EFFECTS OF NEUROPEPTIDE Y ON SINGLE NEURONAL FIRING PATTERNS IN A GENETIC RAT MODEL OF ABSENCE EPILEPSY

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ABSTRACT
Absence seizures are most common type of generalized seizures where brain goes into abnormal pathological rhythm characterized by bilateral and synchronous 3Hz spike and wave discharges on EEG. Currently available anti-epileptic drugs do not have adequate seizure control and have adverse side effects, which increased the desire for development of novel therapeutic options for treating absence seizures. Previous studies demonstrated that neuropeptide Y (NPY) successfully suppresses absence seizures in genetic rat model of absence epilepsy but the underlying neuronal mechanisms for seizure suppression still remain unknown. Recent developments in electrophysiology enabled the quantification of neuronal firing patterns in vivo and many researchers have already demonstrated differential characteristic firing patterns of neurons in thalamo cortical circuit in the brain that plays a critical role in absence seizures. This study was designed to investigate the effects of intracerebroventricular (ICV) and locally administered NPY on neuronal firing patterns in thalamocortical structures, which play a critical role in the generation of absence like seizures in GAERS as well as effects on seizure induction threshold in the cortical S2 region.

METHODS
All experiments were conducted on 8-12 weeks old male GAERS rats. This involved performing in vivo electrophysiological recordings under neurolept anaesthesia. In these experiments single neuronal firing activity was recorded from different regions of thalamocortical circuit. Once stable electrophysiological recordings are achieved, NPY was infused both ICV and focally at the recording site and alterations in the neuronal firing patterns were assessed before and after the drug intervention. In another experiment cortical region S2 was stimulated by external electrical stimulation to induce absence
like seizure and effects of ICV administration of NPY on seizure triggering threshold in the S2 region was assessed in GAERS.

**Results**

NPY suppressed absence seizures in GAERS by reducing total seizure length and percentage of time spent in seizures under neurolept analgesia. In addition, we also demonstrate that NPY mediated reductions in the seizure levels (absence) were associated with increased firing frequency of the NRT neurons interictally in these rats. ICV and focal administration of NPY increased the mean firing frequency of the NRT neurons in interictal periods. Control experiments were performed with saline injections. The saline infusion data showed no change in the neuronal firing patterns of the NRT cells after ICV and focal administered. Furthermore NPY decreased the waveform correlation of local field potentials between NRT and cortical regions. In addition to effect on neuronal firing activity, NPY significantly increased the seizure induction threshold for triggering seizures by external electrical stimulation in the S2 region of cortex in GAERS. Control experiments with saline did not alter seizure-inducing threshold in GAERS. Seizures were not triggered in response to similar external stimulations in non-epileptic control rats.

**Conclusions**

In conclusion, NPY is an endogenous neuropeptide which suppresses absence seizures. These results suggest that NPY mediates its anti-epileptic response through alterations in firing patterns of NRT neurons. It also strengthens the argument that S2 might be focus for origin of SWDs and implicates the NRT as the key target structure of the thalamocortical circuit by which NPY suppresses seizures. These findings are associative in nature and further investigation using NPY receptor Y2 and Y5 subtype selective agents may clarify cellular molecular reasons explaining the antiepileptic effects of NPY.
Finally, NPY associated mechanisms may provide novel therapeutic options for absence seizures and other generalized epilepsy syndromes.
DECLARATION

This is to certify that:

The thesis comprises only my original work towards the PhD

Due acknowledgement has been made in the text to all other material used,

The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed……………………………. Date…………………………
PUBLICATIONS/CONFERENCES

Manuscripts arising from this thesis:


Arun Gandrathi, Idrish Ali, Thomas Zheng, Chris French, Margaret J Morris, Terence J O’Brien Suppression of absence seizures in the genetic rat model by Neuropeptide Y is associated with effects in thalamocortical circuit. (Ready for submission)
Conference proceedings:

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**Arun Gandrathi**, Terence J O’Brien, Margaret Morris, Didier Pinault, Chris French Suppression of absence seizures by neuropeptide Y is associated with the increase in the interictal firing frequency of NRT neurons. Asian ocean epilepsy congress, Melbourne, Australia (Poster).

