Neurophysiological and Pharmacological Study of Carbamazepine on Physiological and Pathological GABAergic-Dependent Thalamocortical Oscillations

Thomas Wen-Juan Zheng

Submitted in total fulfillment of the requirements of the degree of Doctor of Philosophy under a Cotutelle arrangement with The University of Melbourne and Université de Strasbourg

August, 2010

Department of Medicine
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne
&
INSERM U666
Faculty of Medicine
Université de Strasbourg

Abstract

Background

Neurological and psychiatric disorders, including epilepsy and schizophrenia, are associated with abnormal synchronization of brain rhythms. Both the neocortex and thalamus are prominently involved in consciousness, perception and cognition. They are reciprocally connected and work in tandem to generate physiological and pathological rhythms. The thalamus is reciprocally connected with the thalamic reticular nucleus (TRN) and with the cerebral cortex. The thalamocortical (TC) and corticothalamic (CT) neurons are glutamatergic and cross the TRN where they give off axon collaterals. The TRN contains only GABAergic neurons, which project to all nuclei of the dorsal thalamus (Pinault, 2004).

Absence seizures or "Petit Mal" are a common type of seizure occurring in patients with idiopathic generalized epilepsy with genetic causes (Gloor and Fariello, 1988; Crunelli and Leresche, 2002). Absence seizures are non-convulsive with loss of consciousness, perception and responsiveness. The electroencephalogram (EEG) reveals that absence seizures are associated with generalized and bilaterally synchronous, high-voltage oscillations (~3-6 Hz), termed spike-and-wave discharges (SWDs). These absence-related SWDs are generated in the somatosensory CT system (Vergnes et al., 1990; Pinault, 2003). Studies conducted in genetic rodent models suggest that SWDs originate from a restricted focus in the somatosensory cortex (Meeren et al., 2002; Pinault, 2003; Polack et al., 2007), although the precise nature of this postulated "focus" remains unclear. One aim of work completed as part of my thesis was to investigate the spatiotemporal dynamics of neuronal activities to better understand the cortical focus theory of SWD generation.

Medical treatments for the absence epilepsies primarily rely on pharmacotherapy that suppresses seizures. However, in many cases anti-epileptic drugs (AEDs) provide inadequate seizure suppression and are often associated with significant adverse effects (Hosak and Libiger, 2002). In some cases AEDs can paradoxically aggravate seizures. Carbamazepine (CBZ) and CBZ-like drugs are commonly prescribed AEDs that are recognized to aggravate many generalized seizure types, including absences (Zheng et al., 2006). CBZ is also used in psychiatry as a mood stabilizer and in schizophrenic patients (Hosak and Libiger, 2002).

The neuronal mechanisms underlying the anti-epileptic and mood stabilizing actions of CBZ or CBZ-like drugs are unknown. While it has been reported that CBZ exerts a positive allosteric modulation on GABAA receptors (Granger et al., 1995), the neurophysiological consequences of this remain unknown. In the thalamus, the neuronal mechanisms underlying absence-related SWDs involve GABAAreceptor-mediated inhibitory postsynaptic potentials (IPSPs) in TC neurons (Pinault et al., 1998). Experiments presented in my thesis tested the hypothesis that CBZ aggravation of absence seizures is due to increased GABAA-receptor-mediated inhibition.

Studying the neuronal impact of CBZ *in vivo* will improve our understanding of the pathophysiology of TC systems, absence epilepsy and of the therapeutic properties of CBZ-like molecules, with the hope to improve treatments for patients in neurology and psychiatry.

Working Hypothesis

Carbamazepine is known to aggravate absence seizures in genetic rodent models of absence epilepsy. On the basis of recent experimental evidence, we hypothesize that CBZ enhances GABAA receptor-mediated activities within the TC and CT systems.

Major goals

This project is part of a French-Australian collaboration, initiated in 2006 when Prof Terence J O'Brien came to Strasbourg for a sabbatical in the laboratory of Dr Didier Pinault. Our goal is to use a neurophysiological and pharmacological strategy to understand the neuronal mechanisms underlying the initiation and maintenance of SWDs in genetic models of absence epilepsy, and their modulation by AEDs. More specifically, we investigated the pharmacological and neurophysiological effects of CBZ on physiological and pathological TC/CT oscillations.

We tested our hypothesis with two major strategies: 1) Directly *in vitro* on GABAA receptors and 2) *in vivo* on physiological and pathological GABAA receptor-mediated TC oscillations.

Strategy and Methods

1) In the frame of a Australia-French cotutelle thesis, I have been working for 3 years at the Department of Medicine, Royal Melbourne Hospital, The University of Melbourne (Co-directors: Terence J O'Brien, Chris Reid, UoM, and Margaret Morris of The University of New South Wales, with regular interactions with Didier Pinault, UdS) and 1 year at Inserm U666 (head: JM Danion), Faculty of Medicine, University of Strasbourg (Co-directors: Terence J O'Brien and Didier Pinault). My stay at the laboratory of Didier Pinault was supported by a bourse d'excellence Eiffel doctorat (2008).

2) To test the action of CBZ directly on GABAA receptors, we used an *in vitro* approach in Xenopus oocytes expressing recombinant GABAA receptors.

3) To test CBZ's action on physiological and pathological GABAA receptor-mediated TC oscillations *in vivo*, CBZ was microinjected, via implanted cannulae, into the somatosensory thalamus and the related TRN region in free-moving epileptic rats. The effects were evaluated using EEG recordings and behavioural observation. The specificity of the impact on GABAA receptors was investigated using systemic injection of CBZ and intra-thalamic microinjection of bicuculline, a GABAA receptor antagonist.

We used the Genetic Absence Epilepsy Rats from Strasbourg (GAERS), a well-established model of absence epilepsy (Vergnes et al., 1982). As in humans, SWDs in GAERS start and end abruptly on a normal EEG background during quiet wakefulness that are accompanied by behavioural immobility, rhythmic twitching of the vibrissae, facial muscles and unresponsiveness to mild sensory stimuli. Our laboratory has previously demonstrated an aggravation of absence seizures in GAERS by CBZ (Wallengren et al., 2005). The non-epileptic control (NEC) rats are derived from the same colony of Wistar rats. These NEC rats do not display the EEG and behavioural epileptic phenotypes.

4) To test the effect of CBZ in the somatosensory cortex, a major difficulty was to identify the location where CBZ should be applied. Experiments conducted in WAG/Rij rats, another well-established genetic model of absence epilepsy, have yielded new findings supporting the hypothesis that SWDs are initiated in a restricted region of the somatosensory cortex (Meeren et al., 2002). Therefore, it was important to identify in GAERS the cortical region in the somatosensory cortex that is critical in the electrogenesis of absence-related SWDs. We

conducted multi-site cellular, multiunit, local field potential (LFP) and cortical EEG recordings in free-moving and lightly anesthetized GAERS and NEC rats. Intra- and juxtacellular recordings were performed with sharp glass micropipettes that contained a neuronal tracer to label the recorded neurons (Pinault, 1996).

5) We also evaluated *in vivo* the impact of CBZ administration on thalamic oscillations, in particular on TC spindle oscillations, which can be recorded under anesthesia, and which prominently involved GABAAreceptor-mediated IPSPs. For this we used LFP, intra- and juxtacellular recordings of TC and TRN neurons along with the EEG of the related cortex (Pinault, 2003).

Results

1) CBZ acts directly on GABA receptors, enhancing Cl- flux

To investigate a direct interaction between CBZ and GABAA receptors, recombinant GABAA receptors were expressed in Xenopus oocytes. CBZ potentiated GABAA receptor mediated chloride current at therapeutic doses. These results demonstrate that CBZ directly interacts with GABAA receptor, most likely acting as a positive allosteric modulator.

This work is published:

Zheng TW, Liu L, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The Mechanism of Carbamazepine Aggravation of Absence Seizures. J Pharmacol Exp Ther 319: 790-798.

2) CBZ modulates GABAA receptors specifically in the somatosensory thalamus to aggravate absence seizures

To test the region specific effect of CBZ within the thalamus, cortical EEG recordings were performed in freely moving GAERS after drug injections. CBZ aggravated absence seizures after either systemic or intrathalamic injections. Bilateral injections in the somatosensory thalamus aggravated seizures in a dose-dependent manner, while injections into the TRN had no effect, indicating the regional selectivity of CBZ. Furthermore, aggravation of SWDs in GAERS

induced by systemic injection of CBZ was completely blocked by bilateral microinjection of the GABAA receptor antagonist bicuculline in the somatosensory thalamus. These findings provided evidence that the predominant site and mechanism by which CBZ acts to aggravate seizures in GAERS is by enhancing GABAA receptor-mediated activity in the somatosensory thalamus.

This work is published:

Zheng TW, Liu L, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The Mechanism of Carbamazepine Aggravation of Absence Seizures. J Pharmacol Exp Ther 319: 790-798.

3) Oxcarbazepine (OXC), a CBZ analogue that potentiates GABAA receptor-mediated activity, aggravates absence seizures but its active metabolite MHD, which does not have effects on GABAA receptors, does not.

Here we tested the hypothesis that OXC; but not its major active metabolite monohydroxy (MHD) derivative; aggravates seizures. The effects of systematic injections of OXC, MHD and CBZ were compared in freely moving GAERS. As anticipated OXC aggravated seizures in GAERS, similarly to the effect of CBZ. Conversely, MHD did not aggravate seizures. This demonstrates that the ability to potentiate of GABAA receptor-dependent activity is critical to the seizure aggravating effects of CBZ-like molecules in GAERS.

This work is published:

Zheng TW, Clarke AL, Morris MJ, Reid CA, Petrou S, and O'Brien TJ (2009) Oxcarbazepine, not its active metabolite, potentiates GABAA activation and aggravates absence seizures. Epilepsia 50:83–87.

4) Precursor rhythmic neuronal activities in the secondary somatosensory (S2) and insular (IC) cortices during the initiation of genetically determined absence-related SWDs

To investigate the impact of CBZ on SWDs in the somatosensory cortex, CBZ needs to be applied at the right location since this sensory cortex is a large area. The intracortical origin of bilateral synchronous SWDs that accompany absence seizures has been widely debated.

Therefore we characterized the interictal, preictal and ictal neuronal activity in the primary and secondary cortical regions (S1, S2) and in the adjacent IC in GAERS using multi-site cellular and network recordings. SWDs were preceded by field potential 5-9 Hz oscillations, which were detected first in S2 and IC relative to S1. These oscillations were also recorded in NEC rats but did not trigger SWDs. In GAERS, SWDs could be triggered following a 2-s train of 7Hz electrical stimuli with a lower current intensity in S2 than in S1. In S2 and IC, subsets of neurons displayed rhythmic firing (5-9 Hz) in between seizures. During every cycle prior to the spike component of the SW complex, short-lasting high-frequency oscillations (~120 Hz) consistently occurred in IC ~20 ms before S1. S2 and IC layers V and VI neurons fired during the same time window, while in S1 layer VI neurons fired before layer V neurons. These findings demonstrate the presence of precursor cellular and network rhythmic activities in S2 and IC cortices during the generation of absence-related SWDs. These important findings tell us exactly where CBZ should be applied to probe its cortical effect on SWDs.

These findings have led to the preparation of a manuscript that has just been submitted:

Title: Precursor rhythmic neuronal activities in secondary somatosensory and insular cortices during the initiation of genetically determined absence-related spike-and-wave discharges

Authors: Zheng TW, Morris MJ, Jovanovska V, van Raay L, Gandrathi AK, Reid CA, O'Brien TJ and Pinault D

5) Local application of CBZ in S2 suppresses absence seizures

CBZ was microinjected, in separate experiments, into S2, the adjacent S1 and remote cortical areas (primary motor) of GAERS. We obtained evidence for a region selective effect of CBZ in seizure suppression within S2. These data provide further evidence that S2 is likely a region involved in seizure initiation and elucidate a potential target region for future pharmacotherapy for absence epilepsy. In sum, CBZ aggravated absence seizures when administered systemically and also focally (microinjection) into the somatosensory thalamus, but in contrast suppressed seizures when applied into S2. This finding is interesting since CBZ is usually used against focal epilepsies. However, further studies are required to better understand the paradoxical findings we observed in GAERS after intrathalamic and intracortical CBZ micro-injection. So far, the present

recordings provide an indirect argument in favor of the cortical focus theory in the initiation of absence-related SWDs.

6) CBZ increases sleep spindles in the somatosensory thalamus

Here we performed cellular, LFP and EEG recordings to investigate the effect of CBZ on normal thalamic network and cellular activity in lightly anesthetized NEC rats. Cortical EEG and thalamic LFP recordings revealed that the incidence of spindles was remarkably increased after systemic injection of CBZ. Thalamic spindle oscillations are generated in the thalamus (Contreras et al., 1997) and an electrophysiological marker of $GABA_A$ receptor mediated activities. Furthermore, CBZ also increased thalamic 5-9 Hz oscillations, which are also in part mediated by $GABA_A$ receptor-mediated IPSPs and which are generated in the somatosensory cortex (Pinault, 2003). Of importance, spindle oscillations occurred either in isolation, or immediately after a few 5-9 Hz waves, but were never followed by 5-9 Hz oscillations. The recorded LFP oscillations and their changes induced by systemic injection of CBZ were perfectly correlated with cellular firing patterns and membrane potential oscillations (rhythmic hyperpolarizations) in TC and TRN neurons.

These results strongly indicate that CBZ contributes to the drowsiness side effects, which are to all appearances due to an enhancement of $GABA_A$ receptor activities at least in the TC system.

Of importance, an independent high-density EEG investigation revealed significant deficits in spindle oscillations in schizophrenic patients (Ferrarelli et al., 2007). My thesis work strongly suggests that the mood stabilizing action of CBZ operates in part through enhancement of $GABA_A$ receptors-mediated CNS activities, increasing especially TC spindle oscillations.

These findings have led to the preparation of a manuscript that is under preparation:

Title: Carbamazepine increases thalamic spindle oscillations

Authors: Zheng TW, Morris M, O'Brien TJ and Pinault D

Conclusion

CBZ is a widely prescribed anticonvulsant used for the treatment of focal epilepsy and psychiatric disorders. However it is also known for its broad spectrum of action on several molecular targets contributing to common and severe side effects. CBZ directly interacts with GABAA receptors, which play a critical role in the generation of physiological and pathological TC/CT oscillations.

My thesis work provides strong evidence that CBZ affects the firing and oscillation properties of thalamic neurons, at least in the somatosensory system, through enhancement of GABAA receptor-mediated activities, the likely mechanisms that underlie the aggravation of absence seizures. These CBZ-induced neuronal effects might also play a role in the mood stabilizing action of CBZ (bipolar disorders and schizophrenia).

The work presented in this thesis also provides several important leads to mechanisms underlying the initiation and propagation of absence-related SWDs. The present findings demonstrate the presence of precursor cellular and network rhythmic activities in S2 and IC during the generation of absence-related SWDs. Therefore it is tempting to put forward the assumption that S2 and IC cortical areas contain a critical circuit from which excitation spreads to interconnected S1, motor and more frontal cortical areas. This spreading caudo-rostral excitation might be a key neuronal mechanism in the initiation of absence seizures. To all appearances CBZ is effective in suppressing absence-related SWDs only when it is injected close to or at the presumed initiation site of this spreading caudo-rostral excitation.

It is also important to stress that we have demonstrated that brain rhythms can actually be used as electrophysiological markers to study the CNS impact of therapeutic substances. We have also demonstrated that combining pharmacological and multi-scales neurophysiological approaches is an excellent strategy to study the pathophysiological properties of complex neuronal systems, like those formed by the cortex and the thalamus.

References

Bourassa J, Pinault D, Deschenes M (1995) Corticothalamic projections from the cortical barrel field to the somatosensory thalamus in rats: a single-fibre study using biocytin as an anterograde tracer. Eur J Neurosci 7:19-30.

Contreras D, Destexhe A, Sejnowski TJ, Steriade M (1997) Spatiotemporal patterns of spindle oscillations in cortex and thalamus. J Neurosci 17:1179-1196.

Crunelli V, Leresche N (2002) Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci 3:371-382.

Ferrarelli F, Huber R, Peterson MJ, Massimini M, Murphy M, Riedner BA, Watson A, Bria P, Tononi G (2007) Reduced sleep spindle activity in schizophrenia patients. Am J Psychiatry 164:483-492.

Gloor P, Fariello RG (1988) Generalized epilepsy: some of its cellular mechanisms differ from those of focal epilepsy. Trends Neurosci 11:63-68.

Granger P, Biton B, Faure C, Vige X, Depoortere H, Graham D, Langer SZ, Scatton B, Avenet P (1995) Modulation of the gamma-aminobutyric acid type A receptor by the antiepileptic drugs carbamazepine and phenytoin. Mol Pharmacol 47:1189-1196.

Hosák L, Libiger J (2002) Antiepileptic drugs in schizophrenia: a review. Eur Psychiatry 17:371-378.

Meeren HK, Pijn JP, van Luijtelaar EL, Coenen AM, Lopes dSF (2002) Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci 22:1480-1495.

Pinault D, Leresche N, Charpier S, Deniau JM, Marescaux C, Vergnes M, Crunelli V (1998) Intracellular recordings in thalamic neurones during spontaneous spike and wave discharges in rats with absence epilepsy. J Physiol (Lond) 509 (Pt 2):449-456.

Pinault D (2003) Cellular interactions in the rat somatosensory thalamocortical system during normal and epileptic 5-9 Hz oscillations. J Physiol 552:881-905.

Pinault D (2004) The thalamic reticular nucleus: structure, function and concept. Brain Res Brain Res Rev 46:1-31.

Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S (2007) Deep layer somatosensory cortical neurons initiate spike-and-wave discharges in a genetic model of absence seizures. J Neurosci 27:6590-6599.

Vergnes M, Marescaux C, Micheletti G, Reis J, Depaulis A, Rumbach L, Warter JM (1982) Spontaneous paroxysmal electroclinical patterns in rat: a model of generalized non-convulsive epilepsy. Neurosci Lett 33:97-101.

Vergnes M, Marescaux C, Depaulis A (1990) Mapping of spontaneous spike and wave discharges in Wistar rats with genetic generalized non-convulsive epilepsy. Brain Res 523:87-91.

Wallengren C, Li S, Morris MJ, Jupp B, O'Brien TJ. (2005) Aggravation of absence seizures by carbamazepine in a genetic rat model does not induce neuronal c-Fos activation. Clin Neuropharmacol 28:60-65.

Zheng T, Liu L, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The mechanism of carbamazepine aggravation of absence seizures. J Pharmacol Exp Ther 319:790-798.

Zheng T, Clarke AL, Morris MJ, Reid CA, Petrou S, O'Brien TJ (2009) Oxcarbazepine, not its active metabolite, potentiates GABAA activation and aggravates absence seizures. Epilepsia 50:83-87.

Etude neurophysiologique et pharmacologique de la carbamazépine sur les oscillations thalamocorticales, physiologiques et pathologiques, dépendantes d'activités GABAergiques

Contexte

Les maladies neurologiques et psychiatriques, comme les épilepsies et les schizophrénies sont associées à des anomalies de synchronisation de rythmes cérébraux. Le néocortex et le thalamus sont très impliqués dans la conscience, la perception et la cognition. Ils sont réciproquement connectés et travaillent en tandem pour engendrer des rythmes physiologiques et pathologiques. Le thalamus est réciproquement connecté avec le noyau réticulaire thalamique (TRN) et avec le cortex cérébral. Les neurones thalamocorticaux (TC) et corticothalamiques (CT) sont glutamatergiques et traversent le TRN en y laissant des collatérales d'axones. Le TRN ne contient que des neurones GABAergiques qui innervent tous les noyaux du thalamus dorsal (Pinault, 2004).

L'épilepsie-absences, ou "Petit Mal", est un sous-type d'épilepsies idiopathiques généralisées avec des origines génétiques (Gloor et Fariello, 1988; Crunelli et Leresche, 2002). Les crises d'absence ne presentment pas de convulsions et sont caractérisées par une perte de la conscience, de la perception et une insensibilité à des stimuli sensoriels. L'électroencéphalogramme (EEG) du cortex cérébral révèle que les crises d'absence sont associées à des oscillations de haut voltage, généralisées et synchrones dans les deux hémisphères, appelées décharges de pointe-ondes (DPO). Ces DPO sont engendrées dans le système CT somatosensoriel (Vergnes et coll., 1990; Pinault, 2003). Des études réalisées sur le modèle génétique murin suggèrent que les DPO prennent leur origine dans un foyer cortical aux dimensions restreintes dans le cortex somatosensoriel (Meeren et coll., 2002; Pinault, 2003; Polack et coll., 2007). La nature de ce foyer reste indéterminée. C'est pourquoi dans mon travail de thèse nous avons étudié les dynamiques spatiotemporelles des activités neuronales pour mieux comprendre la théorie du foyer cortical dans la genèse des crises d'absences.

Les traitements médicaux pour les épilepsies-absences se basent principalement sur une pharmacotherapy qui supprime les crises. Cependant, dans beaucoup de cas, les médicaments antiépileptiques (AEDs) ne permettent qu'une suppression inadéquate des crises et sont souvent associés avec des effets contraires importants (Hosak and Libiger, 2002). Dans certains cas, les AEDs peuvent paradoxalement aggraver les crises. La carbamazépine (CBZ) et les molécules apparentées sont des AEDs généralement prescrits qui sont reconnus pour aggraver beaucoup de types de crises généralisées, dont les absences (Zheng et coll., 2006). La CBZ est aussi utilisée en psychiatrie comme un stabilisitateur de l'humeur et chez des patients schizophréniques (Hosak and Libiger, 2002).

Les mécanismes neuronaux sous-tendant les actions antiépileptique et stabilisatrice de l'humeur de la CBZ et de molécules apparentées sont inconnus. Des études on montré que la CBZ exerce une modulation allostérique positive sur les récepteurs $GABA_A$ (Granger et coll., 1995), et ses consequences neurophysiologiques restent inconnues. Dans le thalamus, les mécanismes neuronaux qui sous-tendent les DPO associées aux absences impliquent des potentiels post-synaptiques inhibiteurs (PPSIs) dépendant de récepteurs $GABA_A$ (Pinault et coll., 1998). C'est pourquoi, mon travail de thèse consistait à tester l'hypothèse selon laquelle l'aggravation des DPO associées aux absences est due à une augmentation des inhibitions dépendantes de récepteurs $GABA_A$.

Etudier in vivo l'impact neuronal de la CBZ permettra d'enrichir nos connaissances de la physiopathologie des systèmes TC, des épilepsies-absences et des propriétés thérapeutiques de molécules de type CBZ, avec l'espoir d'améliorer le traitement des patients en neurologie et en psychiatrie.

Hypothèse de travail

La CBZ est connue pour aggraver les crises d'absences dans les modèles génétiques murins d'épilepsie-absences. Sur la base de récentes évidences expérimentales, nous soutenons l'hypothèse que la CBZ augmente les activités engendrées par l'activation de récepteurs $GABA_A$ dans les systèmes TC et CT.

Objectifs majeurs

Ce projet s'intègre dans le cadre d'une collaboration franco-australienne, initiée en 2006 avec le Prof Terence J. O'Brien lors de son séjour à Strasbourg pour un congé sabbatique dans le laboratoire du Dr. Didier Pinault. Notre objectif était de comprendre, grâce à une stratégie neurophysiologique et pharmacologique, les mécanismes neuronaux sous-tendant l'initiation des DPO dans les modèles génétiques d'épilepsie-absences ainsi que leur modulation par des AEDs. Plus précisément, nous avons étudié les effets pharmacologiques et neurophysiologiques de la CBZ sur les oscillations TC/CT, physiologiques et pathologiques.

Nous avons testé notre hypothèse avec deux principales stratégies: 1) directement in vitro sur les récepteurs $GABA_A$ et 2) in vivo sur les oscillations TC, dépendantes du $GABA_A$., physiologiques et pathologiques.

Stratégie et méthodes

1) Dans le cadre de la cotutelle de thèse franco-australienne, j'ai travaillé pendant 3 ans dans le Department of Medicine, Royal Melbourne Hospital, The University of Melbourne (co-directeurs: Terence J O'Brien, Chris Reid, University of Melbourne, et Margaret Morris de l'University of New South Wales, avec une interaction régulière avec Didier Pinault, UdS) et une année à l'INSERM U666 (directeur : JM Danion), Faculté de Médecine, Université de Strasbourg (co-directeurs: Terence J O'Brien et Didier Pinault). Mon séjour dans le laboratoire de Didier Pinault a été soutenu par une bourse d'excellence Eiffel doctorat (2008).

2) Pour tester l'action de la CBZ directement sur les récepteurs $GABA_A$, nous avons utilisé une approche in vitro avec des oocytes de Xenopus exprimant des récepteurs $GABA_A$ recombinants.

3) Pour tester l'action de la CBZ in vivo sur les oscillations TC, physiologiques et pathologiques, dépendantes des récepteurs $GABA_A$, la CBZ a été micro-injectée, via des canules implantées, dans le thalamus somatosensoriel, et dans la région correspondante du TRN chez des rats

épileptiques libres de se mouvoir. Les effets ont été évalués grâce à des enregistrements EEG et des observations comportementales. La spécificité de l'impact sur les récepteurs $GABA_A$ a été étudiée grâce à une injection systémique de CBZ et une micro-injection intra-thalamique de bicuculline, un antagoniste des récepteurs $GABA_A$.

Nous avons utilisé des GAERS (Genetic Absence Epilepsy Rats front Strasbourg), un modèle reconnu d'épilepsie-absences (Vergnes et coll., 1982). Comme chez les humains, les DPO des GAERS débutent et se terminent de façon soudaine sur un EEG normal durant un état de veille calme éveillée qui est accompagnée au niveau comportemental par une immobilité, des tremblements des vibrisses et des muscles faciaux et l'absence de réponse à des stimuli sensoriels légers. Notre laboratoire a démontré précédemment une

aggravation des crises d'absence chez les GAERS par la CBZ (Wallengren et coll., 2005). Les rats contrôles non épileptique (NEC) sont dérivés de la même colonie de rats Wistar. Ces rats NEC ne présentent pas le phénotype épileptique comportemental et EEG.

4) Pour tester l'effet de la CBZ dans le cortex somatosensoriel, une difficulté majeure a été d'identifier la région ou la CBZ devait être appliquée. Les expériences réalisées sur les rats WAG/Rij, un autre modèle génétique d'épilepsie-absences reconnu, ont apporté de nouvelles évidences expérimentales soutenant l'hypothèse selon laquelle les DPO sont initiées dans une région restreinte du cortex somatosensoriel (Meeren et coll., 2002). De fait, il était important d'identifier chez les GAERS la région du cortex somatosensoriel impliquée dans l'électrogenèse des DPO associées aux absences. Nous avons donc effectué des enregistrements multi-sites de type cellulaire, multi-unitaire, potentiel de champ local (LFP) et EEG cortical chez des GAERS et des rats NEC, libres de leurs mouvements ou sous anesthésie légère. Des enregistrements intra et juxtacelluaires ont été réalisés avec des micropipettes de verre pointues contenant un traceur neuronal pour marquer les neurones enregistrés (Pinault, 1996).

5) Nous avons également évalué l'impact de la CBZ sur les oscillations thalamiques, plus particulièrement sur les fuseaux du sommeil, lesquels peuvent être enregistrés sous anesthésie, et lesquels impliquent des PPSIs dépendants de récepteurs $GABA_A$. Nous avons donc utilisé des

enregistrements LFP, intra et juxtacellulaire de neurones TC et TRN, simultanément avec un EEG du cortex correspondant (Pinault, 2003).

Résultats

1) La CBZ agit directement sur les récepteurs $GABA_A$, augmentant le courant des ions Chlore

Pour étudier l'interaction directe entre la CBZ et les récepteurs $GABA_A$, nous avons fait exprimer des récepteurs $GABA_A$ recombinés dans des oocytes de xenopus. La CBZ, à des doses thérapeutiques, potentialisait les courants chlore engendrés par des récepteurs $GABA_A$. Ces résultats démontrent que la CBZ interagit directement avec les récepteurs $GABA_A$, agissant très probablement comme un modulateur allostérique positif.

Ce travail a fait l'objet d'une publication:

Zheng TW, Liu L, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The Mechanism of Carbamazepine Aggravation of Absence Seizures. J Pharmacol Exp Ther 319: 790-798.

2) La CBZ module les récepteurs $GABA_A$ spécifiquement dans le thalamus somatosensoriel et aggrave les crises d'absence Pour tester la spécificité régionale de l'effet de la CBZ dans le thalamus, des EEG corticaux ont été réalisés sur des GAERS libres de se mouvoir après injection de la substance neuroactive. La CBZ aggravait les crises d'absence, après des injections systémiques ou intrathalamiques. Les injections bilatérales dans le thalamus somatosensoriel aggravaient les crises de façon dose-dépendante, alors que les injections dans le TRN n'avait aucun effet, indiquant la spécificité régionale de la CBZ. De plus, l'aggravation des DPO chez des GAERS induites par injection systémique de CBZ était complètement bloquée par microinjection bilatérale d'un antagoniste des récepteurs $GABA_A$, la bicuculline, dans le thalamus somatosensoriel. Ces résultats suggèrent que l'action aggravante de la CBZ sur les DPO implique, dans le thalamus somatosensoriel, des activités dépendantes de récepteurs $GABA_A$.

Ce travail a fait l'objet d'une publication:

Zheng TW, Liu L, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The Mechanism of Carbamazepine Aggravation of Absence Seizures. J Pharmacol Exp Ther 319: 790-798.

3) L'oxcarbazépine (OXC), un analogue de la CBZ qui potentialise les activités dépendantes de récepteurs $GABA_A$, aggrave également les crises d'absence ; mais son métabolite actif (MHD), qui n'a pas d'effet sur les récepteurs $GABA_A$, n'exercent aucun effet aggravant

Ici, nous avons testé l'hypothèse selon laquelle l'OXC, mais pas son métabolite actif monohydroxy-dérivé (MHD), aggrave les crises. Les effets d'injections systémiques d'OXC, de MHD et de CBZ ont été comparés chez des GAERS libre de se mouvoir. Comme prévu, l'OXC aggravait les crises chez des GAERS, des effets comparables à ceux observés avec la CBZ. Inversement, le MHD n'aggravait pas les crises. Cela démontre que la capacité à potentialiser les activités dépendantes de récepteurs $GABA_A$ est essentielle aux effets aggravants des crises d'absence de molécules CBZ-like.

Ce travail a fait l'objet d'une publication:

Zheng TW, Clarke AL, Morris MJ, Reid CA, Petrou S, and O'Brien TJ (2009) Oxcarbazepine, not its active metabolite, potentiates $GABA_A$ activation and aggravates absence seizures. Epilepsia 50:83–87.

4) Activités rythmiques de type précurseur dans les cortex somatosensoriel secondaire (S2) et insulaire (IC) durant l'initiation des DPO génétiquement déterminées et associées aux absences

Pour déterminer l'impact de la CBZ sur les DPO dans le cortex somatosensoriel, la CBZ doit être appliquée à un endroit précis, étant donné que ce cortex sensoriel est une aire très vaste. L'origine intracorticale des DPO, bilatérale et synchrone, qui accompagnent les crises d'absences reste un sujet très débattu. C'est pourquoi nous avons caractérisé les activités neuronales intérictales, préictales et ictales dans les régions corticales primaire et secondaire (S1, S2), et dans le IC, une région corticale adjacente, chez des

GAERS en utilisant des enregistrements multi-sites, cellulaire et plus global. Les DPO sont précédées par des oscillations à 5-9Hz dans le LFP, lesquelles apparaissaient plus tôt dans S2 et IC par rapport à S1. Ces oscillations ont également été enregistrées chez des rats NEC mais ne déclenchaient pas de DPO. Chez des GAERS, les DPO pouvaient être déclenchées par un train (2 s) de stimuli électrique (7Hz), avec un courant d'intensité plus faible dans S2 par rapport à S1. Dans S2 et IC, des sous-populations de neurones présentaient une décharge rythmique (5-9Hz) entre les crises. Dans chaque cycle d'une DPO, de brèves oscillations à haute fréquence (~120 Hz) survenaient régulièrement dans le IC environ 20ms avant S1. Les neurones des couches V et VI de S2 et IC déchargeaient durant la même fenêtre temporelle, alors que les neurones de la

couche VI dans S1 déchargeaient avant la couche V. Ces faits démontrent la présence d'une activité rythmique précurseur au niveau cellulaire et des circuits correspondants, dans S2 et IC, Durant l'électrogenèse des DPO associées aux absences. Cette découverte importante nous indique exactement où la CBZ devait être appliquée pour déterminer son effet sur les DPO au niveau cortical.

Ces résultats ont fait l'objet d'un manuscrit qui vient d'être soumis:

Titre: Precursor rhythmic neuronal activities in secondary somatosensory and insular cortices during the initiation of genetically determined absence-related spike-and-wave discharges

Autheurs: **Zheng TW,** Morris MJ, Jovanovska V, van Raay L, Gandrathi AK, Reid CA, O'Brien TJ and Pinault D

5) Une application locale de CBZ dans S2 supprime les crises d'absence

La CBZ a été micro-injectée, dans différentes expériences, dans S2 et S1 (aires corticales adjacentes), et ans une aire corticale plus éloignée (moteur primaire) chez les GAERS. Nous avons mis en évidence un effet sélectif au niveau régional de la CBZ dans la suppression des crises dans S2. Ces données soutiennent que S2 est probablement une région impliquée dans l'initiation des crises et mettent en relief une région-cible potentielle pour de futures thérapies pharmacologiques, pour les épilepsies-absences. En résumé, la CBZ aggrave les crises d'absence quand elle est administrée de façon systémique, et elle supprime les crises quand elle est appliquée localement dans S2. Cette découverte est intéressante car la CBZ est utilisée

régulièrement contre les épilepsies focales. Cependant, des études supplémentaires sont nécessaires pour une meilleure compréhension des résultats paradoxaux que nous avons obtenus avec les micro-injections intrathalamique et intracorticale chez des GAERS. Jusqu'à ce jour, nos enregistrements présentent des arguments indirects en faveur de la théorie du foyer cortical dans l'initiation des DPO associées aux absences.

6) La CBZ augmente les fuseaux du sommeil dans le thalamus somatosensoriel

Nous avons réalisé des enregistrements cellulaires, LFP et EEG pour étudier l'effet de la CBZ sur les activités thalamiques normales, au niveau cellulaire et global, chez des rats NEC légèrement anesthésiés. Des enregistrements EEG corticaux et LFP thalamiques ont révélé que la fréquence des fuseaux était remarquablement augmentée après injection systémique de CBZ. Les fuseaux thalamiques sont engenders dans le thalamus (Contreras et coll., 1997) et sont un marqueur électrophysiologique d'activités dépendantes de récepteurs $GABA_A$. De plus, la CBZ augmente également les oscillations 5-9Hz thalamiques, qui sont en partie due aux PPSIs causés par l'activation de récepteurs $GABA_A$, et qui sont engendrées dans le cortex somatosensoriel (Pinault, 2003). Les fuseaux thalamiques survenaient soit de façon isolée, soit immédiatement après quelques ondes à 5-9Hz, mais n'étaient jamais suivis d'oscillations à 5-9Hz. Les oscillations enregistrées et leurs changements induits par l'injection systémique de CBZ étaient parfaitement corrélées avec les patrons de décharges cellulaires et les oscillations du potentiel de membrane (hyperpolarisation rythmique) de neurones TC et TRN.

Ces résultats indiquent fortement que la CBZ contribue aux effets secondaires de somnolence, qui sont à priori dus à une augmentation des activités sous-tendues en partie par l'activation de récepteurs $GABA_A$, au moins dans le système TC.

Il est important de dire qu'une étude indépendante utilisant des enregistrements EEG de haute densité a révélé un déficit significatif des fuseaux du sommeil chez des patients schizophrènes (Ferrarelli et coll., 2007). Mon travail de thèse suggère fortement que l'action stabilisatrice de l'humeur de la CBZ est due en partie à l'augmentation des activités du SNC dépendantes de récepteurs $GABA_A$, augmentant surtout les fuseaux TC du sommeil.

Ces nouvelles données font l'objet d'un manuscrit en cours de préparation:

Titre: Carbamazepine increases thalamic spindle-like oscillations

Autheurs: **Zheng TW**, Morris M, O'Brien TJ and Pinault D

Conclusion

La CBZ est un anticonvulsivant largement prescrit, utilisé dans le traitement des épilepsies focales et de troubles psychiatriques. Cependant, il est connu que son large spectre d'action sur différentes cibles moléculaires contribue à des effets secondaires communs et sévères. La CBZ interagit directement avec les récepteurs $GABA_A$, qui jouent un rôle critique dans l'électrogenèse d'oscillations TC/CT physiologiques et pathologiques.

Mon travail de thèse offre des arguments solides pour dire que la CBZ module les propriétés de décharge et d'oscillations des neurones thalamiques, au moins dans le système somatosensoriel, au travers d'une augmentation des activités dépendantes de récepteurs $GABA_A$ Ce mécanisme semble être le plus vraisemblable dans l'aggravation des absences. Ces effets de la CBZ expliqueraient son action stabilisatrice de l'humeur (troubles bipolaires et schizophrénies).

Le travail présenté dans cette thèse offre aussi d'importantes clés pour comprendre les mécanismes soustendant l'initiation et la propagation des DPO liées aux absences. Ces résultats mettent en évidence la présence d'activités de type précurseur dans S2 et IC durant l'électrogenèse des DPO. Il est donc tentant de mettre en avant l'hypothèse selon laquelle les aires corticales S2 et IC forment un circuit critique à partir duquel une excitation se propage dans des aires corticales interconnectées : S1, des aires corticales motrice et plus frontales. La propagation de cette excitation caudo-rostral pourrait être un élément neuronal clé dans l'initiation des crises d'absences. La CBZ est efficace chez les GAERS pour supprimer les DPO relatives aux absences uniquement lors d'une injection proche ou au niveau du site d'initiation présumé de cet embrasement excitateur caudo-rostral.

Il est également important de souligner que nous avons démontré que les rythmes cérébraux peuvent être utilisés comme marqueurs électrophysiologiques pour étudier l'impact de substances thérapeutiques sur le SNC. Nous avons également démontré que la combinaison d'approches pharmacologiques et neurophysiologiques multi-échelles est une excellente stratégie pour étudier

les proprieties physiopathologiques de systèmes neuronaux complexes, tels que ceux formés par le cortex et le thalamus.

References

Bourassa J, Pinault D, Deschenes M (1995) Corticothalamic projections from the cortical barrel field to the somatosensory thalamus in rats: a single-fibre study using biocytin as an anterograde tracer. Eur J Neurosci 7:19-30.

Contreras D, Destexhe A, Sejnowski TJ, Steriade M (1997) Spatiotemporal patterns of spindle oscillations in cortex and thalamus. J Neurosci 17:1179-1196.

Crunelli V, Leresche N (2002) Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci 3:371-382.

Ferrarelli F, Huber R, Peterson MJ, Massimini M, Murphy M, Riedner BA, Watson A, Bria P, Tononi G (2007) Reduced sleep spindle activity in schizophrenia patients. Am J Psychiatry 164:483-492.

Gloor P, Fariello RG (1988) Generalized epilepsy: some of its cellular mechanisms differ from those of focal epilepsy. Trends Neurosci 11:63-68.

Granger P, Biton B, Faure C, Vige X, Depoortere H, Graham D, Langer SZ, Scatton B, Avenet P (1995) Modulation of the gamma-aminobutyric acid type A receptor by the antiepileptic drugs carbamazepine and phenytoin. Mol Pharmacol 47:1189-1196.

Hosák L, Libiger J (2002) Antiepileptic drugs in schizophrenia: a review. Eur Psychiatry 17:371-378.

Meeren HK, Pijn JP, van Luijtelaar EL, Coenen AM, Lopes dSF (2002) Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci 22:1480-1495.

Pinault D, Leresche N, Charpier S, Deniau JM, Marescaux C, Vergnes M, Crunelli V (1998) Intracellular recordings in thalamic neurones during spontaneous spike and wave discharges in rats with absence epilepsy. J Physiol (Lond)
509 (Pt 2):449-456.

Pinault D (2003) Cellular interactions in the rat somatosensory thalamocortical system during normal and epileptic 5-9 Hz oscillations. J Physiol 552:881-905.

Pinault D (2004) The thalamic reticular nucleus: structure, function and concept. Brain Res Brain Res Rev 46:1-31.

Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S (2007) Deep layer somatosensory cortical neurons initiate spike-and-wave discharges in a genetic model of absence seizures. J Neurosci 27:6590-6599.

Vergnes M, Marescaux C, Micheletti G, Reis J, Depaulis A, Rumbach L, Warter JM (1982) Spontaneous paroxysmal electroclinical patterns in rat: a model of generalized non-convulsive epilepsy. Neurosci Lett 33:97-101.

Vergnes M, Marescaux C, Depaulis A (1990) Mapping of spontaneous spike and wave discharges in Wistar rats with genetic generalized non-convulsive epilepsy. Brain Res 523:87-91.

Wallengren C, Li S, Morris MJ, Jupp B, O'Brien TJ. (2005) Aggravation of absence seizures by carbamazepine in a genetic rat model does not induce neuronal c-Fos activation. Clin Neuropharmacol 28:60-65.

Zheng T, Liu L, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The mechanism of carbamazepine aggravation of absence seizures. J Pharmacol Exp Ther 319:790-798.
Zheng T, Clarke AL, Morris MJ, Reid CA, Petrou S, O'Brien TJ (2009) Oxcarbazepine, not its active metabolite,
potentiates $GABA_A$ activation and aggravates absence seizures. Epilepsia 50:83-87.

Declaration

This is to certify that

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,
ii. due acknowledgement has been made in the text to all other material used,
iii. the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Signed…….Date…….

Publications

Manuscript arising from this thesis

1. **Zheng T.W.,*** Liu L.,* Morris M.J., Wallengren C., Clarke A.L., Reid C.A., Petrou S., and O'Brien T.J. "The Mechanism of Carbamazepine Aggravation of Absence Seizures." J Pharmacol Exp Ther. 2006 Nov; 319 (2):790-8. (*Co-first author)

2. **Zheng T.W.,** Clarke A.L., Morris M.J., Reid C.A., Petrou S. and O'Brien T.J. "Oxcarbazepine not its Active Metabolite MHD potentiates $GABA_A$ Receptor Activation and Aggravates Genetic Absence Seizures in the Rat." Epilepsia. 2009 Jan;50(1):83-7

3. **Zheng T.W.,** Morris M.J., Jovanovska V., van Raay L., Gandrathi A.K., Reid C.A., O'Brien T.J. and Pinault D. "Precursor rhythmic neuronal activities in secondary somatosensory and insular cortices during the initiation of genetically determined absence-related spike-and-wave discharges". Submitted for publication

4. **Zheng T.W.,** Reid C.A., Morris M.J., O'Brien T.J. and Pinault D. "Carbamazepine increases thalamic spindle oscillations". Submitted for publication

5. **Zheng T.W.,** Morris M.J., Reid C.A. and O'Brien T.J. "Cortical-area selective block of genetically determined absence seizures by carbamazepine." Submitted for publication

Conference Proceedings

Oral Presentations

1. **Zheng T.W.,** Liu L., Wallengren C., Morris M.J., and O'Brien T.J. "Carbamazepine Aggravates Absence Seizures in GAERS Rats Within the Ventrobasal Thalamus via a $GABA_A$ Mediated Mechanism." Annual Meeting, Australian Neuroscience Society, Sydney, 2006

2. **Zheng T.W.,** Clarke A.L., Morris M.J., Reid C.A., Petrou S., O'Brien T.J. "Modulation of $GABA_A$ Receptors by Carbamazepine." Annual Meeting, Epilepsy Society of Australia, Melbourne, 2006

3. **Zheng T.W.,** O'Brien T.J. and Pinault D. "Mechanisms Underlying Carbamazepine Modulation of Genetically Determined Absence Seizures" Annual Meeting, Australian Neuroscience Society, Canberra, 2009

Poster Presentations

1. **Zheng T.W.,** Liu L., Wallengren C., Morris M.J., and O'Brien T.J. "Carbamazepine Aggravates Absence Seizures in GAERS Rats Within the Ventrobasal Thalamus via a $GABA_A$ Mediated Mechanism." Annual Meeting, Australasian Society for Clinical and Experimental Pharmacologists and Toxicologists, 2006

2. **Zheng T.W.,** Morris M.J., Reid C.A. and O'Brien T.J. "Oxcarbazepine Aggravates Seizures in a Genetic Absence Epileptic Rat Model." Annual Scientific Meeting, Epilepsy Society of Australia, Melbourne, 2006

3. **Zheng T.W.,** Clarke A.L., Morris M.J., Reid C.A., Petrou S., O'Brien T.J. "Mechanism of Seizure Aggravation by Carbamazepine." 4th Federation of Asian-Oceania Neuroscience Society Congress, Hong Kong, 2006

4. **Zheng T.W.,** Morris M.J., Reid C.A. and O'Brien T.J. "Oxcarbazepine Aggravates Seizures in a Genetic Absence Epileptic Rat Model." 4th Federation of Asian-Oceania Neuroscience Society Congress, Hong Kong, 2006

4. **Zheng T.W.,** Morris M.J., Reid C.A. and O'Brien T.J. "Oxcarbazepine, but not its Active Metabolite, MHD, Aggravates Genetic Absences Seizures in the Rat and Potentiates $GABA_A$ Receptor Activation". Annual meeting, American Epilepsy Society, Philadelphia, 2007

5. **Zheng T.W.,** O'Brien T.J. and Pinault D. "Electrophysiological Characterisation of the Modulation of Cortico-thalamo-cortical Oscillations by Carbamazepine *in vivo*". 6th FENS forum of European Neuroscience, Geneva, 2008

6. **Zheng T.W.,** Morris M.J., Jovanovska V., van Raay L., Gandrathi A.K., Reid C.A., O'Brien T.J. and Pinault D. "The Intra-cortical Origin of Absence-like Seizures in the GAERS model is the S2 Region of the Somatosensory Cortex" Annual meeting, American Epilepsy Society, Boston, 2009

7. **Zheng T.W.,** Morris M.J., Jovanovska V., van Raay L., Gandrathi A.K., Reid C.A., O'Brien T.J. and Pinault D. "Precursor rhythmic neuronal activities in S2 somatosensory and insular cortices during the initiation of genetically determined absence-related spike-and-wave discharges" 8th Asian & Oceanian Epilepsy Congress, Melbourne, 2010

Awards and Scholarships

The thesis was supported by:

- Nancy Frances Curry Postgraduate Research Scholarship, Faculty of Medicine, University of Melbourne (2006-2008)

- Henry & Rachel Ackman Travelling Scholarship, Faculty of Medicine, University of Melbourne (2007)

- Travel Scholarship, Epilepsy Society of Australia (2007)

- Student Travel Award, Australian Neuroscience Society (2008)

- Eiffel Doctorate Scholarship, French Ministry of Foreign Affairs (2007/2008)

Preface

Understanding the functional mechanisms of the brain, both in health and diseases, presents one of the greatest challenges in biological sciences. Over the past decades, revolutionary research methods in electrophysiology, molecular biology and computational neuroscience led to a significant increase in the knowledge of our own state of consciousness, the mental processes by which we perceive and act, the pathophysiology underlying disorders of the brain and therapeutic approaches to treat these diseases.

The work described in this thesis investigated the fundamental mechanisms of normal and epileptic brain oscillations. More specifically, the aim of the research was to understand brain mechanisms underlying the paradoxical seizure aggravating effect of anti-epileptic drugs on the common, genetically based absence seizures, as well as the cellular and network mechanisms underlying seizure initiation. The thesis describes original experimental data and their interpretations from March, 2006 till September, 2010, unless otherwise stated.

Neuroscience research is a dynamic process involving multiple research scientists with complementary backgrounds and skills, sharing common research goals and exercising concerted physical and mental effect in designing, carrying out experimental procedures. This cotutelle thesis is part of a French-Australian collaboration between the laboratory of Professor Terence J. O'Brien, Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Australia and Dr Didier Pinault INSERM U666, Faculty of Medicine, University of Strasbourg, France.

Studies on the interaction between anti-epileptic drugs and $GABA_A$ receptors (part of Chapters 3 and 4) were completed in collaboration with the laboratory of Dr Steve Petrou, Centre for Neuroscience, University of Melbourne. Experiments were performed by Dr Alison Clarke.

The following experiments were also carried out by colleagues and collaborators:

- CBZ microinjection into VB and TRN (Chapter 3, section 3.3.1) was performed by Dr Lige Liu
- The multi-site depth EEG recording methods (Chapter 2, section 2.5) were developed and optimized by Ms Valentina Jovanovska
- Six animal surgeries and EEG recordings after cortical microinjection of CBZ (Chapter 7) was performed by Ms Leena van Raay
- LFP and juxtacellular recordings in the S2 and S1 region of GAERS were performed by Professor Terence O'Brien, in the laboratory of Dr Didier Pinault

This thesis would not have been possible without the collaborative effect of the colleagues and collaborators both in Melbourne and Strasbourg

Acknowledgements

The main setting for this research work has been at the Department of Medicine, University of Melbourne, Australia and INSERM U666, Faculty of Medicine, University of Strasbourg, France. The path towards this thesis spans several years of work since 2006, many people have been involved and contributed to the presented ideas and understanding gained. I am forever in debt to those who have helped along the way and influenced my understanding of the physiological mechanisms of the brain, pathophysiology of absence seizures and neuroscience research in general.

