intestines, and placenta (Potter et al., 1992, Boorse and Denver, 2006, Vitoratos et al., 2006). In rats, CRF-BP expression is restricted to the brain. Immunohistochemistry and in situ hybridization studies reveal that CRF-BP is expressed in various areas of rat brain including cerebral cortex, amygdala, hippocampus, and sensory relay nuclei associated with auditory, olfactory, vestibular, and trigeminal systems (Potter et al., 1992). Thus, the differential distribution of brain CRF-BP and CRF receptors presents multiple distinct

sites of interaction with CRF (Behan et al., 1993a). CRF-BP is possibly involved in the brain maintenance of the synaptic CRF concentrations either by presynaptic uptake or by modulating the quantity of neuropeptide that activates CRF receptors at the membrane interface (Turnbull and Rivier, 1997).

In the periphery, CRF-BP binds to CRF and dimerizes to prevent CRF-binding receptor bioavailability (Behan et al., 1995a). In the brain, CRF-BP is membrane-associated (Behan et al., 1995). The interaction between CRF and CRF-BP is possibly due to maintenance of synaptic CRF concentrations either by presynaptic uptake or modulation of neuropeptide availability that activates CRF receptors (Turnbull and Rivier, 1997). The role of CRF-BP is not completely understood. However, the differential distribution of CRF-BP shows that the protein has much broader potential for buffering, inhibiting, or enhancing the effects of CRF family of peptides binding to its receptors. Furthermore, the type of effect depends on localization and concentration (Seasholtz et al., 2002), similar to the receptors. CRF-BP binds CRF and urocortins (except Ucn_3) with affinity similar to or greater than CRF receptors, and inhibits CRF-induced ACTH releasing properties of CRF receptor agonist *in vitro* in a dose-dependent fashion (Lowry et al., 1996).

CRF-mediated second messenger signaling. Both CRF_1 and CRF_2 are primarily coupled to G_s proteins resulting in activation of adenylyl cyclase (AC) and increase in levels of cAMP, which activates PKA (Dautzenberg and Hauger, 2002, Grammatopoulos and Chrousos, 2002, Hauger et al., 2006). CRF receptors also have various degrees of



Adopted from (Kuperman and Chen, 2008)

Figure 6. Schematic representation of the mammalian CRF–urocortin family of peptides, receptors and binding proteins.(Kuperman and Chen, 2008). Colored arrows indicate the receptors and binding proteins with which each ligand interacts.
Dotted arrow indicates relatively lower affinity, as compared with unbroken arrow.
CRF has relatively lower affinity for CRF_2 compared with its affinity for CRF_1. Ucn1 has approximately equal affinity for both receptors; Ucn2 and Ucn 3 are selective for CRF_2.
The signaling cascade also includes CRF-BP and the recently identified $sCRF_{2a}$. Both CRF-BP and $sCRF_{2a}$ bind to CRF and urocortin 1 with high affinity.

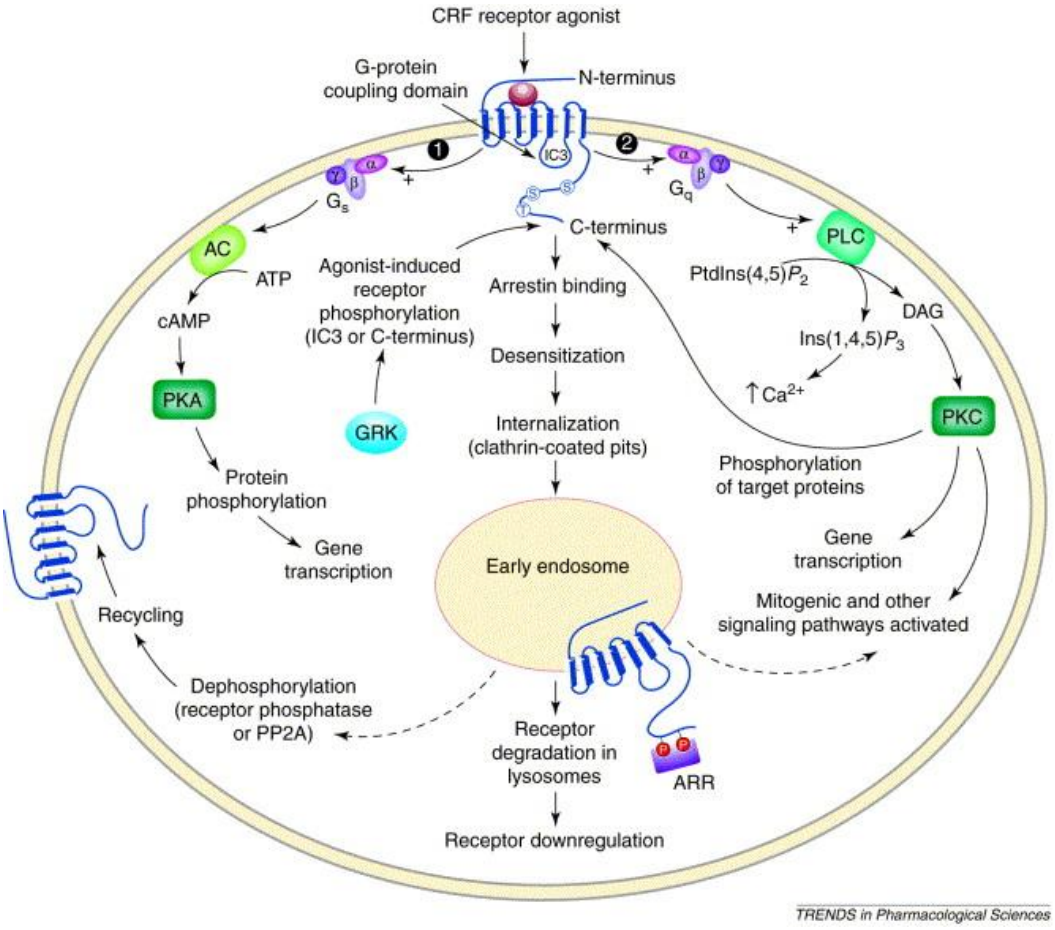
coupling competence and potency to interact with other G-protein systems including G_q, G_i, G_o, $G_{i1/2}$, and G_z (Grammatopoulos et al., 2001). Thus CRF can modulate various signaling cascades and kinases comprising of protein kinase B (PKB), protein kinase C (PKC), mitogen-activated protein (MAP) kinases and intracellular Ca^{2+} concentrations in a tissue-specific and concentration dependent manner (Dautzenberg and Hauger, 2002,

Grammatopoulos and Chrousos, 2002, Hauger et al., 2006). The biological actions of CRF are likely to be mediated by these CRF receptors and their intracellular signals.

CRF/receptor complex internalization and sensitivity. Following CRF receptor binding, G-proteins are phosphorylated and the α-subunits are dissociate from βγ to stimulate second messenger cascades. CRF receptors are then rapidly desensitized by G-protein related kinases (GRKs) via phosphorylation of serines and threonines in C-terminus (Dautzenberg et al., 2002, Kohout and Lefkowitz, 2003, Krasel et al., 2005, Moore et al., 2007, Kelly et al., 2008) and the receptor signaling is then terminated. CRF_1 and CRF_2 form complexes with β-arrestins, which are bound to clathrin and β-adaptin and form vesicles and are internalized. (Oakley et al., 2007, Markovic et al., 2008). These desensitized receptors are then dephosphorylated, resensitized by specific phosphatases and are recycled to the plasma membrane.

Functions of CRF and CRFRs. Hypothalamic CRF coordinates neuroendocrine, autonomic and behavioral responses to stress (Behan et al., 1993b, Behan et al., 1995b, Behan et al., 1996, Burrows et al., 1998a, Bale et al., 2000, Chan et al., 2000, Bale et al., 2002, Chatzaki et al., 2002, Bale and Vale, 2004, Boorse et al., 2006). The complexity of the stress-related mechanisms endows CRF with both positive as well as detrimental effects. CRF promotes survival under acute or short stressful conditions, lasting seconds to minutes, by potentiating synaptic plasticity (Behan et al., 1995b, Cortright et al., 1995, Chen et al., 1996a, Henry et al., 2005, Florio et al., 2007b, Holsboer and Ising, 2008), yet its excess and/ or dysregulation can contribute to a number of detrimental effects (Linton et al., 1990, Behan et al., 1995b, Behan et al., 1996,

Karolyi et al., 1999, Hsu and Hsueh, 2001, Lewis et al., 2001). These results are further corroborated by overexpression of CRF in forebrain which led to learning and memory defects (Linton et al., 1993).



Adopted from (Dautzenberg and Hauger, 2002)

Figure 7. CRFR signaling pathways and their regulation.
CRF_1 and CRF_2 receptors signal mainly through cAMP/PKA signaling.
CRFRs also signal via the PLC/PKA pathway.
GRK3mediated CRFR desensitization, internalization and recycling (might differ based on the specific cellular and/or neuronal background).

Hippocampal synaptic potentiation involves CRF-CRF_1 signaling. Mice lacking CRF_1 showed attenuated synaptic plasticity (Lovejoy et al., 1998), and have memory deficits (Contarino et al., 1999).

The extra hypothalamic effects of CRF within the CNS are predominantly excitatory in various brain regions such as locus ceruleus (Valentino et al., 1983), hippocampus (Siggins et al., 1985) cerebral cortex, hypothalamus and in lumbar spinal cord motor neurons (Dunn and Berridge, 1990, Owens and Nemeroff, 1991). CRF modifies sensory stimulus (Valentino et al., 1983), induces behavioral excitation, stimulating activities such as rearing and grooming (Siggins et al., 1985, Britton et al., 1986).CRF has inhibitory actions in the lateral septum, thalamus, and hypothalamic PVN (Dunn and Berridge, 1990, Owens and Nemeroff, 1991). CRF increases GABA release via CRF_1 in rat amygdala and hypothalamic slices (Bagosi et al., 2008, Bagosi et al., 2012). CRF increases vigilance and decreases slow-wave sleep at doses below those affecting locomotor activity or pituitary-adrenal function and higher doses can be epileptogenic (Ehlers et al., 1983). CRF-mediates increase of presynaptic glutamate release (Lovenberg et al., 1995a), as well as enhanced postsynaptic excitability which is potentially related to CRF-induced suppression of after-hyperpolarization (Aldenhoff et al., 1983). CRF released by IL-1 (Berkenbosch et al., 1987, Sapolsky et al., 1987, Busbridge et al., 1989) mediates fever, thermogenesis, and ACTH release (Naitoh et al., 1988, Busbridge et al., 1989). CRF in the leukocytes may also induce ACTH and endorphin release (Blalock, 1989) which are responsible for natural killer activity (Irwin

et al., 1988) and inflammatory responses. CRF family is also a novel angiogenic regulator in endogenous and inflammatory conditions (Im et al., 2010).

To help determine the role of CRFRs mediating various biologic actions of CRF, a number of selective peptide agonists and antagonists were discovered and developed, based on the receptor differential ligand affinities. For example, Ucn III, has a higher affinity to CRF_2 than CRF_1 and is commonly used as a selective CRF_2 agonist (Hsu and Hsueh, 2001, Lewis et al., 2001). Similarly, ovine CRF is often used as a CRF_1 specific agonist as its affinity to CRF_1 is two orders greater than CRF_2 (Ruhmann et al., 1998a). Through mutation and artificial structural constraints, receptor-specific peptide agonists and antagonists were developed (Ruhmann et al., 1998a, Rivier et al., 2002b, Tezval et al., 2004, Rivier et al., 2007). For example antalarmin (ANT), and anti-sauvagine-30 specifically binds CRF_1 and CRF_2 respectively (Chen et al., 1996b, Schulz et al., 1996, Webster et al., 1996, Holsboer and Ising, 2008).

Stress responses in general, are mediated by the activation of CRF_1 receptors (Owens and Nemeroff, 1991, Chen et al., 1996b, Webster et al., 1996, Chen et al., 1997, Smith et al., 1998), whereas CRF_2 receptors might be involved in the initiation of secondary-stress responses to regain homeostasis (see review (Bale and Vale, 2004, Gysling, 2004). These differential roles may in part be attributed to the activation of the distinct central sources of CRF (Bagosi et al., 2008, Bagosi et al., 2012).Central administration of CRF have anorexic effects (Vaughan et al., 1995, Spina et al., 1996, Smagin et al., 1998) and peripheral administration has been shown to slow gastric emptying and decrease food intake (Asakawa et al., 1999, Nozu et al., 1999).These

effects are mediated by CRF_2 receptors. Activation of CRF_1 receptors in the brain can suppress feeding independently of CRF_2 receptor-mediated mechanisms and independent time-courses (Hotta et al., 1999, Reyes et al., 2001, Inoue et al., 2003). Stress stimulates colonic motility via CRF_1 receptor activation and does not involve CRF_2 receptors (Tache et al., 2001, Martinez et al., 2002, Tache et al., 2002). CRF_2 receptors are predominantly involved in stress related feeding behavior (Keck et al., 2005), energy balance (Bakshi et al., 2002), altering glucose metabolism and decreasing insulin sensitivity in skeletal muscle, altering pancreatic β-cell (Bale et al., 2003, Chen et al., 2006, Li et al., 2007) and cardiovascular function (Hashimoto et al., 2004, Boonprasert et al., 2008). CRFRs play differential roles in mediating gastrointestinal regulation following stress (Stengel and Tache, 2010). While CRF_2 inhibits gastric emptying and small intestine motility to slow digestion, CRF_1 increases colonic motility. In some stress related responses such as stress-induced relapse of drug seeking and stress-mediated inhibition of reproduction, both the CRFRs may contribute to an observed response (Sarnyai et al., 2001, Li et al., 2006, Kalantaridou et al., 2007, Wise and Morales, 2010).

The role of CRF and CRFRs is further elucidated by various genetically engineered mouse models. a. CRF deletion mutant –CRF-knock-out (CRF KO): CRF KO mice are phenotypically normal and display behavioral responses similar to the control mice (Muglia et al., 1995, Dunn and Swiergiel, 1999, Weninger et al., 1999), have low levels of basal plasma concentrations of CORT. (Muglia et al., 1995). In both the WT and KO, stress-induced behaviors were attenuated by CRF_1 antagonists. CRF KO mice generated from heterozygotes show adrenal insufficiency and those from the homozygous

mating died within 24 hours due to improper lung function caused by glucocorticoid deficiency. b. CRF overexpressing (CRF-OE) mice: Constitutive CRF-OE resulted in elevated plasma CORT, ACTH, altered HPA axis and Cushingnoid phenotypes (Stenzel-Poore et al., 1992a, Dirks et al., 2002, Lu et al., 2008); reduced locomotor activity and increased anxiety and stress (Stenzel-Poore et al., 1992a, Dedic et al., 2012), reduced sensitivity to the anxiolytic effects of CRF_1 antagonists, GABAA and glutamate receptor agonists in response to stress-induced hyperthermia (Vinkers et al., 2012).

To explore the functional significance of CRFR binding sites, single and double mutant mice were generated by knocking out CRFRs (Bale et al., 2002) Koob 2001. a. CRF_1 mutant mice: CRF_1 null mutants are normal and fertile when born from heterozygotes. However, progeny from homozygous female died within two days after birth due to lung dysplasia. The CRF_1 mutant mice have low plasma concentration of CORT and showed reduced anxiogenic-like responses compared to the control littermates (Smith et al., 1998, Contarino et al., 1999). Diminished neuronal activity and reduced anxiety-like behaviors were also observed in conditional CRF_1 KOs, including the neuronal circuitries of the anterior forebrain and limbic system, indicating that these behaviors are independent of the HPA axis (Muller et al., 2003, Nguyen et al., 2006). b. CRF_2 mutant mice: These mice exhibit normal fertility with no gross abnormality. These mice show increased anxiety-like behavior, hypertension, increased blood vessel density and are hypersensitive to the HPA axis mediated stress responses (Bale et al., 2000). CRF_2 KO mice show attenuation of stress-coping behaviors and a reduced duration of Ucn-1 induced anorexia. c. Double mutant mice: Mice with the CRF_1 and CRF_2

receptors knocked out show decreased basal CORT and ACTH, increased PVN, CRF and AVP. These double mutants also show an altered reactivity of the HPA axis (Preil et al., 2001, Bale et al., 2002), as well as gender differences in exploratory emotionality and non-genomic transmission of stress-coping traits from mothers to male offspring (Bale et al., 2002). These results corroborate the pharmacological evidence in rats thus supporting a role of endogenous CRF family peptides and receptors in regulation of homeostasis.

Functional roles of CRF-BP. Multiple functional roles have been suggested for CRF-BP, which are controlled by the specific cellular or physiological context (Seasholtz et al., 2002, Westphal and Seasholtz, 2006). CRF-BP can have an inhibitory role on the activity of CRF or Ucn1 by sequestration from the receptors, or by mediating clearance or degradation of the complex. This has been supported by the studies which show that CRF-BP binds to placental CRF and prevents the inappropriate activation of CRF_1 expressing pituitary corticotropes, during pregnancy (Linton et al., 1990, Florio et al., 2007a). Furthermore, CRF binding might trigger the clearance of CRF: CRF-BP complex when levels of CRF increase during late pregnancy or through CRF injection and thus decrease the plasma CRF levels (Linton et al., 1990, Woods et al., 1994). The inhibitory role of CRF-BP is supported by in vitro cell culture assays in which pre-incubation of the CRF with mouse or human CRF-BP showed a reduced CRF_1-mediated ACTH release (Potter et al., 1991, Cortright et al., 1995) and cAMP (Boorse et al., 2006). The inhibitory role of CRF-BP is further confirmed by CRF-BP mutant mouse models (for review see (Seasholtz et al., 2002). CRF-BP-overexpression in the anterior pituitary show unaltered concentrations of ACTH, but increased CRF and arginine-vasopressin levels, suggesting

a compensatory role of the hormones (Burrows et al., 1998b). When CRF-BP transgene was overexpressed in the brain, pituitary, kidney, heart, kidney, spleen, lung, adrenals, and liver, CRF-BP accumulated in plasma similar to that of humans (Lovejoy et al., 1998). An increased inhibition of CRF or UCN activity was suggested in these animals due to impaired stress response to lipopolysaccharide injection and increased weight gain (Spina et al., 1996). In CRF-BP deficient mouse, CORT and ACTH were normal. However, these mice showed a decreased food intake and weight gain, increased anxiety-like behavior in elevated plus maze and defense withdrawal tests were exhibited by male animals (Karolyi et al., 1999).

In contrast, studies support that CRF-BP could enhance or prolong ligand activity by increasing its half-life and delivering it to the receptors. CRF potentiates N-methyl-D-asparate (NMDA) receptor-mediated synaptic transmission and induced cocaine-seeking behavior in VTA, via CRF_2, and these effects were blocked by CRF-BP-specific antagonist, CRF6-33 (Ungless et al., 2003, Wise and Morales, 2010). While CRF-BP exhibit an enhancing effect involving CRF_2 signaling, and all the studies demonstrating an inhibitory role for CRF-BP involved $CRF_{1,}$ these differential effects of CRF-BP on CRF at these two receptors requires further investigation. In addition to the above roles, CRF-BP could also mediate ligand or receptor-independent activity via interactions with other unknown receptors or binding proteins (Chan et al., 2000).

Role and Relevance of CRF in Epilepsy

CRF is known to change the neuronal function in a rapid and reversible manner. CRF has been implicated in a variety of neurological diseases including affective

disorders and epilepsy (Baram and Hatalski, 1998, Hauger et al., 2006). CRF is highly expressed in brain regions associated with developmental seizures, such as the hippocampus and amygdala (Gray and Bingaman, 1996). For example, intracerebroventricular injection of CRF induces seizures (Ehlers et al., 1983, Weiss et al., 1986a, Marrosu et al., 1987, Marrosu et al., 1988) and seizures alter the expression of CRF (Greenwood et al., 1997, Smith et al., 1997, Takahashi et al., 1997, Piekut and Phipps, 1998, Jinde et al., 1999, Wang et al., 2001b), CRF-BP (Smith et al., 1997, Wang et al., 2001b, Park et al., 2003) and CRF receptors (Wang et al., 2001b, An et al., 2003, Park et al., 2003) supporting the notion that CRF is the most potent epileptogenic peptide (Baram and Hatalski, 1998). CRF immunoreactivity has been detected in the cortex (Cha and Foote, 1988, Arzt and Holsboer, 2006), locus coeruleus (Cha and Foote, 1988), olfactory bulb (Bassett et al., 1992) and the limbic structures including the EC (Bassett et al., 1992, Park et al., 2003), hippocampus (Yan et al., 1998, Park et al., 2003) and amygdala (Bassett and Foote, 1992). CRF may also contribute to seizure-related neuronal loss (Ribak and Baram, 1996). Additionally, picomolar amounts of CRF have been shown to induce prolonged limbic seizures involving amygdala and the hippocampus (Baram et al., 1992, Baram and Hatalski, 1998). The proconvulsant effects of CRF are largely age-specific with infants being more susceptible to seizures than adults (Baram and Schultz, 1991a).

