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**STUDY OF AMYGDALA KINDLING-INDUCED ALTERATIONS IN
GABA_A RECEPTOR MEDIATED INHIBITION IN THE PIRIFORM
CORTEX OF RAT.**

by

Cezar Gavrilovici, B.Sc.

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Psychology in partial fulfillment of the requirements for the degree of Master of Science
(Neuroscience Specialization)

Department of Psychology
Carleton University
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Abstract

The piriform cortex is primarily involved in olfactory processing but makes interconnections with other limbic structures (amygdala, entorhinal cortex, hippocampus) that are involved in memory processing. Temporal lobe epilepsy studies have suggested that the piriform cortex is also implicated in the pathophysiological generation and/or spread of excitatory waves. However, little is known about the mechanisms that make piriform cortex a seizurogenic brain region. One possibility is that an imbalance between excitation and inhibition might be an important factor that could lead to epileptic discharges. Here we tested the hypothesis that in the kindling model of temporal lobe epilepsy GABA_A receptor mediated inhibition is altered in piriform cortex. To do this, whole cell patch-clamp recordings were performed in piriform brain slices obtained from unkindled and amygdala kindled adult rats after the establishment of stage 5 seizures. We found that kindling induced an increase in inhibition of nonpyramidal neurons of the anterior piriform cortex. This alteration was not observed on pyramidal (excitatory) cells. We also found that the efficacy to enhance inhibition by the positive modulator THDOC was significantly reduced in nonpyramidal cells of kindled group and THDOC had no effect in the pyramidal neurons after kindling. These data indicate that amygdala kindling caused a shift in the inhibition “balance” between the pyramidal and nonpyramidal cells towards the latter, leading to the dis-inhibition of pyramidal cells. The increased GABA efficacy (increased amplitude and time constant of mIPSCs) suggests that an increase in the number of GABAergic receptor and/or a subunit change might occur in this population after kindling.

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I dedicate this Thesis to my mother. Even she was apart from me for over two years, I felt her in my heart all the time.

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Introduction

Epilepsy is one of the most prevalent neurological diseases in humans, with a prevalence of about 0.6% and a high incidence in young children and elderly (Sundqvist, 2002). The epileptic seizure is by definition a paroxysmal and excessive electrical neuronal discharge in the brain that results from too much excitation or too little inhibition in the area in which the abnormal discharge starts (McIntyre et al., 2002b). This alteration in excitation/inhibition can lead to neuronal hyper-synchronization that then permits the seizure to fully develop and propagate throughout the brain.

All factors that can affect the brain (head traumas, neoplasms, degenerative diseases, infections, ischaemia and haemorrhages) can predispose a person to epilepsy (Vinters et al., 1993). Also, genetic factors (mutations that cause abnormal brain development, progressive neuroregeneration, disturbed energy metabolism and abnormal function of ion channels) contribute to aetiology in up to 40% of patients with epilepsy (Sander et al., 1997). Independent of aetiology all epileptic seizures can be classified clinically into 2 major categories: 1) partial-seizures (those that originate in a small group of neurons that constitute a seizure focus) and 2) generalized-seizures (simultaneous disruption of normal brain activity in both cerebral hemispheres). Partial seizures become secondary generalized when they spread to involve the majority of both cerebral hemispheres (Chabolla, 2002).

Of particular interest are seizures that develop from a few neurons that lie in what is termed a seizure focus. From the seizure focus, local paroxysmal depolarizing shifts (PDS) of membrane potential (large, long-lasting depolarization) associated with high

frequency bursts are thought to spread and cause the seizure to generalize (Matsumoto and Marsan, 1964). PDS are inhibited by alpha-amino-3hydroxy-5methylsoxazole-4-propionate (AMPA), N-methyl-D-aspartate (NMDA) channels receptor antagonists as well as drugs which block voltage-dependent Ca^{2+} channels ceasing transmitter release. The afterhyperpolarisations that follow PDS (GABA receptor activation and voltage- and calcium dependent K^{+} channels) at first reflect the feedback and feed forward inhibition of local neuronal circuit. However, these mechanisms are eventually overwhelmed and the spatial containment (surround inhibition) of the PDS activity is lost permitting the seizure to spread and generalize (Lothman et al., 1985). Nevertheless it seems clear that the development of seizures that generalize often reflects the loss of balance between the magnitude and temporal summation of the excitation/inhibition within a relatively small region (Lothman and Collins, 1990). Most areas of the brain can have a seizure focus, but it was recognised early on that some areas are more seizuregenic than others. In particular, the limbic regions of the brain including the hippocampus, entorhinal, piriform and perirhinal cortex and their underlying structure (endopiriform nucleus for example) are highly seizuregenic (McIntyre and Poulter, 2001). They are often the site of foci in human epilepsy and thus, these areas (particularly the hippocampus) have been scrutinized by researchers for over thirty years as being central to partial complex seizure generation.

Piriform cortex – Structure and limbic connections

Although studied for many years, the piriform cortex has been the subject of recent interest as seizures easily spread from this area and lesions to it can attenuate seizure genesis (Schwabe et al., 2004; de Guzman et al., 2004). Piriform cortex (PC) is a phylogenetically old structure involved not only in olfactory perception and discrimination in mammals but also in memory processing and spread of the excitatory waves (Loscher and Ebert, 1996). Piriform cortex is located at the basolateral surface of the forebrain ventral to the rhinal fissure. Although the piriform cortex is usually divided in two parts: anterior and posterior PC. A central part of PC -a transition zone between the anterior and posterior piriform cortex- has also be delineated (Haberly and Price, 1978) and suggested to play a role in the epileptic network (Lehmann et al., 1998b; Schwabe et al., 2000).

The piriform cortex has three layers: plexiform layer (layer I) which contains few GABAergic neurons (horizontal cells) is further divided in a superficial part (layer Ia) comprised of afferent fibres of the olfactory bulb and a deep part (layer Ib) containing dendrites from deeper cortical layers. Layer II consists of a high number of cell bodies of glutamatergic pyramidal and semilunar cells as well as GABAergic cells with globular somata (Haberly and Price, 1978). Semilunar cells are found mainly in the superficial part of layer II (layer IIa) and are characterised by a lack of basal dendrites but an extensive apical dendritic arborisation in layer I (similar to that of pyramidal cells). Layer III contains both pyramidal (in the superficial part of the layer) and nonpyramidal cells (GABAergic multipolar cells). GABAergic neurons were found in about equal abundance in layer II and III but their axons have an extensive arborisation in layer II where the

pyramidal cells are concentrated (Ekstrand et al., 2001). GABAergic cells provide both feedforward inhibition (lateral olfactory tract activates horizontal cells from layer I which give rise to inhibitory synapses on pyramidal cells) and feedback inhibition (pyramidal cells activate GABAergic multipolar cells that in turn inhibit surrounding pyramidal cells) controlling excitatory firing patterns in piriform cortex.