In particular, I wish to express my deepest gratitude to my four supervisors for their guidance, support, suggestions and criticisms:

Professor Terence O'Brien, Department of Medicine (RMH), The University of Melbourne; for providing the opportunity to complete this cotutelle thesis in both Melbourne and Strasbourg so that I have been able to see life and science research in their full depth; for leading by example with your energy, dedication and optimism.

Dr Didier Pinault, INSERM U666, University of Strasbourg, for teaching me everything I know about *in vivo* electrophysiology, for the frequent and stimulating scientific discussions, for your overwhelming support for me and my work during my stay in Strasbourg and beyond, and for telling me with every experiment that "today may be the experiment of the century".

Dr Chris Reid, Centre for Neuroscience, University of Melbourne, for your constant encouragement, the forever thought-provoking discussions, assistance with experimental set up, general advice in work and life. Your wisdom and knowledge inspired and motivated me.

Professor Margaret Morris, Department of Pharmacology, University of New South Wales, for your continued encouragement and invaluable advice since my undergraduate years, for showing me the attention to detail required in the field of neuroscience research.

I would also like to thank the following people for their assistance throughout my thesis work:

Dr Steve Petrou and Dr Alison Clarke, Centre for Neuroscience, The University of Melbourne, for their role in the collaborative work in designing and carrying out the voltage-clamp experiments in Xenopus oocytes.

Ms Valentina Jovanovska, Department of Medicine, The University of Melbourne, a.k.a. aunty Val, Thankyou for the technical assistance throughout my PhD and for your care, support and friendship.

Thank you to Ms Leena van Raay for your technical assistance and much needed encouragements over the latter part of my PhD.

I would also like to express my gratitude to Dr Chris French, Dr Kim Powell, Dr Nigel Jones and Dr Jeremy Kennard for many stimulating conversations, for your patience in answering my questions in your areas of expertise.

I am grateful to Ms Lucy Vivash and Ms Lisa Cardamone for reviewing parts of this thesis and for your support and friendship over the years.

To all past and present members of the O'Brien laboratory at the Department of Medicine, the University of Melbourne, thank you all for your invaluable support throughout my PhD. You have made our laboratory a dynamic, exciting and coherent place to work in.

To my colleague and friend Julien Gaudias, thank you for trying to teach me French, for assisting to translate my thesis summary to French, for introducing me to Picon and the SIG and for your assistance inside and out of the lab. I cannot thank you enough for your friendship, especially during the time I spent in Strasbourg.

I would also like to thank Ruo-Tian, Jiang, for assisting to translate my thesis summary to French, for our weekly gatherings and stimulating discussions in science and everything else under the sun, and for your friendship that led us to many parts of Strasbourg, Alsace and Europe.

On a personal note, I would like to thank my family:
To my parents whose unconditional love and support have enable me to get to where I am today. I could not have done this without you

To Sai, my soulmate, for your love and support, for being there for me every step of my journey.

Table of Contents

List of Tables and Figures

Abbreviations

AED	Anti-Epileptic Drugs	**JME**	Juvenile Myoclonic Epilepsy
AMPA	α-amino-3-hydroxyl-5-methyl-	**LFP**	Local Field Potential
	4-isoxazole-propionate	**M1**	Primary Motor Cortex
AP	Action Potentials	**MHD**	Mono-Hydroxy Derivative
BIC	Bicuculline	**NEC**	Non-Epileptic Control
CAE	Childhood Absence Epilepsy	**NMDA**	N-methyl-D-aspartic acid
CBZ	Carbamazepine	**OXC**	Oxcarbazepine
CDP	chlordiazepoxide	**PFA**	Paraformaldehyde
CT	Corticothalamic	**PTZ**	Pentylenetetrazol
CTC	Cortico-Thalamo-Cortical	**S1**	Primary Somatosensory Cortex
DMSO	Dimethyl Sulfoxide	**S1FL**	S1 Forelimb Region
EEG	Electroencephalogram	**S1po**	S1 peri-Oral region
EPSP	Excitatory Post-Synaptic Potential	**S1ULp**	S1 Upper Lip
ETX	Ethosuximide	**S2**	Secondary somatosensory Cortex
FPGE	Feline Penicillin Generalized Epilepsy	**s.c.**	subcutaneous
GABA	Gamma-Aminobutyric Acid	**SM**	somatosensory
GAERS	Genetic Absence Epilepsy Rats	**SWD**	Spike-and-Wave Discharges
	From Strasbourg	**TC**	Thalamocortical
HFO	High Frequency Oscillations	**THIP**	4, 5, 6, 7 tetrahydroxyisoxazolo
IC	Insular Cortex		(4,5,c) pyridine 3-ol
ILAE	International League Against Epilepsy	**TRN**	Thalamic Reticular Nucleus
i.p.	Intraperitoneal	**VB**	Ventrobasal

Chapter 1 Pathophysiology of Absence Seizures

1.1 General Introduction

The human brain is considered to be the most complex of all nature's creations. Understanding its functional mechanism presents the greatest of all challenges in biological sciences. Through increasing knowledge of the nervous system, medical research in neuroscience aim to improve diagnosis and therapy of many neurological or psychiatric diseases such as epilepsy, schizophrenia and Alzheimer's disease.

As one of the common chronic neurological disorders, absence seizures or "Petit Mal" occur in several forms of epilepsy with genetic causes (Gloor and Fariello, 1988; Crunelli and Leresche, 2002). Seizures are characterized by frequent and sudden loss of consciousness, perception and responsiveness for up to hundreds of times a day. Over the past decades, insights into the aetiology and the pathogenesis of absence seizures have been accrued from many human and animal research studies. However, the exact underlying neurophysiological mechanisms of absence seizures are not fully understood. The electroencephalogram (EEG) reveals that absence seizures are associated with generalized and bilaterally synchronous, high-voltage oscillations (~3-6 Hz), termed spike-and-wave discharges (SWDs). These absence-related SWDs are generated in the somatosensory system (Vergnes et al., 1990; Pinault, 2003). Studies conducted in genetic rodent models suggest that SWDs originate from a restricted focus in the somatosensory cortex (Polack et al., 2007), although the precise physiological nature and the pathological role of this postulated "focus" in absence seizures remain unclear.

Current medical treatment for absence seizures rely on anti-epileptic drugs (AEDs) which do not 'cure' the disorder and are not effective in seizure suppression in up to 50% of patients (Glauser et al., 2010). In many cases, AEDs are often associated with significant adverse effects, including the paradoxical aggravation of many seizure types (Chaves and Sander, 2005). Carbamazepine (CBZ) and CBZ-like drugs are commonly prescribed medication for the

treatment of focal seizures and as mood stabilizer in schizophrenic patients (Hosak and Libiger, 2002). They are recognized to aggravate many generalized seizure types, including absences (Chaves and Sander, 2005; Sazgar and Bourgeois, 2005). The neuronal mechanisms underlying the anti-epileptic and seizure aggravating actions of CBZ or CBZ-like drugs are unknown.

This thesis aimed to investigate the compelling and critical issues regarding the pathogenesis and pharmacotherapy of absence seizures raised above. The current chapter examines these issues in several ways and reviews the background and recent developments in relevant fields, expands on these themes and discusses the reasoning that led to the hypotheses and experimental approaches of the original research contained within this thesis.

1.2 The Brain and Neural Oscillations

The complex set of operations carried out by the brain underlies the biological basis of consciousness and mental processes by which we perceive, act, learn and remember. As the centre of the human nervous system, the brain monitors and regulates motor behaviour such as walking and running, as well as the more complex cognitive actions such as thinking, feeling and creating. The complexity of these behaviours is controlled by nerve cells, or neurons, which are anatomically organized into regions and circuits, each of them specialized for different functions. Neurons can be classified into three broad functional groups: 1) sensory neurons that are activated by physical modalities and convert these environmental stimuli into internal stimuli; 2) motor neurons that carry the demands of the brain to the relevant muscles fibres or glands; 3) interneuron that convey signals within brain regions or circuits. The integrated neural signaling between interconnected neuronal networks processes information and mediates behavioral responses.

Neural oscillations are a prominent feature of single neuron and network activity. They refer to periodic variations in neuronal activity in relation to the membrane potential of single neuron or a population of neurons. The phenomenon of neural oscillations and synchronization has been

extensively studied since the first electrical activity of the human brain was recorded using the EEG in 1924 by Hans Berger (Berger, 1929).

Using the EEG he was also the first to describe the different waves or rhythms which were present in the normal and abnormal brain, as well as EEG alterations during an epileptic seizure. It is now known that neurons within interconnected networks oscillate in synchrony to unite different features of an object (Singer and Gray, 1995). Synchronization of oscillations reflects the temporally precise interaction of neural activity and underlies mechanisms for neural communication between brain networks and regions (Schnitzler and Gross, 2005). Alterations in patterns of synchronization between interconnected neurons lead to dramatic changes in behaviour. Abnormal synchronization processes are known to be associated with several neurological, psychiatric and movement disorders including epilepsy, schizophrenia and Parkinson's diseases (Lopes da Silva, 1991; Hammond et al., 2007; Uhlhaas and Singer, 2010). Over the past decades, extensive research on neural oscillations has lead to important advances in our understanding of the functional significance of neural synchronization. However, the exact cellular and network mechanisms that generate physiological and pathological neural patterns remain a tantalizing mystery.

1.2.1 Physiological neural oscillations in the brain

Neural oscillations vary with activity, both in humans and other animals. It can be generated by environmental stimuli, electrical resonance of individual cells or intrinsic activity in recurrent networks. The mechanisms that translate single cell activity such as voltage or transmitter release into behavioural responses rely on the generation of specific types of oscillating neural rhythm. This repetitive neural activity can be recorded in the EEG, which acquires a summation of neuronal dendritic electrical events in real time and provides the possibility to analyze oscillatory brain activity. Several physiological oscillatory frequency bands can be found that range between 0.05 to 500Hz (Buzsaki and Draguhn, 2004). Studies over the past decades have described different types of oscillations based on their frequency, amplitude and instantaneous phase (Sauseng and Klimesch, 2008). These rhythms of different frequency show different neural

generators, functionality and are broadly catagorized as delta, theta, alpha beta and gamma (Table 1.1). Amplitude variations of the individual frequency band can selectively indicate sustained activation/deactivation in various sensory or cognitive modalities (Neuper and Pfurtscheller, 2001). Variations in the instantaneous phase of brain oscillations represent neuronal firing patterns with relation to time and indicate functional coherence between multiple brain regions. Neighbouring frequency bands within the same network can be overlapping and are typically associated with different brain states (Engel et al., 2001), while several rhythms can also co-exist within the same network to interact with each other (Steriade, 2001). The spatio-temporal balance of synchronization and desynchronization patterns in various frequencies is directly linked to physiological or pathological behaviour.

Oscillatory types	**Frequency (Hz)**	**Networks involved**	**Physiological Function**
Delta	Up to 4	neocortical, thalamocortical	cortical integration, attention, language
Theta	4-8	cortex, hippocampus, thalamus	code locations in space, memory
Alpha	8-13	cortex, thalamus	behavioural deactivation, perception, attention, memory
Beta	14-30	Cortex	motor activity, attention, cognition
Gamma	30-80	Cortex	sensory processes

Table 1.1 The origin and function of different types of neural oscillations (Basar et al., 2001).

1.2.2 Dynamic mechanisms that generate rhythmic oscillations

Neural oscillations emerge from a dynamic interplay of both intrinsic cellular mechanisms and larger scale network activities involving neuron clusters or more distant brain structures. At the single cell level, neurons generate action potentials (APs) which are predominant way of neuron to neuron communication and are underlain by changes in the membrane conductance to Na^+ and K^+ ions through their respective channels (Hodgkin and Huxley, 1945). APs are conducted down the cell's axon, where it initiates communication with other cells by stimulating the release of a chemical transmitter from the cell, facilitating responses from neighbouring neurons. In addition to APs, subthreshold membrane potential oscillations could also contribute to the rhythmic oscillatory activity by facilitating synchronous activity of interconnected neurons (Llinas, 1988). In local neuronal populations or network, voltage fluctuations detected from a group of neurons are known as local field potentials (LFPs). LFPs are essentially a measure of excitatory post-synaptic potential (EPSP) and inhibitory post-synaptic potential (IPSP).

The frequency of cellular and network oscillations may also depend on both cellular pacemaker mechanisms and neuronal network properties. Depending on neuronal types, network structures promote oscillatory activity at distinct and specific frequencies. Synchronization between large groups of neurons from adjacent and remote brain regions are observed throughout the central nervous system and play an important role in neurocognitive functions.

1.2.3 The cortico-thalamo-cortical (CTC) network as neural oscillators

In the CTC networks, the cortical and the thalamus interact to generate a variety of rhythmic oscillatory states that governs the information transfer in multiple neuronal pathways. It is thought that neural oscillations in the high frequency ranges (beta and gamma) establish precise synchronization within local cortical networks, whereas oscillations in the lower frequencies (theta and alpha) preferentially establish synchronization over longer distance (e.g. cortex to thalamus) (von Stein et al., 2000). In the perceptual system, neural oscillations have been hypothesized to be involved in functions such as somatosensory and odor perception, amongst many others. Changes in environmental stimuli lead to different subsets of neurons firing on

different sets of oscillatory cycles (Wehr and Laurent, 1996). Oscillations are also used as a neural mechanism for movement, temporal and cognitive control, which can be regulated by changes in the quantity of specific oscillatory frequency as well as coupling between different frequency bands(Buzsáki, 2006). Abnormalities in neural oscillations are implicated in various forms of pathology including epilepsy (Pinault et al., 1998; Pinault et al., 2006), schizophrenia (van der Stelt et al., 2004), Alzheimer's disease (Jeong, 2004) and attention deficit hyperactive disorder (Sukhodolsky et al., 2007). This thesis investigates the cellular and network mechanisms underlying absence epilepsy, a typical example of abnormal neural oscillation leading to pathological neural synchrony.

1.2.3.1 Anatomy of CTC Circuitry

Understanding of the anatomy and functioning of the CTC network is crucial in gaining further insight to the pathophysiological mechanisms in absence epilepsy. The term "CTC circuitry" involves the mutual interconnections between the thalamus and cortex (Jones, 1985). The thalamus is the major source of input to the cerebral cortex, relaying sensory information from the periphery. It involves many distinct nuclei defined by their different architecture, function and connections (Figure 1.1). Each of them receive afferents from one major functional source and send efferents to one major cortical area. The relay thalamus includes nuclei where the cerebral cortex receives most of its subcortical afferents (Sherman and Guillery, 1996). It receives a large amount of inhibitory innervations from the thalamic reticular nucleus (TRN) which resides within the ventral thalamus. The TRN is located on the pathway that links the relay thalamus and the cerebral cortex, receiving its main afferents from both structures. The somatosensory cortex encompasses multiple cortical areas including the primary (S1) and secondary (S2) somatosensory cortices. Both regions can be subdivided into different cytoarchitectonic areas with different functional roles (Fitzgerald et al., 2004; Tommerdahl et al., 2010). Like other areas of neocortex, the somatosensory cortex consists of cortical layers I to VI, each containing a characteristic distribution of neuronal types and connections to other cortical and subcortical regions.

The corticothalamic (CT) and thalamocortical (TC) neurons are mainly glutamatergic (Fonnum et al., 1981). Most TC axons terminate in the cortical layers IV and II before information is transmitted to other cortical areas via cortical-cortical interneurons (Jones, 1985). Reciprocally, the pyramidal cells from cortical layers V and VI feed back to the relay thalamus (Jones, 1985; Bourassa et al., 1995). In addition, all thalamic relay nuclei receive GABAergic inhibitory projections from the TRN, the primary source of GABA in the rat thalamus (Jones, 1985; Pinault et al., 1995; Cox et al., 1996). In contrast to the relay thalamus, the TRN provides no direct connection to the cortex and receives its main excitatory afferents from both TC and CT axons.

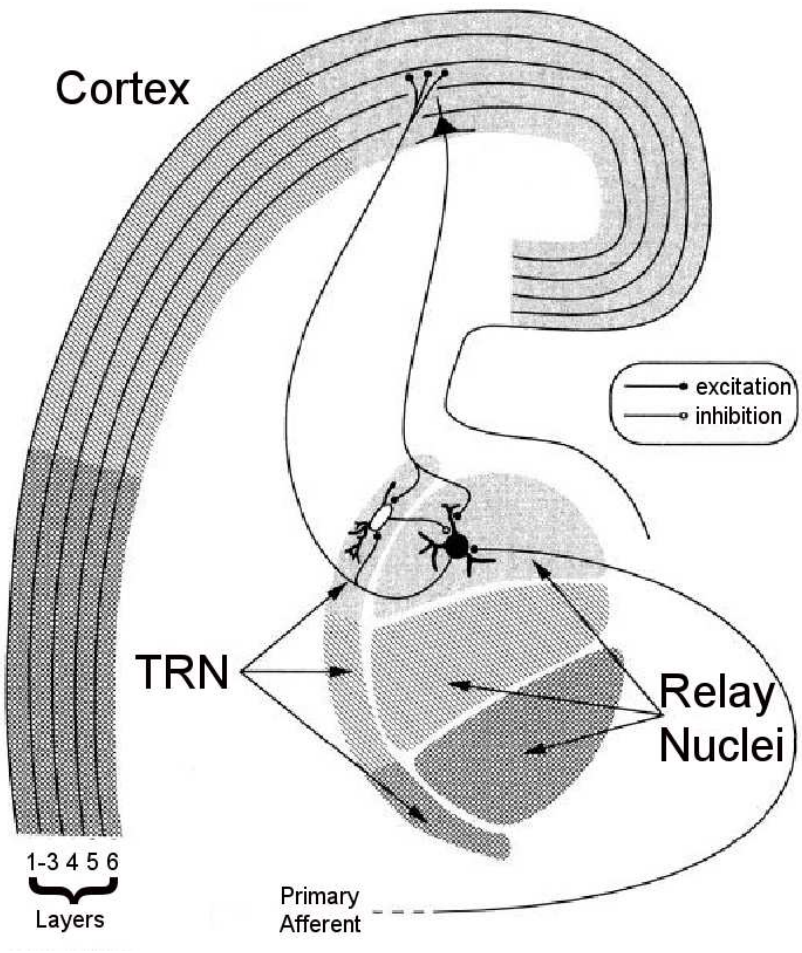

Figure 1.1 Schematic view of major components involved in TC interactions. Three relay nuclei of relay nuclei, three regions of the TRN, and three areas of cerebral cortex (cortex) are shown. Associated regions relay nuclei, TRN, and cortex share the same shading pattern. A primary afferent innervates relay cell on proximal dendrites, and relay the cell projects to layer 4 of cortex. As the axon of relay cell passes through TRN, it gives off a collateral that innervates reticular nucleus whose cells, in turn, project to relay cell. Axons from cortical cells in layer 6 innervate relay cells on their distal dendrites, and as these axons pass through TRN, they also provide collateral innervation of reticular cell. As indicated by key, all connections shown are excitatory except for the connection from the reticular cell to the relay cell, which is inhibitory. (Adapted from (Guillery, 1995))

1.2.3.2 Physiological oscillations within the CTC circuitry

The network connecting the cortex, relay thalamus and the TRN is concerned with almost all functional modalities including sensory, motor and limbic sectors. The thalamus was thought to be a relay station for sensory information gating and is now known to be endowed with intrinsic electrical properties that gave them specific functional dynamics. It is thought that spindles, delta and the slow oscillations (0.5 to 1 Hz) occur during slow-wave sleep, while the quicker beta and gamma oscillations are sustained during the active brain state of waking and rapid eye movement sleep (Steriade, 2006). The spindle oscillations are recognized to be generated from the thalamus and paced by the GABAergic neurons of the TRN (Fuentealba et al., 2004; Traub et al., 2005). Two components are involved in the generation of the sleep related delta waves. It is thought to be of a cortical nature as these rhythms are recorded in both the cortical pyramidal and inhibitory neurons without the involvement of the cortex (Steriade et al., 1993b). The thalamic component arises from the intrinsic properties of the TC neurons. They can be synchronized by CT volleys which activate TRN neurons to hyperpolarize the TC cells to adequate membrane potentials at which delta oscillations are generated (Steriade et al., 1991). In contrast, the faster beta and gamma rhythmicity occur at relatively depolarized membrane potential (Llinas et al., 1991; Steriade et al., 1996). They are thought to be generated and synchronized by CT neurons that are located throughout cortical layers 2-6, and are linked to other cortical and thalamic generators of these oscillations (Steriade et al., 1998).

The thalamus is traditionally thought to spontaneously exhibit two different modes of intrinsic oscillatory activity during normal physiological cycles of wakefulness and sleep (McCormick and Bal, 1997). In periods corresponding to wakefulness and arousal, the thalamus exhibit a *relay* of firing associated with fast, sodium/potassium mediated action potentials. This occurs when thalamic neurons are depolarized from resting membrane potential levels positive to -55 mV. This allows the sensory information to be relayed to the cortex where it is perceived and processed (Llinas et al., 1998). During stages of drowsiness or sleep, the thalamus can be switched from the relay mode into *oscillatory firing mode* by intra-thalamic, TC aminergic and cholinergic systems capable of modulating membrane and synaptic properties (McCormick and von Krosigk, 1992). As a result of membrane hyperpolarization to levels negative to -60 mV, the de-inactivation of a Ca^{2+} conductance triggers an inward current through T-type Ca^{2+} channels

(Chemin et al., 2002). If sufficiently prominent, this calcium current can become regenerative and activate Ca^{2+} -dependent spikes that activate high frequency bursts of Na^{+} dependent action potentials (Steriade and Deschenes, 1984). The hyperpolarization is thought to be mediated by the GABAergic inhibitory neurons in the TRN, which provides rhythmic, synchronous inhibitory postsynaptic potentials (IPSPs) to the thalamic neurons. This synchronous burst firing has been demonstrated to be reinforced by the glutamatergic projections from the cortex (Contreras and Steriade, 1996). Oscillatory bursts commonly occur as sleep spindles during slow-wave sleep, when the relay and the TRN cells are hyperpolarized, and rarely during wakefulness. Under these circumstances, sensory information from the periphery are not relayed to the cortex, and are therefore not perceived, resulting in reduced responsiveness of the organism during sleep or drowsiness. This can be overridden if the stimulations are strong and the firing pattern switches back to a tonic mode of firing.

It is now known that the electrophysiological behaviour of the relay and TRN cells can vary from one moment to the next (Steriade et al., 1986; Steriade et al., 1993a). The thalamic oscillatory mode can be further classified into the wake-related medium voltage synchronized oscillations and the sleep/drowsiness related spindle oscillations. TRN cells display long-lasting 5-9 Hz burst firing during medium voltage oscillations and short-lasting 11-16 Hz firing during spindle oscillations. These two firing patterns are easily distinguishable and are based on distinct cellular mechanisms (Pinault et al., 2006). The 5-9 Hz bursting is thought to be due to the synchronous functioning of the cortical pyramidal cells and inhibitory interneurons (Silva et al., 1991). A thalamic hyper-resonance to this physiological rhythm generated from the somatosensory cortex can give rise to the pathological, absence-related seizures (Pinault et al., 2001; Pinault, 2003).

1.3 Epilepsy

Epilepsy is one of the most prevalent brain diseases worldwide that can develop at any age, regardless of gender, social class, ethnic group or geographical boundaries. It is characterized by unprovoked recurring seizures as a result of abnormal electrical discharges from brain cells. Clinically, seizures can take form of non-convulsive, brief lapses of attention or prolonged convulsions involving violent and involuntary muscle contractions.

1.3.1 Epidemiology, Burden, types and causes of epilepsy

Epilepsy accounts for 1% of the global burden of all diseases, 85% of whom live in developing countries (Atlas, Epilepsy Care in the World, 2005). Recent study that described the prevalence of global life time epilepsy show that the estimated cases could be much higher than the figure of 50 million estimated by the World Health Organization (Ngugi et al, 2010). The median prevalence of the disease was 5.8 per 1000 people in the developed countries. This can increase to 15.4 per 1000 people in the rural areas of developing countries (Ngugi, et al 2010} due to inadequate medical treatment, poor socio-economic status and high incidence of trauma and infectious diseases affecting the brain. In addition to their epilepsy, more than 40% of individuals with epilepsy have one or more additional co-morbid neurological or psychiatric disorders (Boro and Haut, 2003). Conversely, up to half of patients with learning disabilities have seizure disorders (Lhatoo and Sander, 2001). People with epilepsy are also at increased risk for death from multiple causes including status epilepticus, sudden unexpected death in epilepsy (Walczak et al., 2001), trauma and suicide (Bell et al., 2009).

People with epilepsy commonly suffer social discrimination and stigma, and epilepsy has been considered as one of the most dreaded and misunderstood diseases throughout the history of mankind. For centuries, epilepsy was often view as being caused by divine punishment, witchcraft, poisoning or an infliction or possession by supernatural power (Jilek-Aall, 1999), especially when manifested as a convulsive seizure involving violent tremors of the body. In most cultures and religions, patients with epilepsy have been stigmatized, shunned and even imprisoned. Although epilepsy is now recognized as a physical illness, the discrimination and

social stigma surrounding epilepsy worldwide are difficult to overcome. The constant risk of becoming unconscious, sustaining injuries from falling and social embarrassment can lead to psychiatric conditions such as depression, anxiety and psychoses in people with epilepsy. They are often devalued and bear a heavy psychosocial burden which lead to lower income and decrease quality of life, lower school attendance and performance for children and difficulties in finding spouse. There is no doubt that epilepsy imposes a substantial burden on individuals affected by it, their families and communities worldwide.

1.3.2 Types and causes of epilepsy

Epilepsy broadly describes a large group of neurological syndromes and diseases that have a multitude of different manifestations and causes, all of which lead to spontaneous recurrent seizures. Effective classifications of epileptic syndromes are crucial for appropriate diagnosis, treatment and prognosis. Based on expert opinions, modern neuroimaging, genomic technologies and concepts in molecular biology, advances in our knowledge of epilepsy syndromes have resulted in evolving changes in their classification. The most recent report from the The International League against Epilepsy (ILAE) classified and organized epileptic seizures into three categories: generalized, focal or unclassified epileptic seizures.

Generalized epilepsies are now considered to "originate at some point within, and rapidly engage, bilaterally distributed networks" (ILAE, 2009). Seizures can involve both cortical and subcortical structures in both hemispheres but may be asymmetric and do not necessarily involve the entire cortex. Focal seizures are formally known as "localization related seizures" (1989) are now considered to originate within networks limited to one hemisphere, which may be discretely localized or more widely distributed. Seizures may originate from subcortical structures but may have more than one epileptogenic network. For each seizure type, ictal onset is consistent from one seizure to another with preferential propagation patterns which can involve a specific seizure origin from the contralateral hemisphere (ILAE, 2009). There are also other cases where patients can have both focal and generalized seizures in succession, rendering their classifications unclear.

All seizures are believed to be caused by a disruption in the balance between neuronal excitation and inhibition in the brain. (Scharfman, 2007). The underlying aetiology of the epilepsies is complex and has been traditionally classified as idiopathic, symptomatic or cryptogenic. As new information are acquired and new investigative technologies developed, a recent report from the ILAE has abandoned all three terms and redefined the concepts of underlying causes into the following three groups (Berg et al., 2010):

1) Genetically based form of epilepsy as a direct result of a known or presumed genetic defect(s) that result in recurrent seizures. Examples of these include childhood absence epilepsy, autosomal dominant nocturnal frontal lobe epilepsy and Dravet syndrome. There is often a family history of epilepsy and can sometimes affect entire families, although environmental factors can contribute to the expression of the diseases. Defects in genes encoding a number of voltage- or ligand gated ion channels are associated with epilepsy syndromes. Mutations of the sodium channels (Wallace et al., 1998), potassium channels (Biervert et al., 1998) and $GABA_A$ receptors (Baulac et al., 2001), among several others (Heron et al., 2007) have all been shown to be a contributing factor in seizure expression.

2) "Structural/metabolic" Seizures can often be a result of structural or metabolic changes following an insult to the brain. These patients have other conditions or diseases that are associated with increased risk of developing epilepsy. These may include head trauma, haemorrhagic/ischaemic stroke, brain tumours, parasitic infections or neurodegenerative diseases such as Hungtington's (Ullrich et al., 2004) and Alzheimers's disease (Hommet et al., 2007). Although the primary disease may involve genetic factor, epilepsy is a separate disorder that appears to be interposed between the genetic defect and resultant seizures. Environmental factor such as exposure to lead, carbon monoxide and other chemicals as well as recreational drugs and alcohol may also contribute to epilepsy.

3) Unknown cause. The nature of the underlying cause of epilepsy is unknown. It could be a result of a fundamental genetic defect or as a consequence of a separate, unrecognized disorder or a combination of these factors. Examples of these syndromes include migrating partial seizures of infancy and myoclonic epilepsy in infancy (Engel, 2006). Possible genetic factors are involved in some of these syndromes which increase the likelihood of seizure occurrence after an insult to the brain.

As such, there is no single disease termed “epilepsy”. Rather, it can be viewed as abnormal reactions of the brain or parts of the brain caused by a large number of diseases that result in recurrent seizures. Work described in this thesis investigates the underlying mechanisms of absence seizures — a common type of seizures seen in a number of genetic based epilepsy syndromes, in particular childhood absence epilepsy, juvenile absence epilepsy and juvenile myoclonic epilepsy.

1.4 Absence Seizures – An overview

Absence seizures are generalized non-convulsive seizures thought to be genetically determined and are neurophysiologically, pharmacologically and developmentally unique compare to other forms of seizures in many aspects (Berkovic et al., 1987). Absence seizures are integral part of several idiopathic generalized epilepsies including Childhood Absence Epilepsy (CAE) and Juvenile Absence Epilepsy (JAE) and are also seen in at least 30% of patients with juvenile myoclonic epilepsy (JME). CAE accounts for up to 10% of epilepsy in children, with girls at twice the risk than boys and occurs at between 4-10 years of age. Children with this condition are otherwise neurologically normal, with more than 40% of the cases remitting by adulthood. In JAE, the incidence and prevalence in the general population are not known. Seizures begin near or after puberty at between 10-17 years of age, both males and females are equally affected and there are a less likelihood of remission (1989; Crunelli and Leresche, 2002; Mattson, 2003). Both types of epilepsy are characterized by severe and frequent typical absence seizures of around 10 seconds and up to 200 episodes per day (Lennox and Lennox, 1960).

1.4.1 History and hallmarks of absence epilepsy

The first description of what is now known as generalized absence seizures was by Poupart in 1705, who stated in a report to the Académie Royal des Sciences: "At the approach of an attack the patient would sit down in a chair, her eyes open, and would remain there immobile and would not afterward remember falling into this state. If she had begun to talk and the attack interrupted her, she took it up again at precisely the point at which she stopped and she believed she had talked continuously." Similar clinical symptoms was later described and termed as petits accès or petits in the late 18th century by Tissot. Introduced by Calmeil in 1824 and Esquirol in 1838, the terms "absences" and "petit mal" (French word for "little illness") have been interchangeably used since the early 19th century. Prior to the advent of the EEG – a measure of electrical field potentials from various region of the scalp or brain, absences were believed to be non-epileptic (Fridmann 1906). Sauer introduced the term "pyknolepsy" (from the Greek word "pyknos", meaning crowded or densely packed) in 1916, as absence attacks tend to occur in clusters rather than randomly distributed throughout the day.

The introduction of EEG showed that absence seizures were associated with a bursts of bilaterally synchronous SWDs with an oscillation frequency of 3 Hz (range 2.5 – 4 Hz) lasting around 10 seconds (range 4 – 20s) (Gibbs et al., 1935). They consist of large amplitude slow waves of at least 200-300μV that alternate with single or double spikes. Scalp EEG suggest that seizures are of a bilateral, widespread distribution, with maximum amplitude in the front midline region (Ebersole and Pedley, 2003). Rhythmic SWDs arise from a normal, low amplitude, desynchronized background EEG, starts and ends abruptly, with slightly slower frequency towards the terminal phase of the seizure (Figure 1.2). The EEG returns to baseline state and patients resumes their on-going activity.

SWDs have an abrupt onset associated with a complete loss of awareness and responsiveness to environmental stimuli and cessation of on-going activity. Patients would commonly have a blank stare with or without eye rolling and/or mouth twitching. The attack lasts from a few seconds to half a minute, ending as abruptly as it has commenced. In contrast to generalized convulsive or partial seizures, absences leave no postictal depression. Patients are alert immediately afterwards and have no recollection of the seizure episodes.

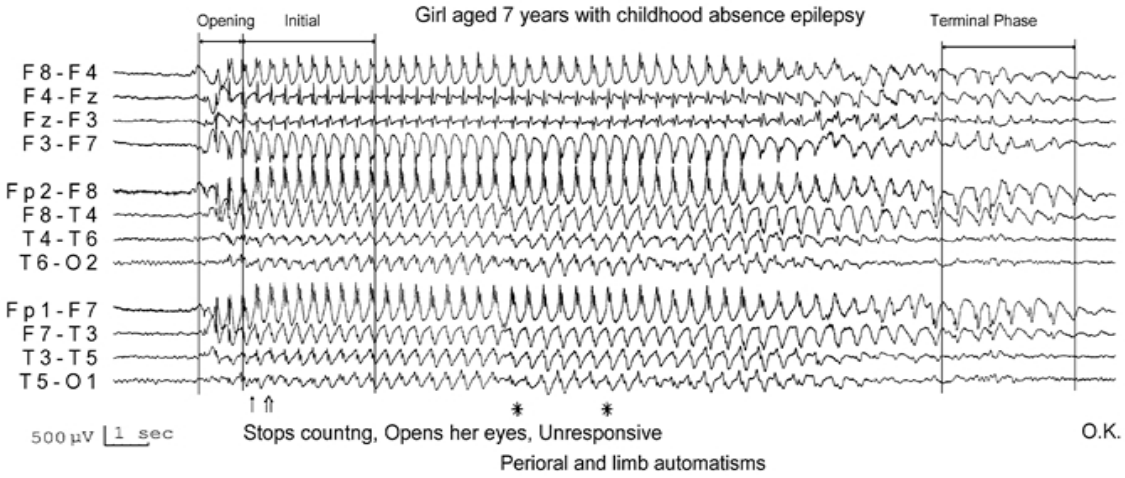

Figure 1.2 EEG traces showing a typical absence seizure episode from a 7 year old girl with childhood absence epilepsy. Seizures are accompanied by SWDs at 2-3 Hz. Seizure arised during hyperventilation with breath counting. She stopped counting (black arrow), opened her eyes (open arrow) and became unresponsive. Marked perioral automatisms occurred later (*). She abruptly recovered at the end of the discharge (O.K.). (Figure adapted from C. P. Panayiotopoulous, "The Epilepsies: Seizures, Syndromes and Management", Chapter 10, Figure 10.2)

1.4.2 Genetic causes of absence epilepsy

It is now well recognized that genetic abnormalities in both human and animal models of absence epilepsy is a predisposition to the expression of seizures (Scheffer and Berkovic, 2003). Studies in twins showed that typical absence seizures develop in 75% of monozygotic pairs of twins and 16 times less likely in the dizygotics (Lennox and Lennox, 1960). Despite decades of study, the precise mode of inheritance and the exact genes involved in seizure generation remains largely unidentified. Genome-wide scans have revealed several loci as potentially carrying genetic polymorphisms implicating ion channels and membrane receptors that render susceptibility to absence seizures (Crunelli and Leresche, 2002). A polymorphism in the GABRB3 in chromosome 15q11 was found to be associated with patients of 50 families with CAE, suggesting a possible involvement of the gene in the underlying pathophysiology (Feucht et al., 1999). Mutations of the CACNA1H gene in highly conserved residues of the T-type calcium channel have also been found in patients with CAE (Chen et al., 2003) which results in the expression of calcium channels with altered biophysical properties (Khosravani et al., 2004). However, conclusive evidence show that none of the mutations discovered so far independently account for the absence epilepsy trait (Robinson et al., 2002). The cause of epilepsy in most patients appears to be more complex and most likely to be polygenic with multiple genes involved (Steinlein, 2004).

1.5 Pharmacotherapy for absence epilepsy

1.5.1 Treatment of absence seizures in epileptic patients

The treatment for epilepsy is primarily by the administration of anti-epileptic medication aimed to prevent recurrent seizures which can lead to improved quality of life. Patients diagnosed with epilepsy are prescribed with one or more anti-epileptic drugs (AEDs) in attempt to prevent the onset and spread of seizures. However, current medication does not 'cure' epilepsy and seizures will recur if the medication is ceased. Furthermore, up to 50% of patients report inadequate seizure control or intolerable side effects despite carefully monitored treatments using multiple AEDs, resulting in increased morbidity and mortality from epilepsy (Glauser et al., 2010).

Over the past three decades, the main therapeutic agents for the treatment of absence epilepsy have been sodium valproate and ethosuximide. However, the mechanism of action by which these two drugs suppress seizures in unclear. A significant group of patients (up to 50%) have seizures that are refractory to current available treatment (Glauser et al., 2010).

1.5.2 Adverse effects of anti-epileptic drugs

AEDs are often grouped according to their presumed mechanisms of anti-epileptic action. The primary groups include "sodium channel blockers", "calcium channel inhibitors", "gamma-aminobutyric acid (GABA) inhibitors", "neuronal vesicle inhibitors", as well as those with unknown molecular targets. However, many AEDs may have several different actions on multiple receptors, and so classifying drugs into these discrete groups is largely a gross over simplification. As a result of their multiple cellular effects, AEDs may have many different adverse effects, which may be severe, including motor disturbance, sedation, memory deficit, weight gain/loss and paradoxically aggravation of many seizure types. The impairment of quality of life from these side effects is considered by patients to be at least as important as that due to recurrent seizures, and they contribute to a large proportion of AED treatment failures. The efficacy for many AEDs for control of seizures is equivalent, and the selection of an AED is often determined by the severity of side effects. However, the exact mechanisms of action for many AEDs are not well understood, their adverse effects from controlled studies are often

lacking. Uncovering the pharmacological mechanisms and defining risk factors for the development of adverse effects for AEDs remains a great challenge for improving epilepsy therapy.

1.5.3 Seizure aggravation by anti-epileptic drugs

A body of evidence have indicated that some AEDs used in certain epilepsies may frequently cause worsening of seizures in certain types of epilepsy syndromes, resulting in a paradoxical increase in the frequency and severity of existing seizures, emergence of new types of seizures or the occurrence of prolonged seizures (status epilepticus) (Berkovic, 1998; Cerminara et al., 2004). The ability of many AEDs to worsen seizures is often overlooked in clinical practice where they may not be identified as the causative agents (Perucca et al, 1998). The mechanisms underlying seizure aggravation is poorly understood but can be related to non-specific manifestation of drug toxicity, drug-induced encephalopathy, inappropriate choice of AED for a particular seizure type and paradoxical or inverse pharmacodynamic effect (Gayatri and Livingston, 2006). A better understanding of the mechanisms underlying seizure aggravation have important implications on many levels, including a better understanding of the underlying pathophysiology of absence seizures, guiding more targeted and rational drug design, and in allowing an improved clinical selection of AEDs for individual patients that minimises the likelihood of seizure exacerbation.

1.5.4 Absence seizure aggravation by carbamazepine and oxcarbazepine

One of the most common AEDs reported to aggravate seizures in clinical practice is carbamazepine (CBZ) – a first line treatment for the treatment of focal seizures, but also used to treatment neuropathic pain and as a mood stabilizer. The first report of seizure aggravation by CBZ was by Snead and Hosey, in children with complex partial seizures. All patients developed either atypical absences or generalised convulsive seizures following CBZ treatment (Snead OC 1985). Subsequent clinical reports, have confirmed that CBZ can cause an increase in the frequency and severity of typical and atypical absence seizures, as well as myoclonic, atonic and tonic seizures, and also on occasions generalised tonic-clonic seizures (Perucca 1998). In

particular, CBZ exacerbated seizures in those with high voltage generalized slow spike-and-wave (1-2 Hz) and exacerbated atypical absence seizures in those with bilaterally synchronous SWDs (2.5-3 Hz). Of these, the aggravation of absence seizures is the most predictable and, because of the availability of good animal models, the most amenable to scientific study (Snead 1985).

In agreement with human studies, CBZ has also been demonstrated to exacerbate pentylenetetrazol (PTZ) induced SWDs in mice after systemic administration (McLean et al., 2004). EEG quantification following the central injection of CBZ in the well-validated Genetic Absence Epilepsy Rats from Strasbourg (GAERS) (Wallengren et al., 2005) showed a significant increase in seizure duration, suggesting that CBZ is acting directly, rather than via a metabolite, in the brain to aggravate seizures.

The mechanism of seizure aggravation by CBZ is poorly understood. The anti-epileptic action of CBZ is believed to primarily occur due to a use dependant blockage of voltage-gated Na^+ channels, thus reducing the likelihood of burst firing of depolarised neurons (Willow et al., 1985). However, the CBZ is also seizure aggravation properties may occur via other neuropharmacological actions of CBZ. One of the less well known, but well documented, other actions of CBZ is an allosteric enhancement of $GABA_A$ receptor activity (Granger et al., 1995). CBZ known to interact with several other channels and receptors including K^+ channels, L-type Ca^{2+} channels and adenosine binding sites in the brain (Gasser et al., 1988; Schirrmacher et al., 1995). It is unclear whether these interactions may contribute to the anti or pro-epileptic properties of CBZ. Our group has proposed that aggravation of seizures may occur via a GABAergic effect of CBZ. If this were true, an analogue of CBZ that retains the Na^+ channel blocking properties, but not its GABAergic effects would therefore be a better therapeutic choice.

Oxcarbazepine (OXC) is one of the structural analogues of CBZ that was found to be equally effective in treatment of focal seizures, and generally accepted as a better tolerated drug than CBZ (Schmidt and Elger, 2004). It is now a commonly used AED in clinical practice world

wide. OXC is a keto analogue of CBZ, with an extra keto group on the central azepine seven-membered ring**.** In humans, OXC is rapidly metabolized to its monohydroxy derivative (MHD) via which it mediates most of its anti-epileptic effect (Schutz et al., 1986). The exact mechanism of action of OXC is unclear, although stereochemical comparisons indicated that OXC may utilize the same mechanisms as CBZ and phenytoin for its anti-convulsive properties, i.e. blockage of sodium channels. However, there is evidence that the spectrum of other pharmacodymamic neuronal effects, such as on high and low threshold activated calcium channels, do differ between these drugs (Stefani et al., 1995; Schmidt and Elger, 2004). OXC exhibits a pharmacological anti-convulsant spectrum and potency similar to that of CBZ, but with a lower frequency and severity of side effects. Recent clinical reports have indicated several cases where OXC also aggravated seizures in generalized type seizures, in particularly myoclonus and absences (Gelisse et al., 2004; Chaves and Sander, 2005; Sazgar and Bourgeois, 2005), although this anecdotally appears to possibly be less common than with CBZ. Whether OXC aggravates seizures in animal models of generalised epilepsy awaits further investigation.

1.6 Animal models of absence seizures and epilepsy

As absence epilepsy is not amenable to surgical treatment, invasive studies can not be done in humans for ethical reasons. Studies in animal models of the disease are essential for gaining valuable insights and generating hypotheses in the pathophysiological processes underlying absence epilepsy, as well as testing the efficacy of new AEDs. Over the past decades, studies from different animal models have produce contrasting findings, drawing divergent conclusions regarding the underlying pathophysiology of the disease. Despite the conflicting ideas, each animal model resembles different aspects of human epilepsy and seizure-related phenomena, providing varying insight into fundamental mechanisms underlying seizure generation. Animal studies include *in vitro* experiments on single cell preparations, cultures and acute brain slices, all of which provided valuable tools for exploring the cellular and molecular processes involved in the generation of a seizure. However, an epileptic seizure can only derive from neuronal networks that are topographically extensive, with reciprocal connections between adjacent and remote brain regions. *In vivo* studies in live animals with an intact brain remain the most important component in epilepsy research.

A valid animal model for absence epilepsy should meet a series of criteria that is representative of the human condition (Snead, 1995). These include: close correlation of EEG characteristics during seizures and associated behaviour, pharmacological profile of SWD suppression or exacerbation that mirrors the human condition, and the demonstration of SWDs in the cortex and thalamus, but not in the hippocampus. Table 1.2 lists some of the commonly used animal models for absence epilepsy, showing some common seizure characteristics. Many models of absence seizure and epilepsy have been described and can be divided broadly into two catagories: 1) models of seizures, where symptoms of the seizures are either chemically or electrically replicated in a non-epileptic animal; 2) models of epilepsy, where animals are selectively bred to be genetically predisposed to absence epilepsy.

Chemically induced animal model of absence epilepsy			
Model	Species	Onset and Seizure characteristics	SWD Frequency (Hz)
Penicillin	cat	Seizures begin 1 h after injection of 300 000 IU kg^{-1}, i.m.,and last 6–8 h	3.00
Low-dose PTZ	rat	20 mg kg^{-1}, i.p. produces bilateral, synchronous SWD from 3 weeks of age	7-9
THIP	rat	5–10 mg kg^{-1}, i.p. produces bilateral, synchronous SWD lasting 1–7 s	4-6
Genetic mouse models (single gene mutations)			
Model	Chromosomes and associated Gene product	Phenotype	SWD Frequency (Hz)
Lethagic (Ih/Ih)	Chromosome 2, gene encoding Ca^{2+} channel β_4-subunit	Lethargy and ataxia, focal motor seizures and cortical SWDs	5-7
Stargazer (stg/stg)	Chromosome 15, gene encoding the Ca^{2+} channel γ_2-subunit	ataxia, neck dystonia SWDs from cortex and thalamus	5-7
Tottering (tg/tg)	Chromosome 8, gene encoding the Ca^{2+} channel α_{1A}-subunit	Ataxia and motor seizures; cortical SWDs	5-7
Genetic rat models (Polygenetic)			
Model	Onset	Remission with age	SWD Frequency (Hz)
GAERS	SWDs start at 30–40 days; all animals show SWD at 13 weeks	no	7-12
WAG/Rij	SWD in all animals at 4 months	no	7-11

Table 1.2 Comparison of the characteristics of some common chemically induced and genetic animal models of absence epilepsy compared to absence seizures in children (Adapted and modified from (Pitkänen et al., 2006))

1.6.1 Animal models of absence seizures

The use of acute models of seizures has expanded our understanding of the cellular and molecular mechanisms in the generation and propagation of absence seizures. Seizures are induced in these models by maximal electric shock, or acute administration of a specific agent to an animal, usually a rat or a mouse. Examples of these seizure models include penicillin epilepsy (Fisher and Prince, 1977), low dose PTZ model (Marescaux et al., 1984) and the 4, 5, 6, 7 tetrahydroxyisoxazolo (4,5,c) pyridine 3-ol (THIP) model (Fariello and Golden, 1987). The systemic administration of these agents provokes acute bilaterally synchronous SWDs, usually within 5 minutes of the injection. SWDs are accompanied by behavioral arrest, facial myoclonus and vibrissal twitching. EEG recordings from these animals can reliably measure seizure duration, frequency and severity (Depaulis et al., 1989) for a limited period of time depending on the half-life of the compound. Most of the drug induced models exhibit similar pharmacological profile to that of the human condition: seizures are attenuated or abolished by ethosuximide and valproic acid and worsened by carbamazepine (Marescaux et al., 1984). The models are easily reproducible and results in virtually no mortality after seizure induction. Therefore, the pharmacological models are useful tools in studying the neuropathological changes associated with absence epilepsy as well as screening putative antiepileptic drugs for their anti-absence seizure efficacy.

1.6.2 Animal models of absence epilepsy

While the chemically induced models are similar in the expression of seizure phenotype, they do not model the underlying pathology of absence epilepsy which is essentially genetically acquired. The lack of spontaneous recurrent seizures in these models does not allow us to study the development of the disease. Unlike the induced seizure models which may have pharmacologically altered neurotransmitter systems, genetic models that manifest spontaneous recurrent seizures can be argued to more closely reproduce the state of chronically recurrent spontaneous seizures observed in the human condition. Spontaneous genetic mutations in mice have provided tools in identifying the gene defects and key molecular mechanisms that lead to the epileptic phenotype. Examples of a few commonly studied mouse mutants include lethargic, stargazer and totterer mice (Table 1.3). However, most genetic mouse models are of single gene

mutations, and are also associated with other neurological abnormalities which may limit their usefulness for behavioural or physiological studies of absence epilepsy.

The observation of spontaneous occurrence of SWDs in untreated rats lead to the development and characterization of two genetic rat models of absence epilepsy: 1) the Genetic Absence Epilepsy Rats from Strasbourg (GAERS), an inbred strain of Wistar rats developed in France (Vergnes et al., 1982); 2) the Wistar Albino Glaxo kept in Rijswijk (WAG/Rij) (van Luijtelaar and Coenen, 1986). Both rat strains show similar seizure characteristics, physiological and pharmacological profile to that of the human condition. Studies using these models over the past 20 years using multiple approaches dramatically improved our understanding of the underlying pathophysiology of absence epilepsy.

1.6.3 The Genetic Absence Epilepsy Rats from Strasbourg (GAERS)

GAERS is a well validated rodent model of absence epilepsy originated in the Centre of Neurochemistry in Strasbourg, France, where thirty percent of the Wistar rats from the initial breeding colony presented bilaterally synchronous SWDs on EEG recording (Vergnes et al., 1982). Animals that displayed SWDs were selectively inbred resulting in a strain in which all animals exhibit absence-type seizures accompanied by SWDs. A control strain free of spontaneous SWDs were bred and maintained from the same Wistar colony and was named non-epileptic control (NEC) rats.