Despite a tremendous volume of descriptive work that supports the involvement of CRF in stress-mediated epileptogenesis; several essential issues regarding the roles of CRF in epilepsy have not been addressed. For example, intracerebroventricular

Table 1. Terminology and definitions.

Terminology	**Definition**
Epilepsy	Brain disorder characterized by uncontrolled, excessive, synchronous neuronal activity resulting in spontaneous, recurrent seizures
Seizure	Clinical condition associated with a transient hyper-synchronous neuronal discharge
Epileptogenesis	Complex process, which alters a normal brain circuit into hyperexcitable network, leading to spontaneous, recurrent seizures. (Clark and Wilson 1999)
Epileptiform-activity	The spike waves, sharp waves, spike and wave activity, or other rhythmic waveforms that may be associated with epilepsy
Homeostasis	The property of a system that regulates its internal environment and tends to maintain a stable, relatively constant condition of properties such as temperature or pH
ictogenesis	generation of epileptic seizures
Seizure-precipitant	Any stimuli that can precipitate a seizure in a person with or without epilepsy
Stress	Any disruption of homeostatic balance (Robert Sapolsky)
Stressor	A specific threat to the body
Stress response	Attempt of the body to deal with the stressor
Stress hormones	Mediators which address the specific aspects of a stressor
Inter-ictal state	Interval between seizures or convulsions
Post-ictal state	Altered state of consciousness after a seizure
Status-epilepticus	A state of continuous seizure activity
POU proteins	Eukaryotic transcription factors containing a bipartite DNA binding domain referred to as the POU domain. The acronym POU is derived from the names of three transcription factors, the pituitary-specific Pit-1, the octamer-binding proteins Oct-1 and Oct-2, and the neural Unc-86 from Caenorhabditis elegans (Clerc et al., 1988, Finney et al., 1988, Ingraham et al., 1988, Sturm et al., 1988, Herr, 1998) .

application of CRF can influence almost all brain regions. However, this raises several important questions; 1) what is the action site in the brain for the effects of CRF on epilepsy? 2) Which type of CRF receptors is involved in CRF-mediated facilitation of epilepsy? 3) What are the signaling molecules required for CRF-mediated facilitation of epilepsy? Further understanding of these mechanisms will advance our knowledge about CRF-mediated integration of the synaptic signals, the firing rates of neurons, and consequently, the input to the hippocampus via TA pathway as well as to other cortical regions.

Ion channels modulating epileptiform activity and neuronal excitability

Neuronal hyperexcitability plays an important role in epileptogenesis. Neuronal excitability is regulated by a number of active membrane properties such as voltage-gated or ligand-gated ion channels, passive membrane properties such as resting membrane potential, resistance, capacitance etc. An increase in action potential (AP) frequency enhances neuropeptide release.

Hyperpolarization Activated Cyclic Nucleotide Channels and the Cation Current, I_h

Changes in the expression and function of ion channels, particularly the hyperpolarization activated cyclic nucleotide (HCN) channels (Chen et al., 2001, Wang et al., 2001a, Bender et al., 2003, Powell et al., 2008, Marcelin et al., 2009) are known to be mechanistically linked to epilepsy. Therefore better understanding of the mechanisms that lead to abnormal activity is important as it provides molecular targets for intervention in the pathological disease process.

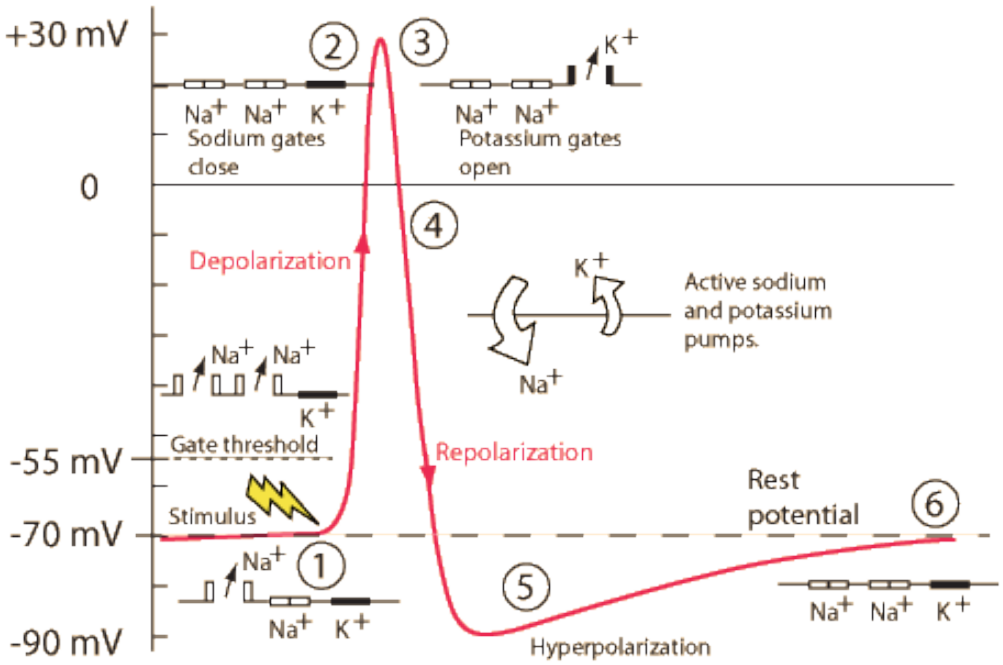


Figure 8. Schematic representation of AP firing of a neuron.
1. A stimulus causes the Na^+ channels to open. Once the action threshold is reached, more Na^+ channels open. Influx of Na^+ causes depolarization, as the cell membrane becomes more positive.
2. The Na^+ channels close and the K^+ channels open. Due to slower kinetics of the K^+ channels, the depolarization takes longer to be completed. This results in opening of both Na^+ and K^+ channels at the same time. Thus the membrane becomes neutral preventing another AP.
3. With the K^+ channels open, the membrane begins to repolarize back toward its rest potential
4. The repolarization typically overshoots the rest potential to about -90 mV. This is called hyperpolarization which prevents the neuron from AP firing, especially in the opposite direction. assureing the signal is proceeding in one direction.
5. After hyperpolarization, the Na^+/K^+ pump brings the membrane back to its resting state of -70 mV. (Adopted from (K. X. Charand;2002)).

HCN channels are cation channels and contain six-transmembrane helices (S1-S6). HCN channels generate inward cationic currents designated as I_h, when the membrane potential is hyperpolarized (Hofmann et al., 2005, Wahl-Schott and Biel,

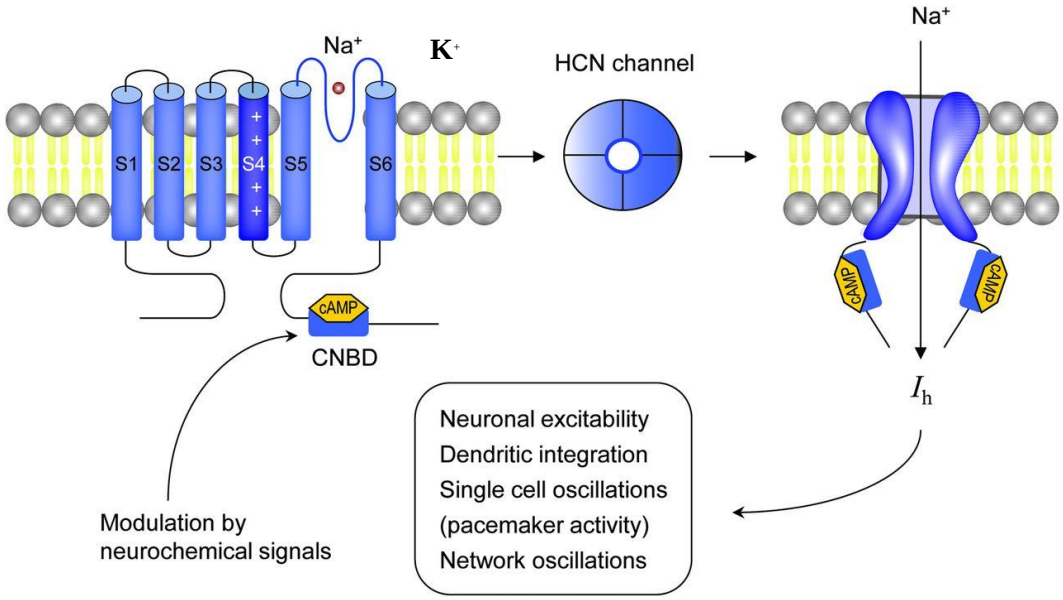
2009) and de-activates on depolarization (Robinson and Siegelbaum, 2003). I_h produces a depolarizing current carried by Na^+ and K^+ ions. I_h is critically involved in the maintenance of the neuronal resting membrane potential, passive membrane properties, pacemaker activity, rebound burst firing in heart and brain, reduction of dendritic summation, as well as governing neuronal network responses (Robinson and Siegelbaum, 2003); Accilli et al. 2002; Robinson & Siegelbaum, 2003; S**a**ntoro & Baram 2003; Poolos, 2004; Maccaferri and McBain 1996; Luthi and McCormick 1998; Magee 199; Santoro and Baram 2003; Biel et al 2009; Hofmann et al 2005).

Its presence in stellate cells was initially established from the observation of a sag-like response during injection of negative current steps (Alonso and Llinas, 1989, Jones, 1994). Subsequent voltage-clamp experiments have investigated the kinetics, voltage dependence, and pharmacology of I_h in stellate cells (Richter et al., 1997, Dickson et al., 2000, Richter et al., 2000, Nolan et al., 2007, Giocomo and Hasselmo, 2008).

HCN channels are regulated at several levels by intracellular signaling cascades including but not limited to cAMP, PIP_2, TRIP8b, as well as voltage-gated potassium channels. HCN channels assemble in tetramers HCN1, HCN2, HCN3, HCN4 (Hofmann et al. 2005) which are expressed heterologously. They are non-selective cation channels that are distributed differentially throughout the brain (Luthi and McCormick 1998) and exhibit developmental expression patters unique to the HCN isoforms with distinct I_h. For example, kinetics of HCN1>HCN2 or HCN4, whereas in terms of sensitivity to cAMP HCN1< HCN2 or HCN4. The S4 segment of the channel is positively charged and

serves as a voltage sensor, while the C terminus contains a cyclic nucleotide binding domain that confers regulation by the cyclic nucleotides cGMP and cAMP, which stimulates by direct interaction with the HCN channel protein and not by protein phosphorylation (Hofmann et al. 2005). HCN channels are blocked by ZD7288 (Richter et al., 1997, Dickson et al., 2000, Nolan et al., 2007) and Cs^+ (Alonso and Llinas, 1989, Klink and Alonso, 1993, Dickson et al., 2000, Richter et al., 2000). I_h in stellate cells, but not mEC pyramidal cells, can be activated by the cAMP analog 8-Bromo-c-AMP (Richter et al., 2000). Consistent with these electrophysiological data, gene expression data from the Allen Brain Atlas indicates that mRNA levels of HCN1 and HCN2 are particularly high in layer II of the mEC. Antibody labeling also suggests strong HCN1 expression in superficial layers of the mEC (Notomi and Shigemoto, 2004, Nolan et al., 2007), whilst HCN2 and HCN3 show moderate expression (Notomi and Shigemoto, 2004). However, antibody labeling could reflect HCN1 channels expressed in the dendrites of pyramidal cells with somata in layers III and V (Shah et al., 2004, Rosenkranz and Johnston, 2006). Studies demonstrate that a reduced I_h and changes in HCN channel expression are observed in epileptic patients and animal models of epilepsy. HCN subunits can co-associate and form heterologous systems (Much et al., 2003). In the hippocampal tissue, heteromerization is markedly enhanced by seizures resulting in altered properties of I_h, significantly enhancing network excitability (Chen et al 2001a; Simeone et al. 2005). I_h depolarizes the resting membrane potential (Biel et al 2009; Hofmann et al 2005; Robinson & Sielgelbaum 2003) and hence a decline in I_h might be expected to reduce excitability. However, a decrease in I_h was accompanied

after enhanced neuronal excitability during kainate-induced MTLE in the EC layer III (Shah et al. 2004) and in the hippocampus of pilocarpine-epileptic animals (Jung et al., 2007, Marcelin et al., 2009)**.** This inhibition of I_h has been suggested to enhance pyramidal cell dendrite excitability by increasing the availability of Ca^{2+} channels (Tsay et al. 2007), as well as by amplifying the membrane resistance (Magee 1998; Stuart and Sprutson 1998).



Adopted from: Eduardo E. Benarroch, Neurology 2013;80;304-310 (Benarroch, 2013)

Figure 9. Structure and functions of hyperpolarization-activated cyclic nucleotide-gated channels.
The four subunits of HCN channels form the central pore.
Each subunit consists of a six-transmembrane core and the cytosolic amino (N)-terminal and carboxy (C)-terminal domains.
The proximal portion of the cytosolic C-terminal domain contains the cyclic nucleotide-binding domain (CNBD), which mediates modulation by cyclic nucleotides, such as cAMP.
The HCN channels are permeable to Na^+ and K^+ and conduct a mixed cation current,I_h.

I_h blockers, such as ZD7288 and CsCl have been shown to decrease electrically induced paroxysmal discharges in vivo, suggesting antiepileptic effects for compounds that decrease the I_h (Kitayama et al. 2003).

CRF and I_h

CRF can act on a number of ionic conductances including IK(Ca) (Aldenhoff et al., 1983). IKir (Kuryshev et al., 1997) and I_h (Qiu et al., 2005b). CRF augments I_h, and neuronal firing in the VTA dopamine neurons (Wanat et al., 2008).

Overall Hypothesis, Approach and Outcomes

Abundant evidence suggests that CRF is a potent epileptogenic neuropeptide and has been reported to facilitate epilepsy in various other regions of the brain, such as the hippocampus, amygdala etc. In this regard, CRF mRNA was observed in the EC (Lovenberg et al., 1995b), but the role and relevance of CRF and its receptors in the EC is not well established. In other brain regions CRF can increase neuronal firing rate through activation of the cAMP-PKA pathway (Aldenhoff et al., 1983, Haug and Storm, 2000, Jedema and Grace, 2004), and can act on a number of ionic conductances including, but not limited to I_h (Qiu et al 2005). Since the EC is an important structure involved in TLE and mRNA of CRF receptors have been detected in the EC by in situ hybridization (Lovenberg et al., 1995b), we hypothesized that CRF would directly interact with its receptors and facilitates epileptiform activity via modulation of the HCN channels, and I_h and cAMP/PKA signaling mechanisms.

Specific Aims

The work presented in this dissertation was conducted to extend the current knowledge on the role and relevance of CRF in facilitating epilepsy. The main objective of this project was to determine the role of CRF in facilitating epileptiform activity in the EC using the well-established picrotoxin (PTX)-slice seizure model of epilepsy. The specific aims were:

- To investigate the role of CRF in facilitating epileptiform activity in the EC.
- To determine the signaling mechanisms involved in CRF-mediated facilitation of epileptiform activity.
- To determine the role of HCN channels in CRF-mediated facilitation of epileptiform activity.
- To determine the signaling mechanisms involved in CRF-mediated modulation of HCN channels and I_h current.
- To determine the role of CRF in modulating neuronal excitability.

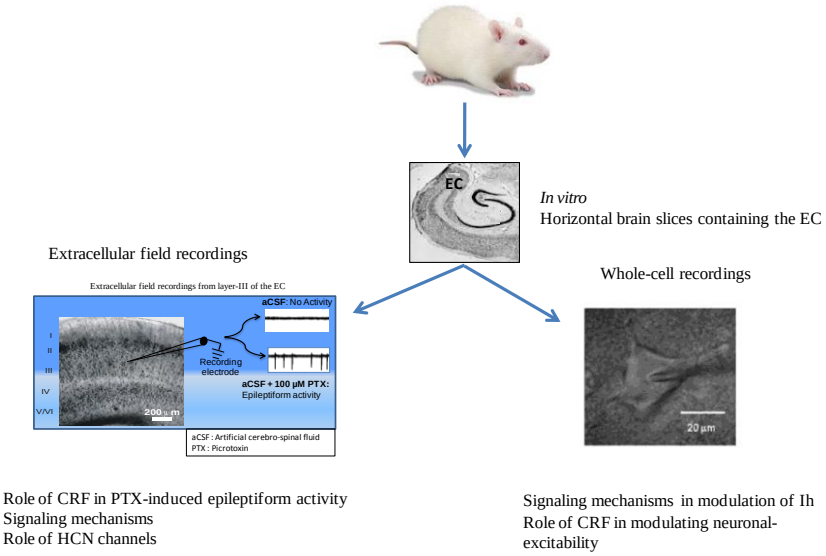


Figure 10. Summary of specific aims.

To test our central hypothesis, we first examined the presence of CRF and its receptors in the EC using immunocytochemistry and western blot techniques. We found that the EC expresses CRF and CRF_2 receptors. However, no detectable CRF_1 was found. Using extracellular field recordings, we examined the effects of CRF on PTX-induced epileptiform activity recorded from the entorhinal slices. The PTX-induced seizure model resembles the simple partial and generalized forms of human epilepsy (Fisher, 1989, Sierra-Paredes and Sierra-Marcuno, 1996, Sarkisian, 2001). PTX is a $GABA_A$ receptor antagonist and blocks the chloride channels thus acting as a convulsant.

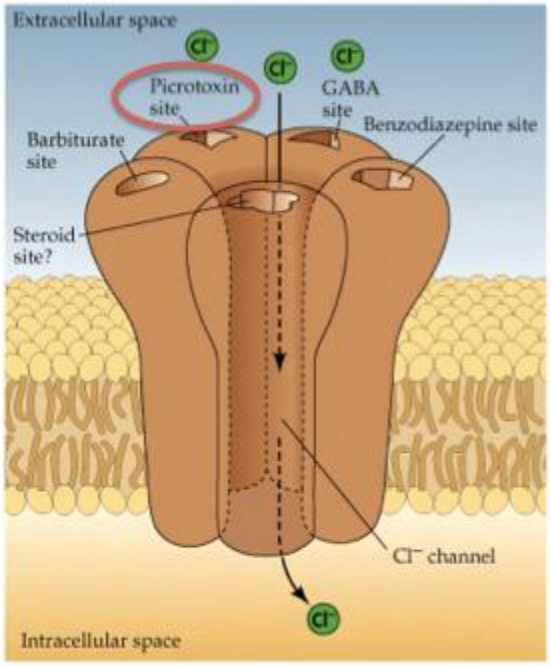


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Figure 11. Schematic representation of the GABAA receptor complex. Five subunits form a transmembrane chloride-gated pore. The receptor complex has binding sites for GABA, Benzodiapene, Barbiturates and Picrotoxin.

Furthermore, clinical studies (Du et al., 1993) as well as experimental models of TLE supported the preferential loss of layer III pyramidal neurons in the mEC (Schwob

et al., 1980, Clifford et al., 1987, Du et al., 1995, Ribak et al., 1998, Jutila et al., 2001, Bartolomei et al., 2005). Hence, we studied the role of CRF in facilitating the PTX-induced epileptiform activity in layer III of the mEC. Our results demonstrate that CRF facilitated the induction of epileptiform activity in the presence of subthreshold concentration of PTX which normally would not elicit epileptiform activity. Bath application of the inhibitor for CRF-BPs, CRF6-33, also increased the frequency of PTX-induced epileptiform activity suggesting that endogenously released CRF is involved in epileptogenesis. CRF-induced facilitation of epileptiform activity was mediated via CRF_2 receptors because pharmacological antagonism and knockout of CRF_2 receptors blocked the facilitatory effects of CRF on epileptiform activity. Application of the AC inhibitors blocked CRF-mediated facilitation of epileptiform activity and elevation of intracellular cAMP level by application of the AC activators or phosphodiesterase inhibitor increased the frequency of PTX-induced epileptiform activity. These data demonstrate that CRF-induced increases in epileptiform activity are mediated by an increase in intracellular cAMP. However, application of selective PKA inhibitors reduced, but not completely block CRF-induced enhancement of epileptiform activity suggesting that PKA is only partially required.

