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Evidence that the Phosphorylation of GABAa receptors regulates neuro-steroid efficacy after Kindling

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Evidence that the Phosphorylation of $GABA_A$ receptors regulates neuro-steroid efficacy after Kindling

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Abstract

Aim It was previously reported that the efficacy of the neuroactive steroid (THDOC) was reduced after the induction of stage 5 amygdala kindled seizures. As the phosphorylation has some implications on THDOC pharmacology such as altered desensitization rate of $GABA_A$ receptors, we hypothesized that kindling induced (long term) changes in the phosphorylation of $GABA_A$ receptors. **Methods** In order to test this hypothesis we manipulated phosphorylation state of $GABA_A$ receptors by employing agents that either activated PKC (PMA, 100 nM), inhibited phosphatase (FK-506,100 nM) or activated phosphatase (Li^+ Palmitate, 100 nM). Through patch clamp recordings in layers II of the piriform cortex prepared from kindled and non-kindled male Sprague Dawley rats (200-250 gm), $GABA_A$ synaptic responses were measured. We also carefully monitored the extra-synaptic currents to determine whether the extra-synaptic transmission was affected. We applied THDOC (100 nM) for a minimum of 10 minutes, a protocol that was shown previously to enhance currents by as much as 100 %. **Findings** We found that PMA and FK-506 prolonged the deactivation of the mIPSCs. The subsequent application of THDOC reduced the amplitude of mIPSCs, but THDOC prolonged mIPSCs decay time further. However, the net affect was that THDOC did not enhance charge transfer, an outcome that was identical to those found in kindled tissue. Both the inactive phorbol (α-PMA, 100 nM) and the PKC antagonist (BIS I, 100 nM) had no effect on the THDOC synaptic modulation. Interestingly, Li^+ palmitate reversed the effect of kindling on THDOC modulation in synaptic transmission, but it showed no effect on extrasynaptic transmission. **Conclusion** taken together these data indicated that phosphorylation had profound effects on THDOC modulation and kindling reduced the THDOC efficacy by potentially inducing the phosphorylation of $GABA_A$ receptors.

Key words: $GABA_A$ receptor mediated currents; mIPSCs; Synaptic transmission; tonic inhibition; THDOC; neurosteroids; pyramidal neurons; kindling

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Introduction

Epilepsy is a common neurological disorder involving almost 1% of the population worldwide (about 50 million people) (Elger, 2002;Fisher et al., 2005). Epilepsy is classically defined by recurrent (two or more) seizures which are unprovoked by any immediate identified cause (Hauser and Kurland, 1975). The increased risk of comorbidity and mortality associated with epilepsy makes it a high priority public health problem (Chang and Lowenstein, 2003).

Based on clinical studies, recurrent spontaneous epileptic seizure is the result of a transient period called epileptogenesis (Fig 1). Classically, Epileptogenesis is a pathological process which starts from transient status epilepticus or febrile seizure and ends in epilepsy. The common epileptogenic factors include traumatic brain injury (TBI), stroke, and cerebral infections (Engel, Jr. et al., 2003).

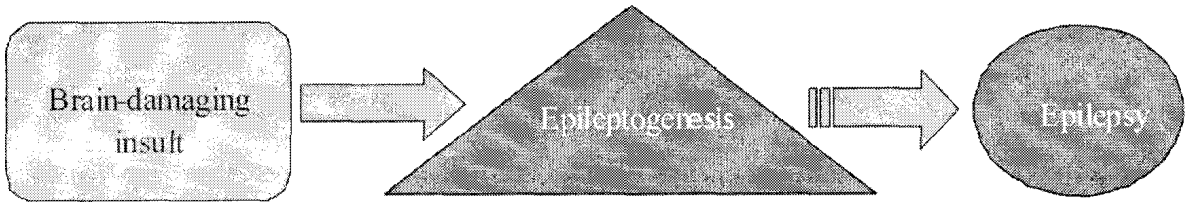

Figure 1 Epileptogenesis: from brain insult to epilepsy

Although surgical methods have provided the opportunity of taking biopsy specimens from epileptic patients, which permits assessment of some of the neuropathological and molecular alterations involved in human epilepsy, obtaining human tissue biopsies from the epileptogenesis period is not possible. This limitation is the main reason that animal models have been developed in order to study the detailed aspects of the epileptogenesis

and epilepsy. In this thesis the kindling model of epilepsy is employed to study some of the biochemical changes that may impact neurosteroid activity before and after seizure generation.

The present thesis attempts to narrow the pathophysiology of epilepsy down from the observed clinical manifestation to cellular level and tries to summarize related concepts and theories which have been published. Then it looks at the specific part of the whole picture which constructs the rational of our research hypothesis. It will be followed by the experimental setting and findings. At the end we will try to generalize our findings to the real situation and will propose perspectives for our new generated hypotheses.

Neuropathological view of Temporal Lobe epilepsy

In many epileptic patients, the seizure originates in the temporal lobe and is called Temporal Lobe Epilepsy (TLE) (French et al., 1993). TLE usually shows the highest incidence in children and young adults. By definition, TLE is a generic term including multiple syndromes with distinct symptoms, triggers, and therapeutic options. A wide spectrum of neuropathology from impairment of neuroplasticity to specific lesion patterns such as Ammon's horn sclerosis (AHS) has been reported in TLE patients (Blumcke et al., 2002;Sloviter et al., 2004;Thom et al., 2005). Moreover, different levels of astrogliosis (Kim et al., 1990;Blumcke et al., 2002) and focal cortical dysplasia such as ganglioglioma, dysembryoblastic neuroepithelial tumors (DNT), and glioneuronal malformations have been reported frequently in epileptic patients (Wolf and Wiestler, 1993;Becker et al., 2001;Blumcke et al., 1999;Luyken et al., 2004;Palmini et al., 2004).

In many TLE patients, cellular damage has been found in the entorhinal cortex, amygdala, and hippocampus. For example, segmental neuronal loss in CA_1 and CA_3/CA_4 areas has been reported while CA_2 and dentate gyrus (DG) granule cells seem to be more resistant to cell death process (Pitkanen et al., 1998;Yilmazer-Hanke et al., 2000;Dawodu and Thom, 2005). An interesting point about the TLE patient is that, although 35% of TLE patients demonstrate a focal lesion in temporal lobe area, the hippocampus proper does not show pronounced segmental neuronal cell loss and substantial structural reorganization.

Surprisingly, neuroanatomical data showed that the widespread neuronal network changes in many brain regions are not limited to the adjacent seizure focus but is also seen in remote structures including basal ganglia (Nolte et al., 2006). For instance, many studies reported that the superior colliculus (SC) and the pedunculopontine tegmental nucleus (PPN) in basal ganglia, ,which are connected reciprocally with the substantia nigra pars reticulata (SNr), are affected. Among several neuro circuit candidates it has been shown that temporal frontal pathway is a critical pathway in propagation of epileptic activity in TLE (Kelly et al., 1999;McIntyre et al., 1996;Mohapel et al., 2001). Further studies disclose that there are extensive projections both divergent and convergent from the perirhinal / insular cortex to the frontal motor regions. All together these observations suggest that temporal lobe epileptic seizure recruits specific motor pathways involving both local and distant circuits (Du et al., 1993;McIntyre and Wong, 1986;Kelly et al., 1999;McIntyre and Racine, 1986;Loscher and Ebert, 1996;Lothman, 1991;McIntyre et al., 1993).

Besides neurocircuit changes and neuronal loss it seems that long lasting changes in receptors such as NMDA and GABA also play a critical role in the pathophysiology of TLE. It has been shown that kindling model of TLE results in meta-plasticity particularly in NMDA and $GABA_A$ receptors. These types of receptors are actively involved in shaping synaptic activity. So any alteration in these receptors results in an increase or decrease of the synaptic strength (Kornau et al., 1995).

Overall, the pathological changes reported in human TLE tissue are multiple and related to structural and cellular reorganization of the involved regions such as the hippocampal formation. For example clinical and pre-clinical studies reported selective neurodegeneration, acquired changes of expression and distribution of neurotransmitters, receptors, and ion channels in temporal and frontal areas. It appears that the pathologic process in TLE results in altered expression of a variety of neurotransmitter-producing enzymes, neurotransmitter receptors, neuropeptides, and calcium-binding proteins. Consequently, the lesion and peri-lesional reorganization phenomenon play an active role in the pathogenesis of epileptic seizures (Wolf et al., 1996;Cepeda et al., 2003;Wolf and Wiestler, 1995).

Interestingly, clinical evaluations also revealed that a significant number of TLE patients are resistant to anti epileptic drug (AED) therapy (Clusmann et al., 2004;Spencer et al., 1994;Zentner et al., 1995;Wiebe et al., 2001;Engel, Jr., 2003).Pharmacoresistance to anti-epileptic drugs is a frequent phenomenon in the patients with temporal lobe epilepsies (TLE). This phenomenon is considered to be the result of an underlying pathophysiology which brings up two major theories: "Multi Drug Transporter (MDT) hypothesis" and "Target hypothesis". Both of these theories proposed innovative research

ideas, developing new therapies to control epileptic seizures. The MDT hypothesis implies that the drug level decreases at their brain targets due to an increased brain expression of drug efflux transporters such as P-glycoprotein (P-gp). On the other hand, "Target hypothesis" suggests that target alterations in epileptogenic brain tissue make the AEDs ineffective (Loscher and Potschka, 2005;Remy and Beck, 2006). Alteration in $GABA_A$ receptors is an example of a mechanistic link between these two theories. So many studies have been designed to explore the pathophysiology of altered modulation of $GABA_A$ receptor and their role in seizure severity and drug failure (Rogawski and Loscher, 2004).

Kindling as a model of TLE

Goddard et al., 1969 found that he could induce seizures in rats by repeatedly stimulating the brain with electrodes implanted in either the hippocampus or amygdala. This model was named the kindling model of epilepsy. Indeed, this stimulation was found to be most effective in temportal lobe structures (Goddard et al., 1969;Coulter et al., 2002). He also noticed that having a seizure increased the probability of a subsequent one and that each seizure lowers the threshold for the subsequent seizure. This gave rise to the statement that "seizures beget seizures" (McNamara, 1994) correlating well to progression of epilepsy in humans. Furthermore, as the seizures produced by electrodes in the temporal lobe structure started in one spot and the secondarily generalized to cause tonic clonic seizures. Kindling modeled many aspects of temporal lobe epilepsy (TLE). (i.e. it produced partial complex seizures). These observations led to the wide use of kindling to study seizuregenesis, as it pertains to TLE.

Neuronal loss as a consequence kindling makes the relevance of kindling for human epilepsy controversial. Quantitative studies often report neuronal loss in human TLE particularly in the hippocampal formation (Babb et al., 1984;Kim et al., 1990;Sagar and Oxbury, 1987). However these observations are made from patients who had a long history of epilepsy and it is often the case that cellular loss may result from the seizures themselves. So it is not clear if cell loss is the initial trigger of seizures or a result of the disease process. Kindling in its initial phases does not seem to produce much cell loss (Bertram et al., 1990;Mathern et al., 1997). However, kindling for protracted periods of time does cause progressive neuronal loss primarily in the hilar region of dentate gyrus.

Another important feature of kindling with respect to TLE is its ability to affect remote brain structures that end with a generalized motor convulsion. In both kindling and TLE, seizures originate from temporal lobe, but then involve remote brain structures such as basal ganglia and brain stem nuclei. For example, kindling and human TLE involves the PedunculoPontine tegmental Nucleus (PPN) and shifts PPN oscillation patterns toward lower frequencies (Nolte et al., 2006). PPN connects basal ganglia to limbic structures, and the PPN involvement mediates the seizure-gating mechanisms of the substantia nigra pars reticulate (SNr) (Mena-Segovia et al., 2004;Patel et al., 1987;Okada et al., 1989;Garant and Gale, 1987;Danober et al., 1994;Deransart et al., 1998;Faingold, 2004;Depaulis et al., 1994;Lee et al., 2000;Temkin et al., 1995b). Another example is dorsal midbrain involvement, which is known as an anticonvulsant zone. Dorsal midbrain is under inhibitory control of monosynaptic afferents from the SNr (Gale, 1988;Depaulis et al., 1994). Outlasting activity changes of SNr neurons have been reported after kindling (Beckstead and Frankfurter, 1982;Gerfen et al., 1982;Garcia-Rill, 1991;Granata

and Kitai, 1991;Temkin et al., 1995a;Lee and Wurtman, 2000;Fujimoto et al., 2003;Mena-Segovia et al., 2004;Gernert et al., 2004). Both involvement (PPN and SNr) lead to reduced GABAergic inhibitory input from SNr to the superior colliculus (SC), which is traditionally considered as an anticonvulsant zone, and the disinhibition can be considered as a seizurogenic mechanism (Garant and Gale, 1987). Indeed the alterations in the network level affect brain homeostasis and function and thereby it makes the brain more susceptible for seizure initiation and propagation (Depaulis et al., 1994;Kondratyev and Gale, 2004). Together, these similarities strongly suggest that kindling models the pathology of limbic epilepsy.

What does kindling do?

As reported in many studies, kindling results in several modifications at a wide range of levels including neural networks, single neurons, synapses, and single channels. These modifications are apparently long lasting and only a slight reduction in the kindling response have been reported over long periods of time, including those that were stimulation free (McNamara, 1995;Mody et al., 1988;Goddard et al., 1969;Hiyoshi and Wada, 1992).

Interestingly, many studies have provided consistent evidence showing that amygdala kindling induces long lasting changes in synaptic efficacy in the ipsilateral piriform cortex (PC). These alterations result in spontaneous discharges and enhanced susceptibility to evoked burst responses. Taken together, several studies demonstrate that PC is a part of an epileptic network which manifests enhanced excitability. This may occur due to prolonged excitatory post synaptic potentials (EPSPs), shortened inhibitory post synaptic potentials (IPSPs), and multiple action potentials produced from a single

stimulation (Callahan et al., 1991;Kaura et al., 1995;Loscher and Schwark, 1987). Strong correlations between these functional alterations and the changes reported in receptors and channels propose a multi-factorial etiology in the epileptic animals (Rempe et al., 1997;Mangan et al., 2000;Bertram et al., 2001;Mathern et al., 1997). Importantly these alterations occur before any changes in the hippocampus suggesting that due to the strong interconnectivity between the the PC and hippocampus. It may regulate of seizuregenesis of hippocampal formation.

The concept that epilepsy reflects an imbalance between excitatory (E) and inhibitory (I) transmission is an obvious explanation as to why seizures occur (Bradford, 1995;Gale, 1992;Olsen and Avoli, 1997). While this concept is supported by the epileptogenic effects of glutamate receptor agonists particularly kainic acid in generating epileptic seizures (Ben Ari et al., 1980;Sperk, 1994), there is little evidence that glutamatergic activity is increased by kindling/epilepsy. Although, it has also been shown that an enhanced phosphorylation state occurs in NMDA receptors after kindling (Kornau et al., 1995) the impact of this is unclear.

Properties of $GABA_A$ receptors

$GABA_A$ receptors are heteromeric ligand-gated chloride channels that mediate most of the rapid synaptic inhibition in brain. Their activation causes membrane hyperpolarization and therfore a reduction in excitability (Hevers and Luddens, 1998;Mehta and Ticku, 1999). $GABA_A$ receptors are permeable to Cl^- and bicarbonate (Twyman and Macdonald, 1991;Delorey and Olsen, 1992;Macdonald and Twyman, 1992).

Structurally, $GABA_A$ receptors belong to the homologous cys-loop super-family of ion channels including the nicotinic acetylcholine, 5-hydroxytryptamine type-3, glycine receptors, and the Zn^{2+} activated ion channels. The chloride channel is integral to the protein complex. The $GABA_A$ receptor contains specific binding sites not only for GABA but for other drugs such as picrotoxin, barbiturates, benzodiazepines, anesthetics and neuro- steroids. (Twyman and Macdonald, 1991;Delorey and Olsen, 1992;Macdonald and Twyman, 1992).

Synaptic and extrasynaptic $GABA_A$ receptor activation

GABA release generates two distinctive responses which result in two kinds of inhibition, phasic or synaptic and tonic or extrasynaptic transmission. The synaptic response is characterized by the brief exposure to a high concentration of GABA that activates the postsynaptic $GABA_A$ receptors for a short period of time. The tonic inhibition is characterized by the prolonged exposure of low concentrations of GABA. Phasic or synaptic transmission is different from extrasynaptic as it arises from brief transient of GABA within the synaptic cleft, whereas extrasynaptic transmission arises from GABA "spillover" from the synaptic cleft that activates perisynaptic $GABA_A$ receptors on the same or adjacent neurons. These low concentrations of GABA exert an extrasynaptic response resulting in a persistent or tonic inhibition (Mody et al., 1994;Semyanov et al., 2004;Semyanov, 2008;Farrant and Nusser, 2005).

GABA mediated Synaptic or Phasic Inhibition

Synaptic activation of inhibitory receptors is termed as phasic activation because the currents last for brief periods of time. The release of GABA is triggered by a local calcium influx into the nerve terminal due to the arrival of an action potential. Thousands

of GABA molecules are released from each vesicle into the synaptic cleft generating a peak of GABA concentration in the milli Molar (mM) range within 10-100 μs (Mody et al., 1994). This rapid increase in the GABA concentration triggers the near-synchronous opening of $GABA_A$ receptor ion channels. The high concentration of GABA lasts for only a few milliseconds and is an important determinant of the phasic nature of this kind of synaptic activity. In absence of an action potential, spontaneous miniature inhibitory postsynaptic currents (mIPSCs) can be recorded. These mIPSCs represent GABA released from a single synaptic vesicle. Typically, mIPSC is activated within a millisecond which reflects the proximity of the receptors to the site of GABA release and the rate at which the receptors activate (Jones and Westbrook, 1995;Burkat et al., 2001;Maconochie et al., 1994;McClellan and Twyman, 1999).

