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Reorganization of inhibitory synapses in experimental epilepsy

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REORGANIZATION OF INHIBITORY SYNAPSES IN EXPERIMENTAL EPILEPSY

Monograph

by

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Abstract

The integrity and stability of interneurons in a cortical network is essential for proper network function. Loss of interneuron synaptic stability and precise organization can lead to disruptions in the excitation/inhibition balance, a characteristic of epilepsy. This study aimed to identify alterations to the GABAergic interneuron network in the piriform cortex (PC: a cortical area believed to be involved in the development of seizures) after kindling-induced seizures. Immunohistochemistry was used to mark perineuronal nets (PNNs: structures in the extracellular matrix that provide synaptic stability and restrict reorganization of inhibitory interneurons) and interneuron nerve terminals in control and kindled tissue. Results indicated that the expression of fully formed PNNs was significantly decreased in kindled tissue and was correlated with an increased expression of matrix metalloproteinase 9 (MMP9: a protease known to degrade extracellular matrix structures). Inhibition of MMP9 by the anti-inflammatory drug, Doxycycline, provided an antiepileptic effect against amygdala kindling. Additionally, we observed a layer specific increase in interneuron release sites. These data show an important loss of interneuron stability in the PC after seizures. Our data suggests that under pathophysiological conditions interneuron wiring patterns are plastic and their synaptic rearrangement may contribute to the development of epilepsy.

Keywords

Epilepsy, seizure, piriform cortex, kindling, immunohistochemistry, GABA, interneuron, perineuronal net, matrix metalloproteinase 9, doxycycline

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List of Abbreviations

ADAM:	A disintegrin and metalloproteinase domain
AGG:	Aggrecan
ANOVA:	Analysis of variance
CB:	Calbindin
CBP:	Calcium binding protein
ChABC:	Chondroitinase ABC
CNS:	Central nervous system
CR:	Calretinin
CSPG:	Chondroitin sulfate proteoglycan
DOX:	Doxycycline
Eph:	Erythropoietin-producing hepatocellular
GABA:	Gamma-Aminobutyric acid
IL-1β:	Interlukin 1 Beta
IL-6:	Interlukin 6
L1:	Layer 1
L2:	Layer 2
L3	Layer 3
LOT:	Lateral olfactory tract
LTP:	Long term potentiation
MMP:	Matrix metalloproteinase
PC:	Piriform cortex
PNN:	Perineuronal net
PTZ:	Pentylenetetrazol
PV:	Parvalbumin

TIMP:	Tissue inhibitor of metalloproteinase
TLE:	Temporal lobe epilepsy
TNFα:	Tumor necrosis factor alpha
VGAT:	Vesicular GABA transporter
WAG/ij:	Winstar Albino Glaxo Rijswijk
WFA:	Wisteria floribunda agglutinin

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1 Introduction

1.1 Epilepsy

The human brain is an assembly of billions of interconnected cells. It controls all organs of the body and allows us to think, perceive, remember, and communicate. The functional unit of the brain is the neuron and in order for the brain to perform these tasks properly, neurons must be highly interconnected and organized. Failure to remain organized often leads to disorders of the nervous system and neurodegenerative diseases. Epilepsy is a common neurological disorder that typically manifests as recurrent, unprovoked bursts of neuronal activity known as seizures. It is one of the world's oldest recognized disorders having been described by Hippocrates in the 5th century BC (Pitkanen & Lukasiuk, 2011). Today global prevalence is estimated at approximately 65 million people, of which 90% of cases are from low-income countries (Thurman *et al.*, 2011). Even with the currently available anti-epileptic drugs, approximately 30% of people with active epilepsy are drug refractory (WHO, 2009) requiring surgical brain resection for seizure control. Therefore there is an urgent need for basic research to understand the complex molecular mechanisms of this disease and to elucidate preventative or superior therapeutic strategies.

Epilepsy is a diverse disorder with over 40 recognized types (McCormick & Contreras, 2001). Most of these types can be broadly classified as partial (focal) or generalized depending if the seizures have a local or distributed origin, respectively (Morimoto *et al.*, 2004). Generalized seizures do not appear to originate from any one area in the brain but instead hypersynchrony occurs simultaneously in both cerebral hemispheres (Chang & Lowenstein, 2003). In contrast, partial seizures usually initiate in a small region of the cerebral hemisphere. The focus is thought to be the initiation source and surgical resection of this area can usually abolish seizures (Chang & Lowenstein, 2003). The cortex as well as limbic structures of the mesial temporal lobe (e.g. the hippocampus, entorhinal and perirhinal cortex) appear to be most vulnerable to hypersynchrony and are often the seizure foci (McIntyre & Poulter, 2001). Partial seizures can be further categorized into simple or complex partial seizures depending if there is a loss of

consciousness. Complex partial seizures may secondarily generalize into tonic (sustained contractions) and/or clonic (oscillating contractions and relaxations) seizures. Temporal lobe epilepsy (TLE) represents the most common and drug-resistant type of epilepsy (Morimoto *et al.*, 2004) and is the focus of this study. It is characterized by simple or complex partial seizures that can secondarily generalize (Engel *et al.*, 1997; Chang & Lowenstein, 2003).

Causes of epilepsy can be symptomatic or idiopathic. Symptomatic forms can result from events such as head trauma, infections, ischemia, febrile seizures and hemorrhages (Vinters *et al.*, 1993). Causes of epilepsy can also be genetic or age-dependent. Although there may be a great diversity in the pathologies leading to epilepsy, seizure activity does not spread randomly throughout the brain but rather appears to be generated and propagated by specific anatomical routes (Loscher & Ebert, 1996; Lothman *et al.*, 1991; Gale, 1988). Therefore it is believed that the actual generation of seizures occurs through common cellular mechanisms and networks (McCormick & Contreras, 2001).

Epileptogenesis is the process by which the brain is altered from a normal neural network to a hyper-excitable, epileptic one (Clark & Wilson, 1999). Following an initial injury, there may be an initial period of months or years before onset of the first seizure, which is known as the latent period (Pitkanen & Lukasiuk, 2011). During this latent period, it is believed that there are progressive changes in neuronal structure, connections and functions that eventually lead to seizures. This reconstruction or modification of neural networks can change circuit properties (McIntyre *et al.*, 2002b; Racine *et al.*, 2002) and ultimately create an imbalance of excitation and inhibition (Tasker & Dudek, 1991; Colmers & El, 2003; Morimoto *et al.*, 2004; McNamara, 1994) often leading to excessive hypersynchrony. Prevention and treatment of epilepsy relies on understanding what happens to the brain during epileptogenesis.

1.2 Animal Models of Epilepsy

The study of epilepsy and epileptogenic mechanisms requires the use of animal models that closely mimic the disease. Animal models attempt to imitate human epileptic

disorders by having similar aetiology, behaviour, anti-epileptic drug mechanisms and pharmacological resistance; however, no single model can completely recapitulate all aspects of seizures in humans (Loscher, 1997). The most common animal models are: (1) the post-status epilepticus model (2) genetic phenotype models and (3) the kindling model (Morimoto *et al.*, 2004; Sharma *et al.*, 2007). In all of these cases seizure discharges can produce many effects including changes in expression of mRNA and proteins, neuron firing properties and circuit reorganization in response to the excess excitation (Morimoto *et al.*, 2004).

The post-status epilepticus models work by injecting a chemical convulsant such as pilocarpine (muscarinic acetylcholine receptor agonist) or kainic acid (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; a kainate receptor agonist). These convulsants produce a prolonged period of acute seizures known as status epilepticus, and after a latent period the animal usually has secondarily generalized spontaneous seizures. Post status epilepticus models are useful because they mimic human TLE patients in that there is observed neuronal loss in the CA1, CA3 area of the hippocampus, dentate gyrus neurogenesis, and mossy fiber sprouting (Turski *et al.*, 1983; Ben-Ari, 1985; Cronin & Dudek, 1988). However the chemical convulsants also produce extensive damage in the neocortex, olfactory cortex, amygdala, thalamus, and substantia nigra which is not typical of patients with TLE (Sharma *et al.*, 2007). Additionally, when using this model a substantial proportion of the animals die during the acute seizure phase.

Several inbred or selectively bred rat lines have been used to study epilepsy. These include the Wistar Albino Glaxo/from Rijwijk (Wag/Rij) rats, the genetic absence epilepsy rats from Strasbourg (GAERS), the genetically epilepsy-prone rats (GEPR), the swim lo-active rats (SwLo) and strains of fast and slow kindling rats. Additionally, many more mouse strains have been developed. All of these genetic animal models were selectively bred based on their epilepsy-like phenotypes.

In this study we used the widely accepted kindling of the basolateral amygdala as a model to recapitulate TLE and activity-dependent synaptic plasticity. Kindling represents a progressive and permanent development of convulsive motor seizures following daily

electrical stimulations of a particular subcortical brain area (Goddard, 1967). In electrical kindling, electrodes are implanted in the brain and current is delivered to induce a focal seizure. Typically, the first stimulation does not evoked a behavioural convulsion, but with repeated stimulations the afterdischarge increases in duration and complexity accompanied by behavioural convulsions of increasing severity (Goddard, 1967; Morimoto *et al.*, 2004). An important feature of kindling is that there is increased propagation of epileptic waves from the epileptic focus to other areas in the brain and these new sites are recruited into the discharge, eventually leading to increased sensitivity to the focal electrical stimulation (Sato *et al.*, 1990).

Five stages of seizures have been characterized in the kindling model (Racine, 1972). The stages are described as: (1) mouth and facial movements, (2) head nodding, (3) forelimb clonus, (4) rearing, (5) rearing and falling (Racine, 1972). The fifth stage is analogous to a full tonic-clonic motor seizure and at this stage the animal is considered fully kindled. However, if kindling is continued several weeks after stage 5, known as the over-kindling protocol, the animal eventually will display spontaneous seizures (Pinel & Rovner, 1978). The kindling model is not associated with significant cell loss but over-kindling to the spontaneous seizure stage is accompanied with substantial neuronal loss, reminiscent of the status epilepticus models. The same cellular mechanisms that establish and maintain kindling in animals have been suggested to be involved in partial epilepsy in humans (Sato *et al.*, 1990; Adamec, 1990).

Some advantages to using the kindling model over post-status models are that specific sites can be targeted for focal activation, the development of chronic epileptogenesis can be observed, and there is little to no brain damage that might contribute confounding influences (Racine *et al.*, 1999). Additionally, interictal (between seizure) spikes are often observed in kindled animals. These spikes are synchronous discharges of a group of neurons in the region of the epileptic focus and can be used to diagnose epilepsy in humans (Staley & Dudek, 2006). However the disadvantages of this model are that basic kindling does not produce spontaneous seizures or hippocampal sclerosis as often seen in humans with epilepsy. Additionally, kindling takes considerably more time to produce an "epileptic" rat.

The importance of various brain structures to the spread of epileptic waves can be assessed by their intrinsic kindling abilities, the required afterdischarge threshold, and by the appearance of interictal spikes (Loscher & Ebert, 1996). Many areas can be used as the seizure focus in kindling, however the piriform, perirhinal and insular cortices have been shown to kindle at very high rates (McIntyre & Plant, 1993).

1.3 Piriform Cortex

The piriform cortex (PC) is the area of focus for this study. It is a phylogenetically old area of the brain in the paleocortex. It is located at the basolateral surface of the forebrain, extending over 5 mm ventral to the rhinal fissure in rodents (Paxinos & Watson, 1986). The PC is part of the limbic system and is main area responsible for olfactory perception and discrimination, receiving direct input from axons of the olfactory bulb carried through the lateral olfactory tract (LOT) (Neville & Haberly, 2004). It has also been implicated in learning, memory processing, and the development of seizures (Racine *et al.*, 1988;Loscher & Ebert, 1996).

There is similar cellular morphology, physiology and circuitry between the six-layered neocortex and the PC. However the PC is characterized by a simple, three-layered laminar structure, serving as a simple model of the more complex neocortex. Layer 1 (L1) contains many afferent fibers from the olfactory bulb and dendrites from pyramidal cells in deeper cortical layers but only few GABAergic interneurons (Loscher & Ebert, 1996;Neville & Haberly, 2004). Layer 2 (L2) is densely packed with the somata of glutamatergic pyramidal cell bodies and some GABAergic cell bodies with extensive interneuron arborization (Ekstrand *et al.*, 2001). Layer 3 (L3) contains both pyramidal cells and a large number of GABAergic cell bodies (Ekstrand *et al.*, 2001). Each layer of the PC has a different density and distribution of interneurons (Gavrilovici *et al.*, 2010).

The major source of afferent input to the PC comes from cells of the olfactory bulb via the LOT and stimulation of the LOT can activate almost all parts of the PC (Loscher & Ebert, 1996). The PC also receives intrinsic synaptic connections and projections from the anterior olfactory cortex, basal forebrain, thalamus, hypothalamus, and brainstem.

Importantly for basolateral amygdala kindling, strong projections from the amygdala indirectly connect to the PC via the entorhinal cortex. Output from the PC goes back to the olfactory bulb and to areas of the limbic system. Therefore the PC has many reciprocal connections with the olfactory system, neocortex, limbic system, hypothalamus, and motor systems (Loscher & Ebert, 1996; Luskin & Price, 1983). This complex integrative circuitry may be responsible for mediating functions related to integrating odour cues with behaviour and memory (Luskin & Price, 1983). In addition to its strong limbic connections, the PC also has a high degree of intrinsic interconnections (Haberly, 2001a).

For the investigation of epilepsy, we chose to study the PC because of its highly epileptic nature and its innate ability to support seizures (Piredda & Gale, 1985; Hoffman & Haberly, 1993; Demir *et al.*, 1999; Ekstrand *et al.*, 2001). The PC contains the most susceptible neural circuit of all forebrain regions for the development of limbic seizures (for review see Losher & Ebert, 1996; Racine *et al*., 1989). During electrical stimulation of other limbic structures large afterdischarges can be observed in the PC indicating that the structure is active early in kindling. Additionally, using the expression of immediate-early genes (e.g. c-fos) the PC was again shown to activate early during limbic stimulation as it was the first region to exhibit the induction of these genes (Loscher & Ebert, 1996). Furthermore, interictal discharges with the largest amplitude originate in the PC independent of which structure is being stimulated (Racine *et al.*, 1988). The PCs strong connections to many limbic structures and its massive recurrent excitatory connections with reliance upon inhibition for its regulation may also contribute to the seizurogenic environment (Loscher & Ebert, 1996; Haberly, 2001b; Luskin & Price, 1983). Finally, the PC has the ability to have synaptic connections strengthened or weakened with repetitive activation, which may be relevant for the spread of limbic seizures. Therefore the PC appears to play a role in initiating and maintaining seizure propagation in the brain. A better understanding of this structure and how it is affected by seizure induction will assist in our understanding of epileptogenesis.

1.4 GABAergic Interneurons

As in other cortical areas the PC contains glutamate-releasing principal neurons and a smaller number of gamma-aminobutyric acid (GABA)-releasing inhibitory neurons (Suzuki & Bekkers, 2007). Pyramidal cells are the most abundant type in the cortex and form excitatory synapses on other neurons. These cells have a pyramidal shaped soma, and dendrites covered with spines. In human TLE there appears to be a massive loss of pyramidal cells, but in contrast GABAergic interneurons are quite well preserved (Babb *et al.*, 1989; Cossart *et al.*, 2005; Sloviter *et al.*, 1991). Nevertheless, alterations to the GABAergic system have been associated with epileptic disorders including changes to GABA neurotransmission, receptor subunits, firing properties, and synaptic connections. The function and alterations of these inhibitory interneurons are the focus of this study.