As in humans, SWDs in GAERS start and end abruptly on a normal EEG background during quiet wakefulness. This is accompanied by behavioural immobility, rhythmic twitching of the vibrissae, facial muscles and unresponsiveness to mild sensory stimuli. SWDs in well developed SWDs from adult animals are typically around 9 Hz, last for 17 ± 10s and occur 1.3 times per minute on average. The SWD frequency of 9 Hz in GAERS are faster than that seen with human absence seizures (approximately 3 Hz.), but this difference is thought to represent a species-dependent difference (Snead, 1978). Similar to the human condition, SWDs can be interrupted by strong and unexpected sensory stimulations or during performance of various motivated tasks

(Vergnes et al., 1991). Spontaneous activity, exploration, feeding, social interactions or learning of positively or negatively reinforced tasks are not impaired in GAERS (Vergnes et al., 1991), although they show an elevated level of anxiety and depressive behaviour compared to the NECs (Jones et al., 2008). The seizures in GAERS commence in early adolescence (30-40 days post-natal), are fully expressed by approximately 4 months and persist for the lifetime of the animal. The ontogeny of the epilepsy therefore is more analogous to Juvenile Absence Epilepsy (JAE) than Childhood Absence Epilepsy (CAE). The pharmacological response of the seizures in GAERS is very similar to that of the human absence epilepsies. Seizures are inhibited by common anti-absence drugs such as ethosuximide and valproate (Micheletti et al., 1985) and exacerbated by carbamazepine (Wallengren et al., 2005). Because most characteristics of absence seizures in GAERS are highly reminiscent of human absence seizures, it has been used as a reference for studying the mechanisms underlying SWDs and is used for the studies in this thesis.

1.7 Anatomical and functional mechanisms underlying absence seizures

1.7.1 Historical views of absence seizures

Since the 1940s, contrasting theories concerning the pathogenesis of SWDs have been proposed. It was first hypothesized that by Jasper and Kershman who analyzed the EEG from patients with absence seizures and found that seizures were characterized by abrupt onset of highly synchronous spike-wave activity that are detected simultaneously from both hemispheres (Jasper and Kershman, 1941). The authors concluded that seizures must originate from a subcortical pacemaker with widespread and diffuse projections into the cortex. This was later termed as the "centrencephalic" theory (Penfield, 1952). Consistent with this theory, studies by Buzsaki recorded rhythmic SWD like events in the intrathalamic network without the involvement of the cortex, and concluded that the rhythmic discharges occur as a result of emergent properties of the relay thalamus and the nucleus reticularis network (Buzsaki, 1991). Although the thalamic hypothesis was well supported at the time, separate experimental evidence resulted in the introduction of the "cortical theory" by Gibbs and Gibbs, who suggested that SWDs are generated within the cortex depending on diffuse cortical processes (Gibbs and Gibbs, 1952). In their experiments in patients with absence epilepsy, injection of the convulsive drug PTZ into the carotid artery, supplying the cortex, produced generalized SWDs (Bennett, 1953). No response was observed when PTZ was injected into the vertebral artery which supplied the diencephalon and brain stem (Bennett, 1953). In the corticoreticular theory first proposed by Gloor et al, (Gloor, 1968), critical roles in the genesis of seizure discharges were assigned to both the cortex and the thalamus. In the "feline penicillin generalized epilepsy" (FPGE) model that was considered a model for human primary absence epilepsy (Avoli et al., 1983), SWDs was produced after diffuse cortical application of penicillin but not in the thalamus. It was later elaborated that SWDs occur as a result of an increase in the excitability of the cortex, which then produces, through corticofugal projections, excitation of the GABAergic reticular neurons, which in turn, induces a prolonged postsynaptic inhibition of the TC neurons (Steriade, 1995). Based on the FPGE cat model, sleep spindles and SWDs are found to share many common cellular features (van Luijtelaar and Coenen, 1986; Drinkenburg et al., 1991). Oscillatory nature of the SWDs further prompted the hypothesis that the thalamic circuits underlying SWDs are the same as those generating spindles and that spindles give rise to SWDs (Gloor, 1968; van

Luijtelaar, 1997). The TRN was proposed to be a pacemaker for both spindles and SWDs (Avanzini et al., 1992). However, this theory does not explain the bilateral and widespread generalization of SWDs. Furthermore, rhythmic discharges from the FPGE model occurs as a result of chemically induced cortical excitability. Therefore, it is arguable whether the same mechanism that generates TC rhythmicity in this model may also apply to the generalization of the human absence epilepsy. SWDs in humans and genetic rat models, which are related to quiet wakefulness, do not occur during the same state of vigilance as sleep spindles. Recent accumulating evidence supports the theory that SWDs develop from the cortically generated sensorimotor oscillations and not from the thalamically generated sleep spindles (Pinault et al., 2006), with the thalamus playing an important role in the synchronization of the cortical rhythm (Meeren et al., 2002; Pinault, 2003).

In contrast to the conventional view of generalized seizures, in which seizures are thought to arise simultaneously from a wide area of the brain from both hemispheres (1989), recent experiments in the genetic models of absence epilepsy lead to the proposal of the "cortical focus" that resides within the cortex from which seizure activity generalizes over the cortex and subsequently, the thalamic regions. Using planar multi-site EEG recordings from the rat cortical surface and non-linear association analysis in the WAG/Rij rats, Meeren and colleagues reported that the focus was located in S1 where nose and upper lip are represented (Meeren et al., 2002). In the GAERS model, EEG mapping have demonstrated that SWDs consistently recorded from lateral frontoparietal cortex and the posterolateral thalamus but never from the hippocampus or in any limbic structures (Vergnes et al., 1987, Vergnes, 1990 #250, Marescaux, 1992 #54). Paired cortical and thalamic juxtacellular recordings revealed that S1 layer VI neurons play a leading role in the generation of SWDs since they triggered rhythmic synchronized activities in related TC and TRN neurons (Pinault et al., 2001; Pinault, 2003). In addition, the local cortical injection of the anti-absence drug ethosuximide in the GAERS suppressed SWDs, whereas injection in to the thalamus had little effect (Manning et al., 2004), further demonstrating the leading role of the cortex in the generation of absence seizures. However, the intracortical location of the postulated seizure generator remains unknown. Although the site of the primary dysfunction in absence epilepsy is still widely debated, all experimental studies on animal

models agree that absence seizures are generated in a circuitry that involves the cerebral cortex and the thalamus.

1.7.2 Absence related SWD generation in the CTC circuitry in GAERS

Understanding the pathophysiology underlying absence epilepsy poses a great challenge as patients show no anatomical brain abnormalities within the CTC network. In GAERS, mapping of the seizure network found that SWDs of maximal amplitude were recorded in the sensorimotor cortex (Vergnes et al., 1990). It was subsequently demonstrated that SWDs develop from medium voltage 5-9 Hz oscillations, a naturally occurring somatosensory cortical rhythm that emerges from a desynchronized EEG (Pinault et al., 2001). Short lasting 5-9 Hz oscillations (<3 seconds) are recorded interictally in GAERS as well as in the NEC rats, the control inbred strain from the same original colony that does not exhibit SWDs, indicating that it is in itself not sufficient for the generation of SWDs (Pinault et al., 2001). Both the 5-9 Hz oscillations and SWDs can only occur when the animal remain in quiet immobile state during wakefulness (Vergnes et al., 1982) and can be interrupted by sensory stimuli (Pinault et al., 2001). These rhythms are now thought to be sensorimotor rhythm corresponding to 7-12 Hz oscillatory neural activity in awake rats, which appears first in the S1 cortex and later in the relay thalamus (Nicolelis et al., 1995; Fanselow et al., 2001). They are often accompanied by small amplitude negative spike components (<0.5 mV) which may be precursors to the negative spike of the SWDs (Pinault et al., 2001). SWDs evolve from these oscillations, probably as a result of genetically mediated dysfunction in the TC network.

1.7.2.1 Cellular and network mechanisms of absence seizures

Single cell recordings from GAERS also support the notion that SWDs arise from cortical rhythms. During SWDs, CT neurons in layer VI of the somatosensory cortex phase lead the TRN and TC cells by an average of 7ms (Pinault, 2003). These cells are glutamatergic and innervate both TC and TRN cells (Bourassa et al., 1995). They have thin axons and give rise to a large number of terminals dispersed in large thalamic territories and in slabs within the TRN. This would allow large numbers of thalamic cells to be recruited on each SW cycle. On the basis of these electrophysiological and morphological data, layer VI CT cells are thought as leaders in the generation, synchronization and spread of SWDs to other cortical regions and the thalamus.

Since absence seizures are thought to arise from cortical rhythms, the generation of SWDs has also been hypothesized to be related to excessive cortical neuronal excitability. This has been demonstrated in a number of animal models: During SWDs in the FPGE model, rhythmic neuronal firing first occurred in the cortex followed by the thalamus 2 or 3 cycles later (Avoli et al., 1983). In another model of absence seizures, the stargazer mice, layer V cortical pyramidal neurons were shown to display an increased excitability (Di Pasquale et al., 1997). This could be due to an increase in expression of the transmembrane AMPA receptor Regulatory Protein (TARPs), stargazing, also found in the somatosensory cortex of GAERS (Powell et al., 2008). Indeed, neurons in layers V and VI of the SM cortex in GAERS show a higher firing rate before and during seizures, as well as an enhanced bursting activity compared to NEC rats (Polack et al., 2007). This is likely a result of the excitatory NMDA receptor hyperfunction that has been reported in both the GAERS and WAG/Rij rats (Pumain et al., 1992; D'Antuono et al., 2006). The changes in both NMDA and AMPA receptors could contribute to cortical hyperexcitability in the epileptic animals and increase the likelihood of switching cortical activity from non-rhythmic state to the more pro-epileptogenic, rhythmic state. However, the cellular and network mechanisms that transform the physiological oscillations into pathological hypersynchrony remain unknown.

During SWDs in GAERS, TRN and TC neurons have a propensity to fire in a synchronous, phase-locked manner, milliseconds after the cortical AP (Pinault, 2003). With every cycle of the

SW complex, both cell types exhibit depolarization followed by a short lasting hyperpolarization (Pinault et al., 1998). The CT cells trigger simultaneous excitatory post-synaptic potentials (EPSPs) both the relay thalamus and the TRN, the summation of which triggers low-threshold Ca^{2+} potentials, resulting in a high-frequency burst of APs (Pinault, 2003).

As SWDs are initiated, background membrane oscillations in the thalamic relay neurons are replaced by rhythmic 5-9 Hz oscillatory activity, superimposed on tonic hyperpolarization (Pinault, 2003). In these neurons, each cycle consist of rhythmic occurrence of a depolarizing wave followed by a hyperpolarizing wave. The depolarizing wave includes a barrage of EPSPs which may trigger low-threshold Ca^{2+} spikes leading to bursts of APs. The hyperpolarizing wave is mediated by Cl^- conductance caused by TRN induced $GABA_A$ receptor mediated inhibitory post-synaptic potentials (IPSPs) (Pinault and Deschenes, 1998; Pinault, 2003). In the TRN cells, each cycle of the SWDs are associated with a long lasting hyperpolarization which may then potentiate the de-inactivation of T-type Ca^{2+} channels results in the generation of rhythmic burst firing at the onset of a SW cycle (Tsakiridou et al., 1995) Both the relay and TRN neurons, recurrent depolarizing wave can be attributed to low-threshold Ca^{2+} potential as a result of a summation of depolarizing potentials (Slaght et al., 2002). Both neuron types discharge in synchrony about 12 ms before the spike component of the SWD complex, suggesting that both cell types are driven by a common cortical input (Pinault et al., 2001).

1.7.2.2 SWDs as a result of CTC hypersynchrony

As described previously, SWDs occur as a result of hypersynchrony of rhythmic oscillatory resonance between the cortex and the thalamus. The cellular and network mechanisms underlying the transition between physiological somatosensory oscillations to hypersynchronized SWDs remain poorly understood. SWDs have a sudden onset from normal desynchronized EEG background, although studies have suggested that the pre-ictal period just preceding (<5 sec) the occurrence of SWDs may represent a transitional state where the CTC network is switched from normal background activity to rhythmic SWDs (Inouye et al., 1990). The processes involved in the generation of SWDs include: 1) cellular and network activity that generate physiological sensorimotor rhythm and 2) a cellular or network trigger that transforms normal oscillations into

pathological SWDs. It is likely that both processes involve widespread alterations in neurotransmission in the epileptic individual that predispose them to CTC hypersynchonization associated with absence epilepsy.

On the basis of current knowledge, a cellular scenario for the generation of SWDs may be as follows: 1) cells within layer VI of the somatosensory cortex maintains the 5-9 Hz physiological oscillation (Pinault et al, 2001); 2) the TRN cells resonates with the cortical neurons at the same frequency and generate $GABA_A$ dependent IPSPs in their target relay cells (Pinault et al 2003); 3) the relay cells are hyperpolarized by the IPSPs and trigger low threshold Ca^{2+} currents in a subset of cells (Pinault et al 2003), leading to rhythmic AP bursts; 4) AP discharges reinforces the reciprocal interactions between the relay and the TRN neurons and recruits new units at both the cortical and thalamic level (Bal and McCormick, 1993).

1.7.3 Role of GABAergic transmission in absence seizures

A large body of literature has examined the role of neurotransmitters in the CTC circuitry in the generation of absence seizures. TC and CT projections are mainly glutamatergic and excess activation of NMDA receptors may contribute to SWD generation. Several intracortical and intrathalamic GABAergic projections have been describe and believed to play a pivotal role in the generation of rhythmic TC activity and SWDs. Mutations in the neuronal T-type Ca2+ channels have also been demonstrated to contribute to the expression of SWDs (Powell et al., 2009). Of these, enhancement of GABAergic activity has been demonstrated to be a common pathophysiological mechanism in several genetic and pharmacological models of absence epilepsy (Cope et al., 2009).

The thalamus receives inhibitory projections from the TRN which is exclusively composed of GABAergic inhibitory neurons (Houser et al., 1980). Different types of GABAergic receptors are involved in the IPSPs recorded in the relay thalamus. Reciprocal connection between the TC and the TRN is thought to control the synchronisation of TC oscillations through the activation of the ionotrophic $GABA_A$ receptors (Wang and Rinzel, 1993). Activation of the metabotrophic

$GABA_B$ receptors can also contribute to rhythmic activity by causing membrane hyperpolarisation which can de-inactivate the low-threshold Ca^{2+} channels (Crunelli and Leresche, 1991). In GAERS, the cellular correlates of the SWDs in the thalamus have been demonstrated to involve rhythmic sequences of an EPSP followed by an IPSP, which is mediated by $GABA_A$ receptors (Pinault, 2003). A recent study have also demonstrated an increase in $GABA_A$ receptor dependent 'tonic' inhibition in the thalamic neurons due to compromised GABA uptake in a number of genetic and pharmacological absence seizure models (Cope et al., 2009).

A series of pharmacological studies have also highlighted the pivotal role of GABAergic transmission in the regulation of absence seizures. Drugs such as vigabatrin and tiagabine, both of which increase the level of GABA in the brain, exacerbate both clinical and experimental absence seizures (Hosford and Wang, 1997). Drugs acting directly at both $GABA_A$ and $GABA_B$ receptors have profound effect on absence seizures: intraperitoneal (i.p.) injection of the $GABA_A$ agonists such as muscimol and THIP induced a dose dependent increase in the duration of SWDs in several animal models of absence seizures (Meldrum and Horton, 1980; Vergnes et al., 1984; Marescaux et al., 1992a). Similarly, the systemic or central injection of R-baclofen, a $GABA_B$ agonist increased SWDs GAERS (Marescaux et al., 1992a). Interestingly, $GABA_A$ receptor agonists injected centrally have variable effects on seizures depending on the site of administration. Injection of the $GABA_A$–mimetics into the thalamic relay nuclei aggravated seizures, while the bilateral application of $GABA_A$–mimetics into the TRN suppressed SWDs (Liu et al., 1991). Furthermore, then systemic and thalamic injection of both $GABA_A$ and $GABA_B$ receptor agonists induced paroxysmal rhythmic oscillations the resemble SWDs in non-epileptic animals (Fariello and Golden, 1987; Marescaux et al., 1992a). The pattern of activation of GABA receptors in the thalamus is therefore, critically important in the generation and regulation of physiological and pathological thalamic oscillations.

1.8 Rationale, aims and Hypotheses

1.8.1 Rationale of the study

Over the past decades, studies in animal models of absence epilepsy have significantly advanced our understanding of the neurophysiology of absence seizures. While it is well known that the interplay between the thalamus and cortex is crucial for the generation of SWDs, the cellular and network properties that generate pathological hypersynchrony is not well understood. The clinical and experimental observation of the paradoxical aggravation of absence seizures by AEDs raises some interesting research questions. As CBZ is a widely prescribed anti-focal seizure drug that aggravates generalized type seizures, investigating the seizure aggravating mechanisms of CBZ and its analogues will not only be clinical relevant in minimizing the likelihood of seizure aggravation, but also provide new insights into the pathophysiological processes important for generation and maintenance of absence seizures. In this thesis, I ask the following questions:

- In what region of the CTC circuitry does CBZ act to aggravate seizures?
- How are SWD initiated and propagated from the relevant regions?
- What are the cellular and network mechanisms underlying seizure aggravation by CBZ?

Answers to these questions will improve our understanding of the fundamental mechanisms underlying absence epilepsy, as well as unveiling novel therapeutic strategies to minimize the likelihood of seizure aggravation. A better understanding of the cellular and molecular mechanisms that trigger SWDs will also provide leads to the pathophysiological mechanisms underlying a range of illnesses characterized by CTC dysrhythmia.

1.8.2 Primary Hypothesis

The primary hypothesis tested in this thesis is that the paradoxical CBZ-mediated exacerbation of absence epilepsy is via direct activation of $GABA_A$ receptors within the thalamus which acts to promote a pathological hyper-resonant and synchronized response to normal TC rhythms, thereby aggravating the tendency of these to transform into SWDs and absence seizures.

1.8.3 Specific Aims and Outline of Research Plan

The work of this thesis is part of a French-Australian collaboration between the laboratory of Professor Terence J O'Brien (Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Australia) and the laboratory of Dr Didier Pinault (INSERM U666, Faculty of Medicine, University of Strasbourg, France). Within the frame of a cotutelle thesis, I have worked for 3 years in Melbourne and 1 year in Strasbourg.

Utilizing neurophysiological and pharmacological strategies, the major goal of this project is to understand the neuronal mechanisms underlying the initiation and propagation of SWDs and their modulation by AEDs, specifically CBZ and related compounds. Using the GAERS model, I investigated the effect of CBZ and its analogues on physiological and pathological CTC oscillations.

The thesis aimed to test the following specific hypotheses:

1 a) CBZ aggravation of absence seizures is due to the potentiating the activation of thalamic $GABA_A$ receptors

This part of the study aimed to identify the neuroanatomical site of action of CBZ within the CTC circuitry and to explore the neuropharmacological mechanisms underlying seizure aggravation. CBZ was injected systemically or centrally via implanted cannulae, into the VB thalamus and the related TRN in freely-moving GAERS. The effects were evaluated using EEG recordings and behavioural observations. The specificity of the impact on $GABA_A$ receptors was

investigated using systemic injection of CBZ and intra-thalamic microinjection of bicuculline, a $GABA_A$ receptor antagonist.

1 b) CBZ interacts directly with $GABA_A$ receptors

To test the action of CBZ directly on $GABA_A$ receptors, we used an *in vitro* approach in Xenopus oocytes expressing human recombinant $GABA_A$ receptors

2) That structural analogues of CBZ may differ in their effect on $GABA_A$ receptors and therefore their propensity to aggravates seizures in GAERS

This series of experiments tested whether OXC and its active metabolite MHD differed from CBZ in their potency of effects on $GABA_A$ receptor function and their propensity to aggravate seizures in GAERS.

3) CBZ modulates physiological TC oscillations via $GABA_A$ receptor mediated mechanisms

In vivo experiments were conducted to investigate the impact of CBZ administration on thalamic oscillations, in particular on TC spindle oscillations, which can be recorded under anesthesia, and which prominently involved $GABA_A$ receptor-mediated IPSPs. For this we used LFP, intra- and juxtacellular recordings of TC and TRN neurons along with the EEG of the related cortex (Pinault, 2003).

4) SWDs are triggered by 5-9 Hz frequency oscillatory rhythms that arise within a localized region of the somatosensory cortex

Experiments conducted in WAG/Rij rats, a well-established genetic model of absence epilepsy, have yielded findings supporting the hypothesis that SWDs are initiated in a restricted region of the somatosensory cortex (Meeren et al., 2002). Therefore, it was important to identify in GAERS the cortical region in the somatosensory cortex that is critical in the electrogenesis of

absence-related SWDs. We conducted multi-site cellular, multiunit, local field potential (LFP) and cortical EEG recordings in free-moving and lightly anesthetized GAERS and NEC rats. Intra- and juxtacellular recordings were performed with sharp glass micropipettes that contained a neuronal tracer to label the recorded neurons (Pinault, 1996).

5) Localised injections of CBZ in the cortical region from which the pre-cursor somotosensory 5-9 Hz rhythm is generated will suppress, rather than aggravate, absence seizures in GAERS

It is hypothesized CBZ is acting to aggravate absence seizures by promoting a hyper-resonant and hyper-synchronized response of the thalamus to normal TC rhythms. Therefore the focal injection of CBZ into the somatosensory cortex from where the precursor 5-9 Hz rhythmic is postulated arise would be expected to result in the suppression of seizures in GAERS, rather than aggravation. To test this hypothesis CBZ was microinjected into the S2, S1 and motor cortex, in separate cohorts of freely moving GAERS and the effect on seizure expression measured.

Chapter 2 General Materials and Methods

2.1 Study Design

The current studies were designed to investigate the neuronal mechanisms underlying the initiation and propagation of SWDs. More specifically, the effects of the anti-focal and pro-absence drug CBZ on physiological and pathological CTC oscillations are investigated. To test the series of hypothesis previously outlined in Chapter 1, section 1.7.3, the studies can be divided into two major categories based on strategies used: 1) to test *in vitro*, the direct effect of CBZ and its analogues on $GABA_A$ receptors; and 2) to investigate *in vivo*, the effect of CBZ on physiological and pathological oscillations.

The key aim for the *in vitro* study was to investigate whether there is a direct interaction between CBZ (and its analogues) and $GABA_A$ receptors. This part of the study was completed in collaboration with Dr Steve Petrou, Centre for Neuroscience, University of Melbourne. Experiments were carried out by Dr Alison Clarke from the same research unit. Human recombinant $GABA_A$ receptors were expressed in Xenopus oocytes, GABA induced Cl^- currents were measured using voltage-clamp techniques with and without the presence of CBZ, OXC and MHD. The drug doses in the study were based on the cerebral spinal fluid (CSF) concentration of the drugs in human patients chronically treated with the drug, as well as previous studies in animal models of epilepsy.

The *in vivo* part of the study, the GAERS model of absence epilepsy and its NEC counterparts were utilized. This study can be further divided into two categories of experiments:

1) EEG recordings and behavioural observation in freely moving GAERS. This series of experiments is completed at my home institution, Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Australia, in the laboratory of Professor Terence J. O'Brien. In these experiments, EEG and/or microEEG recordings were performed via chronically implanted electrodes in multiple brain regions in freely moving rats. CBZ, OXC and MHD were

injected at multiple doses either systemically or via chronically implanted cannulae to test their effect of seizure activity in GAERS. Seizures from the recording periods following drug injections were quantified for data analysis.

2) Multisite cellular, multiunit, local field potential (LFP) and cortical EEG recordings in lightly anaesthetized, immobile GAERS and NECs. This series of experiments were performed at INSERM U666, Faculty of Medicine, University of Strasbourg, France, in the laboratory of Dr Didier Pinault. In this laboratory, I was able to perform single-unit and multi-unit recordings between the principal elements of the seizure associated network along with the global EEG. Using advance surgical and electrophysiological techniques, single cell and local network activity was correlated. CBZ was systemically injected to test its effect on physiological CTC oscillations. The spatio-temporal dynamics of the cortical network was examined to further our understanding on the intracortical mechanisms for seizure generation. The identity of the recorded neurons was then established following juxtacellular labeling (Pinault, 1996).

Using a combination of the above techniques, the experiments were designed to: 1) locate the site of action of CBZ within the thalamic network and to test whether CBZ is aggravating seizures via $GABA_A$ receptor mediated mechanisms; 2) to test the effect of CBZ analogues, OXC and MHD, to investigate whether these compounds differ from CBZ in their effects on $GABA_A$ receptor function and propensity to aggravate seizures at the EEG level; 3) to investigate in the thalamus at the single cell level, the effect of CBZ on thalamic oscillations that involve $GABA_A$ receptor mediated IPSPs; 4) to identify the intracortical structure and functions responsible for the initiation of rhythmic oscillations and absence seizure activity; and 5) to investigate whether CBZ has a region-selective effect to modulate absence seizures within the intracortical network.

2.2 Animal Subjects

2.2.1 GAERS and NEC rats

All animal experiments described within this thesis were performed using the GAERS and NEC rats. As previously described (see Chapter 1.4.3), the GAERS and NECs are progenies of the original Wistar rat colony from Strasbourg and were selected for the presence and absence, respectively, of absence seizure phenotype (Vergnes et al., 1982). All procedures were approved by the University of Strasbourg or University of Melbourne Animal Ethics Committee, where applicable, and performed in accordance with the guidelines published by the Australian National Health and Medical Research Council (NHMRC) and European Union for use of animals in research. Every precaution was taken to minimize stress and the number of animals used in each series of experiments. Rats were at least 13 weeks of age at the start of the experiment, when absence-type seizures accompanied by generalized SWDs are observed in 100% of the population. All rats were born, raised and kept in standard conditions and maintained on a 12 hour day/night cycle, with food and water ad libitum.

2.2.2 Xenopus laevis

Female *X. laevis* were purchased from the South African Xenopus Facility (Noordhoek, Republic of South Africa) and maintained at 18 °C on a 12 h light/12 h dark cycle. All animal experimental procedures adhered to the guidelines of Australian NHMRC Code for the Care and Use of Animals for Scientific Purposes, and were approved by the Animal Experimentation Ethics Sub-Committee of The University of Melbourne

2.3 Experiment 1: Examination of AED modulation of $GABA_A$ current (Melbourne)

2.3.1 Oocyte preparation

Oocytes from adult female *Xenopus laevis* were prepared using a previously published method (Goldin, 1992). The frog was anaesthetized before being placed on a clean surgical platform. A small incision of approximately 1 cm was made in the abdomen from medial to lateral towards the head. The ovaries were visible after the skin and the underlying fascia was cut through. Oocytes were removed by pulling out the lobes of the ovary with a pair of forceps. After a sufficient number of oocytes have been removed, the incision was closed with one stitch. Individual oocytes were dissected, placed in 96 well plates and incubated in ND96 medium (in millimolar; NaCl 96, KCl 2, $CaCl_2$ 0.1, HEPES 5, pH 7.5) and positioned with their brown animal pole upwards. They were then placed into an incubator at 19°C for several hours to allow adhesion to the well bottom. Oocytes were classified into six stages of development, with stage I being the earliest stage. Stages V and VI oocytes are the most mature at 1000-1300 μm in diameter. Only the healthiest Stage V and VI cells were used for the experiments.

2.3.2 Injection of oocytes

The injection procedures were carried out using the Roboocyte Robot (Multi Channel Systems, Reutlingen, Germany). cRNA-encoding human α1, β2 and γ2L $GABA_A$ receptor subunits was filled into an injection pipette with a tip opening of between 5-10 μm and a diameter of 10-12 μm. The pipette was then inserted into a holder mounted on the injection axis of the Roboocyte and connected to the air pressure line. The injection pipetter was positioned to reach the oocyte under the guidance of a microscope, the subunits were injected (~20-30 ng) into the cytoplasm of oocytes and stored at 18 °C for 1-2 days prior to experimentation.

2.3.3 Electrophysiology

To start a recording session, two glass electrodes containing 1.5 M potassium acetate and 0.5 M KCl were positioned onto the electrode holder on the Roboocyte. The 96 well plate was then

mounted onto the plate holder. Oocytes were continually perfused with a ND96 solution using a Gilson 222 XL Liquid Handler and Gilson Minipuls 3 Peristaltic Pump (Gilson Inc., WI, USA) and were impaled with two glass electrodes and held at a membrane potential of −80mV. $GABA_A$ currents were measured with the two-electrode voltage clamp mode of the Roboocyte. To investigate the effect of CBZ on $GABA_A$ receptor mediated Cl^- currents, oocytes were perfused with a 20 second application of bath solution containing GABA (EC_{20}; $6x10^{-6}$ M) followed by a 1 min application of bath solution alone in which the current levels returned to baseline levels. This GABA application was repeated 3 times to determine a baseline response to GABA. This was followed by a 20 s application of solution containing both GABA (EC_{20}; $6x10^{-6}$M) and various concentrations of CBZ (0.1, 1, 10 or 100 mM). The effect of CBZ on the GABA induced current was then expressed as the relative change in EC_{20} GABA current caused by the addition of CBZ according to the formula $(I_{GABA+CBZ}-I_{GABA})/I_{GABA}$ and termed the *potentiation ratio*.

2.4 Experiment 2: EEG recordings in freely moving rats (Melbourne)

2.4.1 Electrode and cannulae preparation

EEG recordings were necessary to quantify seizure frequency and duration following drug administration. For cortical surface recordings, multiple extradural electrodes need to be chronically implanted. Electrodes were prepared by soldering gold “male” connector electrodes (Farnell In One) onto nickel alloy jeweler screws. Stainless steel guide cannulae for intracerebral ventricular injections (22 Gauge, inner diameter (i.d.) 0.39mm, outer diameter (o.d.) 0.71mm), for intracerebral injections (9mm length from pedestal, 26 Gauge, i.d. 0.24mm, o.d. 0.46mm), its dummy cannulae and internal cannula for injection (33 Gauge, i.d. 0.1mm, o.d. 0.2mm) were made to order (Plastics One, Australia).

2.4.2 Surgeries

Adult female rats (13-15 weeks) were first placed under general anaesthesia (xylazine, 10mg/kg and ketamine, 75mg/kg, intraperitioneal, (i.p.)). When sufficient anaesthesia had been achieved (absence of foot pad withdrawal and eye blink responses), the rats’ head and the flanks were

shaved with a pair of clippers. An incision of approximate 2.5cm was made from anterior to posterior along the flank and ovarectomies were performed via the opening. The muscle layer, fat pad and the skin were sutured separately. During the same anaesthesia, a midline incision was made through the skin of the head, the periosteum scraped away to reflect the skull. Bleeding of the scalp was stopped using a diathermy (Medtronics Solan, Florida, USA). Two holes (1.4mm diameter) were drilled bilaterally in the frontal-parietal bone (approximately 1mm anterior to the coronal suture) and two in the parietal bone (approximately 2mm anterior to lambdoid suture) using a low speed electrical drill (Arlec Engraver). Care was taken to not pierce the dura when drilling. The head of each rat was fixed to a stereotaxic frame (Kopf instruments^{TM}, Germany). Bregma was taken as a reference point for placement of all implantation sites (Paxinos and Watson, 2005). For i.c.v. injections, a single catheter was implanted into the right lateral ventricle via a hole drilled 1.5 mm to the right and 1.0 mm posterior to bregma, and a 22G guide cannula and injecting needle were lowered into the hole to a depth of 3-3.5 mm ventral to the dura, until it entered the right lateral ventricle (confirmed by drawback of CSF in the injecting line). For subsequent experiments intracerebral microcatheters were inserted bilaterally into either the relay thalamus or the TRN, with coordinates based on a previous study from our group that developed and validated a method for accurately and reliably administering small volumes (0.2-0.5 µl) of drugs to these structures in female GAERS of the same age, (i.e. relay thalamus - 3.0 mm posterior and 2.6 mm lateral from bregma, and 5.5 mm ventral from dura; TRN - 3.0 mm posterior and 3.6 mm lateral from bregma, and 5.8 mm ventral from dura) (Lohman et al., 2005). The left cannula was first inserted based on these stereotaxic coordinates. The electrodes and cannulae were then held in place with a two-component dental cement (Vertex, The Netherlands). The cement was carefully applied to the left side only, not covering bregma or the midline. The co-ordinates were re-measured to confirm the point of cannula entry on the right side of the brain and the implantation procedures were repeated.

To verify the cannulae locations after completion of the experimental procedures, the animals were injected with 0.2 µl of methylene blue while freely moving and then terminally anaesthetised (xylazine 20mg/kg and ketamine 150mg/kg i.p.). The animals were transcardially perfused with 0.1 M Phosphate Buffered Saline pH 7.4, followed by 4% paraformaldehyde (PFA). The brain was then extracted and post-fixed in 4% PFA at 4ºC for 4 hours. After being

submerged in a cryoprotecting 20% sucrose solution at 4ºC, for 48 hours the brain was then snap frozen. 50 µm coronal sections were cut on a cryostat. The site and extent of the methylene blue staining in the brain was identified under light microscope by a reviewer who was blinded to the results of the EEG recordings.

2.4.3 Postoperative care of animals

Animals were given the carprofen analgesic Rimadyl (4mg/kg) (Pfizer, Australia) and housed individually post-surgery. Rats were placed on their sides in a new cage with fresh bedding and left on a heat mat for the 24 hours post surgery. Rats were frequently monitored for signs of discomfort. All rats were individually housed post surgery and were allowed a recovery period of 7 days prior to the recordings being acquired. From the third day post surgery, rats were gently handled for 5 minutes daily. The cannula dummy was screwed off and on for the rats to familiarize with the experimental conditions.

2.4.4 EEG recordings and Drug injections

The recording sessions took place in a well-lit, quiet room with the rats in their home cages. Rats were connected via wires attached to the electrodes by gold crimp pins to a computer running Compumedics^{TM} EEG acquisition software (Melbourne, Australia). Rats were given a habituation time of 15 minutes. If no spontaneous SWDs occur within the 15 minutes, rats were allow time until the appearance of the first SWD before recording. A 30-minute baseline recording was acquired. Both of the compounds injected – CBZ and BIC (Sigma-Aldrich, Australia) were dissolved in vehicle containing 40% Propylene Glycol, 10% ethanol, and 50% saline. This solvent has been well documented in the literature for similar *in vivo* studies e.g. (Korolkiewicz et al., 1996; Graumlich et al., 1999) and has no adverse effects on seizure activity. Injection into the parenchyma of the brain was performed by removing the EEG leads and dummy cannula from the cap after baseline recording. A polyethylene injection tube was attached to the injection cannula needle (33G, Plastics One, Australia) on one end and a 1.0µl glass syringe (SGE, Australia) on the other end. The needle was gently pushed into the cannula in the cap and locked in place. Drugs were injected using syringe pumps (0.2 µl in 5 minutes).

The EEG recording period was then continued for 90 minutes during which time the animals were able to move freely around their cage with access to food and water. Animals were constantly monitored by an investigator during the recording period to ensure that they did not fall asleep. The second and subsequent treatment arms were performed at the same time of the day for each animal, with at least a two day interval between treatments. The order of the administered of the treatments was randomized for each experiment.

2.4.5 EEG analysis

The seizure expression for the 90-minute post-injection EEG recording was quantified by visual inspection of the EEG using the Compumedics software. The nature of the drug administered was blinded by a second investigator. Standard criteria described for adult GAERS were utilized to classify the seizures, i.e. a SWD burst of amplitude of more than three times baseline, a frequency of 7-12 Hz and a duration of longer than 0.5s (Marescaux et al., 1992b; Dedeurwaerdere et al., 2005). The start and end of each seizure was determined by manually marking the beginning and end of each SWD using Compumedics. From this the total percent time spent in seizure over the 90-minute post-injection EEG recording was determined (% Time in Seizure) - the primary outcome variable for comparison of the effect of the treatments on seizure expression. Two secondary outcome variables were also determined: (i) the mean number of seizures occurring per minute, and (ii) the mean duration of each seizure. These variables were compared between the different treatments arms for each of the experiments.

2.5 Experiment 3: Multisite depth EEG recordings in freely moving rats (Melbourne)

2.5.1 Electrode preparation

Depth intracortical EEG electrode was prepared using insulated stainless steel wires (diameter 100 μM). They were cut into 4 cm pieces and tightly twisted to form a straight bundle. Superglue and was used to hold the wires in place. The tips of the 4 wires within the bundle were cut to expose the tips at different lengths such that the recording tips were 1.0, 3.0 and 5.0 mm respectively, from the most distal contact. Each of the 4 wires was also soldered onto a gold "male" connector electrode (Farnell In One) at the opposite end. Two bundles of electrodes were prepared for each rat. For depth recordings in the primary motor cortex, an insulated silver wire (~0.5 cm) was soldered onto gold "male" connector electrodes. The extradural ground and reference electrodes were prepared by soldering gold "male" connector electrodes (Farnell In One) onto nickel alloy jeweler screws.

2.5.2 Surgeries - Electrode implantation

Adult male rats were deeply anaesthetized using isoflurane in 50% air and 50% oxygen mixture. A total of 7 burr holes (~ 1.4mm diameter) were drilled on the skull for the implantation of electrodes; bilateral implantation of depth cortical electrode bundles [anteroposterior (AP) 0.2mm, medio-lateral (ML) ±3.6mm and dorsal-ventral (DV) 6mm, 20° angle] such that the four contacts reached the S1FL, S1ULp, S2 and the insular cortex (IC) at 1.0, 3.0, 5.0 and 6.0 mm respectively from the cortical surface, two single contact depth electrode electrodes were implanted into the motor cortices bilaterally (AP 2.7mm, ML ±2.6mm and DV 1.8mm); two extradural electrodes were implanted into the parietal bone (AP -4 mm, anterior of the lambdoid suture) to be used as ground and reference points. An additional hole was also drilled for an anchoring screw to be placed (AP -3mm, next to midline suture). Using this implantation set up, depth EEG recordings can be performed from all electrode contracts simultaneously from10 interconnected region of the brain network. Surgical procedures and post-operative care were identical to those described in sections 2.3.2 and 2.3.3.

2.5.3 Intracortical electrical stimulation

Intracortical electrical stimulations were applied at different locations (S1, S2 and IC) in freely moving GAERS and NEC rats in their home cages. Stimulations were delivered using the same steel electrode bundles that were used for depth EEG recordings through each of the recording contacts. Stimulus trains were delivered using a stimulator isolator that allows the delivery of constant currents and was connected to a stimulator accupulser (A365 and A360, World Precision Instruments). Trains containing 1ms square pulses at 7Hz were delivered to each of the S1FL, S1ULp, S2 and IC regions for 2 seconds in a randomized order. The chosen frequency (7 Hz) was similar to that of the naturally occurring 5-9 Hz rhythmic activity that gives rise to SWDs (Pinault et al., 2001). The intensity of the stimuli was initially 50 μA with the rat in quiet wakefulness. As long as the stimulation train did not trigger in the EEG typical generalized SWDs lasting more than 1 s, the intensity of the stimuli was increased by increments of 25-100 μA (up to 600 μA) and reapplied every 30 seconds until a seizure was triggered or a maximum amplitude of 600μA was reached. The order in which the electrodes in the different cortical regions were stimulated was randomized.

2.5.4 EEG recordings

EEG recordings were performed on freely moving implanted rats in their home cages. The electrodes were connected to the Compumedics EEG system (Melbourne, Australia) and the EEG acquired at 256 Hz (band filtered with a low cut-off 0.1 Hz and high cut-off 150 Hz). At least two 60 minute recordings were acquired from each rat. At the end of recording sessions, the position of the recording electrodes was determined by marking the lesions by passing a positive DC current (2mA for 5 seconds). Rats were then euthanized with pentobarbitone (Lethabarb, 300mg/kg i.p.) and transcardially perfused with phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS with 0.5% potassium ferricyanide to visualise the electrode recording tip positions on cryostat cut axial sections.

2.6 Experiment 4: electrophysiology recordings in vivo (Strasbourg)

2.6.1 Anaesthesia, surgeries and animal maintenance

Surgeries were performed in GAERS and NEC rats under deep general anaesthesia induced by pentobarbital (40mg/kg, i.p., Sanofi, Libourne, France) and ketamine (50mg/kg, i.m., Merial, Lyon, France). The dorsal penile vein was used for intravenous injection as it is easily accessible. The technique was originally described in mice but can also be used in the rats (Salem et al., 1963). The tip of the penis is everted with thumb and index finger and the dorsal vein was readily visible. The vein was gently separated from the surrounding tissue and a snip was made on the vein such that a saline filled polyethylene injection tube (connected to a 3ml saline filled syringe) could be inserted. Extreme care was taken to not completely sever the vein. The tube was gently inserted through the vein opening until resistance was met. To ensure that the tube was in place, the syringe was slightly drawn back, with the aspiration of blood confirming the success of the procedure. Knots were tied on the vein and the tube to prevent any movement of the tube throughout the experiment.

Tracheotomy was also systemically performed for each experiment following vein catheterization. A 3 cm longitudinal incision was made at the neck level through the skin and muscle layers with a scalpel blade. The muscles were separated with retractors until the trachea was reached and exposed. A Czerny retractor was placed under the trachea so that suture threads could be slid under it. A small orifice was then made between two cartilaginous rings. A plastic catheter was inserted through the opening with the beveled end head towards the lungs, leaving the other end free. Knots were made with the threads, tying the trachea onto the catheter so that it was fixed in the correct position. Any lung secretion was suck out with a tube connected to a 50ml syringe to prevent blockage of the airway. The cannula was then connected to a ventilator (SAR-830/P, CWE, INC., BIOSEB, Chaville, France) to control and monitor the breathing of the rat. For all remain surgical and recording procedures, the breathing was maintained by the ventilator at 60 breaths per minute with a mixture of 50% air and 50% oxygen. The rectal temperature was maintained at 36.8 -37.3 ºC throughout. The animal was secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) installed on a vibration-isolated platform

(Newport Corporation, Irvine, CA, USA, Micro-Controle, Evry, France). The rat's back was held at the same height as the neck to minimize the likelihood of airway blockage.

At the end of the surgical procedures, neuroleptanalgesia was initiated before the end of the general pentobarbital-ketamine anesthesia. This was induced by an intravenous bolus injection (0.3ml) of the following mixture: glucose (12.5mg, Sigma-Aldrich), d-tubocurarine chloride (0.2mg; Sigma-Aldrich, Saint-Quentin Fallavier, France), fentanyl (0.22µg; Jassen, Boulogne-Billancourt, France) and haldo (25 µg; Janssen). The rat was maintained by a continuous injection (0.4-0.6ml/hr) of the same mixture (per hour per kg): glucose (83mg), D-tubocurarine chloride (1.4mg), fentanyl (1.5 µg) and haldo (167 µg). Animals were given at least 2 hours after the initiation of neurolept-analgesia before recordings commenced. The rectal temperature was progressively increased at the completion of surgeries, and maintained at 37.8–38.3 ºC. The heart rate (300-350 beats/minute), breathing rate (60 breaths/minute, pressure: 8-12cm H2O) and the surface EEG were continuously monitored to ensure stable and adequate depth of anaesthesia.

2.6.2 Craniotomy and duratomy

A stabilizing craniotomy and duratomy technique developed by Pinault (Pinault, 2005) was used to allow access to deep brain structures with recording micropipettes. The surgical procedures allow a small opening of the dura mater on a thin bone membrane, exposing the brain cortical surface (Figure 2.1). The physiological conditions and volume of the brain were kept constant throughout the experiment. The technique allows precise stereotaxic approach of the recording micropipette to the target region and a high success rate of single cell recordings in a live brain.

Rats were transferred and fixed to a stereotaxic frame on an air table. A midline incision was made between the eyes and the ears through the skin. The cranium surface was then cleaned and dried with a chisel blade. All remaining procedures were carried out under the guidance of a stereoscopic microscope (SMZ-2B, Nikon France, Champigny-sur-Mame, France). The target region was determined using the stereotaxic frame and marked with a pencil. A large area of the

skull (5mm x 5mm, approximately) was drilled above the target region using a dental drill (Technobox, Bien Air France SARL, Paris) over a period of 5-10 minutes. A stable and consistent hand control of the dental drill was always maintained to ensure steady advancements through the bone. The drilling procedure was performed in stages, lasting no more than 2 seconds to avoid heating up of the brain surface. Extreme care was taken when bone veins were met during the drilling to avoid excessive bleeding. Bone dust was frequently removed to maintain a clear view of the cranium. Drilling continued until only a thin bone membrane remained. The membrane is thin enough to be soft and transparent, through which the brain's vascular network can be clearly seen under the microscope. A gelatin sponge soaked in saline was frequently applied to ensure that the bone membrane maintains its transparency and softness. Short lasting drilling steps (<1s) was continued in the middle of the bone membrane, until it was thin enough such that a small pressure with thin, sharp forceps punctures the bone without touching the brain. The points from which the recording glass electrodes were to be inserted were stereotaxically marked on the drilled surface using a micropipette (tip diameter 5-10 μm) filled with 4% pontamine Sky Blue in 0.5M NaCl). It was made sure that there was no blood or bone dust on the brain surface. A small opening was made (<0.8 mm in diameter) was made using thin forceps to gently puncture and tear out the bone membrane at the previously marked stereotaxic location.

A blue marking was then stereotaxically applied with pontamine sky blue filled micropipette on the meninges to indicate accurately where the recording pipettes were to be inserted. A 26 Gauge needle (0.45mm x 12mm) was mounted on a 1ml syringe and its tip gently scratched against a hard piece of metal such that a miniature hook was made. The meninges were gently incised with the hook to allow a small opening exposing the brain surface. In cases where the opening was not large enough for the micropipette to be inserted, the edge of the dura and pia maters were gently picked up and moved away from each other using microsurgery forceps. It was made sure that the brain surface was as clean as possible, free of blood and bone dust. Surgical sponges impregnated with 0.9% saline were applied around the opening to prevent dessication of the exposed cortical areas.

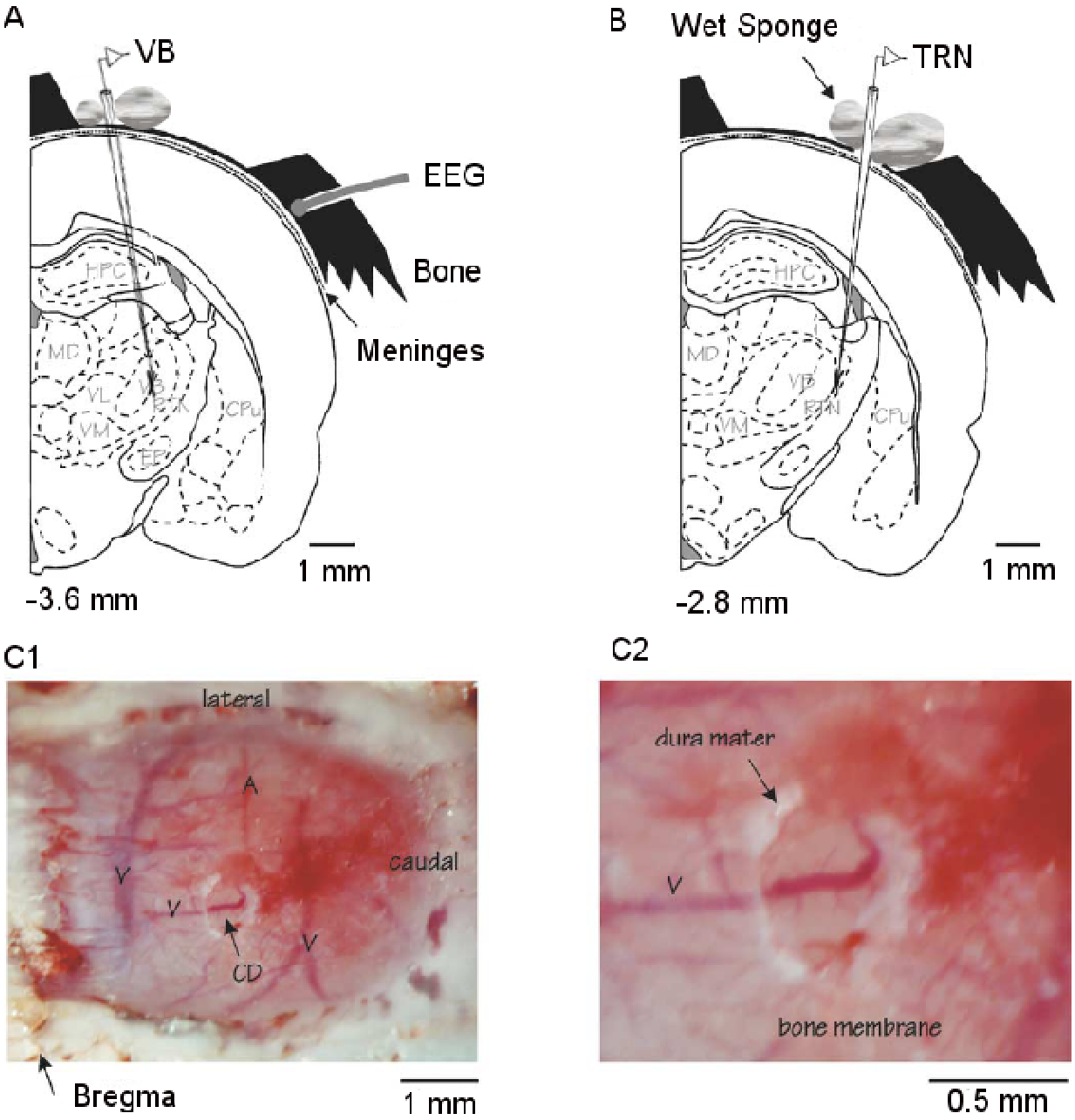

Figure 2.1 An illustration of craniotomy-duratomy technique. A and B, target recording sites for the two micropipettes. Note the minute incision in the meninges, which include the dura and pia maters. Small surgical sponges soaked with saline are laid down on the two cranium openings. C1& C2: Dorsal macrophotographs showing a typical micro-craniotomy–duratomy, allowing the insertion of micropipettes. The transparency of the bone membrane makes veins (V) and arteries (A) visible. (Adapted and modified from (Pinault, 2005))

2.6.3 Electrophysiology (Strasbourg)

2.6.3.1 Preparation of recording micropipettes

Micropipettes were prepared from glass capillaries (1.2- 1.5mm in diameter) containing a microfilament (A-M systems). They were pulled on a vertical pipette puller (Narishege PE-2) such that all pipettes had a consistent, long and thick shaft (Figure 2.2). A small volume of pure water (~1ml) was injected at the top of the capillary and was allowed 10-15 minutes to run to the tip of the thin shaft. The pipette was placed under microscopic observation to make sure that no bubbles remain.