The release of GABA has a number of important outcomes such as the net entry of anion (Cl-) and the increase in conductance making the neurons hyperpolarized and reducing the effectiveness of excitatory inputs. GABA activity also briefly, stops interneuron activity and this likely plays an important role controlling neuronal synchronization and oscillatory behavior (Mohler et al., 1996). Indeed, the timing of the synaptic events is thought to play an important role controlling the rhythmicity of connected interneuron networks and may be a basic element controlling information transfer in the brain(Carrillo et al., 2008).

Tonic or non synaptic transmission

Generally, the term "tonic transmission" means the continuous activation of receptors localized peri- or extra- synaptically. It is typically observed in whole cell patch clamp recordings by a reduction in holding current (typically 10-100 pA) after the application of

a $GABA_A$ receptor antagonist. Tonic transmission represents a significant portion of GABA-mediated inhibition and therefore it has a strong influence on neuronal excitability (Farrant and Nusser, 2005;Mody and Pearce, 2004;Semyanov et al., 2004) (Fig 2). Charge transfer during the activation of tonically active $GABA_A$ receptors is more than three times larger than that produced by phasic inhibition, even when the frequency of the phasic events is high (Nusser and Mody, 2002;Semyanov et al., 2003;Rossi et al., 2003).

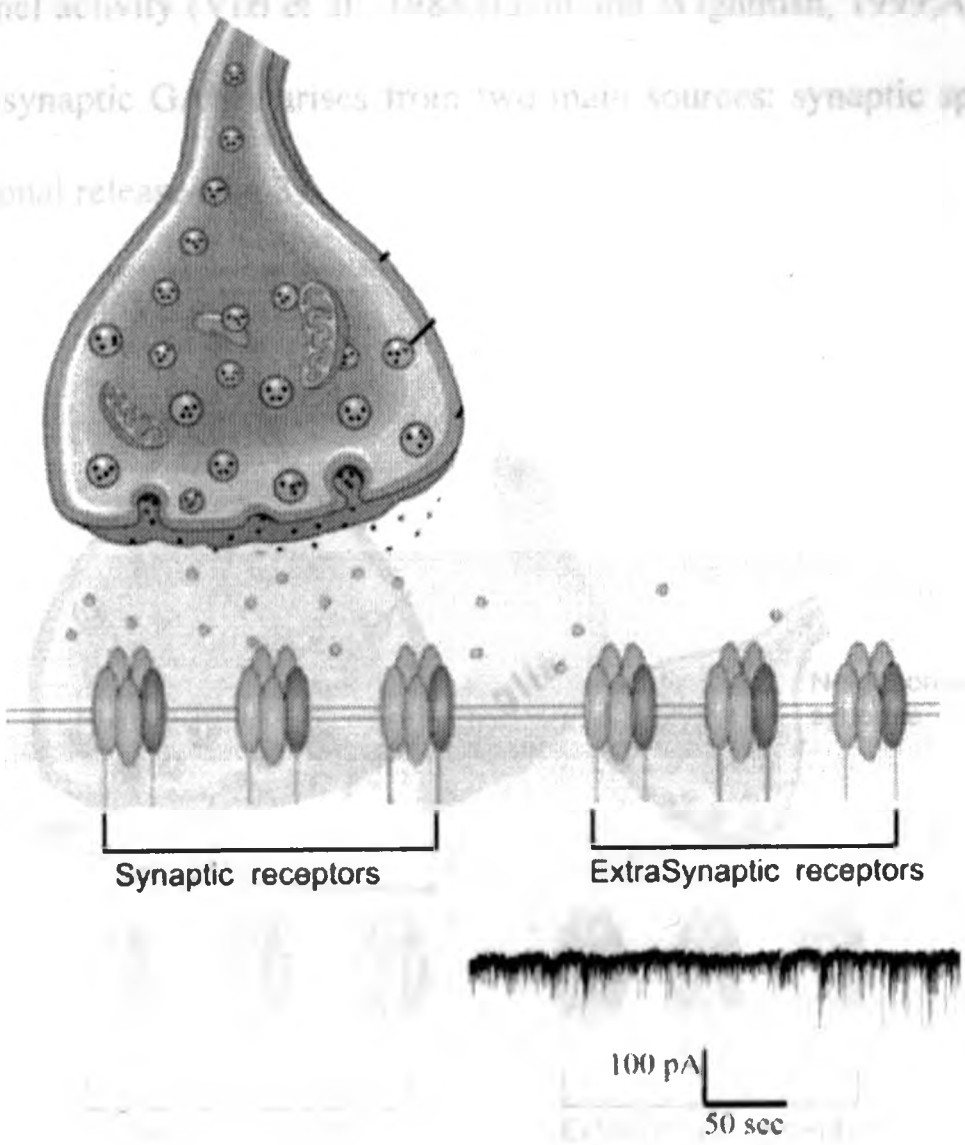

Figure 2 a low concentration of GABA tonically activates high affinity extrasynaptic $GABA_A$ receptors and generates tonic inhibition

Salin and Prince (1996) first described tonic $GABA_A$ current in layer III cells of somatosensory cortical slices. Shortly afterwards, it was reported in cerebellar granule cells (Brickley et al., 1996;Wall and Usowicz, 1997;Salin and Prince, 1996). The source of GABA was not only from vesicular release but came from non neuronal sources such as astrocytes (Parpura et al., 1994;Kozlov et al., 2006). The involvement of the non-neuronal cells supported the concept of volume transmission, which was the idea that extracellular GABA concentration was kept at concentrations high enough to maintain a low level of channel activity (Vizi et al., 1984;Bunin and Wightman, 1999;Agnati et al., 2006). Thus extrasynaptic GABA arises from two main sources: synaptic spillover and non-synaptic neuronal release (Fig 3).

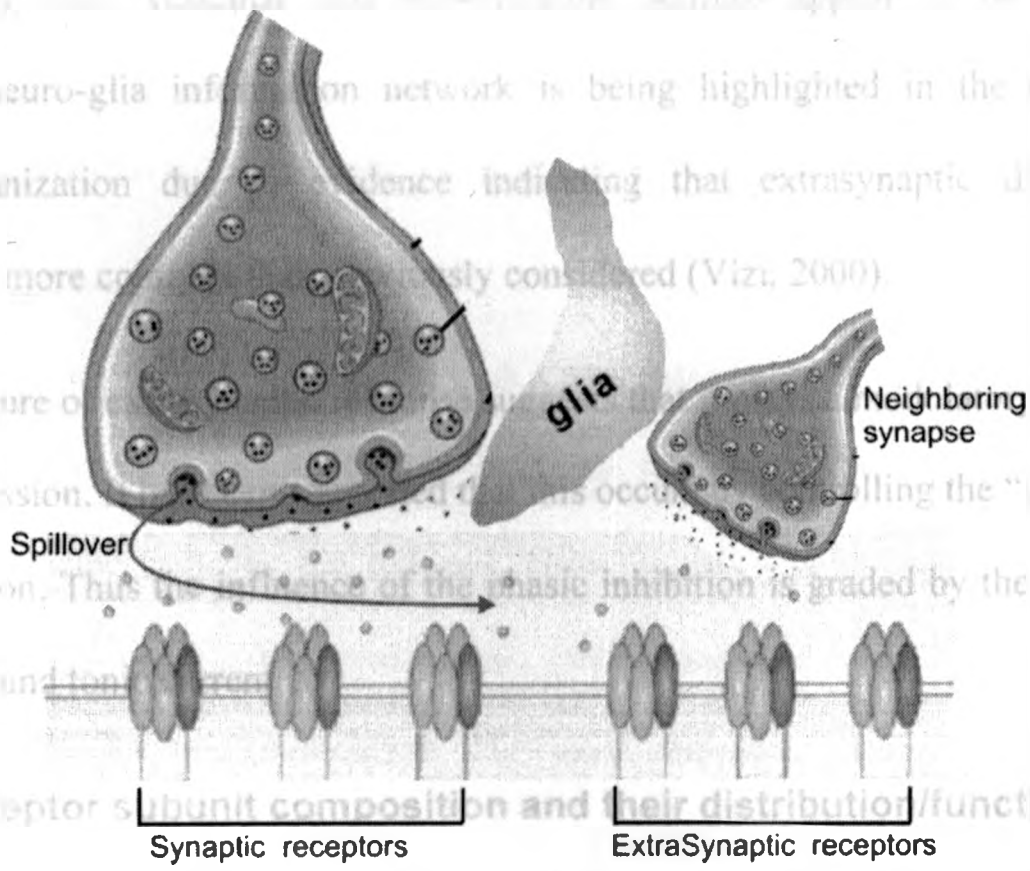

Figure 3 synaptic and extrasynaptic signals. It shows that volume transmission and spillover play important role in extrasynaptic transmission. However non-neuronal sources such as glial cells keep GABA concentration high enough to maintain a low level channel activity.

The size of the tonic currents rely on the concentration of extracellular GABA, the expression of extrasynaptic receptors, subtype identity of the $GABA_A$ receptor expressed, and either the presence or absence of various $GABA_A$ receptor modulators. Inhibition of GABA transporters (GAT-1, 2 and 3) results in a significant rise in tonic inhibition (Rossi et al., 2003). Surprisingly, inhibition of each transporter individually results in a much greater rise in tonic currents than blocking all three transporters but how this occurs is not clear (Keros and Hablitz, 2005). While it is clear that synaptic spillover from vesicular release contributes to the tonic current, synaptic spillover also arises from other non vesicular sources. This concept is based on data showing that blocking the uptake of GABA into synaptic vesicles by concanamycin has no effect on the magnitude of tonic currents. Thus, both vesicular and non-vesicular sources appear to be important. Recently, a neuro-glia information network is being highlighted in the concept of synaptic organization due to evidence indicating that extrasynaptic diffusion of transmitters is more complex than previously considered (Vizi, 2000).

The slow nature of extrasynaptic response suggests that it plays a modulatory role to the phasic transmission. It has been suggested that this occurs by controlling the "gain" of the phasic inhibition. Thus the influence of the phasic inhibition is graded by the magnitude of the background tonic current.

$GABA_A$ receptor subunit composition and their distribution/function

The $GABA_A$ receptor is thought to be a pentameric structure that is composed from distinct subunit classes. They have been designated: α (1–6), β (1–3), γ (1–3), δ, ε, θ, and π (Davies et al., 2003;Connolly and Wafford, 2004).

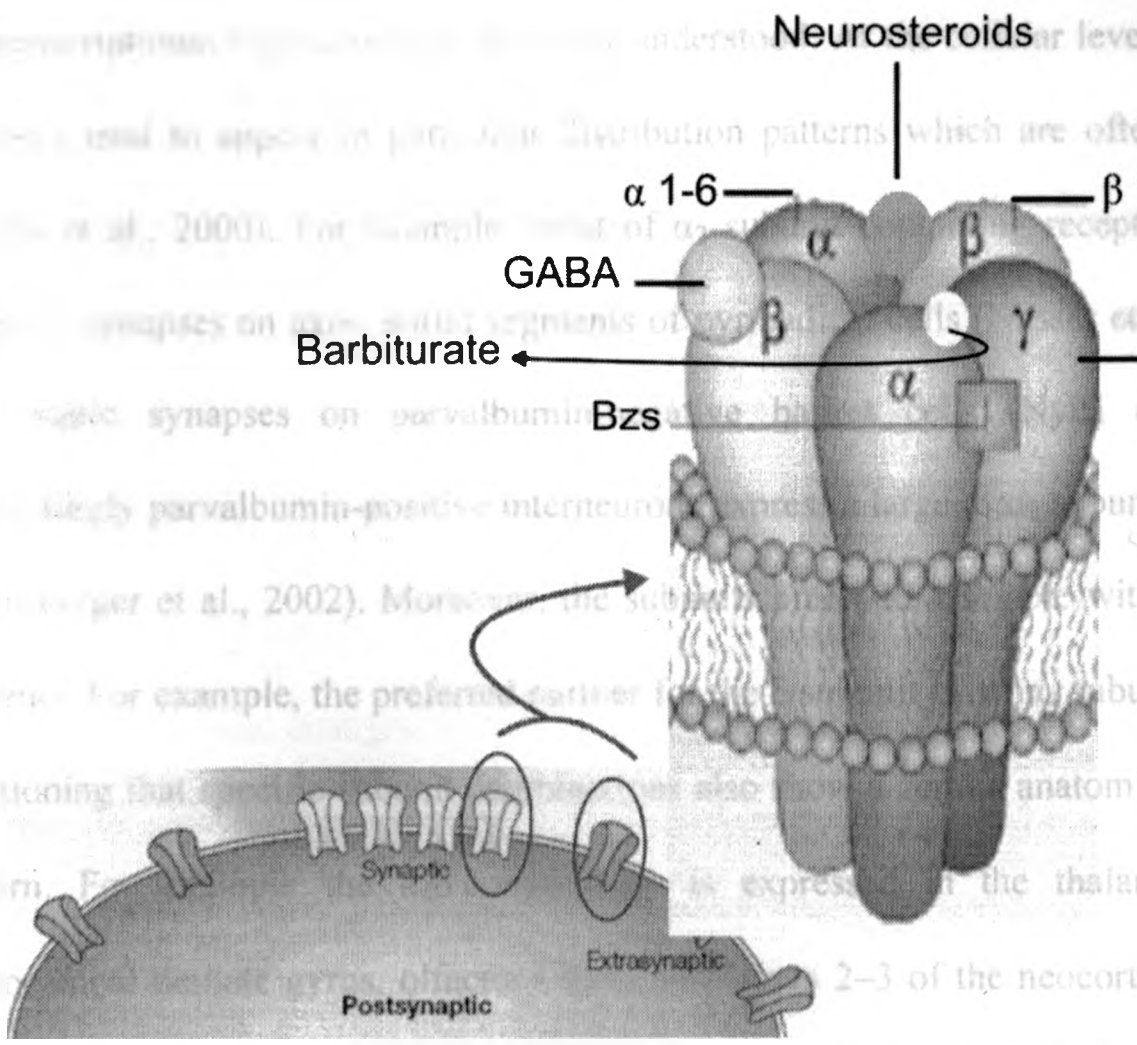

Figure 4 $GABA_A$ receptor structure and potential subunit combination.

The pentameric structure is arranged in such a way that it forms a central pore which is the chloride ion channel. Each subunit has four trans-membrane-spanning domains (TM1-4). TM2 helps form the ion channel. There is also a large intracellular domain located between TM3 and TM4 (Rabow et al., 1995;Macdonald and Olsen, 1994) which often contains site for phosphorylation. The most common combination of $GABA_A$ receptors reported in the mammalian brain is the one made of $2\alpha_1$, $2\beta_2$ and $1\gamma_2$ subunits. (the $\alpha_1\beta_2\gamma_2$ subunit combination) (Hevers and Luddens, 1998;Mody and Pearce, 2004).

The subunits are not uniformly expressed in the brain having both a cellular and anatomical diversity in expression. At the anatomical level, the expression is controlled

by transcriptional regulation that is poorly understood. At the cellular level, the different subunits tend to appear in particular distribution patterns which are often overlapping (Pirker et al., 2000). For example, most of α_2 subunit containing receptors have been found at synapses on axon initial segments of pyrmadical cells (Nusser et al., 1996) and at somatic synapses on parvalbumin-negative basket cells (Nyiri et al., 2001). Interestingly parvalbumin-positive interneurons express a large α_1:α_2 subunit ratio as well (Klausberger et al., 2002). Moreover, the subunits prefer to assemble with certain other subunits. For example, the preferred partner for the δ subunit is the α_4 subunit. It is worth mentioning that specific subunit combinations also show a certain anatomical expression pattern. For example the $\alpha_4\delta$ combination is expressed in the thalamus, striatum, hippocampal dentate gyrus, olfactory bulb, and layers 2–3 of the neocortex (Wei et al., 2003b;Stell et al., 2003;Jia et al., 2005;Drasbek et al., 2006;Glykys and Mody, 2006).

Subunit composition and $GABA_A$ receptor synaptic kinetics

Subunit composition affects receptor/synaptic kinetics through altering affinity as well as the activation/deactivation and desensitization. Each of these attributes can vary with subunit composition and have an impact on the peak amplitude, decay time, and charge transfer (Pirker et al., 2000;Moss and Smart, 2001;Chen et al., 2000). In particular, subunit expression controls one of the most important variables, which is deactivation. It largely controls the duration of the synaptic current. As described above, GABA is only briefly in the synaptic cleft, but the synaptic current lasts many times longer. This is because once bound to the receptor GABA dissociates relatively slowly thus the mIPSC decay time is prolonged. This deactivation has been shown to be influenced by subunit composition. Although other factors including: variations in vesicle size and content, the

nature of vesicle fusion, the geometry of the synaptic cleft, the number and spatial arrangement of GABA transporters postsynaptic receptors in relation to the site of transmitter release may play a role as well. (Weiss and Magleby, 1989;Twyman et al., 1990;Jones and Westbrook, 1995;Jayaraman et al., 1999;Haas and Macdonald, 1999;Burkat et al., 2001). The α_2-subunit for example provides for $GABA_A$ receptors that decay more slowly than the α_1 subunit (McClellan and Twyman, 1999;Vicini et al., 2001).