Since layer II is spared and becomes hyperexcitable in various experimental models of epilepsy and HCN channels are highly expressed in layer II stellate neurons, we examined the role of HCN channels in CRF-mediated facilitation of epileptiform activity. Application of ZD 7288, a blocker of the HCN channels, significantly reduced the frequency of epileptiform activity but increased the numbers of the synchronizing events within single epileptiform activity and the duration of individual epileptiform-

activity. In the presence of ZD 7288, CRF failed to increase the frequency of epileptiform activity but still augmented the numbers of synchronizing events and duration in an epileptiform activity suggesting that part of the effects of CRF on epilepsy is mediated via HCN channels. CRF increased I_h currents recorded from layer II stellate neurons via activation of CRF_2 receptors. cAMP, not PKA was responsible for CRF-mediated facilitation of I_h. Our results provided a cellular mechanism to explain the effects of CRF in epilepsy. Furthermore, at the cellular level, CRF depolarized the membrane potential and increased action potential firing rate of the stellate neurons thus resulting in facilitation of epileptiform activity in the EC.

CHAPTER II

MATERIALS AND METHODS

Slice Preparation

Horizontal brain slices (350 μm) including the EC, subiculum and hippocampus were cut using a vibrating blade microtome (VT1000S; Leica, Wetzlar, Germany) from Sprague-Dawley rats (13- to 18-day-old), wild-type (WT) and CRF_2 knockout (KO) mice (1 month) as described previously (Xiao et al., 2009b, Deng et al., 2010b, Wang et al., 2011, Wang et al., 2012). After being deeply anesthetized with isoflurane, animals were decapitated and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 5.0 $MgCl_2$, and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4). Slices were initially incubated in the above solution at 35°C for 40 min for recovery and then kept at room temperature (~24°C) until use.

Recordings of Epileptiform Activity from the Entorhinal Slices

Slices were bathed in the extracellular solution comprised (in mM) 130 NaCl, 24 $NaHCO_3$, 5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4). Spontaneous epileptiform activity was induced by including the $GABA_A$ receptor blocker PTX (100 μM) in the preceding extracellular solution (Deng et al., 2006, Wang et al., 2013). An electrode containing the extracellular solution was placed in layer III of the EC to record epileptiform activity. After stable spontaneous epileptiform activity occurred, which

usually took ~20 min, CRF was applied in the bath. The epileptiform events were initially recorded by Clampex 9.2 and subsequently analyzed by Mini Analysis 6.0.1.

Extracellular field recordings from layer-III of the EC

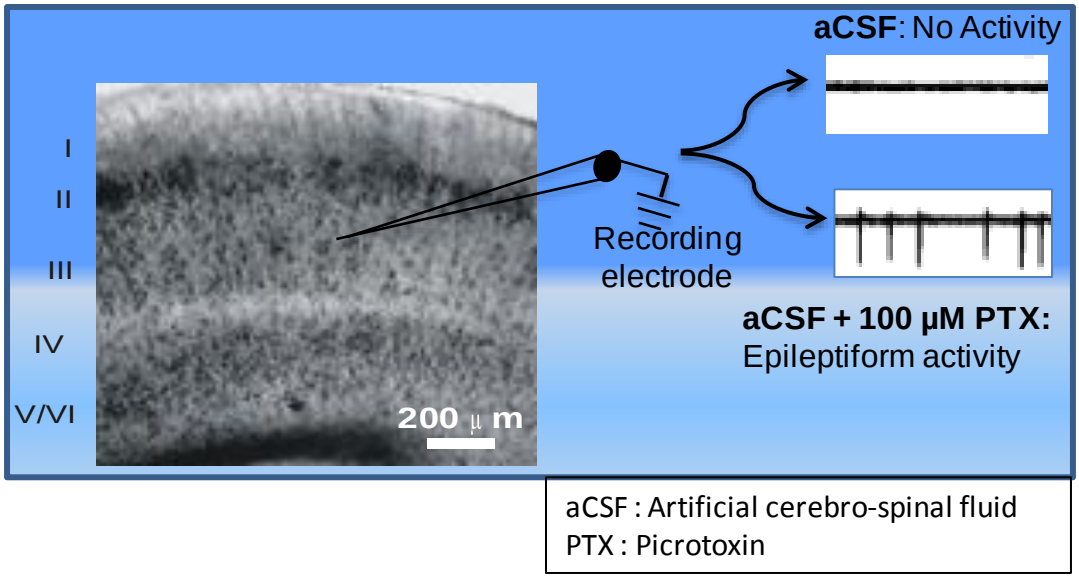

Figure 12. Extracellular field recordings from layer III of the EC.
Recordings were made from layer III of the EC. aCSF produced no epileptiform activity.
Whereas, addition of 100 µM PTX to the aCSF induced epileptiform activity in the brain slices.

Whole-Cell Recordings

Whole-cell recording is a well established and most commonly used patch clamp technique. Whole-cell configuration offers lower resistance and complete exchange between molecules in the pipette solution and the cytoplasm, due to disruption of the membrane patch. Whole-cell configuration has two main modes of recording: the voltage-clamp mode, in which the voltage is held constant allowing the study of ionic currents, and the current-clamp mode, in which the current is controlled enabling the study of changes in membrane potential. However, the disadvantage of this technique is

electrode dialyzing, which is the replacement of the soluble cell contents by the contents of the pipette solution resulting in alteration of cell properties.

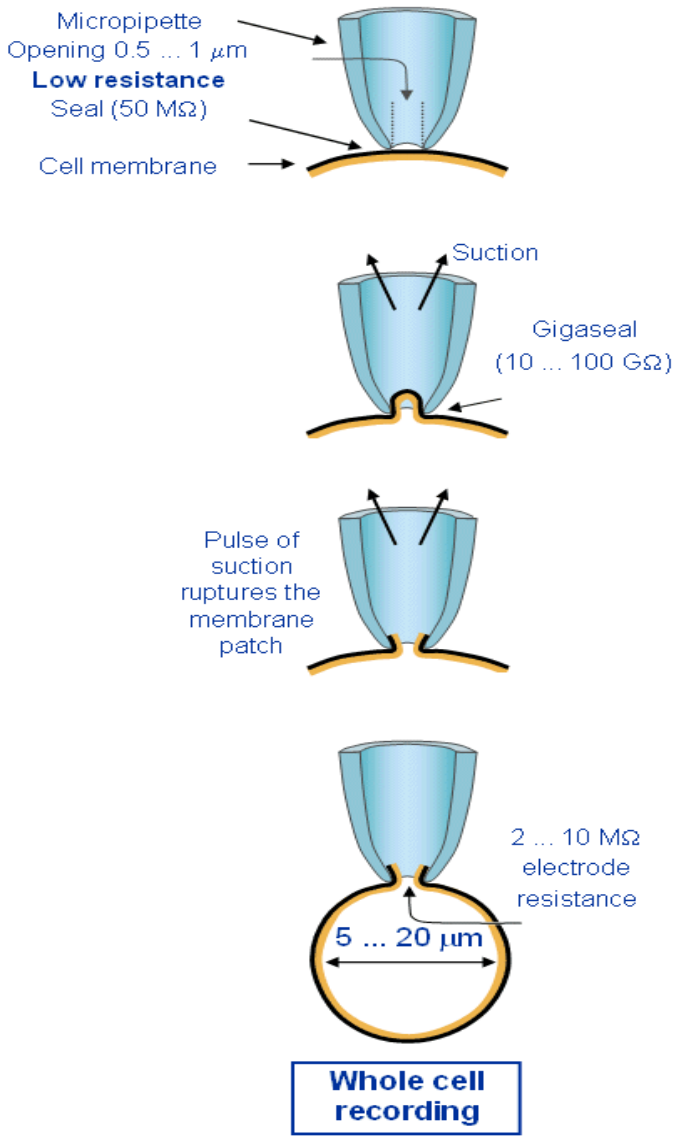

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Figure 13. Schematic representation of the whole-cell configuration.
1. The glass pipette is in contact with the cell membrane.
2. A tight gigaohm seal is formed by gentle suction; 3, more suction causes the membrane to rupture resulting in the whole-cell configuration.
4. Exchange of pipette and cellular contents.

In the current project, whole-cell recordings using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in current- or voltage-clamp mode were made from the stellate neurons in layer II of the medial EC, visually identified with infrared video microscopy (Olympus BX51WI) and differential interference contrast optics. The recording electrodes were filled with (in mM) 100 potassium (K^+)-gluconate, 0.6 EGTA, 2 $MgCl_2$, 8 NaCl, 2 ATP_2Na, 0.4 GTPNa, 40 HEPES, and 7 di-tris-phosphocreatine (pH 7.4, 290–300 mOsm/L). The extracellular solution comprised (in mM) 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$, and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4).

Action potentials (APs) were recorded in the preceding extracellular solution supplemented with bicuculline (10 μM) and CGP55845 (1 μM) to block $GABA_A$ and $GABA_B$ responses, respectively, and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) and DL-2-amino-5-phosphonopentanoic acid (DL-APV, 50 μM) to block glutamatergic transmission. For most of the cells, a positive current injection was required to bring the resting membrane potential (RMP) to ∼−50 mV to induce AP firing. CRF was applied after the AP firing had been stable for 5∼10 min. To avoid potential desensitization induced by repeated applications of CRF, only one cell was recorded from each slice. Data were filtered at 2 kHz, digitized at 10 kHz, acquired online, and analyzed after line using pCLAMP 9 software (Molecular Devices). Frequency of APs was calculated by Mini Analysis 6.0.1 (Synaptosoft, Decatur, GA).

Holding currents (HCs) at −60 mV were recorded from layer II stellate neurons. The preceding extracellular solution was supplemented with TTX (0.5 μM) to block AP firing. HCs at −60 mV were recorded every 3 s and then averaged per minute. We

subtracted the average of the HCs recorded for the last minute prior to the application of CRF from those recorded at different time-points to zero, the basal level of HCs, for better comparison.

Perforated Patch Clamp Recordings

Perforated patch clamp method is very similar to the whole-cell configuration. In this method the patched membrane is not ruptured by suction after the formation of the gigaohm seal. The electrode solution contains small amounts of an antifungal or antibiotic agent, such as amphotericin-B, nystatin, or gramicidin, which diffuses into the

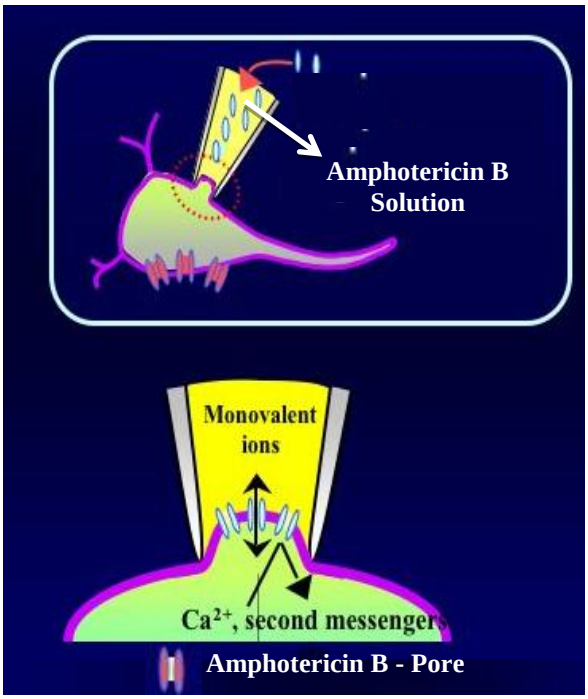

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Figure 14. Illustration of perforated patch clamp recording.
The electrode solution contains small amounts of antibiotic agent, such as amphotericin-B, which diffuses into the membrane patch and forms small pores in the membrane.
Only monovalent ions can pass through the pore and divalent and second messenger components are retained within the cell interior.

membrane patch and forms small pores in the membrane thus providing electrical access to the cell interior (Linley, 2013).

The advantage of the perforated patch clamp technique is that it allows equilibration of only small monovalent ions between the patch pipette and the cytosol. However, larger divalent ions such as Ca^{2+} and signaling molecules such as cAMP, cannot permeate through the pores. This helps in the maintenance of endogenous levels of most intracellular signaling molecules, and reduced current rundown as in cell-attached recordings (Linley, 2013). On the other hand, the disadvantages of using the perforated patch are: it can take a significant amount of time for the antibiotic to perforate the membrane; due to the perforation process the patch may rupture resulting in whole-cell mode and thus the antibiotic contaminates the entire cell contents; perforated patch offers a higher access resistance compared to that of the whole-cell recordings as the pipette tips are occupied by the cell membrane which may decrease current resolution and increase recording noise.

In our current experiments, we used perforated-patch recordings to record HCN-channel currents (I_h) from layer II stellate neurons in horizontal slices as described previously (Deng and Lei, 2007, Deng et al., 2010a). The extracellular solution comprised (in mM) 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4). Tetrodotoxin (0.5 μM) was included in the extracellular solution to block action potentials. Recording pipettes were tip-filled with the intracellular solution comprising 100 K^+-gluconate, 0.6 EGTA, 5 $MgCl_2$, 8 NaCl, 2 ATP_2Na, 0.3 GTPNa and 40 HEPES (pH 7.3) and then back-filled with freshly prepared K^+-gluconate intracellular solution containing amphotericin B

(200 µg/ml, Calbiochem, San Diego, CA). Patch pipettes had resistance of 6-8 MΩ when filled with the preceding solution. A 5-mV hyperpolarizing test pulse was applied every 5 s to monitor the changes of the series resistance and the process of perforation. Stable series resistances (50-70 MΩ) were usually obtained ~30 min after the formation of gigaohm seals. For those cells showing abrupt reduction in series resistance during membrane perforation suggesting the simultaneous formation of whole-cell configuration, experiments were terminated. Perforated-patch configurations were verified by examining the series resistance again at the end of the experiments. Data were included for analysis only from those cells showing <15% alteration of series resistance.

Western Blot

Brain tissues for western blot experiments were taken from 10 rats (18-day-old). For each rat, horizontal brain slices were cut initially and the medial EC region was punched out from the slices under a microscope. The isolated brain region was lysed in tissue protein extraction buffer containing protease inhibitors (Pierce, Rockford, IL). The lysates were centrifuged at 10,000×g for 10 min to remove the insoluble materials and protein concentrations in the supernatant were determined (Bradford, 1976). An equivalent of 40 µg total protein was loaded to each lane. Proteins were separated by 12% SDS–PAGE and transferred to the polyvinylidene difluoride (PVDF, Immobilon-P, Millipore, Billerica, MA) membranes using an electrophoretic transfer system (BioRad, Hercules, CA). Blots were blocked with 5% powdered milk, and then incubated with individual primary antibodies (anti-CRF, anti-CRF_1 or anti-CRF_2, 1:500) overnight at 4°C followed by incubation with the secondary antibody (donkey anti-goat IgG-HRP, 1:2000) for 1 h at room temperature. Tris-buffered saline with 1% Tween-20 was used to

wash the blots 3 times (10 min each) after incubation with both primary and secondary antibodies. β-actin was used as a gel loading control for the tissue homogenates. The blots were developed with enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Immunoreactive bands were visualized and detected on polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Biospectrum Imagining System (UVP, Upland, CA).

Table 2. Antibodies used for western blot.

Antibody	**Source**	**Dilution**
anti-CRF	Goat	1:500
anti-CRF_1	Goat	1:500
anti-CRF_2	Goat	1:500
donkey anti-goat IgG-HRP	Donkey	1:2000

Immunocytochemistry

The detailed procedures for immunocytochemistry were described previously (Lei et al., 2007, Deng and Lei, 2008, Deng et al., 2009, Xiao et al., 2009a, Deng et al., 2010a). Briefly, rats (18-day-old) were anaesthetized with pentobarbital sodium (50 mg/kg) and then perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains were rapidly removed and postfixed in the same fixative for an additional 2 h. After postfixation, brains were cryoprotected with 30% sucrose in PBS for 12 h and then cut into 20 μm slices in thickness horizontally in a Leica cryostat (CM 3050 S) at −21°C. Slices were washed in 0.1 M PBS and then treated with 0.3% hydrogen peroxide (H_2O_2) for 30

minutes to quench endogenous peroxidase activity. After being rinsed in 0.1 M PBS containing 1% Triton X-100 and 1.5% normal donkey serum for 30 min, slices were incubated with the primary antibodies (goat anti-CRF antibody, sc-1761; anti-CRF_1 antibody, sc-12381; anti-CRF_2 antibody, sc-20550; Santa Cruz Biotechnology Inc.) at a dilution of 1:100 at 4°C for 12 h. Slices were incubated at room temperature with biotinylated donkey anti-goat IgG (ABC Staining System, Santa Cruz Biotechnology Inc.) for 1 h and then with avidin-biotin complex (ABC Staining System) for 30 min. After each incubation, slices were then washed three times for a total of 30 min. Diaminobenzidine (ABC Staining System) was used for a color reaction to detect the positive signals. Finally, slices were mounted on slides, dehydrated through an alcohol range, cleared in xylene and covered with cover-slips. Slides were visualized and photographed with a Leica microscope (DM 4000B). We stained 5–6 nonadjacent sections and each staining was repeated by using 3 rats.

Table 3. Antibodies used for immunocytochemistry.

Antibody	**Source**	**Dilution**
anti-CRF	Goat	1:100
anti-CRF_1	Goat	1:100
anti-CRF_2	Goat	1:100
biotinylated donkey anti-goat IgG	Donkey	1:100

Statistical Analysis

All data were presented as the means ± S.E.M. For statistical analysis of the effects of CRF on epileptiform activity, the averages of 3-5 min of the frequency of

epileptiform activity before and after the application of CRF were compared. CRF concentration-response curves were fitted by the Hill equation: $I = I_{max} \times \{1/[1 + (EC_{50}/[ligand])^n]\}$, where I_{max} is the maximum response, EC_{50} is the concentration of ligand producing a half-maximal response, and n is the Hill coefficient. I_h were determined by subtracting the instantaneous currents (I_{Ins}) from the steady-state currents (I_{ss}). Student's paired or unpaired *t* test or analysis of variance (ANOVA) was used for statistical analysis as appropriate; P values were reported throughout the text and significance was set as $P<0.05$. N number in the text represents the slices or cells examined.

Chemicals

CRF was purchased from American Peptide Company (Sunnyvale, CA). The following reagents were products of TOCRIS (Ellisville, MO): K41498, astressin 2B, NBI 27914, CP 154526, MDL 12330A, SQ 22536, forskolin, 3,7-dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione(IBMX), KT 5720, Rp-cAMPS, CGP55845, bicuculline, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and *dl*-2-amino-5-phosphonopentanic acid (*dl*-APV). The other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Antibodies

Goat anti-CRF antibody, sc-1761; anti-CRF_1antibody, sc-12381; anti-CRF_2 antibody, sc-20550; Santa Cruz Biotechnology Inc., biotinylated donkey anti-goat IgG (ABC Staining System, Santa Cruz Biotechnology Inc.), avidin-biotin complex and Diaminobenzidine (ABC Staining System).

Table 4. Signal transduction and ion channel activators and inhibitors.

Compound	Concentration Used	Target
K41498	0.1 µM	CRF_2 antagonist
astressin 2B	0.1 µM	CRF_2 antagonist
NBI 27914	1 µM	CRF_1 antagonist
CP 154526	1 µM	CRF_1 antagonist
CRF6-33	1 µM	CRF-BP inhibitor
MDL 12330A	50 µM	AC inhibitor
SQ 22536	400 µM	AC inhibitor
Forskolin	20 µM	AC activator
IBMX	500 µM	PDE inhibitor
KT 5720	1 µM	PKA inhibitor
Rp-cAMPS	100 µM	PKA inhibitor
ZD7288	100 µM	HCN- channel blocker

Animals

Sprague-Dawley rats were purchased from Harlan Laboratories. CRF_2 homozygous KO mice (Stock number: 010842; Strain name: B6; 129-*crhr2*^{tm1jsp}/J) and WT mice (from the same colony) were bought from Jackson Laboratories. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of North Dakota (0702-2). All efforts were made to minimize suffering.

CHAPTER III

RESULTS

Expression of CRF and CRF_2 in the EC

Since mRNA of CRF receptors has been detected in the EC by in situ hybridization (Lovenberg et al., 1995b) we first examined the expression of CRF and CRF receptors in the EC of rats using immunocytochemistry and western blot analysis. The anatomical location of the EC and the divisions of individual layers in slice of rats were described previously (Deng et al., 2007, Xiao et al., 2009a). Strong immunoreactivity for CRF (Figure 15 A, upper panel) and CRF_2 (Figure 15C, upper panel) were detected in the EC whereas there was no detectable immunoreactivity for CRF_1 in the EC (Figure 15B, upper panel). Western blot demonstrates that a band of ~20 kDa (Figure 15A, lower panel) close to the reported molecular mass of CRF (Lauber et al., 1984, Watabe et al., 1991, Saoud and Wood, 1996) and a band of ~63 kDa (Figure 15C, lower panel) close to the reported molecular mass of CRF_2 (Miyata et al., 1999) were detected in the lysate of the EC. The specificities of the antibodies were confirmed by preabsorption of the antibodies with their corresponding blocking peptides blocked the detection of the bands (Figure 15, lower panel right). Whereas the molecular mass of rat brain CRF_1 was found to be 76–80 kDa (Radulovic et al., 1998, Spiess et al., 1998) there was no conspicuous band within this range (Figure 15B, lower panel) demonstrating that there is no expression of CRF_1 in the EC. Together, these data demonstrate that the EC

expresses CRF and CRF_2 with no detectable expression of CRF_1 (Kurada et al., 2014), consistent with previous results obtained by in situ hybridization (Lovenberg et al., 1995b).