Areas of the brain such as the thalamus, basal ganglia and cerebellum exhibit a low degree of interneuron variability. However, in the cortex there are many identified heterogeneous populations of interneurons, constituting approximately 20% of all neurons in the cortex (Buzsaki *et al.*, 2004). They cannot be studied the same way as their glutamatergic releasing counterpart due to their heterogeneity in biophysical, electrophysiological, and neurochemical properties which may be selective for a specific morphology, possibly reflecting a division of labour into their functional "responsibilities" (Cossart *et al.*, 2005). However, despite this heterogeneity there are common traits among all types of interneurons. Firstly, all interneurons are GABAergic meaning their main neurotransmitter released upon activation is GABA. The release of GABA opens GABAa receptors on the post-synaptic membrane and depending on the chloride gradient, this allows for passage of chloride into the cell. There is usually a higher concentration of extracellular chloride, therefore causing an influx of negative charge. This causes a transient hyperpolarization and an inhibitory effect on the target membrane. GABA can also bind to GABAb receptors on the post-synaptic membrane to stimulate the opening of K^+ channels, preventing Na^{2+} channels from opening and ultimately also causing a hyperpolarization. Secondly, interneurons usually lack the long apical dendrite and projecting axons typical of pyramidal cells, therefore exerting their

effects locally. Interneurons also fire at high frequencies compared to pyramidal cells, activate at low thresholds, and are highly interconnected (Buhl *et al.*, 1994).

In the PC, interneurons exert their effects by participating in inhibitory loops according to their laminar location. The feed forward inhibitory loop begins with lateral olfactory tract activation of layer 1 or layer 2 interneurons with a dendritic tree expanded in layer 1. This gives rise to inhibitory synapses on adjacent pyramidal cells. The feedback inhibitory loop occurs when pyramidal cell activation is spread along its collaterals, exciting the surrounding interneurons which in turn inhibit the pyramidal cells. GABAergic interneurons are capable of having a variety of synaptic connections on pyramidal cells and each other, such as axo-somatic, axo-dendritic, dendo-dendritic etc.

Interneurons use these inhibitory loops to perform a range of functions including basic preservation of an action potential, signal integration, synaptic plasticity, network oscillation, and synchronization (Cobb *et al.*, 1995; Klausberger *et al.*, 2003; Whittington & Traub, 2003). Simply, interneurons prevent excess excitation by regulating excitability and rhythmicity of neural networks. This is possible because many pyramidal cells are contacted by each interneuron. Therefore any alteration to the GABAergic network may lead to an imbalance of the inhibition/excitation balance resulting in the generation of "runaway" excitation (McCormick & Contreras, 2001).

Interneurons can be characterized many different ways such as their morphology, physiology, axonal targets or by their neurochemical content. However none of these classifications are entirely satisfactory given the overlap of some features. Here we used calcium binding proteins (CBPs) as molecular markers. CBPs are useful in differentiating mostly non-overlapping subtypes (McBain & Fisahn, 2001) and are characterized by the presence of a variable number of helix-loop-helix motives binding Ca^{2+} ions with high affinity. The CBPs parvalbumin (PV), calbindin (CB), and calretinin (CR) are expressed in neurons that also express GABA, but are not present in neurons that express glutamate (McBain & Fisahn, 2001). Each of these interneuron subtypes has its own innervation pattern and unique behaviour within the PC (Gavrilovici *et al.*, 2010). A recent study in our lab characterized the distribution and innervation patterns of these interneuron

subsets within the PC (Gavrilovici *et al.*, 2010). PV labeled cell bodies are found predominantly in layer 3 of the PC but many can also be found in layer 2. These interneurons are generally multipolar cells with a circular dendritic shape. CB labeled cell bodies are also predominantly found in layer 3 but a few can be found in layer 2. These interneurons can have various cell types but are horizontally shaped and their dendrites are usually confined to the same layer as their soma. Their broad dendritic arbour likely allows these cells to integrate inputs from a large area in layer 3. CR cell bodies can be found in all three layers of the PC. They have a large presence in layers 1 and 2 but few can be found in layer 3. These are bipolar or bitufted cells with vertical or oval shaped dendritic trees usually traversing the layers. Their restricted horizontal expansion but large vertical expansion likely allows them to integrate processes between the layers. All GABAergic interneuron presynaptic nerve terminals can be marked using the vesicular GABA transporter (VGAT). This protein is responsible for loading GABA into synaptic vesicles before being exocytosed (McIntire *et al.*, 1997).

It was recently shown that kindling results in a loss of interneuron functional diversity (Gavrilovici *et al.*, 2012). Normal interneurons have the ability to fire in five different patterns (non-adapting very high frequency, adapting high frequency, adapting low frequency, strongly adapting low frequency, and weakly adapting low frequency). However, after kindling these interneurons lose diversity and are only able to fire in three different patterns (adapting low frequency, adapting high frequency, and strongly adapting low frequency) (Gavrilovici *et al.*, 2012). These changes may be accounted for by a significant increase in the expression of the Kv 1.6 potassium channels on kindled interneurons. This demonstrates functional plasticity in interneurons. However it still remains unknown whether these interneurons' wiring patterns are also plastic after kindling.

Given the crucial role of GABAergic interneurons in orchestrating the activity of pyramidal cells within the cortex, perturbations to these cells can cause serious neurological deficits.

1.5 Perineuronal nets

Aberrant plasticity has been postulated to underlie the formation of epileptic foci in the temporal lobe (Wilczynski *et al.*, 2008) therefore molecules involved in synaptic plasticity may give clues to a better understanding of the disease.

During development the extracellular matrix (ECM) is essential for many neuronal processes such as proliferation, migration, synaptogenesis, synaptic stability and cell signalling (Dityatev & Schachner, 2003; Dityatev & Schachner, 2003; McRae & Porter, 2012). The ECM of the nervous system is comprised of a complex mixture of proteoglycans, glycoproteins, tenascin, fibronectin and hyaluronan (Galtrey & Fawcett, 2007). In the mature ECM these molecules can either be diffusely dispersed or can form tight net-like structures (Deepa *et al.*, 2006). The net like structures surround cell bodies, dendrites and axon initial segments leaving open "holes" at sites of synaptic contact (Zaremba *et al.*, 1989; Celio & Blumcke, 1994; Hockfield & McKay, 1983; Wang & Fawcett, 2012). These structures are called perineuronal nets (PNNs) and are found in virtually all regions of the CNS but only surrounding a subset of neurons. PNNs are most often displayed around inhibitory GABAergic neurons, specifically those interneurons that also express the calcium binding protein PV (Bruckner *et al.*, 1993; Dityatev, 2010). It has been suggested that PNNs are required specifically around PV expressing interneurons to maintain high rates of action potential firing observed from these cells as PNNs are capable of controlling diffusion of ions (Morris & Henderson, 2000).

PNNs are mainly comprised of hyaluronan, link proteins, tenacin-R and proteoglycans (Koppe *et al.*, 1997; Deepa *et al.*, 2006; Wang & Fawcett, 2012). Hyaluronan synthase on the cells membrane anchors PNNs to the cell and is responsible for synthesizing and secreting hyaluronan to the pericellular environment. Hyaluronan is a disaccharide polymer made of repeating N-acetlyglucosamine and glucuronic acid. The N-terminal of proteoglycans binds to the hyaluronan polymer chains and link proteins appear to stabilize these interactions (McRae & Porter, 2012). The C-terminal of proteoglycans then in turn bind to tenacin-R allowing for cross-linking and the formation of large aggregates on cell membrane surfaces (Wang & Fawcett, 2012). Proteoglycans contain a

core protein with covalently linked glycosoaminoglycan side chains of varying number and length. These chains can be made of heparin, keratin or chondroitin sulfate (Karetko & Skangiel-Kramska, 2009). Chondroitin sulfate proteoglycans (CSPG) are also known as lecticans and are the most abundant protein of the ECM in the CNS. The family of lecticans includes: aggrecan, versican, neurocan and brevican, differentiated mainly by their number of glycosaminoglycan side chains (Yamaguchi, 2000). Aggrecan appears to be an important lectican because it is present in almost all PNNs, where other lecticans are only found in subpopulations of PNNs (Galtrey *et al.*, 2008; Wang & Fawcett, 2012). PNNs can be visualized in a variety of ways. Here we have used both a lectin from *Wisteria floribunda agglutinin* (WFA), which has an affinity for N-acetylgalatosamine, and a primary antibody that recognizes the core protein aggrecan (AGG).

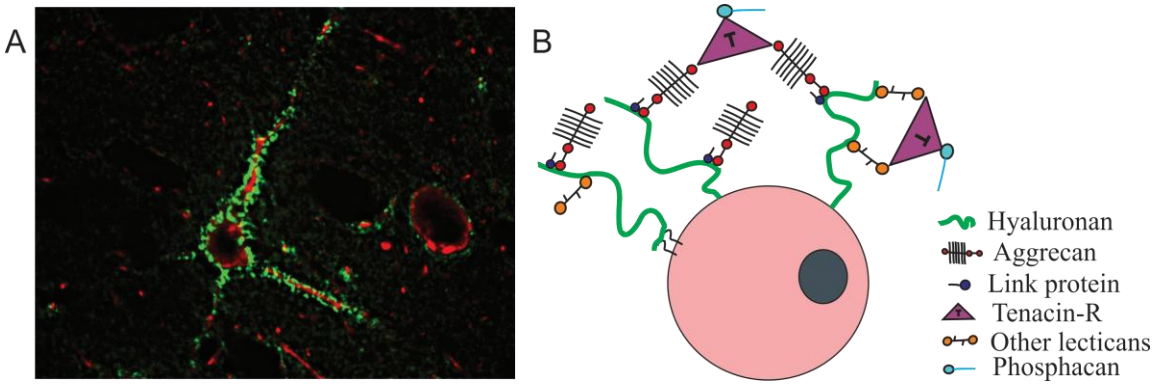

Figure 1. Structure of a perineuronal net in the piriform cortex. (A) WFA stained PNN (green) surrounds the soma and proximal dendrites of a GABAergic interneuron (red) in the PC. (B) A cartoon of the structural composition of a PNN in the CNS. The protein hyaluronan is bound to the cell membrane via hyaluronan synthase. The N-terminal of different proteoglycans is then bound to hyaluronan which is stabilized by link proteins and cross-linked with tenacin-R. The cartoon PNN was adapted from Wikipedia.

Many components of the PNN can be detected in neonatal animals. However, tight aggregation around neurons does not form until later in life. PNNs temporal formation corresponds to the end of the "critical period." The critical period signifies a time when essential experiences drive synaptic formation, refinement and maturation, leading to the organization of neuronal networks (Blue & Parnavelas, 1983; Bignami *et al.*, 1993;

Pizzorusso *et al.*, 2002; Nowicka *et al.*, 2009). The critical period is therefore considered to be a time when the CNS is particularly plastic and when a specific repertoire of synaptic connections is selected for from a wider range and made permanent. PNN formation is not only temporally dependent but also activity dependent. Lack of essential experiences, such as dark rearing an animal during early development or whisker trimmer, has been shown to cause inappropriate synaptic stabilization and a reduced the number of PNNs (Zaremba *et al.*, 1989; Pizzorusso *et al.*, 2002; McRae *et al.*, 2007).

The exact function of PNNs has yet to be determined. However, its temporal appearance, repellent properties against approaching axons/dendrites and highly negatively charged microenvironment leads us to believe that it is likely involved in synaptic stabilization (Karetko & Skangiel-Kramska, 2009). An important study by Pizzorusso *et al.* 2002 showed that in the visual cortex, the organization of chondroitin sulfate proteoglycans into PNNs coincided with the end of the critical period. However, when animals were reared in the dark the end of the critical period for the visual cortex and the appearance of PNNs could be delayed. Additionally, they showed that monocular deprivation was able to cause an ocular dominance shift in adult rats after chondriotinase-ABC (ChABC), a bacterial enzyme isolated from Proteus vulgaris, was used to degrade chondroitin sulfate proteoglycans (Pizzorusso *et al.*, 2002). Without prior degradation of the proteoglycan containing PNNs, monocular deprivation did not cause any effects in adult rats. Another study showed that fear conditioning (by pairing an ambient stimulus with a painful stimulus) can be reduced or erased in early post-natal animals but not in mature animals. Concurrently, the establishment of PNNs in the basolateral amygdala corresponds with the inability to erase fear memories, suggesting that PNNs protect fear memories in adults. The use of ChABC therefore allows subsequent elimination of fear memories in adults (Gogolla *et al.*, 2009; Wang & Fawcett, 2012). Therefore these studies showed that the degradation of PNNs can reactivate cortical synaptic plasticity, giving further evidence for the role of PNNs in synapse stabilization and plasticity prevention.

The mature ECM is inhibitory for activity-dependent plasticity, however an injury may reactivate mechanisms that had previously been in operation during development. During epileptogenesis it is believed that the brain undergoes synaptic rearrangement which

requires permissive extracellular space. Therefore alterations occurring in the ECM, specifically those that effect PNN structure may contribute to the plasticity necessary for epileptogenesis and eventually seizures.

1.6 Ephrins

During development axons navigate towards specific targets. They reliably find these targets by following attractive or repellent cues directed from axon guidance molecules. The axon guidance molecules ephrin and their erythropoietin-producing hepatocellular (Eph) receptors are the largest family of receptor tyrosine kinases. They are distributed widely in the CNS during development and to a lesser extent in the adult brain. These proteins are localized both pre- and post-synaptically and are also expressed by glia (Hruska & Dalva, 2012). During development these molecules are implicated in cell migration, axonal outgrowth and path finding, topographic mapping and axon fasciculation (Knoll & Drescher, 2002). In the adult brain Eph receptors and their ligands may play important role in the maintenance of neuronal connections such as synapse formation and regulation, although exact mechanisms remain unclear.

The family of Eph receptors included 14 members which are subdivided into A-subclass (EphA1-A8, A10) and B-subclass (EphB1-B4, B6). These receptors are assumed to have partially redundant functions in the CNS (Kullander & Klein, 2002). The corresponding membrane-bound ephrin ligands are also subdivided into A-subclass (A1-A5) and B-subclass (B1-B3) based on their binding motifs. Ephrin As are glycosylphosphatidylinositol (GPI) anchored and lack a cytoplasmic domain, whereas ephrin Bs are transmembrane tethered with a short cytoplasmic tail. In general, ephrin As bind to EphA receptors and ephrin Bs bind to EphB receptors however, there may be some degree of promiscuity (Kullander & Klein, 2002).

The ephrin ligands must be membrane bound for proper function. The containment of ephrins in raft microdomains allows for clustering and unique functional properties of the proteins. When the Eph receptors and their membrane bound ligands interact, intracellular signals can be propagated through both the Eph receptor containing cell

(forward signaling) and the ephrin ligand containing cell (reverse signaling). This bidirectional signaling is mediated by tyrosine kinase activity on the Eph receptor cell and by the ability of ephrins to recruit signaling effectors on the other cell (Bruckner *et al*., 1997; Kullander & Klein, 2002).