Altered decay time changes the total amount of charge transfer and it leads to altered synaptic strength. As well, binding of the ligand produces a closed conformational state in spite of the continuing presence of the ligand which is shown to be influenced by subunit expression(Bianchi et al., 2002;Bianchi and Macdonald, 2002;Tia et al., 1996). This state is called ''desensitization'' and it has been reported in many receptors besides the $GABA_A$ receptors including the nicotinic acetylcholine receptors (nAChR), the glycine receptors, and the ionotropic receptors for serotonin (5-HT3) (Dopico and Lovinger, 2009). If desensitization is sufficiently rapid (< 5 ms time constant) it will cause the synaptic current to decay more quickly. However, this build up of desensitized receptors results in the expression of a long-lasting component of the IPSC, because as the receptor recovers from desensitization re-opening of the channel occurs (Jones and Westbrook, 1995). However, it should be mentioned parenthetically that the ability of postsynaptic receptors to respond to repetitive activation can be reduced due to desensitization (Harney and Jones, 2002). Indeed, the exact relationship between structure and kinetic attributes is still not completely understood and more work is

required to fully understand how synaptic function is controlled by varying these parameters through subunit heterogeneity.

Based on both direct (anatomical) and indirect (pharmacological) evidence, between ten and hundred $GABA_A$ receptors are active at synapses resulting in a significant variation in their occupancy by GABA released into the cleft (Cherubini and Conti, 2001;Mody et al., 1994;Gaiarsa et al., 2002). Variation in the receptor occupancy is important as it can change the effectiveness of even a saturating concentration of GABA in the synaptic cleft. These small changes in GABA-mediated inhibition are able to change neuronal excitability profoundly (Mody et al., 1994). Variation in the receptor occupancy is likely influenced by subunit expression, since differing combinations have differing affinities for GABA and this would be predicted to influence occupancy profiles. (Nusser and Somogyi, 1997;Mozrzymas et al., 2003;Frerking and Wilson, 1996;Perrais and Ropert, 1999;Hajos et al., 2000).

Role of Subunit composition in tonic transmission

The extrasynaptic current is controlled by biophysical factors related to binding (how the GABA interacts with the receptor) and gating (how the channel opens and closes in response to GABA). Studies have consistently shown that each of the receptor subunits involved in phasic transmission are also found in the extrasynaptic plasma membrane and no $GABA_A$ receptor subunit type has yet been found to have an exclusive synaptic location (Nusser et al., 1995). However, there are subunits that seem to be excluded from the synaptic membrane. For example, it was shown by electron microscopy that the δ subunit is excluded from synaptic sites (Nusser et al., 98 A.D.;Wei et al., 2003a;Nusser et al., 1998). Specifically, these studies demonstrate that δ subunit containing GABA

receptors are located in the perisynaptic region , just outside the synaptic cleft and are ideally located to sense GABA spillover after a synaptic release ((Nusser et al., 1998;Nusser et al., 98 A.D.). The δ subunit is mainly coupled with the α_4 subunit in either the thalamus and dentate gyrus or the α_6 subunit in cerebellar granule cells, and makes the receptor diazepam-insensitive. However, these $GABA_A$ receptors still modulated by the GABA agonists , gaboxadol and muscimol, as well as neurosteroids (Peng et al., 2002;Sun et al., 2004;Jones et al., 1997;Kapur and Macdonald, 1996;Makela et al., 1997;Drasbek and Jensen, 2006;Storustovu and Ebert, 2006;Belelli and Herd, 2003;Belelli and Lambert, 2005;Stell et al., 2003;Krogsgaard-Larsen et al., 2004). As well, δ subunit containing $GABA_A$ receptors show high affinity for GABA. They are also activated by low concentrations of the transmitter and have little desensitization (Saxena and Macdonald, 1996;Wallner et al., 2003;Bianchi and Macdonald,). Certain subtype of α subunit may be extrasynaptic which provides high affinity receptor as well. For example, α_6 containing receptors (along with δ) play a prominent role in tonic transmission in the cerebellum, as the α_6 subunit makes a very high affinity receptor (EC_{50} ~ 100 nM) that enhances tonic inhibition. Subunit expression has influence on the receptor kinetics that may have an impact on tonic inhibition as well. For example the epsilon containing $GABA_A$ receptors are extrasynaptic in the hypothalamus, amygdala and locus coereleus. They have slow deactivation and spontaneous openings making them efficient mediators of tonic inhibition (Wagner et al., 2005). Thus tonic inhibition is controlled by number of factors related to subunit expression. These include the synaptic subunits that may be extrasynaptic and those that are uniquely extrasynaptic having

properties that make them well suited to sense GABA and respond for long periods of time.

Physiological implications of phasic and tonic inhibition

Phasic inhibition is characterized by the rapid synchronous opening of relatively small number of channels clustered at the synaptic junction, whereas tonic is an apparently random and temporally variable activation of $GABA_A$ receptors distributed over the neuronal surface. These two fundamentally different kinds of inhibition are thought to have equally divergent neurophysiological impacts.

Phasic inhibition plays a central role in generating brain rhythms. These include the generation of rhythmic activities such as delta, theta and gamma oscillations in various neuronal networks (Buzsaki and Chrobak, 1995;Singer, 1996;Somogyi and Klausberger, 2005;Freund, 2003;Whittington and Traub, 2003;Jonas et al., 2004;Cobb et al., 1995). Phasic seems to be most important on interneurons that in turn inhibits projection neurons. This occurs because the timing of the phasic inhibition briefly and synchronously inhibits the interconnected interneuron network "releasing" the excitatory (projection neurons from) inhibition. This mechanism is shown to be important in a number of brain regions including thalamus , hippocampus, neocortex and olfactory bulb (Laurent, 2002).

Unlike the phasic inhibition, tonic inhibition results in a persistent increase in a cell's input conductance which results in reducing the size and duration of the excitatory postsynaptic potential (Farrant and Nusser, 2005). Cell-type specificity for tonic inhibition has been seen in different subcortical structures. For example, in hippocampus

tonic inhibition is bigger in interneurons than pyramidal cells in the same subfield (Semyanov, 2003). In hippocampus, tonic currents are small and are mediated predominantly by spill over from synaptic transmission. It seems that the size of the tonic current can have important implications for the network excitation and inhibition (E/I) balance and cell to cell communication (Kullmann et al., 2005;Ruiz et al., 2003). Moreover, the magnitude of the tonic current will also influence the membrane time constant, which will change the integration of signals originating from distant dendritic compartments(Chance et al., 2002;Mitchell and Silver, 2003;Semyanov et al., 2004). Tonic inhibition is also able to decrease "network noise" by inhibiting the firing of neurons that do not receive adequate input. Thus, unlike phasic inhibition tonic inhibition appears to play a role in limiting/enhancing the effectiveness of synaptic activity (Towers et al., 2004).

Modulation of phasic and tonic inhibition

$GABA_A$ receptors have arguably the most diverse and complex pharmacology and physiological activity in the CNS. Both of these two types of inhibitory transmission may to varying degrees be affected by physiological and pharmacological activity. Moreover the plasticity of these processes may be differentially altered by outside stimuli resulting in an altered physiological and pharmacological profile. Some of the best-known modulators are drugs that include, but are not limited to: benzodiazepines, GABA uptake blockers, competitive and noncompetitive $GABA_A$ receptor antagonists (Semyanov et al., 2004). Many are highly effective agents that enhance or block tonic and phasic inhibition. Important to this thesis are endogenous neuro-active steroids, which either poteniate or inhibit both tonics and phasic inhibition (Brown et al.,

2002;Adkins et al., 2001;Belelli et al., 2002;Wohlfarth et al., ;Wohlfarth et al.,). Modulation may occur due to number of physiological stimuli including hormonal fluctuations and G protein coupled receptor signaling. Neuropathological conditions such as depression and epilepsy (please see below) (Peng et al., 2004;Follesa et al., 2004) may also effect tonic and phasic inhibition. In particular, the activation of protein kinases is well established as a mode of modulation for both types of the transmission. Thus, the modulation of phasic and tonic inhibition may occur at many different levels ranging from subunit expression, physiologic stimuli and pharmacological effects, all of which may be altered by neurological disorders.

$GABA_A$ receptors mediated neurotransmission and Epilepsy

Impairment of GABAergic inhibition particularly $GABA_A$ receptor mediated inhibition has been reported in a number of of models of epilepsy (Morimoto et al., 2004). Differences in expression and function may have two differing consequences. The first are alterations in function that may produce seizures as opposed to those that may occur as result of seizure activity. For example, Poulter et al., 1999 proposed that over expression of α subunits (α_2,α_3 and α_5) selectively makes rats seizure prone whereas over-expressed α_1 subunit makes rats seizure resistant (Poulter et al., 1999;McIntyre et al., 1999). On the other hand, some studies showed that there was a strong association between reduced $GABA_A$ receptors expression and epileptiform activity. This reduction occured progressively after pilocarpine injection and resulted in increased pharmacoresistance to barbiturates and benzodiazepines (Kapur and Macdonald, 1997). Similarly, kindling induced changes in expresson of some GABA receptor subunits (Kamphuis et al., 1994a;Meguro et al., 2004;Gavrilovici et al., 2006), and increased

clustering of receptors in the postsynaptic densities (Nusser et al., 1998;Cohen et al., 2003;Gavrilovici et al., 2006).

Seizures/epilepsy alter $GABA_A$ receptor expression and $GABA_A$ mediated synaptic transmission (Brooks-Kayal et al., 1999;Losher W et al., 2005;Bouilleret et al., 2000;Brooks-Kayal et al., 1998;Fritschy et al., 1999;Titulaer et al., 1995;Kamphuis et al., 1994b;Kokaia et al., 1994;Loup et al., 2000;Nusser et al., 1998;Redecker et al., 2000;Sperk et al., 1998). However, not all models of epilepsy give similar changes in expression. For example, in the pilocarpine model of temporal lobe epilepsy (TLE), loss of α_1 subunit expression in the hippocampus is observed, although this likely reflects both the loss of interneurons that highly express the α_1 subunit and a reduction of α_1 expression in the dentate granule cells. However, pilocarpine also increased the expression of α_4 and δ subunits. By contrast, in the commissure kindled rat model, where there is limited neuronal loss, α_1 reactivity increased on dentate granule cells (Loup et al., 2000;Nusser et al., 1998). In hippocampal tissue obtained from humans with intractable TLE, α_1 subunit expression is also reduced, paralleling the pilocarpine model where significant cell loss occurs (Loup et al., 2000). This study also shows that α_2 immunoreactivity is increased, while other subunits are unchanged. Studies have also found that a transient change of α_1 subunit expression occurred after kindling, while β subunit increases were more permanent (Titulaer et al., 1995;Kamphuis et al., 1994b). What is striking is that no particular pattern of expression has emerged that is highly correlated to seizure status, although each pattern suggests that inhibitory plasticity plays a role in causing seizures. The variability may reflect the starting point from which the epilepsy emerges (mature or immature brain for example), the genetic background or the

nature of a previous injury (stroke or blunt force trauma). Of course the response to the injury may also have a genetic component as well.

$GABA_A$ receptor modulation and Neurosteroids

Neurosteroids are widely accepted as anticonvulsant, anxiolytic, sedative, and hypnotic agents. They also induce general anesthesia (Gasior et al., 1999). Neurosteroids are metabolites of ovarian steroids, such as progesterone and adrenal steroids, such as corticosterone (Belelli and Lambert, 2005). They are synthesized in the central nervous system by specific enzymes such as 5α-reductase and 3α-HSOR and released from glial cells and certain neurons (Fig 5) (Majewska et al., 1986;Belelli and Lambert, 2005;Paul and Purdy, 1992;Reddy, 2003). As well, in the CNS they seem to be at higher concentration compared to non-neuronal systems and produced independently from peripheral steroidogenic tissues such as the gonads and the adrenals (Baulieu, 1997;Bixo et al., 1997;Concas et al., 1999;Reddy and Rogawski, 2002).

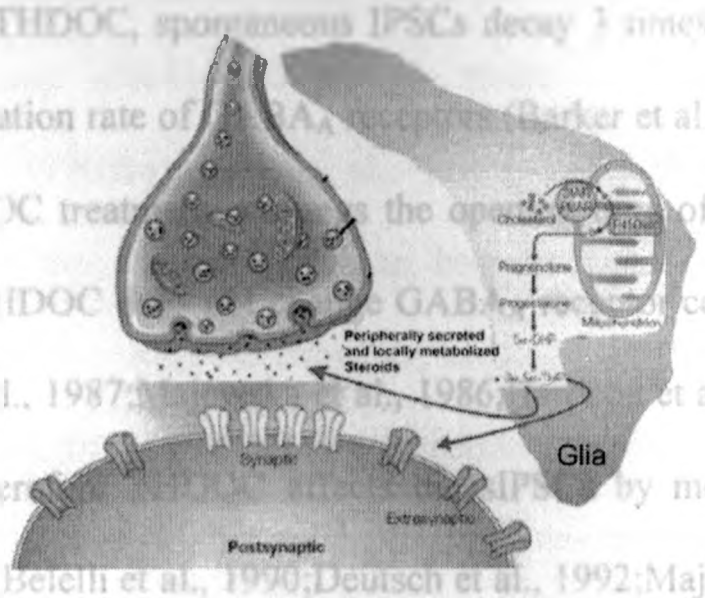

Figure5 Modulation of $GABA_A$ receptor by neuroactive steroids.The neurosteroids are produced and released in neurons and glia cells. Their precursors originate in peripheral steroidogenic organs. This schematic diagram shows that cholesterol and mitochondria play critical role in the synaptic and extrasynaptic modulation.

An endogenous tone of neurosteroids has been reported in central nervous system. Pinna et al., 2000 found that treating neurons with 5α-reductase inhibitor, which inhibits the brain 5α3α activity, decreases the level of neurosteroids. It also results in reduced efficacy of the $GABA_A$ receptor agonist muscimol. This finding suggests that in untreated animals there is an endogenous neurosteroid tone (Puia et al., 2003;Gasior et al., 1999;Pinna et al., 2000).

Many studies showed that tetrahydrodeoxycorticosterone (THDOC) is one of the most potent positive allosteric modulator of $GABA_A$ receptors and is effective at concentrations in the nanomolar (nM) range (Harrison et al., 1987;Poisbeau et al., 1997;Rupprecht and Holsboer, 1999;Lambert et al., 2001). Acute stress increases THDOC concentration to 4-20 fold in plasma and by 10-20 nM in the brain (Purdy et al., 1991). Studies also show that THDOC affects synaptic efficacy (Bianchi and Macdonald, 2003). In presence of THDOC, spontaneous IPSCs decay 3 times more slowly. It also increases the desensitization rate of $GABA_A$ receptors (Barker et al., 1987;Lambert et al., 1989). Although THDOC treatment prolongs the opening time of $GABA_A$ receptors it has been shown that THDOC does not change $GABA_A$ receptor conductance (Barker et al., 1987;Callachan et al., 1987;Majewska et al., 1986;Harrison et al., 1987;Twyman and Macdonald, 1992). Therefore THDOC affects the sIPSCs by modulation of $GABA_A$ mediated transmission (Belelli et al., 1990;Deutsch et al., 1992;Majewska, 1992;Harrison et al., 1987;Gage and Robertson, 1985).

The physiological effect of THDOC is rapid and fully reversible and this effect is influenced by subunit composition (Zhu and Vicini, 1997). Various subunit compositions

exert different sensitivity to THDOC. For example, the δ subunit has a high sensitivity to THDOC and β subunits do not show high sensitivity to same concentration of THDOC (Zhu et al., 1996;Brown et al., 2002;Wohlfarth et al., ;Bianchi and Macdonald, 2003). So subunit composition plays an important role in the efficacy of neurosteroids particularly THDOC (Wohlfarth et al., ;Bianchi and Macdonald, 2003;Stell et al., 2003;Maguire et al., 2005;Wohlfarth et al., ;Lovick, 2006;Mostallino et al., 2006).

It has also been demonstrated that protein kinases alter allosteric modulation of the $GABA_A$ receptors (Leidenheimer et al., 1993). Lambert et al., 1995 showed that same dose of THDOC can produce different effects in CA_1 and DG of the hippocampus. This result suggested the endogenous kinases can be considered as a contributor in shaping the THDOC response selectivity(Lambert et al., 1995).

$GABA_A$ receptor modulation and phosphorylation

Many studies have shown that $GABA_A$ receptors are phosphorylated by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) (Kirkness et al., 1989;Browning et al., 1990;Browning et al., 1993). Phosphorylation occurs on the intracellular domain in the cytoplasmic loop between the third and fourth trans-membrane region of $GABA_A$ receptors(Browning et al., 1993). The amino acids that are phosphorylated by different protein kinases include serine, threonine and tyrosine residues (Brandon et al., 2002). Studies on the recombinant receptors revealed that β and γ subunits are the usual targets for a number of protein kinases. For example the serine residues on the β1 subunit (Ser-409 or Ser-410) show a high affinity for phosphorylation by PKC, PKA, Ca^{2+}/Calmodulin dependent protein kinase (CamKII), and cGMP-dependent protein kinase (Moss et al., 1992;Krishek et al., 1994;McDonald and Moss,

1997). $GABA_A$ receptors have also been found as a substrate of the tyrosine kinases SRC which predominately phosphorylates Tyr-365 and Tyr-367 within the γ_2 subunit (Moss et al., 1995;Valenzuela et al., 1995). γ_2 subtype is also a substrate for being phosphorylated by PKC at serine site (Whiting et al., 1990). By contrast, little phosphorylation of α subunits has been demonstrated and so the primary sites of phosphorylation seem to be the β and γ subunits(Moss et al., 1992;McDonald and Moss, 1997;Bell-Horner et al., 2006).