The solution for filling of the micropipettes contained 1.5% N-(2 amino ethyl) biotin amide hydrochloride (Neurobiotin, Vector Labs, Burlingame, CA, USA) dissolved in 1M CH_3COOK. The pH of the solution was 6.3. It was separated into 0.5ml aliquots, stored in the freezer for up to 3 months and thawed on the day of the experiment. The pipette was filled with the solution, allowed to rest overnight to allow ions and tracer molecules to diffuse to the tip.

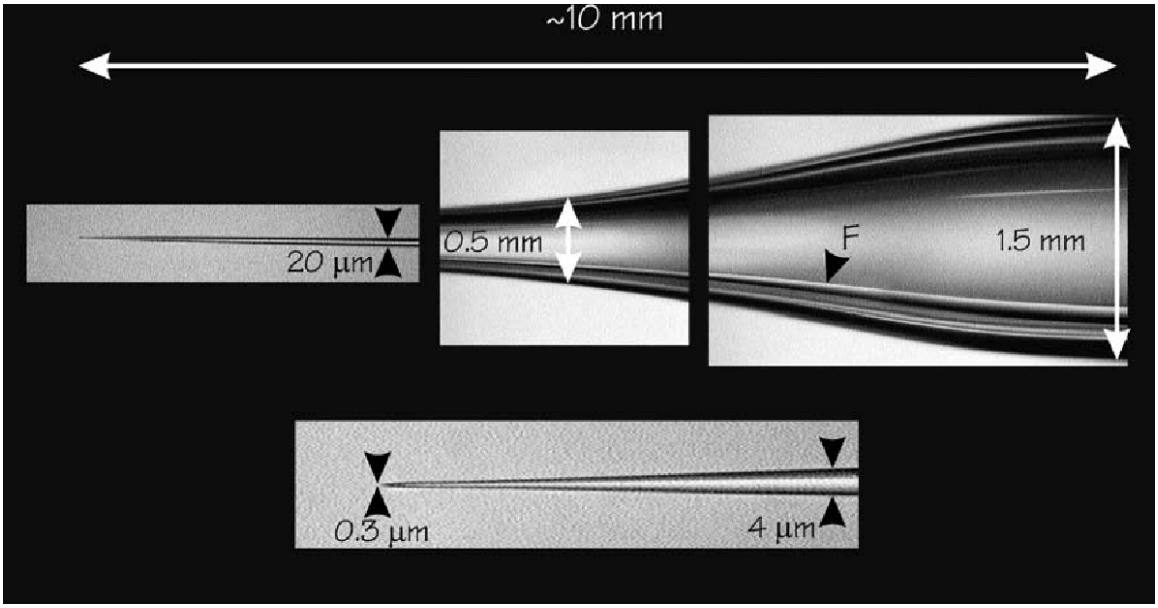

Figure 2.3 Photo taken under a microscope showing the main physical features of a typical sharp-tipped glass micropipette for intracellular recording. F denotes filament. (Adapted from (Pinault, 2005))

2.6.3.2 Stereotaxy, electrophysiology, data acquisition and signal conditioning

Before the insertion of micropipettes into the brain for recordings, micropipettes were placed under microscopic observation, their tips gently touched or rubbed such that the external tip diameter of the pipette is of a desired width (table 2.1).

	Extracellular	Juxtacellular	Intracellular
Tip diameter (μm)	5 – 8	~ 1	<0.8
Resistance (MΩ)	< 10	20-50	> 50
Signal conditioning (bandpass, Hz)	0.1-1200	0-6000	0-6000

Table 2.1 Tip diameter, resistance requirements and signal conditioning for different recording types

The micropipettes were clamped onto holders attached to stepping micro-drivers (Burleigh, Fisher, NY, USA) which were used as micro-manipulators that were able to advance the micropipettes into the brain in well-controlled steps ranging from 1-3μm to reach target regions. Silver chloride wires (silver wires presoaked in bleach) connected to an intracellular preamplifier were inserted into the micropipettes to allow contact with pipette solution. It was, in turn, connected to a signal conditioner (allowing optimal gain and bandpass), an oscilloscope and an analog to digital converter for the storage of voltage and current signals into a computer for online and offline analyses. Signals were digitized at a sampling rate of 20 kHz. For intracellular recordings, a current pulse in the range of -0.2 to -0.5 nA was applied every 2 seconds to keep the Wheatstone bridge balanced.

2.6.4 Identification of recorded regions or neurons

2.6.4.1 Juxtacellular iontophoresis

At the completion of extracellular recordings, a histochemical marker (Neurobiotin) was electrophysiologically applied using an effective single-cell labeling methods to identify the location and anatomy of the recorded cell (Pinault, 1996). Continuous electrophysiological control was maintained throughout the filling procedures. A positive rectangular nanocurrent pulse (0.5-8 nA; 200 ms duty cycle) of increasing intensity was applied to the cell. After a delay of a few seconds to a few minutes, the background noise level of the recording increased indicating that the extracellular pulses became juxtacellular, when the charged surface of the cell membrane was adjacent to the micropipette tip. The neuron usually tonically fired during the 200ms on phase of the current pulse and appeared to oscillate in a rhythmic manner in phase with the current pulses (Figure 2.3). Once the juxta-position and cell driving conditions were reached, pulse intensity was decreased (usually to between 1 and 5 nA) to prevent damage to the neuron. The same current conditions were maintained for 5 minutes to allow the internalization of the tracer molecules into the neuron being recorded.

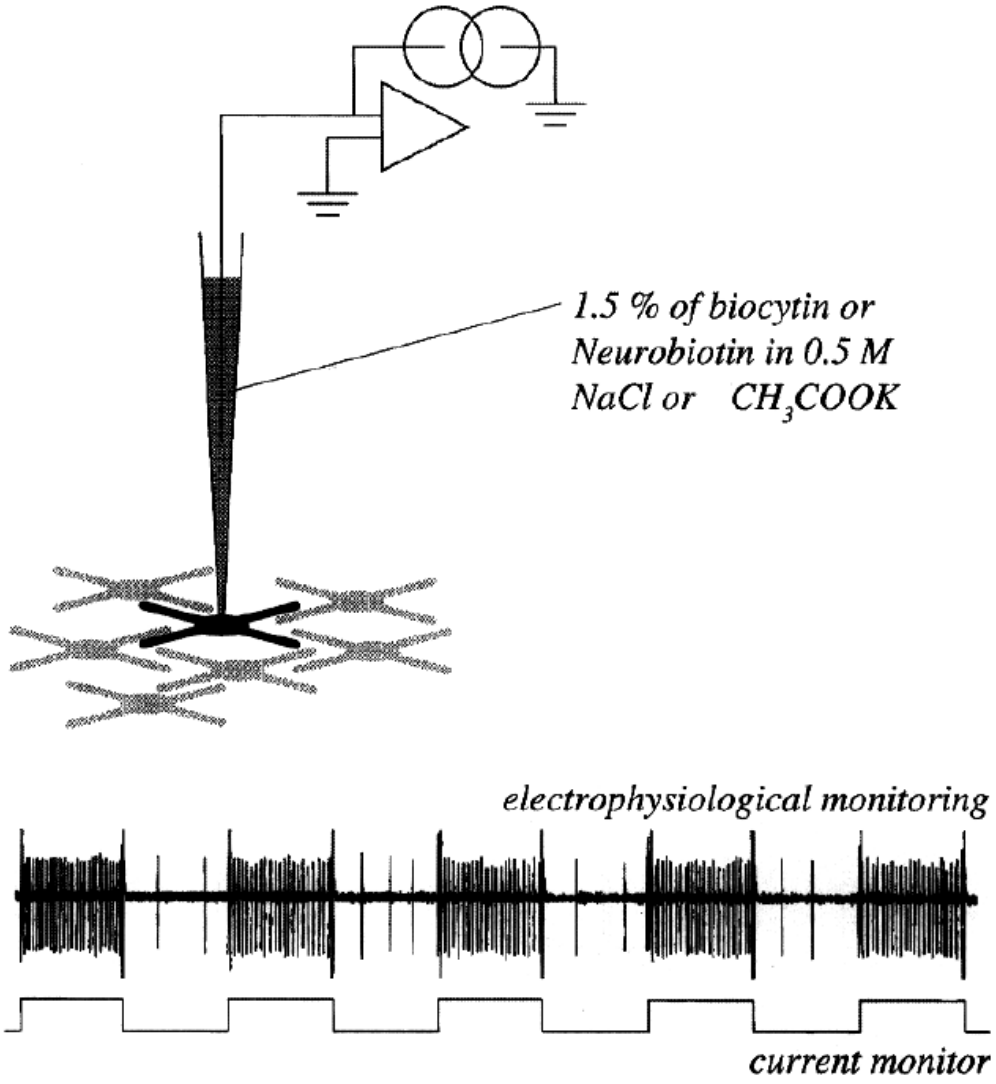

Figure 2.3 Experimental protocol to fill a neuron using a juxtacellular approach. Once a neuron was extracellularly recorded and well isolated, the juxtacellular contact occurred while the tracer was systematically applied with current pulses (200 ms on/200 ms off) under continuous electrophysiological monitoring. The effective current pulses (intensity < 10 DA) modulated the electrical behavior of the neuron under physiological study. (Adapted from (Pinault, 1996))

2.6.4.2 Histology

After a survival period ranging from a few minutes to 3 hours, the animals received a terminal dose of pentobarbital. Rats were then transferred to the fume hood and were transcardially perfused with physiological saline (0.9%, 200ml) followed by 500ml of a fixative containing 4% paraformaldehyde (PFA) and 0.5 % glutaldehyde in 0.1M phosphate buffer (PB, pH 7.4). Sagittal brain sections were cut at 100 μm with a vibratome and serially collected in a 24 well plate in PBS. Slices were thoroughly washed in PBS 3 times before being incubated with avidin-biotin-peroxidase complex (ABC; Vector labs) overnight. The injected tracer, bound to this enzymatic complex, was revealed with the appropriate substrate and chromogen, the hydrogen peroxide and the 3, 3'-diaminobenzidine tetrahydrochloride respectively, according to a previously described method (Horikawa and Armstrong, 1988) and nickel intensification (Adams, 1981). A DAB kit was used for this part of the experiment (Vector Labs). Brain slices were then mounted onto gelatin coated slides. Neurons filled juxtacellularly can be permanently and solidly visualized by the enzymatic reaction product. Tracer filled neurons were examined with a light microscope (E600, Nikon France, Champigny-sur-Marne, France). The location of marked cells was ascertained by referring to a stereotaxic atlas (Paxinos and Watson, 1998).

Chapter 3 The mechanism underlying carbamazepine aggravation of absence seizures

3.1 Introduction

As previously outlined in Chapter 1 (section 1.3.3), the aggravation of seizures by many commonly prescribed AEDs poses important clinical problem that is often overlooked in practice (Lerman, 1986; Perucca E et al., 1998). One of the drugs most implicated in seizure aggravation is CBZ, an anti-focal seizure AED that paradoxically causes an increase in a variety of seizure types, including typical and atypical absence seizures, myoclonic, atonic and tonic seizures, and also on occasions generalised tonic-clonic seizures (Chapter 1, section 1.3.4). Of these, the aggravation of absence seizures is the most predictable, and with the availability of the GAERS model of absence epilepsy (Chapter 1, section 1.4.3), the most amenable for mechanistic studies.

GABAergic mechanisms within the thalamus play a pivotal role in the regulation of rhythmic TC activity and of absence seizures (Chapter 1, Section 1.6.1). $GABA_A$ agonists injected into the thalamus have variable effects on absences seizures depending on the site of administration. Microinjections of muscimol into the ventrobasal (VB) thalamus enhance the seizures, while paradoxically microinjections into the TRN inhibit them (Hosford DA et al., 1997). The activation of $GABA_A$ receptors at different brain regions is, therefore, critically important in the regulation of the TC oscillatory activity and absence seizures.

Early work competed by our group demonstrated that serial i.c.v. injections of CBZ administered to GAERS resulted in an increased percentage time in seizure compared with vehicle controls (Liu et al., 2006). These results show that seizure aggravation in GAERS following i.c.v. injection of CBZ was of similar magnitude to that previously reported with i.p. injections by our group and others (Micheletti et al., 1985; Wallengren et al., 2005), and demonstrate that CBZ acts directly on the brain to aggravate seizures, without requiring initial conversion into a

metabolite. In addition, this study demonstrated that higher CBZ dose (30mg/kg) resulted in excessive sedation while the lower dose (15mg/kg) resulted in little obvious behavioral effects. Therefore the 15mg/kg dose was chosen for the randomised CBZ vs. vehicle study in this chapter.

To extend this finding I investigated the precise neuroanatomical site of action of CBZ, determining if seizure aggravation occurred either through the VB thalamus or the TRN. A second component of this study was to devise a series of experiments determining that CBZ was having its impact directly on $GABA_A$ receptors. This included in vivo pharmacology and testing CBZ on exogenously expressed $GABA_A$ receptors. This work was published in (Liu et al., 2006). Based on recent publications and current data, we hypothesise that CBZ is acting via $GABA_A$ receptor within the VB thalamus to aggravate seizures. More specifically, experiments were designed to: 1) investigate *in vivo* whether CBZ aggravate seizures via $GABA_A$ receptor mediated mechanisms in GAERS; 2) test *in vitro* whether there is a direct interaction between CBZ and $GABA_A$ receptors.

3.2 Materials and Methods

Animals used, surgeries and EEG recording protocols have been described in detail in chapter 2, section 2.3.

3.2.1 Data analysis

The seizure expression for the 90-minute post-injection EEG recording was quantitated by visual inspection of the EEG while the nature of the drug administered was blinded. by visual inspection of the EEG**.** The start and end of each seizure was determined by manually marking the beginning and end of each SWD on the EEG (Figures 3.1 & 3.5). From this the total percent time spent in seizure over the 90-minute post-injection EEG recording was determined (% Time in Seizure) - the primary outcome variable for comparison of the effect of the treatments on

seizure expression. Two secondary outcome variables were also determined: (i) the mean number of seizures occurring per minute, and (ii) the mean duration of each seizure. These variables were compared between the different treatments arms for each of the experiments.

3.2.2 Statistical analysis

Statistical analysis was performed using the software package Statistica™ (StatSoft, Inc., Tulsa, OK, USA). Where there were two treatments administered the matched-pairs *t*-test was used to assess for the level of statistical difference. Where more than two treatments had been administered to each animal, ANOVA for repeated measures was used, with a subsequent planned comparisons analysis to compare two specific treatments. For the comparison between the seizure aggravation following CBZ microinjections into the VB versus TRN, Students *t*-test was performed. All data were expressed as mean ± standard error and *P*-values less than 0.05 were considered significant.

3.3 Results

3.3.1 CBZ aggravates seizures in GAERS in the VB but not the TRN

To determine the anatomical region within the TC circuitry at which CBZ acts to aggravate absence seizures in GAERS, either CBZ or vehicle alone was microinjected into either the VB or TRN to determine if CBZ acted directly at one or both of these regions to aggravate seizures. Bilateral microinjections of CBZ or vehicle were administered into either the VB (n=7) or, in a separate cohort of rats into the TRN (n=7). Each rat received two injections (CBZ and vehicle) in a randomized order separated by at least two days. The CBZ was administered (0.75μg in 0.2μL), with the volume based on that used in our previous validation study of focal VB or TRN injections (Lohman et al., 2005). Significant seizure aggravation was seen after microinjection of CBZ into the VB (mean increase in %Time in Seizure = 53%, p=0.03, n=7) compared to vehicle, but not after the TRN injections (mean increase in %Time in Seizure = -8%, p=0.61, n=7) (Figure 3.1). There were also a significantly greater number of seizures per minute following the VB injections of CBZ versus vehicle injections (2.7 vs. 1.9, p=0.03), but not for the TRN

injections (1.8 vs. 2.0, p=0.31). There were no significant differences in the mean duration of individual seizures between the CBZ and vehicle arms for either the VB injections (5.2 vs. 4.9 seconds, p=0.61) or the TRN injections (4.7 vs 5.4 seconds, p=0.61).

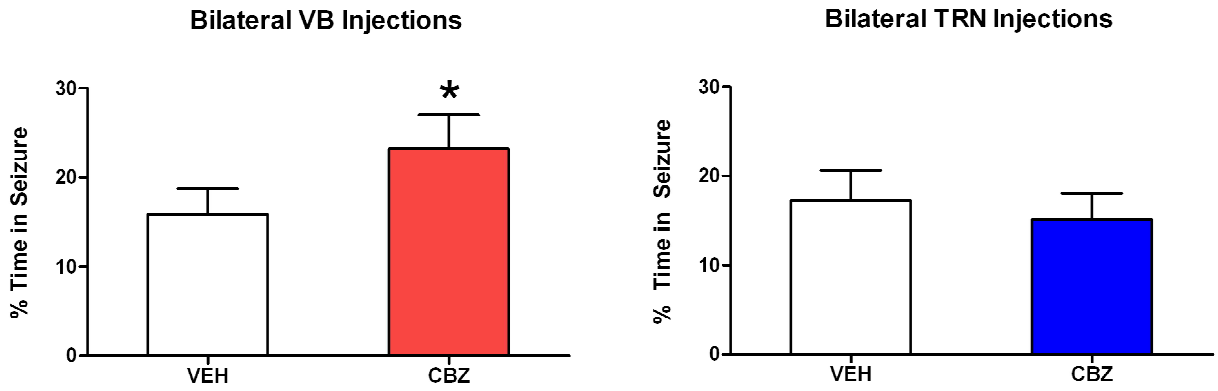

Figure 3.1 Mean percent time in seizure (±S.E.) in GAERS over a 90 minute EEG recording following bilateral microinjection of vehicle or CBZ (0.75 µg in 0.2 µl) showing significant seizure-aggravating effects (versus vehicle injections) following CBZ injection into the VB (n = 7, 54%; * p = 0.01, matched pairs Student's t test) but not the TRN (n = 7, -8%, p = 0.43). The seizure aggravation was significantly different between the VB and TRN injections (p = 0.01, Student's t test).

3.3.2 CBZ aggravate seizures within the VB in a dose-dependent manner

Following the observation that CBZ aggravates seizures following microinjection into the VB, a dose-response study was performed to further explore this effect. A separate cohort of three rats received serial microinjections of CBZ into the VB (in a random order) at (i) 0.1µg, (ii) 0.4µg and (iii) 0.75 µg in 0.2µl of vehicle, as well as (iv) vehicle alone. Repeated measures ANOVA showed a significant effect of treatment on % time in seizure. Vehicle alone did not affect seizure activity (18.3% before and 17.0% after injection). Injection of the two lower doses of CBZ (0.1µg and 0.4µg) increased the % time in seizures, but this did not attain statistical significance compared to vehicle alone (% time in seizure 21.7±3.28, 23.96±1.66, respectively). However, the highest (0.75µg) dose of CBZ resulted in a significant increase in % time in seizures compared to vehicle (% time in seizure 36.59±4.32, p= 0.005) (Figure 3.2).

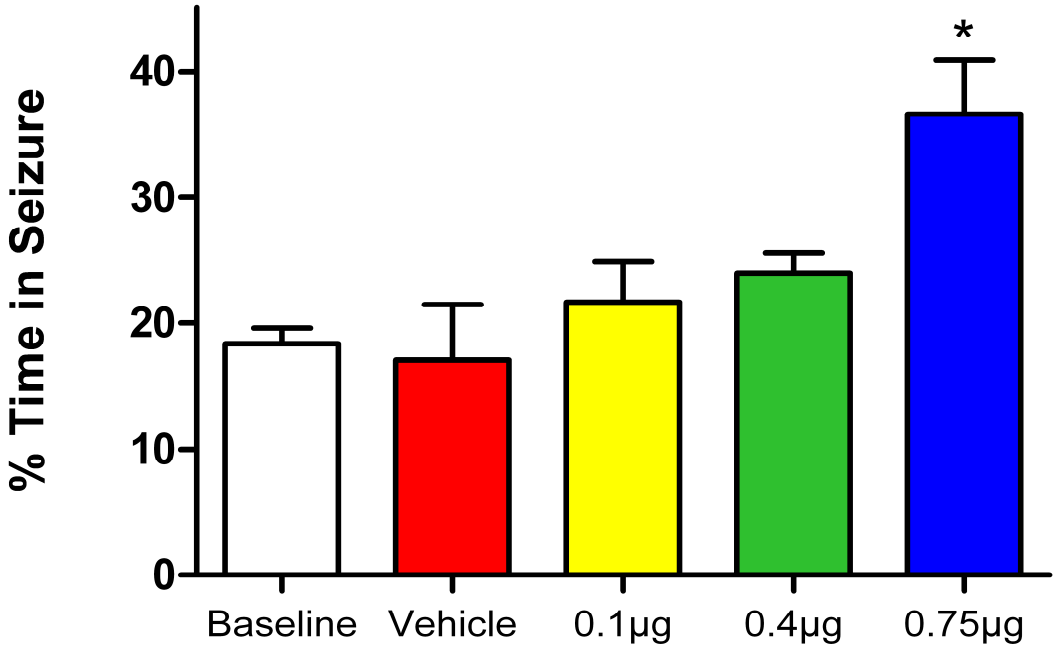

Figure 3.2 Dose-response study for CBZ microinjected into the VB in GAERS, showing mean percent time rats spent in seizure (±S.E.) over a 90-min EEG recording following CBZ injection compared with vehicle alone and with the baseline (pre-injection). Repeated measures ANOVA demonstrated a significant effect of the treatments ($n = 3$, $p<0.005$). Planned comparison show an increase in percent time in seizure after the 0.75µg dose of CBZ (*, $p = 0.005$) compared with vehicle.

3.3.2 Co-administration of a $GABA_A$ antagonist blocks seizure aggravation by VB injection of CBZ

This experiment aimed to test the hypothesis that the aggravation of seizures in GAERS by focal injection of CBZ bilaterally into the VB was occurring via a $GABA_A$ mechanism. One prediction is that the impact of CBZ on seizure activity would be blocked by co-administration of the $GABA_A$ receptor antagonist, bicuculline (BIC). Two cohorts of rats were studied, both of which received three sequential injections into the VB bilaterally, Group A (n=7) was treated with (i)

CBZ (0.75 µg in 0.2 µL), (ii) a mixture of CBZ (0.75 µg) and BIC (0.037µg), or (iii) vehicle alone. Group B received (i) BIC (0.037µg), (ii) a mixture of BIC (0.037µg) and CBZ (0.75µg), or (iii) vehicle. The dose of BIC was chosen on the basis of previous studies demonstrating effective blockage of $GABA_A$ injection following central injections (Sanudo-Pena and Walker, 1997). The injection of CBZ alone aggravated seizures, while the co-injection of CBZ and BIC did not (Figures 3.3 & 3.4). A significant difference in the time rats spent in seizures was found between the treatments for Group A ($p=0.004$, Repeated Measures ANOVA), but not Group B ($p=0.21$) (Figure 3.4). Planned comparison analysis demonstrated a significant increase of the mean %time in Seizure following the CBZ injection, but not following BIC or CBZ-BIC. These data demonstrate that BIC acts within the VB to antagonise CBZ-mediated aggravation of seizures implicating a $GABA_A$ receptor-mediated-mechanism. They do not exclude the possibility that CBZ was also acting at other sites within the brain to exert this effect.

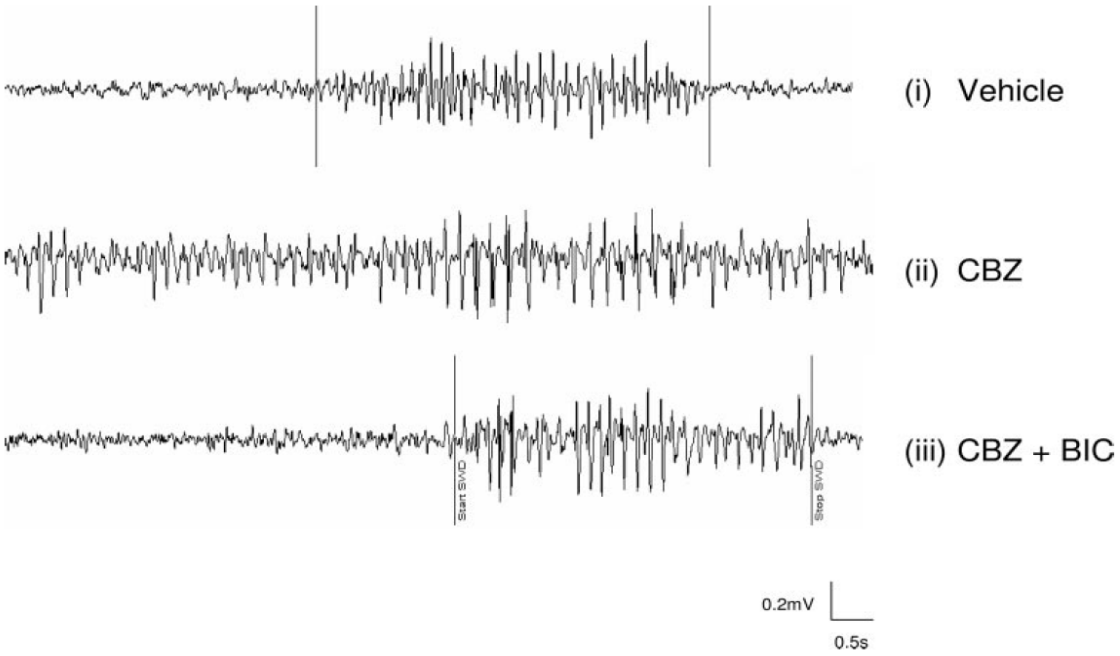

Figure 3.3. A representative 10s EEG trace from GAERS following bilateral microinjection of: (i) 0.2 µl of vehicle, (ii) 0.75 µg of CBZ in 0.2 µl of vehicle and (iii) CBZ and BIC (0.037 µg) in vehicle. CBZ prolonged the total time in seizure over the 90 minute post-injection EEG recording, which was blocked by the co-administration with BIC.

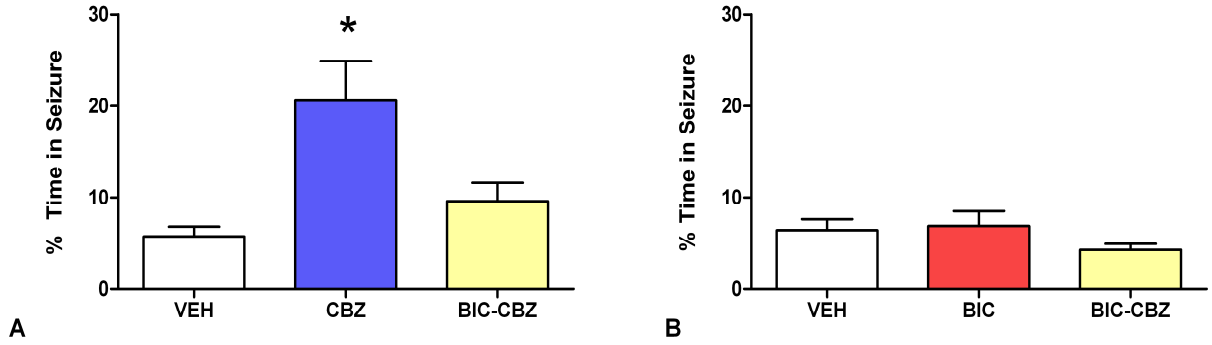

Figure 3.4 Mean percent time in seizure (±S.E.) in GAERS over a 90 min EEG recording following bilateral microinjection into the VB of CBZ (0.75 µg), BIC (0.037 µg), a mixture of BIC and CBZ (BIC-CBZ) or vehicle. There was a significant difference between the treatments for group A (n=7, p = 0.004, repeated measures ANOVA) but not group B (n=7, p = 0.21). Planned comparison analysis showed significant seizure aggravation following the CBZ injection (versus vehicle) (+256.8%, * p = 0.01) (A) but not following BIC (-7.0%, p = 0.77) (B) or CBZ-BIC (A and B combined) (21%, n =14, p = 0.47).

3.3.3 Administration of a $GABA_A$ receptor antagonist into the VB blocks seizure aggravation following systemic administration of CBZ

This experiment aimed to investigate whether injection of BIC bilaterally into the VB would block seizure aggravation in GAERS following i.p. injection of CBZ. This would provide strong evidence that $GABA_A$ activation in the VB is critical in the seizure aggravating effects of systemically administered CBZ in this model. Rats that had been implanted with bilateral VB

cannulae were injected centrally with either with BIC (0.037µg in 0.2 µL of vehicle) or vehicle alone, and i.p. with either CBZ (15 mg/kg in 2 mls of vehicle) or vehicle alone. The rats therefore received four sequential treatments, in a random order, separated by at least 2 days: (i) vehicle VB, CBZ i.p., (ii) BIC VB, CBZ i.p., (iii) vehicle VB, vehicle i.p., (iv) BIC VB, CBZ i.p.. The dose of CBZ administered i.p. was that which we and others had previously demonstrated to aggravate seizures in GAERS (Marescaux C et al., 1984; Micheletti et al., 1985; Wallengren et al., 2005)

Significant seizure aggravation occurs following i.p. CBZ injection, which was completely blocked by the VB injection of BIC (Figure 3.5). The VB injection of BIC alone had no significant effect on % Time in Seizure. These results indicate that activation of $GABA_A$ receptors within in the VB is critical to the mechanism of aggravation by CBZ of seizures in GAERS.

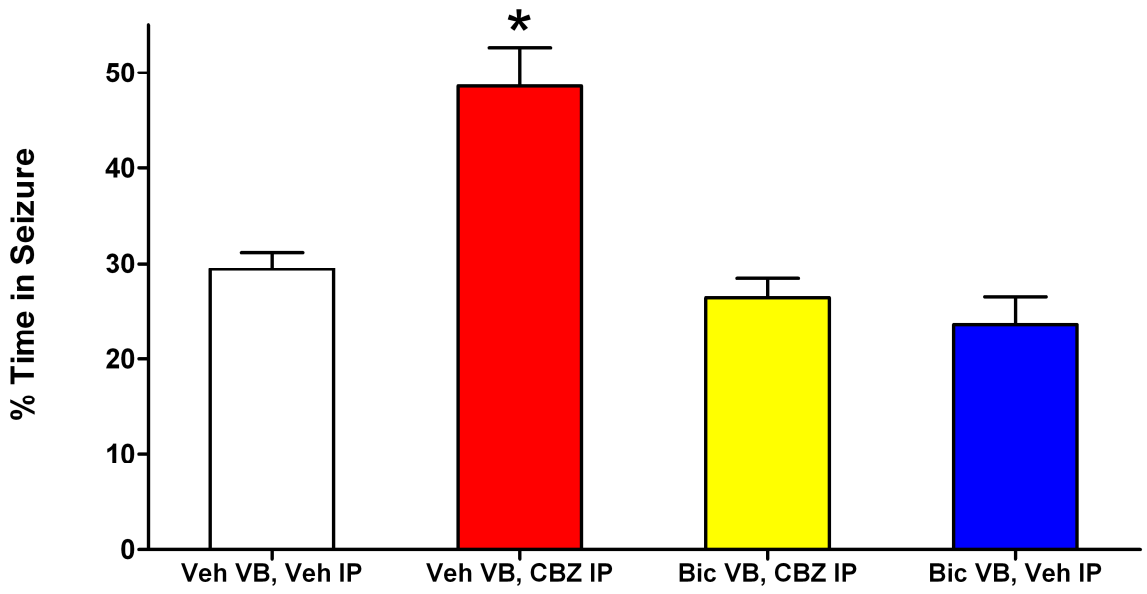

Figure 3.5 Mean percent time in seizure (±S.E.) in GAERS over a 90 min EEG recording following randomized drugs treatments of vehicle injections into the VB and vehicle injection i.p. (Veh VB, Veh IP); vehicle injections into the VB, CBZ injection i.p. (15mg/kg) (Veh VB, CBZ IP); BIC (0.037 μg) microinjection into the VB followed by CBZ i.p. (BIC VB, CBZ IP); and BIC microinjection into the VB and injection of vehicle IP (BIC VB, Veh IP). Repeated measures ANOVA showed a significant difference between the treatment arms ($n = 8$, $p < 0.001$), with planned comparison analysis showing that Veh VB, CBZ IP injection was followed by significantly longer time in seizures than the other three treatments (*$p < 0.001$), which did not significantly differ from each other ($p > 0.05$).

3.3.4 In vitro CBZ potentiates $GABA_A$ receptor current expressed in Xenopus oocytes

To confirm earlier *in vitro* studies demonstrating CBZ potentiation of $GABA_A$ receptor current, we tested whether there was a direct interaction between CBZ and $GABA_A$ receptors. This part of the study was performed in collaboration with Dr Steve Petrou at the Howard Florey Institute, University of Melbourne, Australia. All experiments were performed by Dr Alison Clarke from the same research unit. Recombinant $GABA_A$ receptors with the subunits $\alpha1\beta3\gamma2$ were expressed in *Xenopus* oocytes. Typical traces demonstrating the potentiation of GABA currents by 10 and 100 μM CBZ are shown in Figure 3.6. CBZ produced a dose-dependent potentiation of the GABA current in a physiologically relevant concentration range (1-100 μM, Figure 3.7). CBZ potentiation was clearly apparent and reproducible at 10 and 100 μM as shown. At 1 μM, the potentiation was smaller and could only be reliably determined by averaging multiple responses. Current records were averaged at a range of physiologically relevant CBZ concentrations and plotted (1–100 μM, Figure 3.7)

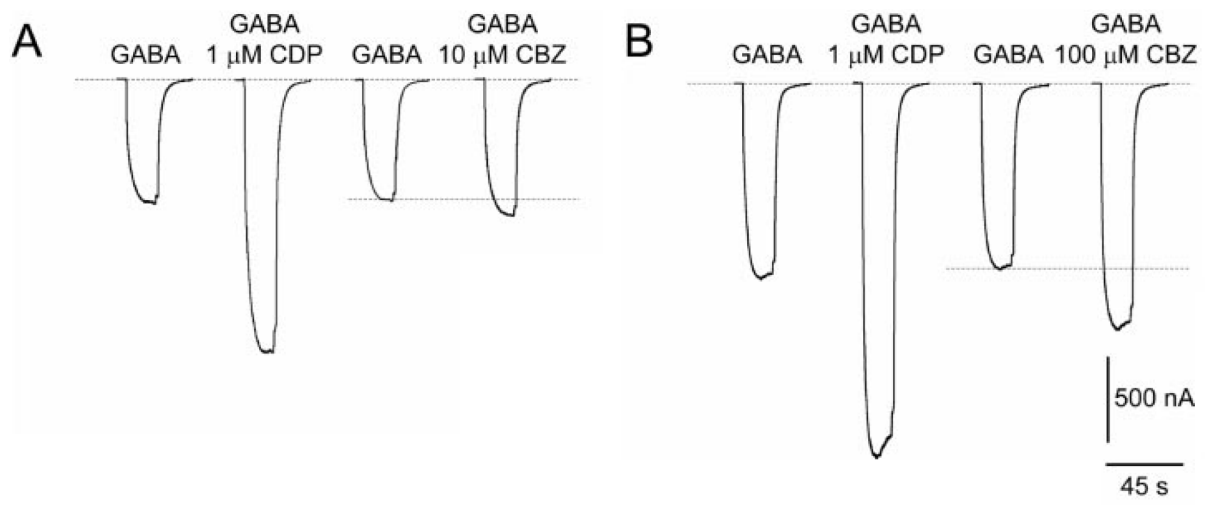

Figure 3.6 Typical current traces showing potentiation of the EC_{20} GABA response by 10 μM (A) and 100 μM CBZ (B) obtained in two electrode voltage-clamped *Xenopus* oocytes held at -80 mV. In each case, the four traces represent a series from the same oocyte showing first the response to GABA, then a reference 1 μM chlordiazepoxide (CDP) potentiation, followed by a recovery GABA response and finally the modulation by CBZ. CDP, a strong benzodiazepine site agonist, was used as a reference to ensure $GABA_A$ γ2 expression. The upper dashed line represents the leak corrected zero current level, and the lower dashed line is a guide to gauge the CBZ potentiation. Time and current scale bars apply to both figures.

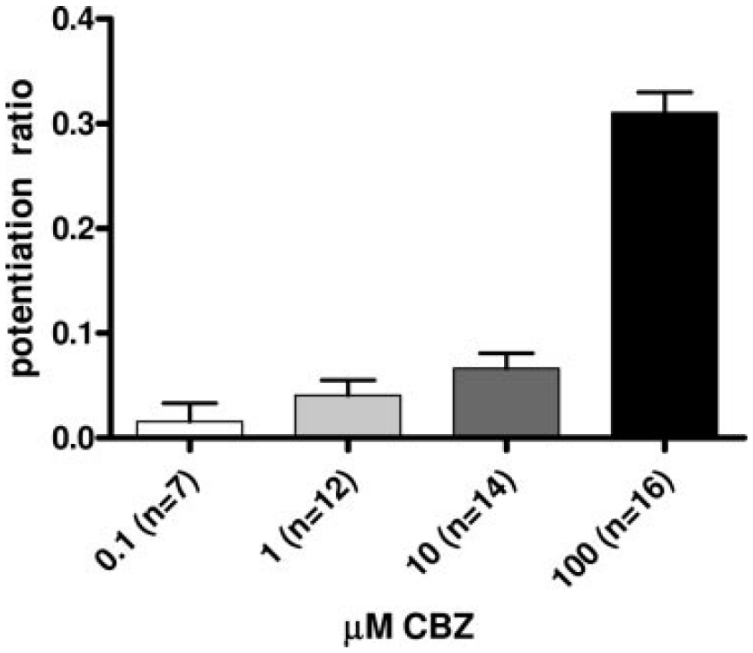

Figure 3.7 Dose dependent $GABA_A$ current potentiation by CBZ. Mean potentiation ratio (with SEM) of GABA induced current in human α1, β3, γ2L $GABA_A$ subunit containing receptors by 4 concentrations of CBZ (n values given in parenthesis.)

3.4 Discussion

The results of this study support our hypothesis that CBZ acts to aggravate absence seizures in GAERS by enhancing $GABA_A$ receptors in the VB thalamus. $GABA_A$ receptors have been a major focus in absence epilepsy research due to their importance in synchronisation and de-synchronisation of the TC circuitry. Recent *in vivo* neurophysiological studies in GAERS as well as the phenotypically similar genetic model, the WAG/Rij rat strain have demonstrated the critical role played by $GABA_A$ receptor, rather than $GABA_B$ receptor, activation in the generation of spontaneous SWDs (Pinault et al., 1998; Staak and Pape, 2001; Pinault, 2003).

Previous studies have demonstrated that the $GABA_A$ receptor antagonist, BIC, when injected systemically induce seizures in non-epileptic rats and enhance seizures in GAERS (Vergnes et al., 2000). Other $GABA_A$ receptor antagonists including penicillin and pentylenetetrazol have also been used to induce chemical models of absence seizures via systemic injections (Ostojic et al., 1997; McLean et al., 2004). In this study we found that the local microinjection of BIC into the VB thalamus did not affect the amount of seizures in GAERS, which is consistent the results of previous studies (Danober et al., 1998). The contrasting effect seen with the systemic injection of $GABA_A$ receptor antagonists is likely due to differential effects in different brain regions, with microinjections of BIC into the TRN shown to aggravate seizure in GAERS (Aker et al., 2002). Microinjection studies in animal models have also demonstrated contrasting effects of $GABA_A$ receptor agonists in different subregions of the thalamus. Injection into the VB thalamus of the GABA transaminase inhibitor, γ-vinyl GABA, which blocks breakdown of GABA and thereby increases its effect, has been shown to aggravate the absence-like seizures in GAERS (Liu Z et al., 1991). Conversely injection into the TRN increases intra-TRN inhibition and therefore reduces the inhibitory input into the thalamic relay nuclei, thus resulting in inhibition of SWDs. Similarly, microinjection of the $GABA_A$ receptor agonist muscimol into the VB region of the lh/lh genetic mouse model increased seizures, while injection into the TRN has the opposite effect (Hosford DA and Wang Y, 1997). It is therefore well established that drugs that enhance $GABA_A$ receptor activity within the VB aggravate experimental models of absence seizures. The results of this current study demonstrate that CBZ acts via this mechanism to aggravate absence seizures in GAERS also by enhancing $GABA_A$ receptor activity in the VB regions of the thalamus.

It had previously been shown that CBZ is positive allosteric modulator of $GABA_A$ receptors by single cell recordings in cultured cortical neurons (Granger et al., 1995). In this study we have re-confirmed, using neurophysiological recordings in-vitro in Xenopus oocytes expressing $GABA_A$ receptors, that CBZ has a dose-dependant effect to enhance the response of $GABA_A$ channels to the application of GABA (Figure 3.7). Our *in vivo* data in GAERS suggest that CBZ has a regionally specific effect on $GABA_A$ channels in the brain, acting on those expressed in the VB neurons but not those expressed on neurons in the TRN. Enhancement of $GABA_A$ receptor activity in the VB would be expected to result in hyperpolarization of the TC neurons. This

would promote de-inactivation of calcium T-channels, thereby enhancing oscillatory TC activity and absence seizures. In contrast, if CBZ were to act on $GABA_A$ channels in the TRN, inhibition within this structure would be enhanced, and GABAergic output to the VB reduced. The expected effect of this would be an inhibition of absence seizures as a result of the decreased $GABA_A$ receptor-mediated hyperpolarization of TC neurons.

Allosteric modulators act on binding sites on receptors different from the (orthosteric) sites activated by the primary agonists for the receptor. They, therefore, offer an increased opportunity for subtype-selectivity in their effects because of the greater divergence in the domains that compose allosteric sites than in the domains that interact with the primary endogenous agonist. The differential effects of CBZ on thalamic subregions may be explained by the molecular heterogeneity of $GABA_A$ receptors in the thalamus. The $GABA_A$ receptor is a heteromeric pentamer consisting of a combination of 8 classes of subunits (DeLorey and Olsen, 1992). Different combinations of these subunits produce a wide array of $GABA_A$ receptor subtypes with varying pharmacological properties. The subunit composition differs significantly in different regions of the TC circuitry. The thalamic relay nuclei, such as the VB, contain high levels of α1, α3-5, β2 and δ subunits and are virtually devoid of α3 and β3 subunits. In contrast the TRN is rich in α3, β1, β3 subunits and expresses very little α4, β2 and δ subunits and less α1 subunits than other brain regions (Pirker S et al., 2000). These different subunit combinations in these two regions present functionally distinct receptor types. Although not directly tested in this study, the results of the studies reported here would implicate α1, α4, β2 or δ as being important in CBZ action on $GABA_A$ channels in the VB.

Subunit composition of the $GABA_A$ receptors strongly influences the physiological and pharmacological properties of the receptor. One prominent example is the lack of GABA potentiation effect of clonazepam in the thalamic relay nuclei as compared to the TRN. This is due to major differences in α and β subunit distributions between the two regions which determines clonazepam selectivity due to the high level of benzodiazepine-insensitive $GABA_A$ channels expressing α4 and δ subunits in the thalamic relay nuclei while the TRN expresses higher levels of benzodiazepine-sensitive subunits, particularly γ2 and α3 (Pirker S et al., 2000;

Browne et al., 2001; Wong C G-T and Carter Snead III O, 2001). In contrast, the 3α-hydroxy ring A-reduced metabolites of progesterone, allopregnanolone and pregnanolone, which are powerful enhancers of $GABA_A$ receptor-mediated responses (Hawkinson JE et al., 1996), are well established to exacerbate absence seizures in the genetic rodent models (Budziszewska B et al., 1999; Snead et al., 1999). Microinjection studies have shown that these substances aggravate experimental absence seizures in the rat when injected into thalamic relay nuclei (i.e. the VB), while injection into the TRN had no effect (Banerjee PK and Snead OC III, 1998). This suggests that the mechanism of absence seizure aggravation by these progesterone metabolites may be via selective activation of a $GABA_A$ receptor subtype that is differentially expressed in the thalamic relay nuclei compared with the TRN. The results of our study suggest that CBZ may also be selective for $GABA_A$ receptor subunits differentially expressed within the VB as compared with the TRN. CBZ has also been shown in HEK 293 cells transfected with α1β2γ2 subunits to potentiate GABA-induced current but not the other subunit combinations tested (α3β2γ2 or α5β2γ2) (Granger et al., 1995). The direct effect of CBZ on $GABA_A$ receptors to enhance GABA action at physiologically relevant concentrations has been confirmed by the results of our studies *in vitro* in Xenopus oocytes (Figures 3.7 & 3.8). The α1,β2,γ2 subunit combination is expressed in the VB thalamus, but not at all in the TRN (Pirker S et al., 2000; Browne et al., 2001; Wong C G-T and Carter Snead III O, 2001). The β3 subunit included in the recombinant $GABA_A$ subunits tested in tour Xenopus oocytes gives essentially the same modulation of benzodiazepine as the β3 subunit. It is also possible that other subunits that show increased expression in the VB compared to the TRN, such as α4 and δ subunits, may be even more important in mediating the differential effect of CBZ on $GABA_A$ receptors – but this needs to be investigated in future studies.

Prior to extrapolating the results of a pharmacological study in an animal model to the human situation, it is important to have an understanding of the relevance of the doses examined relative to those used in clinical practice. The dose of CBZ administered i.p. (15 mg/kg) has been shown by our group and others to aggravate absence-like seizures in rats (Marescaux C et al., 1984; Micheletti et al., 1985; Wallengren et al., 2005) without causing significant sedation or other adverse behavioral effects. This dose has also been shown to be anti-convulsive in many models

of convulsive and limbic seizures (Graumlich et al., 1999). Additionally, the serum levels obtained by following the administration of this dose to rats (Graumlich et al., 1999), are within the ranges seen in human epilepsy patients treated with CBZ (Rambeck et al., 2006). The i.c.v. doses chosen were based on a previous pharmacokinetic study where microdialysis was used to determine plasma and cerebrospinal fluid concentrations of CBZ following i.p. injections at anticonvulsant doses in rodent epilepsy models (Graumlich et al., 1999). We initially performed a study of two different i.c.v. doses of CBZ (15µg and 30µg in 4µL of vehicle), demonstrating that the lower dose resulted in seizure aggravation without significant sedation, similar to those seen with the i.p. injections. For the intracerebral microinjections we use the same concentration of CBZ, but a lower volume (0.2µL). It is obviously difficult to determine the concentration of CBZ that neurons within the VB received following these microinjections, which would depend on the distance of the neuron from the epicentre of the injection and the extent of diffusion of the CBZ. However, the results of the study of Granger et al., (1995), and our new data from Xenopus oocytes expressing $GABA_A$ receptors, indicate that CBZ enhances $GABA_A$ activity at physiologically relevant concentrations (1–100 µM). These concentrations are in the range measured in CSF following the injection of 12mg/kg of CBZ to rats (i.e. 5µM) (Graumlich et al., 1999). Similarly, human studies have demonstrated CSF concentrations of CBZ in patients with chronic epilepsy of 7-14 µM, and brain concentrations of 38-69 µM (Rambeck et al., 2006). The amplitude of potentiation of $GABA_A$ receptor responses by CBZ was 30-50%, which is comparable to those obtained by partial benzodiazepine agonists and is therefore likely to affect function (Granger et al., 1995).

In summary, the results of this study have localised the VB as the specific subregion of the thalamus at which CBZ acts to aggravate absence seizures in a genetic rat model. Bilateral microinjections of BIC demonstrated that activation of $GABA_A$ receptors is critical to this action. We cannot exclude the possibility that CBZ is acting on a distinct target and the BIC blockade of the $GABA_A$ receptors inhibits the seizure aggravation because these receptors are involved downstream in the expression of the phenomenon. However, the weight of converging evidence from the in-vivo and in-vitro studies would make this explanation seem less likely. A better understanding of the cellular mechanisms of underlying seizure aggravation will aid an improved clinical selection of AEDs for individual patients, and guide more targeted drug design to

incorporate the beneficial anti-epileptic action without the seizure aggravating effects. The demonstration that CBZ is acting to aggravate seizures by a mechanism other than that which is believed to be its primary anti-epileptic action (i.e. a use dependant blockade of voltage-gated sodium channels) provides support for this proposition.