Piriform cortex interconnection with other limbic structures (amygdala, entorhinal cortex, hippocampus) might be relevant for the spread of limbic seizures. Two main circuits interconnect piriform cortex with amygdala (a reciprocal connection from amygdala to PC is made through endopiriform nuclei) and with hippocampus (via entorhinal cortex and subiculum) (Luskin and Price, 1983). Also, due to the limbic output provided by amygdala and subiculum, the seizure activity generated in limbic system can be spread to many other brain regions (Loscher and Ebert, 1996).

Animal models of epilepsy: amygdala kindling and the role of piriform cortex in epilepsy.

Animal models of epilepsy have contributed to our understanding of epileptogenesis, spread of epileptic discharge and action mechanisms of many antiepileptic drugs (Coulter et al., 2002). Different animal models reflect certain kinds of epilepsy with varying accuracy and no model seems to entirely recapitulate all aspects of seizures seen in humans. Models can be classified according to the methodology used to generate the seizures. The first are chemical or pharmacological models that employ convulsant drugs (such as pilocarpine or kainate) the second uses direct brain stimulation and is known as

kindling. Also, in recent years, genetic manipulations have produced a number of different models (by selective breeding or gene knock-out).

Kindling -the model used in this thesis- is a widely accepted model that produces complex partial seizures that secondarily generalize (as in temporal lobe epilepsy). Kindling itself is described as a progressive development of electrographic and convulsive motor seizures that follow brief, repeated daily electrical stimulation of a discrete forebrain site (Goddard, 1967). One important feature of kindling represents the propagation of the epileptic discharge to other sites and the possible recruitment of those sites into the discharge, leading to enhanced sensitivity to the focal electrical stimulation. The severity of kindled seizures is rated by a 5 stage scale of Racine: 1. Immobility, eye closure, facial clonus; 2. Head nodding associated with more severe facial clonus; 3. Unilateral clonus of forelimb; 4. Rearing and bilateral forelimb clonus; 5. Rearing with loss of balance and falling accompanied by general clonic seizures (Racine, 1972).

Amygdala was the first structure kindled but subsequent studies demonstrated that other structures (piriform cortex, perirhinal cortex) have a higher susceptibility for kindling while hippocampus requires a much larger number of stimulations to kindle (McIntyre and Plant, 1989; McIntyre et al., 2002b). The role of PC in generation, facilitation and/or spreading the epileptic seizures was observed in several clinical and experimental studies (McIntyre and Wong, 1986). In kindled animals the amygdala and/or piriform cortex showed the earliest onset of interictal discharges regardless of the site of stimulation. Other structures involved in generation of interictal discharges were perirhinal, entorhinal cortex, septal area, ventral hippocampus (McIntyre et al., 2002b). It has also been shown that amygdala stimulation in amygdala –piriform cortex slices from

kindled animals increased the duration of evoked burst events in piriform cortex compared to non-kindled animals (McIntyre and Wong, 1986). These studies also showed spontaneous discharges in the piriform cortex of kindled rats that were not seen in normal unkindled brain.

Pharmacological experiments have shown that both microinjections of muscimol (a GABA_A receptor agonist) or 2-amino-5-phosphonovaleric acid (APV which is a NMDA antagonist) in anterior PC reduced the afterdischarge duration of amygdala-kindled seizures by 20% (Morimoto et al., 1986). Generalized seizures were elicited by administration (microinjections) of low doses of bicuculline in anterior, central and posterior PC (Ebert et al., 2000) but, in contrast, these same drugs were relatively ineffective when injected into amygdala.

One immunohistochemistry study demonstrated that amygdala kindling, (Lehmann et al., 1998a) produced a 20 % decrease in GABA immunoreactive neurons in the ipsilateral central piriform cortex (which might explain the alteration in excitability of central PC pyramidal neurons. The same study also showed a 35% decrease in GABA neurons of both (ipsi- and contralateral) basolateral amygdala (Lehmann et al., 1998a). Another study showed that a bilateral lesion of central piriform cortex with microinjections of the neurotoxin ibotenate retarded amygdala kindling by decreasing progression from stage 3 to 5 compared to anterior and posterior piriform cortex (Schwabe et al., 2000).

The measurement of cerebral metabolism has indicated the brain structures that display a high metabolic activity during the progression of seizures. In early stages (1 and 2) elicited by amygdala kindling, increased ¹⁴C-2-deoxyglucose (2-DG) uptake was observed in ipsilateral piriform cortex, entorhinal cortex, frontal limbic field (sulcal area),

medial septal area, suggesting the early contribution of these structures in the spreading and amplification of the excitatory waves (Engel et al., 1978).

GABA involvement in epilepsy and epileptogenesis

γ -aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the cerebral cortex. The action of GABA is mediated by ionotropic GABA_A (reviewed in (Barnard et al., 1998) and metabotropic GABA_B receptors (reviewed in (Bowery et al., 2002). GABA_A receptors mediate fast inhibitory transmission by gating Cl⁻ ions which hyperpolarize the membrane and inhibit action potentials but they are also involved in the maintenance of rhythmic activities in brain networks (Traub et al., 1999).

Although GABA_A receptors are constructed from a repertoire of at least 21 subunit types (α_{1-6} , β_{1-4} , γ_{1-4} , δ , ϵ , π , ρ_{1-3}) only 5 subunits are used to form a pentameric heteroligomer comprised most usually from 2 α subunits 2 β subunits and one γ (Bormann, 2000). GABA_B receptors are G-protein coupled receptors that hyperpolarize the neuron by increasing the potassium conductance, decreasing Ca²⁺ currents, having a slow inhibitory effect (Bowery et al., 2002). They are less heterogeneous than GABA_A receptor, being comprised of only two differing subunits one which may be alternatively spliced.

Alterations in GABA_A receptor expression in epilepsy have been postulated almost since the heterogeneity of GABA_A receptor was first recognized. The reorganization of GABA_A receptor subunits has been investigated in several labs and alterations in expression have been implicated in the generation of seizures (Brooks-Kayal et al., 1999;

Brooks et al., 1999; Kamphuis et al., 1995; Loup et al., 2000; Fritschy et al., 1999; Nusser et al., 1998; Sperk et al., 1998). Many studies have reported a loss of $\alpha 1$ subunit expression, perhaps reflecting the loss of the neurons that highly express $\alpha 1$ subunit. In commissure kindled rat brain, where there is little or no neuronal loss, $\alpha 1$ reactivity increased on dentate granule cells (Kamphuis et al., 1995; Nusser et al., 1998). Another study that examined hippocampal tissue obtained from humans with intractable temporal lobe epilepsy showed that $\alpha 1$ subunit expression was decreased, similar to animal models where significant cell loss occurs (Loup et al., 2000). However, the same study also showed $\alpha 2$ immunoreactivity went up while $\alpha 3$ immunoreactivity was unchanged. The changes documented thus far do not indicate any particular pattern that is highly correlated to seizure genesis.

Genetic epilepsy and genetic models of epilepsy bring support a role for GABA in epilepsy.