The effects of phosphorylation on inhibitory transmission are both presynaptic and postsynaptic. For example, it has been shown that the inhibition of kinase activity decreases GABA release from the presynaptic terminals (Warrier and Hjelmstad, 2007). Postsynaptically, phosphorylation seems to modify $GABA_A$ receptor configuration and results in altered the receptor properties such as desensitization rate (Jones and Westbrook, 1997), the receptor open probability (Moss et al., 1995), and the receptor trafficking (Wang et al., 2003). Thus phosphorylation generates a highly complex cascade of events that may have diverging effects on synaptic transmission

In particular, there is substantial evidence supporting the role of PKC in epileptogenesis and this may occur through alterations in GABAergic function (Guglielmetti et al., 1997;Niimura et al., 2005;Silva et al., 2007). For example, PKC activation also alters allosteric modulation of the $GABA_A$ receptor (Leidenheimer et al., 1993) and altered allosteric modulation has been reported after kindling as well (Kapur et al., 1989;Gavrilovici et al., 2006;Schwabe et al., 2005). Moreover, some anticonvulsants like valporate have an inhibitory effect on PKC (Toth, 2005), thereby reversing the effects of

PKC activation by seizures. Thus, it has been suggested that modulation of PKC activity may prevent epileptic seizures(Brussaard and Koksma, 2003;Koksma et al., 2003).

PKC was discovered as a lipid independent proteolytic product by Nishizuka in 1977 (Inoue et al., 1977). The PKC family comprises ten members in the AGC kinase branch of the kinome (Newton, 2003). All the members have a highly conserved kinase core at the carboxyl terminus (C1 and C2) and an amino-terminal autoinhibitory pseudosubstrate peptide. They are classified based on their divergent amino terminal regulatory regions (Newton, 2003;Sossin, 2007). The regulatory domain interacts with calcium, phosphatidylserine, and Diacylglycerol, and C1/C2 interacts with ATP and protein substrate binding sites. It has been shown that PKC specifically binds sn-1,2 phosphatidyl-L-serine which results in a conformational change in the enzyme and releasing an auto-inhibitory pseudo-substrate sequence and thus activation. The activation of PKC depends on phosphorylation (Newton, 2008).

At least ten isoenzymes of PKC have been currently identified including PKCs-α, -β_I, -β_{II},-γ, -δ, -ε, -ζ, -η, -θ, -λ, which can been divided into three families on the basis of their different requirements for activation (Nishizuka, 1992;Stabel and Parker, 1991;Osada et al., 1992;Asaoka et al., 1992;Nishizuka, 1992;Stabel and Parker, 1991;Osada et al., 1992;Asaoka et al., 1992). The conventional PKC family including PKCs-α, -βI, -βII,-γ is Ca^{2+}- and phospholipid- dependent and are activated by diacylglycerols and exogenous phorbol esters. The new PKC family including PKCs-δ, -ε -η, -θ is Ca^{2+} independent, but still activated by DAG and phorbols. The last group is the atypical PKC family including PKC-ζ and PKC-λ which contains a single C1 domain that is insensitive to DAG(Newton, 2003;Sossin, 2007). For example PKC-ζ is insensitive to stimulation by

Ca^{2+}, diacylglycerol and phorbol esters, but it's activated by phosphatidylinositol 3,4,5-trisphosphate (IP3) (Stabel and Parker, 1991;Davis et al., 1992).

Stimulation of phospholipase-C results in hydrolysis of phosphoinositides (Fig 5). Two second messengers, inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG) are the products of the hydrolysis. IP3 mobilizes Ca^{2+} from intracellular stores and in the presence of phospholipids and Ca^{2+} , DAG activates PKC (Toullec et al., 1991; Burgess et al., 1984; Nishizuka, 1984).

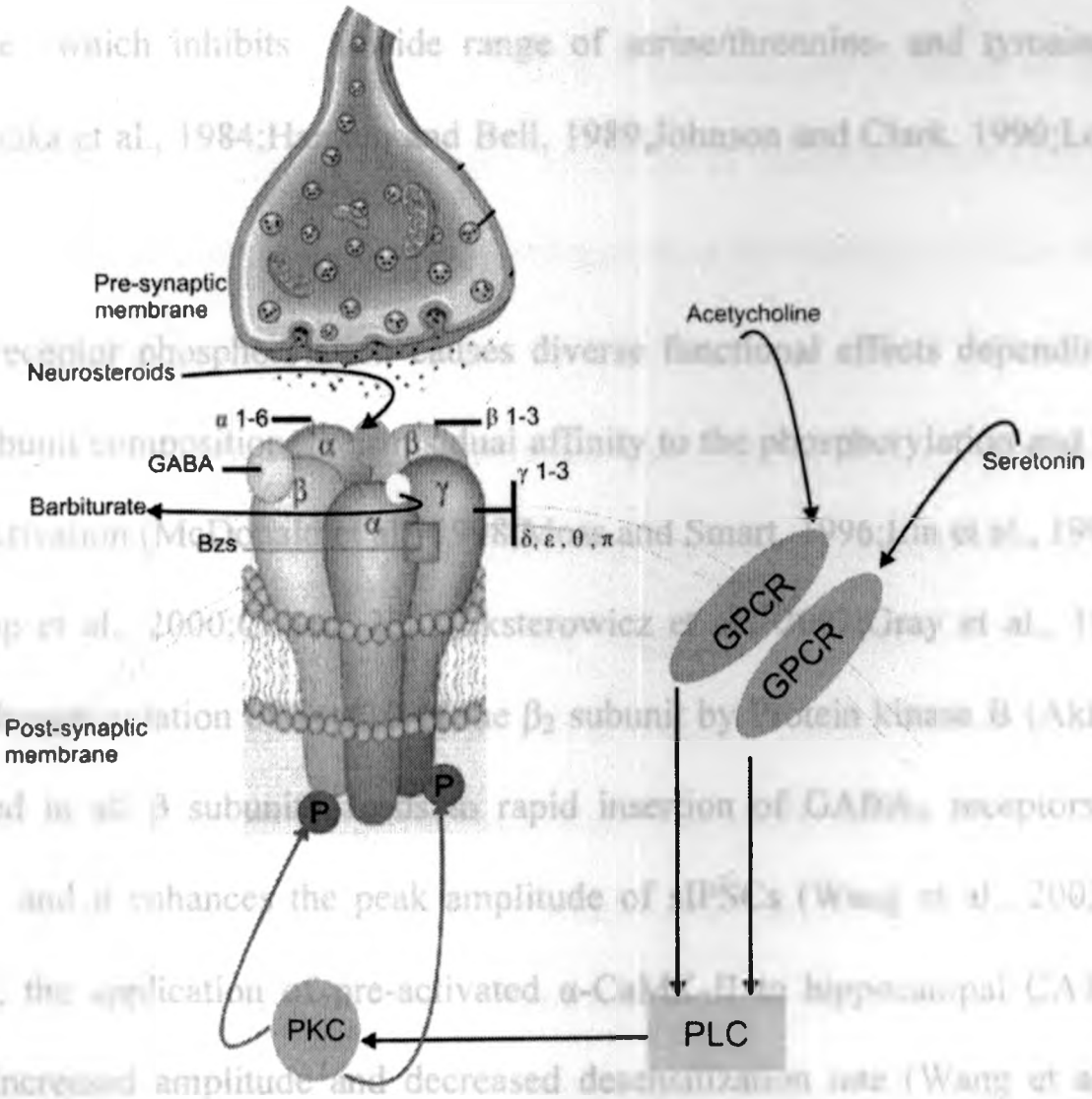

Figure 6 functional talk between G Protein Coupled Receptor (GPCR) and GABA receptor. PKC mediated phosphorylation is mediated by the activation of appropriated GPCR. The direct interaction of β subunit and the activated C kinase (RACK-1) result in the γ subunit phosphorylation.

PKC can be endogenously activated through distinct lipid activators such as arachidonic acid, diacylglycerols, and phospholipids (Kittler and Moss, 2003;Swartz, 1993). Phorbol 12-myristate 13-acetate (PMA) , a diacylglycerol mimetic, is one of the most important PKC activating drugs that binds to the C1 domain and mimics DAG (Kobayashi et al., 1989;Hannun and Bell, 1988;Loomis and Bell, 1988;Hidaka et al., 1984;Tamaoki et al., 1986). PKC can also be inhibited exogenously but the problem is most of them are nonselective. For example, the indolocarbazole (staurosporine) is a potent but nonselective which inhibits a wide range of serine/threonine- and tyrosine protein kinases(Hidaka et al., 1984;Hannun and Bell, 1989;Johnson and Clark, 1990;Love, Jr. et al., 1989).

$GABA_A$ receptor phosphorylation causes diverse functional effects depending on the $GABA_A$ subunit composition, their residual affinity to the phosphorylation and the mode of kinase activation (McDonald et al., 1998;Moss and Smart, 1996;Lin et al., 1996;Smart, 1997;Bishop et al., 2000;Cohen, 2002;Eksterowicz et al., 2002;Gray et al., 1998). For example, phosphorylation of Ser410 of the β_2 subunit by Protein kinase B (Akt) , which is conserved in all β subunits, leads to rapid insertion of $GABA_A$ receptors into the membrane and it enhances the peak amplitude of sIPSCs (Wang et al., 2003).On the other hand, the application of pre-activated α-CaMK-II to hippocampal CA1 neurons results in increased amplitude and decreased desensitization rate (Wang et al., 1995). Therefore the functional effects of $GABA_A$ receptor phosphorylation are diverse and both enhancement and reduction of $GABA_A$ mediated currents have been reported.

Statement of Problem and Hypothesis

In a previous study our lab reported that after kindling the activity of the neurosteroid THDOC was altered in way that reduced its ability to enhance inhibitory synaptic signaling. The reduction in activity occurred only on pyramidal neurons. Specifically it was found that THDOC reduced the amplitude of sIPSCs and prolonged their duration. While the prolongation of the sIPCS would tend to enhance synaptic strength, this effect was offset by the reduction in the sIPSC amplitude. The net result was that no change in the total amount of inhibition was observed. Thus after kindling THDOC was unable to potentiate $GABA_A$ receptor mediated inhibition. Parenthetically it should be added that THDOC effects were unchanged on cells identified as non-pyramidal after kindling. Thus, the purpose of this thesis was to investigate how the alteration in the efficacy in THDOC activity may occur after kindling.

Two potential explanations could be proposed for THDOC action; the first on was focused on altered subunit composition. However, we found that there were no changes in the kinetics of the sIPSCs, which would suggest no change in expression. We confirmed this in a separate study, not part of this thesis, finding no change in subunit expression in the pyramidal cells. The next and most likely possibility was that post-translational alteration of $GABA_A$ receptor which could be responsible for the altered kinetics. Indeed, as the previous work demonstrated that the alteration of $GABA_A$ receptor kinetics by THDOC could not be explained by changes in subunit therefore we hypothesized that receptor phosphorylation was involved.

Thus it our formal hypothesis is that **"Kindling induces (long term) changes in the phosphorylation of $GABA_A$ receptors and this regulates neurosteroid efficacy."**

To test this hypothesis we performed two kinds of experiments.

1) We used agents that would promote phosphorylation in non-kindled tissues to see if we could mimic the THDOC activity after kindling

2) We used agents that would enhance dephosphorylation in kindled tissue to see if we could revert the THDOC pharmacology to its "normal" activity

To evaluate these effects we performed patch clamp recordings from pyramidal cells before and after kindling. Both synaptic and extrasynaptic activity was followed (see Materials and Methods for a complete description). To test the hypothesis, the effect of neuro-steroid (THDOC) on the synaptic (mIPSCs) and extrasynaptic events has been evaluated before and after phosphorylation in kindled and non-kindled rats. In order to activate or inhibit the kinase or phosphatase activity we apply:

- PMA (100nM) as a PKC activator
- FK506 (100nM) as a protein phosphatase inhibitor
- Li^+ palmitate (100nM) as a phosphatase activator
- 4α-PMA (inactivated PMA,100nM) as a control
- BIS-I (Bisindolylmaleimide-1,100nM) as a control

We anticipated that if kindling increases the phosphorylation state of $GABA_A$ receptors, PKC activator and phosphatase inhibitor should generate a similar THDOC response as that seen in kindling. Likewise phosphatase activator should have little effect in non-kindled rats but restore THDOC efficacy in kindled ones.

Methods & Materials

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and protocols approved by University of Western Ontario Animal Care Committee.

Animal, surgery, and kindling protocol

Male Sprague Dawley rats weighing 175-200 g at the time of the initial surgery are used. They are housed individually with free access to food and water under a continuous 12-h/12-h light/dark cycle.

Animals are anaesthetized with Ketamine –domitor combination [0.1 ml/100 gm] and implanted with two bipolar stimulating/recording electrodes bilaterally in the basolateral amygdala with the following coordinates: 2.6 mm posterior to Bregma, 4.5 mm lateral to midline and 8.0 mm ventral (Paxinos and Watson, 1986). The electrodes are made of two twisted strands of 0.127-mm diameter Diamel-insulated Nichrome wire and were attached to male Amphenol pins. The electrodes are implanted and secured to the skull with jeweller's screws. The electrode assembly is fixed to the skull by dental acrylic cement (Molino and McIntyre, 1972).

The rats are allocated to two main groups: control (sham) and kindled group. The control group (sham) included the rats which had implanted electrode but they were never kindled. The kindled group included the rats which received the kindling process 10 days after the electrode implantation. All the electrodes were implanted in basolateral amygdala, and the After Discharge Threshold (ADT) was determined in each amygdala by delivering a 2-s 60-Hz sine wave stimulus of progressively increasing intensity (15,

25, 35, 50, 75, 100 ,150 , 200 , and 250 µA) until an ADT was triggered seizure discharge outlasting the stimulus by 2 or more seconds. The rats were stimulated daily until six generalized stage 5 convulsions were elicited. Seizure severity and duration were recorded daily during the kindling acquisition. The kindled rats were decapitated 48 hours after the last kindling (Racine, 1972;McIntyre and Plant, 1993).

Slice preparation and electrophysiology

Brain slices were isolated from the sham and fully kindled rats. After the rats were deeply anaesthetized with cocktail of Ketamine- Domitor [0.1 ml/100 gm] , they were perfused through the heart with an ice-cold Ringer's solution in which sodium was replaced by choline [containing (in mm): choline Cl, 110; KCl, 2.5; NaH2PO4, 1.2; NaHCO3, 25; CaCl2, 0.5; MgCl2, 7; Na pyruvate, 2.4; ascorbate, 1.3; dextrose, 20] (McIntyre et al., 2002b). After perfusion, the brain was rapidly removed and the temporal lobe area was excised as a block. The block was sliced coronally with a Vibratome (400 µm-thick sections). The slices were obtained from 1 to 0.3 mm relative to bregma. The slices were incubated at 37°C for 30 min and subsequently moved to a room-temperature (22°C) bath for at least 45 minutes. The 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, and equimolar NaCl was replaced instead of Choline [consisting of (in mM) 120 NaCl, 2.5 KCl, 1.2 NaH2PO4, 25 NaHCO3, 0.25 CaCl2, and 3 MgCl2, pH 7.3–7.4 and osomolarity adjusted to 290–300 mosM] . In order to decrease the miniature inhibitory post-synaptic current (mIPSC) frequency Ca Cl_2 (0.2 mM) and $MgSo_4$ (3 mM) were applied. All solutions were maintained at pH 7.4 and bubbled with 5% CO2/95% O_2 (Carbogen).

Patch electrodes were pulled from borosilicate glass capillaries and filled with KCl solution [composition (in mM) of: 145 KCl, 10 NaCl, 2 CaCl2, 10 EGTA (yielding a free Ca2_ concentration of 100 nM), 2 MgATP, 10 D-Glucose, 0.3 Na-GTP and 10 HEPES; the solution was 290–300 mosM, pH adjusted to 7.3–7.4]. The resistance of these electrodes was 3–8 MΩ. Whenever it was needed to use intracellular drugs, they were added to the recording solution. Recordings from pyramidal neurons in layers II of the Piriform cortex were made with an EPC-9 / 2 amplifier (HEKA, Lambrecht, Germany). In the kindled rats recordings were made in the piriform cortex of both the ipsilateral (IPS) and contralateral (CLS) hemisphere relative to the amygdala which was kindled. Series resistance compensation is performed in all recordings (100 μS, 50- 80%). All experiments were performed at 32° C (Fig 7).

Figure 7 pyramidal cell in the layer II of the piriform cortex. The picture has been taken during whole cell patch clamp recording.

Spontaneously occurring mIPSCs were recorded after blocking of Na+, N-methyl-d-aspartate, α-amino-3-hydroxy-5-methylisoxazole-4-propionate and kainate channels with a low Ca^{2+} Ringer's solution (described above) containing 200 nM tetrodotoxin (TTX),

20 μm dinitroquinoxaline-2,3-dione (DNQX) and 50 μm 2-amino phosphonovaleric acid (APV). The neurosteroid THDOC were dissolved in 100% ethanol at a concentration of 10 mM. All data were collected 10 minutes after drug switch.

Morphology reconstruction and cell image analysis

In order to reconstruct the morphology and understand where the recordings were made, 13 mM biocytin were added to the patch electrodes. After the completion of a recording, the slice was removed from the microscope chamber and fixed in 4% Paraformaldehyde (PFA) for at least 24 h. The sections were rinsed in phosphate-buffered saline (PBS) 3 times each time for 5 minutes and after incubation for 12 hours in straptavidin antibody they were mounted on microscope slides for viewing on a confocal microscope. Under the appropriate illumination and emission, individual confocal images were gathered and then projected onto a single two-dimensional image to show the morphology of the cell. The criteria using for the classification of pyramidal vs. non-pyramidal cells are based on: (i) soma morphology; (ii) projection of the axon to deeper layers for pyramidal cells vs. non-projecting non-pyramidal cells; (iii) presence of dendritic spines for pyramidal cells; as well as (iv) spiking properties (McIntyre et al., 2002b;Schwabe et al., 2005).

Data analysis plan

THDOC enhanced $GABA_A$ receptor function by increasing the duration of IPSCs. This prolongation was quantitatively described by fitting the deactivation phases of the synaptic events with exponential functions. In order to remove confounds due to non-temporal summations and other factors related to the extended morphology of the cells only mIPSCs with a fast rise time of less than or equal to < 1.5 ms were used. Figure 8 shows that there is no relationship between rise time and decay time constant. Thus

mISPCS likely originated near the recording electrode site and dendritic filtering and other confounds were minimized.