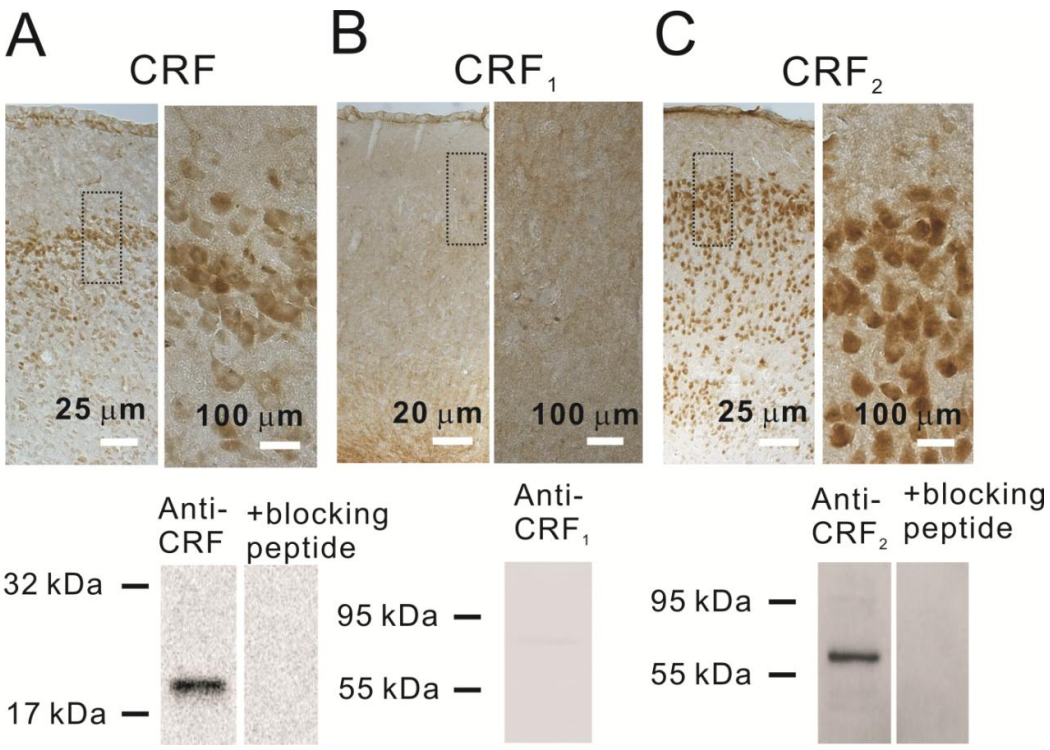


Figure 15. The entorhinal neurons express CRF and CRF_2 receptors but not CRF_1 receptors.
A: Immunoreactivity for CRF (*upper*) and detection of CRF by western blot (*lower*). Upper right: high magnification of the region marked in the left.
B: Lack of immunoreactivity (*upper*) and protein band (*lower*) for CRF_1 receptors.
C: The entorhinal neurons showed immunoreactivity (*upper*) for CRF_2 receptors and western blot detected a band close to the molecular mass of CRF_2 receptors in the lysates of the EC (*lower*).

CRF Facilitates Epileptiform Activity Recorded from the EC in Horizontal Slices

We studied the roles of CRF in epilepsy by recording PTX-induced epileptiform activity from layer III of the EC in horizontal slices (Figure 16) as described previously by Wang et al., stable epileptiform events occurred in ~20 min after bath perfusion of PTX (Wang et al., 2013). We therefore began to record basal epileptiform activity after perfusion of PTX for ~20 min. In this *in vitro* slice seizure model, application of CRF (0.1 μM) in the perfusion solution significantly increased the frequency of the epileptiform activity (n = 7 slices, $P<0.001$, Figure 16).

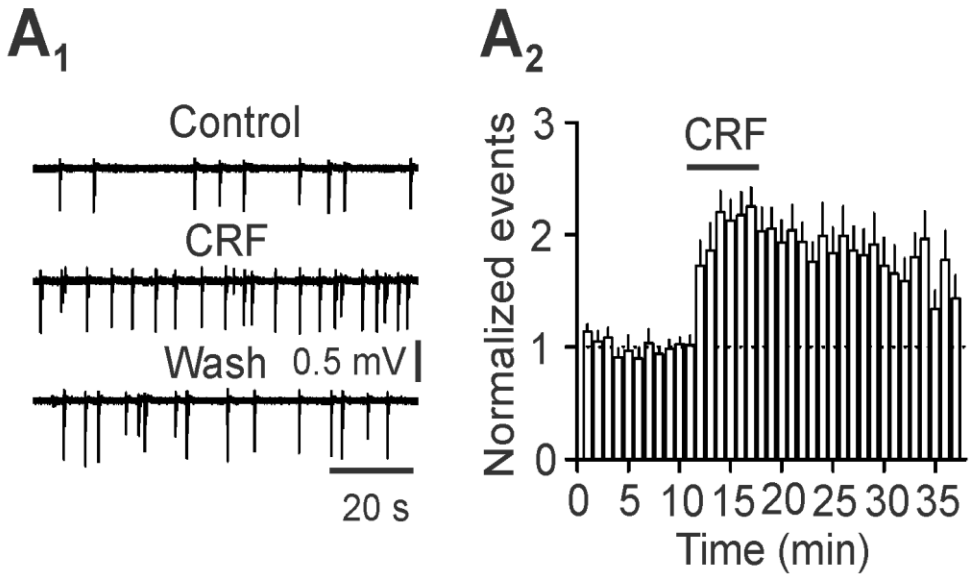


Figure 16. CRF increases the frequency of epileptiform activity induced by PTX.
A_1: Bath application of CRF increased the frequency of epileptiform activity recorded from layer III of the EC in horizontal slices.
A_2: Normalized events showing an increase in epileptiform events after the application of CRF
Control : 100 μM PTX in aCSF, CRF : Control + 0.1 μM CRF,
Wash : 100 μM PTX in aCSF.

CRF facilitates epileptiform activity recorded from the EC in mini-slices

The above experiments were performed in the horizontal slices containing the EC, hippocampus and other cortices. Whereas the connections among the EC and other brain regions such as the hippocampus are unlikely to be complete after cutting of the slices, we still tested whether the effects of CRF on epileptiform activity were due to the action of CRF on structures other than the EC. We therefore cut the medial EC out under a microscope and recorded PTX-induced epileptiform activity from layer III of the EC in this "mini slice" (Figure 17).

Figure 17. Micrograph of the mini-slice.

As shown in Figure 18 A_1-A_2, bath application of CRF (0.1 μM) still significantly increased the frequency of the epileptiform activity in the mini slices (n = 8, P = 0.001, Figure. 18 A_1-A_2) excluding the possibility that the action site of CRF is outside of the EC. Because CRF-induced increase in epileptiform activity recorded from

the horizontal slices was statistically indistinguishable from that recorded from the mini slices, (P = 0.98, two-way ANOVA), we used the horizontal slices for the rest of the

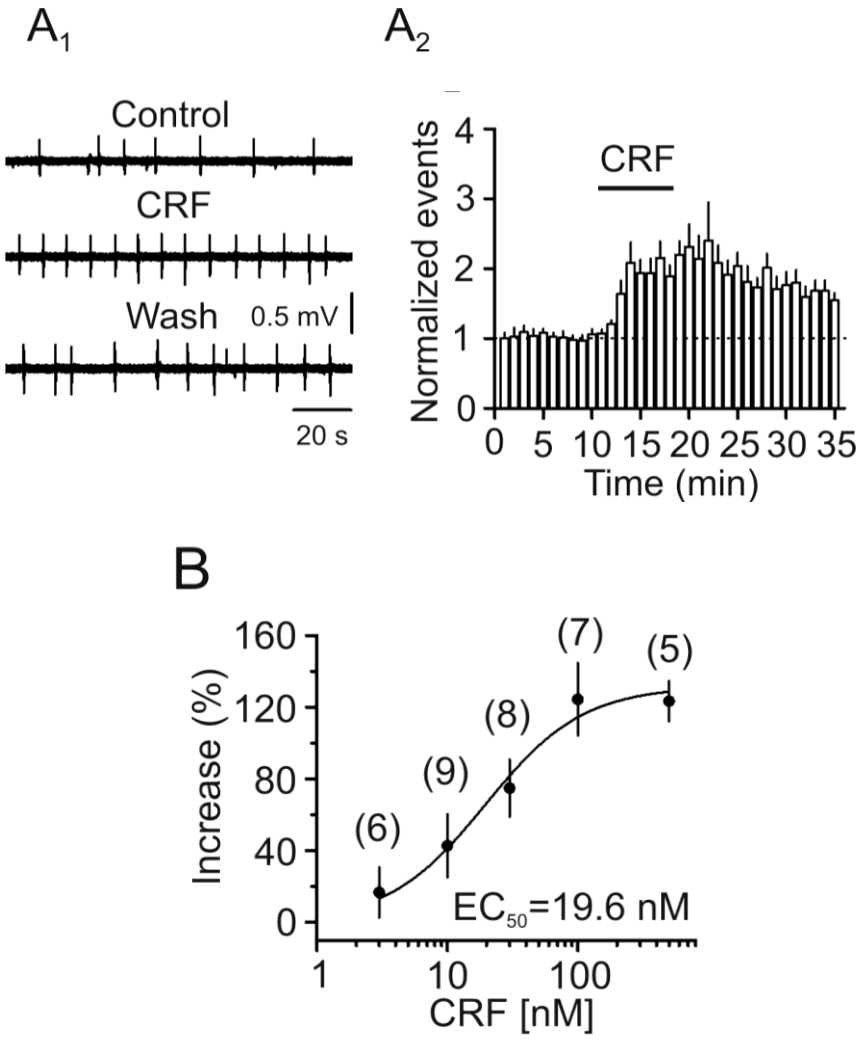


Figure 18. CRF increases the frequency of epileptiform activity induced by PTX in mini-slices.
A_1 : Bath application of CRF increased the frequency of epileptiform activity recorded from layer III of the EC in "mini slices" for which the hippocampus and other cortices were cut away.
A_2 Normalized events showing an increase in epileptiform events after the application of CRF.in "mini slices"
B: Concentration-response curve of CRF-induced facilitation of epileptiform activity. Numbers in the parentheses were numbers of slices recorded.

experiments simply for the convenience of experiments. The EC_{50} for CRF was measured to be 19.6 nM Figure. 18B. Because the maximal effect of CRF could be observed at 0.1 μM, we used this concentration of CRF for the rest of experiments.

CRF Facilitates the Susceptibility of Epilepsy in the EC

We then tested whether CRF facilitates the susceptibility of epilepsy. Bath application of the subthreshold concentration of PTX (10 μM), instead of 100 μM PTX, used in previous experiments, for 30 min did not induce epileptiform activity (Figure 19. A_1-A_2), but subsequent co-application of CRF (0.1 μM) induced robust epileptiform activity (Figure 19. A_1-A_2), suggesting that CRF increases the susceptibility of epilepsy.

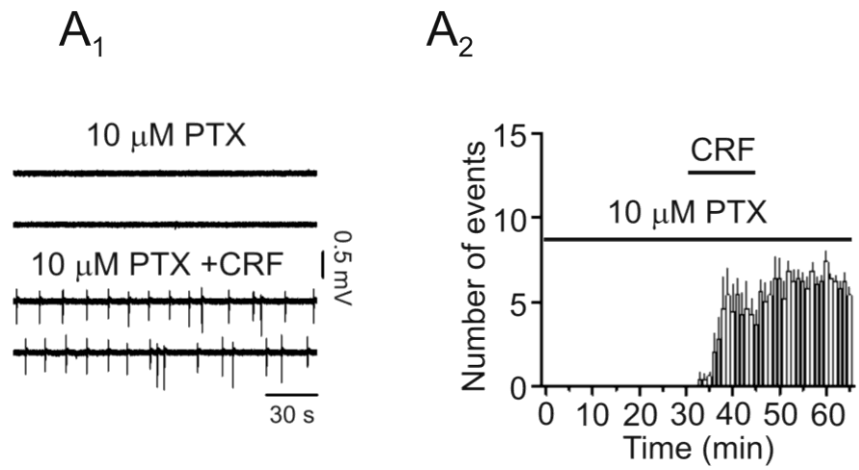


Figure 19. CRF facilitates the susceptibility of epilepsy.
A_1: Bath application of subthreshold concentration of PTX (10 μM) for 30 min failed to induce epileptiform activity whereas co-application of CRF induced robust epileptiform activity.
A_2: Normalized events showing CRF induced epileptiform activity in the presence of subthreshold PTX.

Endogenously Released CRF Facilitates Epileptiform Activity

The above results suggest that endogenously released CRF may play a role in epileptogenesis. As shown in Figure 15A, high density of CRF immunoreactivity was detected in the EC. Since there is evidence that the neuronal release of neuropeptides requires higher stimulation frequencies than that required by monoamine neurotransmitters colocalized in the same neuron (Lundberg and Hokfelt, 1983, Consolo et al., 1994) we hypothesized that PTX (100 µM)- induced epileptiform activity that may have increased CRF release, which further facilitates the epileptiform activity. We therefore tested this hypothesis by probing the roles of endogenously released CRF in PTX-induced epileptiform activity. Because CRF binds to the CRF-BP which buffers the amount of free CRF in the extracellular compartment (Jahn et al., 2005), we superfused slices with CRF6-33 (1 µM), a comparative inhibitor of the CRF-BP This peptide was used successfully to test the endogenous role of CRF in facilitating intracellular Ca^{2+} release in midbrain dopamine neurons (Riegel and Williams, 2008). Bath application of CRF6-33 significantly increased the frequency of epileptiform activity induced by PTX (236±39% of control, n = 4, P = 0.04, Figure 20 A_1-A_2). As will be shown below, CRF-mediated increases in epileptiform activity were mediated by activation of CRF_2 receptors. Pre-incubation of slices with and continuous bath application of the selective CRF_2 antagonist, K41498 (0.1 µM) blocked CRF6-33-induced augmentation of epileptiform activity (98±9% of control, n = 5, P = 0.82, Figure 20 B). These data together demonstrate that endogenously released CRF facilitates epileptiform activity.

CRF Increases Epileptiform Activity via Activation of CRF_2 Receptors

We next probed the roles of the receptors and the signaling mechanisms involved in the CRF-mediated facilitation of epileptiform activity, using various pharmacological challenges (Figure 21, Table 5). In order to overcome the problem of non-specificities of the inhibitors used in the blockade of the signaling cascade, we took care of the following parameters: i). Used more than one type of the inhibitor ii). Carefully chose the effective concentrations and IC_{50} values of the inhibitors given in the literature. iii).To ensure pharmacological effectiveness, we pre-treated the slices with the inhibitors as described in the literature prior to the application of CRF. Pretreatment of slices with and continuous bath application of K41498 (0.1 μM), a CRF_2 antagonist, significantly reduced CRF-induced increases in epileptiform activity (122±10% of control, n = 11 slices, $P<0.001$ vs. CRF alone, Figure 22 A). Application of astressin 2B (0.1 μM), another CRF_2 antagonist, in the same fashion blocked CRF-induced increases in epileptiform activity (113±9% of control, n = 7, $P = 0.22$ vs. baseline, Figure 22 B). We further confirmed the role of CRF_2 receptors by using CRF_2 receptor KO mice. Application of CRF (0.1 μM) increased the epileptiform activity in WT mice (n = 14 slices from 4 mice, $P<0.001$, Figure 22 C) but did not facilitate the epileptiform activity in CRF_2 receptor KO mice (n = 12 slices from 3 mice, $P = 0.59$, Figure 22 D) further confirming the requirement of CRF_2 receptors.

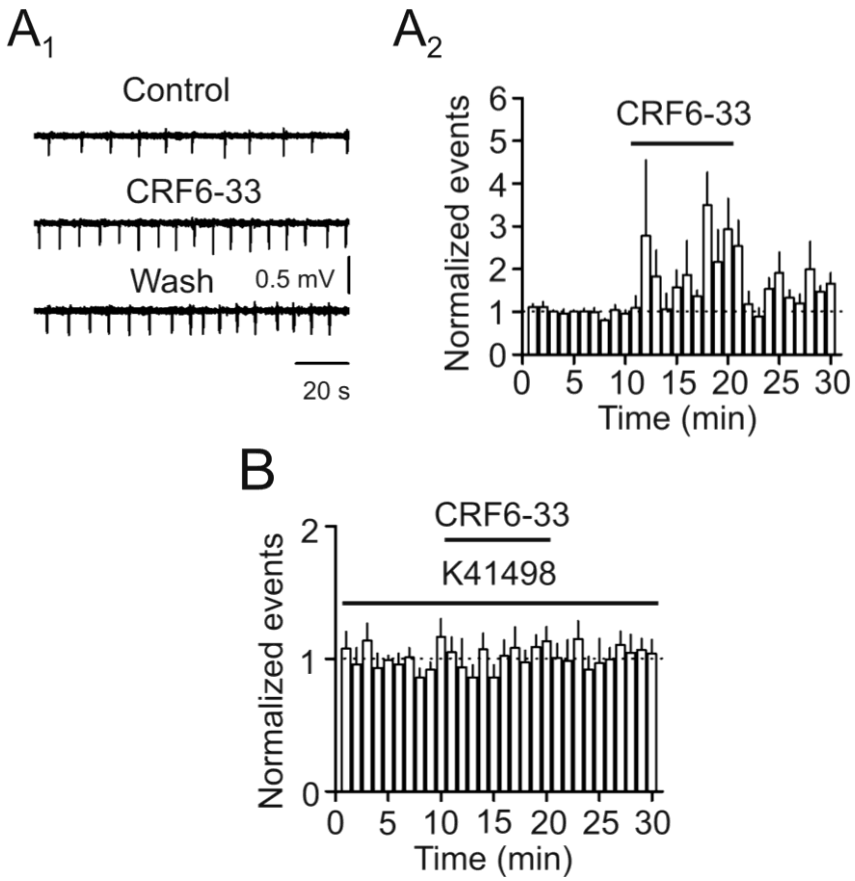
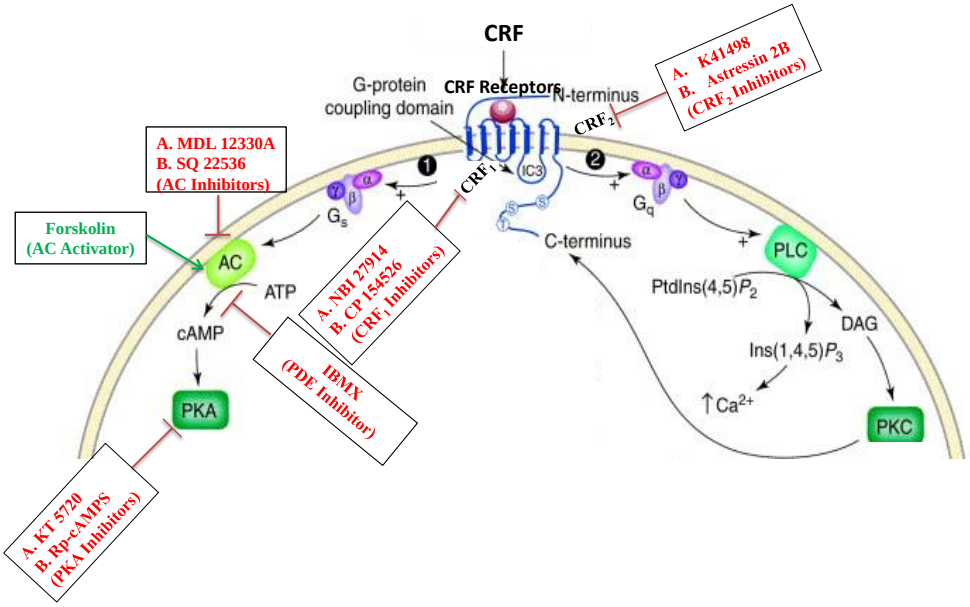


Figure 20. Endogenously Released CRF Facilitates Epileptiform Activity..
A_1-A_2: Bath application of CRF6-33, a comparative inhibitor of the CRF-binding protein, significantly increased the frequency of epileptiform activity via activation of CRF_2 receptor s.
B: Pre-application of K41498, a selective CRF_2 antagonist, blocked CRF6-33-induced increases in the frequency of epileptiform activity.