Epilepsy appears to have a genetic component or reflect the alteration of gene expression. This may be due to the inheritance of a mutation or the perhaps-inappropriate expression due to a perturbation (for example brain injury). Many differences in gene expression have been documented, including changes in potassium channels and metabolic enzymes (Morimoto et al., 2004).

GABA_A receptor expression has been altered in various knockout mice models having varying impacts. For example a relatively severe effect was found in GABA $\beta 3$ -deficient mice that displayed frequent myoclonus and occasional epileptic seizures,

documented by electroencephalographic recording (Homanics et al., 1997). Another model shows elevated levels of GABA $\alpha 2$ and $\beta 1$ -subunit mRNA in tottering cortex (Tehrani et al., 1997). These mice display ataxia, sporadic focal myoclonic seizures and generalized cortical spike-wave discharges. Another animal model of seizure predisposition, which shows reduced responses to GABA_A receptor agonists and deficit in GABA function, is the genetically epilepsy-prone rat (GEPR) (Molnar et al., 2000). Abnormal overexpression of GABA receptor subunits observed in kindling-prone ($\alpha 2$, $\alpha 3$ and $\alpha 5$) and kindling-resistant ($\alpha 1$) rats may result in the incapacity of the brain to prevent hypersynchrony in the neural networks (Poulter et al., 1999). These seizure resistant and prone rats do not display spontaneous seizures but do have other behavioural manifestations that are co-morbid with epilepsy like attention deficit hyperactive syndrome (McIntyre and Anisman, 2000).

Genetic evidence for the involvement of GABA_A receptors in human epilepsy has been shown by Baulac et al. (2001) who have reported a point mutation in the GABA_A receptor $\gamma 2$ -subunit gene. This mutation segregates in a family with a phenotype closely related to generalized epilepsy with febrile seizures plus (GEFS+). Analysis of recombinant $\alpha 1\beta 2\gamma 2$ receptors expressed in *Xenopus laevis* oocytes confirmed the predicted effect of the mutation, a decrease in the amplitude of GABA-activated currents. Another mutation in a gene encoding a GABA_A receptor $\gamma 2$ subunit was found in two families with febrile seizures (FS) and childhood epilepsy. This mutation expressed in *Xenopus* oocytes suppressed diazepam potentiation of GABAergic currents (Wallace et al., 2001).

Pharmacology of GABA_A receptors in Epilepsy

Evidence from experimental and clinical sources involving GABA agonists and antagonists provide support for a role of GABA in the mechanism and treatment of epilepsy. Drugs that facilitate activity in GABA systems including benzodiazepines, barbiturates, neurosteroids have been shown to elicit anti-seizure effects (Rupprecht and Holsboer, 2001). These drugs have distinct binding sites on GABA_A receptors augmenting GABA-mediated responses by increasing the mean channel opening time induced by GABA (barbiturates), increasing the affinity of the receptor for GABA as well as the frequency of GABA-activated channel openings (benzodiazepines) and intensifying both the channel open frequency and channel open duration (neurosteroids) (reviewed in (Sieghart, 1995)).

Inhibition of GABA is frequently epileptogenic: GABA antagonists (bicuculline, picrotoxin) are described as proconvulsant and GABA-synthesis inhibitors (4-deoxyprydoxine, isoniazid, thiosemicarbazide) can cause seizures (Treiman, 2001). In human, GABA_A receptors were found to be reduced in CA1 and CA4, but unchanged in dentate gyrus (DG) of individuals with medically refractory temporal lobe epilepsy (McDonald et al., 1991). Using positron emission tomography (PET) and the benzodiazepine (BZ) ligand [11C] flumazenil, (Savic et al., 1998) showed that the BZ ligand binding was reduced in the epileptic foci of patients with partial epilepsy.

Neurosteroids are modulators of GABA_A receptor mediated inhibition

It has been known for some time that neurosteroids alter GABA_A receptor mediated inhibition (Harrison et al., 1987; Barker et al., 1987; Cooper et al., 1999; Jorge et al., 2000) and recent evidence indicates that they may be endogenous acute and perhaps long-term modulators of GABA_A receptor activity and synaptic inhibition (Bitran et al., 1995; Calixto et al., 1995; Fahey et al., 1995; Fernandez and Picazo, 1995; Frye, 1995; Frye and Sturgis, 1995; Lambert et al., 1995; Poisbeau et al., 1997; Poulter et al., 1997; Puia et al., 1990; Yu and Ticku, 1995; Zhu and Vicini, 1997; Zhu et al., 1996). These compounds, which are metabolites of steroid hormones, have modulatory effects on GABA_A receptor function by a number of mechanisms, including enhancement of affinity of GABA receptors for GABA and by alteration in the desensitisation kinetics (Lambert et al., 2003).

One class of neurosteroids having both positive and negative modulatory effects on GABA_A receptors are metabolites of pregnanolone. The best characterized is the positive modulator allopregnanolone, which is synthesized in numerous body tissues (Mickan and Zander, 1979; Compagnone et al., 1995). Evidence suggests that allopregnanolone levels may rise and fall depending on the progesterone concentration. In particular the “crash” in progesterone plasma concentrations after parturition has been implicated in increased susceptibility to drug induced seizures (Smith et al., 1998a; Smith et al., 1998b). Allotetrahydrodeoxycorticosterone (THDOC) is another neurosteroid of interest that is synthesized from deoxycorticosterone (DOC). DOC is released during stress under the control of CRH and ACTH (Reddy, 2003). Thus it has been suggested that psychological stressors may cause a modulation of GABA_A receptor activity. Although an attractive

hypothesis, in the presence of stress, plasma concentrations of THDOC rise to levels that would have only small effects on GABA_A receptor activity (Reddy, 2003). So while one may surmise that these compounds are endogenous modulators of inhibition (synaptic or extrasynaptic), conclusive evidence that they fulfil an important physiological function so far has not been presented.

Nevertheless other evidence suggests that pharmacological doses of THDOC may be effective anticonvulsants in young rats and for the treatment of infantile seizures in humans. In this way, administration of adrenocortical hormones may act by enhancing the levels of THDOC via the release of its precursor DOC (Edwards et al., 2002a; Edwards et al., 2002b).

The molecular pharmacology of neurosteroids is complex and may vary from one steroid to the next (for review (Lambert et al., 2003). Some neurosteroids (e.g., allopregnanolone) seem to have little selectivity, while others like THDOC may show significant, albeit not fully appreciated selectivity. This selectivity may determine neurosteroid activity in synapses having different subunit compositions as well as between synaptic versus extrasynaptic sites.