Fits and subsequent analysis of the data were performed automatically by use of Mini Analysis Software (Synaptosoft Inc., Leonia, NJ, USA). In all analyses, mIPSCs were sorted to exclude events with 10–90% rise times of > 1.5 msec, half-width durations of < 3 msec (i.e., events that are too brief to be considered genuine GABAergic synaptic transients), and events that are not considered to be caused by a single mIPSC.

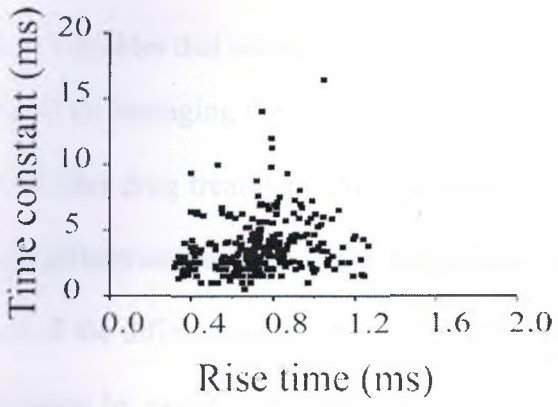

Figure 8 scatter platter shows that there is no statistical association between rise time and decay time.

In order to evaluate the efficacy of THDOC on $GABA_A$ mediated synaptic transmission, peak amplitude, deactivation times (fast and slow components), and total charge transfer of averaged mIPSCs were assessed (Fig 9). For a given recording, all the acceptable mIPSCs were averaged and were therefore composed of mono- and bi-exponential events. We also calculated the total charge transfer from the mIPSCav (McIntyre et al., 2002a). In order to calculate total charge transfer we used the formula $(Amp_1 * Tau_1)+(Amp_2 * Tau_2)$. In this formula Amp_1 is the amplitude of the first exponential component and Amp_2 is the amplitude of second exponential component .

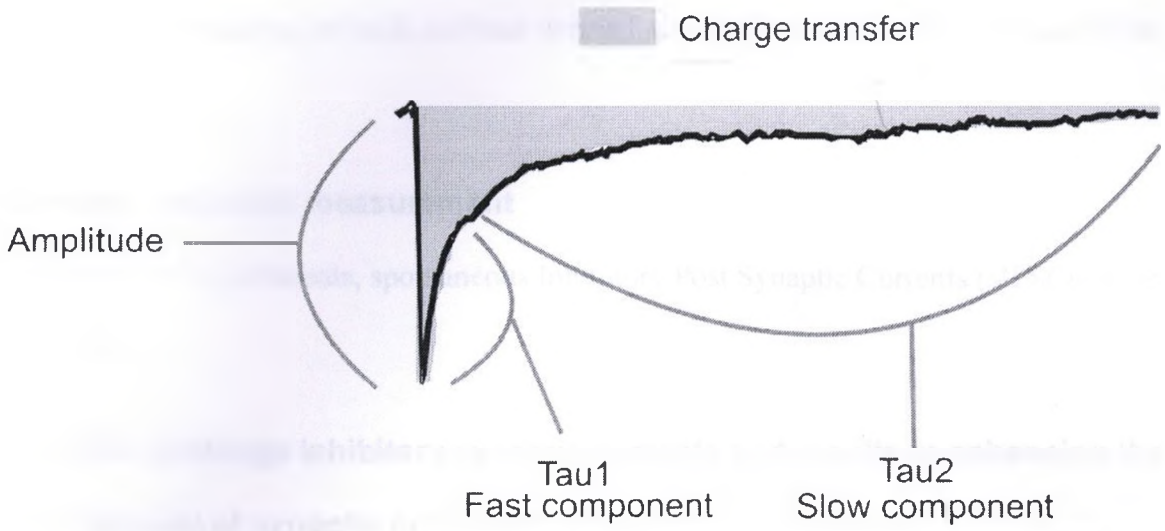

Figure 9 Variables that measure the effect of THDOC

Drug effects were compared by averaging the change in the mean values for the attributes of the $mIPSC_{av}$ before and after drug treatment. All representative data were reported as mean ± SE. The average difference between these values was then compared using a paired t-test. Significance of the differences is set at $P < 0.05$. The THDOC efficacy on tonic inhibition was evaluated by monitoring the amount of inward or outward shift of holding current(Schwabe et al., 2005). We applied percent of change in total charge transfer before and after THDOC as an identifier of THDOC efficacy.

Results

Data reported here are from 128 patch recordings from a total of 268 made from Piriform Cortex (PC) pyramidal cells in kindled and non-kindled rats. Only recordings from pyramidal neurons having a holding current between −150 and 150 pA and having action potentials with an over shoot of at least +20 mV were acceptable. As well the

pyramidal morphology of each cell was verified according the procedures outlined in the Methods.

Synaptic response measurement

In this set of experiments, spontaneous Inhibitory Post Synaptic Currents (sIPSCs) were measured.

THDOC prolongs inhibitory synaptic currents and results in enhancing the total amount of synaptic inhibition

First, we examined the effect of THDOC (100 nM) on the sIPSC in sham rats (control group). After 10 minutes of switching to ACSF containing THDOC, peak amplitude of mIPSCs did not change significantly (mean ± SEM; n = 6) (-49.0 ± 2.4 vs. -49.4 ± 3.5 pA) (Fig 10A), whereas synaptic events showed a significant slowing in decay time for both fast (2.1 ± 0.1 vs. 3.6 ± 0.3 ms, $P<0.01$) and slow components (6.0 ± 0.3 vs. 14.9 ± 0.6 ms, $P<0.001$) (Fig 10B). This significant increase in decay time results in an increase charge transfer which can be estimated according to the analysis outlined in the methods. Here we found that under normal conditions the charge transfer was -153 ± 8.4 versus 296.3 ±17.5 pC for THDOC-treated cells $p<0.001$. Thus, THDOC increased the synaptic inhibition by 96 ± 11.9 percent (Fig 10C).

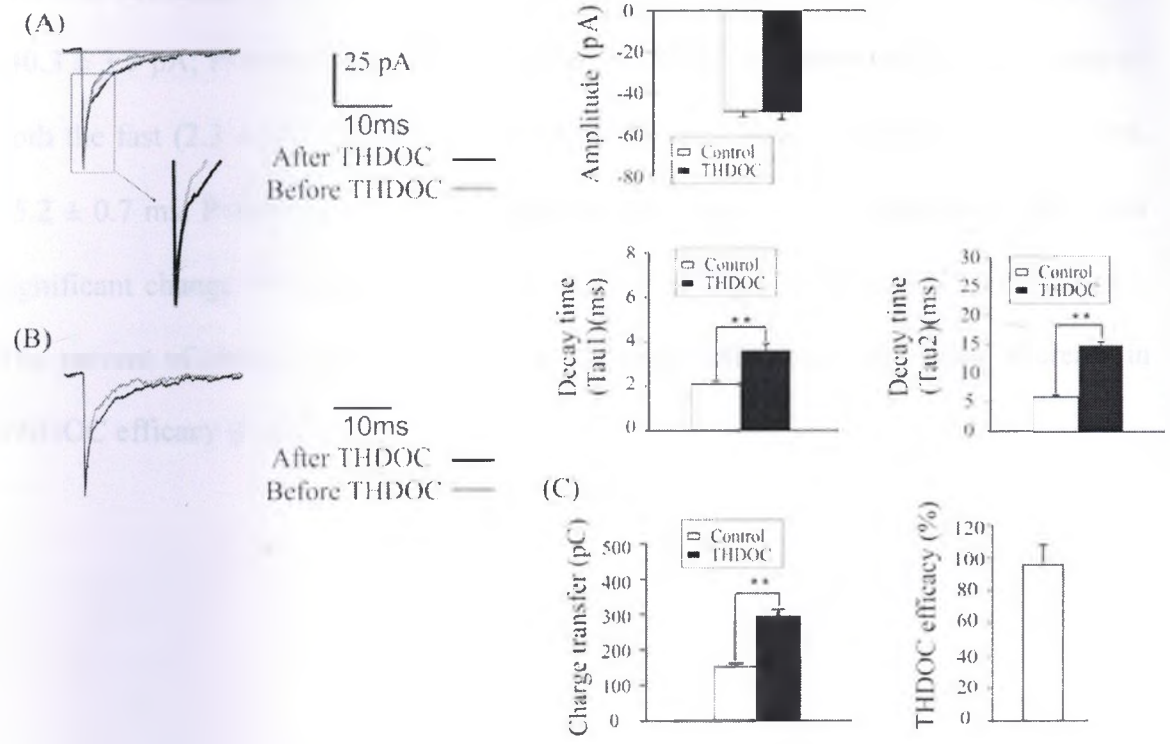

Figure 10 THDOC enhances total amount of synaptic inhibition by altering $GABA_A$ receptor kinetics. A, in the presence of THDOC (100nM) peak amplitude of mIPSCs were not changed. B, in presence of THDOC mIPSCs delayed slower in both fast (τ_1) and slow (τ_2) components.

Kindling reduces THDOC efficacy on pyramidal neurons

After kindling we found that THDOC modulation of $GABA_A$ receptors wa.. THDOC decreased the peak amplitude of the mIPSCs (mean ± SEM; n=6; -65.3 ± 5.9 vs. -40.3 ± 3.9 pA; P<0.001)(Fig 11A). However, THDOC still prolonged the decay time of both the fast (2.3 ± 0.1 vs. 4.1 ± 0.3 ms, P<0.001) and slow components (7.1 ± 0.2 vs. 15.2 ± 0.7 ms, P<0.01)(Fig 11B). An estimate of the total charge transfer did not show significant change following THDOC application (-272.5 ± 26.1 vs. -273.4 ± 22.0 pC). The percent of change was only 10.8 ± 6.5 percent, reflecting a significant decrease in THDOC efficacy (Fig 11C).

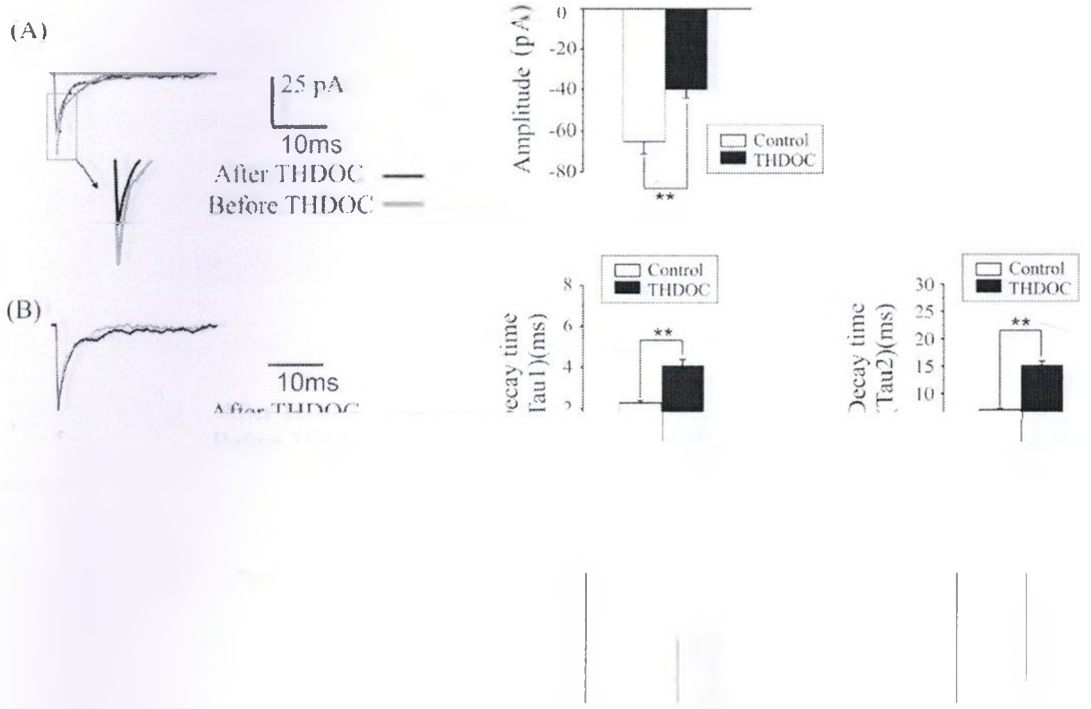

Figure 11 Kindling reduces THDOC efficacy on $GABA_A$ receptor synaptic transmission in pyramidal cells. A, after kindling THDOC (100 nM) reduced peak amplitude of mIPSCs ($P<0.001$,$n=6$). B, both fast (1) and slow (2) components delayed slower than control. C, after kindling THDOC had no effect on charge transfer and the efficacy of THDOC was reduced from 96% in non-kindled cells to 10% in kindled cells.

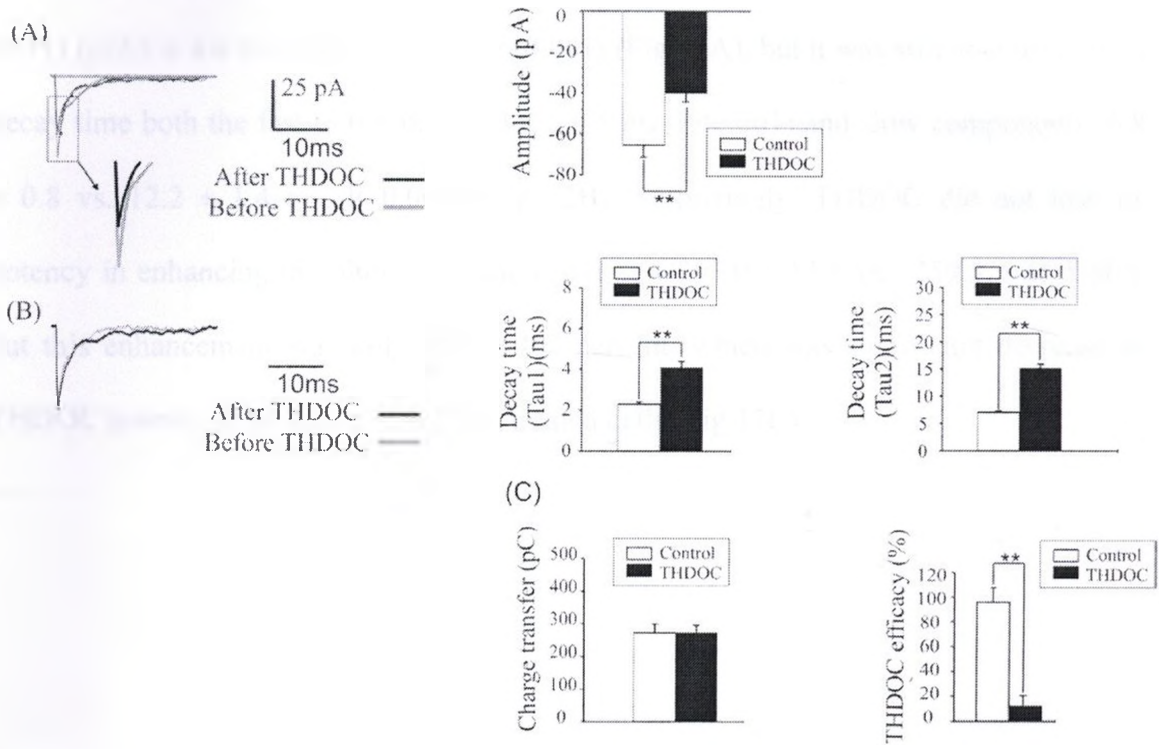

Figure 11 Kindling reduces THDOC efficacy on $GABA_A$ receptor synaptic transmission in pyramidal cells. A, after kindling THDOC (100 nM) reduced peak amplitude of mIPSCs ($P<0.001$,$n=6$). B, both fast ($\tau 1$) and slow ($\tau 2$) components delayed slower than control. C, after kindling THDOC had no effect on charge transfer and the efficacy of THDOC was reduced from 96% in non-kindled cells to 10% in kindled cells.

Enhancing phosphorylation reduces THDOC efficacy in non-kindled rats

PMA (100 nM), a phorbol ester which activates PKC, changed the THDOC modulation of $GABA_A$. In presence of PMA, THDOC decreased the peak amplitude (mean ± SEM; n=11) (-52.3 ± 4.4 vs. -41.4 ± 3.4 pA; P<0.001) (Fig 12A), but it was still able to prolong decay time both the fast (3.0 ± 0.3 vs. 4.9 ± 0.5 ms, P<0.001) and slow components (6.8 ± 0.8 vs. 12.2 ± 1.4 ms, P<0.001) (Fig 12B). Surprisingly, THDOC did not lose its potency in enhancing inhibitory synaptic response (-203.0 ± 14.3 vs. -259.3 ± 15.5 pC), but this enhancement was only 30.5 ± 8.2 percent, which was significant decrease in THDOC potency compared to not PMA treated cells (Fig 12C).