Adopted from : Holmes, A. et al., *Trends Pharmacol Sci* **24** (11), 580 (2003)

Figure 21. Elucidation of signaling mechanisms using various inhibitors (in red) and activators (green).

Table 5. Peptide and small-molecule CRFR antagonistsa (Zorrilla et al., 2003).

Common name	CRF receptor binding affinity		Chemical structure	References
	CRF_1 (nm)	CRF_2 (nm)		
CP154526	2.7	>10 000	N-Butyl-N-[2<comma>5-dimethyl-7-(2<comma>4<comma>6-trimethylphenyl)-7H-pyrrolo[2<comma>3-d]pyrimidin-4-yl]-N -ethylamine	(Seymour et al., 2003)
NBI27914	2[c]	>10 000[c]	5-Chloro-N-(cyclopropylmethyl)-2-methyl-N-propyl-N′-(2<comma>4<comma>6-trichlorophenyl)-4<comma>6-pyrimidinediamine	(McCarthy et al., 1999)
Astressin2-B	>500	1.3	Cyclo(31–34)[d-Phe11<comma>His12<comma>CαMeLeu13<comma>39<comma>Nle17<comma>Glu31<comma>Lys34]acetyl-sauvagine8–40	(Rivier et al., 2002a)
K41498	425	0.7	[d-Phe11<comma>His12<comma>Nle17]-sauvagine11–40	(Ruhmann et al., 1998b, Lawrence et al., 2002)

a. Binding data represent Ki values (inhibition constants) at rat or human CRF receptors as reported in the associated table reference, unless otherwise indicated.
b.50% inhibitory binding concentrations (IC_{50}) from (Rivier et al., 2002a) using cloned human CRF_1 and murine $CRF_{2(b)}$ receptors with ^{125}I-[Tyr^0,Glu^1,Nle^{17}]-sauvagine as the competitive radio ligand.
c. K_i value for rat CRF receptors as reviewed in (Gilligan et al., 2000).

Table 6: Signaling cascade inhibitors and activators used.

Common name	Chemical structure	Target	References
MDL 12,330A	(±)-*N*-[(1*R**,2*R**)-2-Phenylcyclopentyl]-azacyclotridec-1-en-2-amine hydrochloride	AC Inhibitor	(Guellaen et al., 1977, Hunt and Evans, 1980, van Rossum et al., 2000)
SQ 22536	9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine	AC Inhibitor	(Harris et al., 1979, Fabbri et al., 1991, Hourani et al., 2001)
Forskolin	[3*R*-(3α,4aβ,5β,6β,6aα,10α,10aβ,10bα)]-5-(Acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1*H*-naphtho[2,1-*b*]pyran-1-one	AC Activator	(Awad et al., 1983, Seamon et al., 1983, Laurenza et al., 1989, Kim et al., 2005)
IBMX	3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1*H*-purine-2,6-dione	PDE Inhibitor	(Freitag et al., 1998, Lepski et al., 2013)
KT 5720	(9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, hexyl ester	PKA Inhibitor	(Kase et al., 1987, Gadbois et al., 1992, Cabell and Audesirk, 1993)
Rp-cAMPs	(*R*)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium	PKA Inhibitor	(Van Haastert et al., 1984, Rothermel and Parker Botelho, 1988, Dostmann et al., 1990, Fu et al., 2008)
ZD 7288	[4-(N-Ethyl-N-phenylamino)-1,2 dimethyl-6-(methylamino) pyrimidinium chloride ICI-D7288 N-Ethyl-1,6-dihydro-1,2-dimethyl-6-(methylimino)-N-phenyl-4-pyrimidinamine hydrochloride]	HCN channel Inhibitor	(BoSmith et al., 1993, Harris and Constanti, 1995, Green et al., 1996, Klar et al., 2003)

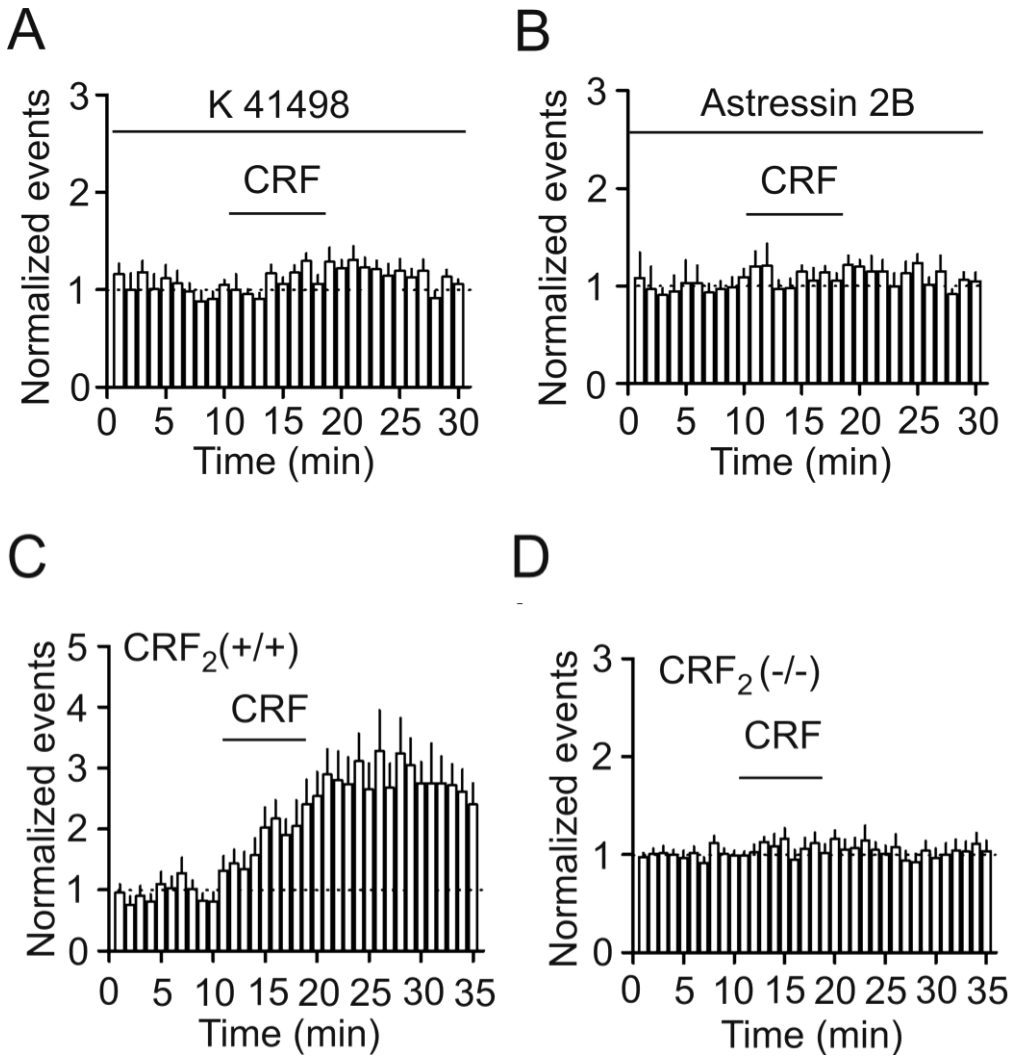


Figure 22. CRF facilitates epileptiform activity via activation of CRF_2 receptors.
A: Pretreatment of slices with and continuous bath application of K41498, a selective CRF_2 antagonist, blocked CRF-mediated increases in epileptiform activity.
B: Pretreatment of slices with and continuous bath application of astressin 2B, another selective CRF_2 antagonist, blocked CRF-mediated increases in epileptiform activity.
C: Application of CRF increased epileptiform activity in WT mice.
D: Application of CRF did not induce an increase in epileptiform activity in CRF_2 KO mice.

Whereas these data demonstrate the requirement of CRF_2 receptors, we also examined the roles of CRF_1 receptors. CRF-induced increases in epileptiform activity

were not altered significantly (vs. CRF alone) in slices treated with NBI 27914 (1 µM, n = 8,*P*= 0.78, Figure 23 A) or CP 154526 (1 µM, n = 12,*P*= 0.87, Figure 23 B), two selective CRF_1 antagonists.

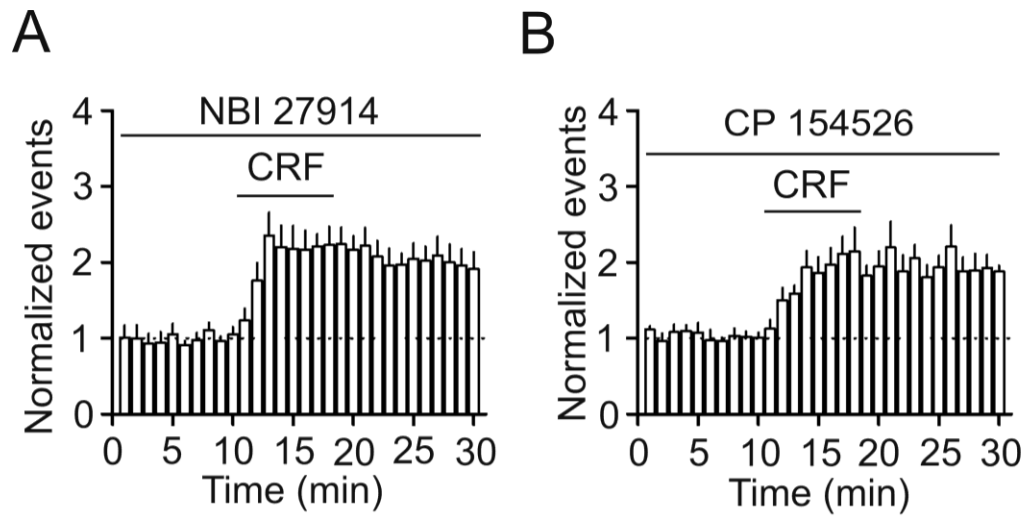


Figure 23. CRF-mediated facilitation of epileptiform activity does not involve CRF_1 receptors.
A: Pretreatment of slices with and continuous bath application of NBI 27914, a selective CRF_1 antagonist, failed to alter significantly CRF-mediated increases in epileptiform activity.
B: Pretreatment of slices with and continuous bath application of CP 154526, another selective CRF_1 antagonist, did not change the facilitatory effect of CRF on epileptiform activity.

Roles of the AC/cAMP/PKA Pathway in CRF-Induced Increases in Epileptiform- Activity

Because CRF_2 receptors are coupled to AC/cAMP/PKA pathway and there is strong evidence demonstrating that cAMP and PKA signals exert a tonic control of epilepsy (Boulton et al., 1993, Yechikhov et al., 2001, Higashima et al., 2002, Vazquez-Lopez et al., 2005, Ure and Altrup, 2006, Ristori et al., 2008), we tested the roles of this pathway in CRF-mediated facilitation of epileptiform activity (Figure 24). Slices were pretreated with the selective AC inhibitor MDL 12330A (50 µM) for ~20 min and the

same concentration of MDL 12330A was included in the PTX-containing extracellular solution and applied in the bath before and during the application of CRF. In this condition, bath application of CRF (0.1 μM) did not significantly increase the frequency of the epileptiform activity (n = 11, P = 0.14, Figure 24 A). Similarly, application of SQ 22536 (400 μM), another AC inhibitor, in the same fashion also blocked CRF-mediated facilitation of the frequency of epileptiform activity (n = 7, P = 0.2, Figure 24 B). These data together indicate that CRF increases epileptiform activity via activation of AC. Activation of AC increases the generation of cAMP. We next tested whether elevation of cAMP level mimics the effect of CRF. Bath application of forskolin (20 μM), an AC activator, significantly increased the frequency of epileptiform activity (170±12% of control, n = 13, P<0.001, Figure 24 C). Moreover, application of IBMX (500 μM), a phosphodiesterase (PDE) inhibitor to inhibit the degradation of cAMP, also significantly increased the frequency of epileptiform activity (253±23% of control, n = 15, P<0.001, Figure 24 D). These data together demonstrate that CRF-induced increases in the frequency of epileptiform activity are related to an increase in intracellular cAMP level.

We next tested the roles of PKA in CRF-induced facilitation of epileptiform activity. Slices were pretreated with the selective PKA inhibitor KT 5720 (1 μM) for ~20 min and the same concentration of KT 5720 was included in the PTX-containing extracellular solution and applied in the bath before and during the application of CRF. In this condition, bath application of CRF (0.1 μM) induced a statistically smaller increase in the frequency of epileptiform activity (130±8% of control, n = 10, *P* = 0.005 vs. CRF

alone, Figure 25 A). Application of Rp-cAMPS (100 µM), another specific PKA inhibitor, in the same fashion also significantly diminished CRF-mediated facilitation of

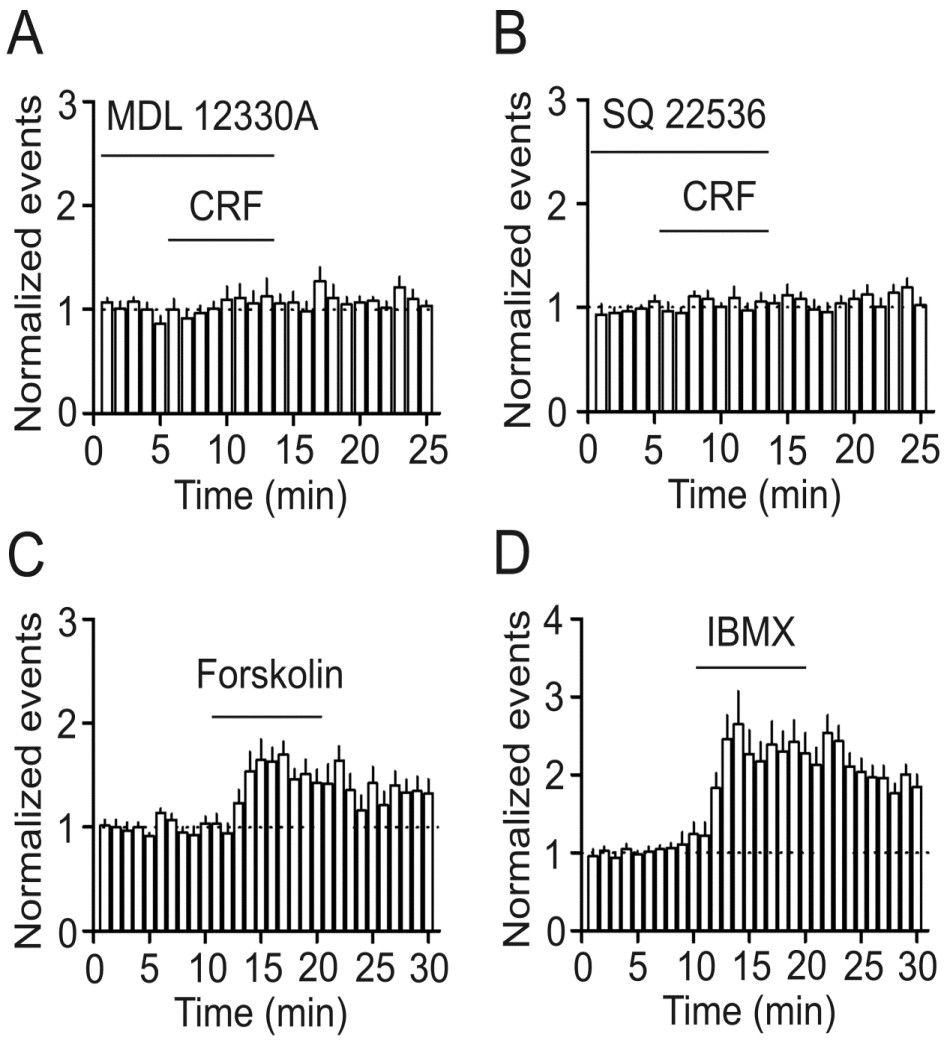


Figure 24. CRF facilitates epileptiform activity via activation of AC/cAMP pathway.
A: Pretreatment of slices with MDL-12330A, a selective AC inhibitor, blocked CRF-mediated increases in epileptiform activity.
B: Pre-application of SQ-22536, another AC inhibitor, blocked CRF-mediated facilitation of epileptiform activity.
C: Bath application of forskolin, an AC activator, increased the frequency of epileptiform activity.
D: Bath application of IBMX, a PDE inhibitor, enhanced the frequency of epileptiform activity.

the frequency of epileptiform activity (133±4% of control, n = 9, *P* = 0.004 vs. CRF alone, Figure 25 B). These data together demonstrate that PKA also plays a role in CRF-mediated increase in epileptiform activity.

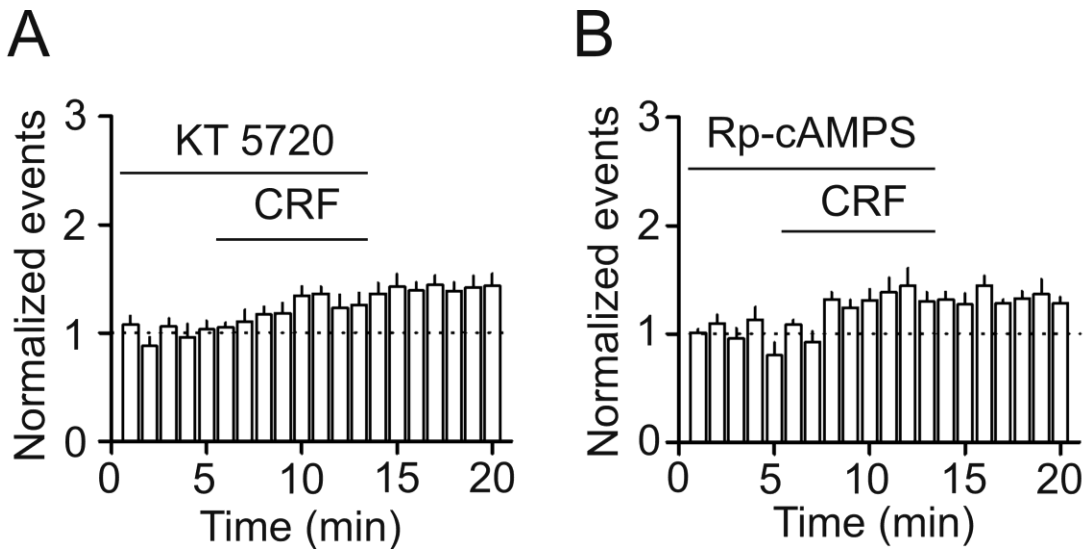


Figure 25. CRF facilitates epileptiform activity via partial involvement of PKA pathway.
A: Pretreatment of slices with KT 5720, a selective PKA inhibitor, partially blocked CRF-mediated enhancement of the frequency of epileptiform activity.
B: Pretreatment of slices with Rp-cAMPS, another specific PKA inhibitor partially blocked the facilitatory effect of CRF on epileptiform activity.

Roles of HCN-Channels in CRF-Mediated Facilitation of Epileptiform Activity

We tested whether HCN channels, in general, are involved in CRF-induced facilitation of epileptiform activity for the following reasons. First, CRF has been shown to facilitate HCN-channels (Qiu et al., 2005a, Wanat et al., 2008, Giesbrecht et al., 2010). Second, entorhinal neurons express robust HCN-channels (van der Linden and Lopes da Silva, 1998, Dickson et al., 2000). Third, HCN-channels are involved in epilepsy in other regions of the brain (Huang et al., 2009, Noam et al., 2011). Bath application of the

selective HCN-channel blocker, ZD 7288 (100 μM) significantly reduced the frequency of epileptiform activity to 54±7% of control (n=9, P<0.001, Figure 26 A, B) and bath application of CRF in the presence of ZD 7288 did not further elevate the frequency of epileptiform activity (53±12% of the base line before the application of ZD 7288, n=9, P=0.94 vs. ZD 7288 alone, Figure 26 A, B). These data suggest a role for HCN-channels in CRF-induced facilitation of epileptiform activity.

However, further analysis by measuring the numbers of synchronizing events, reminiscent of the interictal activity (between-seizures), in single ictal (seizure)-like epileptiform discharges and the duration of individual epileptiform activity demonstrated that ZD 7288 significantly increased the numbers of synchronizing events in single epileptiform activity (322±59% of control, n=9, P=0.009, Figure 26 A) and the duration of individual epileptiform activity (230±34% of control, n=9, P=0.002, Figure 26 A) suggesting that HCN-channels alter the patterns of epileptiform activity. In the presence of ZD 7288, bath application of CRF further increased the number of synchronizing events in single epileptiform activity (568±92% of control, n=9, P<0.001 vs. ZD 7288 alone, Figure 26 C) and the duration of individual epileptiform activity (366±62% of control, n=9, P=0.008 vs. ZD 7288 alone, Figure 26 C). These data suggest that CRF facilitates epileptiform activity via multiple mechanisms and HCN-channels contribute to but are not the sole performer in CRF-mediated facilitation of epilepsy.