In the mature brain Eph receptors and their ligands may have important roles in synaptic plasticity. For example, pervious work has shown that presynaptic ephrin Bs are necessary for the induction of long term potentiation (LTP) in the hippocampus (Armstrong *et al.*, 2006). When ephrin Bs cytoplasmic domain was mutated, deficits in LTP were observed. It was suggested that signaling downstream from ephrin B3 might recruit more neurotransmitter vesicles to presynaptic active zones and thus increase synaptic transmission and induction of LTP (Hruska & Dalva, 2012). Therefore mutating the protein would disrupt this recruitment. Additionally, the postsynaptic expression of ephrin B3 may play a role in controlling synaptic density. Neurons with a greater postsynaptic expression of ephrin B3 appeared to have a competitive advantage enabling the reception of more synapses (McClelland *et al.*, 2010). Furthermore, another study showed that antagonizing EphA receptors impaired the performance of mice in two behavioural paradigms (Gerlai *et al.*, 1999). Conversely, the activation of EphA receptors enhanced the performance of mice on these tasks. Therefore EphA receptors are likely necessary for learning and LTP (Gerlai et al., 1999).

The synaptic localization and ability to coordinate events on both sides of the synapse suggests that Eph receptors and their ligands have important roles at the synapse and are involved in the regulation of synaptic function. Indeed deficits in these proteins have been found in a number of neurological disorders. Xu *et al* (2003) demonstrated that EphAs and ephrin As were perturbed in an epilepsy model. By antagonizing EphA receptors they were able to delay the development of behavioural seizures by perforant path stimulation. Inversely, by antagonizing ephrin A ligands they showed a dose dependent effect, where the higher doses assisted in the development of seizures. Therefore Eph receptors and their ligands appear to be necessary not only in axon guidance during development but also in synaptic plasticity and perturbations to their actions may contribute to plasticity observed in acquiring seizures.

1.7 Matrix Metalloproteinase 9

Metalloproteinases are a large family of proteases that include matrix metalloproteinases (MMPs) and proteins with a disintegrin and metalloproteinase domain (ADAMs). MMPs are structurally related, zinc-dependent proteinases. Their shared structure has four domains: the propeptide, catalytic, haemopexin-like and transmembrane domains. These proteins are initially expressed as inactive pro-MMP zymogens but are activated when the cysteine-zinc interaction is disrupted and their catalytic site is exposed. MMPs are essential for normal biological processes such as embryonic development, morphogenesis and tissue remodeling (Yin *et al.*, 2011) and may play an important role in synaptic plasticity, learning and memory (Szklarczyk *et al.*, 2002; Meighan *et al.*, 2006; Nagy *et al.*, 2006). They are considered to be major players in ECM remodeling (Birkedal-Hansen *et al.*, 1993; Chakraborti *et al.*, 2003; Wilczynski *et al.*, 2008), degrading adhesion and signaling molecules, neurotransmitters, and growth factors (Nagase & Woessner, Jr., 1999; Michaluk *et al.*, 2009; Takacs *et al.*, 2010). However, MMPs have also been implicated in many neurodegenerative pathologies including ischemia, trauma and Alzheimer's disease (Yong, 2005). Since epileptogenic plasticity involves extensive nervous tissue remodeling MMPs may play an essential role in epilepsy (Ben-Ari, 2001).

The expression of MMPs are largely absent from the normal CNS and their activity is tightly regulated due to their potent proteolytic potential. They are first regulated at the transcription level. Since MMPs are not constitutively expressed, the promotor region of inducible genes that encode MMPs generally contain binding sites for transcription factors that are responsive to oncogenes and cytokines. For example, tumor necrosis factor alpha (TNFα) can induce the transcription of MMP9 (Yong *et al.*, 2001). Their transcription can also be promoted by growth factors, chemokines, and cell-cell or cell-matrix interactions (Yong *et al.*, 2001). MMPs are also regulated post translationally since they are initially expressed as inactive zymogens. Lastly, they are controlled by the endogenous tissue inhibitor of metalloproteinases (TIMPs).

MMP2 and MMP9 are the most abundantly expressed MMPs in the brain and have received attention because of their role in injury and repair (Rivera *et al.*, 2010). MMP9

appears to be primarily but not exclusively expressed by neurons, whereas MMP2 originates primarily from glia cells (Szklarczyk *et al.*, 2002). MMP9 has attracted attention because it was shown to cleave ECM molecules in and around the synaptic cleft. It is therefore assumed to be necessary in the cascade of events leading to the formation of a new synapse. This MMP was also shown to be essential for long-term potentiation. Specifically, the inhibition of MMP9 prevented the acquisition of spatial memories during the Morris water maze (Wright *et al.*, 2007; Meighan *et al.*, 2006; Mizoguchi *et al.*, 2011).

There is evidence now to suggest that MMP9 is involved in epileptogenesis. Wilczynski *et al.* (2008) showed that MMP9 facilitated chemical kindling in vivo. They demonstrated that MMP9 knockout mice were insensitive to pentylenetetrazol (PTZ) kindling and MMP9 transgenic rats with a constitutive neuronal MMP9 overexpression had an increased susceptibility to epileptogenesis. Additionally, in studies with children suffering from febrile seizures and convulsive status epilepticus, MMP9 and the ratio to its endogenous inhibitor, tissue inhibitor of metalloprotinase-1 (TIMP1), were shown to be increased in the serum (Suenaga *et al.*, 2008). Therefore it seems that MMP9 plays an important role in the development of epileptic seizures and associated plasticity.

Neuroinflammation can contribute to the pathogenesis of epilepsy. Insults to the CNS cause neuroinflammation which can increase neuroexcitability and enhance seizure susceptibility. The tetracycline derivative Doxycycline (DOX) is a commonly prescribed antimicrobial drug that has anti-inflammatory actions independent of its antimicrobial actions. It is the most potent broad-spectrum MMP inhibitor (Golub *et al.*, 1991). Although it is nonselective, it predominantly inhibits MMP2 and MMP9 (Lokeshwar *et al.*, 2002; Seftor *et al.*, 1998). Various studies have shown that DOX reduces inflammation by binding to the active zinc site on MMPs causing a conformational change and a loss of enzymatic activity. DOX has been shown to be neuroprotective in a variety of disease states. For example, pre-treatment with DOX reduced the total infarct volume following cerebral ischemia in rats (Burggraf *et al.*, 2007). It also was shown to help control inflammatory responses associated with neurocysticercosis (Alvarez *et al.*, 2009). Furthermore, DOX is protective against pilocarpine-induced convulsions

(Nogueira *et al.*, 2011). Specifically, pre-treatment of DOX prior to pilocarpine injection in rats significantly increased latency to the first convulsion and latency to death (Nogueira *et al.*, 2011). Therefore, the inhibition of MMPs by DOX may have potentially therapeutic properties in the prevention of seizures.

1.8 Rationale and Hypothesis

It has recently been discovered that there is a loss of PNN expression in the hippocampus after the experimental induction of a post-status seizure. However the effects of a less invasive seizure model on the PC have yet to be determined. Additionally, to our knowledge no one has looked at the effects of kindling and PNN loss on the GABAergic inhibitory release sites.

We hypothesize that kindling induces a breakdown in the extracellular matrix (specifically of PNNs) in the PC and the loss of PNNs is associated with an alteration in the innervation patterns of GABAergic inhibitory synapses. Specifically we asked: (1) Is the extracellular matrix of the piriform cortex plastic after kindling-induced seizures? (2) Are there changes to the layer specific inhibitory synaptic innervation after kindling-induced seizures? (3) If so, what is/are the mechanism(s) responsible for the rearrangement and can they be reversed?

2 Materials and Methods

2.1 Animals and Surgery

Male Sprague Dawley rats from Charles River weighing approximately 200g at the time of initial surgery were used. They were housed in standard plastic cages with free access to food and water under a continuous 12h/12h light/dark schedule.

2.1.1 Electrode implantation

Animals were anaesthetized with ketamine (100mg/kg, i.p)/domitor (10mg/kg, i.p) and implanted with two bipolar stimulating/recording electrodes bilaterally in the basolateral amygdala using the following coordinates: 2.6 mm posterior to Bregma, 4.5 mm lateral to midline and 8.0 mm ventral (Paxinos & Watson, 1986). Electrodes were made from two twisted strands of Diamel-insulated Nichrome wire with a diameter of 0.127 mm. They were attached to male Amphenol pins. Once implanted the electrodes were secured to the skull with jewellers screws and the electrode assembly was fixed to the skull using dental acrylic cement (McIntyre & Molino, 1972).

2.1.2 Kindling

The kindling procedure began at least 10 days after surgery. An afterdischarge threshold (ADT) was determined in each amygdala by delivering a 3s 60-Hz sine wave stimulus of progressive intensity (25, 50, 75, 100, 250, 500, 750 μA) until an afterdischarge and behavioural response was triggered (McIntyre & Plant, 1993). The rats were stimulated unilaterally daily until three generalized stage 5 seizures occurred. The stimulated side of the brain (ipisilateral) was considered the “kindled” tissue where as the unstimulated side of the brain (contralateral) was considered “partially kindled” tissue. The partially kindled tissue was evaluated since some kindling effects have been observed on the contralateral side even without direct stimulation. Though, contralateral afterdischarges do not develop in parallel with the ipsilateral side, at least before seizures become generalized. A minimum of 10 days were passed post-kindling before kindled rats were sacrificed.

2.2 Immunohistochemistry

2.2.1 Tissue Preparation and Fixation

Male Sprague Dawley rats were deeply anesthetised with ketamine (100mg/kg, i.p)/domitor (10mg/kg, i.p) and perfused intracardially with heparinized saline, followed by LANA's fixative (4% paraformaldehyde, 20% picric acid). The brain was quickly removed and post fixed in LANA's fixative for 24 hours, then transferred to a 30% sucrose phosphate buffer for 48 hours. Brains were then flash frozen in -80°C isopentane for 50 seconds and stored at -20°C until sectioning. The tissue was sectioned on a cryostat at -15°C in the coronal plane into 60 µm sections. Free-floating sections were placed in tissue culture dishes with a cryoprotectant solution of 35% sucrose and 35% ethylene glycol in 0.1M phosphate buffer, and stored at -20°C until they were ready to be processed.

2.2.2 Immunohistochemistry

Slices were washed 2 x 5 minutes in 0.5% triton x-100, then blocked with 10% donkey or goat serum in 0.025% triton x-100 in 1% BSA in 1 x PBS for 1 hour at room temperature. Slices stained with aggrecan (AGG) antibodies were pre-treated with 0.2 U/ml Chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) at 37°C for 40 minutes prior to blocking. The primary antibody solutions were diluted in 1% BSA in 1 x PBS and pipetted into tissue culture dish wells. Sections were incubated overnight at 4°C. Sections were then washed 2 x 5 minutes in 0.5% triton x-100, followed by an application of secondary antibody diluted in 1% BSA in 1 x PBS and incubated for 1 hour at room temperature under low-light conditions. Sections were then washed 3 x 10 minutes in 1 x PBS. To reduce lipofusion autoflorescence, sections were placed in 1% Sudan Black B dissolved in 70% ethanol for 1.5 minutes. Sections were then washed 2 x 1 min in 70% ethanol, and 1 x 2 min in 70% ethanol. This was followed by 2 washes in 1 x PBS for 5 min each. All washes and incubations for immunohistochemistry took place on an agitator to ensure thorough washes and maximal antibody binding. Sections were wet mounted onto Fisher SuperFrost Plus slides and mounted with glass coverslips in Prolong

gold Antifade with DAPI mounting medium (Molecular Probes, Eugene, OR). Slides were stored protected from light at -20°C until they were imaged.

2.2.3 Antibodies

Free-floating coronal sections were labeled with the following antibodies (refer to Table 1.): Mouse antiVGAT, guinea pig antiPV, mouse antiPV, rabbit antiCB, rabbit antiCR, lectin for WFA, mouse antiAGG, rabbit antiEFNA5, rabbit antiEFNB3, and rabbit antiMMP9.

Table 1. Primary antibodies and the concentrations used in this study

Protein	**Primary Antibody**	**Cat No.**	**Immunogen**
Vesicular GABA Transporter/Parvalbumin	AntiVGAT; mouse; 1:200; Synaptic Systems, Germany. AntiPV; guinea pig; 1:2500; Chemicon, Canada	131 011 /AB9314	Synthetic peptide (aa 75-87 in rat) coupled to kethole limpet hemocyanin/Rat muscle parvalbumin
Vesicular GABA Transporter/Calbindin	AntiVGAT; mouse; 1:200; Synaptic Systems, Germany. AntiCB; rabbit; 1:2500; Chemicon, Canada	131 011/AB1778	Synthetic peptide (aa 75-87 in rat) coupled to kethole limpet hemocyanin/Recombinant mouse calbindin
Vesicular GABA Transporter/Calretinin	AntiVGAT; mouse; 1:200; Synaptic Systems, Germany. AntiCR; rabbit; 1:1000; Chemicon, Canada	131 011/AB5054	Synthetic peptide (aa 75-87 in rat) coupled to kethole limpet hemocyanin/Recombinant rat calretinin

Wisteria floribunda/Parvalbumin	Lectin for WFA, biotin conjugate; 1:500; Sigma-Aldrich, Canada. AntiPV; mouse; 1:2500; Swant, Switzerland	L1516/235	Purified protein, Wisteria florbunda/Purified carp muscle parvalbumin
Wisteria floribunda/Calbindin	Lectin for WFA, biotin conjugate; 1:500; Sigma-Aldrich, Canada. AntiCB; rabbit; 1:2500; Chemicon, Canada	L1516/AB1778	Purified protein, Wisteria florbunda/Recombinant mouse calbindin
Wisteria floribunda/Calretinin	Lectin for WFA, biotin conjugate; 1:500; Sigma-Aldrich, Canada. AntiCR; rabbit; 1:1000; Chemicon, Canada	L1516/AB5054	Purified protein, Wisteria florbunda/Recombinant mouse calretinin
Aggrecan/Parvalbumin	Anti-aggrecan Abcam; mouse; 1:1000. AntiPV; guinea pig; 1:2500; Chemicon, Canada	AB78292/AB9314	Feline brain proteoglycan/Rat muscle parvalbumin
EphrinA5/Parvalbumin	AntiEphrinA5; rabbit; Abcam, Canada; 1:500; AntiPV; guinea pig; 1:2500; Chemicon, Canada	AB70114/AB9314	Synthetic peptide derived from internal sequence of human ephrin A5/ Rat muscle parvalbumin
EphrinB3/Parvalbumin	AntiEphrinB3;	AB53063/AB9314	Synthetic peptide from N

	rabbit; Abcam, Canada; 1:500. AntiPV; guinea pig; 1:2500; Chemicon, Canada		terminal domain/ Rat muscle parvalbumin
MMP9	AntiMMP9; rabbit; Abcam; Canada; 1:1000	AB38898	Full length MMP9 native protein (mouse)

2.3 Image Acquisition and Analysis

Confocal images were taken on an Olympus IX 60 inverted microscope outfitted with a Perkin Elmer Spinning Disk Confocal attachment with either a 60x (Numerical Aperture = 1.4) immersion oil, 40x (N.A = 0.75), 20x (N.A = 0.5), or 10x (N.A = 0.3) objective. The microscope was equipped with a Hamatsu Orca ER CCD camera (1300 x 1030 pixels) and images were acquired using Velocity software. Each image represented a stack of 10 images spaced 0.2 μm apart in the z-plane, for each wavelength.