**Arun Gandrathi**, Terence J O’Brien, Margaret Morris, Didier Pinault, Chris French Suppression of absence seizures by neuropeptide Y is associated with the increase in the interictal firing frequency of NRT neurons. Australian neuroscience society, Auckland, New Zealand (oral presentation).
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<td>Action potential</td>
</tr>
<tr>
<td>APFP</td>
<td>AP firing proportion per EEG spike</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
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<tr>
<td>CF</td>
<td>Cycle frequency</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebro spinal fluid</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3 P-diaminobenzidine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>EPSP</td>
<td>excitatory post-synaptic potential</td>
</tr>
<tr>
<td>FC</td>
<td>Febrile convulsions</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transforms</td>
</tr>
<tr>
<td>GABA</td>
<td>γ- Aminobutyric acid</td>
</tr>
<tr>
<td>GAERS</td>
<td>Genetic absence epilepsy rats from Strasbourg</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization gated cation</td>
</tr>
<tr>
<td>IC</td>
<td>Insular cortex</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebro ventricular</td>
</tr>
<tr>
<td>IBF</td>
<td>Intra burst frequency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ILAE</td>
<td>International League against Epilepsy</td>
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<tr>
<td>IPSP</td>
<td>Inhibitory post-synaptic potential</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic acid</td>
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<tr>
<td>LFP</td>
<td>Local field potential</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MAP</td>
<td>Mean number of AP’s per EEG spike</td>
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<tr>
<td>MAPB</td>
<td>Mean number of AP’s per burst</td>
</tr>
<tr>
<td>MAPF</td>
<td>Mean action potential firing frequency</td>
</tr>
<tr>
<td>MDS</td>
<td>Mean duration of seizure</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MTLE</td>
<td>Mesial temporal lobe epilepsy</td>
</tr>
<tr>
<td>MxAPB</td>
<td>Maximum number of AP’s per burst</td>
</tr>
<tr>
<td>NEC</td>
<td>Non-epileptic control</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NRT</td>
<td>Nucleus of reticular thalamus</td>
</tr>
<tr>
<td>PABD</td>
<td>Percentage of AP’s in burst</td>
</tr>
<tr>
<td>PB</td>
<td>Percentage of burst with EEG spike</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic peptide</td>
</tr>
<tr>
<td>PTS</td>
<td>Proportion of recording time of seizures</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SI</td>
<td>Synchronization index</td>
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<tr>
<td>SUDEP</td>
<td>Sudden unexpected death due to epilepsy</td>
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<tr>
<td>SWDs</td>
<td>Spike and wave discharges</td>
</tr>
<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
</tr>
<tr>
<td>TLS</td>
<td>Total length of seizures</td>
</tr>
<tr>
<td>VB</td>
<td>Ventrobasal</td>
</tr>
<tr>
<td>WAG/Rij</td>
<td>Wistar albino glaxo kept in Rijswijk</td>
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Chapter 1

Literature Review

1.1 Introduction:

1.1.1 EPILEPSY

Epilepsy is a neuro pathological disorder characterized by recurrent seizures because of abnormal and irregular neuronal activity in the brain. A seizure is a consequence of abnormal and irregular neuronal discharges in the brain, it is also referred as a convulsion or fit. Seizure episodes are characterized by impaired consciousness, loss or excess muscular activity, or an abnormal sensation. The excessive neuronal discharges may be restricted to small region of the brain (a lesion or focus) resulting in partial seizures or focal seizures or initiate in small area (focus) and spread to the whole brain resulting in generalized seizures. Seizures may occur during any time of life and can occur intermittently or frequently. Some epilepsies are limited to specific age groups; some patients suffer from epilepsy for a limited time, and some for their whole lifetime. Epilepsies that develop after an identifiable event or cause incident (e.g., asphyxia, head injury, meningitis are referred to as symptomatic epilepsy or epilepsies developing from unknown cause are referred as idiopathic epilepsy.

1.1.1.1 Prevalence of epilepsy

Epilepsy is a chronic condition with a high prevalence rate. The prevalence of epilepsy is defined as the number of patient’s diagnosed with epilepsy at a given point of time. When examining age-adjusted studies conducted in developed and developing countries, the prevalence of epilepsy is more in developing countries. The age adjusted prevalence was 41.0 per 1000 in
Nigeria (Olumide A.e · Oyediran A.B.O.d · Pearson C.A.d · Bolis C.L.f 1982) and 22.0 per 1000 in Ecuador (Cruz et al., 1985) much higher when compared to the developed countries, prevalence in New York, United States was 7.1 per 1000 (Haerer et al., 1986) and in prevalence in Europe ranged from 3.7 to 3.3 in 1000 in studies conducted in Italy (Rocca et al., 2001, Banerjee et al., 2009). Environmental and social differences play a significant role in the development of epilepsy compared to racial differences (Noronha et al., 2007). Generally, 5-10 people in each 1,000 around the world are identified with epilepsy. Two to five per cent of the overall population has experienced epilepsy at least once in their lifetime. 40 to 50 million people are detected with epilepsy, and a significant section (~30%) have treatment refractory epileptic situation (Wiebe, 2000).