Objectives

The main objective of the study was to determine whether the amygdala kindling induces an alteration of GABA-mediated inhibition in anterior piriform cortex and whether neurosteroid pharmacology is altered by kindling/seizures. These experiments are designed to answer the following different questions:

- 1) One mechanism that might produce a decrease of inhibition in PC after kindling is related to a possible increased synaptic efficacy of GABAergic neurons (increased number of GABA_A receptors on GABA neuron or/and an increase in GABA release) that might lead to an enhanced inhibition of GABAergic network in PC due to connection between interneurons. I can test for this possibility by comparing the amplitude and perhaps the frequency of mIPSCs in the differing cell populations.
- 2) Kindling may also change the expression of the GABA_A receptors. Previous studies have shown that alterations in receptor expression are manifested by changes in the time course of mIPSCs. Thus following the time course of miniature inhibitory postsynaptic currents (mIPSCs) will permit evaluation of this possibility. A change in timing implies that the structure of the receptor has changed.
- 3) High excitability of pyramidal neurons of PC after amygdala kindling might be explained by a decreased inhibition. One mechanism that might account for this behavior could be a decrease of GABAergic neurons in PC. Thus after kindling the direct or feedback inhibition of pyramidal cells may be reduced. A loss in inhibitory neurons may be seen as a reduced mIPSCs frequency thus I may be able to compare the mIPSC frequency on nonpyramidal and pyramidal cells to test this hypothesis.

These questions will be examined by making patch clamp recordings from either nonpyramidal cells from layer III and pyramidal cells from layer II of kindled and nonkindled anterior piriform cortex.

Materials and Methods

All experiments are conducted in accordance with the guidelines of the Canadian Council on Animal Care and protocols approved by The Carleton University.

Animals and surgery:

Male Sprague Dawley rats weighing 200g at the time of the initial surgery were used. They were housed individually during the whole experiment, with free access to food and water under a continuous 12-h/12-h light-dark cycle.

Electrode implantation: Animals were anesthetized with pentobarbital (60mg/kg, i.p.) and implanted with two bipolar stimulating/recording electrodes bilaterally in the basolateral amygdala with the following coordinates: 2.6 posterior to Bregma, 4.5 mm lateral to midline, 8.0 ventral (Paxinos and Watson, 1986). The electrodes were constructed of two twisted strands of 0.127 mm-diameter Nichrome wire, Diamel-insulated, and were attached to male Amphenol pins. The electrodes were implanted and secured to the skull with jeweler's screws. The electrode assembly was fixed to the skull by dental acrylic cement (McIntyre and Molino, 1972)

Kindling and slice preparation

The rats were allocated to 2 groups: the experimental rats were kindled one week after the surgery. The afterdischarge-threshold was determined in each amygdala by delivering a 2-s 60-Hz sine wave stimulus of progressively increasing intensities (15, 25, 35, 50, 75, 100, 150, 200, 250, 300 and 350 μ A until an AD threshold was triggered

(McIntyre and Plant, 1993). The rats were stimulated daily until six generalized stage-5 convulsions were elicited. Seizure severity and duration were recorded daily during the kindling acquisition. 5 days after the last stage 5-seizure the AD threshold was determined again (post-kindling ADT) using the same protocol as for pre-kindling ADT.

The control group (nonkindled) were implanted but never kindled.

After 1-10 weeks from the kindling brain slices were isolated from experimental and control group. After anesthesia with sodium pentobarbital (60 mg/kg, i.p.) the rats were intracardiac perfused with an ice-cold Ringer solution in which sodium was replaced by choline (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).

The intracardiac perfusion was performed by opening the thoracic cavity, clamping off the descending aorta, and cutting the right atrium. The left ventricle of the heart was perfused with 50 ml of the ice-cold choline solution. After perfusion, the brain was rapidly removed and the temporal lobe area was excised as a block. The block was sliced (coronally) with a Vibratome (300- μm -thick sections). The slices were obtained from the following stereotaxic levels: anterior slices (1.5 to -0.3 mm relative to bregma). The slices were incubated at 37°C for 30 min and subsequently moved to a room-temperature bath for at least 45 minutes. Slicing, incubation, and storage were all performed in the choline solution. The Ringer's solution used during electrical recordings was similar to the choline solution except that pyruvate and ascorbate were removed, equimolar NaCl replaced the choline Cl, and CaCl_2 and MgCl_2 were both used at a 2 mM concentration (McIntyre et al., 2002a). Both solutions were maintained at pH 7.4 and bubbling with 5% CO_2 / 95% O_2 (carbogen).

Patch electrodes were pulled from borosilicate glass capillaries and filled with KCl solution having an internal composition (in mM): KCl 145, NaCl 10, CaCl_2 2, EGTA 10, MgATP 2, dextrose 10, and HEPES 10 (300-320 mOsm; pH: 7.3-7.4). The resistance of these electrodes was 3-8 MOhm.

Recordings from neurons in layer II and III of anterior piriform cortex were made with a Heka EPC-9/2 amplifier. In the kindled rats recordings were made in the piriform cortex of both ipsilateral (IPS) and contralateral (CLS) hemisphere (relative to the amygdala which was kindled). Series resistance compensation was performed in all recordings. The initial access was <20 MOhm and compensated by 70-80%.

Individual neurons were visually identified by using differential phase contrast optics (Dodt, Zeiss) on a modified Zeiss Axioscope upright microscope. Voltage-gated currents and excitability of the cell were monitored by means of voltage-clamp and current-clamp protocols. Spontaneously occurring miniature inhibitory post synaptic currents (mIPSCs) were recorded after blocking of Na^+ channels, NMDA, AMPA and kainate channels with a Ringer's solution (described above) containing 250 nM tetrodotoxin (TTX; Sigma), 10 μM dinitroquinoxaline-2,3-dione (DNQX; Research Biochemicals), and 20 μM 2-amino phosphonovaleric acid (APV; Research Biochemicals).

Histochemistry

In order to reconstruct the morphology and understand where the recordings were made patch electrodes included 0.3% Lucifer Yellow. After the completion of a recording the slice are removed from the microscope chamber and post fixed in Lana's solution after rinsing in PBS 24-48 hours. The sections were mounted on a microscope slide for

viewing under epifluorescence light. The slide is illuminated with 488 nm light and the Lucifer Yellow then emits a 520 nm. In order to reconstruct the morphology of the cell a series of digital photos in the z plane are taken. They are subsequently deconvolved using commercially available software. (EPR, Scanalytics, Mc Clean Va. U.S.A.). The individual confocal images are then projected onto a signal 2-D image to show the morphology of the cell. Criteria for classification of pyramidal versus nonpyramidal cells were based on: 1) Soma morphology; 2) Projection of the axon to deeper layers for pyramidal cells versus non-projecting nonpyramidal cells; 3) Presence of dendritic spines for pyramidal cells; 4) Spiking properties. On this basis, the neurons were classified as pyramidal and nonpyramidal and included in appropriate groups (nonkindled -NK, kindled contralateral-CLS and kindled ipsilateral- IPS).

Analysis of mIPSCs.