Chapter 4 Oxcarbazepine and not its active metabolite potentiate $GABA_A$ receptors and aggravate absences seizures in GAERS

4.1 Introduction

In Chapter 3, I have demonstrated that the mechanism underlying the CBZ aggravation of seizures in the genetic absence rats from Strasbourg (GAERS) involves a $GABA_A$ receptor-mediated action within the VB thalamus (Liu et al., 2006). Further *in vitro* studies using recombinant $GABA_A$ receptors in *Xenopus* oocytes showed direct dose dependent enhancement of GABA effects at these channels by CBZ (Liu et al., 2006). We predict that a structural analogue of CBZ that retains its primary anti-focal seizure Na^+ channel blocking properties without the $GABA_A$ receptor potentiating effect would reduce the likelihood of seizure aggravation.

Oxcarbazepine (OXC) is a structural (10-keto) analogue of CBZ (Figure 4.1) and one of the newer generation AEDs on the market. OXC is advocated to be overall better tolerated than CBZ with less drug-drug interactions, while maintaining efficacy against partial seizures (Schmidt and Elger, 2004). OXC is rapidly metabolized in humans to its monohydroxy derivative (MHD) which mediates most of its anti-epileptic effect (Schutz et al., 1986). The primary mechanism of action of OXC/MHD is thought to be via a use-dependant blockade of voltage-dependent Na^+ channels, similar to that of CBZ. However, these drugs differ in many aspects including their metabolism and pharmacodynamic neuronal profiles (Schmidt and Elger, 2004). The significant interaction of CBZ with calcium channels is thought to be mediated via L-type calcium currents (Ambrosio et al., 1999), whereas OXC or MHD exerts its effect via N-, P- and R-type calcium currents (Stefani et al., 1995). While there are reports that OXC may also aggravate generalized type seizures, in particularly myoclonus and absences (Gelisse et al., 2004; Chaves and Sander, 2005; Sazgar and Bourgeois, 2005; Vendrame et al., 2007), this anecdotally appears to be less common than with CBZ (Carignani and Rosso, 1997). The present study utilized a combination

of *in vitro* and *in vivo* techniques to investigate whether OXC or MHD differed from CBZ in their potency of effects on $GABA_A$ receptor function and propensity to aggravate seizures in GAERS. The individual effects of OXC and MHD were able to be studied *in vivo* as rats lack the enzyme that converts OXC to MHD in humans (Tecoma, 1999).

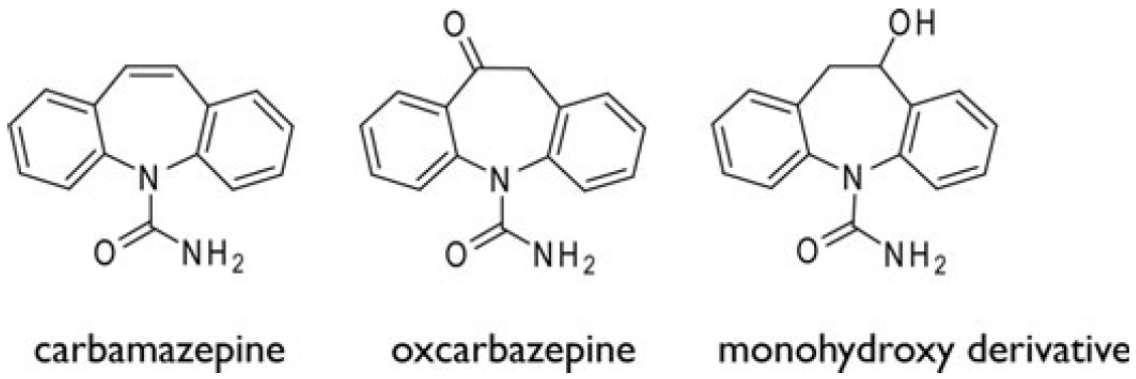

Figure 4.1 Chemical structures of carbamazepine, oxcarbazepine and its metabolite, monohydroxy derivative (MHD) (Adapted from (Zheng et al., 2009))

4.2 Materials and Methods

4.2.1 Examination of $GABA_A$ current modulation

Detailed protocol for oocyte preparation and electrophysiology have been described in Chapter 2, section 2.3. cRNA-encoding human α1, β2 and γ2L $GABA_A$ receptor subunits was injected into the cytoplasm of stage 5 or 6 oocytes using the Roboocyte Robot and stored at 18°C for 1 to 2 days prior to experimentation. For the two-electrode voltage clamp recordings, oocytes were impaled with two glass electrodes containing 1.5 M potassium acetate and 0.5 M KCl and held at a membrane potential of -80 mV and $GABA_A$ currents were measured. Oocytes were continually perfused with a ND96 solution (96 mM NaCl, 2 mM KCl, 0.1 mM CaCl2, and 5 mM HEPES, pH 7.5). Oocytes were perfused with a 20-s application of bath solution containing GABA (EC20; 6 x 10^{-6} M) followed by a 1-min application of bath solution alone in which the current levels returned to baseline levels. This GABA application was repeated three times to determine a baseline response to GABA. This was followed by a 20s application of solution containing both GABA (EC20; 6 x 10^{-6} M) and various concentrations of OXC, MHD and CBZ (1, 10, or 100 μM). The effect of the drug tested on the GABA induced current was then expressed as the relative change in EC20 GABA current caused by the addition of the drug according to the formula $(I_{GABA+drug} - I_{GABA})/I_{GABA}$ and termed the potentiation ratio.

4.2.2 Effect of OXC and MHD on absence seizures in GAERS

Protocols for animal surgeries and EEG recordings were described in detail in chapter 2, section 2.4. CBZ was obtained from Sigma-Aldrich (St. Louis, MO). OXC was a gift from Sinochem (Tianjin, China). MHD was a gift of Norvatis Pharmaceuticals (Basel, Switzerland). For the *in vivo* studies, all drugs were dissolved in 100% DMSO and then diluted such that the final vehicle for i.p. injections contained 10% DMSO, 40% propylene glycol and 50% saline. EEG recordings commence after the 7 day recovery period. After a 15 minute habituation, a 30 minute baseline recording was acquired. Drug/vehicle injections were then immediately administered intraperitoneally (i.p.). Ninety minute post-injection EEG recordings were then acquired. The second and subsequent treatment arms were performed at the same time of the day for each animal, with at least a 3-day interval between treatments. The order of the administered of the

treatments was randomized for each experiment. Criteria used to quantify seizures were described in chapter 2, section 2.3.5.

Statistical analysis was performed using the software package Statistica (StatSoft, Inc., Tulsa, OK). For the *in vivo* studies ANOVA for repeated measures was used to compare all four treatment arms, with a subsequent planned comparisons analysis to compare two specific treatments. For analysis of the *in vitro* data a Kruskal-Wallis one way analysis of variance by ranks was performed with the independent variables being the type of drug treatment and the concentrations and the dependant variable the potentiation ratio (i.e. the relative change in EC_{20} GABA current caused by the addition of the test drug). Dunn's multiple comparison procedures were used for posteriori comparisons. All data were expressed as mean ± S.E., and $p \leq 0.05$ was considered significant.

4.3 Results

4.3.1 OXC potentiates $GABA_A$ current in Xenopus oocytes similar to CBZ, while MHD is less effective

To investigate whether OXC and MHD affected $GABA_A$ current these compounds were applied at or below therapeutically relevant concentrations (Clinckers et al., 2005), in the range of 1 to 100μM, on recombinant $GABA_A$ receptors (α1β2γ2L) expressed in *Xenopus* oocytes. These results were compared with those obtained at the same concentration range for CBZ (Figure 4.2). Current records were averaged at each of the three concentrations tested. There was a significant difference between the treatment groups (Figure 4.2A, $p < 0.001$, Kruscal-Wallis one way analysis). Reproducible $GABA_A$ current potentiation by OXC was seen at all concentrations tested and was similar to that obtained with CBZ ($p > 0.05$, Dunn's Method). MHD had no effect on $GABA_A$ current at 1 or 10 μM and while there was some potentiation at 100 μM this was significantly less than that induced by CBZ and OXC ($p < 0.05$, Dunn's Method).

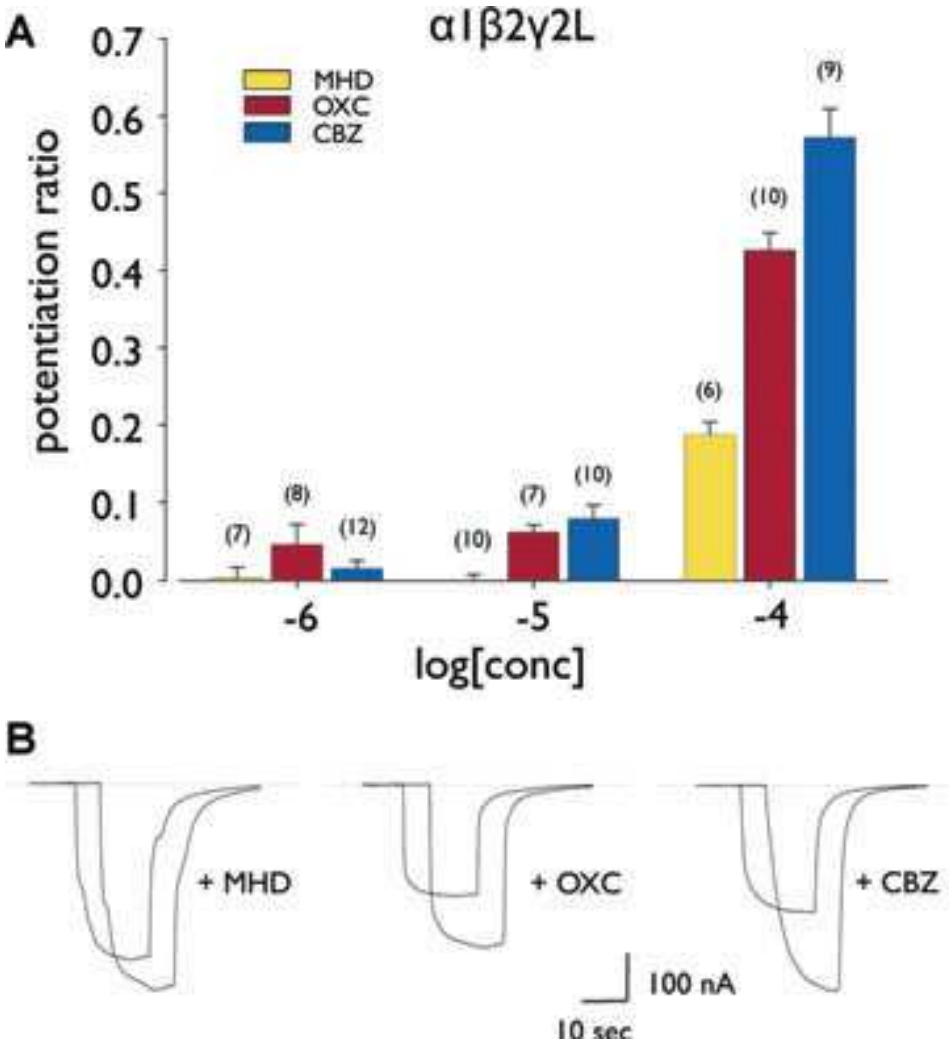

Figure 4.2 (A) Mean potentiation ratio (±SE) of GABA-induced current in human α1, β2, and γ2L $GABA_A$ subunit containing receptors expressed in Xenopus oocytes by three different concentrations of OXC, MHD, and CBZ (n values given in parentheses). There was a group difference between the treatments ($p < 0.001$, Kruschal-Wallis one-way ANOVA by ranks). Similar dose-dependent $GABA_A$ current potentiation was seen by OXC and CBZ ($p > 0.05$, Dunn's posttest), while MHD showed significantly less potentiation ($p < 0.05$, Dunn's posttest). (B) Typical current records showing the potentiation effect of MHD, OXC, and CBZ on GABA-induced currents in Xenopus oocytes expressing the a1b2c2L GABAA receptor subunit combination. The first trace of each figure shows the effect of GABA (EC_{20}; 6 x 10^{-6} M) alone, while the second, slightly offset trace shows the effect of MHD, OXC, and CBZ (10^{-4} M) on this GABA-induced response. (Published in (Zheng et al., 2009))

4.3.2 OXC aggravates absences seizures in GAERS similar to CBZ, but MHD does not

These experiments aimed to investigate whether systemic administration of either OXC or MHD aggravates absence seizures *in vivo* in GAERS, and if so how this compares to that induced by CBZ. Two cohorts of rats were studied, both of which received four sequential intraperitoneal injections. Both OXC and MHD are rapidly absorbed after i.p. injection, and the doses of the drugs were chosen on the basis of previous studies demonstrating effective anti-convulsive effects comparable to that of CBZ in a range of rat models following systemic injections (Kubova and Mares, 1993; Schmutz et al., 1994). Group A (n=8) was treated with CBZ (20mg/kg), OXC (15mg/kg), OXC (30mg/kg) and vehicle only. Group B (n=7) received OXC (30mg/kg), MHD (15mg/kg), MHD (30mg/kg) and vehicle only. For both groups the drugs were given in a random order. Repeated measures ANOVA showed a significant difference between the treatments for both Group A and B ($p=0.004$, $p<0.001$, respectively, repeated measures ANOVA). In the first cohort of rats, both doses of OXC aggravated seizures to a similar extent (149% and 178% of vehicle, respectively), and were not significantly different to the effect of CBZ (Figure 4.3A). Planned comparison analysis demonstrated a significant increase in the mean percentage time in seizures in the 90 minutes following the injections of CBZ and OXC at both doses tested. In the second cohort, OXC at 30 mg/kg was again shown to significantly increase percentage time in seizures. However, its human metabolite MHD had no effect on seizure quantity (Figure 4.3B). Behavioural observations during the post-treatment recording period suggest that all of the drugs tested caused a similar degree of mild drowsiness.

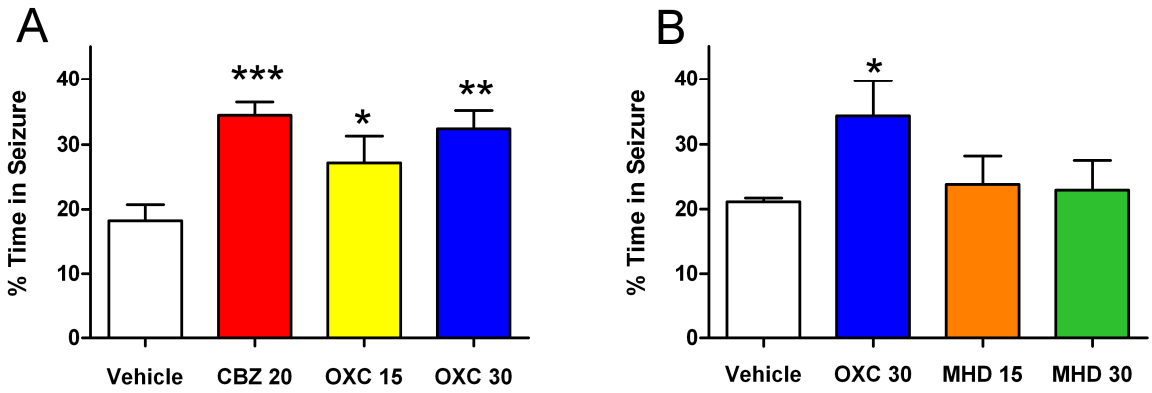

Figure 4.3 Mean percentage time in seizure (±SE) in GAERS over a 90 minute EEG recording following i.p. injection of (A) vehicle only, 20 mg/kg CBZ, 15 mg/kg OXC (OXC15), and 30 mg/kg OXC (OXC30) (n = 8) and (B) i.p. injection of vehicle only, 30 mg/kg OXC (OXC30), 15 mg/kg MHD (MHD15), and 30 mg/kg MHD (MHD30) (n =7). (A) Repeated measures ANOVA showed a significant difference between the treatment arms ($p = 0.003$), with planned comparison analysis showing that i.p. injections of CBZ, OXC (15 mg/kg), and OXC (30 mg/kg) were followed by significantly longer time in seizures than vehicle (***, $p < 0.001$; *, $p < 0.05$; *, $p < 0.005$, respectively). (B) Planned comparison analysis showing that i.p. injection of OXC (30 mg/kg) was followed by significantly longer time in seizures than following the MHD (15 mg/kg and 30 mg/kg) or vehicle treatments (*, $p < 0.05$). There was no significant difference between the two doses of MHD and vehicle (Published in (Zheng et al., 2009))

4.4 Discussion

This study demonstrate that OXC potentiates $GABA_A$ α1, β2 and γ2L receptors in *Xenopus* oocytes at therapeutically relevant doses and in a similar manner to that of CBZ on this receptor subunit combination, as well as that demonstrated previously CBZ on α1β3γ2L subunit combination (Chapter 3, see also (Liu et al., 2006)). In contrast, the clinically active metabolite of OXC, MHD had only a minor effect. Consistent with this, OXC in vivo aggravated absence-like seizures in GAERS to a similar degree to CBZ, while MHD did not. Humans metabolize OXC rapidly via cytosolic aldo-keto reductase to produce MHD (Lloyd et al., 1994). In contrast, it is reported that rats predominantly metabolize OXC by oxidative reactions producing little MHD (Feldmann et al., 1978; Wagner and Schmid, 1987). It is therefore possible, in rats, to compare the independent effects of OXC and MHD. Interestingly, OXC in vivo aggravated absence-like seizures in GAERS similar to CBZ, while MHD did not. Therefore, the use of MHD as a direct therapeutic agent, rather via the metabolism of OXC as is currently the situation, may provide the desired antiepileptic effect with less risk of seizure aggravation. This may represent a clinical advantage, especially for children in whom absence seizures and seizure aggravation are most prominent. In summary, our *in vivo* and *in vitro* data supports seizure aggravation of CBZ and OXC via $GABA_A$ receptor modulation which is absent for MHD.

These results are significant on three levels. Firstly, the study's findings indicate that the substitution of a monohydroxyl group at position 10 of the tricyclic ring that forms the common molecular structural component of CBZ results in a mitigation of the effect to potentiate $GABA_A$ receptors. This structural change alters the electrochemicophysical properties of the molecule, possibly by a 3-dimensional conformational change, so that it is less able to interact with $GABA_A$ receptors. Therefore this region is likely critical for the allosteric binding of tricyclic drugs to the $GABA_A$ receptor. Secondly it provides a further link between the presence of the $GABA_A$ potentiating effects of the tricyclic AEDs and the propensity to aggravate absence-like seizures, although this has not been directly tested in this study. Thirdly, it indicates that the clinical reports of the aggravation of absence seizures in patients taking OXC may primary result from circulating unmetabolised OXC rather than its MHD metabolite which

is responsible for the majority of its therapeutic antiepileptic action. It is important to note that despite the rapid conversion, 4-6 hours are needed for MHD to reach peak plasma concentration (Lloyd et al., 1994). As a result, significant amounts of OXC remain within the plasma, and 9% of the injected dose is excreted as glucuronide of OXC (Schutz et al., 1986). Therefore MHD administered directly could potentially be a drug that has efficacy for partial seizures without the generalized seizure aggravating effects of CBZ or OXC.

As described in chapter 3 of this thesis, CBZ aggravates absence seizures by enhancing $GABA_A$ receptor activity within the VB. Given the structural similarity of OXC to CBZ and its similar effects on recombinant $GABA_A$ channels in oocytes demonstrated in the current study, the possible mechanism of seizure aggravation by OXC is likewise by potentiation of $GABA_A$ receptors in the VB thalamus, although this was not directly tested. Whether GABAergic drugs exacerbate or suppress seizures depends on balance of actions at different sites within the TC circuitry. A relative enhancement of $GABA_A$ receptor activity within the VB thalamus is known to promote absence seizures (Liu et al., 1991; Hosford et al., 1997). In contrast, those that increase local inhibition within the cortex or the reticular thalamic nucleus will suppress seizures. The subunit combination of the $GABA_A$ receptors strongly influences the physiological and pharmacological properties of the receptor. Variation in the expression of different subunits between brain regions likely underlies their differential response to GABAergic drugs. The VB thalamus expresses high levels of benzodiazepine-insensitive $GABA_A$ channels containing α4 and δ subunits, while the TRN expresses higher levels of benzodiazepine-sensitive subunits, particularly γ2 and α3 (Pirker et al., 2000). This may explain why benzodiazepine drugs suppress rather than aggravate seizures. Whether such subunit specificity also exists for the CBZ-like drugs is uncertain, but is the subject of further research. The $GABA_A$ channels tested here consist of the human α1, β2 and γ2L subunits that are naturally expressed within the thalamic relay nuclei and are less abundant in other regions of the brain (Pirker et al., 2000).

Chapter 5 Carbamazepine increases thalamocortically generated spindle-like oscillations

5.1 Introduction

As reported in Chapter 3, *in vitro* experiments showed that CBZ potentiates Cl^- current through a direct interaction with $GABA_A$ receptors. In addition, in freely moving GAERS, CBZ aggravates absence seizures likely through an enhancement of $GABA_A$ receptor function within the VB thalamus. However, the underlying cellular and network mechanisms remain to be determined.

Work in this chapter aim to decipher the mechanisms of action of CBZ on $GABA_A$ receptor mediated activities in the somatosensory thalamus in GAERS and NEC rats. Electrophysiological cellular and network *in vivo* recordings were performed in rats under neuroleptanalgesia – an experimental condition that allows spontaneous generation of 5-9 Hz oscillations and SWDs that are also recorded in freely moving rats. Both oscillation types are regulated by $GABA_A$ receptor mediated mechanisms within the thalamus (Pinault 2006; Steriade, 1995). Therefore, the impact of CBZ on the dynamics of physiological and pathological TC oscillations could be assessed using these methods.

5.2 Methods

Animals, anaesthesia and surgery and histology protocols have been described in chapter 2, section 2.6

5.2.1 Electrophysiology

Glass micropipettes (8-13MΩ for field potential recordings, 25-70 MΩ for single cell extra or intracellular recordings) were filled with a solution containing 1.5 % N-(2 amino ethyl) biotin amide hydrochloride (Neurobiotin®; *Vector Labs, Burlingame, CA,*) dissolved in either 0.5 or 1 M CH_3COOK. The pipette was lowered with a stepping micro-driver (Burleigh, Fishers, NY, USA) into the somatosensory thalamus for field potential recordings or to reach a single TC or TRN neuron for extracellular and/or intracellular recordings (Figure 5.1). EEG recordings from the somatosensory cortex were simultaneously performed with each experiment. The recorded neurons were labeled with Neurobiotin using the juxtacellular labeling technique (Pinault, 1996). The location and morphology of the recorded cells were identified histologically. The location of the field potential recording sites was also identified histologically following extracellular iontophroretic application (500-600 nA, 200 ms on/200 ms off, 5-10 min) of Neurobiotin.

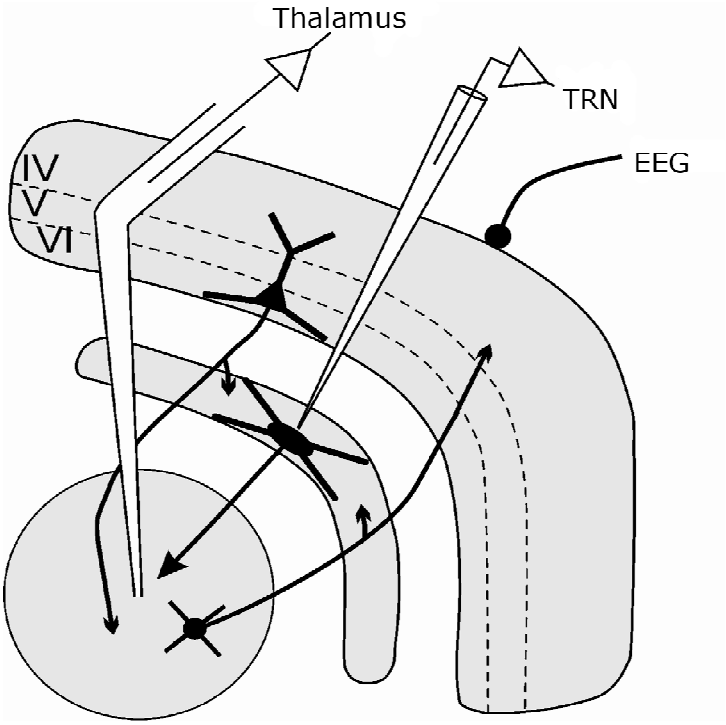

Figure 5.1. Experimental design showing the 3 principal interconnected structures that make up CTC systems: the somatosensory cortex, the TRN and the related thalamus.

5.2.2 Signal conditioning

Electrophysiological data were processed with band passes of 0.1-800 Hz for the EEG, of dc-6 kHz for cellular activity, and of 0.1-6 kHz for multi-unit recordings (Cyber-Amp 380, *Axon Instruments, Foster City, CA, US*). Signals were digitized at a sampling rate of 20kHz. During the intracellular recording session, a current pulse in the range of -0.2 to -0.5 nA was applied every 2 s to keep the Wheatstone bridge well balanced.

5.2.3 Pharmacology

CBZ was dissolved in a mixture of 10% DMSO, 40% propylene glycol and 50% saline no more than 30 minutes before injection. A subcutaneous injection volume of 5ml/kg was used for each

animal. For each experiment, rats receive both vehicle (5ml/kg) and CBZ injection (10mg/kg), separated by at least 2 hours. Recordings were performed following each of the injections.

5.2.4 Data analysis

Electrophysiological recordings were analyzed with the Axon software (*Clampex, v7, AxonInstruments*), and the tracer-filled neurons were examined with a light microscope (*E600, Nikon France, Champigny-sur-Marne, France*). The location of any marked cell was ascertained by referring to a stereotaxic atlas (Paxinos & Watson, 1986).

Fast Fourier Transformations (FFT) was computed using DataWave softwares (*SciWorks, v4, DataWave Technologies, Berthoud, CO, USA*). Total power of each frequency bands was based on 1.6-s epochs, with a resolution of 0.6 Hz, and was applied on at least 1-minute segments of EEG or extracellular field potential signals (re-digitized at a sampling rate of 2.5 kHz).

5.3 Results

5.3.1 CBZ suppresses absence seizures in GAERS under neuroleptanalgesia

Surface EEG recordings were performed in the somatosensory cortex of GAERS under neuroleptanalgesia. GAERS under the influence of neuroleptanalgesia display prolonged, absence seizure related SWD with an amplitude of >1 mV on the EEG (Figure 5.2). SWDs have an abrupt onset from medium voltage 5-9 Hz oscillations (<0.5 mV), which arise from desynchronized background EEG. The duration of each SWD episode can last from a few seconds to continuous SWDs, where seizures can be observed for tens of minutes. In contrast to data obtained in freely moving GAERS, the injection of CBZ (10 mg/kg s.c.) unexpectedly deceased the amount of typical SWDs. The onset of this effect varies between animals (10 and 30 minutes) and last for the duration of the experiment. Figure 5.2 shows representative trace of the global EEG events following CBZ injection. At 30 minute time point, SWDs could still be observed but appear to be disorganized and of small amplitude. At 60 minute time point, no more SWDs were observed. The occurrence of 5-9 Hz oscillations no longer develop into higher voltage SWDs and were interspersed by frequent medium voltage oscillations at spindles frequency (10-16 Hz) as well as slow waves (1-4 Hz).

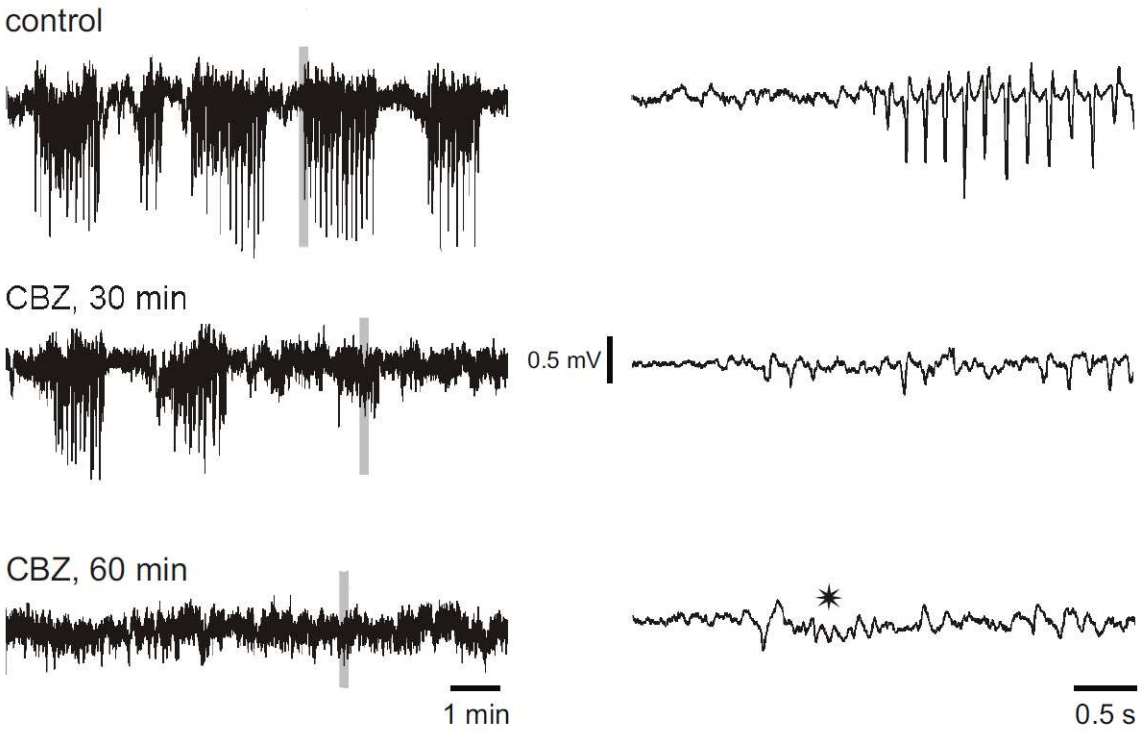

Figure 5.2 Carbamazepine abolishes absence-related SWDs in GAERS under neuroleptanalgesia. Surface EEG from the GAERS somatosensory cortex showing control, 30 minutes and 60 minutes after injection of CBZ (10mg/kg). Grey bars indicate 4s recording periods that are expanded on the right. Stars indicate spindle frequency oscillations.

5.3.2 CBZ increases the amount of cortical 5-9 Hz and spindle frequency (11-16 Hz) oscillations in GAERS and NEC rats under neuroleptanalgesia

Surface EEG recordings were performed in the frontal-parietal cortex of NEC rats (n=8). Recordings under control conditions show that rats alternated between small-voltage fast oscillations (<0.2mV) and medium-voltage slower oscillations (<0.5mV, <16Hz). The systemic injection of CBZ (10 mg/kg, s.c.) consistently transformed the desynchronized fast oscillations into the slower oscillations at frequencies of between 1 – 16 Hz. CBZ induced higher voltage slow waves (≥0.5 mV, 1-4 Hz), spindle-like oscillations (11 – 16 Hz) that are commonly accompanied by slow waves (1-4 Hz) as well as 5-9 Hz oscillations (Figure 5.3A). Spectral analysis was computed on periods of recordings of at least 1 minute for every 15 minutes before and after the injection of CBZ. The value of total power was extracted for the 5-9 Hz and 11-16 Hz oscillations and plotted against time. Fast Fourier transformation analysis (FFT) show that the broadening of the electrical signals after CBZ injection can be attributed to an increase in the amount 5-9 Hz oscillations (Figure 1 B1), as well as spindle frequency activity (Figure 1 B3). The power of both frequency bands is increased and lasted at least 90 minutes.

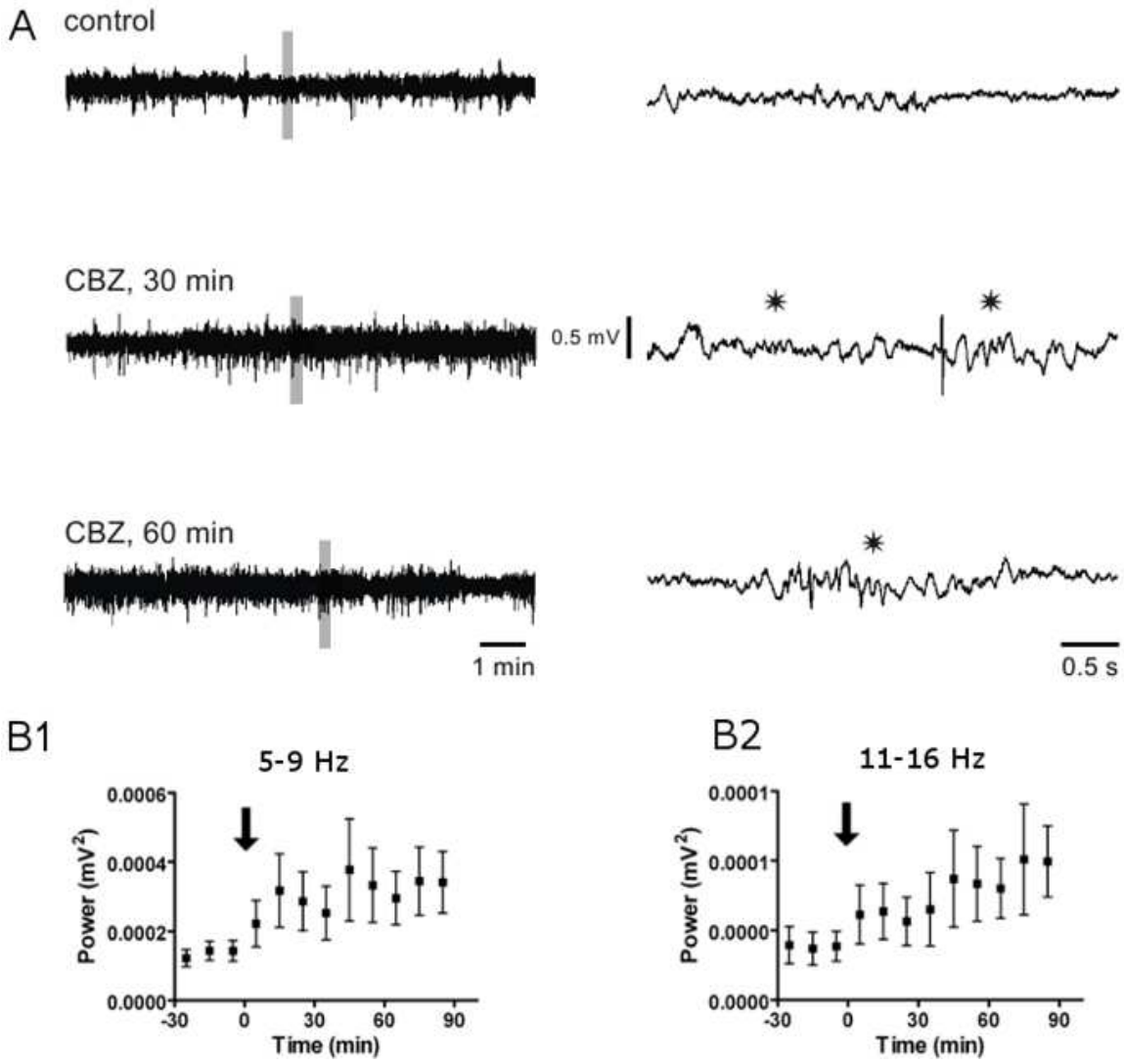

Figure 5.3 CBZ increases the amount of cortical 5-9 Hz and spindle frequency (11-16 Hz) oscillations in NEC rats under neuroleptanalgesia. A. Surface EEG from the NEC rat somatosensory cortex showing control, 30 minutes and 60 minutes after injection of CBZ (10mg/kg). Grey bars indicate 4s recording periods that are expanded on the right. Stars indicate spindle frequency oscillations. Note that spindle-like events can follow slower (~5-9 Hz) waves. B1-2. Spectral analysis showing the total power of 5-9 Hz and 11-16 Hz rhythm (averaged every 15 minutes) 30minutes before and 90 minutes after CBZ injection (Arrows indicate time in injection). Power of both frequency bands are concomitantly increased after CBZ injection (10mg/kg, s.c.)

5.3.3 CT 5-9 Hz oscillations interrupted by spindle frequency (11-16 Hz) oscillations in TC and TRN neurons

To identify the cellular correlates of the EEG oscillations, juxtacellular single-unit recordings of TRN neurons were performed. Two distinct types of TRN neuronal discharges were observed under control conditions: when the EEG displays a low-voltage, desynchronized state, TRN neuronal discharges consist of irregular, non-rhythmic single APs as well as occasional high frequency bursts of APs (7-13 APs at up to 500 Hz). During the occurrence of either 5-9 Hz oscillations or spindle-like oscillations in the related somatosensory cortex, TRN neurons displayed a rhythmic discharge pattern that was characterized by the occurrence of high-frequency bursts of APs that occurred on each cycle of the cortical oscillation on the EEG. These neurons may, on rare occasions, displayed a rhythmic firing pattern that commenced with 5-9 Hz AP bursts that commonly lasted 2-3 cycles and were then replaced by AP bursts in the spindle frequency of 11-16 Hz (Figure 5.4A). These stereotypical firing patterns were occasionally accompanied by oscillations of similar frequency on the surface EEG.

The TC neurons do not fire at every cycle of the TC oscillations. Therefore, intracellular recordings were performed to investigate the normal and CBZ induced changes in thalamic oscillations. The activity of the TC neurons mirrored that of the TRN neurons. Consistent with previous experiments (Pinault 2003), the intracellular 5-9 Hz oscillations of TC neurons consisted of rhythmic subthreshold depolarizing wave-hyperpolarizing wave (Figure 5.5A2). Similar to the TRN neurons, the 5-9 Hz and spindle frequency rhythmic events were readily distinguishable. The quicker, spindle-like oscillations were characterized by 11-16 Hz rhythmic hyperpolarizations that lasted no more than 1.5 seconds (mean, 0.82 ± 0.05 s).

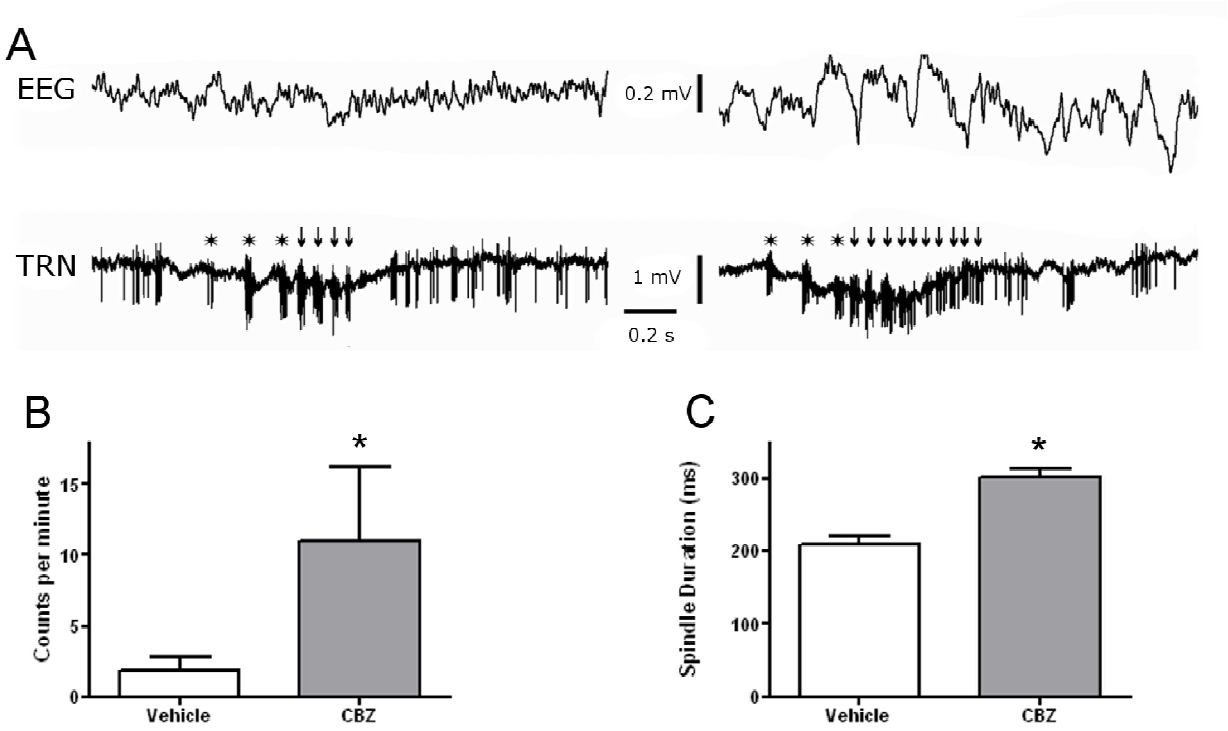

Figure 5.4. CBZ increases both the frequency of the stereotyped 5-9Hz-spindle (11-16Hz) sequences and the spindle duration in TRN cells**. A.** Simultaneous surface EEG from the somatosensory cortex and juxtacellullar recording of a TRN cell before (left) and 30 minutes after (right) CBZ injection. Stars indicate 5-9 Hz burst firing and arrows spindle frequency (11-16 Hz) burst firing. B-C. The frequency of the stereotyped 5-9 Hz-spindle sequences (B) and their duration (C) are significantly increased 30 minutes after CBZ injection (B. counts per minute, vehicle vs CBZ, n=5, $p = 0.03$; C. Spindle duration, vehicle vs CBZ, n=87, $p<0.001$).

5.3.4 CBZ increases both the frequency of the stereotyped 5-9Hz-spindle frequency (11-16Hz) sequences and spindle duration in TC and TRN cells

Recordings of the TRN neurons show that the stereotypical 5-9Hz-spindle oscillations rarely occurred under control conditions and were significantly increased in frequency of occurrence after injection of CBZ (1.85 ± 0.91 min^{-1} vs 11.03 ± 5.21 min^{-1}, n=5, p=0.03, figure 5.4B). The duration of the spindle frequency burst sequence was also increased after CBZ compared to vehicle (CBZ vs vehicle, 210.28 ± 11.0ms vs 302.39 ± 10.32ms, $p < 0.001$, figure 5.4C).

The intracellular events recorded in the TC neurons mirrored the extracellular events recorded in the TRN cells. The injection of CBZ induced the stereotypical 5-9 Hz-spindles firing pattern, where the commonly occurring 5-9 Hz oscillations are interrupted by spindle frequency events (Figure 5.5 A & B). The 5 -9 Hz oscillations continued for 2-4 cycles before being replaced by spindle-like oscillations characterized by a chain of rhythmic hyperpolarizations at between 11-16 Hz (Figure 5.5B2). Although spontaneous 5-9 Hz oscillations were relatively common under control and vehicle conditions, the occurrence of spindle-like oscillations were rare (2 out of 10 cells) and in most instances, immediately after 5-9 Hz oscillations (probability near 0). In the cells recorded after the injection of CBZ, 5 of 6 cells showed frequent spindle-like oscillations (11-16 Hz, up to 12 per minute) and in most instances, follow that of 5-9 Hz oscillations (maximum amplitude, 10.52 ± 0.54 mV). For both the 5-9Hz and the spindle-like oscillations, rhythmic waves occur during a hyperpolarization envelope that began at the onset of the 5-9 Hz oscillations and last till the end of the hyperpolarizing spindle train.

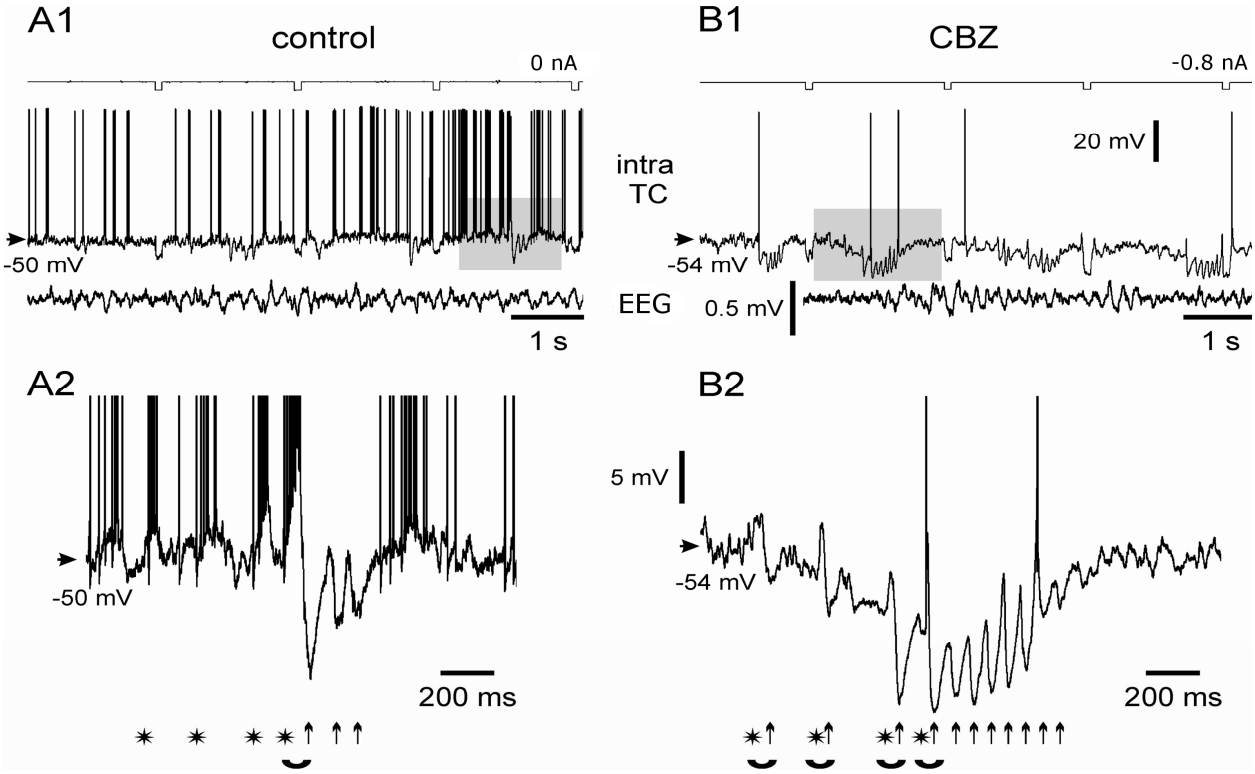

Figure 5.5 TC intracellular correlates of the stereotyped 5-9Hz-spindle sequences. A1 and B1: 8s intracellular recordings of a TC neuron along with the surface EEG of the related somatosensory cortex before (left) and after (right) CBZ injection, respectively. The grey areas are expanded in A2 and B2. A2 and B2: The expanded intracellular records show rhythmic (5-9 Hz) EPSPs (stars), 5-9 Hz EPSP-IPSP sequences (star-arrow sequences), and the following rhythmic (11-16 Hz) IPSPs (series of arrows). Note that the rhythmic spindle IPSPs (series of arrows) immediately followed the rhythmic 5-9 Hz EPSPs or EPSP-IPSP sequences (stars and star-arrow sequences).

5.3.5 In TC neurons the rhythmic 5-9Hz and spindle (11-16 Hz) IPSPs are reversed at a membrane potential of -65/-70 mV

Previous studies have indicated that both 5-9 Hz and spindle IPSPs were, in part, mediated by $GABA_A$ receptors (Pinault, 2003; Pinault et al., 2006). To investigate whether the CBZ induced 5-9 Hz-spindle sequences were a result of a similar $GABA_A$ receptor mediated mechanisms, hyperpolarizing current were injected into the recorded cells. The hyperpolarizing spindle-like IPSPs, whether they occurred alone or following the 5-9 Hz oscillations, were reversed by hyperpolarizing the neuron (~-67mV, Figure 5.6B). The oscillations became depolarizing when the membrane potential was further hyperpolarized. This is consistent with the reversal potential of Cl^- and thus implicating the involvement of $GABA_A$ receptor in the generation of spindle sequences (~-88mV, Figure 5.6C).

During 5-9 Hz EPSPs, the TC cell could generate an apparent low-threshold Ca^{2+} that triggered a high frequency burst of APs. Our results indicate that the low-threshold Ca^{2+} induced APs could also occur on top of a depolarizing current pulse applied from a holding hyperpolarizing current (Figure 5.6 D), as well as on a reversed 5-9 Hz IPSP (Figure 5.6 B2). On the other hand, during spontaneously occurring spindle oscillations, no TC cells displayed high-frequency bursts of APs induced an apparent low-threshold Ca^{2+} potential (Figure 5.6 C).