1.1.1.2 Incidence of epilepsy
Incidence is defined as the rate at which new patients are diagnosed with a disease within a given time in a given population. In epilepsy, the annual incidence rate is generally calculated per 100,000 populations. The estimated incidence rate of epilepsy is 40–70/100,000 in developed nations and 100–190/100,000 in underdeveloped or developing nations; socially and economically deprived individuals are at higher risk (Sander, 2003). From surveys during the past decade, the epilepsy incidence rate is high in young age groups (<15 years), and in older age groups (>70 years) (Olafsson et al., 2005, Banerjee et al., 2009)

1.1.1.3 Aetiology of epilepsy
Some significant changes were made to the terminology and classification of epilepsy by ILAE after 2011. Novel techniques in neuroscience research have
led to development of rational system for classification of epilepsies based on underlying mechanisms.

According to the ILAE the causative factors for epilepsy could be i) Genetic reasons: Epilepsy is caused because of genetic defects e.g., genetic generalised epilepsies and channelopathies ii) structural or metabolic conditions: Epilepsy is the consequence of a structural or metabolic defect such as brain lesions, malformations during the cortical development (Barkovich et al., 2012) iii) unknown: there are many patients suffering with epilepsy with unidentified aetiology (Berg and Scheffer, 2011) (Hauser, 1997).

1.1.1.4 Mortality
Mortality rate is generally elevated in patients with epilepsy. Mortality rate in epilepsy is low in developing countries compared to underdeveloped or developing countries. A recent study in China revealed that epileptic patients had 3–4 times higher mortality rate than the normal population (Ding et al., 2006). Epilepsy associated mortality rate is considerably increased by 2-3 times in worlds population(Hitiris et al., 2007).

1.1.2 Classification of epilepsies
Many changes have been made to the classification of epilepsy and terminology in recent times. Latest developments and advancements in scientific techniques have improved the understanding of epilepsy. Currently epilepsy is classified into three main types (Berg and Scheffer, 2011, Engel, 2011, Shorvon, 2011) (Commission on classification and terminology of the International League against epilepsy, 2011) (Figure 1.1). The three types are 1) generalised epilepsy, where electrical activity is spread throughout the brain (Figure 1.2) and, 2) focal epilepsy, where the electrical activity is
limited to particular areas of the brain, mostly frontal and temporal regions (Figure 1.3). 3) Unknown, where seizures cannot be diagnosed either a generalised or focal seizures are grouped as unknown such as epileptic spasms or other seizures.

Figure 1.1 above is the current classification of epilepsy. Epilepsy is mainly classified into two major types: Focal epilepsies and Generalised epilepsies, these epilepsies are further classified to other syndrome types based on pathophysiology of the syndrome (adopted from www.epilepsy.org.au).

1.1.2.1 Focal epilepsy

Focal (partial) epilepsies are mostly symptomatic in nature with an identifiable cause. The focal point or focus may reside in frontal, parietal, temporal, occipital lobes or in the motor cortex. These seizures exhibit better responses to treatment compared to the generalised symptomatic seizures.
Efficacious drugs in focal epilepsies are phenytoin and carbamazepine and phenytoin. With the use of these anti-epileptic drugs the seizure become less severe or less frequent. In the case of inadequate improvement with these drugs, additional drugs such as valproate may be used. Maintaining a good and healthy lifestyle is very important: sleep deprivation, emotional stress and alcohol consumption may trigger these seizures. (Nashef et al., 2007). The idiopathic focal epilepsies are rare and display good response (efficient seizure reduction with drugs) to treatment (phenytoin or carbamazepine).

Figure 1.2 These are focal or partial seizures, in these seizure the abnormal electrical activity or discharges start and confined to a localised region in the brain (adopted from www.epilepsy.org.au accessed on 2/10/2013).

1.1.2.2 Generalized epilepsy

The majority of these epilepsies are idiopathic in nature. These epilepsies usually show a good response to anti-epileptic drugs. The most common generalised epilepsies are: absences (treatment: ethosuximide and valproate), generalized tonic-clonic (treatment: carbamazepine, valproate or phenobarbitone) and myoclonus (treatment: benzodiazepines and valproate).
Some of the generalized epilepsies are symptomatic. These may be caused by asphyxia, neonatal brain damage or with inborn errors of metabolism.

Figure 1.3 These are generalised seizures where the seizure arises in both hemispheres of the brain simultaneously (adopted from www.epilepsy.org.au accessed on 2/10/2013).

1.1.3: Prognosis of Epilepsy

Prognosis is used to predict the course or outcome of a medical condition or disorder. For patients with epilepsy, prognosis means the probability of more seizures after an unprovoked seizure or the chance of attaining seizure freedom after recurrent seizures have been established (Sander, 1993). The table below illustrates the relationship between the type of epilepsy, medication available and the prognosis (Sander, 1993, Cockerell et al., 1997, MacDonald, 2001).
1.1.3 The relationship between epilepsy, prognosis and treatment.