If HCN-channels are involved in CRF-mediated facilitation of epileptiform activity, CRF should modulate HCN-channel activity.

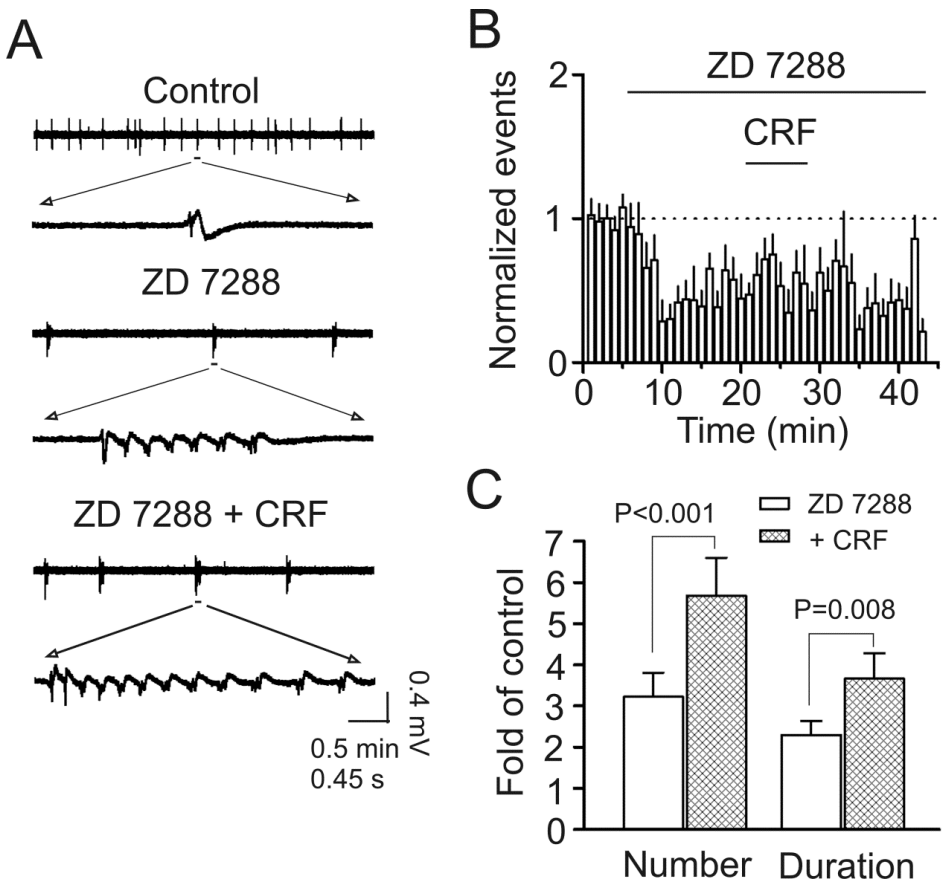


Figure 26. HCN channels are involved in CRF-mediated facilitation of epileptiform-activity.

A: Epileptiform activity recorded from a slice in response to sequential applications of ZD 7288 and CRF. Arrows indicate the expansion of a single epileptiform activity. Note the difference of the time in the scale bar.

B: Summarized time course of the frequency of the epileptiform activity in response to the sequential applications of ZD 7288 and CRF. Note that application of ZD 7288 significantly reduced the frequency of epileptiform activity and application of CRF in the presence of ZD 7288 failed to further increase the frequency of epileptiform activity.

C: Summarized data for the number of synchronizing events in individual epileptiform activity and the duration of single epileptiform activity in response to ZD 7288 and CRF. Data were normalized to the control level before application of ZD 7288. Note that CRF still increased the number of synchronizing events in single epileptiform activity and the duration of epileptiform activity in the presence of ZD 7288.

We next tested whether CRF modulates HCN-channel function by recording I_h from the stellate neurons in layer II of.the EC, because these neurons express robust HCN-channels (van der Linden and Lopes da Silva, 1998, Dickson et al., 2000)

Stellate Neuron Identification:

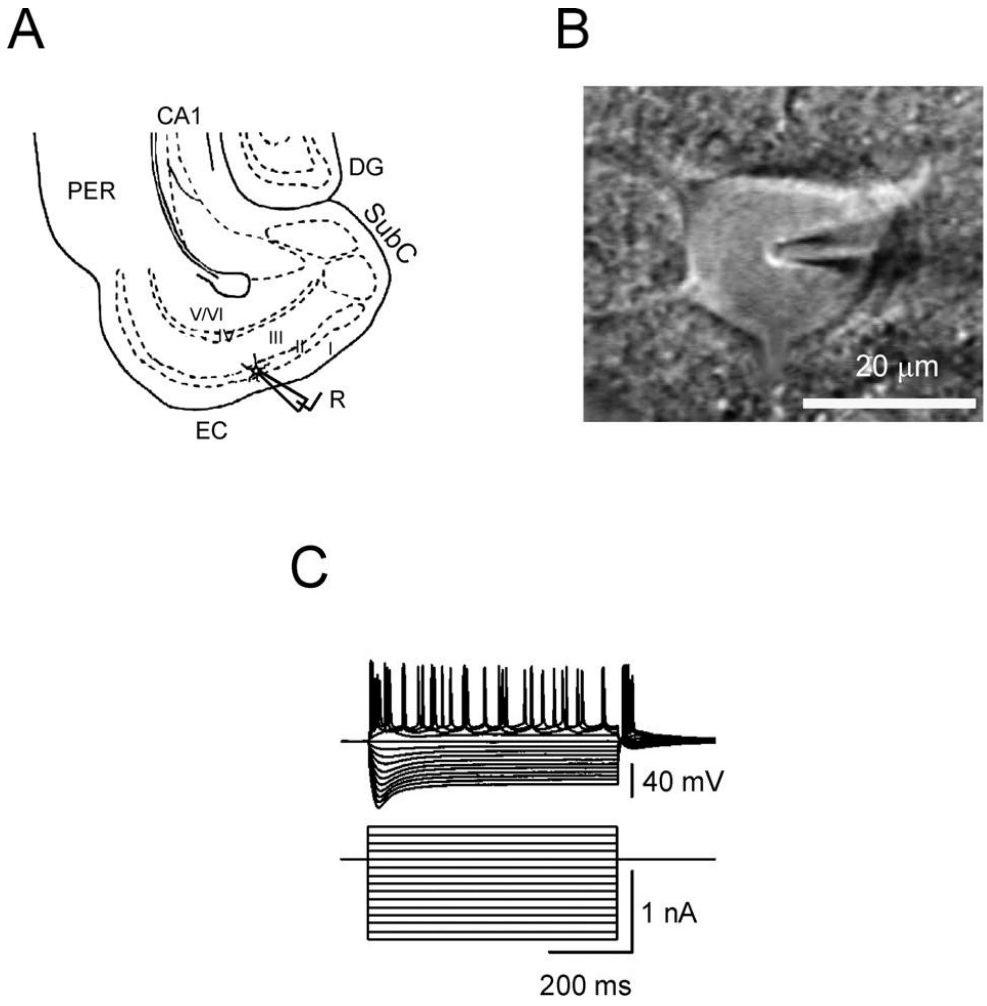


Figure 27. Stellate neuron identification.
A: Schematic illustration of the recording location in the EC. DG, dentate gyrus; Sub, subiculum; PER, perirhinal; R, recording electrode.
B: a stellate neuron identified under an infrared video microscopy.
C: voltage responses (top) generated by current injection from + 0.4 to -1 nA at an interval of -0.1 nA (bottom) recorded from a stellate neuron in layer II. Note the depolarizing voltage sag in response to hyperpolarizing current pulses.

mEC layer II neurons were visually identified using infrared video microscopy (Olympus BX51W1) and differential interference contrast optics. About 70% of the neurons in layer II of the mEC are stellate neurons (Klink and Alonso, 1997). A schematic illustration of the EC appears in Figure 27.

Stellate neurons are the most numerous class of excitatory neurons in layer II. These neurons are usually located in layer II or the border of layer II and III, and they have distinct "star like" morphology with a larger and polygonal soma and a variable number of main dendrites radiating out from the cell body but are devoid of a clearly dominant dendrite (Deng and Lei, 2007) (Figure 27) These neurons have unique electrophysiological properties; i.e., hyperpolarizing current pulse injection always caused the membrane potential to attain an early peak and then "sag" to a steady-state level (Alonso and Klink, 1993, Deng and Lei, 2007).

CRF Augments HCN-Channel Currents in Layer II Stellate Neurons

Because we initially found that I_h recorded by whole-cell configuration underwent significant run-down, we used perforated-patch recordings (Figure 28). Cells were held at -50 mV and a series of hyperpolarizing voltages (from -50 mV to -130 mV at an interval of 10 mV) were applied for 1 s to record the voltage-current relationship before and after the application of CRF (Figure 28A). A single voltage step (from -50 mV to -130 mV) was applied every 20 s to measure the time course of I_h in response to CRF application (Figure 28 C). At the end of experiments, ZD 7288 (100 μM) was applied to corroborate the identity of the recorded I_h (Figure 28). Under these circumstances, bath application of CRF (0.1 μM) significantly increased I_h (133±8% of control, n=9, p=0.002, Figure 28 B) demonstrating that CRF up-regulates I_h.

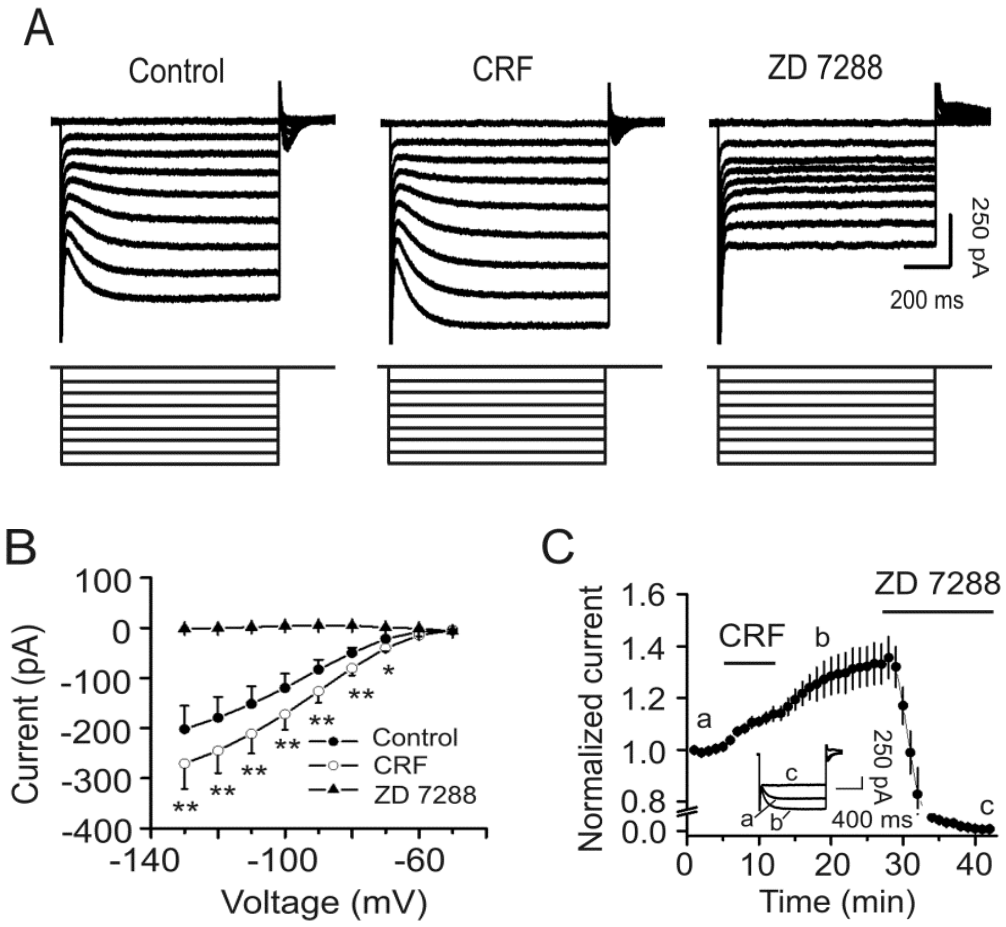


Figure 28. CRF enhances I_h recorded from layer II stellate neurons in perforated-patch recordings.
A: I_h recorded by hyperpolarizing to different voltages from the holding potential of -50 mV for 1 s at an interval of 10 mV before (left) and after (middle) application of CRF. Application of ZD 7288 (100 μM) at the end of the experiments blocked I_h.
B: Voltage-current relationship before and after the application of CRF. * P<0.05, ** P<0.01.
C: Time course of I_h recorded by hyperpolarizing from -50 mV to -130 mV before, during and after the application of CRF. Application of ZD 7288 at the end of the experiments completely blocked Ih. Inset shows the current traces recorded at different time points indicated in the figure.

We further determined the effects of CRF on the voltage-sensitivity of I_h conductance. I_h conductance (G_H) was determined as the amplitude of I_h measured at different potentials (V) divided by the driving force ($V-E_H$), where E_H is the reversal potential of I_h. The

value of E_H was arbitrarily set at -35 mV, which represents the median E_H reported in a variety of cell types (Ghamari-Langroudi and Bourque, 2000). The G_H at different voltages were normalized to the maximal G_H and plotted versus the voltages. The voltage-conductance relationship was fit by the equation $G_{H(V)} = 1/(1+e^{-k(V-V1/2)})$, where $G_{H(V)}$is the fraction of maximal G_H observed at individual voltage, k is the slope factor and $V_{1/2}$ is the half-maximal voltage. CRF shifted the $V_{1/2}$ by 5.7±1.4 mV (n=9, P=0.004) to a more depolarized potential (Figure 29 A). CRF slightly but significantly altered the slope factor (k) (n=9, p=0.025, Figure 29 B).

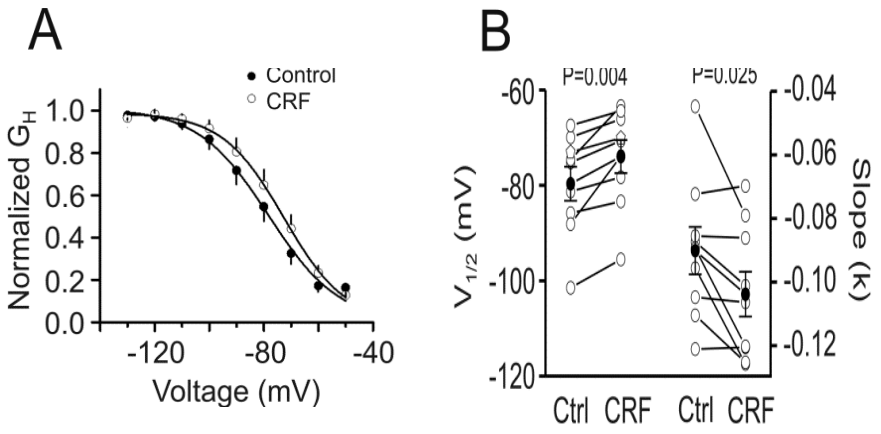


Figure 29. Effects of CRF on the voltage-sensitivity of I_h conductance
A: Voltage-conductance relationship before and after the application of CRF.
B: Summarized $V_{1/2}$ (left) and k (right). The open symbols represent the values for individual cells and the solid symbols indicate the averages. CRF slhifted the $V_{1/2}$ to more depolarized potential and slightly, but significantly altered the slope factor (k).

We then tested the involvement of CRF_2 receptors in CRF-induced increases in I_h. Bath application of the selective CRF_2 antagonist, K41498 (0.1 µM), blocked CRF-mediated augmentation of I_h (n=5, P=0.2, Figure 30 A_1-A_2) whereas application of the selective.

Involvement of cAMP Not PKA in CRF-Mediated Increases in Ih in Layer II Stellate Neurons

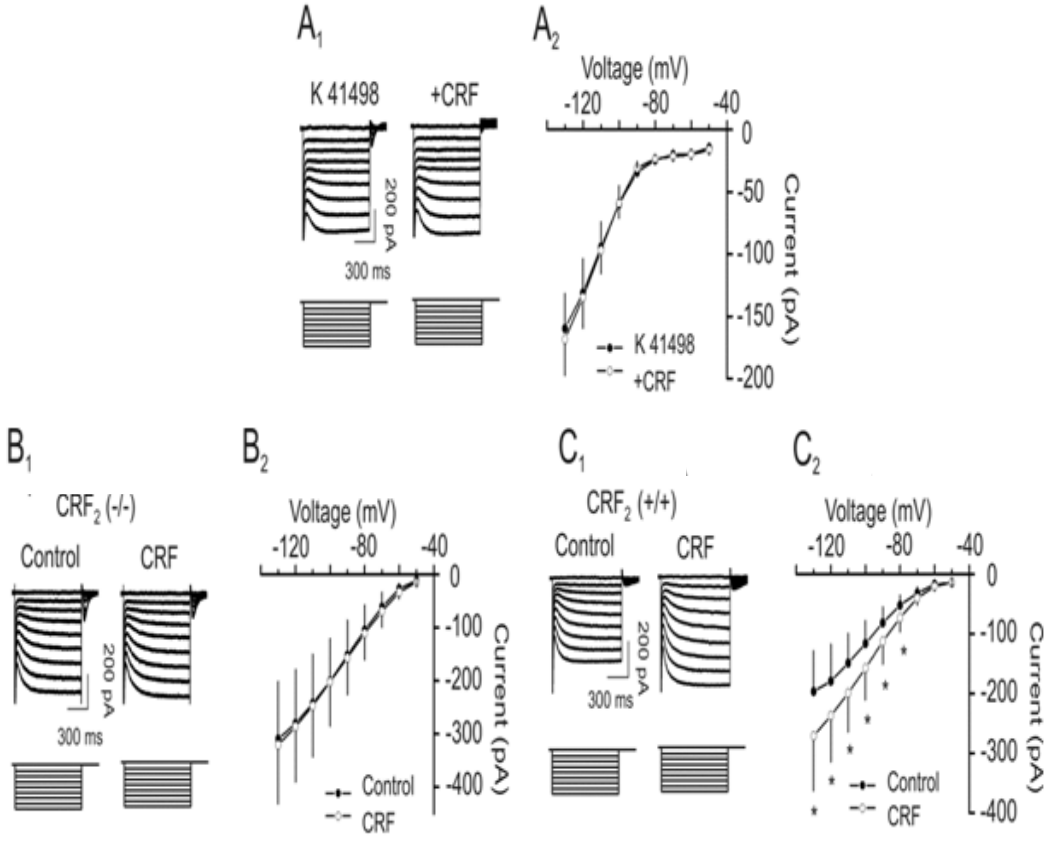


Figure 30. CRF augments I_h via activation of CRF_2 receptors.
A_1-A_2: Application of CRF in the presence of CRF_2 antagonist, K41498, failed to increase I_h.
B_1-B_2: CRF did not enhance I_h in slices cut from CRF_2 knockout mice.
C_1-C_2: Application of CRF increased I_h in slices cut from wild-type mice.

Furthermore, CRF did not increase I_h in slices cut from CRF_2 knockout mice (n=9 cells from 3 mice, P=0.27, Figure 30 B_1-B_2) whereas CRF still significantly increased Ih in slices cut from wild-type mice (n=7 cells from 3 mice, P=0.02, Figure 30 C_1-C_2).

CRF_1 antagonist, NBI 27914 (0.1 µM) still significantly augmented I_h (n=9, P=0.009, Figure 31 A_1-A_2). These data together indicate that CRF increases I_h via activation of CRF_2 receptors.