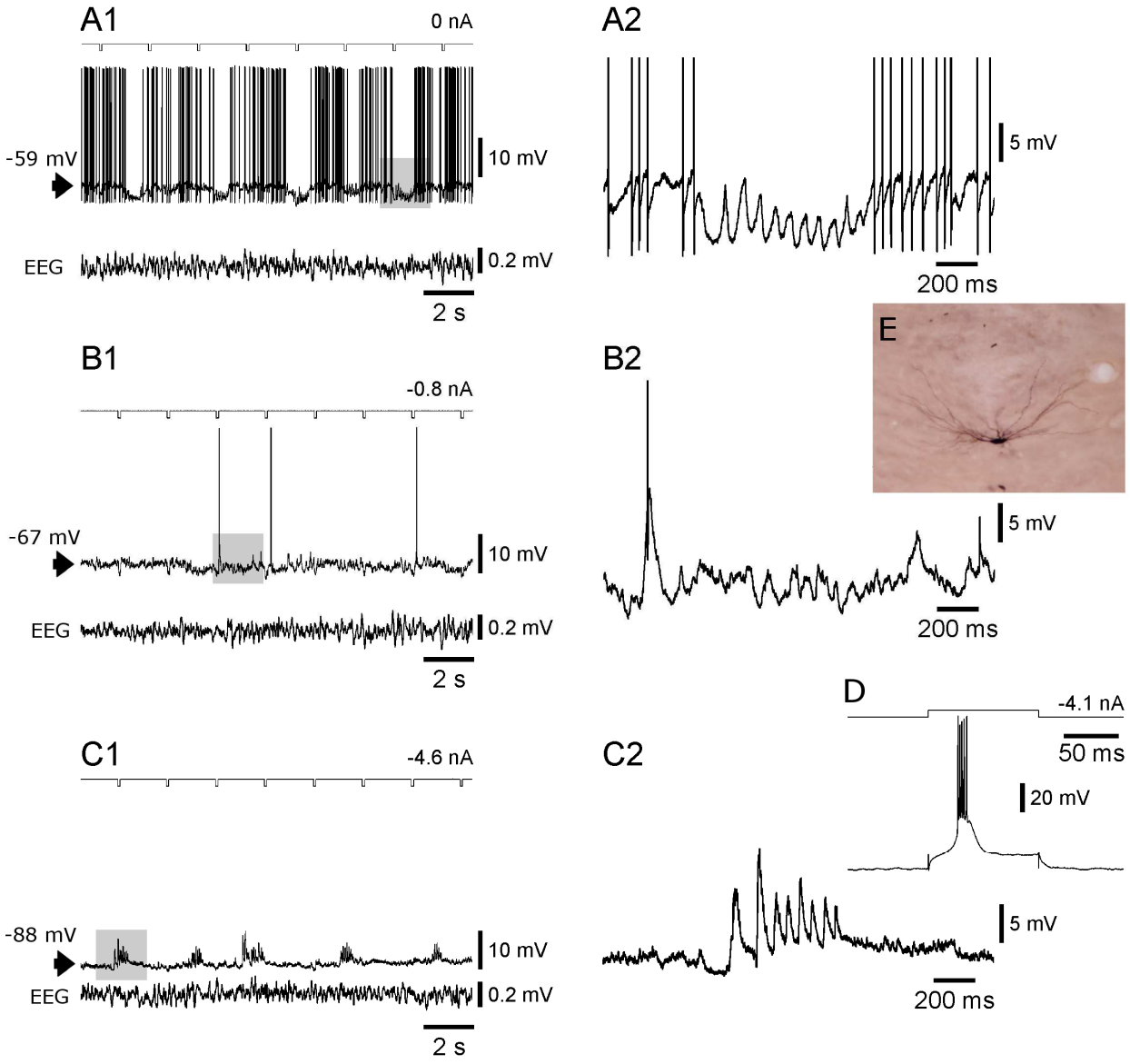

Figure 5.6 In TC neurons the rhythmic 5-9Hz and spindle (11-16 Hz) IPSPs are reversed at the same membrane potential. All the intracellular records (under CBZ condition) are from the same TC neuron that was stained at the end of the experiment (microphotograph). The holding current was changed during this recording session (0 nA in A1-2; -0.8 nA in B1-2; -4.6 nA in C1-2). The recorded IPSPs reverse at ~-65/-70 mV (B1-2). In A1, B1 and C1, the grey bands are expanded on the right panel (A2, B2 and C2, respectively). In B2, a 5-9 Hz EPSP triggers a low-threshold Ca^{2+} potential topped by an action potential discharge. D. A typical intracellularly labeled TC neuron revealed after histological processes. E. A depolarizing current pulse, triggered from a holding hyperpolarizing current, give rise to a low-threshold Ca^{2+} potential topped by a high-frequency burst of APs

5.4 Discussion

The present *in vivo* study in the rat thalamic system suggests that CBZ enhanced the triggering of the TC spindle-like oscillations, which are mainly underlain by rhythmic (11-16 Hz) $GABA_A$ receptor mediated IPSPs. These spindles are accompanied by slower rhythmic activities, including delta and the CT 5-9 Hz oscillations. Of importance, the thalamic spindle oscillations occur either in isolation, or immediately followed a few CT 5-9 Hz cycles, but were never followed by 5-9 Hz oscillations. This suggests that CT cycles evoked spindle frequency resonance in the thalamus. The CBZ induced changes in the global EEG and thalamic LFP were correlated with cellular firing patterns and membrane potential oscillations in the TC and TRN neurons.

5.4.1 CT 5-9 Hz oscillations trigger $GABA_A$ receptor mediated spindle frequency oscillations

Our results indicate that under CBZ influence, the commonly occurring 5-9 Hz oscillations trigger spindle frequency oscillations (11-16 Hz) in rats under neuroleptanalgesia. The cortically generated 5-9 Hz oscillations frequently occurred but lasted for 2-4 cycles before being interrupted by spindle oscillations. The cellular events underlying both 5-9 Hz and spindle oscillations have been well studied. While the two oscillation types may at times overlap in their frequency bands, they have intrinsically different underlying cellular mechanisms (Pinault et al., 2006). During 5-9 Hz oscillations, TC neurons exhibit synchronized depolarization events which are initiated with the summation of I_h currents that trigger an apparent low-threshold Ca^{2+} potential, which in turn, launches high frequency bursts of APs (McCormick and Pape, 1990; Pinault 2006). In contrast, the spindle oscillations have been demonstrated to originate from the thalamus (Morrison et al 1945) and are related to drowsiness and deep stages of sleep. The GABAergic, inhibitory TRN neurons are able to generate spindle frequency rhythmicity without external inputs (Steriade et al., 1985) and exhibit burst firing in a pacemaker-like manner to inhibit the TC neurons. Both *in vivo* and *in vitro* studies indicated that the subsequent spindle IPSPs observed in the TC neurons were due to activation of a Cl^- conductance, suggesting an important role for $GABA_A$ receptors in spindle generation (Steriade and Deschenes, 1984; von

Krosigk et al., 1993). As shown from current and previous data, the TC cells did not fire in a synchronized manner during spindle oscillations, and exhibit IPSP sequence with no apparent low threshold Ca^{2+} currents and no APs.

The CBZ induced transformation from 5-9 Hz to spindle oscillations could be a direct effect of CBZ on $GABA_A$ receptors. As reported in Chapter 3 and 4 of this thesis, CBZ aggravated SWDs in freely moving GAERS via its direct interaction with $GABA_A$ receptors (Chapter 3). *In vitro* data have also demonstrated that CBZ directly interacts with $GABA_A$ receptors to potentiate Cl^- flux (Chapters 3 & 4). Spindle oscillations have been shown to occur at a more hyperpolarized membrane potential than 5-9 Hz oscillations (Pinault, 2006). An interaction between CBZ and $GABA_A$ receptors within the thalamus is likely to result in a marked hyperpolarization of the TC neurons, promoting the occurrence of spindle-like oscillations which disrupted the 5-9 Hz activity in NEC rats and SWDs in GAERS.

The functional role of the 5-9 Hz-spindles sequence remains to be determined, but appears to reflect a pathological state induced by CBZ, as these sequences of events rarely occur under control conditions. As spindles interrupt 5-9 Hz activity and are thought to be related to sleep, the CBZ induced oscillatory sequences may be a brain state of induced drowsiness. Such sequence may be used as an interesting model to evaluate the action of therapeutic substances on $GABA_A$ receptor mediated events.

5.4.2 Possible mechanisms underlying CBZ-induced changes in the dynamics of the CT system

In both human patients and in a number of animal models, including freely-moving GAERS, CBZ is known to increase the expression of absence-related SWDs (Perucca et al., 1998; McLean et al., 2004; Wallengren et al., 2005) On the contrary, in rats under the influence of neuroleptanalgesia, the injection of CBZ is associated with the generation of $GABA_A$ receptor mediated spindle activity, which disrupts the occurrence of SWDs in GAERS and 5-9 Hz oscillations in the NEC rats. Therefore, current data suggest that the CBZ-induced $GABA_A$

receptor mediated spindle activity prevents the development of synchronized discharges from TC neurons with the cortical 5-9 Hz volleys and instead, triggers the thalamic resonance at the spindle frequency, resulting in incoherent behaviour between the TC and CT neurons.

The occurrence of frequent spindle oscillations induced by CBZ could occur as a result of the unique, pro-absence seizure brain state induced by the neurolept-analgesia cocktail which contains, amongst others, fentanyl (analgesic, opioid agonist) and haloperidol (dopamine D1 and D2 receptor antagonist), two drugs that penetrate the blood brain barrier. At the neruoleptanalgesic doses, the injection of fentanyl results in an initial suppression of SWDs followed by a prolonged increase (Inoue et al., 1994). Haloperidol is known to act on both dopamine D1 and D2 receptors to cause a dose-dependent increase in absence seizures (Warter et al., 1988). The combination of the drugs results in SWDs of high frequency and duration in GAERS, and at times, absence status epilepticus. This indicates a highly synchronous, pro-absence state of the TC circuit, where SWDs is associated with a long lasting hyperpolarization trough in both TC and TRN neurons (Pinault et al., 2006). When these cells are about to display spindle-like oscillations, their membrane potential are even more hyperpolarized compared to normal or epilepsy related 5-9 Hz oscillations (Pinault et al., 2006). Therefore, the enhancement of $GABA_A$ receptor activity by CBZ would result in a more marked hyperpolarization of the TC and TRN neurons than those under neuroleptanalgesia, switching the brain from the pro-absence seizure, 5-9 Hz oscillatory state to a state associated with common occurrence of spindle oscillations.

The CBZ induced occurrence of spindle-like oscillations, as well as the slow wave and 5-9 Hz activity suggest that CBZ maintains the TC system in a state closely related to that of drowsiness and early stages of sleep, since both wake-related 5-9 Hz oscillations and sleep-related spindles were recorded. The recorded spindle-like oscillations were short lasting (<1.5s) and occurred in conjunction with high voltage slow waves (1-4 Hz) that are thought to be hallmarks of sleep episodes (Niedermeyer, 1982), but rarely from desynchronized EEG. This is in agreement with previous studies that recorded spindle oscillations in sleeping rats but were absent during wakefulness (Gandolfo et al., 1985; Buzsaki et al., 1990). One of the severe adverse effects of

CBZ is its likelihood to cause sedation and drowsiness in humans (Pandey et al., 2005). As previously mentioned (Chapter 3), a high dose of CBZ (30mg/kg, i.p.) may also result in drowsiness and sedation in freely moving rats. These evidence suggest that the CBZ induced spindle-like oscillations are likely to be related to drowsiness or sleep, where the CBZ induced, excessive TC spindle events over-ride the CT 5-9 Hz activity, switching the brain from the wake to sleep-related state.. Further experiments in freely moving rats are required to validate and ascertain the physiological effect of the CBZ induced 5-9 Hz-spindle oscillation sequences.

5.4.3 Thalamic spindles prevent development of synchronized 5-9 Hz oscillations and SWDs

In GAERS, wake-related CT 5-9 Hz oscillations are more pro-epileptogenic than sleep-related spindle oscillations (Pinault, 2006). This is in contrast with previous studies in ferret thalamic slices and the FGPE model, where spindle-like oscillations are thought to be able to transform into absence-related SWDs (von Krosigk et al., 1993; Kostopoulos, 2000). As demonstrated in the current study, no more SWDs occur in GAERS when 5-9 Hz oscillations are interrupted and replaced by spindle frequency oscillations. This provides further evidence that absence-related SWDs cannot arise from spindle oscillations and that synchronized 5-9 Hz oscillations are pro-epileptogenic and required for the generation of SWDs.

In the present study, spindle-like oscillations occurred either in conjunction with slow waves, or immediately followed that of the 5-9 Hz oscillations under CBZ influence, while the reverse never occurred. Under control conditions, GAERS exhibit spontaneous absence-related SWDs and the NEC rats exhibit medium voltage 5-9 Hz activity under both neuroleptanalgesia (Figure 5.2A) as well as in the undrugged, freely moving state (Pinault 2001). These 5-9 Hz activity are thought to be more wake-related and are phase lead by layer VI cortical-thalamic neurons, before they can be detected in the thalamus, indicating the origin of these normal physiological rhythms reside in the cortex (Pinault 2003). The 5-9 Hz rhythms is not coherent with the hippocampal theta rhythm, indicating that they are not linked with the intrinsic oscillations of the limbic

system (Pinault et al., 2001) and could be a sensorimotor rhythm important in tactile functions that may correspond to the human μ rhythm (Wiest and Nicolelis, 2003).

After the injection of CBZ, our results suggest that the increase in the power of 5-9 Hz frequency band may be due to an apparent increase in frequency of occurrence but not the duration of these oscillations. More importantly, under CBZ influence, the 5-9 Hz oscillations are always short lasting and commonly last 2-3 cycles (<1 second) and are frequently disrupted by the $GABA_A$ receptor mediated spindle oscillations, resulting in a stereotypical firing patterns that are rarely observed under control conditions.

5.4.4 Therapeutic implications and side effects of CBZ in epilepsy and psychiatric disorders

Despite the effective use of CBZ in the treatment for focal seizures, the mechanisms underlying significant side effects of the drug such as drowsiness and severe alteration of sleep architecture (Levy et al., 1985; Yang et al., 1989) remains unclear. Pharmacotherapies that induce drowsiness or sedation, whether as its primary therapeutic mechanism of action or an adverse effect, commonly involve $GABA_A$ receptor mediated neurotransmission. This diverse class of drugs includes benzodiazepines, barbiturates, ethanol and several anaesthetics that have been used either therapeutically or as experimental tools to study sleep physiology. $GABA_A$ agonist such as muscimol are known to induce sleep that is associated with increase in slow waves (0.5-4 Hz) and the generation of spindles (11-16 Hz) in rats (Lancel et al., 1996). Treatments for insomnia have also been dominated by positive modulators of $GABA_A$ receptors such as barbiturates and benzodiazepines, which act at different binding sites on $GABA_A$ receptors to increase spindle frequency activity (Winsky-Sommerer, 2009). The physiological occurrence of these hyperpolarizing spindle oscillations has been well characterized in both *in vitro* brain slices (von Krosigk et al., 1993) and intact brains (Contreras et al., 1997) to be mediated by the activation of $GABA_A$ receptors. Our current study showed that the CBZ induced increase in EEG spindles and the correlated increase spindle-like IPSPs in the TRN and TC neurons and are likely due to enhancement of $GABA_A$ receptor activity. Although the CBZ binding site on $GABA_A$ receptors

is not known, it is demonstrated to be a positive allosteric modulator on $GABA_A$ receptor subtypes that naturally expressed in the thalamus (Granger et al., 1995; Liu et al., 2006; Zheng et al., 2009). As spindle activities are commonly associated with drowsiness or early-phase of slow wave sleep (Gandolfo et al., 1985; Contreras et al., 1997), the sedative adverse effect caused CBZ are most likely due to its direct action on $GABA_A$ receptors.

Aside from its primary use as an anti-convulsant, CBZ is also extensively used in the treatment of both neurological and psychiatric disorders (Lerer et al., 1985). In patients with schizophrenia, CBZ have been used as an adjunct to neuroleptic medications and is effective against symptoms such as violent outbursts, hyperactivity and affective symptoms (Hosak and Libiger, 2002). The mechanism by which CBZ affects schizophrenic symptoms is poorly understood. Early studies have indicated that the diverse actions of CBZ, including its reduction of noradrenaline and dopamine turnover (Post et al., 1992), as well as its effect on GABA receptor systems (Bernasconi, 1982), may play a role in its antipsychotic effects. Interestingly, a recent high density EEG investigation revealed significant deficits in spindle oscillations in schizophrenic patients (Ferrarelli et al., 2007). The present study strongly suggests that the mood stabilizing action of CBZ could operate in part, through the enhancement of the $GABA_A$ receptor mediated TC activities, including the spindles oscillations.

Chapter 6 Precursor rhythmic neuronal activities in S2 somatosensory and insular cortices during the initiation of genetically determined absence-related SWDs

6.1 Introduction

The neural mechanisms underlying the generation of absence-related SWDs have been the subject of many in vitro (McCormick and Contreras, 2001) and in vivo studies (Gloor, 1968; Vergnes et al., 1990; Meeren et al., 2002; Pinault, 2003), giving rise to several concepts that are still widely debated. Recently a body of evidence from genetic rat models of absence epilepsy has suggested that seizures arise from the primary somatosensory (S1) neocortex, more specifically from a topographically localized "focus" in Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats (Meeren et al., 2002) and in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) (Polack et al., 2007). Using planar multi-site EEG recordings from the rat cortical surface and non-linear association analysis, Meeren and colleagues reported that the focus was located in S1 where nose and upper lip are represented. However, close examination of the figures from this study indicates that the proposed focus was stereotaxically located more lateral and ventral than S1 (Paxinos and Watson, 1998), corresponding to the secondary somatosensory cortex (S2). Using the GAERS model, Polack and colleagues used an electrophysiological approach aimed at comparing precursor activities between S1 and the primary motor cortex. They concluded that the focus was in S1 and that SWDs were initiated in layer V (Polack et al., 2007). In contrast, studies using paired cortical and thalamic juxtacellular recordings demonstrated that S1 layer VI CT neurons, which are anatomo-functionally different to layer V pyramidal cells, play a leading role in the generation of SWDs triggering synchronized rhythmic activities in related TC and TRN neurons (Pinault et al., 2001; Pinault, 2003). Furthermore, Pinault (2003) demonstrated that in S1 layer VI neurons fire before layer V neurons during the generation of absence-related SWDs, and that pre-ictal 5-9 Hz rhythmic activity occurs first in layer VI of S1 relative to the interconnected somatosensory thalamus.

Despite these published studies, the cellular and network neuronal activity in the somatosensory cortical region that leads to the onset of the seizure activity remains uncertain. Therefore the major aim for this study was to characterize, in GAERS, spatio-temporal dynamics of intra-cortical network and cellular activities in S1, S2 and the insular cortex (IC) before and during seizures.

6.2 Methods

The experimental strategies used in this chapter include multi-electrode EEG recordings and intra-cortical electrical stimulation in freely moving rats (Melbourne, Australia) and dual electrode single-cell or local network recordings in rats under neurolept-anaesthesia (Strasbourg France). Both methods have been described in detail in Chapter 2, sections 2.5 and 2.6, respectively.

6.2.1 Animals

Experiments were conducted in inbred adult male Wistar rats (30 GAERS and 13 NEC rats). All procedures were approved by the University of Strasbourg and the University of Melbourne Animal Ethics Committee (AL/01/23/11/07, AEC #0810999, respectively) and performed in accordance with the guidelines published by the Australian NHMRC and European Union for use of animals in research, where applicable. All rats were progenies of the original GAERS and NEC rat strains from Strasbourg, and were born and housed in standard conditions.

6.2.2 Multisite depth EEG recordings and electrical stimulation in freely moving rats

Rats were chronically implanted with insulated depth electrodes using stereotaxic guidance into the S1FL, S1ULp, S2 of the somatosensory cortex, insular cortex (IC) and primary motor cortex (M1) (Figure 6.1 A). Two additional extradural electrodes were implanted into the parietal bone (AP -4 mm) as ground and reference points. EEG recordings and electrical stimulations were

performed via the same electrodes. Details of the methods are described in Chapter 2, section 2.5.

6.2.3 Electrophysiological recordings under neuroleptanalgesia

Experiments on rats under neuroleptanalgesia were performed in 25 GAERS and 10 NEC rats. The neuroleptanalgesia set the brain in a state that electrophysiologically corresponded to quiet immobile wakefulness, altering between desynchronized and synchronized episodes, allowing the spontaneous occurrence of SWDs (Pinault et al., 2001). All rats undergo penile vein catheterization for the ease of intravenous injections, tracheotomy for the control of ventilation throughout the experiments and a stabilizing craniotomy and duratomy procedures for the increased precision of reaching single neurons in deep brain structures and optimized recording conditions (Pinault, 2005). The EEG, heart rate and body temperature of the rats were continuously monitored and maintained at physiological level throughout the experiment.

For electrophysiological recordings, glass micropipettes (15-50 MΩ) were filled with a solution containing 1.5% N-(2 amino ethyl) biotin amide hydrochloride (Neurobiotin, Vector Labs, Burlingame, CA, USA) dissolved in 1M CH3COOK. Pipettes were connected to an intracellular recording amplifier (NeuroData IR-283; Cygnus Technology Inc., Delaware Water Gap, PA< USA) and were lowered with stepping micro-drivers (Burleigh, Fishers, NY, USA) to reach neurons in S1, S2 and IC. Data were processed with bandpasses of 0.1- 800 Hz for the EEG and LFP, and 0.1-6000 Hz for cellular activity (Cyber-Amp 380; Axon instruments, Foster City, CA, USA). A sampling rate of >2.5 kHz were used for the EEG and LFP, 20 kHz for single cell recordings per channel (Digidata 1200B; Axon Instruments), using pClamp 10.2 (Axon instruments). All recordings were performed simultaneously with the surface S1 EEG.

LFP recordings were performed with glass micropipettes (tip diameter 4-8 μM). A single pass as made through the S1, S2 and IC regions and recordings performed at multiple depths between 1000 and 6000 μm (AP -0.2mm, ML +4.1-4.2mm, angle 20°), in 3 GAERS and 5 NEC rats. Paired LFP recordings were performed in the S1 (AP -0.2, ML +4.8mm, angle 0°, DV 1.8-2.0mm) and S2/IC (AP -0.2, ML +3.4mm, angle 20°, DV 4.9-5.1mm) in 7 GAERS. The location

of the recording sites was identified histologically following extracellular application of Neurobiotin (500-600nA, 200ms on/off current, 5-10 min). Dual extracellular single-unit recordings of somatosensory cortical cells were performed in 15 GAERS and 5 NEC rats. At the end of the recording sessions, neurons were individually labeled using the juxtacellular technique (Pinault, 1996). In all single cell recording experiments, the juxtacellular labeling procedure was applied only in the last two recorded neurons. At the end of the experiments, rats were euthanized, transcardially perfused and the brains processed using standard histological techniques as described in Chapter 2, section 2.6.4. Tracer-filled regions and/or neurons were examined with a light microscope and the location of the marked region and neurons were ascertained by consulting the rat brain atlas (Paxinos and Watson, 1998).

6.2.4 Data Analysis

EEG recordings were reviewed with the Compumedics system (Melbourne, Australia) and cellular electrophysiological recordings with Axon software (Clampex, v10.2; Axon instruments). Event detections and autocorrelograms of multi- and single unit activity were computed using DataWave software (SciWorks, v5.2; DataWave Technologies, Berthoud, CO, USA). Data are presented as means ± s.e.m. and were evaluated for statistical significance with appropriate tests with the significance level set to 0.05.

6.3 Results

6.3.1 Pre-ictal oscillations in S2/IC cortical areas lead S1 and motor cortical regions at the onset of generalized SWDs

Multi-site depth EEG recordings in freely moving GAERS revealed typical generalized SWDs at 5-9 Hz accompanied by sudden behavior arrests. In 60% of seizures (mean, 60%, range 30-85%, 20 SWDs examined from each rat, n=5), 5-9 Hz medium voltage (0.2-0.5 mV) oscillations were recorded from 1-2 electrodes during the interictal-ictal transition. In 4 and 1 out of 5 rats pre-ictal oscillations were initially seen at the S2 and in IC electrodes, respectively.. Oscillations were then detected progressively, over 1-2 seconds, from the S1ULp, S1FL electrodes and then the M1 electrodes as the discharge transformed into a typical, high-voltage (>1mV) generalized SWDs, which were considered to be the hallmark of the absence-like seizures in GAERS (Figure 6.1B). Natural 5-9 Hz oscillations began in average 1.40 ± 0.08 s (maximum 3.7 s) before the first EEG SW complex. In the other 40% of seizures (40 of 100; range 15-70%, 20 SWDs examined from each rat, n=5), SWDs were observed to start abruptly and simultaneously from all recording electrodes from both hemispheres with no apparent temporal difference between the recorded regions.

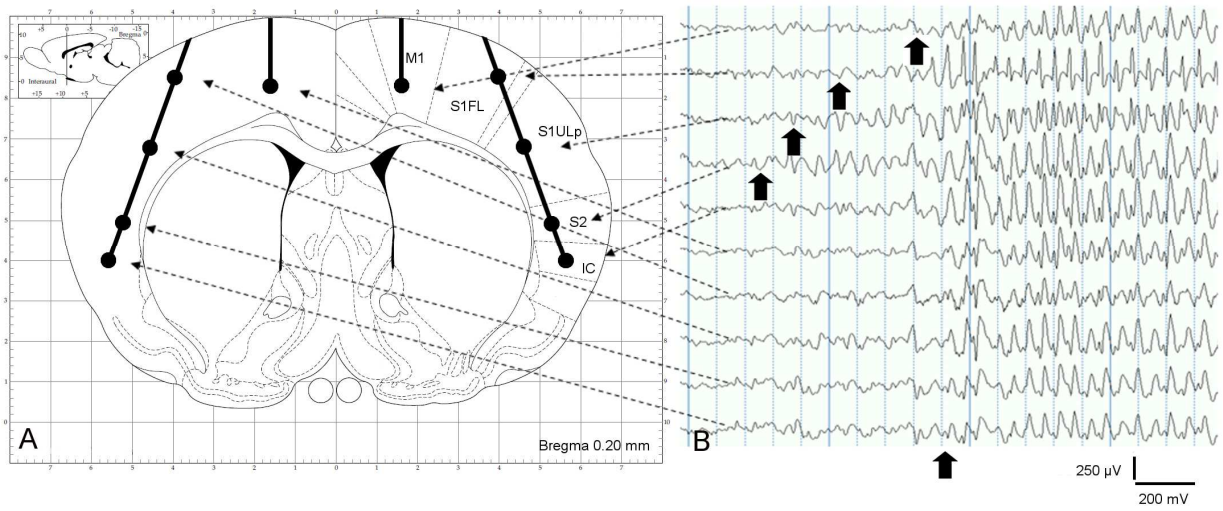

Figure 6.1 SWDs are first detected within the S2 somatosensory cortex. A. Sites of electrode placements of the multi-site EEG recordings acquired in freely moving rats (Atlas adapted from Paxinos and Watson). Electrode coordinates with reference to bregma - S1FL, S1ULp, S2 and IC entry point: Anteroposterior (AP), +0.2mm, medio-lateral (ML), ±3.6mm, angle 20º. Bilateral M1 electrodes: AP +1.0mm, ML±2.6mm B. Typical EEG recording trace demonstrating the oscillatory rhythmic discharge commences unilaterally in at the one S2 electrode (arrowed) before spreading superiorly to the S1ULp and inferiorly to the IC electrodes, and then to the S1FL and then finally the M1 and the electrodes in the contralateral hemisphere.

To further investigate the timing of the onset of oscillatory activity in S2/IC versus S1 during the generation of SWDs in GAERS, paired LFP or single neuron recordings were performed simultaneously in S1 and S2/IC along with the EEG over the frontoparietal cortex in neurolept-analgesied rats (Figure 6.2C, n=7). After filtering (300-800 Hz), LFP recordings revealed short-lasting high frequency oscillations (HFOs, 400-600 Hz) in S1, S2 and IC, which always appeared before each SW complex on the EEG (Figure 6.2). Of importance, HFO in S2/IC occurred at each cycle of the SWD ~20 ms before those in S1 (Figure 6.2B,C. -66.4 ±2.8ms vs -40.7 ±2.4ms; t-test, $p<0.001$, n=7), time zero being the negative component of the SW complex recorded in the surface EEG.

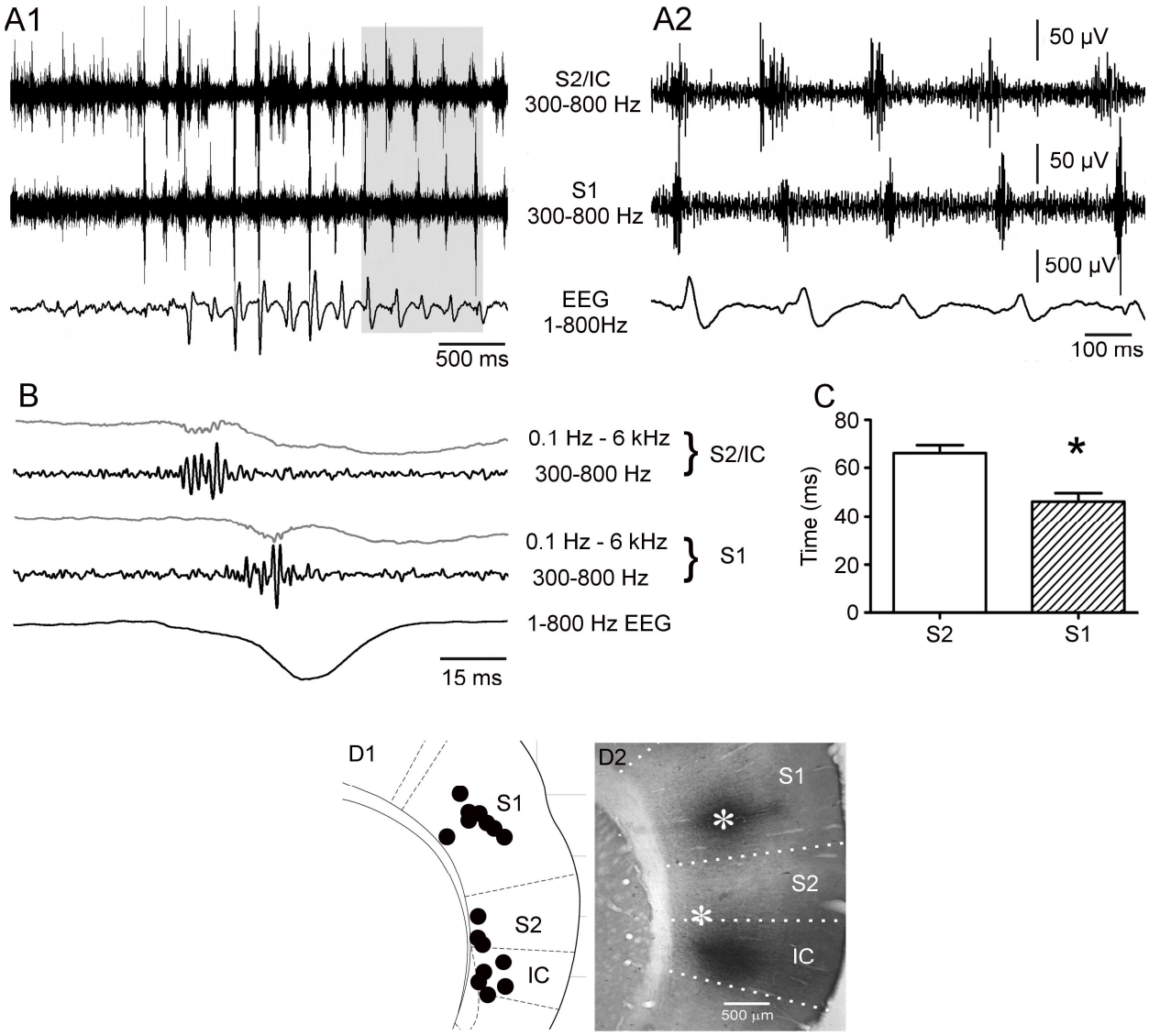

Figure 6.2 High frequency oscillations (HFO) occur in S2/IC before S1 during seizures. A1. Trace of filtered (300-800 Hz) local field potential (LFP) recordings of S2/IC and S1 and EEG of the related cortex. Grey band show a 1 second recording expanded in A2. HFOs occurs earlier in S2 compared to the S1 region (-66.4 ± 2.8ms vs -40.7 ± 2.4ms, with reference to surface EEG, n=140, t-test, $p<0.001$). B. A single spike during a typical seizure showing non-filtered (grey, 0.1 Hz to 6 kHz) and filtered (black, 300-800Hz) LFP from S2/IC and the S1; C. Time between the onset HFO in S2/IC and S1 prior to the EEG spike (* t-matched pair t test, $p<0.001$); D1. Site of paired S1 vs S2/IC recordings were labeled on a rat brain atlas. D2. An example of histological staining revealing site of recording (white stars).

6.3.2 Typical SWDs can be triggered following a brief train of 7 Hz electrical stimuli in the cortex of GAERS at a lower intensity in S2 than S1

The existence of precursor neuronal activities at least in S2 and IC relative to S1 and motor cortex suggests that these regions may be more hyperexcitable. To test this we applied a 2 s train of 7-Hz electrical stimuli (see methods) in each of the cortical regions. In GAERS, SWDs were consistently evoked by a single stimulation train delivered to all 4 cortical regions. The SWDs evoked by the stimulation were identical in morphology and internal frequency to that of typical, spontaneously occurring absence seizures (mean cycle frequency of spontaneous vs. evoked, 7.3 ± 0.1 vs 7.6 ± 0.1 Hz, n=20, p=0.15, Figure 6.3A-B). The duration of the evoked SWDs was also not significantly different to that of the spontaneous events (mean, 12.0 ± 1.35 vs 9.5 ± 0.79 s, p=0.12, Figure 6.3C). The evoked SWDs were accompanied by the same behavioral characteristics, including immobility and head-nodding, to those of spontaneous seizures. It is noteworthy that the stimulation train only induced seizures when rats were in a state of immobile quiet wakefulness accompanied by desynchronized EEG, and not when they were active or sleepy (i.e. stimulations evoked SWDs in the state that spontaneous seizures occur in GAERS). Of importance, a significantly smaller current was required to evoke a self sustained SWD seizure within the S2 region when compared to the adjacent S1ULp region (146 ±31 μA vs 257±56, p=0.02, Figure 6.3D). There was no difference in the current needed to stimulate seizures in S2 and IC (146 ±31 μA vs 179 ±27 μA, p=0.67).

Identical stimulation testing were carried out in NEC rats (100-600 μA, n=3). Stimulation trains could evoke dampening 2-3 cycles (<0.05 s) medium-voltage oscillations immediately followed by the baseline EEG resembling that recorded before stimulation (Figure 6.3E). No self sustained SWD seizures or behavioral manifestations could be induced by the stimulations in the investigated NEC rats.

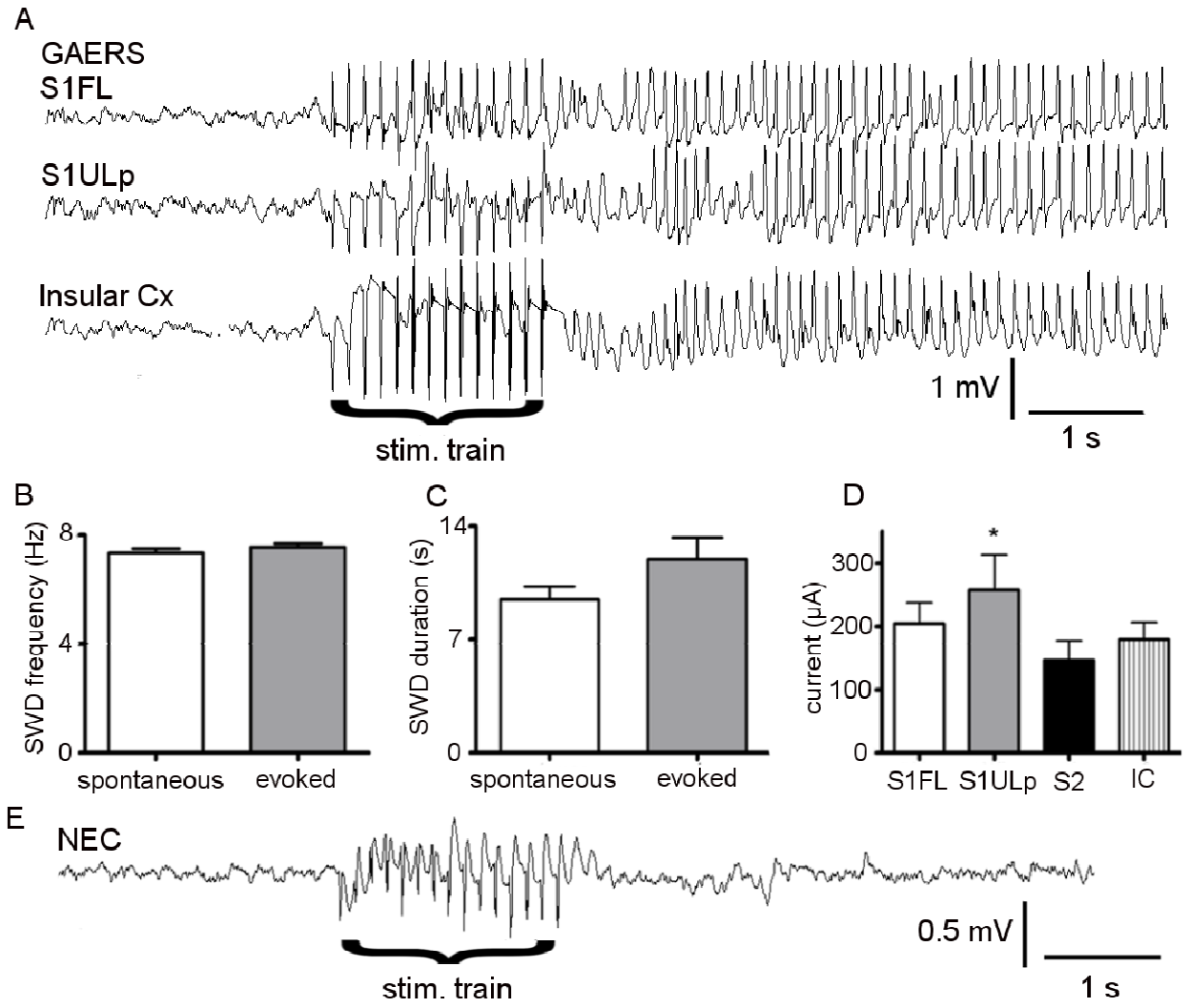

Figure 6.3. Cortical electrical stimulation in GAERS and NEC rats. A. A 10 second trace showing simultaneous depth EEG recording of S1Fl, S1Ulp and IC before, during and after electrical stimulation of S2. Typical SWD events were induced. B. Mean frequency of spontaneous vs evoked SWDs in GAERS (7.3 ± 0.1 vs 7.6 ± 0.1, respectively, unpaired t-test, p=0.15). C. Mean duration of spontaneous vs evoked SWDs (mean, 12.0s ± 1.35 vs 9.5s ± 0.79, unpaired t-test, p=0.12). D. Mean current (μA) required to consistently evoke seizures in the S1Fl, SlUlp, S2 and IC of GAERS. A smaller current is needed in the S2 to evoke SWDs compared to the adjacent S1Ulp (146.4 ± 30.6 vs 257.1 ± 56.4, n=7, unpaired t-test, p=0.025). E. Electrical stimulations in the NECs evoke short episodes of small to medium voltage 7 Hz oscillations but no SWD events occur (n=3).

6.3.3 Preictal and interictal 5-9 Hz rhythmic activities in S2

To understand the spatio-temporal dynamics of the interictal and pre-ictal oscillatory network activities in S1, S2 and IC, multi-unit and LFP recordings were conducted in GAERS (n=3) under neuroleptanalgesia. Glass micropipettes were advanced through S1, S2 then IC and recordings were performed every 200-1000μm, along with simultaneous surface EEG (Figure 6.4). Sweeps were recorded at multiple cortical locations during the same recording session and were time referenced to the negative spike of the SW complex in the EEG. Rhythmic pre-ictal multi-unit and field potential were observed, beginning earliest in S2 (depth 5000μm, represented by "*", Figure 6.4) and occur at the frequency of the SWD (5-9 Hz; see autocorrelation histograms in Figure 6.5) before appearing in the S1 and IC. These rhythmic multiunit discharges could be observed to commence many seconds before seizure onset and could be sustained for minutes throughout interictal periods while the surface cortical EEG remained at a low-voltage (<0.2mV) desynchronized background level (Figure 6.5).

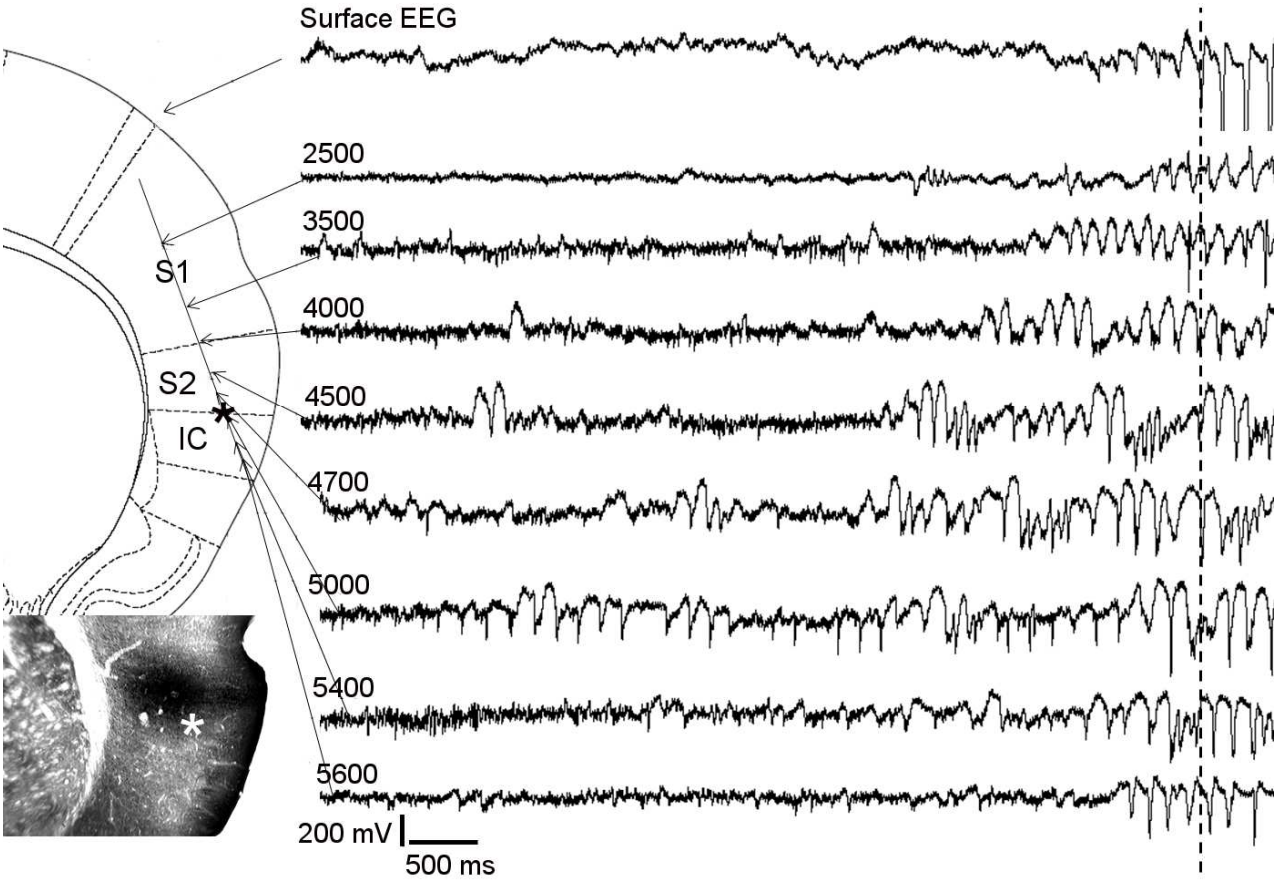

Figure 6.4 Seizure related multi-unit activity and field oscillations are first detected from the S2 - MicroEEG recordings of intracortical sites through the S1, S2 and IC in a GAERS under neurolept-anaesthesia. The lines on the brain atlas (Paxinos and Watson, 1998) demonstrate the trajectory of the electrodes (n=3) and the regions of brain in which the recordings were acquired (every 200 – 1000 μM). Distances are measured from the point of entry of the pipette into the cortex in the high S1 region (AP +0.2 mm, ML 3.6-4.2 mm, angle 20°). All recordings are of 6 seconds prior to the onset of seizure, with the first negative spike of the SWD on the surface EEG (top trace) aligned as zero-time reference. The star "*" indicate a depth of 5000 μM within S2 where persistent rhythmic multiunit potentials were recorded. The onset of the oscillatory field activity begins progressively later in the recordings more superior and inferior to this point.

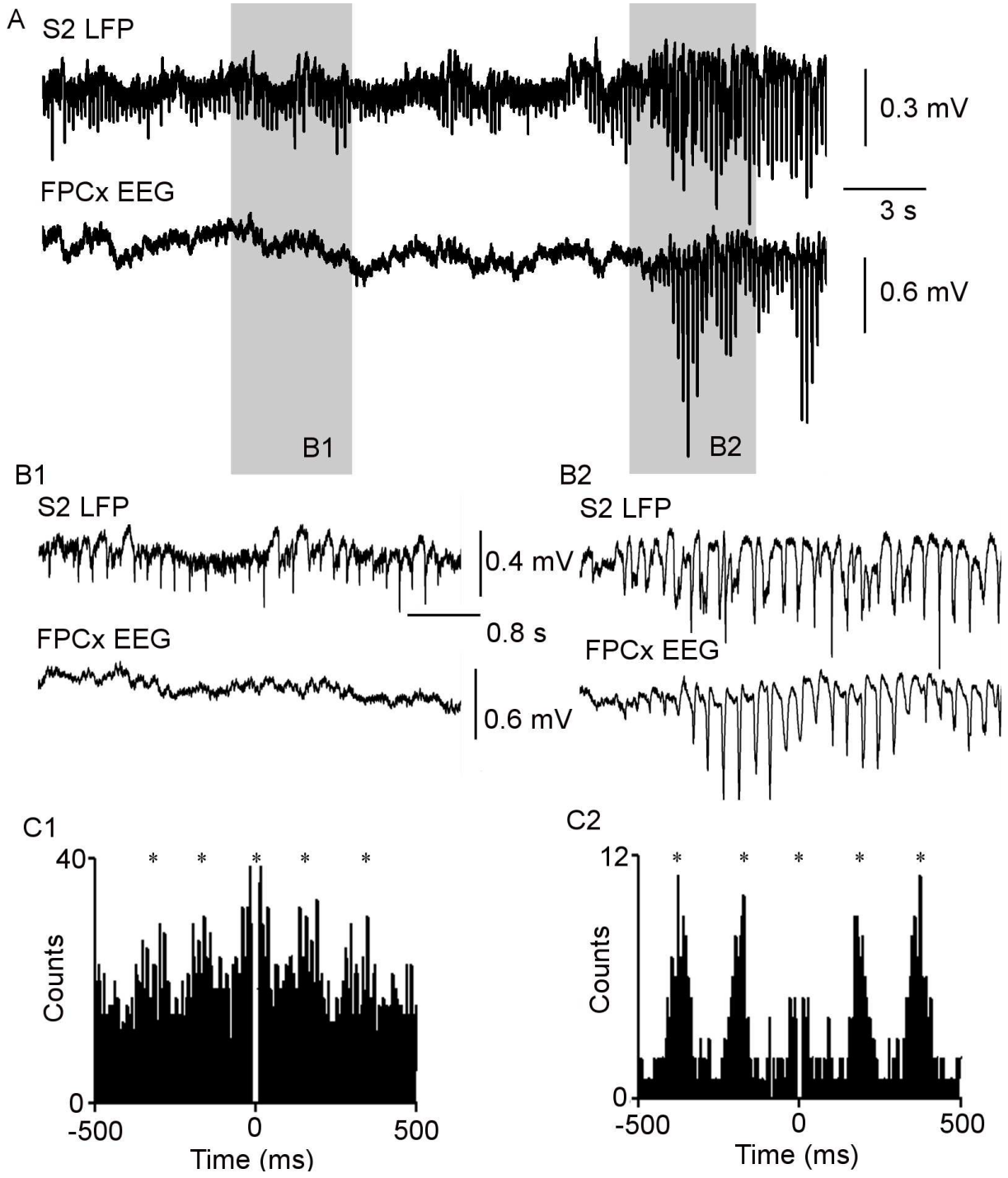

Figure 6.5 Sustained interictal multi-unit activities from the S2 cortex. A. 30 second multi-unit recording trace of the S2 cortex simultaneously with the EEG of the related frontal parietal somatosensory cortex. B1 & 2. A 6s selection of A. preictal and ictal periods, respectively, showing multiunit activity during interictal periods and increase in amplitude during seizures.