In order to evaluate the differential effects on the mono- and bi- exponential mIPSCs, the deactivation phases of the mIPSCs were *individually* fitted with exponential functions. Thus, mono- and bi-exponential fits were performed on each mIPSC. The residual deviations were then compared to decide which of the fits to retain, as we have described previously (Hutcheon et al., 2000). First, *all* mIPSCs that were collected in a recording were sorted to eliminate events with 10-90% rise times > 1.5 ms, half-width durations < 4 ms (i.e., events that were too brief to be considered genuine GABAergic synaptic transients) as well as those temporally non-discrete events. After sorting, about ~80% of the collected events were rejected for further analysis. Our initial screen of the events showed that ~30% of the mIPSC had rise times that were > 1.5 ms, ~10 % were

false events (shifts in the baseline or noise), while another ~40% could not be fitted because another mIPSC occurred during the fitting window.

After categorisation, about 90-150 mIPSCs were selected as a representative sample to include diversity with respect to amplitude and kinetics parameters for each event.

These representative samples were otherwise randomly selected. The median values describing the amplitudes and deactivation kinetics of the selected mono- and bi-exponential mIPSCs from each cell were calculated in each group and then averaged to generate an overall change in behavior for that group. Comparisons within the groups (pyramidal versus nonpyramidal) were made by using t-test while comparisons between the groups (pyramidal mIPSCs attributes comparison between NK, CLS and IPS group, for example) were made by using ANOVA and Tukey multiple comparison as a *post hoc* test where appropriate.

This analysis permitted the comparison of mIPSC attributes within groups (pyramidal vs nonpyramidal) and between the groups (NK vs CLS vs IPS) by generating 6 different distributions:

1. Time constant of mono-exponential mIPSCs
2. The amplitude of mono-exponential mIPSCs
- 3& 4. Fast and slow time constants of deactivation of bi-exponential mIPSCs
5. The amplitude of bi-exponential mIPSCs
6. Total charge transfer (due to mono- and respectively bi-exponential mIPSCs)

Results

These data concern whole cell patch clamp recordings from neurons located in the second and third layer of the piriform cortex. Here we show the analysis 96 of 150 recordings that met the criteria stipulated in the Materials and Methods with regard to low-access resistance, appropriate resistance compensation and morphological identification. In presence of TTX (250 nM), APV (20 μ M) and DNQX (10 μ M) spontaneously occurring miniature inhibitory currents (mIPSCs) were collected for later offline analysis. There were no differences in the mIPSC frequency observed between groups. Thus objective # 3 was shown to be unlikely and our analysis focused upon objectives 1 and 2. So, we analyzed the attributes of the mIPSCs first in the pyramidal and nonpyramidal cells in the nonkindled group (electrode implanted but unstimulated brain slices) then ipsilateral (IPS) to the kindled site and finally contralateral to the kindling. We then compared the activity of the neurosteroid tetrahydrodeoxycorticosterone (THDOC) on the different populations of neurons. These experiments addressed the hypothesis that an imbalance of inhibition between the pyramidal and nonpyramidal cells might be a factor that contributes to generation and/or spread of epileptic activity and that inhibition becomes relatively insensitive to positive modulator drugs.

As reported previously we have identified two different populations of mIPSCs that can be characterized by their amplitudes and kinetic attributes (Hutcheon et al., 2000; McIntyre et al., 2002a). One population is small in amplitude and deactivates at a mono-exponential rate while the other larger amplitude population decays with two exponential

phases. Thus, these populations were sorted and analyzed separately in each cell type in each area examined.

In nonkindled (NK) rats, the amplitudes and the decay time constant of mono- and bi-exponential mIPSCs on pyramidal and nonpyramidal neurons were indistinguishable from each other. ($P > 0.05$, t-test; Figure 1; Table 1).

Figure 1. Comparison of mIPSC attributes in nonkindled piriform cortex. In naive tissue the attributes of both mono-exponential and bi-exponential mIPSCs in pyramidal and nonpyramidal neurons are very similar both in time course and in amplitude. These attributes are summarized in Table 1.

However, in the kindled cortex -ipsilateral to the kindling site (IPS), the amplitude of both mono-exponential and bi-exponential mIPSCs of nonpyramidal cells were ~24% bigger than pyramidal neurons in the same region ($P < 0.001$, t-test; Figure 2). Also, the

decay time of the mono-exponential mIPSCs were significantly longer in IPS nonpyramidal cells versus IPS pyramidal cells ($P < 0.05$, t-test).

Figure 2. In ipsilateral kindled PC, nonpyramidal mIPSCs are larger in amplitude and decay more slowly than those recorded in pyramidal cells of the same region (IPS). Here, we show that both mono- and bi-exponential nonpyramidal mIPSCs are significantly enhanced compared to those in pyramidal neurons (summarized in Table 1).

These differences in mIPSCs attributes observed between the nonpyramidal and pyramidal cells from kindled ipsilateral group were also found when we compared the nonpyramidal cell between the groups (NK vs CLS, vs IPS) while the same comparison for pyramidal mIPSCs showed no significant change.

We found a 38% increase in the amplitude of the mono-exponential mIPSCs on the ipsilateral nonpyramidal cells versus the same type of cell in nonkindled group

($P < 0.001$). The decay time constant of the mono-exponential mIPSCs was also prolonged by 28% in the ipsilateral group versus nonkindled ($P < 0.001$; Figure 5). Thus, overall kindling appeared to have selectively increased the inhibition on the nonpyramidal neurons.

It has been observed that the contralateral to the kindling site in temporal cortex that brain behaves as though it is partially kindled (McIntyre and Poulter, 2001). Thus it was of interest to compare and contrast the status of the inhibition in the contralateral piriform cortex in order to ascertain whether or not concomitant changes in inhibition had occurred in this region. No changes in mIPSCs attributes were found between pyramidal and nonpyramidal cells of CLS ($P > 0.05$, t-test; Figure 3).

Figure 3. Kindling does not significantly alter the inhibition in the contralateral piriform cortex. Here, comparison between mIPSCs attributes (amplitude and decay time) in pyramidal and nonpyramidal cells of CLS showed no significant changes.

The mIPSCs from the pyramidal cells were also indistinguishable from those recorded from either nonkindled control or ipsilateral brain slices ($P > 0.05$). For the nonpyramidal cells the mIPSCs were on average about 20 % larger than those in the NK group but this apparent difference failed to reach statistical significance ($P = 0.052$). However they were significantly different than mIPSCs recorded from the ipsilateral kindled side ($P < 0.05$, Tukey multiple comparison; Figure 4). The time constants of the nonpyramidal contralateral mIPSCs were not statistically different than NK mIPSCs ($P = 0.09$) but they were different from the time courses recorded from the ipsilateral side ($P < 0.05$, Tukey multiple comparison; Figure 5).

Figure 4 shows the distribution of mIPSCs amplitudes for the two different kinds of synaptic responses with the three groups examined showing an upward trend in both mIPSCs to larger amplitude as the seizure progress.

Figure 4. Scatter plots of the median mIPSC amplitudes for individual recording from nonpyramidal neurons of the piriform cortex. The upward trends in the distributions are evident in both types of mIPSC populations.