2.3.1 PNN Acquisition & Analysis

WFA and AGG primary antibodies were used to stain PNNs in the PC. To image the PNNs, three pictures were taken in layer 3 of the PC with a 20x objective. For WFA stained PNNs we assigned the nomenclature “grade 1” to identify fully formed PNNs and “grade 2” to identify diffusely formed PNNs. Counting the number of grade 1 and grade 2 PNNs in each picture determined the changes in PNN expression. The total number of PNNs in each grade was compared separately between control, kindled and contralateral or “partially” kindled tissue using a one-way ANOVA ($p < 0.05$) and Tukey's *post hoc* test.

WFA was co-localized with primary antibodies for three CBPs (PV, CB, CR). To image the co-localization of PNNs with CBPs, three pictures were taken in layer 3 of the PC with a 20x objective. The percent of each interneuron subtype with a surrounding PNN

was found in control tissue and compared to kindled tissue using a two tailed, unpaired t-test.

2.3.2 Nerve Terminal Acquisition & Analysis

To image presynaptic nerve terminals primary antibodies for VGAT were co-localized with three CBP antibodies. Three images were taken in each of the three layers of the PC using a 60x emersion oil objective (9 images per animal). Layer 1 images were taken directly above the lateral olfactory tract, layer 2 images were taken from areas with densely packed pyramidal cells, and layer 3 images were taken directly above layer 2. The analysis of the co-expression of VGAT with PV, CB, and CR, was done using Imaris software (Bitplane, Zurich, Switzerland). The image resolution depended on objective power as both x and y scales were equal to 6.4 μm / Objective magnification; and z resolution was 0.2 μm. Images were then deconvolved with AutoQuant software (AutoQuant Imaging, Burnbury, Ontario, Canada) to eliminate nonspecific background. Images were analyzed using a co-localization module that allowed for pixels in each channel to be windowed according to a set of criteria and only these pixels were included in the co-localization analysis. Targets of interest were intensely labeled; therefore all images were analyzed using only the 5% brightest pixels from each channel as previously described in Hutcheon *et al.* (2004). This isolated the high-intensity fluorescent signal and eliminated the nonspecific or diffuse staining. The juxtaposed segmented pixels in the stack were converted to co-localized 3-dimensional voxels and were represented by a separate channel pseudo coloured white (Figure 1). The number of co-localized voxels were counted in identical fields and averaged amongst the 3 images taken within each layer. The average number of co-localized voxels was compared between control, kindled and partially kindled animals within each layer using a One-way ANOVA ($p < 0.05$) and Tukey's *post hoc* test.

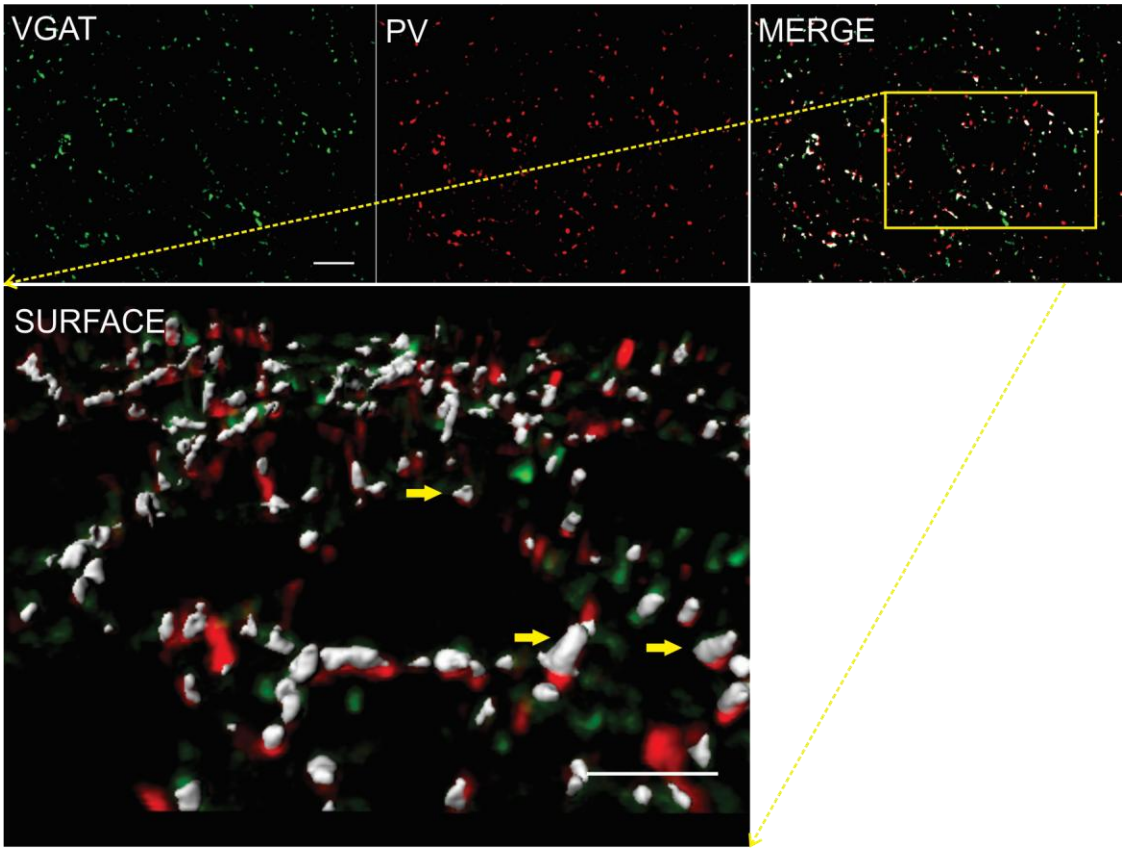

Figure 2. A co-localization module was used to analyze nerve terminal density. The 5% brightest pixels are isolated in the VGAT (green) and the calcium binding protein channel (red). Overlap between the two channels is assumed to be a release site, and is pseudo-coloured white. The average number of co-localized voxels (3D pixel) was counted in each layer. Surfaces can be rendered on the co-localized channel to construct objects that often have the morphology of nerve terminals or *en passant* boutons. Arrows show examples of objects having what appear to have nerve terminal morphology. Scale bar = 10 µm.

2.3.3 Ephrin Acquisition & Analysis

Primary antibodies for Efna5 and Efnb3 were used to stain the proteins. Three images were taken in layers 2 and 3 of the PC. The analysis of ephrin proteins was done using Imaris software (Bitplane, Zurich, Switzerland) after images were deconvolved with AutoQuant software (AutoQuant Imaging, Burnbury, Ontario, Canada). Image stacks were normalized and background subtracted. The amount of protein was found using the

co-localization of the 5% brightest pixels at twice the background level. The number of co-localized voxels was averaged amongst the three images taken and compared between control and kindled tissue using a two-tailed, unpaired t-test.

2.4 Microarray

2.4.1 Tissue Preparation

Male Sprague Dawley rats (sham n = 4, kindled n = 4) were deeply anesthetised with ketamine (100km/kg, i.p)/domitor (10mg/kg, i.p) and perfused intracardially with heparinized saline, followed by ice-cold Ringer's solution in which sodium was replaced by choline [containing (in mm): choline Cl, 110; KCl, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 0.5; $MgCl_2$, 7; Na pyruvate, 2.4; ascorbate, 1.3; dextrose, 20]. After perfusion the brain was rapidly removed and the piriform cortex was sectioned and placed in 1ml of TRIzol per 50-100 mg of tissue.

2.4.2 RNA Extraction

Tissue was homogenized using a power homogenizer and allowed to incubate for 5 min before adding 0.2 ml of chloroform per 1 ml of TRIzol reagent. Tubes were vortexed for 15 seconds and allowed to incubate for 3 min. The samples were then centrifuged at 12 000 x g for 15 min at 4°C. The supernatant was then removed, transferred to a fresh tube with 500 µl of isopropanol, and allowed to incubate for 10 min. Samples were then centrifuged at 12 000 x g for 10 min at 4°C. Supernatant was then removed again and RNA pellet was washed with 100% ethanol and centrifuged at 7500 x g for 5 min at 4°C. The wash and spin were then repeated with 75% ethanol. The RNA pellet was then vacuumed dry and dissolved in RNase-free water. Sample quality was checked using a NanoDrop 1000 Spectrophotometer. All samples had a 260/280 ratio and 260/230 ratio between 1.8-2.1 and a minimum concentration of 500 ng/µl. Equal amount of mRNA from sham rat 1 and 2 were pooled, and equal amounts from sham 3 and 4 were pooled. The same was done for kindled mRNA. The mRNA was given to the London Regional Genomics Center to be processed.

2.4.3 Microarray Analysis

Approximately 29 000 genes on the GeneChip Rat Gene 1.0 ST array were analyzed using Partek Genomic Suite software (St. Louis, Missouri). Two Affymetrix probe-level data sets in CEL files were compared: (1) sham control tissue and (2) kindled tissue. Each set was normalized using the Robust Multichip Average (RMA) method where the raw data were background corrected, log2 transformed and quantile normalized. A linear model was then fit to the normalized data producing an expression measure for each probe set on each array. We then identified pathways of changing genes. Genes were mapped onto pathways and pathway annotation of gene expressions were retrieved from the publicly available Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Appendix Table 2). Some candidate genes from the Axon Guidance KEGG pathway were selected for further analysis.

2.5 Real-time Polymerase Chain Reaction (qPCR)

Extracted RNA (2 μg) was reverse transcribed using the Invitrogen Superscript II protocol with oligo(dT) primers. QPCR reactions were performed in duplicate for each sample using BioRad MyIQ single-color real-time PCR detection system. DNA abundance was detected using SYBR Green (Bio Rad iQ SYBR Green Supermix) following the manufacturer's protocol. A five point 10x dilution series of cDNA (100ng-10pg) was used to optimize primers. PCR primer sets annealed at 55°C. Peptidylprolyn isomerase A (cyclophilin A) was used as a reference gene (Forward: 5'-ATTCATGTGCCAGGGTGGTG-3', Reverse: 5'-CCGTTTGTGTTTGGTCCAGC-3') to normalize gene expression levels. The following primer sequences were used: Efna5 – Forward: 5'-CTGCGACCACACATCCAAAG-3', Reverse: 5'-GAACTTCAGCGGTCCGTTTG-3'; Efnb3 – Forward: 5'-CAAGGTTCTAGTGGGGCTG-3', Reverse: 5'-AAGCACGTAACCACCCTCTG-3'; Epha6 – Forward: 5'-CCCAGAACTGCACCTTCACT-3', Reverse: 5'-GCAGCATCACGAGAGATCCA-3'; Ephb1 – Forward: 5'-CGAAAGGACACGGAGAAGCC-3', Reverse: 5'-GGAGCCTGGGACATTTGGAA-3'; Mmp9 – Forward: 5'GACCTCAAGTGGCACCATCA-3', Reverse: 5'-

AGTCATCGATCACGTCTCGC-3'; Adamts4 – Forward: 5'-CGTTCCGCTCCTGTAACACT-3', Reverse: 5'-TTGAAGAGGTCGGTTCGGTG-3'. Differences in abundance are found by comparing the Δ cycle threshold (C_t) between control and kindled samples ($\Delta C_t = C_{t\ (reference\ gene)} - C_{t\ (gene\ of\ interest)}$). Differences were analyzed using an unpaired, two tailed t-test (significance was determined at $p < 0.05$).

2.6 MMP Inhibition

MMP's are known to be involved in degrading extracellular matrix molecules and remodelling brain tissue. We used the commonly prescribed anti-microbial drug Doxycyline hyclate (Millipore) as non-selective MMP inhibitor to inhibit the degrading effects of MMP's in the CNS. Doxycycline works by binding to the zinc region of MMPs to cause a conformational change and thus inhibit MMP's enzymatic activity. Rats were divided randomly into three groups: (1) saline injected and kindled, (2) doxycycline injected and kindled, (3) doxycycline injected and not kindled. Rats receiving doxycycline received 40mg/kg via i.p. injections daily and saline injected rats received the same volume of saline via i.p. injection daily. The rats who received daily kindling impulses had their seizures scored using the Racine scale: stage 1 (immobility), stage 2 (facial clonus), stage 3 (unilateral forelimb clonus), stage 4 (rearing), stage 5 (rearing and falling seizure) (Racine, 1972). The number of stimulations it took to kindle a rat and the amount of current (in microamperes) necessary to elicit a kindling behaviour were recorded and compared between DOX injected and saline injected/kindled rats. LANA's fixed tissue from all three groups was stained with WFA and PV. The total number of grade 1 and grade 2 PNNs were counted and compared between (1) saline injected and kindled, (2) doxycycline injected and kindled, (3) doxycycline injected and not kindled using a One-Way ANOVA ($p < 0.05$) and Tukey's *post hoc* test.

3 Results

The purpose of this study was to determine the implications of kindling-induced seizures on the organization of interneuron release sites in the piriform cortex (PC). Specifically looking at how kindling affected the plasticity of interneurons and the consequences to their layer specific innervation patterns in the PC.

3.1 The expression of perineuronal nets was decreased after kindling

Perineuronal nets (PNNs) were prominently displayed in the PC and were predominantly located in layer 3 (Figure 2). PNNs may be used as a marker of synaptic stability in the adult central nervous system (CNS) because of their known inhibitory effects on structural rearrangement and axonal growth (Celio & Blumcke, 1994; Pizzorusso *et al.*, 2002; Frischknecht *et al.*, 2009). To determine if experimentally induced epilepsy affected their expression, kindling-induced seizures were produced in the basolateral amygdala of adult male rats. The expression of PNNs labeled with either a lectin from Wisteria floribunda agglutinin (WFA) or a primary antibody that recognizes the chondroitin sulfate proteoglycan core protein aggrecan (AGG), were evaluated. The number of grade 1 and grade 2 PNNs were counted in each field. The expression of both grade 1 and grade 2 WFA stained PNNs were compared separately between control, kindled, and the contralateral side of the kindled brain tissue (called “partially” kindled). The immunohistochemistry results showed that the number of grade 1 PNNs was significantly reduced in the PC of both ipsilateral and contralateral kindled tissue (one way ANOVA, $F = 8.2$, $df = 20$, $p < 0.05$; Figure 3). The expression of grade 2 PNNs were not significantly different from control in either ipsilateral or contralateral kindled tissue. To confirm the decrease in PNNs we also stained control and kindled tissue with AGG and results again showed a significant reduction in overall number of PNNs after kindling induced seizures (Appendix Figure 13).

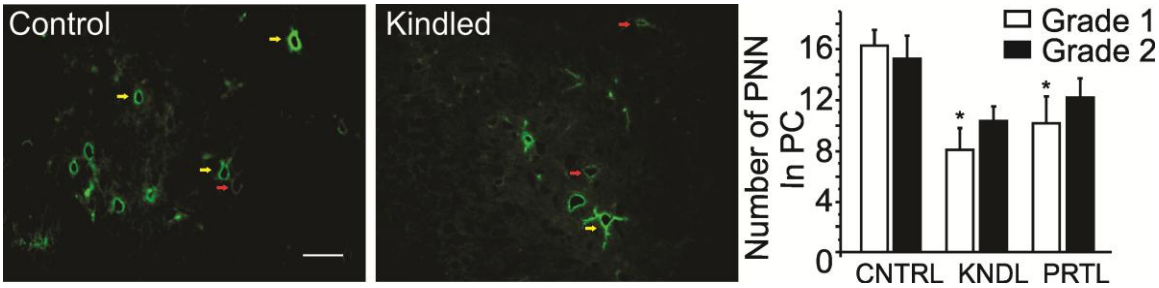

Figure 3. The average number of grade 1 perineuronal nets was decreased in the piriform cortex of kindled rats after kindling-induced seizures. WFA immunoreactivity (green) was reduced in kindled tissue (both ipsilateral [n = 7] and contralateral [n = 7]) compared to control tissue ([n = 8], contralateral tissue not shown). Analysis (one-way ANOVA, Tukey's *post hoc* test) showed there was a significant decrease in the average number of WFA stained grade 1 PNNs after kindling (*$p < 0.05$). Error bars represent the standard error of the mean. Scale bar = 30µm. Grade 1 = fully formed (yellow arrows), grade 2 = diffusely formed (red arrows).