<table>
<thead>
<tr>
<th>Epilepsy</th>
<th>Prognosis</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1. Localization-related</td>
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<td></td>
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<tr>
<td>1.1) Idiopathic</td>
<td>Responsive</td>
<td>Phenytion, Carbamazepine</td>
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<tr>
<td>1.2) Symptomatic</td>
<td>Depends upon the lesion</td>
<td>Phenytion, Carbamazepine</td>
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<td>2. Generalized</td>
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<tr>
<td>2.1) Absence</td>
<td>Responsive</td>
<td>Valproate, Ethosuxmide</td>
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<td>Myoclonus</td>
<td></td>
<td>Valproate, Benzodiazepines</td>
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<td>Generalized Tonic-Clonic</td>
<td></td>
<td>Phenobarbitone, Valproate, Carbamazepine.</td>
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<td>2.2) Cryptogenic/symptomatic</td>
<td>Not responsive</td>
<td>ACTH, oral steroids, Valproate, Benzodiazepines, Prednisolone Valproate, Benzodiazepines</td>
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<td>Infantile spasms</td>
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<td>Phenobarbitone, Valproate, Carbamazepine.</td>
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<td>Lennox- gestaut</td>
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<tr>
<td>2.3) Symptomatic</td>
<td>Not responsive</td>
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<td>3. Special Syndromes</td>
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<tr>
<td>3.1 Situation related Seizures</td>
<td>Responsive</td>
<td>Valproate</td>
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<td>3.2 Febrile Convulsions</td>
<td>Responsive</td>
<td>Benzodiazepines</td>
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1.2 Absence seizures

Absence seizures are the most common type of generalized seizures (Blumenfeld, 2003). These seizures were first designated by Poupart in 1705, and later were described by Tissot in 1770. Calmeil used the term absence for the first time in (Temkin, 1994). Gibbs and his associates described the association of signature 3-Hz spike and wave discharges (SWDs) on electroencephalograms (EEG)(Gibbs FA, 1935). Some seizures are recurrent and brief, limited to few seconds they are termed as pyknoleptic seizures. Contrarily some seizures last for a few seconds to minutes and could occur less frequently in a day, these are termed as non-pyknoleptic absence seizures. Generally absence seizures onset at 2-13 year of age peaking at 6 and females have higher incidence rate of absence than males (Kramer et al., 1998).

1.2.1 Classification of absence seizures

The International League Against Epilepsy (ILAE) Commission has revised the concepts, terminology, and approaches for classifying seizures and epilepsy (1989).

According to ILAE, the classification of absence seizures is as follows:

- Absence seizures - Typical or atypical.
- Absence with special features - which includes myoclonic absence and eyelid myoclonia

1.2.2 Aetiology of absence seizures

The underlying mechanism for generation and propagation of absence seizures is complicated and not completely understood. In 1947, Jasper et al electrically stimulated thalamic nucleus of cats at 3 Hz and triggered bilaterally synchronous SWDs on EEG (Jasper HH, 1947). In 1953,
researchers were able to record SWDs from patient with absence seizures by using depth electrodes in thalamus (Williams, 1953).

Gloor et al in 1977 revealed that 3Hz SWDs associated with absence seizures were generated in the cortex in the feline penicillin model of absence seizures.

Pathological oscillatory rhythms during absence seizures are supposed to initiate in thalamocortical circuit that involves gamma-aminobutyric acid (GABA) mediated inhibition and glutamate mediated excitation.

1.2.3 Age of onset

Absence seizures appear in childhood and may continue to adulthood. The cause for absence seizures may be genetic or a perinatal insult (Hamiwka and Wirrell, 2009).

Five main syndromes are associated with absence epilepsy: Childhood absence epilepsy, onset of this syndrome occurs at age 4-8 years, peaking at the age of 6-7 years (P, 1985). Juvenile absence epilepsy, the onset of this syndrome is around puberty and varies between pyknoleptic absences which occur between 8.3 ± 4.5 years of age or non-pyknoleptic seizures which occur between 14.8 ± 8.3 years (Wolf, 1979). Juvenile myoclonic epilepsy, this syndrome has the age of onset from 8-26 years (Wolf, 1979, Porter, 1993, Loiseau et al., 1995). As the absence seizure episodes are brief, they are often unrecognised.

1.2.4 Morbidity and mortality

An absence seizure per se, does not directly result in deaths. However, if the absence episodes occur while driving or operating dangerous machines, patients may suffer from fatal injuries or death.
1.2.5 Electroencephalography

Electroencephalography is the only diagnostic test for absence seizures. Generalized 3-Hz SWDs are presented during the seizures (Gibbs FA, 1935, Wolf, 1979, Lopes da Silva et al., 2003) (Figure 1.4). The onset and termination of absence seizures are completely unexpected. During an absence episode, mild clonic jerks, facial twitching and rhythmic eye blinks may be seen and automatisms may be present during seizure progression (Penry et al., 1975).