Figure. 5. Comparison of decay time of mono-exponential mIPSCs in nonpyramidal cells of NK, CLS and IPS groups. * $P < 0.05$; ** $P < 0.001$, Tukey multiple comparison

Next, we calculated the change in the total charge transfer that would occur due to these changes in amplitude and time course of mIPSCs recorded on the ipsilateral nonpyramidal cells. In nonkindled animals the average charge transfer for a mono-exponential mIPSC was 295 ± 16 pA pColoumbs (pCs). This value significantly increased to $524 \text{ pCs} \pm 24$ ($P < 0.001$) in nonpyramidal cells on the ipsilateral side, an increase of nearly 80 %. A smaller but statistically significant increase in charge transfer was observed in the contralateral neurons as well (387 ± 17 pA, $P < 0.05$). For the bi-exponential mIPSCs the increase observed in nonpyramidal IPS group was less dramatic, only increasing by 35 % (542 ± 45 pCs in NK versus 732 ± 28 pCs respectively). The

charge transfer due to the bi-exponential mIPSCs in the contralateral side failed to reach significance versus nonpyramidal NK group.

Characterization of THDOC effect on mIPSCs in pyramidal and nonpyramidal cells of piriform cortex

In the next set of experiments, we were interested in determining if the neurosteroid THDOC would differentially affect the nonpyramidal versus pyramidal populations in either nonkindled, ipsilateral and contralateral kindled groups.

We wished to use concentrations of THDOC that are considered to be physiological and so we began by using 50 nM THDOC which has been reported to be effective in other preparations (Cooper et al., 1999). However, this concentration had no effect on mIPSCs in any cell tested. Therefore, we increased the concentration to 100 nM, the upper limit of what is thought to be “physiological”. At this concentration THDOC had significant effects on both the pyramidal and nonpyramidal neurons in the NK slices. First, as expected it significantly prolonged the mono-exponential time constant and slow component of the bi exponential decay in both cell types, but it had no effect on fast component of the bi-exponential population (Figure 6; Table 2, Table 3). Surprisingly, THDOC produced a significant *decrease* in the amplitudes of both the mono- and bi-exponential mIPSCs.

Figure 6. THDOC increases the deactivation rate of all populations of mIPSCs but reduces the amplitude as well (mIPSCs presented here were recorded in ipsilateral pyramidal and nonpyramidal cells)

While increasing the deactivation time constant would tend to enhance inhibition, the reduction in mIPSC amplitude tends to decrease it. So, in order to assess the overall impact of these conflicting effects we calculated the average charge transfer for the individual events in each cell and compared these values to those obtained in the presence of THDOC. Figure 7 shows a summary of these data. First, in pyramidal cells THDOC had no statistically significant effect on the mono-exponential mIPSCs in either control or in ipsilateral (kindled slices). In the contralateral side there was a statistically significant effect ($P < 0.05$, t-test). The total inhibition due to the bi-exponential mIPSCs was increased in both contra- and ipsilateral side but not in nonkindled tissue.

The charge transfer due to mono-exponential mIPSCs in nonpyramidal neurons in unkindled slices was significantly increased ($P < 0.001$, t-test) but no effect was seen in either the contralateral or ipsilateral slices. The charge transfer due to bi-exponential events THDOC was uniformly increased by THDOC ($P < 0.001$, t-test). Overall, in nonpyramidal neurons THDOC failed to increase inhibition in the mono-exponential event while maintaining its efficacy in the other population.

Figure 7. THDOC had variable effects on charge transfer. The combined effects of THDOC (see Figure 6) resulted in variable efficacy in potentiating inhibition. Only weak effects were seen in pyramidal cells where the mono-exponential mIPSC in NK and IPS were unaffected. A similar lack of effect was also seen in nonpyramidal neurons of kindled groups (CLS and IPS) where THDOC failed to significantly increase the charge transfer due to mono-exponential mIPSCs. The nonpyramidal bi-exponential mIPSCs seemed to be uniformly potentiated independent of kindling status. * $P < 0.05$, ** $P < 0.001$, t-test.

Table 1. Comparison of mIPSC attributes in morphologically detected neurons of nonkindled, contralateral and ipsilateral kindled groups.

Group	Nonkindled (NK)		Contralateral (CLS)		Ipsilateral (IPS)	
Cell type	Pyramidal	Nonpyramidal	Pyramidal	Nonpyramidal	Pyramidal	Nonpyramidal
Number of cells	15	14	12	19	8	28
Mono τ_1 (ms)	7.3 ± 0.4	6.5 ± 0.2	7.3 ± 0.2	7.5 ± 0.3	7.0 ± 0.4	8.4 ± 0.1 *,**
Amp (pA)	50.8 ± 2.0	44.9 ± 2.1	52.8 ± 1.9	53.5 ± 2.8	50.3 ± 1.7	62.2 ± 2.4 *,**
Bi τ_1 (ms)	2.8 ± 0.1	2.5 ± 0.1	2.2 ± 1.3	2.4 ± 0.2	2.3 ± 0.3	2.2 ± 0.1
τ_2 (ms)	20.2 ± 1.5	17.8 ± 1.2	17.7 ± 0.7	17.7 ± 0.6	19.4 ± 0.8	18.4 ± 0.4
Amp (pA)	58.8 ± 2.0	53.7 ± 2.4	61.6 ± 2.7	60.4 ± 2.9	57.5 ± 2.1	71.3 ± 2.6 *,**

Significance with respect to neurons of the same morphology in kindled contralateral group:

* $P < 0.05$, Tukey multiple comparisons.

Significance with respect to neurons of the same morphology in nonkindled rats: ** $P < 0.001$,

Tukey multiple comparisons.

Table 2. Change in mIPSC attributes due to the neurosteroid (THDOC) treatment in morphologically identified pyramidal cells in nonkindled, contralateral and ipsilateral kindled groups.

mIPSCs type	Monoexponential		Biexponential		
mIPSC attributes	τ_1 (ms)	Amp (pA)	τ_1 (ms)	τ_2 (ms)	Amp (pA)
NK (n=11)	$1.4 \pm 0.5^*$	$-4.2 \pm 1.7^*$	0.2 ± 0.1	$5.3 \pm 2.0^*$	$-5.7 \pm 2.0^*$
Contralateral (n=9)	$2.2 \pm 0.5^{**}$	$-5.8 \pm 2.0^*$	0.6 ± 0.3	$9.8 \pm 1.9^{**}$	$-8.7 \pm 1.8^*$
Ipsilateral (n=6)	$1.9 \pm 0.3^*$	$-6.1 \pm 2.1^*$	0.4 ± 0.6	$7.9 \pm 1.0^{**}$	$-7.4 \pm 2.7^*$

*P<0.05, **P<0.001, t-test

Table 3. Change in mIPSC attributes due to the neurosteroid (THDOC) treatment in morphologically identified nonpyramidal cells in nonkindled, contralateral and ipsilateral kindled groups.