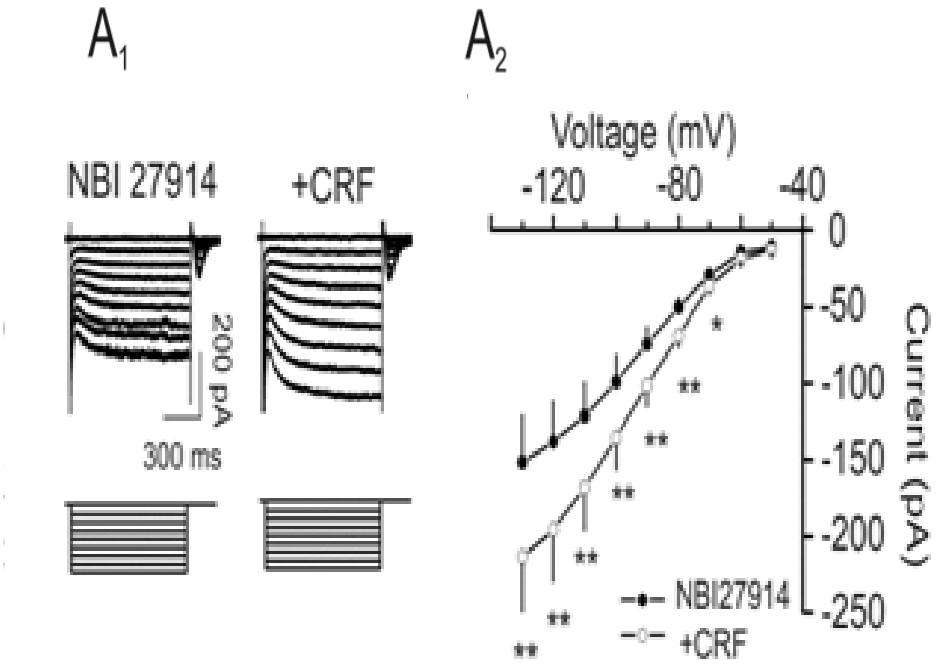


Figure 31. CRF_1 receptors are not involved in Ih increase.
A_1-A_2: Application of CRF in the presence of CRF_1 antagonist, NBI27914, still augmented I_h.

We further probed the roles AC/cAMP/PKA pathway in CRF-induced increases in I_h in layer II stellate neurons. CRF-induced increases in I_h were blocked by application of MDL 12330A (50 µM, 107±4% of control, n=7, p=0.15, Figure 32 A_1-A_2) and SQ 22536 (400 µM, 104±2% of control, n=5, P=0.14, Figure 32 B_1-B_2) indicating that CRF- mediated facilitation of I_h requires the functions of AC and cAMP. However, application of CRF still augmented I_h in the presence of Rp-cAMPS (100 µM, 150±21%

of control, n=6, P=0.008, Figure 32 C_1-C_2) and KT 5720 (1 µM, 127±3% of control, n=5, P=0.026, Figure 32 D_1-D_2) demonstrating that CRF-induced increases in I_h is independent of PKA activity.

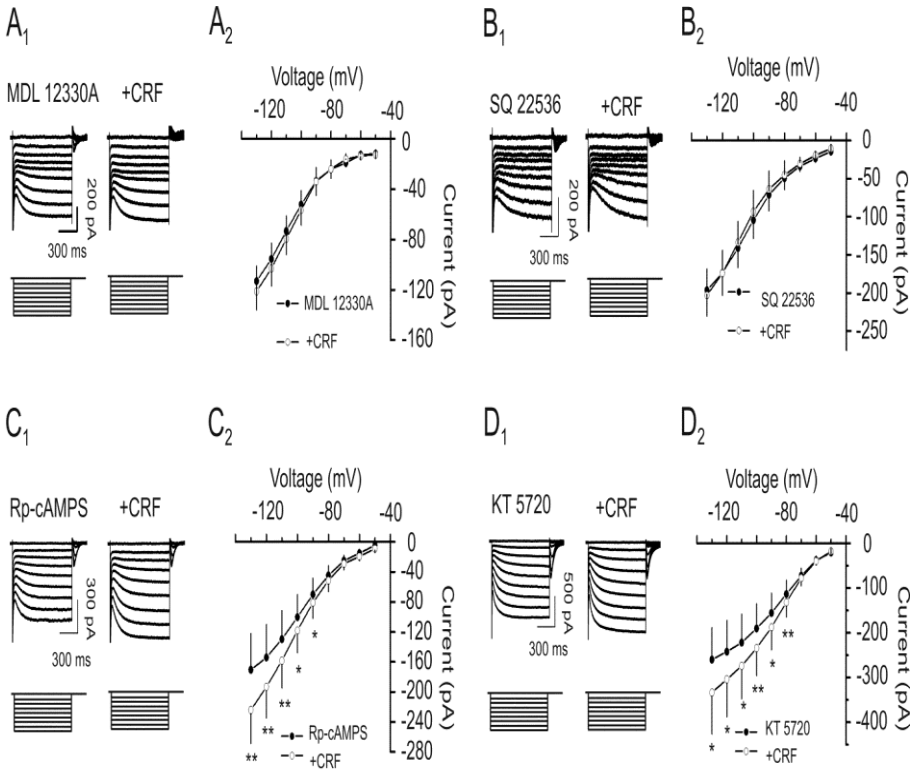


Figure 32. CRF-induced enhancement of I_h is mediated by cAMP but not by PKA.
A_1-A_2: Application of CRF in the presence of AC inhibitor, MDL 12330A, failed to increase I_h significantly.
B_1-B_2: CRF did not enhance I_h in the presence of another AC inhibitor, SQ 22536.
C_1-C_2: Application of CRF in the presence of Rp-cAMPS still increased I_h.
D_1-D_2: Application of KT 5720, another PKA inhibitor, failed to block CRF-induced increases in I_h.

CRF Increases AP Firing Frequency in Layer II Stellate Neurons of the EC

We then tested the effect of CRF on neuronal excitability by recording AP firing from the layer II stellate neurons. Bath application of CRF (0.1 μM) for 7 min. significantly increased the firing frequency of these neurons (control: 1.06 ± 0.16 Hz; CRF: 3.69 ± 0.44 Hz; n = 7; P = 0.001; Figure 33), and the firing frequency was sustained even during the wash, after the application of CRF was stopped.

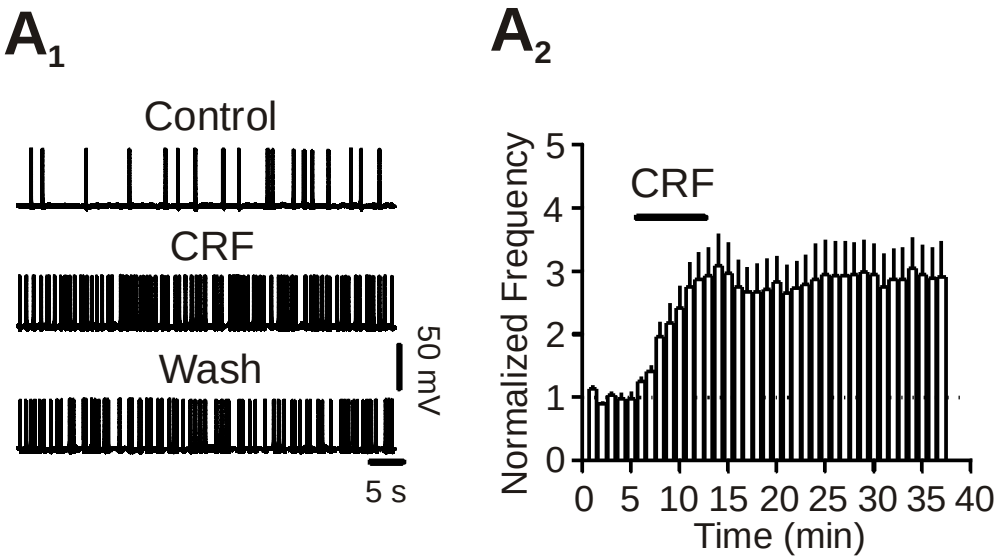


Figure 33. Bath application of CRF increased the firing frequency of APs recorded from layer II stellate neurons in EC.
A_1: APs recorded prior to, during, and after application of CRF (0.1 μM).
A_2: time course of CRF-induced increases in AP firing frequency (n = 7).

CRF Causes Increase in Inward HCs

We next examined the effects of CRF on the inward holding current, in voltage-clamp mode, CRF induced an inward shift of the HCs recorded at −60 mV (−37.1 ± 3.1 pA; n = 9; P < 0.001; Figure), further confirming the membrane depolarizing effect of CRF.

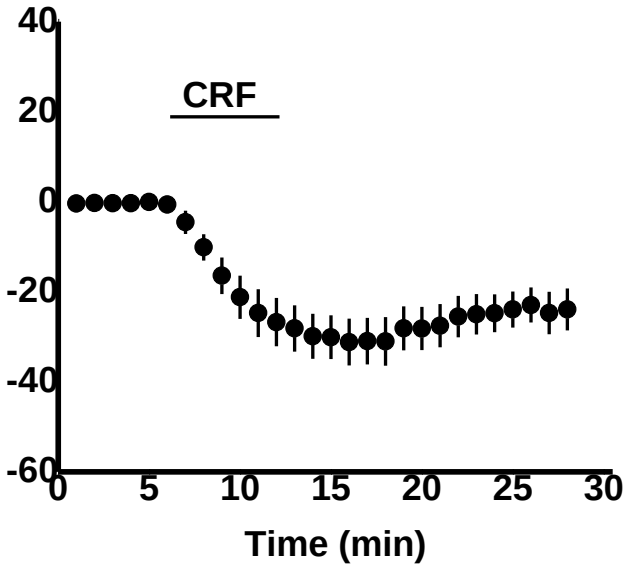


Figure 34. CRF causes increase in inward HCs.

CHAPTER IV

DISCUSSION

A number of brain regions including the hippocampus, the amygdala and the EC are involved in the epileptogenesis. However, the cellular and molecular mechanisms are still not well established. The EC is an important limbic structure involved in the development, maintenance and spread of seizures in the patients with TLE and in epileptic animal models (Spencer and Spencer, 1994, Gloveli et al., 1998, Avoli et al., 2002, Avoli and de Curtis, 2011). Studies show that the EC is more susceptible to seizures than the hippocampus. EC is a six-layered structure and functions as a gateway to the hippocampus and other cortical regions (Witter et al., 1989, 2000). The current study focuses on layers II and III for the following reasons: The layer III pyramidal neurons are hyperactive in vivo after the induction of TLE, and thus play a critical role of epileptogenesis. Layer III neurons are preferentially lost in both human TLE (Kim et al., 1990, Du et al., 1993) as well as animal models of epilepsy (Du and Schwarcz, 1992, Du et al., 1995). With the onset of epilepsy and loss of CA3 neurons and the SC pathway (Ben-Ari and Cossart, 2000), the TA pathway has been suggested as a major excitatory drive to the principal neurons of the CA1 region (Barbarosie et al., 2000, Avoli et al., 2002, Wu and Leung, 2003). Thus altered pyramidal cell excitability in the layer III of the EC is likely to have a greater impact in the development of TLE. The neurons of

layer II are well spared and become hyperexcitable (Bear et al., 1996) due in part to a reduction in inhibitory input (Kobayashi and Buckmaster, 2003), and proexcitatory alterations in sodium channel gating parameters (Hargus et al., 2011).Thus layer II cause an excitatory drive into the DG and the hippocampus (Buckmaster and Dudek, 1997) resulting in seizure induction and propagation. Hence mEC layer II neurons can be a potential site of action of AEDs to suppress neuronal excitability or to reduce the seizure activity once fully evoked (Hosseinmardi et al., 2007).

A number of factors can precipitate seizures in patients with epilepsy (Spector et al., 2000), and stress is amongst the most common triggering factors (Lai and Trimble, 1997, Frucht et al., 2000, Haut et al., 2003, Nakken et al., 2005, Haut et al., 2007, Sperling et al., 2008). Epileptic patients show a dysregulation in the HPA axis (Zobel et al., 2004, Mazarati et al., 2009) and increased basal levels of stress hormones, which further increase after seizures (Abbott et al., 1980, Pritchard et al., 1985, Culebras et al., 1987, Galimberti et al., 2005). CRF is a 41 amino acid neuropeptide initially isolated by Vale and his colleagues (Vale et al., 1981), a hypothalamic factor which activates the HPA axis, initiating the release of ACTH from the pituitary, which in turn causes the release of glucocorticoids from the adrenal cortex, to regain homeostasis (Dunn and Berridge, 1990). CRF mediates the endocrine, autonomic, and behavioral responses to stress (Aldenhoff et al., 1983, Ehlers et al., 1983, Siggins et al., 1985, Swanson et al., 1986, Conti and Foote, 1995, Sawchenko et al., 1996, Baram and Hatalski, 1998). Supporting evidence from various studies using immunolabeling, radioimmunoassay, and mRNA expression have demonstrated that CRF is widely distributed in brain areas of.,

human (Charlton et al., 1987), rat (Fischman and Moldow, 1982), and mouse(Nakane et al., 1986). In addition to the PVN of the hypothalamus, CRF-containing neurons are also found in various other brain regions, and is highly expressed in areas associated with developmental seizures, such as the hippocampus and the amygdala (Gray and Bingaman, 1996, Yan et al., 1998);(Steckler and Holsboer, 1999). The extra-hypothalamic source of CRF has been demonstrated by central administration of CRF which caused anxiety-like behavioral and autonomic effects (Britton et al., 1982, Dunn and File, 1987, Koob and Heinrichs, 1999) and these behaviors persisted in hypophysectomized rats (Eaves et al., 1985). Abnormalities in the HPA- and the extra-HPA axis-CRF homeostasis have been documented with epilepsy. CRF transduces its effects via two B-type G-protein coupled receptors CRF_1 and CRF_2, which show 70% homology (Perrin and Vale, 1999). Free levels of CRF are maintained in the brain as well as periphery by a binding protein called CRF-BP. Both the receptors and the binding protein have distinct pharmacological profiles (De Souza, 1995, Gulyas et al., 1995, Dautzenberg and Hauger, 2002, Chatzaki et al., 2006, Fekete and Zorrilla, 2007), and are distinctly and differentially distributed throughout the brain (Steckler and Holsboer, 1999). Various animal models revealed that intracerebroventricular injection of CRF induces seizures (Ehlers et al., 1983, Marrosu et al., 1987, Marrosu et al., 1988, Weiss et al., 1993) and the levels of CRF and CRF-BP are increased in patients as well as animals models of epilepsy.Despite of the tremendous amount of studies relating the EC and CRF in mediating epilepsy, not much is known about the role and relevance of CRF in the EC.

Based on the available information that CRF, CRFRs and CRF-BP mRNA was shown in various regions including the EC, our overarching hypothesis of this project was that CRF in the EC binds to its receptors and facilitates epilepsy. To begin to test this hypothesis, we initially wanted to examine the presence of CRF and the type of receptors in EC, using immunocytochemsitry and western blot techniques. While strong immunoreactivities were detected for CRF and $CRF_{2,}$ there was no detectable immunoreactivity for CRF_1. These results were further corroborated by westernblot. The reported masses for CRF, CRF_2 and CRF_1 are, ~20 kDa (Lauber et al., 1984, Watabe et al., 1991, Saoud and Wood, 1996) ,~63 kDa (Miyata et al., 1999) and 76–80 kDa (Radulovic et al., 1998, Spiess et al., 1998) respectively. While bands closer to the above respective molecular mass were observed for both CRF and CRF_2, no conspicuous band was observed for CRF_1. These results demonstrated that both CRF protein and CRF_2 receptors are predominantly expressed in the EC (Kurada et al., 2014), as shown in previous in situ hybridization studies (Lovenberg et al., 1995b), suggesting that CRF plays an important role in the EC.

A number of studies have shown CRF to be a potent epileptogenic molecule and CRF contributes to increased excitation in limbic regions. For example, intracerebroventricular injections of CRF produces spontaneous seizures (Ehlers et al., 1983, Weiss et al., 1986b, Marrosu et al., 1988, Baram and Schultz, 1991b). CRF-induced epileptiform activity is initiated in the amygdala which then spreads to other brain regions (Baram et al., 1992). Since our previous results indicate the presence of CRF and its receptor, we then wanted to explore whether CRF facilitates epileptiform

activity in the EC. A number of in vitro and in vivo models of epilepsies have been developed to study the mechanisms underlying different types of epilepsies. Various chemical convulsants are commonly used such as, PTX, Pentylenetetrazol, pilocarpine and kainic acid. These drugs generally act by interfering the normal synaptic transmission (Kupferberg, 2001) .Here we used a well-established in vitro model called PTX-slice seizure model. The PTX-induced seizure model resembles the simple partial and generalized forms of human epilepsy (Fisher, 1989, Sierra-Paredes and Sierra-Marcuno, 1996, Sarkisian, 2001). PTX blocks the chloride channels linked to $GABA_A$-receptors and thereby prevents the conductance of chloride ions into the neuronal membrane. This result in the inhibition of GABA neurotransmission and GABA mediated inhibition of neuronal activity and thereby elicits seizures (Orhan et al., 2012). Using PTX (100 µM) in the acsf, epileptiform activity was induced. Extracellular field recordings were obtained from layer III of the EC, in the horizontal hippocampus-entorhinal slices After stable events, basal or control epileptiform activity was recorded and then CRF (0.1 µM) was applied. Electrophysiological measurements showed that application of CRF to the entorhinal slices robustly increased the frequency of epileptiform activity induced by PTX. The CRF-mediated epileptiform activity was sustained even during wash, after the application of CRF (Kurada et al., 2014), indicating that CRF is triggering a cascade of signaling events resulting in possible receptor and/or channel up regulation. Our results demonstrate that the EC is at least one of the action sites for CRF-mediated facilitation of epilepsy.

The above experiments were conducted in the horizontal slices containing the EC, hippocampus and other cortices. Although it was unlikely that the connections among the EC and other regions to be complete after cutting the slices, it might be possible that the action site of CRF could likely be in the other brain regions other than the EC. In order to confirm whether the action site is the EC, we recorded from the 'mini' slices in which other brain regions except the mEC were cut off. CRF still induced the same level of facilitation of PTX-induced epileptiform activity (Kurada et al., 2014). These results together demonstrate that CRF acting in the EC can facilitate epileptiform activity. Since CRF-mediated increase in epileptiform activity recorded from the horizontal and the mini-slice was statistically indistinguishable, horizontal slices were used for the rest of experiments, for the sake of convenience. The calculated EC_{50} was 19.6 nM. However, since the maximal effect was obtained at 0.1 μM, the same concentration was used for the rest of our experiments.

CRF is known to show pro-convulsant properties. CRF could not elicit seizures by itself in infant hippocampus rats (Hollrigel et al., 1998), when CRF is administered before amygdala kindling, the time to reach the fully kindled state is markedly reduced (Weiss et al., 1986) The proconvulsive role of CRF is further confirmed by studies from the Lewis rats, with reduced CRF expression. These rats take longer to kindle and require more electrical stimulations (Weiss et al., 1993). Based on these studies, we then wanted to test whether CRF by itself can induce epileptiform activity in the EC. In the absence of PTX, when CRF was bath applied, CRF could not induce any epileptiform activity. However, in the presence of sub-threshold concentration (10 μM) instead of (100 μM)

PTX, though the sub-threshold concentration of PTX could not elicit any epileptiform activity, application of CRF could produce epileptiform events. These results are in agreement with the previous studies showing the pro-convulsant actions of CRF in other brain regions. These results corroborate the fact that CRF cannot induce epileptiform activity but itself in the EC, but can increase the epileptiform activity induced by PTX.

Our next question was regarding the mechanisms by which CRF modulates epilepsy. We next wanted to determine the type of receptor, CRF_1 and/or CRF_2, involved in CRF-mediated facilitation of epileptiform activity in the EC. Since our earlier experiments showed strong immunoreactivity for CRF_2, which was further confirmed by the western blot, we pretreated slices with antagonists for CRF_2 receptors. In the presence of CRF_2 antagonists, CRF-mediated facilitation of epileptiform activity was significantly blocked. Multiple antagonists were used to rule out any possibility of antagonist non-specificity. The results obtained from the above pharmacological challenges were further corroborated by CRF_2 KO mice. When CRF was applied to the slices cut from the CRF_2 KO mice, CRF failed to increase the frequency of epileptiform activity. However, CRF still exerted robust facilitatory effects on the frequency of epileptiform activity when applied to slices cut from WT mice. Though the above results suggest that CRF_2 is predominantly expressed in the EC and facilitates the epileptiform activity, it might be possible that CRF_1 receptors were undetected by the methods we employed in our experiments, but may still be functionally active. To rule out this possibility, we tested the role of CRF_1 in CRF-mediated epileptiform activity. In the presence of CRF_1 antagonists, CRF could still significantly augment the frequency of

PTX-induced epileptiform events. Furthermore, CRF binds with high affinity to the CRF_1 but has low affinity for CRF_2 (Dautzenberg and Hauger 2002). Hence it might be possible that CRF_1 receptors, even if present in the EC, might be non-functional. Our results have therefore filled a gap for the effects of CRF in the EC by demonstrating that CRF-elicited facilitation of epileptiform activity is mediated by CRF_2 receptors.