Figure 1.4 An image of human absence seizures displaying characteristic 3 Hz spike and wave discharges. These absence seizures arise abruptly out of normal background and ceases after few seconds on EEG (adopted from (Lopes da Silva et al., 2003)).
1.2.6 Thalamocortical circuit and mechanisms of absence seizures

Many electrophysiological studies support SWDs are initiated in the thalamocortical system that connects the thalamus and the cerebral cortex (Danober et al., 1998, Panayitipoulos, 2005a, Benbadis SR, 2006).

1.2.6.1 Anatomy and Physiology thalamocortical circuit

The thalamocortical circuit consists of the cerebral cortex, VB thalamus, nucleus of reticular thalamus (NRT) and their connecting neurons (Danober et al., 1998) (Figure 1.5). The thalamocortical circuit involves glutamatergic (Fonnum et al., 1981b, Kaneko and Mizuno, 1988) axons projecting from VB thalamus to layers III and IV of the cerebral cortex (Alloway et al., 1993) and glutamatergic axon projections from layers V and VI of the cerebral cortex to the relay nuclei of the thalamus (Bourassa et al., 1995).

The NRT consists of inhibitory GABAergic neurons that innervates the VB and sends projections to its own neurons releasing GABA, a potent inhibitory neurotransmitter forming a loop (Pinault et al., 1995, Cox et al., 1996) (Figure 1.6). The NRT neurons receive projections from the thalamocortical and corticothalamic circuit but do not project to the cerebral cortex (Spreafico et al., 1991, Contreras et al., 1993, Bourassa et al., 1995).
**Figure 1.5** This Figure illustrates coronal section of the GAERs rat brain displaying the major brain structures of the thalamocortical circuit: Cerebral cortex, VB thalamus and NRT that are responsible for absence seizures. (Page12, Line 1-3).

**Figure 1.6** This Figure illustrates the major brain structures of the thalamocortical circuit and the thalamocortical interactions: This circuit consists of reciprocally connected excitatory corticothalamic and thalamocortical glutamergic neurons in cortex and VB and inhibitory GABAergic neurons in NRT that innervates VB forming a pathological oscillatory loop responsible for absence seizures.

+ **Excitatory glutamergic connections**

− **Inhibitory gabaergic connections**
The view of generalized seizures is that, they arise simultaneously from both hemispheres of the brain. Nevertheless, recent experiments in genetic rat models of absence epilepsy, WAG/Rij and GAERS models have proposed that a “cortical focus” within the cerebral cortex from which seizures initiate and spreads over the cortex and subsequently to thalamic regions. Meeren et al described that the focus was located in S1 region of somatosensory cortex (Meeren et al., 2002). A recent study from our lab in GAERS demonstrated that seizures were found to originate within the somatosensory cortex (Zheng et al., 2012). The VB thalamus is the major source of input to the cortex, transmitting sensory information. The cerebral cortex receives its subcortical afferents from VB. The VB receives inhibitory inputs from the NRT, which is located in the pathway that links VB thalamus and cerebral cortex and receiving afferents from both structures.

The thalamocortical and corticothalamic neurons are mostly glutamatergic (Fonnum et al., 1981a). Most thalamocortical axons project to cortical layers IV and II and information is transmitted to other cortical areas via cortical interneurons (Jones, 1985). All sensory information, except smell, is relayed to the cerebral cortex via the thalamus. In return, the pyramidal neurons from cortical layers V and VI projects to VB thalamus (Jones, 1985, Bourassa and Deschenes, 1995). In addition, all VB thalamus neurons receive GABAergic inhibitory projections from the NRT, which is the major source of GABA in thalamus (Jones, 1985, Pinault et al., 1995, Cox et al., 1996). NRT in return receives excitatory inputs from both thalamocortical and corticothalamic axons.

Thalamocortical neurons exhibit two modes of firing patterns associated with sleep/wake cycles: 1) relay mode or tonic mode, this occurs during
quite wakefulness and is associated with faithful transmission of sensory information to the cerebral cortex where it is perceived and processed (Llinas et al., 1998) (Figure 1.7). 2) Oscillatory mode or burst firing mode, this occurs during sleep and is associated with filtering of sensory information (Snead, 1995) (Figure 1.8).