C1 &2. Autocorrelograms (2ms bin width) of interictal and ictal periods confirming the highly rhythmic nature of the multiunit discharge

6.3.4 Inter-ictal and pre-ictal rhythmic firing is predominant in deep layers of S2/IC

To identify the cellular correlates of the pre-ictal and inter-ictal rhythmic multi-unit events described above, juxtacellular recordings of single neurons were performed within S1, S2 and IC. A total of 178 cells from 16 GAERS and 78 cells from 5 NEC rats were recorded. Two distinct types of cellular firing patterns were observed during the 10 second pre-ictal period prior to the commencement of SWDs (Figure 6.6): Type A. *Rhythmic Cells* that predominantly fired at the SWD frequency of 5-9 Hz during interictal periods, either as single APs or AP bursts (21% [37 of 178], see autocorrelation histograms in Fig6.6A2-3). Firing pattern was sustained and persisted for seconds to minutes before, during and often after the occurrence of a SWD event on the surface EEG. A small number of these rhythmic cells (9 of 178) could fire at a higher frequency (8-15) Hz during interictal periods and slowed to 5-9 Hz up to 2 seconds prior to the onset of SWDs. Type B. *Non-Rhythmic cells* that fired irregularly during interictal periods before the abrupt switching to 5-9 Hz rhythmic firing with the commencement of the SWDs on the EEG (79% [141 of 178], Fig. 6.6B1-3). Despite the distinctively different pre/inter-ictal firing patterns, during absence-related seizures all recorded neurons fired in a highly synchronous rhythmic manner, phase-locked with every SW complex (Figure 6.6 A-C). The timing of onset of rhythmic 5-9 Hz cellular firing pre-ictally was measured with reference to the first downward spike of the SWDs on the corresponding ictal surface EEG. Consistent with our observations in the field recordings, S2 neurons exhibited rhythmic firing before S1 neurons (mean ± s.e.m., -0.94 ±0.2s vs. -0.4 ± 0.2, p=0.045). No significant difference was found between the onsets of rhythmic firing between neurons recorded in S2 versus those in IC.

The recorded neurons were juxtacellularly labeled using neurobiotin-filled pipettes and the location of the recorded neurons identified. The stereotaxic locations of all recorded cells were plotted on the rat brain atlas ((Paxinos and Watson, 1998) Figure 6.6D). Neurons that fired rhythmically during pre- and interictal periods (rhythmically firing Type A and B cells, plotted as black squares) were predominantly clustered in a restricted region centered in S2, but extending to include the dorsal IC and ventral S1Ulp regions. Rhythmic cells were found in both layers V and VI and their principal morphological features were not apparently different to those

of the non-rhythmic cells (Figure 6.6E1-2). A similar proportion of rhythmic cells that were found in GAERS were also identified in NEC rats, with 13 of 78 cells (17%) firing rhythmically.

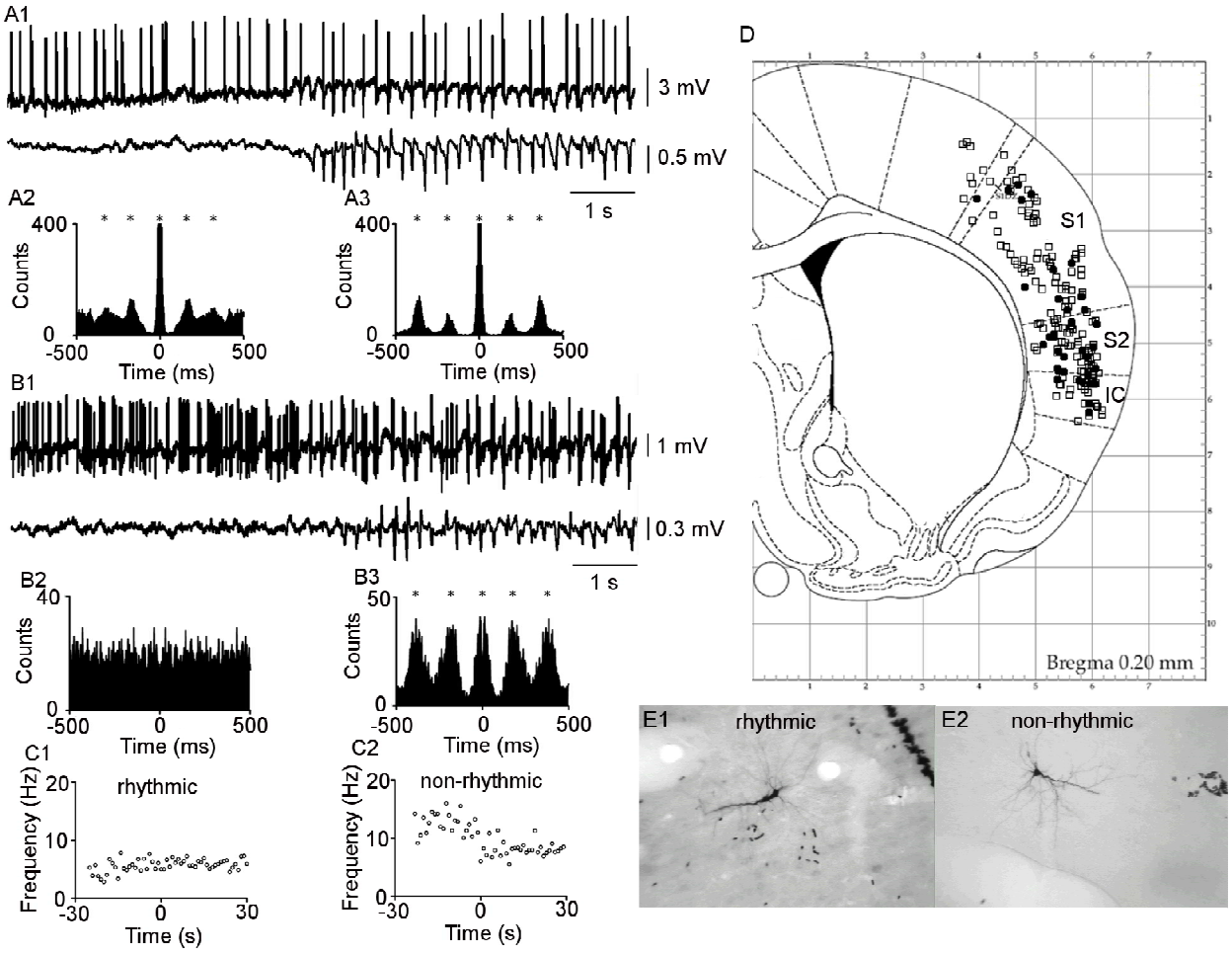

Figure 6.6 Identification of rhythmic and non-rhythmic cell types in GAERS under neurolept-anaesthesia. Juxtacellular recording were performed in the GAERS somatosensory cortex simultaneously with surface EEG during pre- and ictal periods. A. inherently rhythmic cells (21%) – autocorrelogram reveals the 5-9 Hz rhythmicity before and during seizures. B. non-rhythmic cells (79%) – autocorrelogram reveals no rhythmicity before seizures and 5-9 Hz ictal rhythmicity. C1-2. the instantaneous frequency of 2 successive APs from each of the 2 cell types

(1 second bin width). D. Stereotaxic location of all recorded neurons with reference to the Paxino and Watson Rat Brain Atlas. Neurons that show interictal rhythmicity are plotted in black. E. Microphotograph of a rhythmic (left) and non rhythmic (right) cortical layer V neuron.

In addition, GAERS layer V and VI neurons in S2 show greater burst firing than those of NEC rats. The interictal firing characteristics of the juxtacellularly recorded layer V and VI neurons were quantitatively compared between GAERS and NEC rats for S1, S2 and IC (Table 6.1). There was no difference in the neuronal firing rate between GAERS and NEC rats in all cortical regions examined. However, neurons within S2 of the GAERS showed more burst firing compared to those in the same region of NEC rats (3.4% vs 2.4%, $p=0.03$). The amount of burst firing of neurons in the adjacent S1 or IC was not different between the two strains.

6.3.5 Layer VI neurons in the somatosensory cortex play a leading role during seizures

To investigate the cellular correlates of the oscillations that occurred in S2 before the neighboring cortices during both pre- and interictal periods, single cell juxtacellular recordings of the cortical layers V and VI were performed in S1, S2 and IC (n=108 cells from 15 GAERS). During a SWD, all recorded cells fired rhythmically and synchronously with surface EEG spikes. S2 neurons fired before S1 and IC neurons throughout the seizures (Figure 6.7, S2, -39.5 ± 2.5 ms, vs S1, -22.2 ± 2.6 ms & IC, -29.8 ± 3.8 ms compared to the EEG spike). Time latencies between neurons of layer V and VI were also compared for both S1 and S2 (Table 6.2, Figure 6.7B). Layer VI cells fired significantly ahead of layer V cells in S1 during SWDs (layer VI, -31.3 ± 3.7ms, vs layer V, -11.1 ± 5.12 ms, $p<0.01$). However, there was no significant time lag between layers V and VI in S2 (layer V, -40.1 ± 2.5ms vs layer VI, -37.6 ± 4.6ms, $p>0.05$). No significant difference was found between timing of the firing of layer VI cells of S1 and S2 during SWDs (Table 6.2). There was no difference in the firing rate or percentage burst firing between the regions or layers.

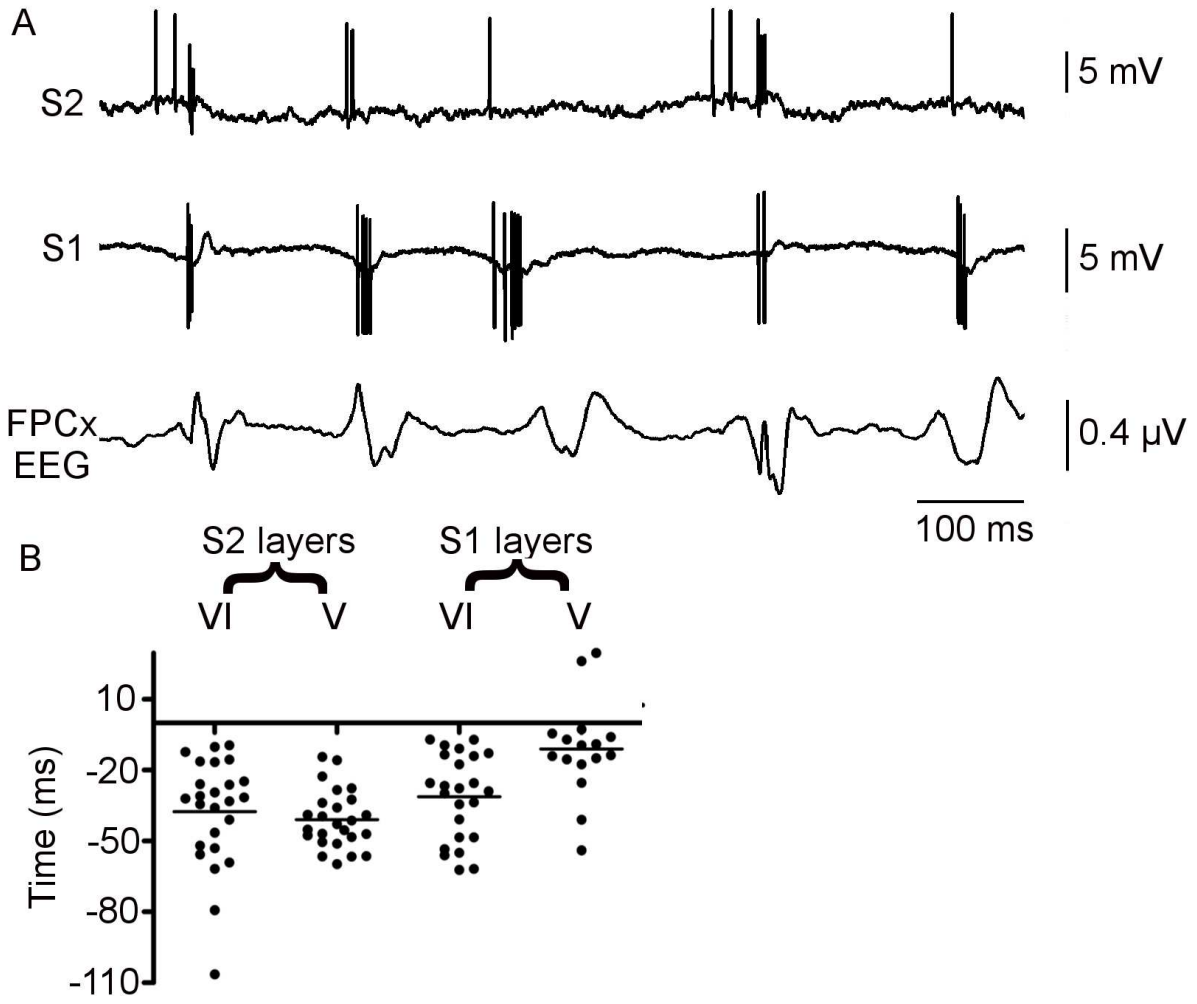

Figure 6.7 A. Representative trace of juxtacellular recording of single cells from the S2 and S1 during seizures. show that S2 cell lead S1 cell firing during a SWD, both of which precede the downward spike of the SW complex on the EEG. B. Dot plot showing the temporal relationship between cells in layers V and VI of the S1 and S2 cortices with reference to the ictal EEG spike (Time zero) during seizures. S1 layer V cells show delayed firing compared to other regions (* Kruscal-Wallis test, $p<0.0001$).

6.4 Discussion

The main observations of the present study are: (1) In GAERS, SWDs arose from brief (<2 sec.) 5-9 Hz oscillations, which were first observed in S2 and IC; they were also recorded in NEC rats but did not trigger SWDs ; (2) Self sustained SWDs could be triggered following a short-lasting train of 7Hz electrical stimuli in GAERS, but not in NEC rats, during periods of quiet wakefulness, with a lower stimulus intensity in S2/IC than in S1; (3) A population of neurons focused around S2/IC showed sustained rhythmic firing during interictal periods and physiological 5-9 Hz oscillations; (4) During SWDs, HFO occurred in S2/IC before S1; and (5) During SWDs, S1 and S2 layer VI cells fired synchronously, followed by S1 layer V cells. Altogether, these results demonstrate the presence of precursor cellular and network rhythmic activities in S2/IC cortices during SWDs generations and suggest that the S2/IC contain a critical circuit from which seizures are initiated.

6.4.1 Precursor oscillations in S2/IC prior to the onset of seizures in GAERS

Linear comparisons of cellular and network activities between the S1 and the primary motor cortex (M1) by Polack and colleagues (Polack et al., 2007) found precursor-like activities in S1 relative to the M1 - a location for the presumed focus that is topographically more dorsal (Fig1 of Polack et al.) to that found by Meeren et al. ((Meeren et al., 2002) see Fig2). Our current study extended Polack et al.'s "linear" strategy by comparing the neural dynamics of four cortical regions: M1, S1, S2 and IC. We have found precursor oscillatory network and cellular rhythmic activities in S2 and IC relative to S1 during preictal and interictal periods in freely-moving rats. The frequency of these oscillations was on average ~5-9 Hz (minimum 4 Hz, maximum12 Hz) in agreement with previous studies (Pinault et al., 2001; Polack et al., 2007). The internal frequency of pre-ictal oscillations can start at 12 Hz then slows down to 5-9 Hz (Pinault et al., 2001; Pinault, 2003), an observation confirmed recently (Polack et al., 2007) and true for the present study. These localized precursor oscillations are also found in NEC rats, suggesting that they are not themselves sufficient to generate SWDs, but represent an important element of the seizure trigger in an epileptic animal. Local field precursor oscillations were consistently recorded first in S2/IC, relative to S1/M1, in 60% of freely moving rats. In the remaining 40%, no apparent precursor activity was detected at a given recording site. This could have resulted from: 1) a

second or extended focus distant from the recording sites, resulting in simultaneous detection of synchronous oscillations; 2) hypersynchronization of the cortical network prior to the onset of SWDs. The current data suggest that the presumed focus is likely located in a cortical region including at least S2 and IC, raising fundamental questions about its size and emergent network synaptic and intrinsic properties.

A sub-population of preictally rhythmic neurons is the likely cellular correlates of the rhythmic oscillations from which SWDs arise. They are found predominantly in layers IV to VI of S1ULp, S2 and granular IC, corresponding to the region where rhythmic oscillations are first detected (Figure 6). These cells show sustained interictal 5-9 Hz firing, with a small number of them firing at a slightly higher frequency (up to 12 Hz), a phenomenon also reported previously (Pinault, 2003). Seizure can only be initiated when the non-rhythmic cells are recruited to fire in synchrony with the rhythmic cells at 5-9 Hz. These findings suggest that rhythmic neurons, mostly found in S2/IC, may drive S1 neurons during the generation of SWDs, perhaps via direct layer VI cortico-cortical connections (Zhang and Deschenes, 1998).

In agreement with previous studies that identified rhythmic electrical events in the non-epileptic rats (Buzsaki et al., 1990; Pinault et al., 2001), the preictally rhythmic cells are present in similar proportions in GAERS and NEC rats. However, we recorded increased burst firing in S2/IC cortex in GAERS, a potential feature that may increase the likelihood of seizure occurrence. Possible mechanisms for this apparent cortical hyperexcitability including the increase in expression of the transmembrane AMPA receptor Regulatory Protein (TARPs), stargazin, that our group has recently reported in the somatosensory cortex of GAERS (Powell et al., 2008). An NMDA receptor hyper-function reported in both GAERS and WAG/Rij compared to the control rats (Pumain et al., 1992; D'Antuono et al., 2006) within the S2/IC region could also increase the likelihood of switching cortical activity from non-rhythmic state to the more pro-epileptogenic, rhythmic state.

6.4.2 Intracortical electrical stimuli trigger typical SWDs in GAERS

Brief (2 s) trains of 7 Hz electrical stimuli in S1, S2, or IC could reliably trigger typical SWDs in GAERS that resemble spontaneously-occurring SWDs. Electrical trains stimulates primarily the axons before cell bodies and dendrites since the current pulse duration that is needed to evoke twice the rheobase current is below 200 μs for axons and in the range of 1-10 ms for cell bodies and dendrites (Ranck, 1975) (Nowak and Bullier, 1998). The elements that could be activated by the stimulation are primarily the axons branches and axon initial segments surrounding the electrode tip (Nowak and Bullier, 1998), including those of TC neurons, as well as the surrounding cell bodies and dendrites. The level of the current required for the stimulations to trigger seizures was significantly lower in S2 than in S1, suggesting that the S2/IC stimulation sites are closer to the site of seizure initiation than the S1 region. However, the same stimulation may activate orthodromically efferent axons from the S2 to distant postsynaptic intracortical regions as well as antidromic activation of afferent axons and of passing fibres. Based on this experiment alone, the involvement of a distant afferent cortical or subcortical structure or a region whose axons of its neurons pass through S2 or IC for SWD initiation cannot be ruled out.

6.4.3 HFOs are seen prior to the SW complexes in GAERS seizures, occurring earlier in S2/IC than S1

Interestingly, we observed in GAERS the existence of rhythmic short-lasting HFOs (400-600 Hz) in deep cortical layers of S1, S2 and IC prior to the SW complex, during the generation of absence-related SWDs. These HFO systematically occurred just before the spike component of the SW complex and resemble fast ripples recorded in the hippocampus (Buzsaki et al., 1992) where they are thought to reflect a strong coupling of several neurons. The HFOs detected in IC and S2 consistently lead S1 by >20 ms. This time lag is remarkably long since the paired recorded sites were relatively close (range of 1-3 mm), suggesting a synchronization mechanism other than direct intracortical recruitment. HFOs in S1 could be a result of direct volume-conducted fast activity from S2/IC or recruited by the thalamus as part of secondary synchronization mechanism. Further experiments are also required to determine the precise origin of these absence-related HFOs and whether they are either intrinsically generated in the

cortex, or the consequence of massive hypersynchronized synaptic inputs originating in the cortex or in subcortical structures. HFOs may also arise from electrically coupled networks via gap junctions between cell axons to trigger synchronized network activity (Draguhn et al., 1998; Traub et al., 1999). The existence of HFOs in genetic models of absence epilepsy is a puzzling issue that requires in depth investigation.

6.4.4 Intracortical spread of rhythmic activity

S1 and S2 are reciprocally connected and receive topographically organized inputs from the ventral posteromedial thalamic nucleus (VPM) and ventral posterolateral thalamus (VPL) (Liao and Yen, 2008). Cells within layer VI of S1 and S2 are known to project to the VPM and the TRN (Allen et al., 1991; Bokor et al., 2008) and are thought to initiate SWDs at least in the primary somatosensory system (Pinault, 2003). The IC receives visceral, gustatory and somatosensory inputs and is reciprocally connected with dorsal thalamus (Allen et al., 1991). Although the IC, S1 and S2 are anatomically interconnected (Saper, 1982), its anatomo-functional properties and its roles in SWD generations remain unclear. The intra-cortical spread and synchronization of the 5-9 Hz rhythmic events from S2/IC may not simply follow the rule of synaptic circuits within the intracortical circuitry, as rhythmic activity may be sustained within the S2/IC for minutes. The initiation of SWDs may involve a CT-induced resonant phenomenon in the thalamus (Pinault, 2003) subsequently to S2/IC rhythmic events. Such a CT resonance would then recruit S1 layer V neurons, which lag behind by >20ms during SWDs. Thus, a mass synchronization processes involving both intracortical spread and thalamic feedback must be present to enable the transformation of the physiological somatosensory rhythm into pathological SWDs.

On the basis of the findings from current and previous studies, we propose a model for the sequence from which normal oscillations develop into absence-like SWDs as follows: 1) the S2 and IC cortical areas contain a critical circuit where rhythm generator cells initiate and sustain the physiological 5-9 Hz. pre-ictal oscillations; 2) excitatory propagation spreads from the S2/IC to the interconnected S1, motor and frontal cortical regions, possibly via a caudo-rostral excitatory pathway (Fujita et al., 2010); 3) Layer VI cells in the S1 and S2 phase locks with thalamic relay and reticular oscillations; 4) The intrinsic resonating properties of the TRN

allows the generation of rhythmic high frequency AP discharges leading to $GABA_A$ receptor mediated IPSPs in their target thalamic cells; 5) The TRN induced hyperpolarization trigger low-threshold Ca^{2+} spikes in a subset of thalamic relay cells that exhibit the H-current, leading to AP discharges. This would be reinforced by a) the reciprocal cellular interactions between the thalamic and TRN cells (Bal and McCormick, 1993); and b) the abnormalities in expression and function of low-threshold calcium channels (Tsakiridou et al., 1995; Talley et al., 2000; Powell et al., 2009), which are functionally mutated in GAERS (Powell et al., 2009). 6) The thalamus, in turn, recruits new units in layer V of the S1 cortex, as well as cells in other cortical regions to fire in synchrony. The cycle is therefore restarted, resulting in cellular hypersynchronization.

	S1			S2			IC		
	GAERS (n=75)	NEC (n=18)	p-value	GAERS (n=63)	NEC (n=42)	p-value	GAERS (n=23)	NEC (n=7)	p-value
Firing Rate (Hz)	6.1 (0.2–27.1)	6.6 (0.1–41.9)	0.93	6.7 (0.1–24.8)	7.0 (0.1– 46.0)	0.18	6.2 (0.4–27.2)	7.3 (0.5–9.9)	0.9
% Burst Firing	3.1 (0–91.7)	3.6 (0.0–50.7)	0.94	3.4 (0–70.8)	2.4 (0–35.6)	0.03	0.7 (0–41.1)	7.9 (1.7–52.5)	0.43

Table 6.1 Single unit juxtacellular recording of layer V and VI neurons in the S1, S2 and IC regions interictally. Firing rate and % Burst firing of neurons were compared between GAERS and NECs in each of the recorded regions

	S1			S2		
	layer V (n=17)	layer VI (n=25)	p-value	layer V (n=24)	layer VI (n=25)	p-value
Firing Probability (%)	74	68	0.41	80	74	0.54
Burst Firing (%)	49	51	0.92	58	54	0.78
Maximum time lag (ms)	-43.3	-78.8	0.0004	-97.9	-88.2	0.17
Median time lag (ms)	-11.4	-32.7	0.0027	-42	-37.6	0.16

Table 6.2 Single unit juxtacellular recordings in the S1 and S2 neurons during seizures. The timing of cell firing was compared to the spike of the SWDs on the concurrent EEG recordings. Layer VI neurons in the S1 fired before layer V neurons during SWDs, whereas in the S2 region there is no difference in the firing timing between layers V and VI neurons. The neuronal firing probability and percentage burst firing were not different between the layers in both the S1 and the S2 region.

Chapter 7 Cortical-area selective block of genetically determined absence seizures by carbamazepine

7.1 Introduction

As discussed in Chapter 1 and 6, the expression of absence seizures is known to involve large scale interaction and synchronization between the cortical and thalamic network, although the relative roles played by the component parts of the CT circuitry is not fully understood. Specific cortical inputs for the initiation and synchronization of absence seizure related SWDs have been long postulated for a number of animal models of absence seizures (Seidenbecher et al., 1998; D'Arcangelo et al., 2002). In the WAG/Rij model of absence epilepsy, using non-linear association analysis of EEG signals from multiple cortical surface and depth thalamic electrodes, the peri-oral region of the somatosensory cortex was found to exhibit seizure activity before other cortical and thalamic regions (Meeren et al., 2002). Pharmacological studies in the same rat model compared the effect of local administration of the anti-absence drug ethosuximide (ETX) in the intracortical regions (Manning et al., 2004). The authors reported seizure suppression in the peri-oral region of the S1 (S1po), with a less marked effect in the adjacent S1 FL and no effect in the M1 cortices. Based on this evidence, the authors concluded that the S1po may be the specific focal origin in the generation of SWDs(Manning et al., 2004). In the GAERS model, layer VI of the primary somatosensory cortex (S1) has been demonstrated to show SWD frequency oscillations several seconds before being propagated synchronously to the related relay thalamus and the TRN (Pinault, 2003). Local field potential and single cell recordings in this thesis have demonstrated the presence of rhythmic SWD precursor activity in the more ventral secondary somatosensory cortex (S2) during the generation of SWDs before spreading to the S1 (Chapter 6), indicating the S2 as the likely critical region for the generation of seizures in GAERS rats.

Cortical-Area Selective block of SWDs by CBZ

As previously described (Chapter 1 & 3), CBZ is an effective anti-focal seizure drug known to aggravate generalized absence seizures with systemic administration. Results reported earlier in this thesis indicate that CBZ is acting via enhancement of $GABA_A$ receptors within the VB thalamus to aggravate absence-like seizures in GAERS (Chapter 3). The experiments reported here aimed to examine the effect of local microinjection of CBZ within the different regions of the cortex of GAERS – somatosensory S2, S1 and primary motor cortex (M1). It was hypothesized that if the precursor somatosensory rhythm arising from the S2 cortex is critical to the initiation of seizures in GAERS then focal injection of CBZ into this region would suppress the occurrence of seizures.

7.2 Methods

Methods for animal surgeries, EEG recordings, analysis and histology are described in detail in Chapter 2, section 2.3.

7.2.1 Electrode and cannulae implantations

In all animals, for the implantation of extra-dural electrodes for EEG recordings, two holes (1.4 mm diameter) were drilled bilaterally in the frontal-parietal bone (approximately 1mm anterior to the coronal suture) and two in the parietal bone (approximately 2mm anterior to lambdoid suture). In separate experiments, bilateral cannulae were implanted either the M1 (mm, relative to bregma, anterior-posterior [AP], 2.7; medial-lateral [ML], 2.6; dorsal-ventral, [DV], 1.6), S1 (AP, 0.2; ML, 3.6; DV, 1.6) and S2 (AP, 0.2; ML, 5.8; DV, 2.75) (Figure 7.1). EEG recording protocols were as described in Chapter 2, section 2.3.4. Upon completion of the experiments, methylene blue solution (0.1%, 1μl) was infused through the cannulae. Rats were euthanized and brains sectioned. Identification of brain regions was achieved with reference to Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998).

7.2.2 Data analysis

SWD incidence was calculated as the percentage of total time spent in seizures during the 90 minute recording period following drug injections (mean ± S.E.M.). Each animal was treated with saline, vehicle and CBZ, thus permitting each to serve as its own control. Drug effects were assessed by repeated measures analysis of variance (ANOVA), with planned comparison between each pair when significant differences ($P \leq 0.05$) were found.

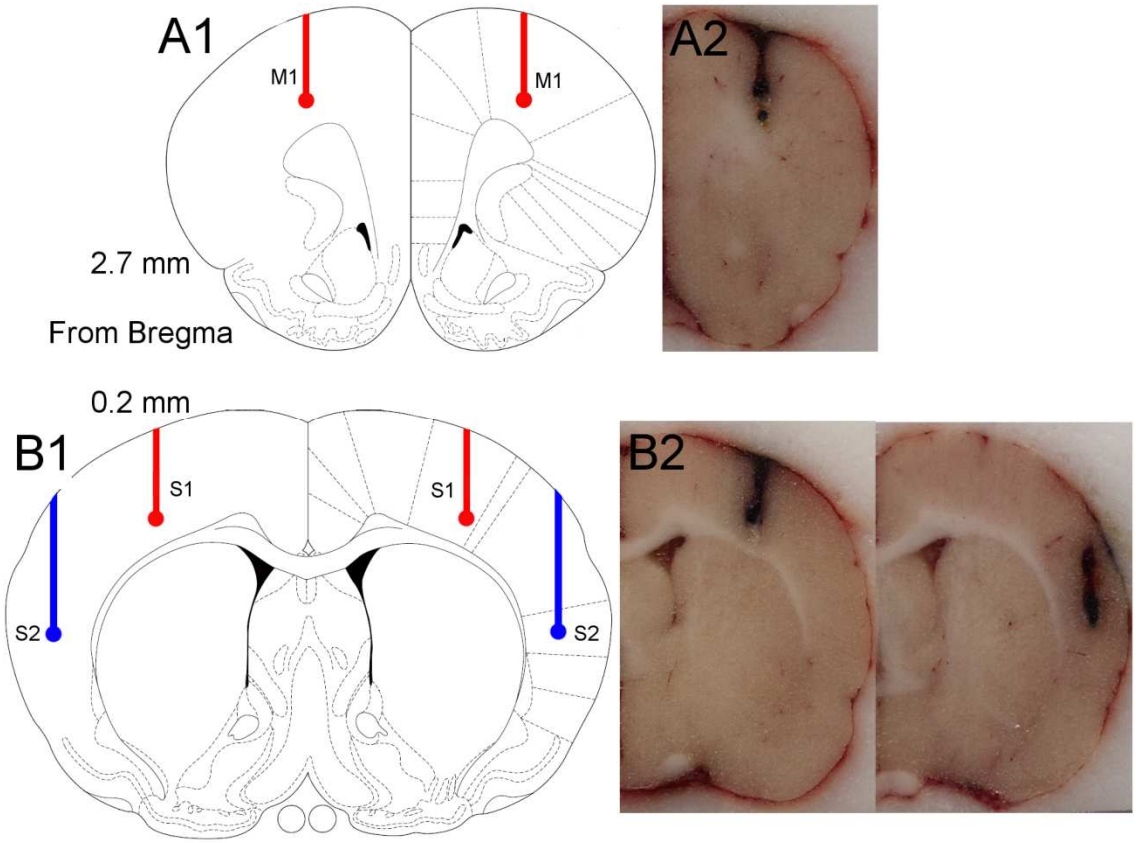

Figure 7.1 A1 and B1. Coronal brain slices taken at +2.7 mm and +0.2 mm, respectively, relative to bregma. The blue and red tracts show the intended site of implantation and injection. (Depiction of brain slices adapted from (Paxinos and Watson, 1998). **A2 and B2.** Brain sections showing methylene blue diffusion post-injection (1 µl) though the M1, S1 and S2 cortices.

7.3 Results

7.3.1 Bilateral microinjection of CBZ into S1, S2 and M1

This experiment aimed to investigate the effect of CBZ on seizure activity after microinjection into the S1, S2 or M1 in 3 separate cohorts of GAERS. Each animal was sequentially injected with saline, vehicle and CBZ (3.75 μg in 1μl, bilaterally) in a randomized order. Absence seizures accompanied by typical SWDs were observed in all rats after saline, vehicle and CBZ injections (Figure 7.2).

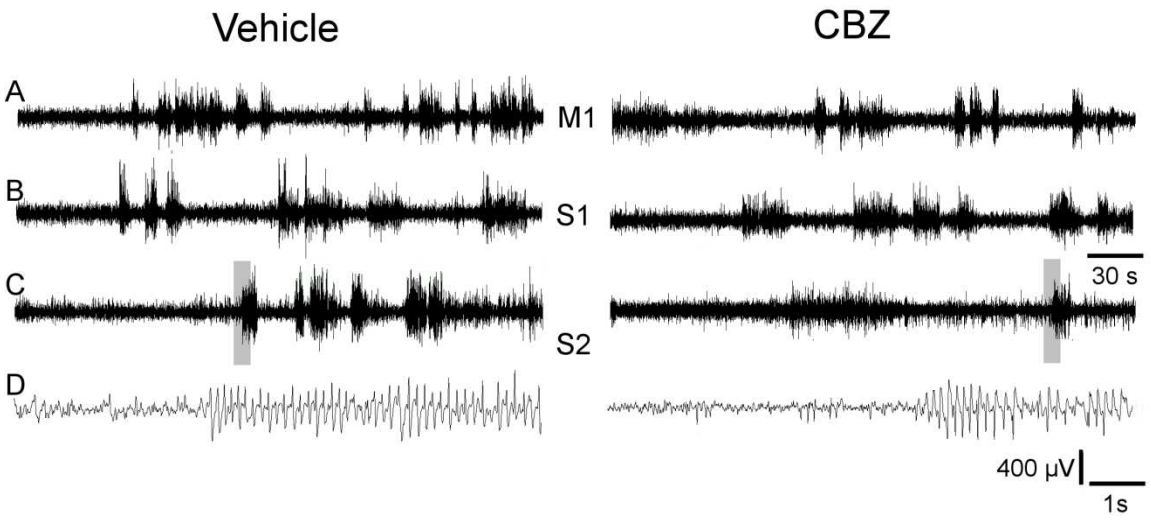

Figure 7.2 A-C. Representative EEG traces showing five minute recording episodes during vehicle (left) and 30 minutes after CBZ injection (right). Typical SWDs were frequently observed in all vehicle conditions and in the M1 and S1 following CBZ injection into the M1 and S1. Very few SWDs were observed after CBZ injection into the S2. **D.** Expansion of grey band from C - Ten second representative EEG trace after vehicle injection (left) and CBZ injection into the S2 (right). SWD morphology was not affected by the injection of CBZ.

Bilateral injection of vehicle into each of the three regions of GAERS produced no significant effects on SWDs compared to saline control. CBZ injection into the S2 region resulted in an immediate and significant decrease in the amount of seizure activity within the 90 minute period post-injection (Figure 7.2). Although rats spent less time in seizures, the morphology of the SWDs were not affected by CBZ. Microinjection of CBZ into either the S1 or the M1 showed no effect on the amount of seizures within the 90 minute period post-injection (Figure 7.2 & 7.3).

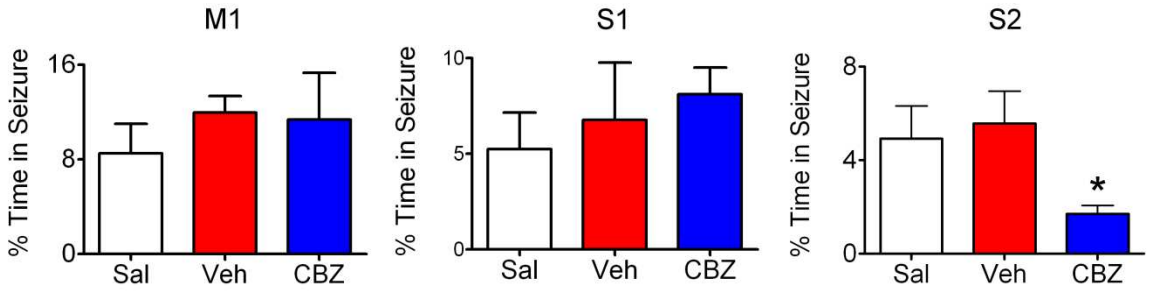

Figure 7.3 Mean percent time in seizure (±S.E.) in GAERS over a 90 min EEG recording following bilateral microinjection into the M1, S1 and S2 (respectively, left to right) of saline, vehicle or CBZ (3.75µg). No significant differences were found between the treatments for injection into either the M1 (n = 5) or the S1 (n = 6). There was a significant difference between the treatments for S2 microinjections (n = 8, p = 0.05). Planned comparison analysis showed significant seizure suppression following CBZ injection (versus vehicle, -69%, *p = 0.01).

7.4 Discussion

7.4.1 Focal injections of CBZ into the S2 cortex suppress SWDs in GAERS

The results of the experiments reported in this chapter show that microinjection of CBZ (3.75μg/side) into the S2 of freely moving GAERS results in a significant suppression of seizure activity. This effect was specific for the S2 cortical region, not being seen with injections into the S1 or M1 cortex.

The effect of the local administration of CBZ into the S2 cortex contrasts with that observed after systemic and focal intrathalamic administration of CBZ, which result in aggravation of seizures in GAERS (Chapter 3).

In GAERS, the cortical network and cellular precursors of absence seizures are the naturally occurring, medium voltage oscillations at 7-12 Hz in freely moving rats and 5-9 Hz in rats under neuroleptanalgesia (Pinault et al., 2001). These oscillations emerge from a desynchronized EEG that is also recorded in the non-epileptic control (NEC) rats (Pinault et al., 2001). These oscillations are thought to be a wake-related sensorimotor rhythm that can be synchronized between the cortex and thalamus and are associated with quiet whisking movements when rats explore their environment. Absence seizures occur as a result of hypersynchronization of these natural rhythms within the cortico-thalamo-cortical circuitry. As demonstrated in Chapter 6 of this thesis, the likely cellular correlates of these cortical rhythms may be a sub-population of neurons that are predominantly found within the S2 region. Therefore, a possible mechanism for suppression of seizures following local injection of CBZ into the S2 would be the suppression in the generation of the rhythmic SWD precursor activity within the S2. CBZ is known to interact with several molecular targets including the use dependent blockade of Na^+ channels, as well as showing a $GABA_A$ receptor enhancing effect as demonstrated in this thesis. These cellular effects would be expected to suppress neuronal firing, and therefore inhibit generation of the somatosensory rhythms if administered locally. Further experiments are needed to identify the exact molecular mechanisms underlying the SWD suppression effect of CBZ within the S2.

In contrast when CBZ is administered focally into the thalamus its effect to enhance neuronal hyperpolarisation, via effects on $GABA_A$ receptors, deinactivates low threshold Ca^{2+} channels and thereby promoting a hyper-resonant burst firing pattern in response to the hypersynchronized CT afferent volley from the somatosensory rhythm. The resultant effect is aggravation of the absence seizures. The effect of systemic CBZ injection is the net balance between the pro-absence effects in the thalamus versus the anti-absence effects in the somatosensory cortex, which in most cases favours the former.

7.4.2 S2 cortex may form the focal origin in the generation of absence epilepsy

Both clinical and experimental findings have indicated that the somotosensory cortex may be the site of initiation of absence seizures. In patients with childhood absence epilepsy and other types of idiopathic generalized epilepsies, SWDs were observed to start first within the frontal cortex (Ferri et al., 1995; Niedermeyer, 1996). Experiments in several animal models have demonstrated that the cortex leads the thalamus in the initiation of SWDs (Steriade and Amzica, 1994; Meeren et al., 2002; Pinault, 2003). However, the intracortical location of a possible seizure origin has not been demonstrated and is still a matter of debate. Planar multi-site EEG recordings from the cortical surface and non-linear association analysis in the WAG/Rij rats suggested that the focus was located in S1 (Meeren et al., 2002). However, close examination of the figures from this study indicates that the proposed focus was stereotaxically located more lateral and ventral than S1 (Paxinos and Watson, 1998), corresponding to the secondary somatosensory cortex (S2). A subsequent pharmacology study showed a seizure suppression effect of the anti-absence drug ETX in the S1po with less effect in the S1FL, while the S2 was not compared (Manning et al., 2004). In combination with Chapter 6 of this thesis which showed rhythmic precursor activity in the S2/IC region, the finding of the experiments reported in this Chapter provide further support that the S2 of GAERS may play a more critical role in the generation of seizures in GAERS than that of nearby cortical areas such as the S1 and M1. This adds further evidence to support the

involvement of the S2 as a specific focal origin in the generation of genetically determined absence seizures.

Chapter 8 General discussion, future directions and conclusions

8.1 Novel key findings from this thesis

CBZ is a widely prescribed anticonvulsant for the treatment of focal epilepsy and psychiatric disorders. However it has multiple cellular effects which likely contribute to its frequent and at times severe side effects that include a paradoxical aggravation of generalized absence seizures. The research in this thesis investigated neurophysiological and neuropharmacological mechanisms relevant to the aggravation of absence seizures by CBZ in a genetic rat model (GAERS). More specifically, this involved exploring the effect of CBZ on TC oscillations, the role of the interaction with $GABA_A$ receptors in the thalamus, as well as the mechanisms underlying the generation of absence-related SWDs. By conducting *in vivo* experiments in GAERS and *in vitro* experiments in oocytes, work presented in this thesis produced novel data demonstrating that:

1) CBZ directly interacts with a $GABA_A$ receptor subtype that is highly expressed within the VB (somatosensory) thalamus, most likely acting as a positive allosteric modulator to potentiate Cl^- influx (Chapter 3).

2) CBZ is selectively acting within the VB thalamus to aggravate seizures in a dose-dependent manner, and that $GABA_A$ receptors here are critical for this effect (Chapter 3).

3) OXC, a structural analogue of CBZ potentiated $GABA_A$ receptor mediated activity and aggravated absence seizures in GAERS after systemic injection. However, its active metabolite MHD, had no effect on the same $GABA_A$ receptor subunit combination, and consistent with this, did not aggravate seizures in GAERS (Chapter 4).

4) In rats under neuroleptanalgesia, CBZ increased the incidence of spindle oscillations in the thalamus, which are at least, mediated by $GABA_A$ receptor-mediated IPSPs (Chapter 5).

5) SWDs in GAERS were preceded by 5-9 Hz oscillations, which were detected first in the secondary somatosensory cortex (S2) and the insular cortex (IC), relative to the primary somatosensory cortex (S1). In the somatosensory cortex, focused around the S2 and IC, there are subsets of neurons which display a rhythmic firing (5-9 Hz) pattern in between seizures. These may be the cellular substrate of the precursor somatosensory rhythm that appears to trigger the absence seizures in GAERS. In addition, single cell recording during seizures showed that S2 and IC layers V and VI neurons fired during the same time window, while in S1 layer VI neurons fired before layer V neurons (Chapter 6).

6) During every cycle prior to the spike component of the SW complex, short-lasting high-frequency oscillations (~120 Hz) consistently occurred in IC ~20 ms before S1.

7) The bilateral microinjection of CBZ into S2 suppressed absence seizures in GAERS, while the same injection into the adjacent S1 and remote cortical areas (primary motor) of GAERS showed no effect on seizure activity (Chapter 7).

8.2 Interpretation and integration of the findings

8.2.1 Seizure aggravation by CBZ occurs via enhancement of $GABA_A$ receptor activity in the VB thalamus

By studying the underlying mechanisms of absence seizure aggravation by anti-epileptic drugs such as CBZ and OXC, insight can be gained into the fundamental mechanisms of the disorder itself. As previously described, drugs that facilitate GABA action may aggravate absence seizures. Both *in vitro* and *in vivo* experimental data presented in this thesis provided strong evidence that CBZ enhances $GABA_A$ receptor-mediated activity within the relay thalamus, which play a critical role in the modulation of both physiological and pathological TC oscillations. The enhancement of $GABA_A$ receptor activity by CBZ is likely the mechanism underlying the aggravation of absence seizures in GAERS. Consistent with this, the CBZ structural analogue, OXC, was shown to have similar $GABA_A$ receptor potentiating effects and aggravated absence seizures in GAERS, while its metabolite, MHD, had no effect on $GABA_A$ receptors and did not aggravate seizures. This further supports the proposition that the ability to potentiate $GABA_A$ receptor activity is critical to the seizure aggravating effects of CBZ-like molecules in GAERS. The enhancement of $GABA_A$ receptor activity in the VB thalamus would be expected to result in hyperpolarization of the TC neurons. This would promote de-inactivation of calcium T-channels that are expressed in a subset of these neurons, thereby encouraging the resonance phenomenon between the cortical and thalamic network, hence enhancing TC oscillatory activity and absence seizures.

In addition, the selectivity of CBZ for the thalamus and not the TRN suggests that CBZ is selective for a $GABA_A$ receptor subtype combination that is differentially expressed between these two thalamic structures. $GABA_A$ receptors are heteropentamers consisting of two α subunits, two β subunits and a single γ subunit (Sieghart, 2006), with the different subunit compositions producing a wide array of $GABA_A$ receptors subtypes. The relay nuclei mainly express α1, β2, γ2, α 4 and δ, whereas α3, β3, γ2 subunits are present in the TRN (Pirker et al., 2000). It is likely that the action of CBZ on α1 subunit

is critical for seizure aggravation, as it is the primary subunit in TC synapses. Results in this thesis demonstrate that the α1, β2, γ2 subunit composition are sensitive to the enhancement of $GABA_A$ receptor activity by CBZ (Chapter3) although the possible interaction with other $GABA_A$ receptors with other subunit compositions needs to be investigated in future studies.

8.2.2 Absence seizures in GAERS arise from a precursor 5-9 Hz rhythm that originates in a cortical area including parts of the S2 and insular cortices

The conventional view of generalized epilepsy was that seizures arise simultaneously in a wide area of brain in both hemispheres (Commission on Classification and Terminology of the International League Against Epilepsy [ILAE], 1981). Recent advances in epilepsy research have prompted the ILAE to re-classify generalized seizures as "originating at some point within, and rapidly engage bilaterally distributed networks" (Berg et al., 2010). Novel findings reported in this thesis demonstrate the presence of precursor cellular and network rhythmic activities in S2 and IC during the generation of absence seizure-related SWDs (Chapter 6), suggesting that this is likely the primary region involved in the seizure initiation. Further support for this was found by the cortical microinjection studies in GAERS (Chapter 7) that demonstrated that the microinjection of CBZ into the S2 but not into the S1 or the M1 cortices potently suppressed seizures.

It is interesting to note that the precursor activity and interictally rhythmically firing cells were also been detected in the insular cortex, part of the gustatory cortex. While the IC is known to be implicated in focal seizures, especially temporal lobe epilepsy, it has not been implicated as being primarily involved in absence epilepsy. The existence of precursor activity within the IC indicates functional connectivity and propagation of rhythmic excitatory events from this region to the S2 and S1. Further experiments are required to define how this functional connectivity may underlie a pathological role for the IC in the generation of absence seizures.

On the basis of the findings presented in this thesis and previous studies, a model for the sequence from which normal oscillations develop into absence-like SWDs is: 1) the S2 and IC cortical areas contain a critical circuit where rhythm generator cells initiate and sustain the physiological pre-ictal oscillations that are important in sensorimotor function (7-12 Hz in freely moving rats, 5-9 Hz in rats under neuroleptanalgesia); 2) excitatory propagation spreads from the S2/IC to the interconnected S1, motor and frontal cortical regions, possibly via a caudo-rostral excitatory pathway (Fujita et al., 2010); 3) The intrinsic properties of the TRN neurons allow them to resonate before TC neurons, displaying rhythmic, robust high-frequency bursts of APs (Pinault et al., 2001) underlain by low-threshold Ca^{2+} potential (Slaght et al., 2002; Pinault, 2003). 4) TRN AP bursts generate $GABA_A$ receptor mediated IPSPs in their target thalamic cells, leading to a level of hyperpolarization that may trigger low-threshold Ca^{2+} spikes in a subset of thalamic relay cells that exhibit the H-current, leading to AP discharges. This may be reinforced by a) the reciprocal cellular interactions between the thalamic and TRN cells (Bal and McCormick, 1993); and b) the abnormalities in expression and function of T-type Ca^{2+} channels (Tsakiridou et al., 1995; Talley et al., 2000; Powell et al., 2009), which are functionally mutated in GAERS (Powell et al., 2009). 5) The thalamus, in turn, recruits new units at the cortical level to fire in synchrony. The cycle is therefore restarted, resulting in cellular hypersynchronization.

8.2.3 The transformation of the somatosensory 5-9Hz rhythm to SWD is dependent on the state of thalamic resonance

The precursor cortical rhythm originating from the S2/IC are themselves not sufficient for the generation of absence-related SWDs, as they are also found in the non-epileptic rats. For this physiological rhythm to be transformed into epileptic SWDs the TRN and the TC neurons need to resonate with the cortical 5-9 Hz rhythm in a hyper-synchronized manner. Intrinsic cellular and network properties must be present within the TC network in an epileptic animal that facilitate the thalamic cells being engaged to fire at SWD frequency in a hyper-resonant and synchronized state. The firing pattern of the thalamic

relay neurons is dependent on the level of hyperpolarization of the thalamic neuronal membrane potential. Intracellular recordings of the TC neurons show that SWD-related oscillations occur in the trough of a long-lasting hyperpolarization (Pinault et al., 2006). By enhancing $GABA_A$ receptor mediated activity within the thalamus, the i.p. injection of CBZ in freely moving GAERS would hyperpolarise the TC neurons to the level at which the de-inactivation of low-threshold T-type Ca^{2+} channels occurs within a subset of TC neurons. Thus, incoming APs from CT cells result in a low-threshold Ca^{2+} potential, which triggers the TRN, then TC neurons to fire in a bursting manner and synchronizes the firing of other interconnected thalamic neurons. In addition, GAERS are known to have increased amplitudes of T-type Ca^{2+} channels and channel expression (Talley et al., 2000), and have recently been reported to have a gain of function mutation in the gene for the $Ca_v3.2$ low-threshold calcium channel mutation (Powell et al., 2009). Therefore, the mechanism by which CBZ aggravates absence seizures in freely moving GAERS is by enhancing of $GABA_A$ receptor activity, hyperpolarizing the TC neurons, and thereby promoting them to fire in a hyper-resonant manner when engaged by synapsing CT cells.