3.2 The expression of perineuronal nets was reduced around parvalbumin interneurons after kindling

PNNs can be observed surrounding virtually all neurons in the cortex, but are most prominently displayed around GABAergic interneurons that express the calcium binding protein (CBP) parvalbumin (PV) (Celio & Blumcke, 1994). Therefore we wanted to know if the expression of PNNs was specifically being decreased around this subtype of interneuron after kindling. Both WFA and AGG were used to mark PNNs in the PC and primary antibodies for the CBPs: PV, calbindin (CB) and calretinin (CR) were used to mark three distinct subpopulations of inhibitory interneurons. The percent of interneurons with a PNN were compared between control and kindled tissue. In control tissue 29% of CB-immunoreactive neurons, 30% of CR-immunoreactive neurons, and 76% of PV-immunoreactive neurons were surrounded by a PNN. The expression of WFA stained PNNs surrounding CB and CR immunoreactive neurons was not significantly different between control and kindled tissue (t-test $p = 0.62$ and $p = 0.79$). However there was a significant decrease in the expression of WFA stained PNNs surrounding PV-immunoreactive neurons after kindling (two tailed, unpaired t-test, $p<0.05$, $F = 3.7$, df =

21; Figure 4). After kindling only 53% of PV^+ interneurons were surrounded by a PNN. To confirm our data we also stained control and kindled tissue with primary antibodies for AGG and PV and results again showed a significant reduction in PNNs after kindling induced seizures (data not shown). The loss of PNNs was not due to a decreased expression of PV expressing interneurons (interneurons previously counted in our lab and shown to remain the same before after amygdala kindling: (Gavrilovici *et al.*, 2012))

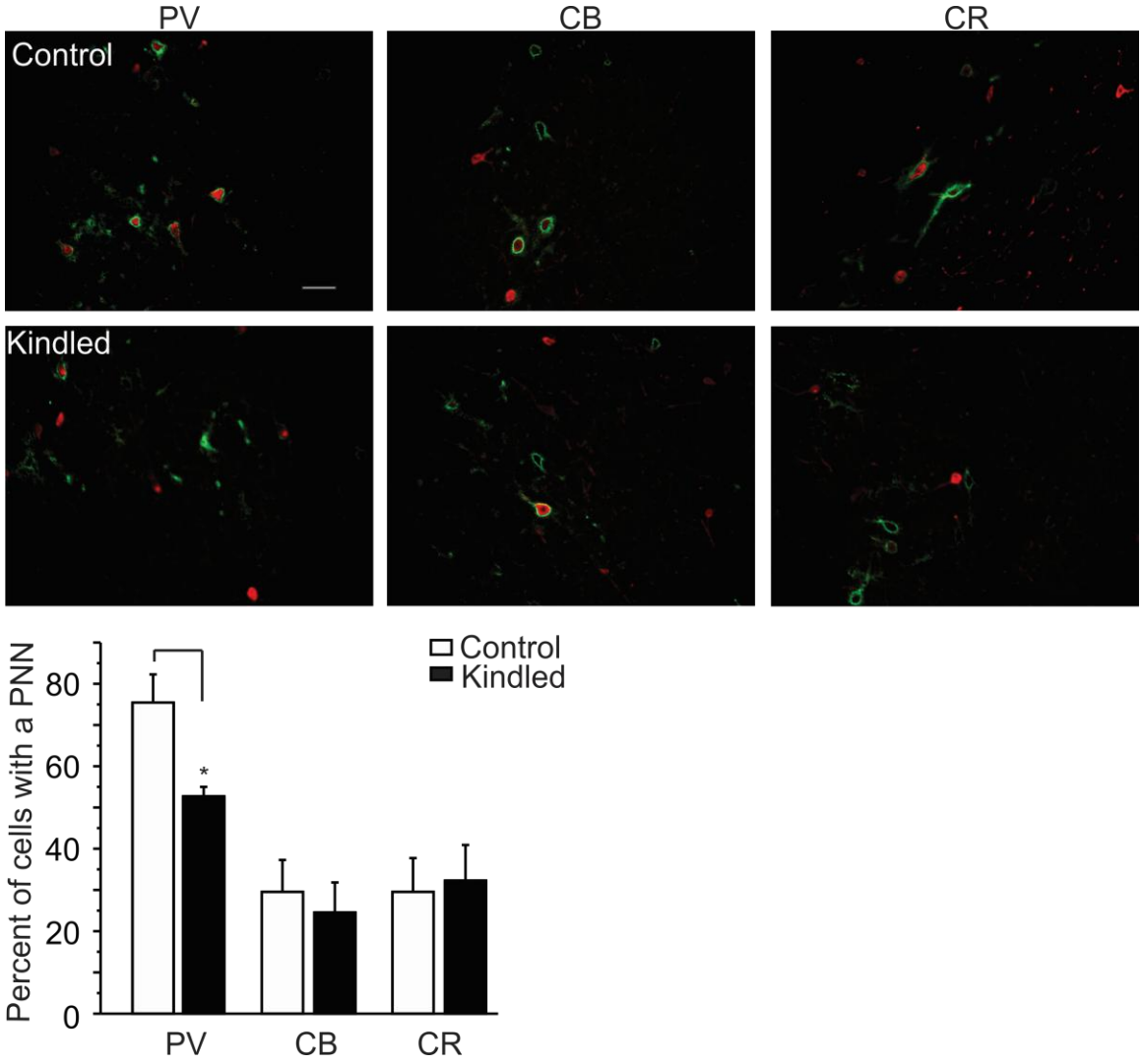

Figure 4. Perineuronal nets surrounding PV^+ interneurons were significantly reduced after kindling. The percent of PNNs (green) surrounding PV^+ interneurons (red; panel 1) was significantly reduced in the kindled tissue (n = 7) compared to control (n = 8). There was no statistical difference between the percent of PNNs surrounding CB^+ (red; panel 2)

and CR^+ (red; panel 3) interneurons after kindling. Analysis showed a significant decrease in the percent of PNNs surrounding PV^+ interneurons after kindling (two-tailed, unpaired t-test, * $p < 0.05$, $F = 3.7$, $df = 21$). Error bars represent the standard error of the mean. Scale bar = 30μm.

3.3 Inhibitory interneuron synapses rearranged after kindling in a layer specific manner

Next we wanted to know if the loss of PNNs after kindling-induced seizures affected the pattern of inhibitory synaptic release sites in the PC. To determine any perturbations to GABAergic interneuron wiring we labeled presynaptic nerve terminals with a polyclonal antibody for the vesicular GABA transporter (VGAT). VGAT was co-localized with the three different CBPs (PV, CB, and CR) to differentiate subtypes of interneurons. The co-localization was assumed to be an inhibitory release site, and they appeared as puncta on dendrites and cell bodies. We examined the number of synaptic release sites for each interneuron subtype in all three layers of the PC using a co-localization module (Figure 1). The number of synaptic release sites was compared within each layer between control, kindled and partially kindled tissue using a One-way ANOVA and Tukey's *post hoc* test. Results showed that there was a layer and interneuron specific increase in inhibitory synaptic release sites after kindling.

PV expressing interneurons had a significant increase in the number of co-localized voxels (co-localized PV and VGAT proteins) after kindling. In the kindled as compared to control tissue there was a 42% increase in layer 1 ($p = 0.01$, $F = 6.0$, $df = 24$) and a 29% increase in layer 3 co-localization ($p = 0.048$, $F = 3.5$, $df = 21$) (Figure 4). CB expressing interneurons had a 31% increase in the number of co-localized voxels in layer 1 ($p = 0.012$, $F = 5.3$, $df = 23$) and 25% layer 3 ($p = 0.008$, $F = 5.8$, $df = 23$) (Figure 5). There was no statistical difference in the co-localization found in layer 2 for these interneuron subtypes. However, in the kindled as compared to control tissue, CR expressing interneurons had a 20% increase in the number of co-localized voxels in layers 1 ($p = 0.034$, $F = 4.2$, $df = 22$) and 36% layers 2 ($p = 0.001$, $F = 9.9$, $df = 23$) (Figure 6). There was no statistical difference in the co-localization found in layer 3 for this interneuron subtype.

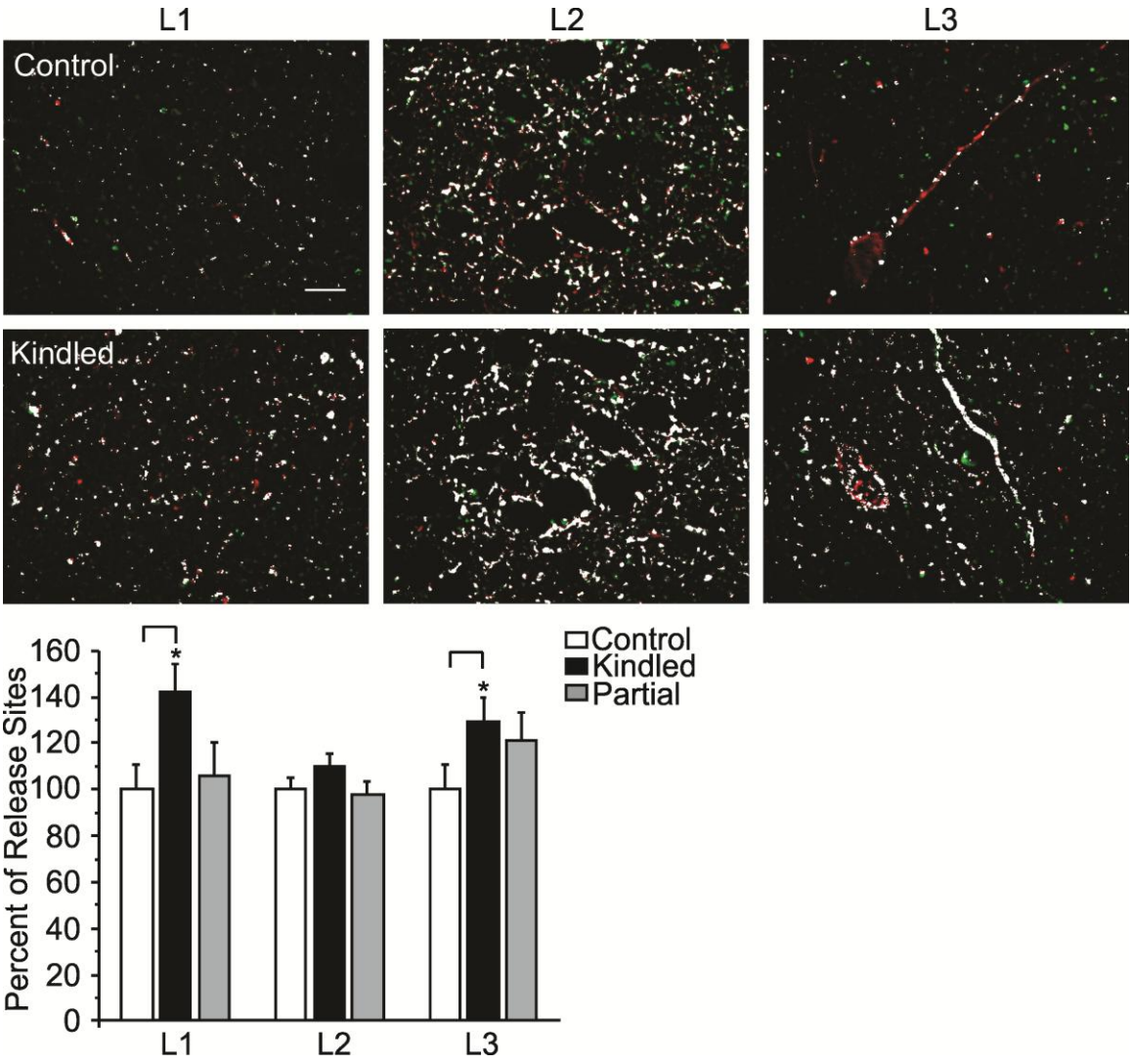

Figure 5. There was a significant increase in PV^{+} interneuron release sites after kindling induced seizures. The pre-synaptic nerve terminal marker VGAT (green) co-localized with PV (red) to mark release sites (white). The percent of release sites was significantly increased in kindled tissue in layers 1 ([n = 9], L1) and 3 ([n = 8], L3), but not in layer 2 ([n = 9, L2) of the PC compared to control ([n = 8 for each layer; One-Way ANOVA, Tukey's *post hoc* test, * $p < 0.05$). Top panels are representative photomicrographs of control and kindled tissues; partially kindled tissue is not shown. Error bars represent the standard error of the mean. Scale bar = 10µm.