During wakefulness the thalamus exhibits relay or tonic mode or firing with fast sodium/potassium mediated action potentials (Figure 1.9). In relay mode thalamic neurons are depolarized from resting membrane potential levels positive to -55 mV. During sleep or drowsiness, the thalamus switches its mode from relay to oscillatory or burst firing mode, where the membrane potential is hyperpolarized to -60 mV. The de-inactivation of a Ca\textsuperscript{2+} conductance causes an inward current through T-type Ca\textsuperscript{2+} channels (Chemin et al., 2002) which may lead to high frequency burst of action potentials (Steriade and Deschenes, 1984b) (Figure 1.9). This hyperpolarization was believed to be mediated by the Gabaergic inhibitory neurons in the NRT that provides rhythmic, synchronous inhibitory postsynaptic potentials (IPSPs) to the VB thalamus. These oscillatory bursts frequently occur during sleep, when the VB thalamus NRT neurons are hyperpolarized. These conditions are suggested to be the functional basis of sleep and drowsiness, where sensory information is not relayed to the cortex, resulting in reduced responsiveness. These bursts are displayed on EEG as spindles and delta waves during slow-wave sleep (Danober et al., 1998). Spindles are frequent rhythmic oscillations of 7-14 Hz that occur every 3-10 seconds slow-wave sleep (Steriade and Deschenes, 1984a).
Figure 1.7 This Figure demonstrates the thalamic relay or tonic mode of firing that happens during quive wake full ness or in non-seizing period that is associated with the reliable transmission of sensory information to the cerebral cortex (modified from (Paxinos et al., 1980)).

Figure 1.8 This Figure demonstrates the oscillatory or burst firing mode that happens during sleep or during seizing period that is associated with filtering of sensory information to the cerebral cortex (modified from (Paxinos et al., 1980)).
Figure 1.9 This Figure demonstrates two modes of firing patterns, showing the transmission between two modes of firing. Here in this picture thalamic neurons fires initially in a tonic mode during interictal period, as the seizures start the firing mode switches to low frequency burst firing mode during a seizure.

The above pathological change in thalamocortical mode of firing from relay to oscillatory is the mechanism underlying the generation of absence seizures (Snead, 1995, Kostopoulos, 2001). The initiation and triggering point of this SWD’s is currently unknown but the possible explanation is that these SWD’s may arise from disruption of delicate balance between inhibitory and excitatory neurotransmitters acting on the Thalamocortical neurons (Slaght et al., 2002). This disruption may result in the hyper-polarization and oscillatory firing of thalamocortical neurons at inappropriate times.

1.2.7 Animal models of absence seizures

Absence epilepsy is a common neurological disorder that mainly occurs in children and is not amendable to surgical intervention. It is ethically not possible investigate the mechanism and pathophysiology of absence seizures in humans. Therefore, many acquired and genetic models of absence epilepsy have been developed to understand the cellular and molecular mechanism underlying the generation and propagation of
absence seizures. In some animal models seizure are induced by external electrical stimulation and in some animal models, seizures are induced by acute administration of a specific agent to an animal for example penicillin epilepsy model (Fisher and Prince, 1977) low dose pentylenetetrazol (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 administration of these drugs induces SWDs accompanied by behavioral arrest, twitching of vibrissae and facial myoclonus. Most of the these models display similar pharmacological profile humans, seizures are diminished on administration of ethosuximide and valproic acid and worsened by carbamazepine (Marescaux et al., 1984). These pharmacological models are play a crucial role examining the neuropathological alterations related with absence epilepsy as well as in screening the efficacy of antiepileptic drugs. The pharmacological models exhibit similarity in seizure phenotype but they do not model the underlying pathology of absence epilepsy to study the development of the disease.

Unlike the chemical induced seizure models, genetic models, which exhibit spontaneous recurrent seizures as witnessed in the human absence seizures. Examples of commonly used mouse mutants include lethargic, stargazer and totterer mice. However, most of these models are the result of single gene mutation and are associated with other neurological abnormalities that limit these models mostly to behavioral and physiological studies.

The spontaneous recurrence of SWDs in untreated rats lead to the development and characterization of two genetic rat models of absence epilepsy: 1) the GAERS, an inbred strain of Wistar rats developed in France (Marescaux and Vergnes, 1995) the Wistar Albino Glaxo kept in Rijswijk (WAG/Rij) (Coenen et al., 1992a). 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 have significantly improved the understanding of underlying mechanism of absence seizures. In the current thesis we have used the GAERS model for our investigations.

1.2.7.1 The Genetic Absence Epilepsy Rats from Strasbourg (GAERS)

GAERS is a genetic rat model of absence epilepsy originated in the Centre of Neurochemistry in Strasbourg, France, where animals that displayed SWDs were selectively inbred to produce a strain that exhibits absence type seizure characterized by SWDs. A control strain, which does not exhibit SWDs were inbred from the same colony and designated as non-epileptic control rats (NEC).