mIPSCs type	Mono-exponential		Bi-exponential		
mIPSC attributes	τ_1 (ms)	Amp (pA)	τ_1 (ms)	τ_2 (ms)	Amp (pA)
NK (n=13)	$2.6 \pm 0.3^{**}$	$-3.7 \pm 1.4^*$	0.2 ± 0.1	$8.0 \pm 1.2^{**}$	$-4.8 \pm 1.8^*$
Contralateral (n=11)	$1.8 \pm 0.6^*$	$-5.3 \pm 2.5^*$	0.2 ± 0.3	$7.9 \pm 1.3^*$	$-5.6 \pm 2.4^*$
Ipsilateral (n=16)	$1.5 \pm 0.5^*$	$-5.3 \pm 1.7^*$	0.3 ± 0.2	$7.8 \pm 1.2^{**}$	$-5.0 \pm 2.0^*$

*P<0.05, **P<0.001, t-test

Discussion

These data indicate an increased inhibition in nonpyramidal neurons in the piriform cortex of kindled rats (ipsilateral site related to the amygdala stimulation). This increase in the charge transfer in nonpyramidal cells (due to significant increase in mIPSCs amplitude and mono-exponential time constant) is reported here for the first time. However, enhanced inhibition after kindling or pilocarpine treatment has been shown to be induced on granule cells of the dentate gyrus (Nusser et al., 1998; Cohen et al., 2003; Otis et al., 1994).

How this increased inhibition develops in our preparation (or others) is not clear. However, some possible mechanisms that could account for our observations have been ruled-out, while for others there is direct evidence. The most obvious mechanism would be if kindling-induced an increase in open state probability (P_{open}). Increased P_{open} would occur if the channels had increased affinity for GABA or if the desensitization that normally develops is attenuated by some biochemical or stoichiometric (subunit) change. The best method for estimating P_{open} is by non-peaked scaled non-stationary fluctuation analysis, which we have been unable to do here (due to a lack of available software). Those that have analyzed P_{open} by this methodology have, however, failed to detect a change in P_{open} (Otis et al., 1994; Nusser et al., 1998). Thus, although we have not ruled out an increase in P_{open} , this mechanism seems unlikely.

The next possibility is enhanced channel conductance. This change suggests that the channels are modified or replaced in the synapse with those that are more permeable to chloride ion. Stationary noise analysis can also estimate this, but as above, we have

been unable to this. Again, when analyzed by others, channel conductance was also found not be altered (Nusser et al., 1998; Otis et al., 1994).

The next possible mechanism is increased GABA release from presynaptic terminals. Increased GABAergic concentration at the synapse by an enhanced GABAergic release might alter the mIPSCs amplitude. This possibility cannot be directly tested by patch clamp measurements methodology, however, several lines of evidence argue against it. First, several studies have shown that GABAergic release was found to be decreased in kindled amygdala (Kaura et al, 1995; Shin et al. 1995). Second, increased release suggests that under normal conditions the synapses are not saturated, a conclusion not supported by most studies (Maconochie et al., 1994; Jones et al., 1998; Perrais and Ropert, 1999). The possibility that the quantal content goes up (i.e. increased probability that more than one vesicle is released on average) seems unlikely as the morphological enlargement of the terminals that has been reported after kindling (Nusser et al., 1998) would account for the reduction (and not an increase) in quantal content. Nusser et al (1998) explained the reduction of quantal content as resulting from reduced efficacy of action potential invasion of the enlarged terminal.

An increased number of postsynaptic GABAergic receptors seems to be the most likely mechanism to explain the increased amplitude of mIPSCs in nonpyramidal cells after kindling. First, recent data has shown that there is an increase in GABA_A subunit expression in amygdala kindling (Meguro R et al., 2004). There is also direct evidence from electron microscope data that shows increased density of immunogold particles in the post synaptic density of dentate gyrus cells after kindling (Nusser et al., 1998). Further support for this mechanism was provided by benzodiazepine binding studies that

have revealed an increase in postsynaptic GABA_A/BNZ binding sites after kindling in amygdala (Tuff et al., 1983) and dentate gyrus (Shin et al., 1985; Clark et al., 1994).

This increased density could also include a subunit switch as the kinetics of the mIPSCs after kindling changed becoming significantly longer (see below). Other possible changes could account for the kinetics, the most likely being phosphorylation status. Phosphorylation of GABA_A receptor is known to change the kinetics of the GABA receptor by enhancing desensitization (Jones and Westbrook, 1997).

Numerous studies have shown that seizures are associated with changes in GABA_A receptor subunit expression (Bouilleret et al., 2000; Brooks-Kayal et al., 1998; Brooks-Kayal et al., 1999; Fritschy et al., 1999; Kamphuis et al., 1995; Kamphuis et al., 1994; Kokaia et al., 1994; Loup et al., 2000; Nusser et al., 1998; Redecker et al., 2000; Sperk et al., 1998; Meguro R et al., 2004). However, it is clear from these studies that these changes are very heterogenous varying from cell population-to-population and region-to-region. Changes in GABA_A receptor configuration that correlate with after hippocampus or amygdala kindling are well documented. These changes were detected as an upregulation of mRNAs for β_3 and γ_2 following hippocampal kindling (Kamphuis et al., 1994) and increased expression of α_2 , β_1 and β_3 subunits in dentate gyrus after amygdala kindling (Clark et al., 1994). Also in pilocarpine model, CA1, CA2 and CA3 mRNA expression of α_2 and α_5 was increased while in dentate gyrus α_5 expression was increased (Rice et al., 1996). In the same model, epileptic rats showed altered expression of GABA_A receptor subunit mRNAs in dentate cells with an increased up-regulation of mRNA for α_4 and β_3 as well as a decreased expression for α_1 , β_1 and δ GABAergic subunit (Brooks-Kayal et al., 1998; Brooks-Kayal et al., 1999). Another study that

examined hippocampal tissue obtained from humans with intractable temporal lobe epilepsy, showed that α_1 subunit expression was decreased, similar to animal models where significant cell loss occurs (Loup et al., 2000). However, the same study also showed α_2 immunoreactivity went up while α_3 immunoreactivity was unchanged. What is striking is that the GABA subunit changes documented thus far do not fit any particular pattern and therefore it appears that expression may depend on the course of the pathology, species and according to our data, cell phenotype. In any case, what seems clear is that no single pattern has emerged that is highly correlated to seizure genesis. Thus, the possibility exists that the expression of GABA_A receptor subunits has been changed here, as well. Overall, these data suggest that there is some alteration subunit expression, at least in abundance and perhaps in identity.

There is no reason to rule out the fact that some combination of effects may be occurring as well. But as some reasons seem unlikely given the evidence from other studies, here, based on the kinetic changes and the increased mIPSC amplitudes, it appears that an increase in subunit expression and an alteration in the identity of the expression are occurring in nonpyramidal population after kindling.