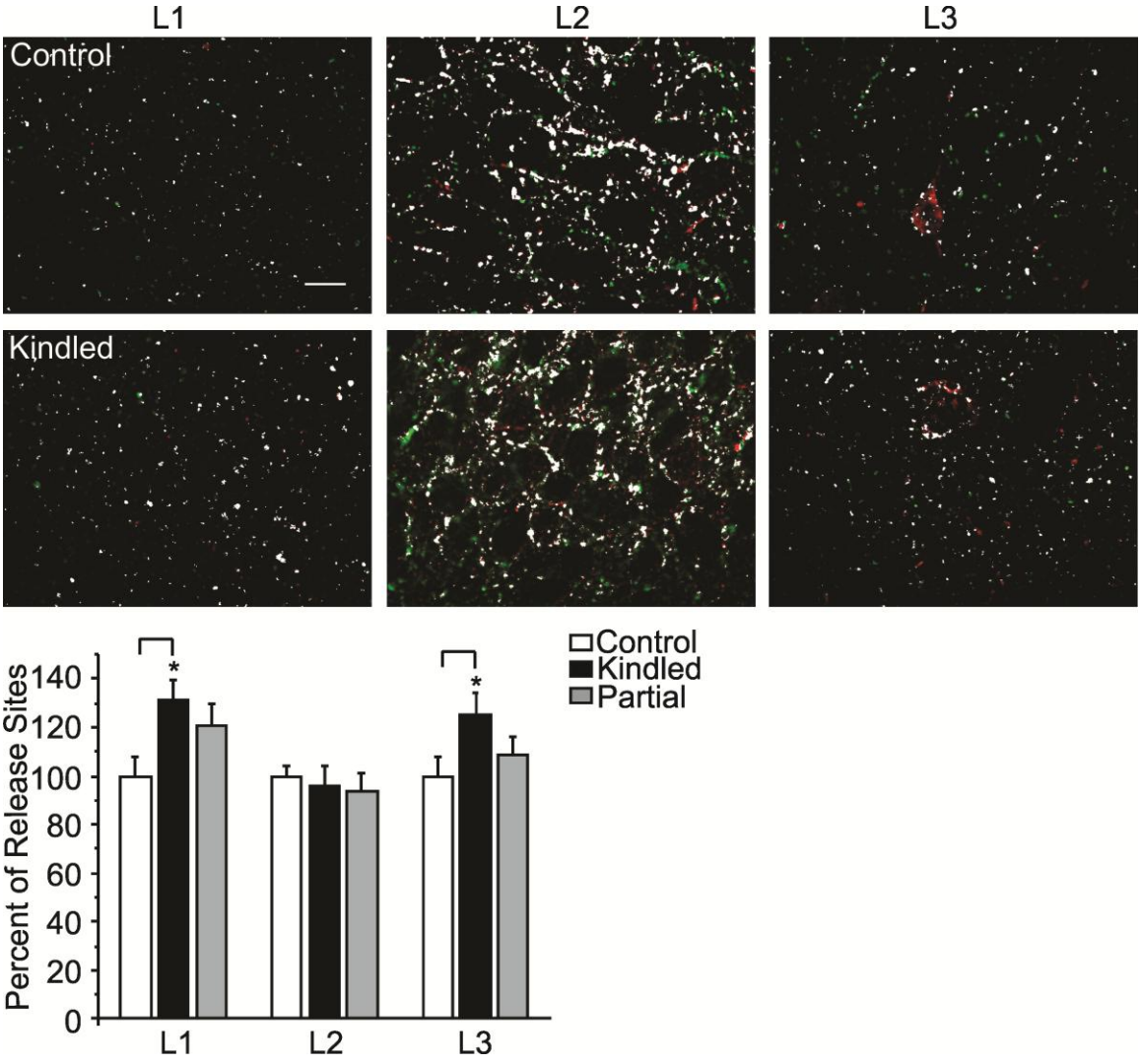

Figure 6. There was a significant increase in CB^{+} interneuron release sites after kindling induced seizures. The pre-synaptic nerve terminal marker VGAT (green) co-localized with CB (red) to mark release sites (white). The percent of release sites significantly increased in kindled tissue in layers 1 ([n = 8], L1) and 3 ([n = 8], L3), but not in layer 2 ([n = 8], L2) compared to control (n = 8 for each layer; One-Way ANOVA, Tukey's *post hoc* test, * $p < 0.05$). Top panels are representative photomicrographs of control and kindled tissues; partially kindled tissue is not shown. Error bars represent the standard error of the mean. Scale bar = 10µm.

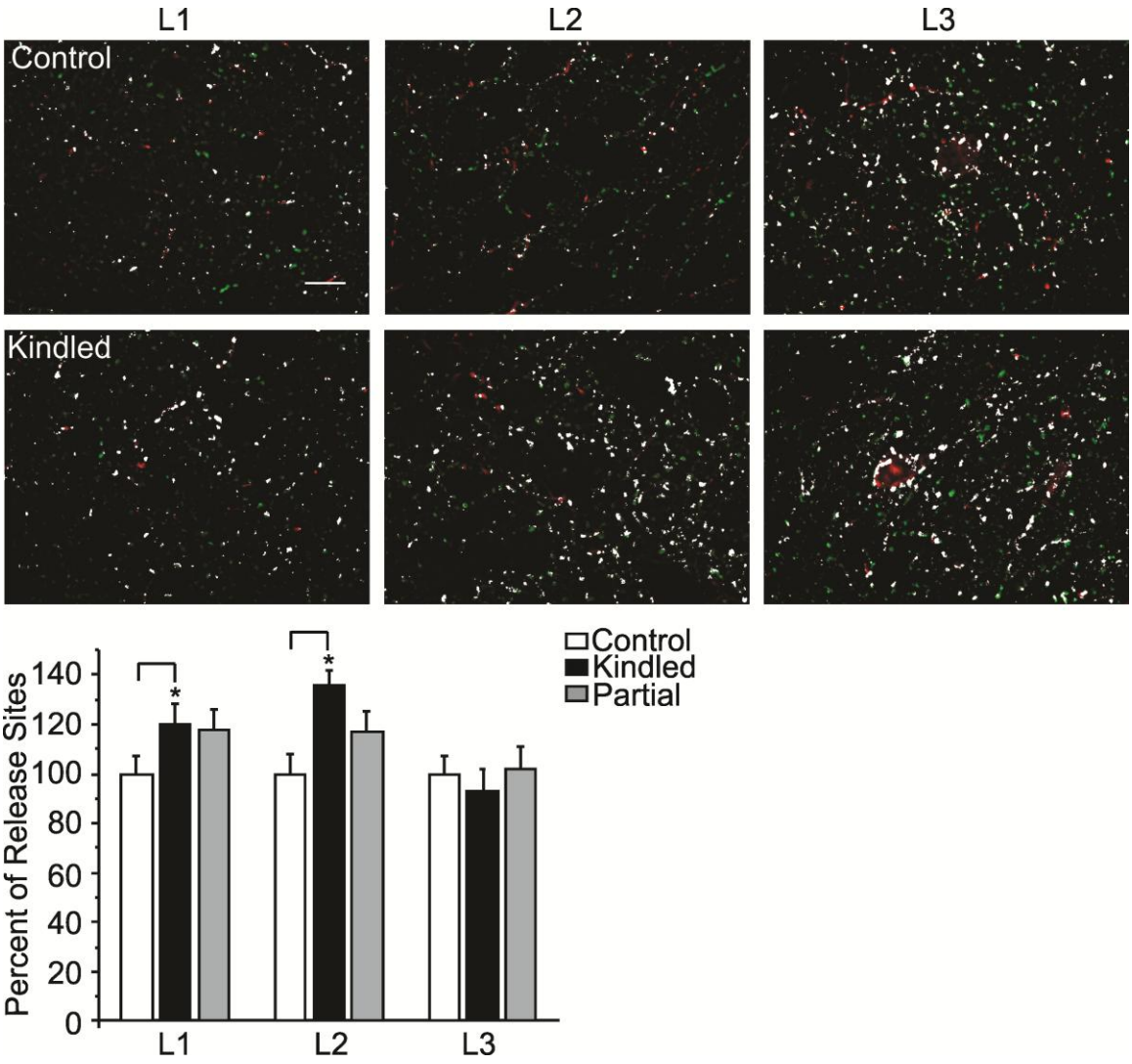

Figure 7. There was a significant increase in CR^{+} interneuron release sites after kindling induced seizures. The pre-synaptic nerve terminal marker VGAT (green) co-localized with CR (red) to mark release sites (white). The percent of release sites significantly increased in kindled tissue in layers 1 ([n = 8], L1) and 2 ([n = 8], L2) but not layer 3 ([n = 8], L3) compared to control (n = 7 for each layer; One-Way ANOVA, Tukey's *post hoc* test, * $p < 0.05$). Top panels are representative photomicrographs of control and kindled tissues; partially kindled tissue is not shown. Error bars represent the standard error of the mean. Scale bar = 10µm.

3.4 The expression of ligands involved in axonal guidance increased after kindling

A microarray was used to bioassay genes that had an altered expression after kindling-induced seizures. To refine our search we used Partek software and slotted genes with a greater than 1.3 fold change (either increase or decrease) into pathways using the publicly available Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. One of the pathways generated was called the Axon Guidance pathway and many of the genes in this pathway were shown to either increase or decrease after kindling. We then selected four candidate genes from the pathway (Efna5, Efnb3, Epha6, and Ephb1) to validate using qPCR. Results showed that the mRNA of Efna5 and Efnb3 significantly increased after kindling-induced seizures (unpaired t-test, $p < 0.05$; Figure 8). Immunohistochemistry was then used to determine if there was also an increased expression of the Efna5 and Efnb3 protein in the PC. Confocal images were taken in layers 2 and 3 of the PC. By co-localizing each protein with itself at twice the background level, we determined that there was a significant increase in Efnb3 protein in layer 2 of the PC after kindling-induced seizures (unpaired t-test, $p < 0.05$; Figure 9). No significant protein differences were found for Efna5 (data not shown).

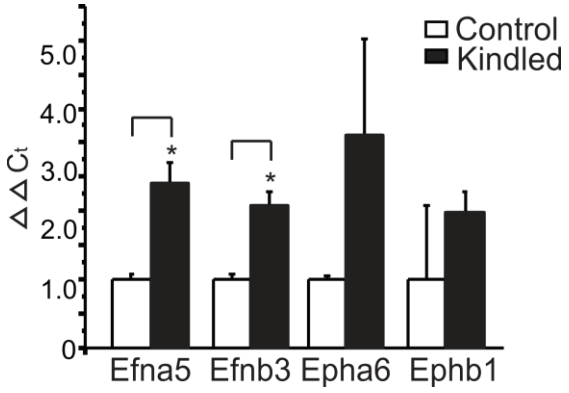

Figure 8. The expression of genes involved in axon guidance increased after kindling. The delta delta C_t was used to show significantly increased mRNA expression of Efna5 and Efnb3 in kindled tissue compared to control (two tailed, unpaired t-test * $p < 0.05$, $n = 6$). Error bars represent the standard error of the mean.

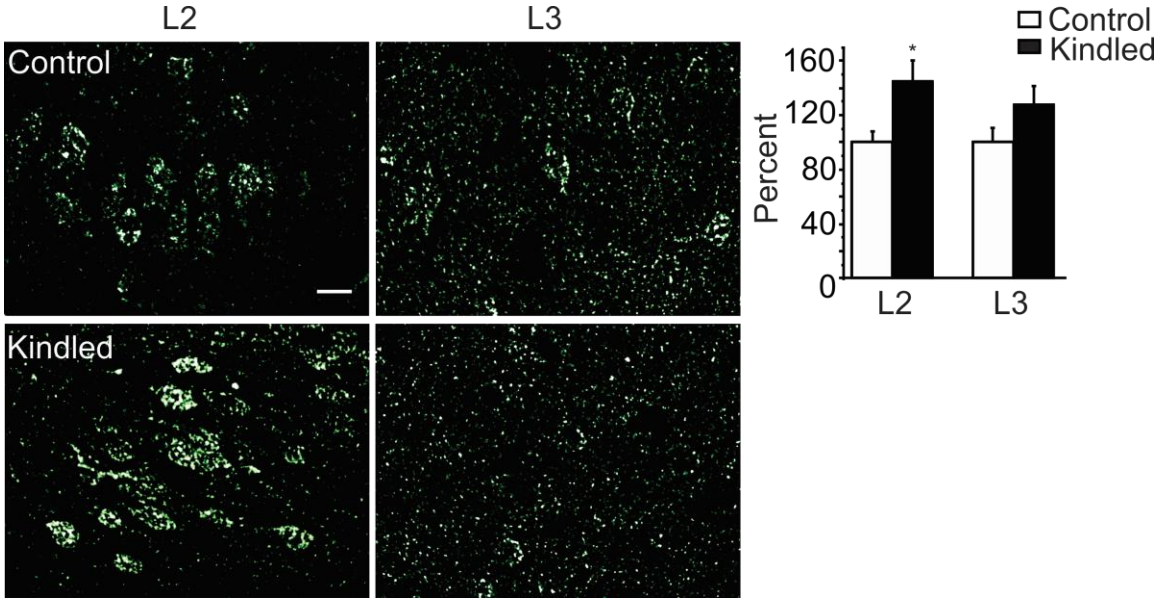

Figure 9. The expression of Efnb3 protein increased after kindling. The percent of Efnb3 (green) protein was found using the 5% brightest pixels co-localized (white) at twice background level was significantly increased in kindled tissue (n = 5) in layer 2 of the PC compared to control (n = 5, two tailed, unpaired t-test, * $p < 0.05$). Error bars represent the standard error of the mean. Scale bar = 10μm.

3.5 The expression of a protease involved in perineuronal net degradation increased after kindling

The microarray assay also indicated a greater than 1.5 fold increase in MMP9 and ADAMTS4, two proteases known to cleave lecticans in PNNs. Immunohistochemistry was used to determine if there was an increased expression of the MMP9 protein in the PC. A primary antibody for MMP9 was co-localized with PV. Using the 5% co-localization module (Figure 1), results showed an increase in the percent of co-localization of the two proteins in layer 2 of the PC after kindling (unpaired t-test, $p < 0.05$; Figure 10). Therefore the expression of MMP9 protein had increased on PV interneurons in layer 2 after kindling induced seizures.

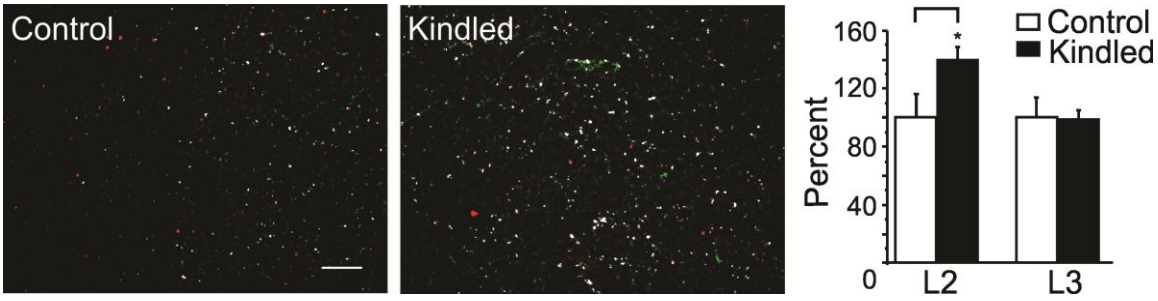

Figure 10. The expression of MMP9 protein on PV cells increased after kindling. The percent of MMP9 (red) and PV (green) co-localization (white) was significantly increased in kindled tissue (n = 7) compared to control tissue (n = 6) in layer 2 of the piriform cortex (two-tailed, unpaired t-test, * $p < 0.05$). No change was observed in layer 3. Scale bar = 10µm**.**

3.6 Doxycycline had a protective effect against kindling-induced seizure

Electrode implanted rats were randomly divided into three groups: (1) Doxycycline (DOX) injected/non-kindled, (2) Doxycycline (DOX) injected/kindled, (3) Saline injected/kindled. DOX injected, not kindled rats received daily i.p injections of DOX for two weeks. Rats in the kindled groups received daily i.p injections of either DOX or saline one hour prior to kindling. The average current (in microamperes) required for rats to elicit a kindling behaviour and their corresponding Racine seizure stage were recorded daily (Figure 11). For saline injected rats the amount of current required to elicit a seizure stage decreased as they progressed through the stages. Specifically, saline injected rats required an average of 330 µA at stage 2 but only 207 µA at stage 4, therefore exhibiting a significant decrease in the amount of required current (paired t-test, $p < 0.05$). For DOX injected rats the current required to elicit a seizure stage did not significantly decrease as they progressed through the stages. Specifically, DOX injected rats required an average of 240 µA at stage 2 and an average of 282 µA at stage 4, therefore no statistical decrease in the amount of required current was observed ($p = 0.6$).

Rats receiving DOX were less sensitive to amygdala kindling than those receiving saline. Of the six DOX injected rats, 1 reached stage 5 by day 16, two reached stage 5 on day 19,

and the other three did not reach stage 5 by day 20 when injections were terminated. Conversely, of the five saline injected rats all reached stage 5 by day 16. To determine if these observations could have occurred by chance we used a CHI squared significant test and got a X^2 value of 5.486, giving us a p value > 0.05.