Similar to human absence, SWDs in GAERS start and end abruptly on EEG background accompanied by behavioral arrest, twitching of the vibrissae and facial muscles. GAERS exhibit SWDs typically around 9Hz faster than human absence seizures (approximately 3Hz) (Figure 1.10). However, this difference in frequency is a species-dependent difference (Snead, 1995). In GAERS SWDs last for 17 ± 10 seconds and occur 1.3 times per minute on average. The SWD frequency of 9Hz in GAERS and can be interrupted by strong and unexpected sensory stimulus. (Vergnes et al., 1991). GAERS start to exhibit seizures in early adolescence (30-40 days post-natal) and are fully expressed by the age of 3 months and persist for rest of their life. GAERS exhibit similar pharmacological responsiveness to human absence epilepsy.

As most characteristic features of absence epilepsy in GAERS are highly similar to human absence epilepsy, GAERS are used as reference for investigating the mechanisms underlying absence seizures.
Figure 1.10 This Figure shows a wistar rat and below is the picture of 5-9 Hz spike and wave discharges exhibited by GAERS on EEG. These spike and wave discharges are very similar to human absence seizures on EEG but vary in frequency of SWDs where humans exhibit 3 Hz SWDs.

1.2.8 Pharmacologic Treatment for absence epilepsies

Most anti-epileptic drugs have significant sedative and cognitive side effects. Some absence epilepsy patients may need long-term medication to control seizures and in some cases for the rest of their life. EEG is used to confirm the presence of spontaneous epileptic seizures and can be documented with longer EEG recordings or EEG video monitoring. Two first-line anti-epileptic drugs have approval from the US Food and Drug
Administration (FDA) for treating patients with absence epilepsy: valproic acid (Depakene, Depacon) and ethosuximide (Zarontin). Ethosuximide can be used for absence seizures only but valproic acid has effectiveness for absence seizures, generalized tonic-clonic seizures, and myoclonic seizures.

Some AEDs can aggravate seizures (Lerman, 1986), treatment with carbamazepine (Snead and Hosey, 1985, Liu et al., 2006) and oxcarbazepine (Vendrame et al., 2007) exacerbates the absence seizures (Liu et al., 2006).

1.3 Introduction to NPY and its receptors:

Neuropeptide Y (NPY) is a naturally occurring 36 amino acid peptide, a member of the pancreatic peptide family discovered from pig brain by Tatemoto in 1982 (Tatemoto, 1982b). NPY is richly found in many brain regions, such as the hypothalamus, amygdala, hippocampus, nucleus of the solitary tract, locus ceruleus, nucleus accumbens and the cerebral cortex (Allen et al., 1983, Chronwall et al., 1985, de Quidt and Emson, 1986). NPY mediates many physiological responses such as feeding behaviour, water consumption, learning and memory, locomotion, body temperature, sexual behaviour, emotional behaviour, neuronal excitability, cardiovascular homeostasis, hormone secretion, circadian rhythms (Chronwall et al., 1985, Wahlestedt and Reis, 1993, Munglani et al., 1996, Baraban et al., 1997, Hokfelt et al., 1998, Inui, 1999, Vezzani et al., 1999), depression, anxiety (Kask et al., 2002, Berglund et al., 2003) and blood pressure (Klapstein and Colmers, 1997).

NPY mediates its response by binding to G-protein coupled heptahelical receptor subtypes, named Y1, Y2, Y4, Y5, and y6 (Stroud et al., 2005)
which inhibits adenylate cyclase and thus decrease intracellular calcium levels (Michel et al., 1998, Berglund et al., 2003).

Receptor subtypes Y1, Y2 and Y5 are predominantly expressed in the mammalian CNS. NPY has been implicated in anabolic activity through circuits in the hypothalamus (Woods et al., 1998, Morris et al., 2007) and seizure modulation in the thalamus (Sperk and Herzog, 1997, Bacci et al., 2002, Xapelli et al., 2008)

1.3.1 Discovery of NPY
Many neuropeptides were discovered and identified basing on the biological processes mediated by them. NPY was first identified by its C-terminal tyrosine amide structure and is isolated from porcine brain extracts (Tatemoto and Mutt, 1978, Tatemoto et al., 1982). This peptide is named as neuropeptide Y as it contains numerous tyrosine residues (Y) in its structure (Tatemoto, 1982a, c).

1.3.2 Primary structure of NPY:
NPY is a 36 amino acid linear polypeptide. NPY shows a greater degree of sequence homology between with PYY compared to PP (Figure 1.11). It was then suggested that NPY, PYY and PP belong to an unrecognised peptide family (Tatemoto, 1982a).