An interesting finding in our study was also related to the pyramidal cells of piriform cortex. Initially, the mIPSCs attributes of pyramidal cells of nonkindled rats do not differ significantly from nonpyramidal cells but after kindling the inhibition of the nonpyramidal cells was nearly twice as large as that of the pyramidal cells. Thus, it seems that an imbalance in inhibition is created between pyramidal and nonpyramidal cells after kindling. Again, it is not clear why or if this apparent balance is important under normal conditions. Altering this apparent balance may have an impact on the timing relationships

that are controlled by the summation of synaptic inputs on the two cellular populations. More importantly, as the feedback inhibition of pyramidal cells would not be affected after kindling, the overall inhibition on the pyramidal cells might be decreased as a result of summated inhibition of the nonpyramidal neurons which is known to be an important generator of cortical rhythms (Traub et al., 1999). This suggests that the generation and/or spreading of epileptic waves through the PC network might be due to the altered balance of the feedback versus inhibitory drive on the interneuronal network.

Our study contrasts all other studies that have found increases in inhibition on the excitatory neurons. Although it has been suggested that the increased inhibition on the dentate cells may enhance synchronization of the hippocampal network, a more facile argument is that it represents an attempt by the system to dampen seizures by reducing the activity of the dentate granule cells. Our findings indicating that these changes were found on the inhibitory cells (or those involved in enhancing inhibition) suggest the opposite. Here the changes should be considered as causing seizures primarily through dis-inhibition.

Another interesting finding was related to the spiny nonpyramidal neurons. Only three out of 60 cells where the morphology was identified were spiny nonpyramidal (excitatory neurons). One of these cells was from the ipsilateral (the other two on the contralateral) side. Interestingly, the inhibition on the spiny nonpyramidal cell from IPS group was more similar to the ipsilateral GABAergic nonpyramidal cells. In the contralateral side the inhibition was indistinguishable from either the contralateral nonpyramidal or pyramidal cells. If this finding is corroborated (in subsequent studies) another mechanism that supports seizure genesis may be at play. As the excitation of

these neurons is thought to support the excitation of the local inhibitory neurons, increasing the inhibition of the spiny nonpyramidal cells may also make seizure more likely.

Effects of neurosteroids

At the physiological concentration of 100nM, THDOC potentiated the GABAergic currents in nonpyramidal cells of piriform cortex but it failed to increase the inhibition in pyramidal cells of nonkindled and ipsilateral kindled groups. After kindling, THDOC had no effect on charge transfer in the mono-exponential mIPSCs but was equally effective in the bi-exponential population. Thus, the potentiation of charge transfer observed in nonpyramidal cells was due primarily to the prolongation in the decay time constants that occurred in the bi-exponential responses.

In nonkindled brain, an enhancement of affinity is the most likely mechanism that accounts for the prolonged deactivation observed in the mono-exponential population (Lambert et al., 2003). For the other population, enhanced desensitization may be occurring as well. THDOC has been reported to enhance the “movement” of the GABA_A receptors into the desensitized state. This creates a higher proportion of receptors that are able to re-open as the receptors deactivate, producing a longer decay. Enhanced desensitization likely explains the decreased amplitude of mIPSCs observed in our study in both pyramidal and nonpyramidal cells. This has not been observed before in synaptic current but a reduced amplitude was seen in applications of GABA and THDOC on outside patches in comparison to control (Zhu and Vicini, 1997). In spite of this mIPSCs amplitude reduction, THDOC increased the strength of inhibition in nonpyramidal cells

as we previously showed (by increasing the decay time). Interestingly, the effect of THDOC is, however, not uniform, the nonpyramidal cells in nonkindled group (NK) showed a greater sensitivity as the charge transfer due to either mono- or bi-exponential mIPSCs were significantly increased while in kindled groups (contralateral and ipsilateral) only the charge transfer for the bi-exponential mIPSCs was enhanced significantly. For comparison, a representative calculation that takes in consideration the relative contribution to the total charge transfer by mono- and bi-exponential mIPSCs on 3:1 ratio implies that THDOC potentiate the inhibition in nonpyramidal cells by ~27% in NK group, ~17% in kindled contralateral but only by ~13% in kindled ipsilateral group.

This differential sensitivity to THDOC in kindled animals versus control group might be explained by a possible alteration in GABA_A subunit configuration in nonpyramidal population after kindling. If this is the case then THDOC could have a lower potency for the new GABA_A subunits. This mechanism is supported by other studies on recombinant receptors that have shown that neurosteroids have some selectivity based on subunit expression (Maitra and Reynolds, 1999; Maitra and Reynolds, 1998; Hauser et al., 1996; Hauser et al., 1995; Srinivasan et al., 1999). For example, the potency of neurosteroids may be greater on α_2 than on α_1 containing receptors while γ_3 containing receptors exhibit a greater sensitivity to modulation by neurosteroids than γ_2 or γ_1 isoforms (Maitra and Reynolds, 1999). Also, incorporation of ϵ subunit decreased GABA-modulatory properties of pregnane steroids while δ subunit incorporation had an opposite effect (Belelli et al., 2002).

The alternative to this is idea that the phosphorylation state of the GABA_A receptors may change due to kindling and this alters THDOC efficacy. Previous data

(Brussaard and Koksma, 2003; Brussaard and Koksma, 2002; Brussaard et al., 2000; Tasker, 2000; Fancsik et al., 2000) showed that the alteration in phosphorylation state of GABAergic receptors could influence the neurosteroid potentiation of GABAergic currents. There is some evidence that various protein kinases can be up-regulated over protracted periods of time due to seizures (Sola et al., 2001; MacDonald et al., 1996). So, although phosphorylation is believed to be labile, up-regulation of kinase over long periods of time may cause more permanent changes in the phosphorylation state. Alternatively, a subunit change to one that is often phosphorylated ($\beta 1$ for example (Krishek et al., 1994) may also make the changes due to phosphorylation more stable. Overall, these data might suggest that a positive modulator such as THDOC becomes less effective in kindled brain in nonpyramidal neurons probably due to a change in GABA_A subunit expression that could be corroborated with an alteration in the phosphorylation status of the receptor.

In conclusion, electrophysiological and pharmacological analysis of piriform cortex GABAergic receptors suggest that kindling might increase GABA efficacy on nonpyramidal neurons via an increase in the number of receptors and/or an alteration in the GABA subunit configuration. However, the quantification of this increase as well as the nature of this alteration remain to be elucidated.

This study suggests that amygdala kindling caused a shift in the inhibition “balance” between pyramidal and nonpyramidal cells of piriform cortex towards the latter. This shift predicts that the interneurons will be more heavily inhibited than in normal brain. The trend towards higher inhibition on the contralateral side suggests that the increase in inhibition follow the progression of the seizures due to the kindling. What

is not clear is how these changes are produced by initial convulsive discharges and/or the seizures. They may be related to alterations in subunit expression and this may tie into the fact that THDOC is less effective in kindled groups. What is clear is that kindling does not produce the equivalent changes in pyramidal cells and therefore excitatory neuron plasticity is fundamentally different from nonpyramidal cell plasticity in the piriform cortex.

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