After rats were either fully kindled or had received twenty injections (whichever came sooner), they were sacrificed, brain slices were made, and the tissue was stained with WFA to visualize PNNs (Figure 12). PNNs were counted and assigned as either grade 1 or grade 2. Results showed that PNNs in DOX injected/non-kindled rat tissue appeared the same as control rats. Using a one-way ANOVA with Tukey's *post hoc* test we showed that there was a significant decrease in the average number of grade 1 PNNs in the saline injected/kindled rats compared to both DOX injected/not kindled rats and DOX injected/kindled rats (Figure 12). Therefore DOX injected/kindled rats were also not significantly different from control rats. DOX injections likely assisted in either protecting the degradation of PNNs or decreasing sensitivity of rats to amygdala kindling so they did not reach stage 5.

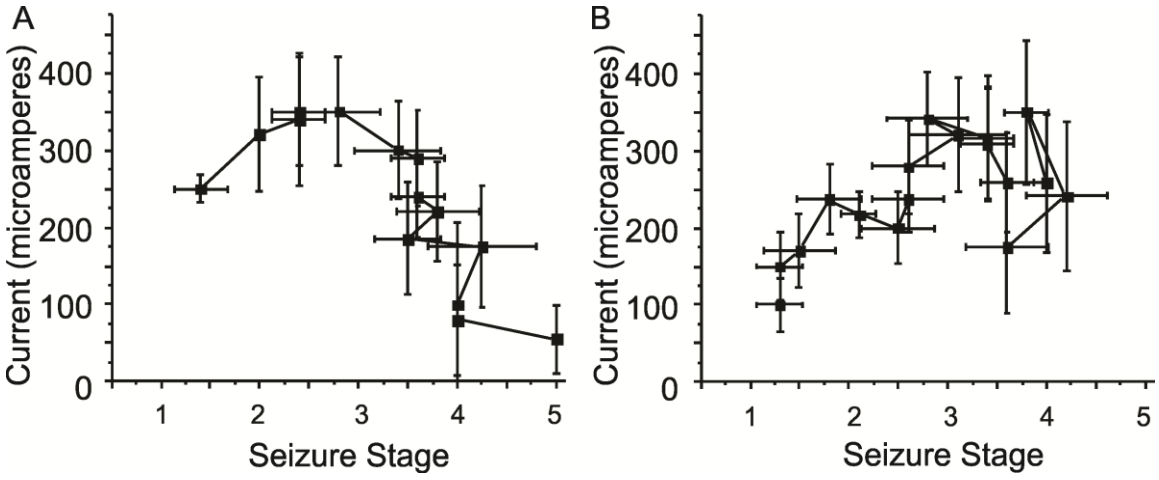

Figure 11. Trends in the current (microamperes) required to elicit a seizure stage differed between saline injected and Doxycycline injected rats. (A) Saline injected rats (n = 4) required less current to elicit a kindling behaviour as the rats progressed to later stages of kindling. (B) There was no observed decrease in the amount of current required to elicit a kindling behaviour in DOX injected animals (n = 6).

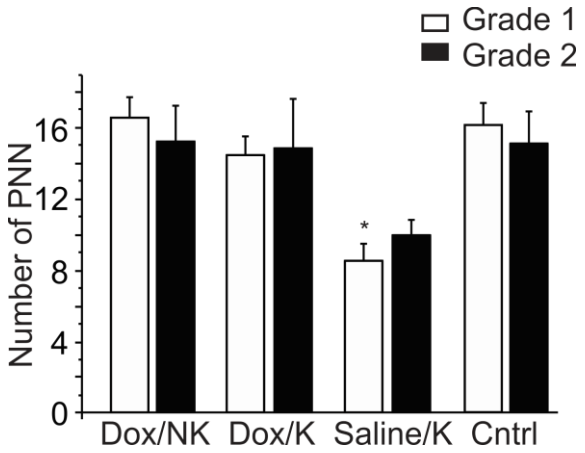

Figure 12. There was no observed decrease in grade 1 PNNs in Doxycycline injected/kindled rats after kindling. After kindling the average number of grade 1 PNNs was significantly decreased in saline injected/kindled animals (n = 4) compared to the DOX injected/non-kindled rats (n = 5) and DOX injected/kindled rats (n = 6). There was no observed difference in the number of grade 2 PNNs. Analysis (One-Way ANOVA, Tukey's *post hoc* test) showed a significant loss of grade 1 PNNs (*$p < 0.05$). Error bars represent the standard error of the mean. Grade 1 = fully formed, grade 2 = diffusely formed. NK = non-kindled, K= kindled.

4 Discussion

4.1 Summary of key findings

The primary goal of this study was to look at the effects of experimentally induced epilepsy on the inhibitory network of the piriform cortex (PC). We aimed to learn more about the role the PC played in epileptogenesis by looking at extracellular matrix structure alterations and rearrangement of interneuron release sites. The novel findings from this project are summarized below:

1) The expression of fully formed perineuronal nets (PNNs) surrounding parvalbumin (PV) interneurons was significantly decreased after kindling-induced seizures in the PC.
2) This loss of PNN expression in the PC was correlated with the increase in matrix metalloproteinase 9 (MMP9), a protease capable of degrading lecticans of the PNN.
3) The non-selective MMP inhibitor, Doxycycline (DOX), was antiepileptic and able to attenuate the progression of amygdala kindling and protect fully formed PNNs.
4) There was a layer specific increase in inhibitory synaptic release sites after kindling. There were increased calbindin (CB) and PV release sites in layers 1 and 3 of the PC, and there were increased calretinin (CR) release sites in layers 1 and 2 after kindling.
5) The increased nerve terminals were correlated with an increased expression of ephrinA5 and ephrinB3, two genes involved in axon guidance, as detected by mRNA and immunohistochemistry.

Stimulating the basolateral amygdala influences the PC because there is both strong connections from PC pyramidal cells to nuclei of the amygdala and a strong indirect connection from the amygdala to the PC via the entorhinal cortex (Loscher & Ebert, 1996). The data found in this study suggest that kindling induced seizures increased the plasticity of the PC by assisting breakdown of PNNs and allowing for rearrangement of synaptic connections. Inhibiting MMP9 may potentially be an important therapeutic target for the protection of extracellular matrix (ECM) structures. These data are

important for understanding the consequences of kindling on the PC and how alterations to the PC assist in the progression of epileptogenesis.

4.2 Kindling induced a breakdown of perineuronal nets

PNNs are lattice like structures made of aggregated ECM constituents. Their exact function remains unknown but they are temporally and spatially positioned to play a role in synapse stabilization. Once an animal reaches maturity, PNNs form around certain cell bodies and proximal dendrites to maintain the formed synapse integrity. Therefore degradation of PNNs can be detrimental to the networks formed during development and stabilized during the critical period. It has been determined that the majority of PNNs in the cortex and hippocampus are formed around GABAergic interneurons. Specifically cells that also express the calcium binding protein (CBP) PV (Bruckner *et al.*, 1993; Dityatev, 2010). These cells typically have a multipolar morphology and are characterized by fast-spiking activity. Therefore the highly negatively charged PNN formations may be necessary around these cells to stabilize synapses, provide local homeostasis and maintain high rates of action potential firing (Morris & Henderson, 2000). Since GABAergic interneurons are necessary for controlling pyramidal cell firing and network synchrony, disruptions to the PNNs ensheathing these cells may result in an imbalance of excitation and inhibition, a hallmark of epilepsy. This disruption may ultimately initiate and/or maintain the hypersynchrony necessary for seizure generation.

In the current study, we used both a lectin from Wisteria floribunda agglutinin (WFA) and a primary antibody that recognized the core protein aggrecan (AGG) to detect PNNs in the PC. We detected grade 1 (fully formed) and grade 2 (diffusely formed) PNNs in layer 3 of the PC. Since we know that layer 3 of the PC is primarily filled with GABAergic interneuron cell bodies it stands to reason that the majority of PNNs in the PC should be found there. Results showed that there was a 50% reduction in the expression of grade 1 PNNs in layer 3 of the PC after kindling induced seizures. The decrease was specifically detected around GABAergic interneurons also expressing PV. We showed that 23% of PV interneurons lost their surrounding PNN after kindling induced seizures whereas no detectable decreases were observed on CB and CR interneurons. Recently McRae *et al* (2012) showed a similar breakdown of PNNs in the

hippocampus. They demonstrated that the proteoglycan aggrecan expressed in the PNN significantly decreased after pilocarpine induced status epilepticus. Specifically, the expression of PNNs had decreased one week after status epilepticus and remained significantly decreased for up to two months after. Interestingly they showed that in the hippocampus there was also an emergence of PNNs with poor structural integrity, suggesting that components of the PNN were being degraded after seizures. Therefore, excessive hypersynchronous neural activity during seizures induced by either amygdala kindling or chemical convulstants likely contributed to the breakdown of PNNs in multiple brain areas. However, the observed loss of PNN components appears to be age-dependent. In another study by McRae *et al* (2010) they demonstrated a transient increase in the expression of aggrecan containing PNNs after the induction of an early life seizure (using kainic acid) at post-natal day 10. They showed that excessive neural activity significantly increased aggrecan expression around PV cells during the development of network synapses in the dorsal hippocampus. Though, this transient increase was resolved by adulthood. Therefore during development and before PNNs have fully formed, the induction of seizures might have the opposite effect seen in mature brain. However, these studies all demonstrate that constituents of the ECM can be influenced by neuronal activity.

4.3 Increased MMP9 expression may assist in seizure generation

The observed decrease in PNN expression after seizure induction led us to believe that there was likely an upregulation of molecules capable of degrading the PNN. Matrix metalloproteinases (MMPs) have been implicated in the study of many pathologies due to their potent proteolytic potential. They are involved in remodeling the pericellular environment and when unregulated they have the ability to degrade collagen, laminin and chondroitinin sulfate proteoglycans in the ECM (Yong *et al.*, 2001). In our microarray data, we observed a > 1.5 fold change in the expression of MMP9 in kindled rats compared to sham rats, although this change in expression was not seen in follow-up qPCR validation. However, we did find that there was an increased expression of MMP9 protein on PV expressing interneurons in layer 2 of the PC after kindling induced seizures. It is probable that we were unable to detect the increased mRNA expression

using qPCR as we were using whole PC homogenate and not just layer 2 of the PC. Therefore the technique may not have been sensitive enough to detect any changes. Further studies using laser caption micro-dissection may be used to validate increased MMP9 mRNA specifically on PV interneurons.

The increased expression of MMP9 protein in layer 2 of the PC may be partially responsible for the observed degradation of PNNs in the PC and assist in the progression of epileptogenesis. Many studies have also found that the expression of MMP9 increased following various epileptiform activities. For example, MMP9 was shown to increase after induction of kainic acid (Szklarczyk *et al.*, 2002; Jourquin *et al.*, 2003), pilocarpine (Kim *et al.*, 2009), 4-aminopyridine (a potassium channel blocker) (Takacs *et al.*, 2010) and in the Wistar Galaxo Rijswijk rats (Takacs *et al.*, 2010). These increases were seen in areas of the brain prone to seizure activity such as the thalamus, prefrontal cortex, and hippocampus. Importantly, Wilczynski *et al* (2008) showed that MMP9 was critical for the development of seizures using the pentylenetetrazol (PTZ) chemical kindling model. First, they used an MMP9 knock out mouse and observed a decreased sensitivity to PTZ kindling compared to wild types. This demonstrated a potentially protective effect of the MMP9 gene knock out. In contrast, they generated rats with a transgenic overexpression of MMP9 and demonstrated an increased susceptibility to PTZ kindling. Furthermore, they demonstrated that MMP9 caused aberrant synaptogenesis in the hippocampus, an effect that was not seen when the protease was absent (Wilczynski *et al.*, 2008). This study confirmed that MMP9 likely plays a prominent role in epileptogenesis. Since in our study we saw an increased MMP9 expression after kindling induced seizures correlated with decreased PNNs, we believe this protease likely contributed, whether directly or indirectly, to their degradation. Additionally the proteases, A Disintegrin and Metalloproteinase with Thrombospondin Motifs 4 or 5 (ADAMTS4/5), may also play a role in degrading PNNs. These proteases, also called aggrecanases, have been shown to cleave various lecticans (particularily aggrecan) in the CNS and show a significant expression increase after administration of kainic acid (Yuan et al., 2002). Therefore the relationship of these proteases to the degradation of PNNs should be investigated further to gain a better understanding of the epileptogenic process.

4.4 Kindling caused an inflammatory response in the piriform cortex

Insults to the CNS induce neuroinflammatory responses that are characterized by activation of microglia and astrocytes, damage to the blood brain barrier, and acute upregulation of proinflammatory cytokines. Experimental models of seizures and clinical cases of epilepsy are known to produce this inflammatory response in the brain (Vezzani, 2005). Proinflammatory cytokines such as interlukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), and interlukin 6 (IL-6) are typically expressed at very low levels in a normal brain but after a seizure their expression rapidly increases, as seen in the brain of animal models of epilepsy (Vezzani, 2005). Some cytokines are only transiently upregulated, but other such as IL-1β have been shown to remain upregulated in the brain for up to 60 days after seizure (De Simoni *et al.*, 2000). The overproduction of these cytokines can enhance neuronal excitability, increase seizure susceptibility and exacerbate the injury (Yin *et al.*, 2011). Specifically, intrahippocampal injection of IL 1β prior to kainic acid injection prolonged seizure activity (Vezzani *et al.*, 2002) by increasing neuronal excitability through its actions in inhibiting GABA receptors, increasing NMDA receptor function and inhibiting potassium efflux (Viviani *et al.*, 2003). Intriguingly, promoter regions on genes that encode MMPs generally contain binding sites for transcription factors which are responsive to oncogenes and cytokines. As such, cytokines are a strong stimulus for MMP release and consequently MMPs are upregulated during inflammation. Therefore inhibition of MMPs may modify the inflammatory response and would therefore help reduce the damage caused by inflammation.

In addition to its proteolytic and pro-inflammatory role, MMP9 may also contribute to epileptogenesis via its actions on extracellular macromolecules. Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family, participates in plasticity and maintenance of neurons in adulthood (McAllister, 1999) and is a substrate of MMP. A recent study showed that the ratio of extracellular pro-BDNF to mature BDNF is controlled by high-frequency neural activity (Nagappan *et al.*, 2009). Of particular importance is the fact that MMPs are responsible for cleaving pro-forms of

BDNF and converting it to mature BDNF. Studies using the PTZ kindling model showed that indeed there was increased mature BDNF levels in the hippocampus after repeated PTZ kindling compared to controls (Mizoguchi *et al.*, 2011). Additionally they showed that MMP9-knockout animals showed reduced levels of mature BDNF during the early stages of kindling, and their rates of kindling were also significantly slower (Mizoguchi *et al.*, 2011). This suggests that MMP9 plays a role in seizure development by converting pro-BDNF to mature BDNF. In other studies $BDNF^{+/-}$ mice showed reduced rates of kindling acquisition and conversely transgenic mice with increased expression of mature BDNF had more severe seizures in response to kanic acid (Kokaia *et al.*, 1995; Lahteinen *et al.*, 2003). Since we know that extracellular BDNF stimulates tyrosine kinase receptor B on mossy fibers to induce axonal sprouting, this may create hyper-excited dentate circuits in parts of the brain. Therefore increased MMP found after kindling may lead to increased mature BDNF and more excited circuits.