The next question was the role of endogenously released CRF in facilitating epileptiform activity in the EC. CRF is released within many other brain regions such as the hippocampus, the amygdala (Roozendaal et al., 2002) locus ceruleus (Valentino and Wehby, 1988, Snyder et al., 2012). Since CRF can travel to distances longer from the regions of origin, within the brain (Bittencourt and Sawchenko, 2000a), it might be possible that CRF from extra-entorhinal source which is transported from the distal brain, here in this case, the hippocampus and other regions in the horizontal slice, and acts on CRFRs in the EC. The levels of free CRF in the extracellular component are regulated by the CRF-BP. We then determined the role of endogenously released CRF by the application of the CRF-BP antagonist CRF6-33, which also increased the frequency of PTX-induced epileptiform activity suggesting that endogenously released CRF is involved in epileptogenesis. Thus, the CRF-BP may hold as a reservoir of CRF and therefore the regulation of the interaction between CRF and CRF-BP may represent a mechanism by which CRF can be released to activate the CRF receptors present in the EC. Taken together these results demonstrate that the CRF endogenously released from the EC facilitates epileptiform activity via activation of the CRF_2 receptors.

What are the signaling mechanisms by which CRF facilitates epileptiform activity in the EC? The biological actions of CRF are likely to be mediated by CRFRs and their intracellular signals. Both CRF_1 and CRF_2 are primarily coupled to G_s proteins, activating the AC/cAMP/PKAcascade (Dautzenberg and Hauger, 2002, Grammatopoulos and Chrousos, 2002, Hauger et al., 2006). CRFRs also have various degrees of coupling competence and potency to interact with other G-protein systems including G_q, G_i, G_o, $G_{i1/2}$, and G_z (Grammatopoulos et al., 2001). Thus CRF can modulate various signaling cascades and kinases comprising of protein kinase B (PKB), protein kinase C (PKC), mitogen-activated protein (MAP) kinases and intracellular Ca^{2+} concentrations in a tissue-specific and concentration dependent manner (Dautzenberg and Hauger, 2002, Grammatopoulos and Chrousos, 2002, Hauger et al., 2006).The AC/cAMP/PKA pathway is predominant Gs activated signaling cascade, increasing the neuronal firing. However, whether PKA is coupled with the CRF in transducing the epileptiform activity is not clear. Elucidating the signal transduction mechanism in the EC, will help in better understanding of the etiology of the disease. To elucidate the signaling mechanisms, various activators and inhibitors of the cAMP/PKA signaling cascade were used. In order to overcome the problem of non-specificities of the inhibitors used in the blockade of the signaling cascade, we took care of the following parameters: i). Used more than one type of the inhibitor ii). Carefully chose the effective concentrations and IC_{50} values of the inhibitors given in the literature. iii).To ensure pharamacological effectivesness, we pre-treated the slices with the inhibitors as described in the literature prior to the application of CRF. Activation of CRF_2 receptors increases the function of AC resulting in

augmentation of cAMP production and subsequent activation of PKA. We demonstrate that AC and cAMP are fully required but PKA may be partially necessary for CRF-mediated facilitation of epileptiform activity based on the following results. AC is an enzyme that converts ATP to cAMP. Hence inhibition of AC should result in blockade of CRF-mediated increases in epileptiform activity. To test this, we used AC inhibitors, MDL 12330A and SQ 22536. In the presence of these AC inhibitors, CRF-induced augmentation of epileptiform activity was completely blocked. Next, elevation of endogenous cAMP level should augment epileptiform activity by itself. To test this, we used Forskolin, a cAMP activator and IBMX, a PDE inhibitor. PDE is the enzyme that degrades cAMP to 5'-AMP and thereby limits the stimulatory effect of the cAMP cascade. By inhibiting PDE using IBMX, the levels of cAMP are therefore increased. Forskolin and IBMX produced significant increases in epileptiform activity by themselves and thus mimic the effects produced by CRF. If PKA is involved in CRF-mediated increases in epileptiform activity, blocking PKA should block the CRF-mediated effects. To test this hypothesis, PKA was inhibited by KT 5720 and Rp-cAMPS. This resulted in significantly reduced but not completely blocked CRF-mediated increases in epileptiform activity. In accordance with our results, tremendous evidence demonstrates that AC/cAMP/PKA pathway plays a facilitatory role in epilepsy (Boulton et al., 1993, Yechikhov et al., 2001, Higashima et al., 2002, Vazquez-Lopez et al., 2005, Ure and Altrup, 2006, Ristori et al., 2008).

Given the direct link among ion channel activity, neuronal excitability and epilepsy, ion channels remain an active area of investigation. What are the ion channels

that are involved in CRF-mediated increases in epileptiform activity? Since HCN channels play an important role in epilepsy (Huang et al., 2009, Noam et al., 2011) and the entorhinal neurons express robust HCN-channels (van der Linden and Lopes da Silva, 1998, Dickson et al., 2000), we tested the roles of HCN-channels in CRF-mediated facilitation of epileptiform-activity. HCN channels maintain the RMP and thus control the neuronal excitability. HCN channels are regulated by neurotransmitters and hormones that act via cAMP, cGMP, or intracellular Ca^{2+}(Pape 1996); cAMP and cGMP modulate HCN channel activity via direct interaction with the cyclic nucleotide-binding domain protein of the C-terminus (Ludwig et al. 1998). HCN channels interact with multiple neurotransmitter systems and are coupled both positively, via the Gs, and negatively, via Gi-proteins, to the cAMP synthesis and thus are up- and down-regulated (Frere and Luthi 2004; Pape 1996). Since our results show the involvement of cAMP in CRF-mediated epileptiform activity, and HCN channels are modulated by cAMP; and the layer II stellate neurons express high levels of HCN channels, we wanted to test the role of these channels in CRF-mediated facilitation of epilepsy. We then probed the role of HCN-channels. HCN-channels by themselves exert three effects on picrotoxin-induced epileptiform activity. First, application of the selective HCN-channel blocker ZD 7288 significantly reduced the frequency of the epileptiform activity suggesting that HCN-channels modulate the rhythm of epilepsy. This is also consistent with the roles of HCN-channels because HCN-channels are involved in rhythmic modulation of neuronal activity. Second, inhibition of HCN-channels results in enhanced number of synchronizing events in single epileptiform activity. Third, HCN-channel inhibition also

enhanced the duration of individual epileptiform activity. It must be noted that the synchronizing events represent the interictal activity, which is a state of continuous neuronal excitability; the above results show that CRF modulates the HCN channel properties.

If CRF plays a role in modulating the HCN channels, CRF should modulate the current flowing through these channels as well. HCN channels carry current known as Ih. We then wanted to probe the role of I_h current in CRF-mediated facilitation of epileptiform activity. Perforated patch clamp recordings were performed from the layer II stellate neurons. Voltage-current relationship revealed that CRF augments Ih. These results were further corroborated by the blockade of HCN channels using ZD7288. Furthermore, CRF increased the conductance and caused membrane depolarization which is indicative of increased neuronal excitability. In order to identify whether CRF modulates the Ih current via CRFRs, using selective CRF_2 inhibitors, we found that CRF augments I_h current via CRF_2 receptors. These results were further corroborated using CRF_2 KO mice, in which CRF-mediated I_h increase was blocked. In the presence of AC/cAMP blockers, CRF could not facilitate the increases in I_h, whereas the PKA inhibitors have no effect on CRF-mediated I_h increase. This shows that CRF augments I_h via cAMP signaling, but PKAis not required. The result that blockade of HCN-channels by ZD 7288 also blocked CRF-mediated increase in the frequency of epileptiform activity suggests that HCN channels contribute to CRF-mediated augmentation of epileptiform activity. However, we do not consider HCN channels as the sole player in CRF-mediated facilitation of epileptiform activity based on the following pieces of

evidence. First, the number of the synchronizing events in single epileptiform activity after inhibition of HCN-channels by ZD 7288 was still significantly increased after application of CRF. Second, the duration of individual epileptiform activity was still enhanced by CRF after inhibition of HCN channels by ZD 7288. Third, our results suggest that CRF facilitates I_h via cAMP without requirement of PKA. If HCN channels are the only performer, inhibition of PKA should not exert any effects on CRF-mediated facilitation of epileptiform activity. However, our results demonstrated that inhibition of PKA significantly reduced CRF-mediated enhancement of epileptiform- activity suggesting that molecules other than HCN channels are still targets of CRF. Further studies will identify other mechanisms underlying CRF-induced facilitation of epileptiform activity.

CRF is known to induce excitability in cortex and the forebrain (Eberly et al. 1983), the hippocampus, Purkinje cells, and the dorsal vagal complex is augmented by CRF-mediated reductions in afterhyperpolarization (AHP) (Aldenhoff et al. 1983; Hollrigel et al. 1998; Lewis et al. 2002; Yamashita et al. 1991).We then wanted to probe the role of CRF at the cellular level and the mechanisms involved. When CRF (0.1 μM) was applied to the stellate neurons, a significant increase of neuronal firing was produced in the presence of CRF and was sustained even when CRF was washed out. Also, CRF produced significant inward holding currents, which could be due to opening of $Na^+/Cl^-/K^+$ channels These results indicate that CRF acts at the neuronal level and increases excitability. Further experiments to identify the ion channels and signaling mechanisms would help to better understand the role of CRF in the EC.

Summary and Significance

CRF is an important neuropeptide and involved in various brain functions, and a potent epileptogenic neuropeptide. The seizure-facilitating effects of CRF have largely been explained based on its actions in various other regions of the brain such as the hippocampus and the amygdala. However, the action sites and mechanism of actions of CRF are not yet determined. CRF and CRFR immunoreactivity was found in the EC, but the role and relevance of CRF in facilitating seizures are not well established. This dissertation provides evidence that CRF facilitates epileptiform activity via activation of CRF_2 receptors. and cAMP signaling, whereas PKA is partially involved. Endogenously released CRF is responsible for the CRF-mediated epileptiform activity, as blockade of CRF-BP by a selective antagonist, CRF6-33, significantly increased the epileptiform activity. CRF-mediated facilitation of epileptiform activity involved the modulation of HCN channels and increases in I_h current activated via CRF_2 signaling, with the involvement of cAMP. PKA was not involved in the enhancement of CRF mediated increases in I_h (Figure 35). Further understanding the mechanisms of pro-convulsant effects of CRF within the EC, will provide valuable information for developing targeted drug moieties that can reduce the seizure activity.

Accumulating evidence suggests that the stress-related hormone CRF and the HPA axis play a critical role in the pathophysiology of TLE. CRF facilitates exacerbation of neuronal excitability by modulating molecular, structural and synaptic functions resulting in decreased seizure threshold and epilepsy. Epilepsy-mediated by stress system, especially by CRF and its receptors have important implications for the therapy

of patients with TLE. Changes in the molecular composition and function of the CRF system and its receptors may alter the modulatory effects of CRF in facilitating epilepsy in various brain regions. A deeper understanding of the mechanisms and specific target regulation of CRF receptor in regionally distinct roles of the CRF receptors in various brain regions would allow specific target receptors in the epileptic neurons and thus increasing efficacy and reducing the side effects. Furthermore, certain neuropeptide characteristics such as discrete neuroanatomical localization, relatively little disruption of normal physiology by the neuropeptide ligands, and the requirement of higher stimulation frequencies for the neuronal release of neuropeptides than those required by monoamine neurotransmitters and GABA colocalized in the same neuron makes CRF an alluring target for epilepsy treatment. Thus, pharmacological alteration of CRF function might normalize pathological activity in circuits mediating stress, such as the HPA axis, without producing unwanted side-effects (Hokfelt et al., 2000)(Hokfelt et al. 2000). Indeed, drugs that are antagonists at CRF receptors might have a particularly low side-effect burden because such compounds would not be expected to disrupt normal physiology in the absence of neuropeptide release. CRF-receptor antagonists have been shown to increase seizure threshold and are effective in animal models of febrile seizures (Toth et al. 1998). This information implies that selective blockers of CRF-receptor activation might be useful anticonvulsants for some types of seizures, which with further investigation might be useful to treat other epilepsy types. However, CRF system affects a wide range of behaviors and physiological processes. Added to these, the complexity of the signaling mechanisms makes it much more complicated to successfully target the CRF-system for

pharmacotherapy. Most importantly, CRF-mediated TLE animal data requires further validation in human tissue to provide us new important information about the role of CRF in TLE. Further insights into the complex role of CRF system in the facilitation and exacerbation of epilepsy would help us identify new molecular targets for novel AEDs, which might help inhibit and/or abrogate the disease pathogenesis.

Additionally, CRF-mediated facilitation of I_h current in the EC might be an important mechanism underlying the generation of high-frequency activity in the hyperexcitable EC and could become an alternative target for defining antiepileptic treatment strategies. In summary, we report that CRF has profound excitatory effects on neuronal excitability in the layer II stellate neurons leading to increases in I_h current and thus facilitating the epileptiform activity. A potential mechanism for this enhanced excitatory effect could be the increase in the I_h current. Since the EC is involved in the initiation and maintenance of seizures, this study provides a rationale for selective targeting of the CRF_2 receptors, through receptor antagonist as a new treatment strategy for the TLE

Future Directions

Since drug delivery systems for therapy cannot target individual brain regions and cross talk between various peptide and neurotransmitter systems makes it much more difficult for single target drug therapies (Arzt and Holsboer, 2006), a deeper understanding of signal transduction pathways mediated by CRF system is quintessential for specific targeting to obtain greatest therapeutic benefits. Focusing on the downstream effects of CRF would help unravel the critical mechanisms underlying the facilitation of

CRF-meditated epilepsy not only in the EC, but other brain regions as well. Further experimentation with intracellular recordings is required to identify other kind of ligand-voltage gated ion channels involved in the CRF-mediated, cAMP/PKA-dependent modulation of epileptiform activity and increases in neuronal excitability.

Since our results show that CRF causes membrane depolarization, which could be due to the activation of cationic conductance or inhibiting background K^+ channels or both, leading to neuronal excitability. Firstly, if CRF-induced depolarization is due to the activation of cationic conductance, influx of extracellular Na^+ should be the major ions responsible for the membrane depolarization. This will be tested by replacement of extracellular NaCl with the same concentration of NMDG-Cl. Then test whether Ca^{2+} is involved, by substituting extracellular Ca^{2+} with the same concentration of Mg^{2+} and including 1 mM EGTA to chelate the ambient Ca^{2+}. Blockade of CRF-induced inward HC due to the above challenges suggests the role of specific cationic channel. If background K^+ channels are involved, CRF induced currents should have a reversal potential close to the K^+ reversal potential. We will measure the reverse potential using a ramp protocol (from −120 mV to 0 mV) to construct the voltage–current curve before and during the application of CRF (0.1 μM).Under these conditions, if CRF induces a current with a reversal potential close to that of theoretical K^+ reversal potential calculated by the Nernst equation, it suggests that CRF produces membrane depolarization by inhibiting background K^+ conductance. Using the classic K^+ channel blockers tetraethylammonium (TEA), 4-aminopyridine (4-AP) or Cesium (Cs+), the properties of K^+ channels involved

in CRF-induced depolarization would then be characterized, based on the different kinds of K^+ channels.

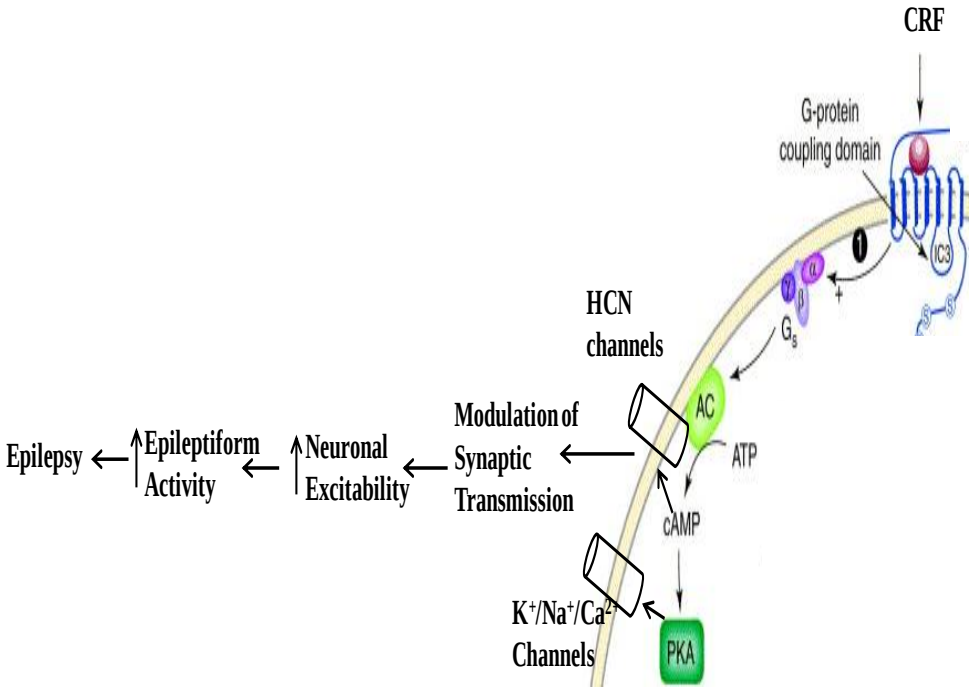


Figure 35. Summary and Future Directions

CRF has been shown to have diverse effects on synaptic transmission, resulting in neuronal excitability and epileptogenesis (Hollrigel et al., 1998). Using whole-cell recording techniques, the actions of CRF on synaptic transmission would be identified. To investigate the actions of CRF on glutamate-mediated synaptic transmission in the EC, spontaneous –miniature and evoked-monosyaptic excitatory post synaptic currents would be recorded from the layer-II stellate neurons. To investigate the actions of CRF on GABA-mediated synaptic transmission, spontaneous inhibitory post synaptic currents will be recorded from the layer-II stellate neurons.

Since our results show that CRF enhances I_h, using surface-protein biotinylation method, we will identify whether HCN channels are upregulated in the EC. EC shows HCN1 and HCN2 type predominantly. Using specific antibodies for these channels, we will identify whether there is CRF is involved in modulation of HCN surface channel expression in the EC.

Limitations of the study:

The in vitro model of epilepsy used in the current study have several advantages such as, ease of application of various concentrations of drugs, relatively intact local circuits of the tissue to visualize and obtain stable intracellular recordings from the layer-II stellate neurons without any anesthetics or immobilizing agents. These features helped to draw important conclusions regarding the role and relevance of CRF and its receptors as well as elucidate some of the molecular mechanisms involved in facilitation of epileptiform activity in the EC. However, the study has certain limitations when extrapolating the results. One of the major limitations is that the in vitro slice preparations lack intact functioning circuits of a normal brain, and are thus isolated from influences of other hormones, neurotransmitters or neuropeptides. Thus the results may differ from intact organism and need to be replicated in vivo, e.g., using CRFR transgenic mice to further elucidate the mechanisms underlying CRF-mediated facilitation of epilepsy in the EC, with further confirmation by human studies. Despite the major limitations, the in vitro slice models of epilepsy have been proven to be very useful as adjunctive approach to other models of epilepsy, due to the challenges involved in human studies.

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ABBREVIATIONS

aCSF	Artificial cerebral spinal fluid
ACTH	adrenocorticotropic hormone.
AEDs	Anti-epileptic drugs
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CA3	Cornu Ammonis
$CaCl_2$	Calcium Chloride
cAMP	cyclic Adenosine 3':5'-cyclic monophosphate
CNS	Central Nervous System
CO_2	Carbon dioxide
CRF	Corticotropin Releasing Factor
CRH	Corticotropin Releasing Hormone
CRF_1	Corticotropin Releasing Factor receptor 1
CRF_2	Corticotropin Releasing Factor receptor 2
DMSO	Dimenthyl sulfoxide
DG	Dentate gyrus
EDTA	Ethyelenediaminetetracetic acid

EGTA	Ethyleneglycoltetraacetic acid
EC	Entorhinal cortex.
FBS	Fetal bovine serum
GABA	gamma-Aminobutyric acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, buffer
HPA	Hypthalamic-pituitary-adrenal axis
HRP	Horseradish peroxidase
KCl	Potassium Chloride
KO	Knockout
$MgCl_2$	Magnesium Chloride
mEC	medial-Entorhinal Cortex
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium dihyrdogen phosphate
NMDA	N-Methyl-D-aspartic acid
O_2	Oxygen
PBS	Phosphate buffer saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PVN	Paraventricular nucleus

PTX	Picrotoxin
PVDF	Polyvinylidene fluoride
RMP	Resting Membrane Potential
SDS-Page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD	Standard Deviation
SEM	Standard error of mean
TBS-T	Tris buffered saline containing Tween 20
TA	Temporoammonic
TEMED	N,N,N’,N’-tetramethylene-diamine
TLE	Temporal lobe epilepsy
UV	Ultraviolet
WT	Wild-type