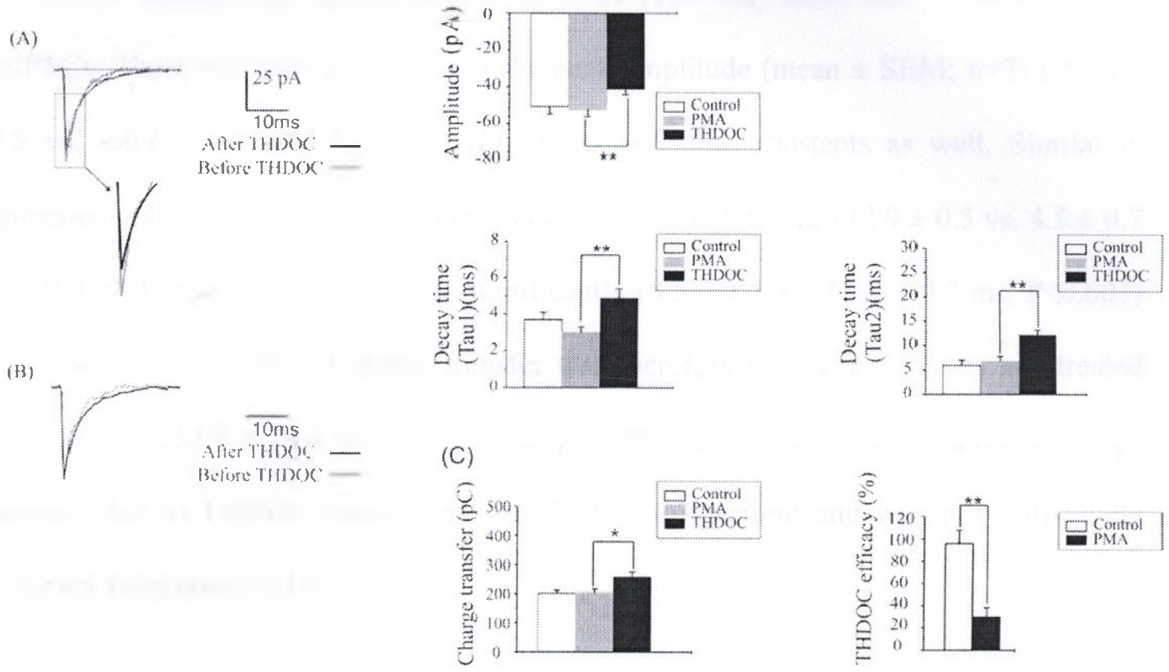

Figure 12 PKC activation by PMA reduced THDOC efficacy on $GABA_A$ synaptic transmission. Here we show traces after PMA and after PMA+ THDOC. A, THDOC reduced peak amplitude of mIPSCs in the presence of phorbol ester, PMA ($P<0.001$, $n=11$). B, PMA had no effect on THDOC modulation on time constant (both fast ($\tau 1$) and slow (τ_2) components). THDOC still prolonged the time constant. C, THDOC enhanced the charge transfer ($P<0.01$), but THDOC efficacy was reduced from 96% in non-kindled cells to 30% in PMA treated cells.

To determine whether the effect of PMA on THDOC potentiation was the result of activation of PKC, experiments were performed with inactive PMA (4α-PMA) and PKC antagonist (BIS-I). The application of 4α-PMA (100 nM) alone had no effect on the mIPSCs. There was also no change in the peak amplitude (mean ± SEM; n=7) (-55.4 ± 4.5 vs. -60.6 ± 2.0 pA; P> 0.05) (Fig 13A) and time constants as well. Similar to untreated cells, THDOC prolonged the decay time of both the fast (2.9 ± 0.5 vs. 4.5 ± 0.7 ms, P<0.001) and slow components significantly (8.5 ± 1.1 vs. 13.8 ± 0.7 ms, P<0.001) (Fig 13B). The amount of charge transfer was therefore the same value as non-treated recordings (-233.8 ± 14.4 vs. -410.2 ± 3.5 pC; P<0.001). The percent change in charge transfer due to THDOC modulation was 75.1 ± 6.3 percent and was not statistically different from control (Fig 13C).

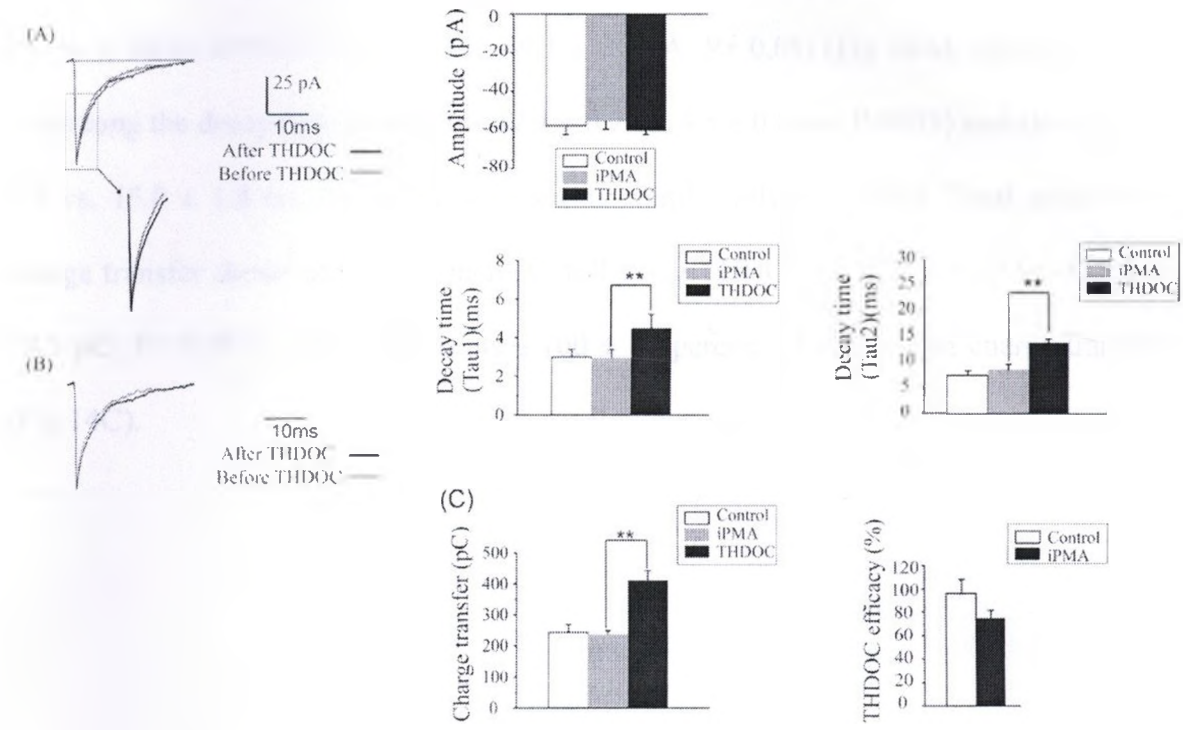

Figure 13 Inactive PMA had no effect on THDOC modulation of $GABA_A$ synaptic transmission. Here we show traces after α-PMA and after α-PMA+THDOC. A, the peak amplitude of mIPSCs did not show significant change ($P > 0.01$; n=7). B, THDOC prolonged time constant (both fast (τ1) and slow (τ2) components). C, THDOC enhanced the charge transfer , and its efficacy did not change significantly.

By co-application of BIS-I (100 nM), which is a PKC antagonist, and PMA we found that THDOC modulation of $GABA_A$ receptors was again identical to non treated cells. In presence of BIS-I and PMA, THDOC had no effect on the peak amplitude of mIPSCs (mean ± SEM; n=5) (-49.4 ± 6.1 vs. -49.1 ± 7.3 pA; $P > 0.05$) (Fig 14A), but it was able to prolong the decay time of both fast (2.4 ± 0.2 vs. 4.5 ± 0.8 ms, $P<0.01$) and slow (5.8 ± 0.7 vs. 15.0 ± 1.4 ms, $P<0.001$) components significantly (Fig 14B). Total amount of charge transfer shows significant increase following THDOC (-167.9 ± 18.7 vs.-335.6 ± 39.5 pC; $P< 0.001$), which represents a 100 ± 12 percent of increase in charge transfer (Fig 14C).

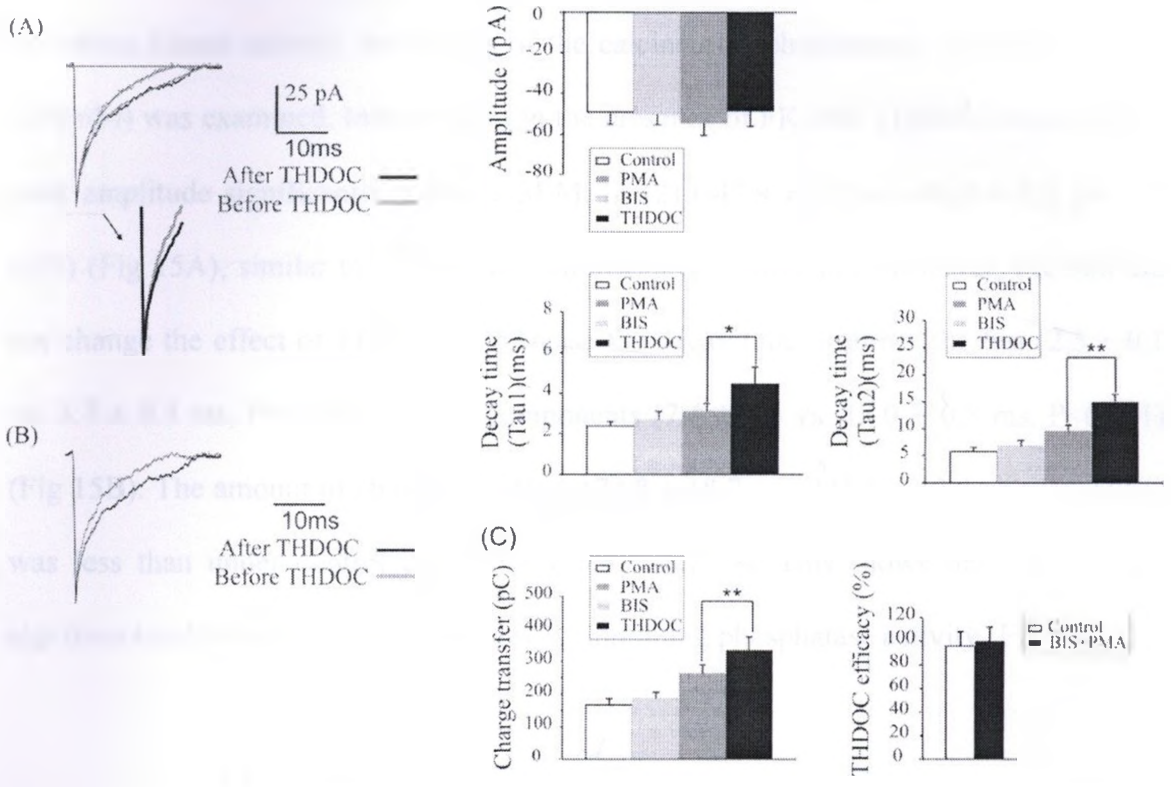

Figure 14 in presence of PKC antagonist, BIS-I, PMA had no effect on THDOC efficacy. Here we show traces after BIS-I+PMA and after BIS-I+PMA+THDOC. A, in presence of BIS-I, PMA had no effect on THDOC modulation over peak amplitude of mIPSCs. B, in presence of BIS-I, PMA still allowed THDOC to prolong the time constant (both fast (τ_1) and slow (τ_2) components). C, in presence of BIS-I, PMA did not affect the charge transfer enhancement following THDOC modulation and THDOC efficacy was similar to non-treated cells.

Phosphatase inhibition mimics the effect of PKC activation in reducing THDOC efficacy

Next to see if the inhibition of phosphatase activity had the equivalent affects as activating kinase activity, the effects of the calcineurin (phosphatase) inhibitor FK 506 (100 nM) was examined. Interestingly, in the presence of FK 506, THDOC decreased the peak amplitude significantly (mean ± SEM; n=12) (-47.9 ± 2.4 vs. -41.4 ± 2.2 pA; P< 0.01) (Fig 15A), similar to the effects seen after PKC activation. However, FK 506 did not change the effect of THDOC to increase the decay time of either the fast (2.5 ± 0.1 vs. 3.7 ± 0.3 ms, P<0.001) or slow components (7.4 ± 0.3 vs. 13.0 ± 0.3 ms, P<0.001) (Fig 15B). The amount of charge transfer (-174.8 ± 18.7 vs. -235.6 ± 13.1 pC; P< 0.001) was less than under control conditions (36.4 ± 8.7 %). This shows that there was a significant reduction in THDOC efficacy by inhibiting phosphatase activity (Fig 15C).

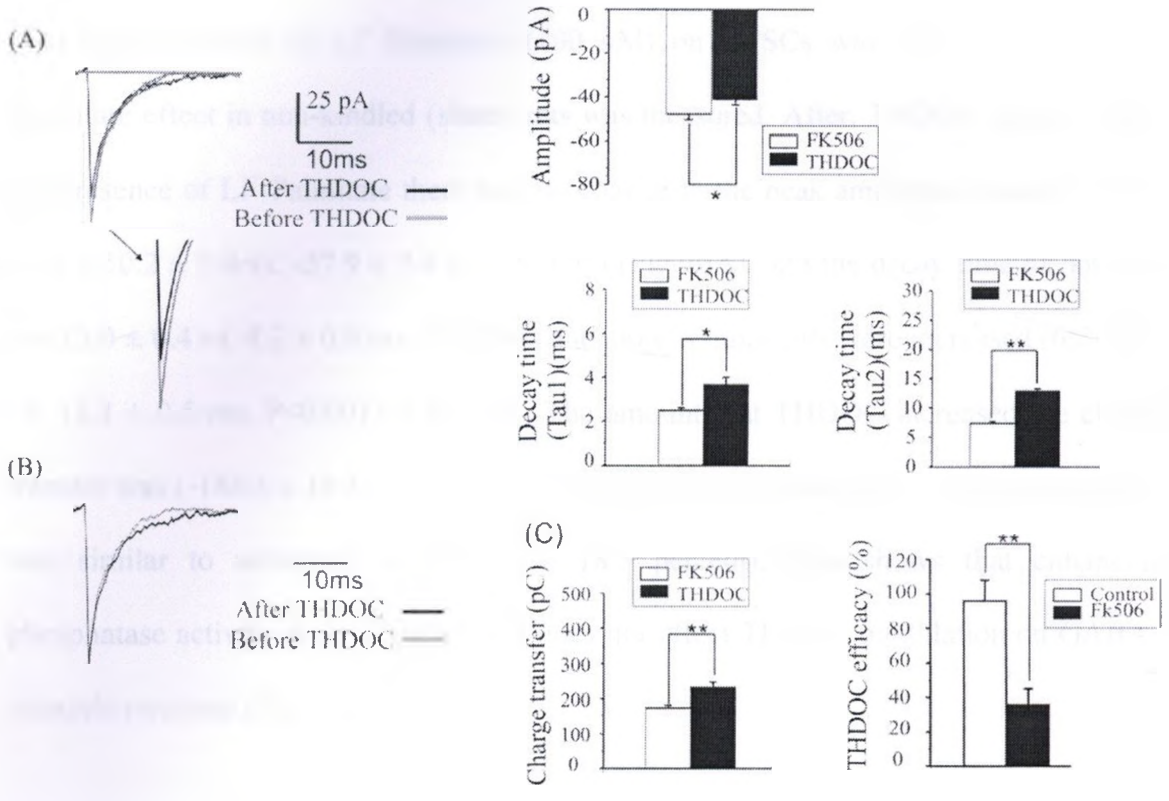

Figure 15 Calcineurin (phosphatase) inhibition by FK506 reduced THDOC efficacy in pyramidal cells. A, in presence of FK506, THDOC decreased peak amplitude of mIPSCs ($P<0.001$; $n=12$). B, Co-application of Li+ Palmitate and THDOC prolonged GABAA receptors time constant (both fast (τ_1) and slow (τ_2) components) significantly ($P<0.001$). C, in presence FK506, THDOC enhanced the amount of charge transfer, but its efficacy was reduced from 96% in non-kindled cells to 36% Phosphatase inhibitor treated cells.

Enhancing dephosphorylation reverts THDOC efficacy to normal after kindling

To examine whether the phosphatase activation restores THDOC efficacy after kindling, the effect of Li^+ Palmitate (100 nM) on sIPSCs was measured. First, Li^+ Palmitate effect in non-kindled (sham) rats was measured. After, THDOC application in the presence of Li^+ Palmitate there was no change in the peak amplitude (mean ± SEM; n=6) (-50.2 ± 5.9 vs. -57.9 ± 5.4 pA; $P>0.05$) (Fig 16A), and the decay time of both the fast (3.0 ± 0.4 vs. 4.2 ± 0.6 ms, $P<0.001$) and slow components were increased (6.7 ± 0.4 vs. 13.3 ± 0.5 ms, $P<0.001$) (Fig 16B). The amount that THDOC increased the charge transfer was (-188.3 ± 18.4 vs. -347.6 ± 15.7 pC; $P<0.001$), and the percent enhancement was similar to untreated cells (93.4 ± 18.5 percent). This shows that enhancing phosphatase activity in non kindled rats does not affect THDOC modulation on $GABA_A$ synaptic response (Fig 16C).