4.5 Inhibition of MMP9 was antiepileptic in amygdala kindling

The design of antiepileptic drugs has started to focus on finding truly antiepileptic drugs to modify the progression of the disease instead of just controlling seizures. Targeting the immune system involvement in epilepsy, attempts have been made to design antiepileptic drugs that prevent effects of brain inflammation. Doxycycline (DOX) is a tetracycline antibiotic with known neuroprotective and anti-inflammatory properties in ischemia (Burggraf *et al.*, 2007), neurocysticerosis (Alvarez *et al.*, 2009) and epilepsy (Nogueira *et al.*, 2011). Its beneficial effects are associated with its ability to decrease microglial activation, and inhibit MMPs, caspases, and cytokines. Here we showed that pretreatment with DOX (via i.p injection) before amygdala kindling, attenuated the progression of seizures and protected fully formed PNNs thereby providing a potentially protective/antiepileptic effect of the drug. Specifically, our rats that received DOX injections required significantly more electrical stimulations to reach stage 5 (Appendix Figure 13), half of which never actually reached stage 5 with 20 stimulations. This was compared to our saline injected rats that required significantly fewer electrical stimulations, all of which reached stage 5. The amount of current required to elicit a seizure stage in a saline injected rat decreased as the rat progressed through the Racine

kindling stages. However, the amount of current delivered to a DOX injected rat did not decrease as the rat progressed through the stages. Meaning, a greater amount of current was required to elicit the same behavioural response in DOX injected rats. After the rats had reached stage 5 or had received 20 electrical stimulations (which ever came sooner) the brain was removed and PNNs were stained using WFA. Results showed that saline injected/kindled rats had significantly fewer grade 1 PNNs compared to DOX injected rats/kindled rats and DOX injected/non-kindled rats. Therefore DOX likely played a role in protecting the fully formed, grade 1 PNNs after amygdala kindling. DOX may have also protected these PNNs by decreasing sensitivity to kindling therefore not all rats reached stage 5 seizures where the majority of PNN degradation may have occurred.

Further evidence in the literature supports our conclusion that DOX is antiepileptic. Nogueria *et al* (2011) showed that pretreatment with DOX prior to pilocarpine induced seizures increased animals latency to their first spontaneous convulsion and increased the animals latency to death compared to wildtypes. This study showed that DOX was also protective in another epilepsy model (Nogueira *et al.*, 2011). Another study showed that DOX was protective in cerebral ischemia. Animals pretreated with DOX had smaller infarct volumes compared to controls after stroke and their microvessels were protected by the inhibition of MMPs and plasminogen activators (Burggraf *et al.*, 2007). Lastly, a study showed that DOX had effects in decreasing the levels of inflammatory cytokines (IL 1β, TNFα) and BDNF present in the frontal cortex, striatum and hippocampus areas after hypoxic ischemia (Jantzie & Todd, 2010). Therefore these studies and our results suggest that DOX may be a beneficial in treating inflammatory diseases associated with excessive MMP activity. The development of novel antiepileptic drugs that target brain inflammation will allow for better targeting of the underlying physiological mechanisms of the disease and not just symptomatic control.

Conversely, the protective effects of DOX might not be observed in all types of epilepsy as some studies have shown conflicting results. In genetic models of absence epilepsy, WAG/Rij rats showed an increased incidence and duration of spike wave discharges with injection of DOX (Kovacs *et al.*, 2011). More research must be done to determine the toxicity of this drug in humans before its therapeutic potential can be explored.

4.6 Kindling increased inhibitory release sites in the piriform cortex

In this study we showed that after kindling induced seizures there was an interneuron subtype and layer specific increase in inhibitory release sites in the PC. By co-localizing VGAT with three CBPs we were able to reliably identify subtypes of inhibitory nerve terminals. Specifically, we showed that after kindling there was an increase in CB and PV nerve terminals in layer 1 and 3, and an increase in CR nerve terminals in layer 1 and 2 in the PC. Correlated with these findings was an increase in the mRNA expression of ephrin A5 and B3, genes known to be involved in axon guidance. These results suggested that inhibitory nerve terminals increased/reorganized in response to kindling which may either assist in causing seizures in the PC or may be a compensatory mechanism.

Interneurons are heterogeneous for morphology, physiology, axonal formation and neurochemical markers. As such, they perform a diverse range of functions and there is likely a "division of labour". This means that morpho-physiologically diverse subtypes control differing network synchronizations (Cossart *et al.*, 2005). These cells target different areas on pyramidal cells and act in separate time windows to manage network firing and ultimately control brain states. Interneurons expressing PV are typically large, fast-spiking, multipolar cells whose axons make a basket-like or chandelier-like formation. Their axons target the soma and proximal dendrites of pyramidal cells as well as other interneurons. This means they can control the output of cells by regulating Na^{2+} dependent action potentials (McBain & Fisahn, 2001). Interneurons expressing CB typically have a horizontal morphology with axons targeting distal pyramidal dendrites. This means that they can control the input to a cell by shunting inputs distal to the soma (McBain & Fisahn, 2001). Lastly, interneurons expressing CR are typically bipolar or bitufted vertical cells whose axons also target pyramidal cell dendrites or other interneurons. Therefore interneurons have specific patterns of innervation and this heterogeneity is important for synchronizing cell firing and supporting oscillations of networks at many different frequencies in the CNS (Buzsaki *et al.*, 2004b). Interneurons can also fire action potentials and release GABA at different time points to distinct areas on the pyramidal cell (Klausberger & Somogyi, 2008). Normal brain function likely

relies on correct organization and firing of these interneurons, therefore any perturbations to the network of highly diverse interneurons likely supports the progression of epilepsy.

The observed loss of fully formed PNNs likely assisted in creating a permissive environment for axon growth and synapse rearrangement in the PC. Here we found a significant increase in the percent of CB and PV positive nerve terminals in layer 3 of kindled tissue. Since we know that layer 3 mostly consists of GABAergic cell bodies that innervate pyramidal cells in layer 2, this means that after kindling there is likely an increased number of inhibitory nerve terminals targeting other interneurons. Increased inhibition would likely reduce the activity of layer 3 and augment the excitability of the network, a process is called disinhibition. This phenomenon works when inhibitory activity increases the efficacy of the excitatory stimulus by reducing the activity of inhibitory cells that innervate the excitatory cells (Toth *et al.*, 1997). Therefore the observed increase in inhibitory nerve terminals in layer 3 may be responsible for causing excess excitation in the PC, and possibly is an integral part of the epileptogenic process. In conjunction with increased inhibitory release sites in layer 3, we also found a significant increase in CR positive nerve terminals in layer 2. This layer consists mostly of excitatory pyramidal cells therefore after kindling there appears to be an increase of inhibitory nerve terminals targeting excitatory cells. Although we do not yet know the exact reason for this increase, we can speculate that this may a compensatory mechanism in response to the excess excitation in the PC.

It is well known that axons of mature neurons can grow and reorganize in response to many physiological/pathological conditions, including epilepsy. Therefore understanding mechanisms of axonal sprouting is important for better understanding this disease. In an attempt to determine mechanism(s) responsible for the observed nerve terminal sprouting and rearrangement we looked at the expression of genes involved in axon guidance in mature animals. After selecting some candidate genes from our microarray data and confirming their altered mRNA expression using qPCR we showed an increased expression of ephrin A5 and ephrin B3 in the PC. Next using immunohistochemistry we determined that the ephrin B3 protein increased specifically in layer 2 of the PC. Through their interactions with Eph receptors, both ephrin A5 and ephrin B3 have previously been

shown to play role in guiding cells using adhesion and repulsion signals depending on their expression in either the pre- or post-synaptic terminal. Therefore because of the observed increase in ephrin A5 and ephrin B3 and their known roles in providing axon guidance cues, these genes must be further investigated to elucidate how they contribute to the layer specific increase in synaptic release sites and ultimately to the process of epileptogenesis.

4.7 Kindling disrupted the inhibitory network of the piriform cortex

Interneurons are responsible for synchronization of pyramidal cells and other interneurons in the network. Synchronization of cells creates network oscillations, which indicate highly coordinated neuronal activity over large areas. Networks can have a wide range of oscillatory activity from 0.05 Hz to 500 Hz, and distinct frequencies are proposed to play a role in different brain areas and are said to be behaviour dependent (Buzsaki & Draguhn, 2004). For example, theta frequency oscillations (3-12 Hz) are important in encoding, consolidating and retrieving information (Ylinen *et al.*, 1995; Buzsaki, 2002). Beta frequency oscillations (10-30 Hz) in the PC have been shown to play a role in odour sampling and memory (Martin *et al.*, 2007). Gamma frequency oscillations (30-80 Hz) are correlated with cognitive functions such as working memory and attention and have received a lot of attention because their relationship to higher brain function is most apparent (Engel & Singer, 2001). The individual firing patterns observed from each interneuron subtype are often well matched to the oscillations in which they participate. For example, basket cells (most of which also express PV) are said to be essential in generating gamma rhythms (Kawaguchi *et al.*, 1987). This is because PV interneurons form synapses both on other PV expressing interneurons and on the soma of pyramidal cells which likely synchronizes the network and distributes the synchronized activity, respectively. Additionally, synchronization of neurons is understood to be much more effective by perisomatic inhibitory post-synaptic potentials than dendritic excitatory postsynaptic potentials (Lytton & Sejnowski, 1991).

Recently our lab showed that after kindling induced seizures interneurons lose the ability to fire at very high frequencies (>100 Hz) (Gavrilovici *et al.*, 2012). Specifically after

kindling, multipolar cells lose the ability to fire at a non-adapting very high frequency but a greater proportion of these cells start to fire at an adapting low frequency. This suggests that changes to multipolar cell firing properties make high frequency oscillations harder achieve and sustain. Therefore there would be a tendency for these interneurons to have lower frequency oscillations after kindling. This loss of high frequency firing is important for understanding epilepsy as slow wave oscillations have been said to be a hallmark of epileptic activity (McIntyre *et al.*, 2002a). Oscillations at ~ 1 Hz have been shown to facilitate the generation, maintenance and propagation of stimulus evoked epileptiform activity in the hippocampus (Wolansky *et al.*, 2006). In addition to the loss of high frequency firing after kindling, interneuron excitability is also reduced due to an increased expression of a potassium channel (Kv 1.6) on PV^+ multipolar cells. This means that after kindling multipolar cells are not able to provide the same level of inhibitory strength (Gavrilovici *et al.*, 2012).

Taken together we know that after kindling there is a loss of interneuron firing diversity, a loss of inhibitory strength, and we now show that there is increased inhibitory nerve terminal innervation within the layers of the PC. The increased nerve terminals likely changed the signalling dynamics and ability of these interneurons to maintain the same oscillatory patterns. Kindling induced alterations to GABAergic interneurons likely caused increased excitation in the PC, helping to initiate seizures. These complex changes implicate interneurons of the PC in the process of epileptogenesis, possibly helping to explain why this area of the brain is highly seizurogenic.

4.8 Conclusions

The data presented here describe changes in the PC of mature rats cause by experimentally induced epilepsy. Decreased PNNs surrounding PV interneurons throughout layer 3 of the PC occurred concurrent with an increased expression of the endopeptidase, MMP9. The inhibition of MMP9 provided an antiepileptic effect during amygdala kindling, and may provide a potentially new therapeutic target for the prevention of epileptogenesis. Increased inhibitory release sites throughout the PC were found in addition to the increased expression of ephrin A5 and ephrin B3, axon guidance ligands. The role of axon guidance molecules in driving seizure progression or seizure

prevention has not yet been determined. This study provides preliminary data for the role of ephrin A5 and ephrin B3 in inhibitory nerve terminal rearrangement. Taken together these data suggest that following kindling induced seizures changes to the PNNs may contribute to a more permissive extracellular environment within the PC providing space for synaptic growth and the progression of epileptogenesis.

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Appendices

Table 2. Top 20 KEGG pathways with a fold change greater than 1.3

Pathway Name	**# of genes in pathway**
Neuroactive ligand-receptor interaction	37
Calcium signaling pathway	23
African trypanosomasis	6
Axon guidance	13
GABAergic synapse	10
Retrograde endocannabinoid signaling	11
Gastric acid secretion	9
Salivary secretion	9
Glycosaminoglycan biosynthesis – heparin sulfate	5
Glutamatergic synapse	11

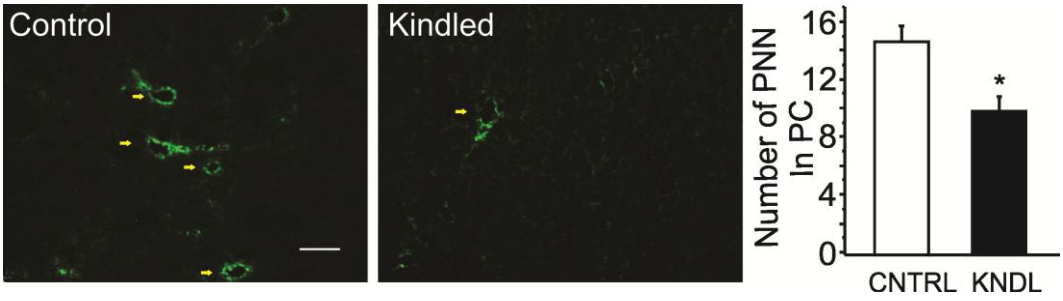

Figure 13. The average number of perineuronal nets was decreased in the piriform cortex of kindled rats after kindling-induced seizures. AGG immunoreactivity (green) was reduced in kindled tissue (ipsilateral) compared to control tissue. Analysis (unpaired, t-test) showed there was a significant decrease in the average number of AGG stained PNNs after kindling (*$p < 0.05$). PNNs were not assigned to either grade 1 or 2 as overall AGG staining was less intense and more diffuse than WFA. Error bars represent the standard error of the mean. Scale bar = 20µm.

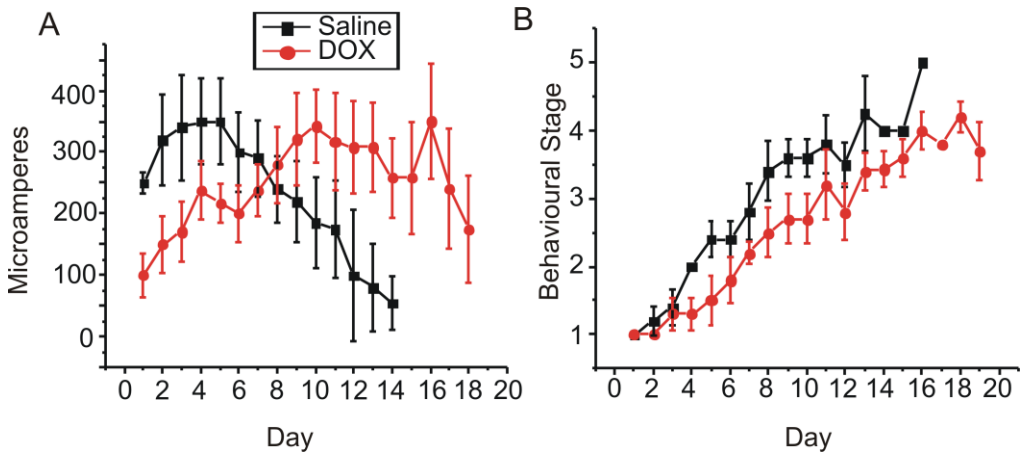

Figure 14. DOX attenuated amygdala kindling. (A) Saline injected rats required less current to elicit a kindling behaviour as they progressed through the seizure stages. There was no observed decrease in the amount of current required to elicit a kindling behaviour in DOX injected rats. (B) Saline injected rats reached stage 5 seizures quicker than DOX injected rats, some of which not reaching stage 5 by day 20.

Curriculum Vitae

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