Absence seizures preferentially occur during relaxed wakefulness and drowsiness when there is a modest level of TC hyperpolarisation. At deeper stages of sleep, when there is more marked hyperpolarization of TC neurons, the 5-9 Hz oscillations and SWDs are ceased and spindle-like oscillations occur. This may be the underlying reason of the paradoxical findings between freely-moving GAERS and those under neuroleptanalgesia, where the systemic injection of CBZ aggravated seizures in freely-moving GAERS and suppressed seizures in the latter. It is also consistent with previous experiments which demonstrated a requirement for a specific level of TC hyperpolarization for the thalamus to resonate with the 5-9 Hz cortical rhythm, thereby generating SWDs and absence seizures. The enhancement of $GABA_A$ receptors by CBZ in freely moving GAERS allow the TC neurons to reach the first level of hyperpolarization, at which they are able to synchronously resonate with the CT volleys. Under neuroleptanalgesia or high dose CBZ, an additional enhancement of $GABA_A$ receptors mediated activity by CBZ would further hyperpolarize the TC neurons, therefore promoting the occurrence of spindle-like

oscillations which disrupted the 5-9 Hz activity in NEC rats and SWDs in GAERS (Chapter 5).

8.3 Future Research Directions

8.3.1 Do absence seizures arise from a topographically restricted "focus"?

The data presented in this thesis (Chapter 6) indicates that the precursor 5-9 Hz frequency somatosensory rhythm that leads to SWDs and absence seizures originates from the S2 and IC cortex, possibly generated by a population of rhythmically firing neurons in these regions. However, whether this represents a topographically restricted "focus" that drives the absence seizures, or alternatively a diffuse region from which the precursor rhythm may arise in a multifocal or distributed manner, is yet to be determined. To date, no studies have conclusively demonstrated a distinct and stationary seizure focus. Neurons from the focus may have intrinsic properties to suddenly transmit their locally synchronized rhythmic oscillations simultaneously to adjacent and remote cortical regions for mass scaled hypersynchronization. To address these questions studies could be done using a three dimensional grid of depth electrode recordings and non-linear association analysis for all relevant regions of the seizure network.

8.3.2 Are the pro-epileptogenic cortical precursor discharges more prominent in GAERS than in non-epileptic rats?

The work reported in this thesis, and previous work (Pinault et al., 2001), demonstrated that the precursor somatosensory rhythm is present in both non-epileptic and epileptic rats. However whether this is the same in both groups of animals has not been systematically investigated. It is possible that the somatosensory rhythm has characteristics that make it innately more pro-epileptogenic in GAERS, such as being longer, of higher amplitude or involving more hypersynchronized neuronal firing. Work reported in Chapter 6 demonstrated an increase in burst firing of neurons within S2 of the

GAERS compared to the same region of NEC rats. Increased NMDA-mediated synaptic excitability and over-expression of Na^+ channels Nav1.1 and 1.6 have all been reported in the cortex of WAG/Rij epileptic rats (Klein et al., 2004; D'Antuono et al., 2006). Whether such changes in ion channel function or expression contribute to the generation of oscillatory neuronal activity remains to be explored.

8.3.3 What are the cellular mechanisms that result in the precursor somatosensory rhythm being transformed into SWDs?

The cellular mechanisms that are responsible for transforming the 5-9 Hz somatosensory rhythm, which is also seen in non-epileptic rats, into epileptiform SWDs remains uncertain. It is thought that certain properties of the TC network in an epileptic brain enables and encourages its hyper-resonant firing. Changes in expression and function of a number of different ion channels and modulators of neuronal function could be responsible, for example T-type Ca^{2+} channels, HCN channels, Ca^{2+}-dependent K^+ channels, as well as neuropeptides such as NPY. Characterizing these in GAERS, and their resultant effect(s) on cellular network resonant firing, may provide important new insights into the fundamental mechanisms of epileptogenesis in the absence epilepsies.

8.3.4 Which $GABA_A$ receptor subtypes are susceptible to the effect of CBZ to enhance their function, thereby aggravating absence seizures?

The work in this thesis demonstrated that CBZ enhances the function of a $GABA_A$ receptor subtype that is highly expressed in the VB thalamus, comprised of subunits α1, β2, and γ2. Whether this is also true of other $GABA_A$ subunit combinations is not known. However the finding that microinjections of CBZ into TRN had no effect on seizures in GAERS while injections into the VB thalamus aggravated the seizures (Chapter 3), suggests that $GABA_A$ receptor subunit types that are differentially expressed in the TRN compared with the VB are resistant to the effect of CBZ. This should be examined in subsequent work. The CBZ binding site on $GABA_A$ receptors is also unknown, and could potentially represent a novel potential therapeutic target. As the primary anti-focal seizure

mechanism of CBZ and OXC are via the use-dependent blockade of Na^+ channels, a structural analogue of these drugs that retains its Na^+ channel effect but without its ability to enhance $GABA_A$ receptor activity could also have an improved therapeutic profile.

8.4 Final conclusions

CBZ is a widely prescribed anticonvulsant used for the treatment of focal epilepsy and as a mood stabilizer in psychiatric disorders such as schizophrenia. However, it is also known for its broad spectrum of action on several molecular targets contributing to common and severe adverse effects. The neuronal mechanisms underlying these therapeutic and adverse actions of CBZ and CBZ-like drugs are poorly understood. Work completed as part of my PhD provides strong evidence that CBZ affects the firing and oscillation properties of thalamic neurons, at least in the somatosensory system, through enhancement of $GABA_A$ receptor-mediated activities and this is the likely mechanism that underlies the aggravation of absence seizures.

The work presented in this thesis also provides important data providing new insights into the mechanisms underlying the initiation and propagation of absence-related SWDs. This includes the demonstration of precursor cellular and network rhythmic activities in S2 and IC prior to the onset of absence-related SWDs. Therefore it is tempting to put forward the notion that S2 and IC cortical areas contain a critical circuit from which excitation spreads widely to interconnected S1, motor and more frontal cortical areas. This caudo-rostral spread of excitation might be a key neuronal mechanism in the initiation of absence seizures. In contrast to its effect when administered systemically, CBZ is effective in suppressing absence-related SWDs only when it is injected into the S2 cortex, the presumed initiation site of this spreading caudo-rostral excitation. In conclusion, the body of work presented in this thesis provides significant insight into the neurobiological mechanisms underlying absence seizures and has potential implication in the development of novel therapeutic strategies for a common neurological disorder.

Chapter 9 References

(1989) Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. Epilepsia 30:389-399.

Adams JC (1981) Heavy metal intensification of DAB-based HRP reaction product. J Histochem Cytochem 29:775.

Aker RG, Ozkara C, Dervent A, Yilmaz Onat F (2002) Enhancement of spike and wave discharges by microinjection of bicuculline into the reticular nucleus of rats with absence epilepsy. Neurosci Lett 322:71-74.

Allen GV, Saper CB, Hurley KM, Cechetto DF (1991) Organization of visceral and limbic connections in the insular cortex of the rat. J Comp Neurol 311:1-16.

Ambrosio AF, Silva AP, Malva JO, Soares-da-Silva P, Carvalho AP, Carvalho CM (1999) Carbamazepine inhibits L-type Ca2+ channels in cultured rat hippocampal neurons stimulated with glutamate receptor agonists. Neuropharmacology 38:1349-1359.

Avanzini G, de Curtis M, Marescaux C, Panzica F, Spreafico R, Vergnes M (1992) Role of the thalamic reticular nucleus in the generation of rhythmic thalamo-cortical activities subserving spike and waves. J Neural Transm Suppl 35:85-95.

Avoli M, Gloor P, Kostopoulos G, Gotman J (1983) An analysis of penicillin-induced generalized spike and wave discharges using simultaneous recordings of cortical and thalamic single neurons. J Neurophysiol 50:819-837.

Bal T, McCormick DA (1993) Mechanisms of oscillatory activity in guinea-pig nucleus reticularis thalami in vitro: a mammalian pacemaker. J Physiol 468:669-691.

Banerjee PK, Snead OC III (1998) Neuroactive steroids exacerbate gamma-hydroxybutyric acid-induced absence seizures in rats. European Journal of Pharmacology 359:41-48.

Basar E, Basar-Eroglu C, Karakas S, Schurmann M (2001) Gamma, alpha, delta, and theta oscillations govern cognitive processes. Int J Psychophysiol 39:241-248.

Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud'homme JF, Baulac M, Brice A, Bruzzone R, LeGuern E (2001) First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet 28:46-48.

Bell GS, Gaitatzis A, Bell CL, Johnson AL, Sander JW (2009) Suicide in people with epilepsy: how great is the risk? Epilepsia 50:1933-1942.

Bennett FE (1953) Intracarotid and intravertebral metrazol in petit mal epilepsy. Neurology 3:668-673.

Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, Engel J, French J, Glauser TA, Mathern GW, Moshe SL, Nordli D, Plouin P, Scheffer IE (2010) Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 51:676-685.

Berger (1929) Über das Elektroenkephalogramm des Menschen. Arch Psychiatr Nervenkr 87:pp. 527-570.

Berkovic SF (1998) Aggravation of generalized epilepsies. Epilepsia 39 Suppl 3:S11-14.

Berkovic SF, Andermann F, Andermann E, Gloor P (1987) Concepts of absence epilepsies: discrete syndromes or biological continuum? Neurology 37:993-1000.

Bernasconi R (1982) The GABA hypothesis of the affective illness influence of clinically effective antimanic drugs on GABA turnover. Amsterdam: Excerpta Medica,.

Biervert C, Schroeder BC, Kubisch C, Berkovic SF, Propping P, Jentsch TJ, Steinlein OK (1998) A potassium channel mutation in neonatal human epilepsy. Science 279:403-406.

Bokor H, Acsady L, Deschenes M (2008) Vibrissal responses of thalamic cells that project to the septal columns of the barrel cortex and to the second somatosensory area. J Neurosci 28:5169-5177.

Boro A, Haut S (2003) Medical comorbidities in the treatment of epilepsy. Epilepsy Behav 4 Suppl 2:S2-12.

Bourassa J, Pinault D, Deschenes M (1995) Corticothalamic projections from the cortical barrel field to the somatosensory thalamus in rats: a single-fibre study using biocytin as an anterograde tracer. Eur J Neurosci 7:19-30.

Browne SH, Kang J, Akk G, Chiang LW, Schulman H, Huguenard JR, Prince DA (2001) Kinetic and pharmacological properties of GABA(A) receptors in single thalamic neurons and GABA(A) subunit expression. J Neurophysiol 86:2312-2322.

Budziszewska B, Van Luijtelaar G, Coenen AML, Leskiewicz M, Lason W (1999) Effects of neurosteroids on spike-wave discharges in the genetic epileptic WAG/Rij rat. Epilepsy Research 33:23-29.

Buzsaki G (1991) The thalamic clock: emergent network properties. Neuroscience 41:351-364.

Buzsaki G, Draguhn A (2004) Neuronal oscillations in cortical networks. Science 304:1926-1929.

Buzsaki G, Laszlovszky I, Lajtha A, Vadasz C (1990) Spike-and-wave neocortical patterns in rats: genetic and aminergic control. Neuroscience 38:323-333.

Buzsaki G, Horvath Z, Urioste R, Hetke J, Wise K (1992) High-frequency network oscillation in the hippocampus. Science 256:1025-1027.

Buzsáki G (2006) Rhythms of the Brain. New York: Oxford University Press.

Carignani M, Rosso D (1997) Clinical and EEG asymmetries in juvenile myoclonic epilepsy (JME). Epilepsia 38:258.

Cerminara C, Montanaro ML, Curatolo P, Seri S (2004) Lamotrigine-induced seizure aggravation and negative myoclonus in idiopathic rolandic epilepsy. Neurology 63:373-375.

Chaves J, Sander JW (2005) Seizure aggravation in idiopathic generalized epilepsies. Epilepsia 46 Suppl 9:133-139.

Chemin J, Monteil A, Perez-Reyes E, Bourinet E, Nargeot J, Lory P (2002) Specific contribution of human T-type calcium channel isotypes (alpha(1G), alpha(1H) and alpha(1I)) to neuronal excitability. J Physiol 540:3-14.

Chen Y, Lu J, Pan H, Zhang Y, Wu H, Xu K, Liu X, Jiang Y, Bao X, Yao Z, Ding K, Lo WH, Qiang B, Chan P, Shen Y, Wu X (2003) Association between genetic variation of CACNA1H and childhood absence epilepsy. Ann Neurol 54:239-243.

Clinckers R, Smolders I, Meurs A, Ebinger G, Michotte Y (2005) Quantitative in vivo microdialysis study on the influence of multidrug transporters on the blood-brain barrier passage of oxcarbazepine: concomitant use of hippocampal monoamines

as pharmacodynamic markers for the anticonvulsant activity. J Pharmacol Exp Ther 314:725-731.

Contreras D, Steriade M (1996) Spindle oscillation in cats: the role of corticothalamic feedback in a thalamically generated rhythm. J Physiol 490 (Pt 1):159-179.

Contreras D, Destexhe A, Steriade M (1997) Spindle oscillations during cortical spreading depression in naturally sleeping cats. Neuroscience 77:933-936.

Cope DW, Di Giovanni G, Fyson SJ, Orban G, Errington AC, Lorincz ML, Gould TM, Carter DA, Crunelli V (2009) Enhanced tonic GABAA inhibition in typical absence epilepsy. Nat Med 15:1392-1398.

Cox CL, Huguenard JR, Prince DA (1996) Heterogeneous axonal arborizations of rat thalamic reticular neurons in the ventrobasal nucleus. J Comp Neurol 366:416-430.

Crunelli V, Leresche N (1991) A role for GABAB receptors in excitation and inhibition of thalamocortical cells. Trends Neurosci 14:16-21.

Crunelli V, Leresche N (2002) Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci 3:371-382.

D'Antuono M, Inaba Y, Biagini G, D'Arcangelo G, Tancredi V, Avoli M (2006) Synaptic hyperexcitability of deep layer neocortical cells in a genetic model of absence seizures. Genes Brain Behav 5:73-84.

D'Arcangelo G, D'Antuono M, Biagini G, Warren R, Tancredi V, Avoli M (2002) Thalamocortical oscillations in a genetic model of absence seizures. Eur J Neurosci 16:2383-2393.

Danober L, Deransart C, Depaulis A, Vergnes M, Marescaux C (1998) Pathophysiological mechanisms of genetic absence epilepsy in the rat. Prog Neurobiol 55:27-57.

Dedeurwaerdere S, Vonck K, Van Hese P, Wadman W, Boon P (2005) The acute and chronic effect of vagus nerve stimulation in genetic absence epilepsy rats from Strasbourg (GAERS). Epilepsia 46 Suppl 5:94-97.

DeLorey TM, Olsen RW (1992) Gamma-aminobutyric acidA receptor structure and function. J Biol Chem 267:16747-16750.

Depaulis A, Snead OC, 3rd, Marescaux C, Vergnes M (1989) Suppressive effects of intranigral injection of muscimol in three models of generalized non-convulsive epilepsy induced by chemical agents. Brain Res 498:64-72.

Di Pasquale E, Keegan KD, Noebels JL (1997) Increased excitability and inward rectification in layer V cortical pyramidal neurons in the epileptic mutant mouse Stargazer. J Neurophysiol 77:621-631.

Draguhn A, Traub RD, Schmitz D, Jefferys JG (1998) Electrical coupling underlies high-frequency oscillations in the hippocampus in vitro. Nature 394:189-192.

Drinkenburg WH, Coenen AM, Vossen JM, Van Luijtelaar EL (1991) Spike-wave discharges and sleep-wake states in rats with absence epilepsy. Epilepsy Res 9:218-224.

Ebersole JS, Pedley TA (2003) Current practice of clinical electroencephalography. 3rd ed Philadelphia, PA: Lippincott Williams & Wilkins.

Engel AK, Fries P, Singer W (2001) Dynamic predictions: oscillations and synchrony in top-down processing. Nat Rev Neurosci 2:704-716.

Engel J, Jr. (2006) ILAE classification of epilepsy syndromes. Epilepsy Res 70 Suppl 1:S5-10.

Fanselow EE, Sameshima K, Baccala LA, Nicolelis MA (2001) Thalamic bursting in rats during different awake behavioral states. Proc Natl Acad Sci U S A 98:15330-15335.

Fariello RG, Golden GT (1987) The THIP-induced model of bilateral synchronous spike and wave in rodents. Neuropharmacology 26:161-165.

Feldmann KF, Brechbuhler S., Faigle J.W., P. I (1978) Pharmacokinetics and metabolism of GP 47680, a compound related to carbamazepine in animals and man. Amsterdam and Lisse: Swets & Zeitlinger B.V., .

Ferrarelli F, Huber R, Peterson MJ, Massimini M, Murphy M, Riedner BA, Watson A, Bria P, Tononi G (2007) Reduced sleep spindle activity in schizophrenia patients. Am J Psychiatry 164:483-492.

Ferri R, Iliceto G, Carlucci V (1995) Topographic EEG mapping of 3/s spike-and-wave complexes during absence seizures. Ital J Neurol Sci 16:541-547.

Feucht M, Fuchs K, Pichlbauer E, Hornik K, Scharfetter J, Goessler R, Fureder T, Cvetkovic N, Sieghart W, Kasper S, Aschauer H (1999) Possible association between childhood absence epilepsy and the gene encoding GABRB3. Biol Psychiatry 46:997-1002.

Fisher RS, Prince DA (1977) Spike-wave rhythms in cat cortex induced by parenteral penicillin. II. Cellular features. Electroencephalogr Clin Neurophysiol 42:625-639.

Fitzgerald PJ, Lane JW, Thakur PH, Hsiao SS (2004) Receptive field properties of the macaque second somatosensory cortex: evidence for multiple functional representations. J Neurosci 24:11193-11204.

Fonnum F, Storm-Mathisen J, Divac I (1981) Biochemical evidence for glutamate as neurotransmitter in corticostriatal and corticothalamic fibres in rat brain. Neuroscience 6:863-873.

Fuentealba P, Timofeev I, Steriade M (2004) Prolonged hyperpolarizing potentials precede spindle oscillations in the thalamic reticular nucleus. Proc Natl Acad Sci U S A 101:9816-9821.

Fujita S, Adachi K, Koshikawa N, Kobayashi M (2010) Spatiotemporal dynamics of excitation in rat insular cortex: intrinsic corticocortical circuit regulates caudal-rostro excitatory propagation from the insular to frontal cortex. Neuroscience 165:278-292.

Gandolfo G, Glin L, Gottesmann C (1985) Study of sleep spindles in the rat: a new improvement. Acta Neurobiol Exp (Wars) 45:151-162.

Gasser T, Reddington M, Schubert P (1988) Effect of carbamazepine on stimulus-evoked Ca2+ fluxes in rat hippocampal slices and its interaction with A1-adenosine receptors. Neurosci Lett 91:189-193.

Gayatri NA, Livingston JH (2006) Aggravation of epilepsy by anti-epileptic drugs. Dev Med Child Neurol 48:394-398.

Gelisse P, Genton P, Kuate C, Pesenti A, Baldy-Moulinier M, Crespel A (2004) Worsening of seizures by oxcarbazepine in juvenile idiopathic generalized epilepsies. Epilepsia 45:1282-1286.

Gibbs F, .Davis H, WG. L (1935) The EEG in epilepsy and in conditions of impaired consciousness. Arch Neurol Psychiatry:1134-1148.

Gibbs FA, Gibbs EL (1952) Atlas of Electroencephalography. : Addison-Wesley Publishers.

Glauser TA, Cnaan A, Shinnar S, Hirtz DG, Dlugos D, Masur D, Clark PO, Capparelli EV, Adamson PC (2010) Ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy. N Engl J Med 362:790-799.

Gloor P (1968) Generalized cortico-reticular epilepsies. Some considerations on the pathophysiology of generalized bilaterally synchronous spike and wave discharge. Epilepsia 9:249-263.

Gloor P, Fariello RG (1988) Generalized epilepsy: some of its cellular mechanisms differ from those of focal epilepsy. Trends Neurosci 11:63-68.

Goldin AL (1992) Maintenance of Xenopus laevis and oocyte injection. Methods Enzymol 207:266-279.

Granger P, Biton B, Faure C, Vige X, Depoortere H, Graham D, Langer SZ, Scatton B, Avenet P (1995) Modulation of the gamma-aminobutyric acid type A receptor by the antiepileptic drugs carbamazepine and phenytoin. Mol Pharmacol 47:1189-1196.

Graumlich JF, McLaughlin RG, Birkhahn D, Shah N, Burk A, Jobe PC, Dailey JW (1999) Carbamazepine pharmacokinetics-pharmacodynamics in genetically epilepsy-prone rats. Eur J Pharmacol 369:305-311.

Guillery RW (1995) Anatomical evidence concerning the role of the thalamus in corticocortical communication: a brief review. J Anat 187 (Pt 3):583-592.

Hammond C, Bergman H, Brown P (2007) Pathological synchronization in Parkinson's disease: networks, models and treatments. Trends Neurosci 30:357-364.

Hawkinson JE, Acosta-Burruel M, Kimbrough CL, Goodnough DB, Wood PL (1996) Steroid inhibition of [3H]SR 95531 binding to the GABAA recognition site. European Journal of Pharmacology 304:141-146.

Heron SE, Scheffer IE, Berkovic SF, Dibbens LM, Mulley JC (2007) Channelopathies in idiopathic epilepsy. Neurotherapeutics 4:295-304.

Hodgkin AL, Huxley AF (1945) Resting and action potentials in single nerve fibres. J Physiol 104:176-195.

Hommet C, Hureaux R, Barre J, Constans T, Berrut G (2007) Epileptic seizures in clinically diagnosed Alzheimer's disease: report from a geriatric medicine population. Aging Clin Exp Res 19:430-431.

Horikawa K, Armstrong WE (1988) A versatile means of intracellular labeling: injection of biocytin and its detection with avidin conjugates. J Neurosci Methods 25:1-11.

Hosak L, Libiger J (2002) Antiepileptic drugs in schizophrenia: a review. Eur Psychiatry 17:371-378.

Hosford DA, Wang Y (1997) Utility of the lethargic (lh/lh) mouse model of absence seizures in predicting the effects of lamotrigine, vigabatrin, tiagabine, gabapentin, and topiramate against human absence seizures. Epilepsia 38:408-414.

Hosford DA, Caddick SJ, Lin FH (1997) Generalized epilepsies: emerging insights into cellular and genetic mechanisms. Curr Opin Neurol 10:115-120.

Hosford DA, Wang Y (1997) Utility of the lethargic (lh/lh) mouse model of absence seizures in predicting the effects of lamotrigine, vigabatrin, tiagabine, gabapentin, and topiramate against human absence seizures. Epilepsia 38:408-414.

Hosford DA, Wang Y, Cao Z (1997) Differential effects mediated by $GABA_A$ receptors in thalamic nuclei in lh/lh model of absences seizures. Epilepsy Research 1997:55-65.

Houser CR, Vaughn JE, Barber RP, Roberts E (1980) GABA neurons are the major cell type of the nucleus reticularis thalami. Brain Res 200:341-354.

ILAE (2009) Revised terminology and concepts for organization of the
epilepsies: Report of the Commission on Classification and
Terminology. Commision Report
Internation League Against Epilepsy.

Inoue M, Ates N, Vossen JM, Coenen AM (1994) Effects of the neuroleptanalgesic fentanyl-fluanisone (Hypnorm) on spike-wave discharges in epileptic rats. Pharmacol Biochem Behav 48:547-551.

Inouye T, Sakamoto H, Shinosaki K, Toi S, Ukai S (1990) Analysis of rapidly changing EEGs before generalized spike and wave complexes. Electroencephalogr Clin Neurophysiol 76:205-221.

Jasper HH, Kershman J (1941) Electroencephalographic classification of the epilepsies. Arch Neurol Psychiatry 45:903-943.

Jeong J (2004) EEG dynamics in patients with Alzheimer's disease. Clin Neurophysiol 115:1490-1505.

Jilek-Aall L (1999) Morbus sacer in Africa: some religious aspects of epilepsy in traditional cultures. Epilepsia 40:382-386.

Jones EG (1985) The Thalamus: Eds P.C. Emson. Raven Press: New York.

Jones NC, Salzberg MR, Kumar G, Couper A, Morris MJ, O'Brien TJ (2008) Elevated anxiety and depressive-like behavior in a rat model of genetic generalized epilepsy suggesting common causation. Exp Neurol 209:254-260.

Khosravani H, Altier C, Simms B, Hamming KS, Snutch TP, Mezeyova J, McRory JE, Zamponi GW (2004) Gating effects of mutations in the Cav3.2 T-type calcium channel associated with childhood absence epilepsy. J Biol Chem 279:9681-9684.

Klein JP, Khera DS, Nersesyan H, Kimchi EY, Waxman SG, Blumenfeld H (2004) Dysregulation of sodium channel expression in cortical neurons in a rodent model of absence epilepsy. Brain Res 1000:102-109.

Korolkiewicz R, Kleinrok Z, Mlynarczyk M (1996) Intracerebroventricular pertussis toxin enhances sensitivity to chemical convulsants and decreases the protective efficacy of carbamazepine in mice. Pharmacol Res 33:211-215.

Kostopoulos GK (2000) Spike-and-wave discharges of absence seizures as a transformation of sleep spindles: the continuing development of a hypothesis. Clin Neurophysiol 111 Suppl 2:S27-38.

Kubova H, Mares P (1993) Anticonvulsant action of oxcarbazepine, hydroxycarbamazepine, and carbamazepine against metrazol-induced motor seizures in developing rats. Epilepsia 34:188-192.

Lancel M, Cronlein TA, Faulhaber J (1996) Role of GABAA receptors in sleep regulation. Differential effects of muscimol and midazolam on sleep in rats. Neuropsychopharmacology 15:63-74.

Lennox WG, Lennox MA (1960) Epilepsy and related disorders. Boston: Little, Brown and Co, :546-574.

Lerer B, Moore N, Meyendorff E, Cho SR, Gershon S (1985) Carbamazepine and lithium: different profiles in affective disorder? Psychopharmacol Bull 21:18-22.

Lerman P (1986) Seizures induced or aggravated by anticonvulsants. Epilepsia 27:706-710.

Levy A, Chong SK, Price JF (1985) Carbamazepine-induced drowsiness. Lancet 2:221-222.

Lhatoo SD, Sander JW (2001) The epidemiology of epilepsy and learning disability. Epilepsia 42 Suppl 1:6-9; discussion 19-20.

Liao CC, Yen CT (2008) Functional connectivity of the secondary somatosensory cortex of the rat. Anat Rec (Hoboken) 291:960-973.

Liu L, Zheng T, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ (2006) The mechanism of carbamazepine aggravation of absence seizures. J Pharmacol Exp Ther 319:790-798.

Liu Z, Vergnes M, Depaulis A, Marescaux C (1991) Evidence for a critical role of GABAergic transmission within the thalamus in the genesis and control of absence seizures in the rat. Brain Res 545:1-7.

Liu Z, Vergnes M, Depaulis A, Marescaux C (1991) Evidence for a critical role of GABAergic transmission within the thalamus in the genesis and control of absence seizures in the rat. Brain Research 545:1-7.

Llinas R, Ribary U, Contreras D, Pedroarena C (1998) The neuronal basis for consciousness. Philos Trans R Soc Lond B Biol Sci 353:1841-1849.

Llinas RR (1988) The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. Science 242:1654-1664.

Llinas RR, Grace AA, Yarom Y (1991) In vitro neurons in mammalian cortical layer 4 exhibit intrinsic oscillatory activity in the 10- to 50-Hz frequency range. Proc Natl Acad Sci U S A 88:897-901.

Lloyd P, Flesch G, Dieterle W (1994) Clinical pharmacology and pharmacokinetics of oxcarbazepine. Epilepsia 35 Suppl 3:S10-13.

Lohman RJ, Liu L, Morris M, O'Brien TJ (2005) Validation of a method for localised microinjection of drugs into thalamic subregions in rats for epilepsy pharmacological studies. J Neurosci Methods 146:191-197.

Lopes da Silva F (1991) Neural mechanisms underlying brain waves: from neural membranes to networks. Electroencephalogr Clin Neurophysiol 79:81-93.

Manning JP, Richards DA, Leresche N, Crunelli V, Bowery NG (2004) Cortical-area specific block of genetically determined absence seizures by ethosuximide. Neuroscience 123:5-9.

Marescaux C, Vergnes M, Depaulis A (1992a) Neurotransmission in rats' spontaneous generalized nonconvulsive epilepsy. Epilepsy Res Suppl 8:335-343.

Marescaux C, Vergnes M, Depaulis A (1992b) Genetic absence epilepsy in rats from Strasbourg--a review. J Neural Transm Suppl 35:37-69.

Marescaux C, Micheletti G, Vergnes M, Depaulis A, Rumbach L, Warter JM (1984) A model of chronic spontaneous petit mal-like seizures in the rat: comparison with pentylenetetrazol-induced seizures. Epilepsia 25:326-331.

Marescaux C, Micheletti G, Vergnes M, Depaulis A, Rumbach L, Warter JM (1984) A model of chronic spontaneous petit mal-like seizures in the rat: comparison with pentylenetetrazol-induced seizures. Epilepsia 25:326-331.

Mattson RH (2003) Overview: idiopathic generalized epilepsies. Epilepsia 44 Suppl 2:2-6.

McCormick DA, von Krosigk M (1992) Corticothalamic activation modulates thalamic firing through glutamate "metabotropic" receptors. Proc Natl Acad Sci U S A 89:2774-2778.

McCormick DA, Bal T (1997) Sleep and arousal: thalamocortical mechanisms. Annu Rev Neurosci 20:185-215.

McCormick DA, Contreras D (2001) On the cellular and network bases of epileptic seizures. Annu Rev Physiol 63:815-846.

McLean KJ, O'Brien TJ, Cook MJ, Vajda FJ (2004) The influence of gender on the aggravation of absence seizures by carbamazepine in the low-dose pentylenetetrazol rat model. Seizure 13:208-216.

Meeren HK, Pijn JP, Van Luijtelaar EL, Coenen AM, Lopes da Silva FH (2002) Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci 22:1480-1495.

Meldrum B, Horton R (1980) Effects of the bicyclic GABA agonist, THIP, on myoclonic and seizure responses in mice and baboons with reflex epilepsy. Eur J Pharmacol 61:231-237.

Micheletti G, Vergnes M, Marescaux C, Reis J, Depaulis A, Rumbach L, Warter JM (1985) Antiepileptic drug evaluation in a new animal model: spontaneous petit mal epilepsy in the rat. Arzneimittelforschung 35:483-485.

Neuper C, Pfurtscheller G (2001) Event-related dynamics of cortical rhythms: frequency-specific features and functional correlates. Int J Psychophysiol 43:41-58.

Nicolelis MA, Baccala LA, Lin RC, Chapin JK (1995) Sensorimotor encoding by synchronous neural ensemble activity at multiple levels of the somatosensory system. Science 268:1353-1358.

Niedermeyer E (1982) Petit Mal, Primary Generalized Epilepsy and Sleep. New York: Academic Press.

Niedermeyer E (1996) Primary (idiopathic) generalized epilepsy and underlying mechanisms. Clin Electroencephalogr 27:1-21.

Nowak LG, Bullier J (1998) Axons, but not cell bodies, are activated by electrical stimulation in cortical gray matter. II. Evidence from selective inactivation of cell bodies and axon initial segments. Exp Brain Res 118:489-500.

Ostojic ZS, Ruzdijic S, Car M, Rakic L, Veskov R (1997) The connection between absence-like seizures and hypothermia induced by penicillin: possible implication on other animal models of petit mal epilepsy. Brain Res 777:86-94.

Pandey CK, Raza M, Tripathi M, Navkar DV, Kumar A, Singh UK (2005) The comparative evaluation of gabapentin and carbamazepine for pain management in Guillain-Barre syndrome patients in the intensive care unit. Anesth Analg 101:220-225, table of contents.

Paxinos G, Watson C (1998) The Rat Brain in Stereotaxic Coordinates. 4th Edition, Academic Press, San Diego, CA.

Penfield W (1952) Epileptic automatism and the centrencephalic integrating system. Res Publ Assoc Res Nerv Ment Dis 30:513-528.

Perucca E, Gram L, Avanzini G, Dulac O (1998) Antiepileptic drugs as a cause of worsening seizures. Epilepsia 39:5-17.

Perucca E, Gram L, Avanzini G, Dulac O (1998) Antiepileptic drugs as a cause of worsening seizures. Epilepsia 39:5-17.

Pinault D (1996) A novel single-cell staining procedure performed in vivo under electrophysiological control: morpho-functional features of juxtacellularly labeled thalamic cells and other central neurons with biocytin or Neurobiotin. J Neurosci Methods 65:113-136.

Pinault D (2003) Cellular interactions in the rat somatosensory thalamocortical system during normal and epileptic 5-9 Hz oscillations. J Physiol 552:881-905.

Pinault D (2005) A new stabilizing craniotomy-duratomy technique for single-cell anatomo-electrophysiological exploration of living intact brain networks. J Neurosci Methods 141:231-242.

Pinault D, Deschenes M (1998) Projection and innervation patterns of individual thalamic reticular axons in the thalamus of the adult rat: a three-dimensional, graphic, and morphometric analysis. J Comp Neurol 391:180-203.

Pinault D, Bourassa J, Deschenes M (1995) Thalamic reticular input to the rat visual thalamus: a single fiber study using biocytin as an anterograde tracer. Brain Res 670:147-152.

Pinault D, Vergnes M, Marescaux C (2001) Medium-voltage 5-9-Hz oscillations give rise to spike-and-wave discharges in a genetic model of absence epilepsy: in vivo dual extracellular recording of thalamic relay and reticular neurons. Neuroscience 105:181-201.

Pinault D, Slezia A, Acsady L (2006) Corticothalamic 5-9 Hz oscillations are more pro-epileptogenic than sleep spindles in rats. J Physiol 574:209-227.

Pinault D, Leresche N, Charpier S, Deniau JM, Marescaux C, Vergnes M, Crunelli V (1998) Intracellular recordings in thalamic neurones during spontaneous spike and wave discharges in rats with absence epilepsy. J Physiol 509 (Pt 2):449-456.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815-850.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) $GABA_A$ receptors: Immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815-850.

Pitkänen A, Schwartzkroin PA, Moshé SL (2006) Models of Seizures and Epilepsy: Academic Press.

Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S (2007) Deep layer somatosensory cortical neurons initiate spike-and-wave discharges in a genetic model of absence seizures. J Neurosci 27:6590-6599.

Post RM, Susan R, Weiss B (1992) Sensitization, kindling, and carbamazepine: an update on their implications for the course of affective illness. Pharmacopsychiatry 25:41-43.

Powell KL, Kyi M, Reid CA, Paradiso L, D'Abaco GM, Kaye AH, Foote SJ, O'Brien TJ (2008) Genetic absence epilepsy rats from Strasbourg have increased corticothalamic expression of stargazin. Neurobiol Dis 31:261-265.

Powell KL, Cain SM, Ng C, Sirdesai S, David LS, Kyi M, Garcia E, Tyson JR, Reid CA, Bahlo M, Foote SJ, Snutch TP, O'Brien TJ (2009) A Cav3.2 T-type calcium channel point mutation has splice-variant-specific effects on function and segregates with seizure expression in a polygenic rat model of absence epilepsy. J Neurosci 29:371-380.

Pumain R, Louvel J, Gastard M, Kurcewicz I, Vergnes M (1992) Responses to N-methyl-D-aspartate are enhanced in rats with petit mal-like seizures. J Neural Transm Suppl 35:97-108.

Rambeck B, Jurgens UH, May TW, Pannek HW, Behne F, Ebner A, Gorji A, Straub H, Speckmann EJ, Pohlmann-Eden B, Loscher W (2006) Comparison of brain extracellular fluid, brain tissue, cerebrospinal fluid, and serum concentrations of antiepileptic drugs measured intraoperatively in patients with intractable epilepsy. Epilepsia 47:681-694.

Ranck JB, Jr. (1975) Which elements are excited in electrical stimulation of mammalian central nervous system: a review. Brain Res 98:417-440.

Robinson R, Taske N, Sander T, Heils A, Whitehouse W, Goutieres F, Aicardi J, Lehesjoki AE, Siren A, Laue Friis M, Kjeldsen MJ, Panayiotopoulos C, Kennedy C, Ferrie C, Rees M, Gardiner RM (2002) Linkage analysis between childhood absence epilepsy and genes encoding GABAA and GABAB receptors, voltage-dependent calcium channels, and the ECA1 region on chromosome 8q. Epilepsy Res 48:169-179.

Salem H, Grossman MH, Bilbey DL (1963) Micro-Method for Intravenous Injection and Blood Sampling. J Pharm Sci 52:794-795.

Sanudo-Pena MC, Walker JM (1997) Role of the subthalamic nucleus in cannabinoid actions in the substantia nigra of the rat. J Neurophysiol 77:1635-1638.

Saper CB (1982) Convergence of autonomic and limbic connections in the insular cortex of the rat. J Comp Neurol 210:163-173.

Sauseng P, Klimesch W (2008) What does phase information of oscillatory brain activity tell us about cognitive processes? Neurosci Biobehav Rev 32:1001-1013.

Sazgar M, Bourgeois BF (2005) Aggravation of epilepsy by antiepileptic drugs. Pediatr Neurol 33:227-234.

Scharfman HE, Pedley, T.A., (2007) Neurobiology of Disease. Elsevier:349-368.

Scheffer IE, Berkovic SF (2003) The genetics of human epilepsy. Trends Pharmacol Sci 24:428-433.

Schirrmacher K, Mayer A, Walden J, Dusing R, Bingmann D (1995) Effects of carbamazepine on membrane properties of rat sensory spinal ganglion cells in vitro. Eur Neuropsychopharmacol 5:501-507.

Schmidt D, Elger CE (2004) What is the evidence that oxcarbazepine and carbamazepine are distinctly different antiepileptic drugs? Epilepsy Behav 5:627-635.

Schmutz M, Brugger F, Gentsch C, McLean MJ, Olpe HR (1994) Oxcarbazepine: preclinical anticonvulsant profile and putative mechanisms of action. Epilepsia 35 Suppl 5:S47-50.

Schnitzler A, Gross J (2005) Normal and pathological oscillatory communication in the brain. Nat Rev Neurosci 6:285-296.

Schutz H, Feldmann KF, Faigle JW, Kriemler HP, Winkler T (1986) The metabolism of 14C-oxcarbazepine in man. Xenobiotica 16:769-778.

Seidenbecher T, Staak R, Pape HC (1998) Relations between cortical and thalamic cellular activities during absence seizures in rats. Eur J Neurosci 10:1103-1112.

Sherman SM, Guillery RW (1996) Functional organization of thalamocortical relays. J Neurophysiol 76:1367-1395.

Sieghart W (2006) Structure, pharmacology, and function of GABAA receptor subtypes. Adv Pharmacol 54:231-263.

Silva LR, Amitai Y, Connors BW (1991) Intrinsic oscillations of neocortex generated by layer 5 pyramidal neurons. Science 251:432-435.

Singer W, Gray CM (1995) Visual feature integration and the temporal correlation hypothesis. Annu Rev Neurosci 18:555-586.

Slaght SJ, Leresche N, Deniau JM, Crunelli V, Charpier S (2002) Activity of thalamic reticular neurons during spontaneous genetically determined spike and wave discharges. J Neurosci 22:2323-2334.

Snead OC, 3rd (1978) Gamma hydroxybutyrate in the monkey. I. Electroencephalographic, behavioral, and pharmacokinetic studies. Neurology 28:636-642.

Snead OC, 3rd (1995) Basic mechanisms of generalized absence seizures. Ann Neurol 37:146-157.

Snead OC, 3rd, Depaulis A, Vergnes M, Marescaux C (1999) Absence epilepsy: advances in experimental animal models. Adv Neurol 79:253-278.

Staak R, Pape HC (2001) Contribution of GABA(A) and GABA(B) receptors to thalamic neuronal activity during spontaneous absence seizures in rats. J Neurosci 21:1378-1384.

Stefani A, Pisani A, De Murtas M, Mercuri NB, Marciani MG, Calabresi P (1995) Action of GP 47779, the active metabolite of oxcarbazepine, on the corticostriatal system. II. Modulation of high-voltage-activated calcium currents. Epilepsia 36:997-1002.

Steinlein OK (2004) Genes and mutations in human idiopathic epilepsy. Brain Dev 26:213-218.

Steriade M (1995) Thalamic origin of sleep spindles: Morison and Bassett (1945). J Neurophysiol 73:921-922.

Steriade M (2001) Impact of network activities on neuronal properties in corticothalamic systems. J Neurophysiol 86:1-39.

Steriade M (2006) Grouping of brain rhythms in corticothalamic systems. Neuroscience 137:1087-1106.

Steriade M, Deschenes M (1984) The thalamus as a neuronal oscillator. Brain Res 320:1-63.

Steriade M, Amzica F (1994) Dynamic coupling among neocortical neurons during evoked and spontaneous spike-wave seizure activity. J Neurophysiol 72:2051-2069.

Steriade M, Domich L, Oakson G (1986) Reticularis thalami neurons revisited: activity changes during shifts in states of vigilance. J Neurosci 6:68-81.

Steriade M, Dossi RC, Nunez A (1991) Network modulation of a slow intrinsic oscillation of cat thalamocortical neurons implicated in sleep delta waves: cortically induced synchronization and brainstem cholinergic suppression. J Neurosci 11:3200-3217.

Steriade M, McCormick DA, Sejnowski TJ (1993a) Thalamocortical oscillations in the sleeping and aroused brain. Science 262:679-685.

Steriade M, Nunez A, Amzica F (1993b) Intracellular analysis of relations between the slow (< 1 Hz) neocortical oscillation and other sleep rhythms of the electroencephalogram. J Neurosci 13:3266-3283.

Steriade M, Deschenes M, Domich L, Mulle C (1985) Abolition of spindle oscillations in thalamic neurons disconnected from nucleus reticularis thalami. J Neurophysiol 54:1473-1497.

Steriade M, Contreras D, Amzica F, Timofeev I (1996) Synchronization of fast (30-40 Hz) spontaneous oscillations in intrathalamic and thalamocortical networks. J Neurosci 16:2788-2808.

Steriade M, Timofeev I, Durmuller N, Grenier F (1998) Dynamic properties of corticothalamic neurons and local cortical interneurons generating fast rhythmic (30-40 Hz) spike bursts. J Neurophysiol 79:483-490.

Sukhodolsky DG, Leckman JF, Rothenberger A, Scahill L (2007) The role of abnormal neural oscillations in the pathophysiology of co-occurring Tourette syndrome and attention-deficit/hyperactivity disorder. Eur Child Adolesc Psychiatry 16 Suppl 1:51-59.

Talley EM, Solorzano G, Depaulis A, Perez-Reyes E, Bayliss DA (2000) Low-voltage-activated calcium channel subunit expression in a genetic model of absence epilepsy in the rat. Brain Res Mol Brain Res 75:159-165.

Tecoma ES (1999) Oxcarbazepine. Epilepsia 40 Suppl 5:S37-46.

Tommerdahl M, Favorov OV, Whitsel BL (2010) Dynamic representations of the somatosensory cortex. Neurosci Biobehav Rev 34:160-170.

Traub RD, Schmitz D, Jefferys JG, Draguhn A (1999) High-frequency population oscillations are predicted to occur in hippocampal pyramidal neuronal networks interconnected by axoaxonal gap junctions. Neuroscience 92:407-426.

Traub RD, Contreras D, Cunningham MO, Murray H, LeBeau FE, Roopun A, Bibbig A, Wilent WB, Higley MJ, Whittington MA (2005) Single-column thalamocortical network model exhibiting gamma oscillations, sleep spindles, and epileptogenic bursts. J Neurophysiol 93:2194-2232.

Tsakiridou E, Bertollini L, de Curtis M, Avanzini G, Pape HC (1995) Selective increase in T-type calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. J Neurosci 15:3110-3117.

Uhlhaas PJ, Singer W (2010) Abnormal neural oscillations and synchrony in schizophrenia. Nat Rev Neurosci 11:100-113.

Ullrich NJ, Riviello JJ, Jr., Darras BT, Donner EJ (2004) Electroencephalographic correlate of juvenile Huntington's disease. J Child Neurol 19:541-543.

van der Stelt O, Belger A, Lieberman JA (2004) Macroscopic fast neuronal oscillations and synchrony in schizophrenia. Proc Natl Acad Sci U S A 101:17567-17568.

van Luijtelaar EL (1997) Spike-wave discharges and sleep spindles in rats. Acta Neurobiol Exp (Wars) 57:113-121.

van Luijtelaar EL, Coenen AM (1986) Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci Lett 70:393-397.

Vendrame M, Khurana DS, Cruz M, Melvin J, Valencia I, Legido A, Kothare SV (2007) Aggravation of seizures and/or EEG features in children treated with oxcarbazepine monotherapy. Epilepsia 48:2116-2120.

Vergnes M, Marescaux C, Depaulis A (1990) Mapping of spontaneous spike and wave discharges in Wistar rats with genetic generalized non-convulsive epilepsy. Brain Res 523:87-91.

Vergnes M, Marescaux C, Boehrer A, Depaulis A (1991) Are rats with genetic absence epilepsy behaviorally impaired? Epilepsy Res 9:97-104.

Vergnes M, Marescaux C, Depaulis A, Micheletti G, Warter JM (1987) Spontaneous spike and wave discharges in thalamus and cortex in a rat model of genetic petit mal-like seizures. Exp Neurol 96:127-136.

Vergnes M, Boehrer A, Reibel S, Simler S, Marescaux C (2000) Selective susceptibility to inhibitors of GABA synthesis and antagonists of GABA(A) receptor in rats with genetic absence epilepsy. Exp Neurol 161:714-723.

Vergnes M, Marescaux C, Micheletti G, Depaulis A, Rumbach L, Warter JM (1984) Enhancement of spike and wave discharges by GABAmimetic drugs in rats with spontaneous petit-mal-like epilepsy. Neurosci Lett 44:91-94.

Vergnes M, Marescaux C, Micheletti G, Reis J, Depaulis A, Rumbach L, Warter JM (1982) Spontaneous paroxysmal electroclinical patterns in rat: a model of generalized non-convulsive epilepsy. Neurosci Lett 33:97-101.

von Krosigk M, Bal T, McCormick DA (1993) Cellular mechanisms of a synchronized oscillation in the thalamus. Science 261:361-364.

von Stein A, Chiang C, Konig P (2000) Top-down processing mediated by interareal synchronization. Proc Natl Acad Sci U S A 97:14748-14753.

Wagner J, Schmid K (1987) Induction of microsomal enzymes in rat liver by oxcarbazepine, 10,11-dihydro-10-hydroxy-carbamazepine and carbamazepine. Xenobiotica 17:951-956.

Walczak TS, Leppik IE, D'Amelio M, Rarick J, So E, Ahman P, Ruggles K, Cascino GD, Annegers JF, Hauser WA (2001) Incidence and risk factors in sudden unexpected death in epilepsy: a prospective cohort study. Neurology 56:519-525.

Wallace RH, Wang DW, Singh R, Scheffer IE, George AL, Jr., Phillips HA, Saar K, Reis A, Johnson EW, Sutherland GR, Berkovic SF, Mulley JC (1998) Febrile seizures and generalized epilepsy associated with a mutation in the Na+-channel beta1 subunit gene SCN1B. Nat Genet 19:366-370.

Wallengren C, Li S, Morris MJ, Jupp B, O'Brien TJ (2005) Aggravation of absence seizures by carbamazepine in a genetic rat model does not induce neuronal c-Fos activation. Clin Neuropharmacol 28:60-65.

Wang XJ, Rinzel J (1993) Spindle rhythmicity in the reticularis thalami nucleus: synchronization among mutually inhibitory neurons. Neuroscience 53:899-904.

Warter JM, Vergnes M, Depaulis A, Tranchant C, Rumbach L, Micheletti G, Marescaux C (1988) Effects of drugs affecting dopaminergic neurotransmission in rats with spontaneous petit mal-like seizures. Neuropharmacology 27:269-274.

Wehr M, Laurent G (1996) Odour encoding by temporal sequences of firing in oscillating neural assemblies. Nature 384:162-166.

Wiest MC, Nicolelis MA (2003) Behavioral detection of tactile stimuli during 7-12 Hz cortical oscillations in awake rats. Nat Neurosci 6:913-914.

Willow M, Gonoi T, Catterall WA (1985) Voltage clamp analysis of the inhibitory actions of diphenylhydantoin and carbamazepine on voltage-sensitive sodium channels in neuroblastoma cells. Mol Pharmacol 27:549-558.

Winsky-Sommerer R (2009) Role of GABAA receptors in the physiology and pharmacology of sleep. Eur J Neurosci 29:1779-1794.

Wong C G-T, Carter Snead III O (2001) The $GABA_A$ receptor: Subunit-dependent functions and absence seizures. Epilepsy Currents 1:1-5.

Yang JD, Elphick M, Sharpley AL, Cowen PJ (1989) Effects of carbamazepine on sleep in healthy volunteers. Biol Psychiatry 26:324-328.

Zhang ZW, Deschenes M (1998) Projections to layer VI of the posteromedial barrel field in the rat: a reappraisal of the role of corticothalamic pathways. Cereb Cortex 8:428-436.

Zheng T, Clarke AL, Morris MJ, Reid CA, Petrou S, O'Brien TJ (2009) Oxcarbazepine, not its active metabolite, potentiates GABAA activation and aggravates absence seizures. Epilepsia 50:83-87.