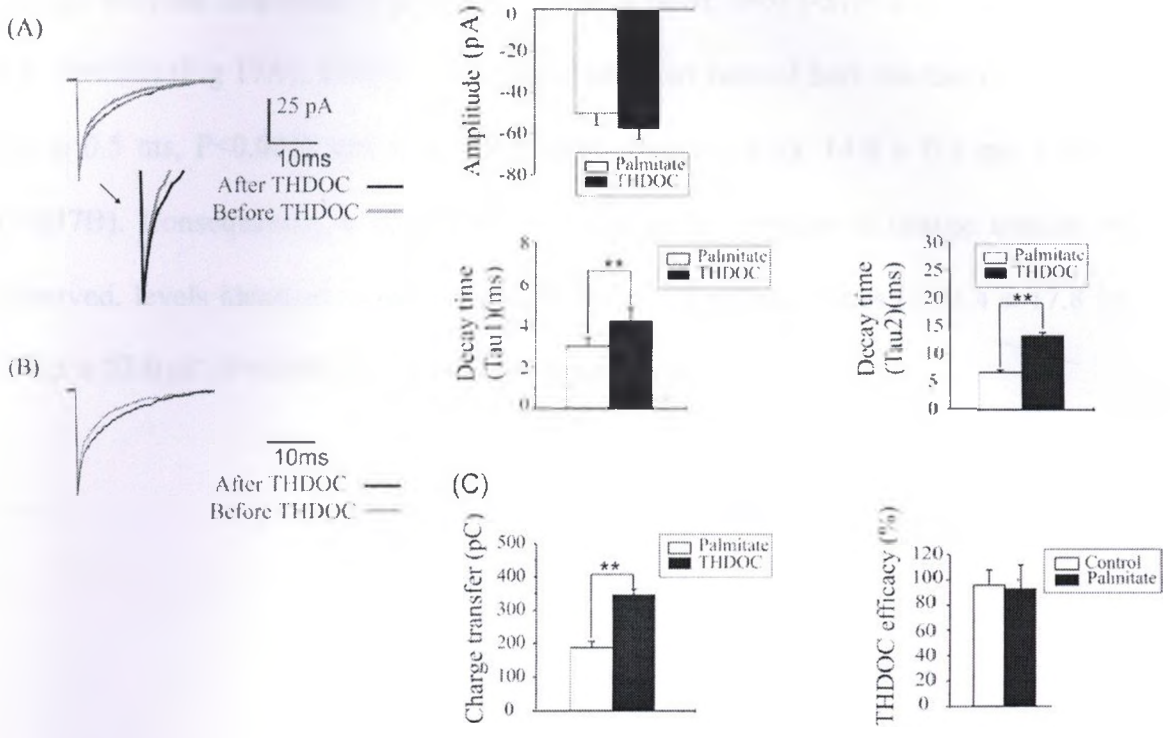

Figure 16 phosphatase activation did not affect THDOC modulation. A, phosphatase activation by using Li+ Palmitate had no effect on THDOC modulation on peak amplitude of mIPSCs. B, co-application of Li+ Palmitate and THDOC prolonged time constant (both fast (τ_1) and slow (τ_2) components) significantly ($P<0.001$; $n=6$). C, in presence of Li^+ Palmitate, THDOC enhanced charge transfer and its efficacy was same as THDOC efficacy on non-treated cells.

Second, we measured the effect of phosphatase activation on THDOC modulation after kindling. As hypothesized, we found that in presence Li^+ Palmitate THDOC did not change the peak amplitude significantly (mean ± SEM; n=5) (-57.9 ± 7.7 vs. -53.6 ± 5.5 pA; $P>0.05$) (Fig 17A). THDOC prolonged the decay time of both the fast (2.1 ± 0.2 vs. 4.5 ± 0.5 ms, $P<0.001$) and slow components (6.9 ± 0.4 vs. 14.8 ± 0.1 ms, $P<0.001$) (Fig17B). Consequently, a significant increase in the amount of charge transfer was observed, levels identical to those found in non-kindled brain slices (-201.4 ± 17.8 vs. -378.5 ± 23.0 pC; $P<0.001$;92.0 ± 14.9 percent) (Fig 17C).

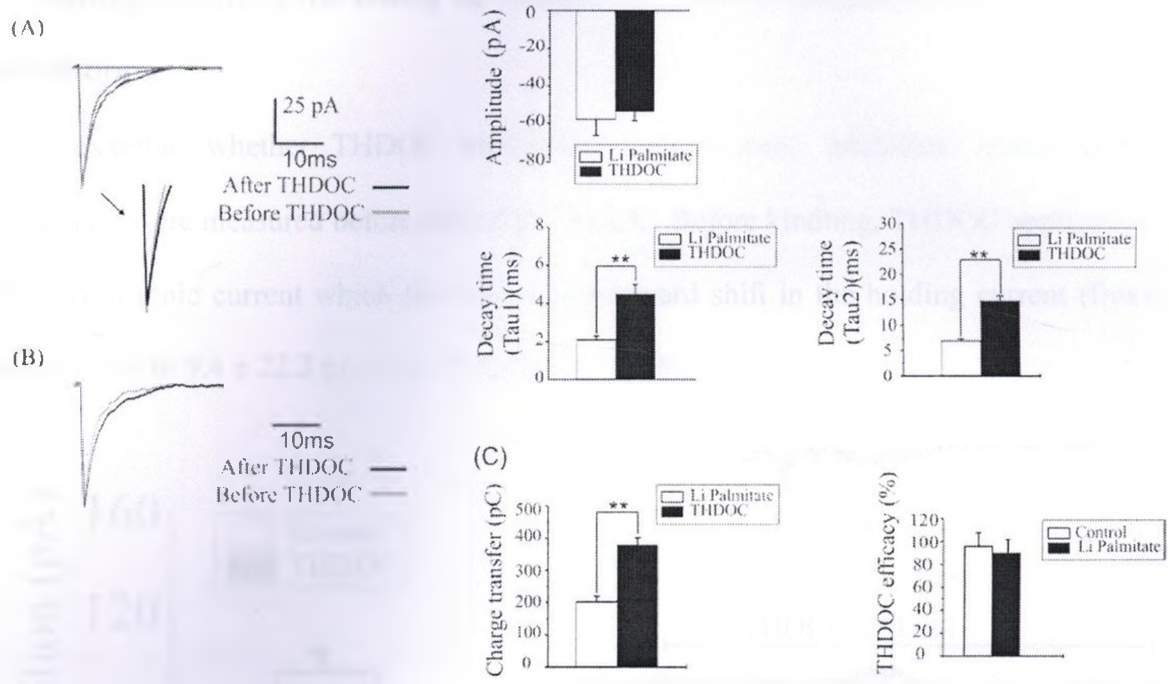

Figure 17 phosphatase activation restores THDOC efficacy after kindling. A, in presence of Li^+ Palmitate THDOC had no effect on peak amplitude of mIPSCs in kindled slices. B, co-application of Li^+ Palmitate and THDOC still prolonged time constant (both fast (τ_1) and slow (τ_2) components) after kindling ($P<0.001$; n=5). C, Li^+ Palmitate restored THDOC potency in charge transfer enhancement after kindling, and THDOC efficacy got increased in the same level as non-treated, non-kindled slices.

Extrasynaptic responses to THDOC modulation after Kindling and phosphorylation

Kindling removes the ability of THDOC to enhance extrasynaptic inhibition

To examine whether THDOC modulation affects tonic inhibition, extrasynaptic responses were measured before and after THDOC. Before kindling, THDOC application induced a tonic current which appeared as an inward shift in the holding current (from 45.6 ± 19.9 to 9.4 ± 22.2 pA; n=8, P< 0.001) (Fig 18).

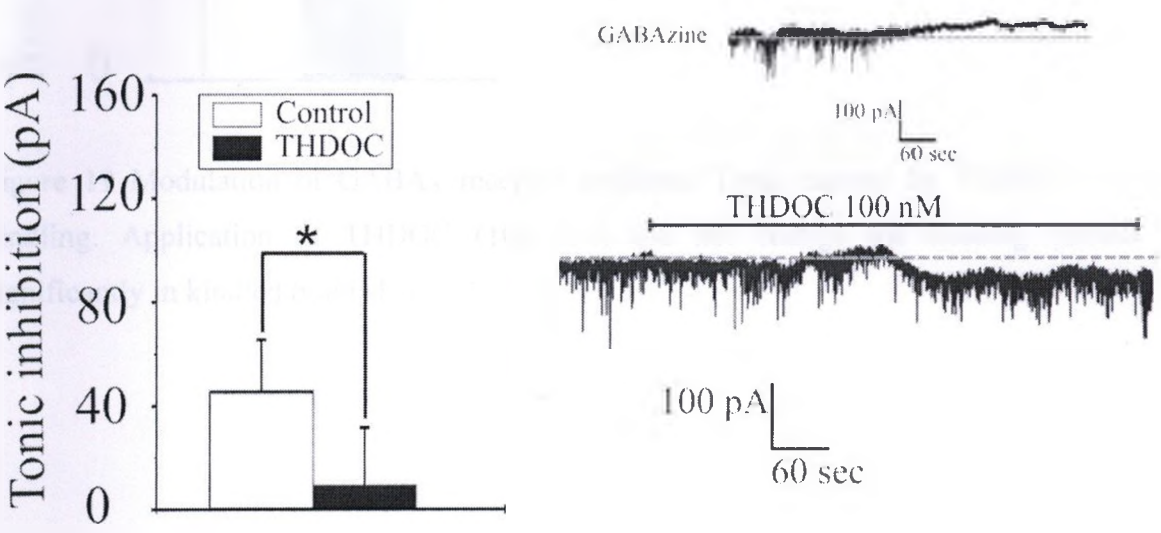

Figure 18 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC. Application of THDOC (100 nM) resulted an inward shift in non-kindled brain slices.

Surprisingly, after kindling, THDOC was not able to induce an inward shift in the extrasynaptic response (from 76.8 ± 18.3 to 92.3 ± 16.8 pA; n=5, P> 0.05). Thus THDOC's efficacy to enhance tonic inhibition after kindling was lost (Fig 19).

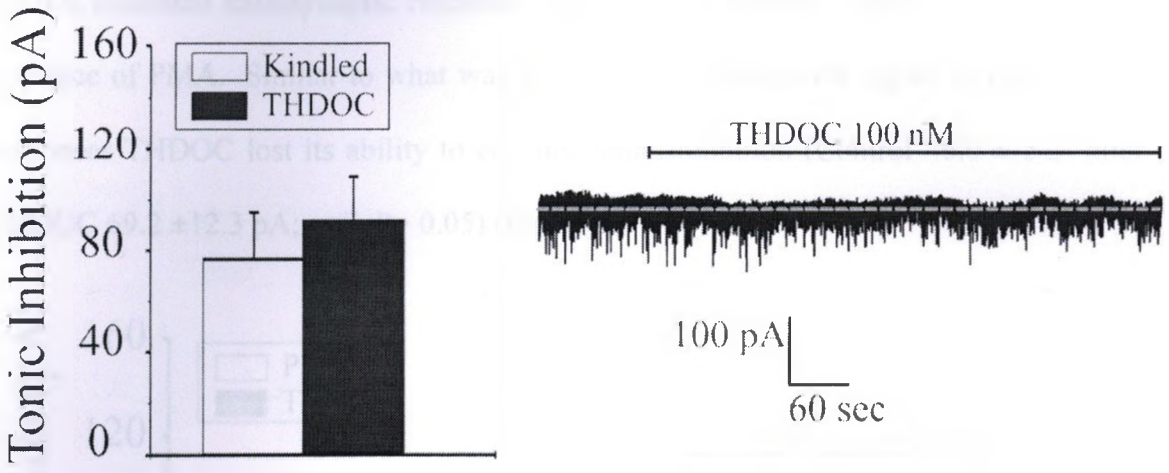

Figure 19 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC after kindling. Application of THDOC (100 nM) did not change the holding current significantly in kindled brain slices

Phosphorylation enhancement mimics kindling effect on THDOC modulation of extrasynaptic inhibition

To characterize the effect of phosphorylation enhancement on THDOC modulation of GABA mediated extrasynaptic response, the effect of THDOC effect was assessed in presence of PMA. .Similar to what was found after kindling with regard to the synaptic responses THDOC lost its ability to enhance tonic inhibition (Control 46.0 ± 5.2 after THDOC 69.2 ±12.3 pA; n=7, P> 0.05) (Fig 20).

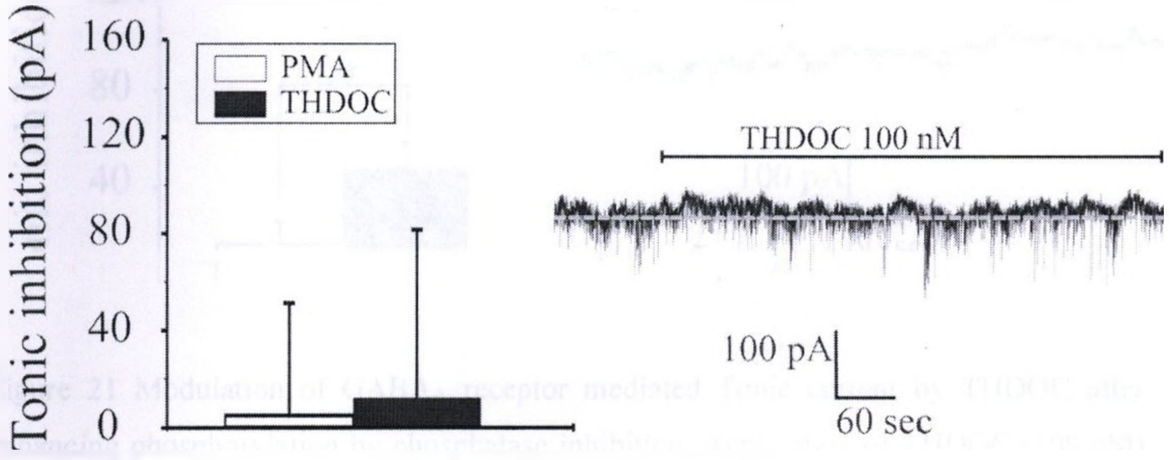

Figure 20 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC after enhancing phosphorylation by PKC activation. Application of THDOC (100 nM) was not able to change the holding current after PKC activation by PMA (100 nM) in non-kindled brain slices.

To further characterize the impact of enhanced phosphorylation over the THDOC modulation of GABA mediated tonic inhibition, we also used FK 506 to inhibit phosphatase inhibition. Surprisingly in the presence of FK 506, THDOC inhibited the tonic current producing a significant outward shift in the current (Control 16.3 ± 9.6 to 48.0 ± 11.0 pA; n=9, P< 0.001) (Fig 21).

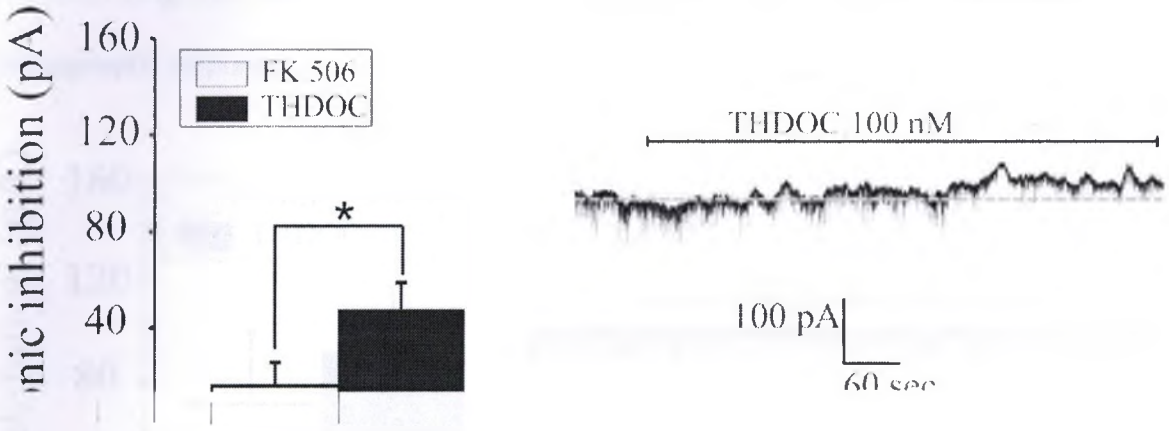

Figure 21 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC after enhancing phosphorylation by phosphatase inhibition. Application of THDOC (100 nM) resulted in outward shift in the holding current after phosphatase inhibition by FK506 (100 nM) in non-k kindled brain slices.

To substantiate whether the PKC activation by PMA is directly responsible for changing the THDOC modulation of GABA mediated tonic inhibition, we measured the extrasynaptic response in presence of PKC antagonist (BIS-I). Surprisingly, PKC antagonist (BIS-I) also did not change the direction of THDOC modulation on GABA mediated extrasynaptic response significantly (from 71.6 ± 28.4 to 92.6 ± 25.3 pA; n=4, P> 0.05) (Fig 22), but it seems that PKC antagonist affect THDOC modulation on extrasynaptic response.

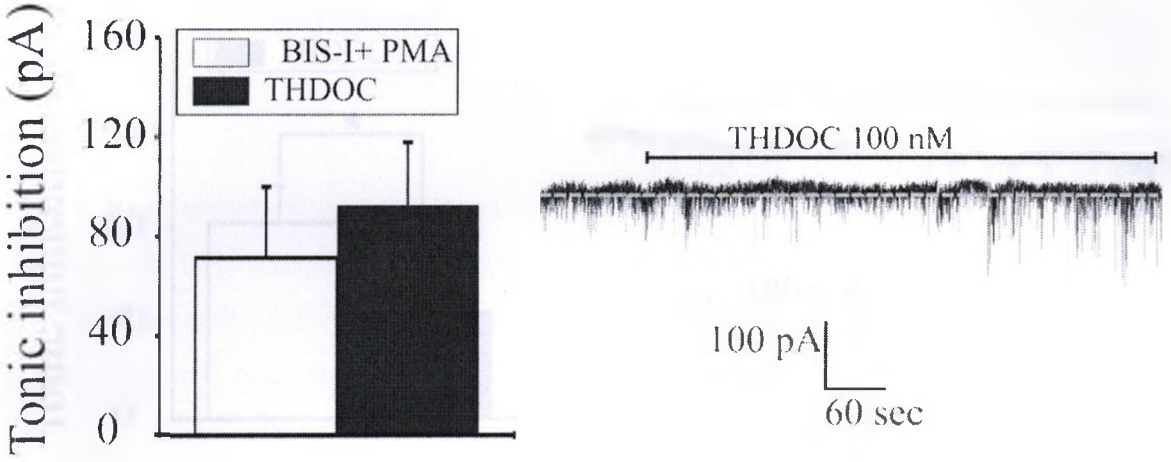

Figure 22 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC in presence of PKC inhibitor, BIS-I (100 nM). Application of THDOC (100 nM) was not able to change the holding current after PKC activation by PMA (100 nM) in presence of BIS-I in non-kindled brain slices.

Dephosphorylation enhancement does not restores THDOC modulation of extrasynaptic inhibition after kindling

To see whether dephosphorylation has an effect on the THDOC mediated extrasynaptic response, we measured tonic extrasynaptic response in presence of phosphatase activator (Li^+ Palmitate) in non-kindled (sham) rats. Consistently, THDOC enhanced tonic inhibition inducing a significant inward shift in GABA mediated extrasynaptic response (from 82.0 ± 15.2 to 44.6 ± 15.0 pA; n=9, P< 0.000) (Fig 23).

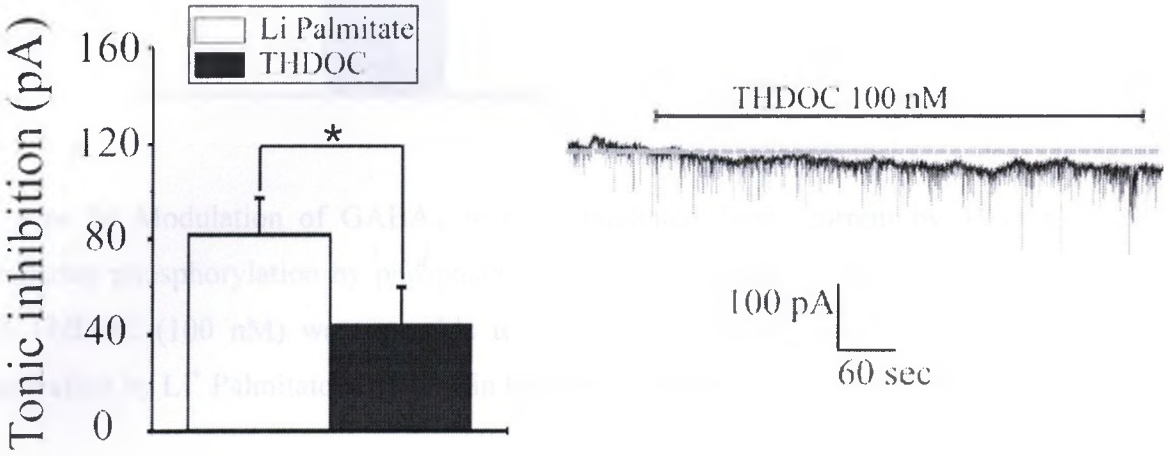

Figure 23 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC after reducing phosphorylation by phosphatase activation. Application of THDOC (100 nM) resulted in inward shift in the holding current after phosphates activator by Li+ Palmitate (100 nM) in non-kindled brain slices.

While phosphatase activation appeared not interfere with THDOC modulation in normal condition after kindling THDOC was still unable to induce an inward shift (from 48.8 ± 14.0 to 15.4 ± 63.6 pA; n=5, P> 0.05) (Fig 24).

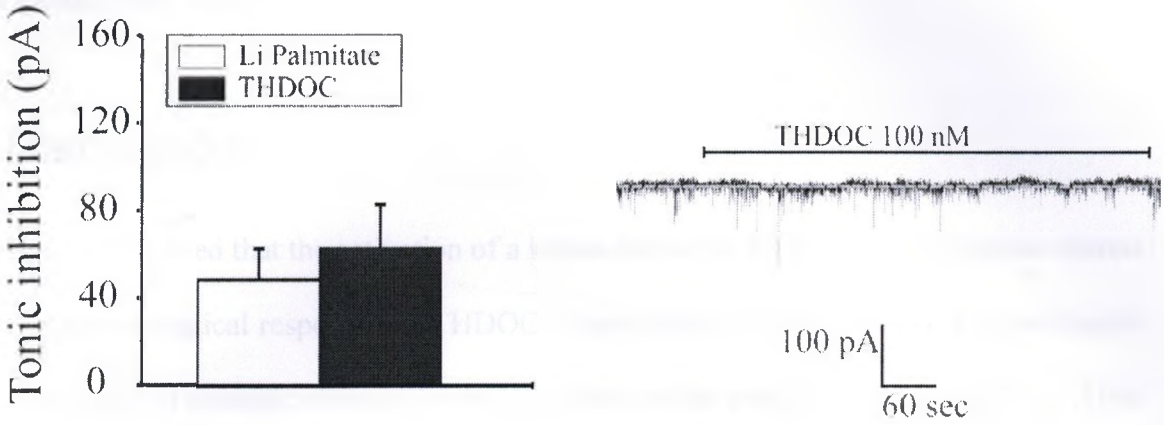

Figure 24 Modulation of $GABA_A$ receptor mediated Tonic current by THDOC after reducing phosphorylation by phosphatase activation in kindled brain slices. Application of THDOC (100 nM) was not able to change the holding current after phosphatase activation by Li^+ Palmitate (100 nM) in kindled brain slices.

In general, we found that phosphorylation enhancement affects Synaptic Plasticity by changing THDOC efficacy in synaptic and extrasynaptic responses. Moreover, dephosphorylation enhancement was able to restore THDOC efficacy only in synaptic responses after kindling.

Discussion

Our data showed that the activation of a kinase or the inhibition of a phosphatase altered the pharmacological responses of THDOC. Under control conditions THDOC prolonged the duration of synaptic currents having no effect on the amplitude of the mIPSCs. After treatment with agents that favor phosphorylation of proteins (including $GABA_A$ receptors) the amplitude of the mIPSCs was reduced while the effects on the duration remained relatively the same. The overall effect was that the amount of inhibition (measured by charge transfer) was reduced. These results were reminiscent of those observed after kindling where THDOC reduced the amplitude of mIPSCs either little or no enhancement of inhibition was observed. The addition of a phosphatase activator had little effect in non kindled rats, but restored the "normal" pharmacology in kindled brain slices. These data suggested that $GABA_A$ receptors in kindled brain were more phosphorylated than in non-kindled brain and this hyper-phosphorylated state was responsible for the altered THDOC pharmacology. We also showed that kindling reduced the THDOC induced enhancement of tonic inhibition and that PMA mimicked this effect as well. However Li^+ palmitate treatment was unable to reverse this effect in the kindled brain. As all these recordings were done in kindled brain slices at least two weeks after

the last seizure, this suggests that there were long term alterations in the phosphorylation state of the $GABA_A$ receptors after kindling.

The hyper-phosphorylation may be a feature of pharmacoresistant epilepsy (PE). PE is prevalent in about 30 % of all epilepsy patients and may originate in the seizure focus of temporal lobe epilepsy. Two distinctive hypotheses have been proposed to account for PE. The first is the Multi Drug Transporter (MDT) hypothesis and second is the Altered Target (AT) hypothesis. The MDT hypothesis suggests that a transporter in the blood brain barrier becomes hyperactive making the "loading" of the brain with medication impossible. The AT hypothesis contends that the drug target has been altered so drug efficacy is reduced. All our recordings are done in brain where the blood barrier has been removed, so the MDT hypothesis cannot be considered here. On the other hand, long lasting changes, like phosphorylation, are likely to occur and so our data support the idea that PE may arise from an altered drug target.

Although our data are consistent with the idea that the phosphorylation state of the $GABA_A$ receptor is being altered by our treatments, we have not directly measured the phosphorylation. Nevertheless, the fact that we have converging observations that show consistent effects when we apply treatments favoring phosphorylation and an opposite effect when phosphorylation is presumably decreased it is reasonable to assume some alterations in phosphorylation is occurring. Therefore we will use the terms enhanced or reduced phosphorylation to discuss various aspects of our data.

The main effect observed after kindling was that the peak amplitude of the mIPSC was reduced due to THDOC application. This reduction in amplitude counteracts the effect of

the prolongation of the mIPSC and thus THDOC is unable to significantly augment inhibition. The mechanism by which this occurs is unconfirmed. One study has suggested that THDOC enhances the rate at which the receptor desensitizes. As mentioned in the introduction, desensitization in this case means the closing of the channel even though the agonist is still bound. Studies indicate that desensitization likely is responsible for the initial decay of mIPSC. Importantly computer modeling has found that the rate of fast desensitization can reduce the probability that the channel will open even on the initial activation of the synaptic response (Elenes et al., 2006). Under normal conditions the rate of desensitization does not seem to be rapid enough to reduce amplitude of mIPSCs. THDOC may be able to enhance entry into the desensitized state after kindling or enhanced phosphorylation reducing the mIPSC amplitude. Phosphorylation has been shown to reduce desensitization, which is consistent with our findings here. We found that PMA treatment slowed the first time constant of the mIPSC. This implies that the ability of THDOC to enhance the entry into the desensitized state is independent of the effect of phosphorylation on the rate of desensitization itself.

Previous studies have shown that the effect of PMA on the $GABA_A$ receptors occurs through activation of PKC (Kusama et al., 1995), while the PKC inhibitor (BIS) and inactive analogs of PMA do not change GABAA receptor function (Sigel and Baur, 1988) (Moran and Dascal, 1989;Sigel et al., 1991;Gillette and Dacheux, 1996). Our observations are largely consistent with these previous observations (except where noted below). We can be reasonably sure that PMA exerts its effects on $GABA_A$ receptors function through activation of PKC. The mechanism is likely phosphorylation of the $GABA_A$ receptor β and γ_2 subunits on serine 410 and serine 327 respectively. Evidence

that subunit composition of the receptor may influence neurosteroid efficacy is not clear (Puia et al., 1993;Lambert et al., 1996). Our data suggests that the heterogeneity of neurosteroid modulation that is often observed may actually arise from a variable phosphorylation state of the receptor.

As mentioned above THDOC seems to alter the desensitization kinetics of $GABA_A$ receptors. Altering the desensitization may contribute to the prolongation of slow component of decay time as it has been shown previously that the slow second component is due to re-openings of the channels as they recover from desensitization (Jones and Westbrook, 1995). Altering the phosphorylation is not expected to alter the primary feature of THDOC activity, which is to prolong the duration of the second component of the mIPSC. Nevertheless the significant decrease in mIPSC amplitude most likely results from the ability of THDOC to enhance the rate of entry into the desensitized state to level that effectively competes with the rate of channel opening. This interpretation has been in fact been modeled in the past where Jones and Westbrook show that by enhancing the rate of entry into the desensitized state the P_{open} is significantly reduced (Jones and Westbrook, 1995).

Changing affinity to THDOC due to phosphorylation enhancement

It is possible that THDOC enhances the affinity of GABA after modification of phosphorylation, although we have not performed experiments with GABA and THDOC under equilibrium concentrations. Phosphorylation may alter the affinity of THDOC for the neurosteroid binding site or perhaps weaken the allosteric coupling between the neurosteroid binding site, the GABA binding site and chloride channel. This suggestion is consistent with previous results indicating that phosphorylation decreases coupling

between the α-adrenergic (Beeler and Cooper, 1993) and β-adrenergic (Bouvier et al., 1991) receptors and their associated heterotrimeric G-proteins . It also affects the interaction between G-protein subunit (Imaizumi et al., 1991). After kindling or after increasing phosphorylation the reduced ability the THDOC's to enhance the tonic current may be due to a loss of THDOC action to enhance GABA affinity.

Phosphorylation enhancement mimics kindling

The main purpose of this study was to determine whether either enhancing phosphorylation in control tissue or reversing phosphorylation in kindled tissue and treating with THDOC decreases the peak amplitude, while conserving THDOC's potency to enhance the duration of the mIPSCs. We also found that activation of phosphatases brought new insight into THDOC modulation. Phosphatase activation did not interfere in THDOC modulation in un-kindled rats. This implies that in the pre-kindled state the basal phosphorylation of $GABA_A$ receptors was low and phosphatase activation was not likely a mechanism which regulates THDOC activity in the absence of seizure induction. On the other hand, our results demonstrated that THDOC efficacy was restored after kindling by activation of phosphatases. This observation supports the idea that phosphorylation may play a critical role in THDOC modulation under normal conditions.

The perisynaptic effect of phosphorylation enhancement on THDOC modulation

Previous studies have suggested that tonic inhibition is enhanced by the increasing affinity of GABA for the extrasynaptic receptors using either benzodiazepine or a neurosteroid like THDOC. Our observations suggest that hyper-phosphorylation of the non-kindled brain slices reduced THDOC's ability to enhance GABA affinity. The other

possibility is that THDOC increased desensitization and this may have lead to more receptors being desensitized after hyper-phosphorylation preventing the augmentation of the tonic current. We found that in the presence FK-506 an outward shift was evident, thus THDOC under these conditions may desensitize to such an extent that the tonic current is reduced from control levels.

A confounding outcome that contradicts this conclusion is that BIS-I (the PKC antagonist) failed to prevent the effects of PMA and phosphatase activation after kindling and did not restore the effects of THDOC on the tonic current. Although BIS is able to antagonize many PKC isoforms, it is unable to antagonize a number of others including the θ, ζ as well some splice variants of the β isoforms (Wilkinson et al., 1993). This implies that different PKCs may regulate the phosphorylation of the synaptic and extrasynaptic receptors. It should be noted that these recordings were done with a dialyzed internal milieu, so our manipulations would only affect membrane bound PKC isoforms. This differential activity may reflect a compartmentalization of differing PKC isoforms associated with $GABA_A$ receptors. Similarly, the lack of effect of the Li^+ palmitate implies that there may be relatively little phosphatase activity "available" in the extrasynaptic compartment but that phosphatases may be more closely linked to the synaptic compartment.

Phosphorylation enhancement through either PKC activation or phosphatase inhibition closely mimicked our observations after kindling. This similarity between kindling and phosphorylation enhancement indicates kindling can result in hyper-phosphorylation of $GABA_A$ receptors in this region of temporal lobe. Consequently, it leads to impaired THDOC modulation of synaptic $GABA_A$ receptor plasticity. Thus hyper-phosphorylation

is the most likely outcome that explains the altered function after kindling. Interestingly, after kindling, dephosphorylation did not restore the THDOC potency in enhancing tonic inhibition. The reason for this may be that subunit composition in synaptic and perisynaptic receptors is different and so they may be less sensitive to phosphatase.

Muscarinic enhancement activates PKC

PKC activation effects both intrinsic cellular properties and synaptic responses. PKC activation can occur through many different G protein coupled receptors (GPCRs), including GPCRs activated due to muscarinic activation (Sim et al., 1992). Acetylcholine (ACh), a neurotransmitter that activates muscarinic receptors, is an important activator of PKC. Neurons arising in the forebrain project to the limbic regions including the hippocampus, entorhinal cortex, and amygdale forming connections that are involved important physiological roles. The medial septum has also been reported as an important provider of cholinergic projections to temporal lobe (Woolf et al., 1984). These projections are considered as modulators of a number of processes such as potentiation of NMDA receptor conductance, inhibition of potassium channels and the stimulation of second-messenger cascades (Krnjevic, 1993;Markram and Segal, 1992).

Seizures strongly activate cholinergic function. Cholinergic modulation of neuronal activity in temporal lobe structures has been shown to decrease the conductance of a number of potassium channels through the activation of G protein-coupled receptors (Bymaster et al., 2003;Benardo and Prince, 1982). *In vivo* application of muscarinic agonists (pilocarpine) and acetylcholinesterase (AChE) inhibitors induce seizures, may lead to status epilepticus, and result in spontaneous seizures following a latent period. There is also evidence that endogenous muscarinic activation supports epileptic seizures

(Turski et al., 1989). The muscarinic antagonist atropine or pirenzepine co-applied with pilocarpine prevent the onset of seizures (Turski et al., 1984). It is clear that a good deal of the PKC activation that is occurring supports seizuregenesis and it is reasonable to speculate that this activation may be the mechanism that hyperphosphorylates $GABA_A$ receptors.

Previous studies have shown that synaptic transmission can also be regulated by intracellular second messenger cascade which is Ca^{2+} dependent (Malenka et al., 1986;Shapira et al., 1987;Hori et al., 1999;Yawo, 1999). In our experiments application of PMA, which is a functional analogue of diacylglycerol (DAG), may influence Ca^{2+} level at both pre- and post-synaptic sites. It has been shown that phorbol esters such as PMA influence Ca^{2+} evoked release (Lou et al., 2005). PKC activation alone results in mitochondrial Ca^{2+} efflux, and presynaptic Ca^{2+} influx (Fu and Huang, 1994;Byrne and Kandel, 1996). This significant increase of intracellular Ca^{2+} is important in triggering $GABA_A$ receptor modulation through calcium and calmodulin-dependent kinase II and calcineuirin activity. Machu et al., (1993) showed that calcium and calmodulin-dependent kinase II normally phosphorylate the intracellular loop of the γ2L subunit of the $GABA_A$ receptors (Machu et al., 1993;Wang et al., 1995;Aguayo et al., 1998;Churn et al., 2000). Together, the findings bring up the possibility of combinatory effects due to activation of PKC and calcium and calmodulin-dependent kinase II in a cascade format. Consequently the heterogeneity of $GABA_A$ receptor subunit expression in synaptic and perisynaptic area reflects profound complexity in THDOC modulation.

Conclusion and future perspectives

Present findings imply that enhancement of phosphorylation prevents the potentiation by THDOC of GABAA receptors after kindling. This conclution suggests that kindling (seizures) activate Ikinase activity (the identity of which is not known). Our observations also suggest that this hyper-phosphorylation state is long lasting as we have observed the effects of kindling on THDOC as long as three weeks after the last seizure. Nevertheless, this altered pharmacology can be reversed by phosphatase activation. This alteration in activity may contribute excitation/inhibition imbalance. As there are long term changes in the state of GABA support the altered target hypothesis of pharmacoresistant epilepsy.

In conclusion, the present findings are consistent with the accumulating molecular, electrophysiological, and behavioral data that highlight the key role of phosphorylation in inhibitory synaptic transmission and in physiologic and pathologic conditions such as temporal lobe epilepsy. It also proposes the possibility of the induction of long-lasting changes in $GABA_A$ receptors after kindling which may be reversible. This reversibility may create the opportunity to evaluate the effects of potential anti -epileptic drugs.

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