After that human NPY was isolated and the primary structure of NPY isolated from human varied from the NPY isolated from porcine peptide only at one location of the 36 residues (Corder et al., 1984) and NPY was subsequently isolated from other animals such as birds, frogs, and others (Larhammar et al., 1993). Dixon and co-workers first identified the cDNA encoding NPY.
1.3.3 Cloning of NPY specific receptor subtypes

Development in biotechnology techniques have led to the identification of five NPY specific receptor subtypes, Y1, Y2, Y4, Y5, and Y6 (Michel et al., 1998). These receptor subtypes exhibited only 30-50% sequence homologies to each other. Moreover, each receptor subtype exhibits distinct tissue localization and differential pharmacological profile.
1.3.3.1 NPY Y1 receptor subtype

The primary structure of the human NPY Y1 receptor was identified in 1992 (Herzog et al., 1992, Krause et al., 1992, Larhammar et al., 1992). It was the first NPY specific receptor subtype to be cloned, it is a 384 amino acid protein. The NPY Y1 receptor is widely distributed in mammalian CNS, heart, colon, kidney, adrenal gland and placenta (Wharton et al., 1993).

1.3.3.2 NPY Y2 receptor subtype

The NPY Y2 receptor subtype is a 381 amino acid protein and exhibits 31% similarity with the Y1 receptor (Gerald et al., 1995, Rose et al., 1995, Gehlert et al., 1996, Rimland JM, 1996). The NPY Y2 receptors are widely expressed in human CNS, and are thought to mediate many physiological responses including anti-epileptic actions. NPY Y2 receptor is widely distributed in human CNS, heart, ileum and colon (Caberlotto et al., 1998b, Ferrier et al., 2002, Jonsson-Rylander et al., 2003), the Y2 receptors are located mostly on NPY-containing neurons (Caberlotto L, 2000).

1.3.3.3 NPY Y3 receptor subtype

There is no evidence for the existence of a NPY Y3 receptor (Michel et al., 1998) and the reported Y3 receptor clone failed to confer NPY binding sites (Rimland et al., 1991, Herzog et al., 1993, Jazin et al., 1993).

1.3.3.4 NPY Y4 receptor subtype

The NPY Y4 receptor is a 375 amino acid G-protein coupled receptor (Lundell et al., 1995). The human NPY Y4 receptor exhibits 43% similarity with the human NPY Y1 receptor (Bard et al., 1995, Lundell et al., 1995, Yan et al., 1996). NPY Y4 receptors are localised in intestine, prostate gland, and pancreas (Lundell et al., 1995), and are lightly expressed in the human
CNS (Parker and Herzog, 1999).

1.3.3.5 NPY Y5 receptor subtype

The NPY Y5 receptor is a 455 amino acid protein (Borowsky et al., 1998), this receptor was originally cloned as a feeding receptor in hypothalamus. The Y5 receptor is broadly localised in the human CNS (Dumont et al., 1998) and is also found in small intestine, colon, testis, spleen, and pancreas (Goumain et al., 1998, Statnick et al., 1998).

1.3.3.6 NPY Y6 receptor subtype

The NPY Y6 receptor is a 290 amino acid protein present in chicken, cow, dog, rabbit, mouse and human (Burkhoff et al., 1998). The NPY Y6 receptors are functionally inactive in primates due to mutations occurred during evolution (Matsumoto et al., 1996).

1.3.4 Distribution of NPY in central and peripheral nervous system

Bloom et al demonstrated that NPY is the most abundant neuropeptide and is widely distributed in the brain (Adrian et al., 1983, Allen et al., 1983). NPY is abundantly found in the paraventricular hypothalamic nucleus, hypothalamic arcuate nucleus, paraventricular thalamic nucleus and suprachiasmatic nucleus (Chronwall et al., 1985). Many in vitro studies have revealed the coexistence of NPY with other neurotransmitters and neuropeptides (Hokfelt et al., 1983). Selective receptor agonist studies revealed that NPY Y1 and Y2 receptors are expressed independently in the human brain and majority of the NPY receptors identified in the brain were Y2 receptors (Aicher et al., 1991).

NPY like immune-reactivity in peripheral system was first identified in 1982 (Lundberg et al., 1982). NPY like immune-reactivity was identified in sympathetic ganglia, heart atrium, spleen and blood vessels. NPY like
immune-reactivity was also found in gut and pancreas (Sundler et al., 1983). NPY is mostly found in the sympathetic neurons (McDonald, 1988) thus seem like an important peptide in sympathetic nervous system.

### 1.3.5 Biological effects of NPY

Several studies revealed that NPY mediates anti depressive, anti-stress, anxiolytic and anti-nociceptive actions. NPY receptor subtype Y1 was found to mediate anxiolytic-like action (Heilig et al., 1993, Wahlestedt and Reis, 1993) and also found to mediate anxiogenic effect via the Y2 receptor subtype (Nakajima et al., 1998). NPY transgenic mice exhibited anxiolytic behaviours (Inui, 1999), additionally these transgenic rats with hippocampal overexpression of NPY were unresponsive to restraint stress and presented reduced spatial learning (Thorsell et al., 2000). NPY Y1 receptor-deficient mice were reported to develop hyperalgesia to pain, and did not show any pharmacological analgesic effects of NPY (Naveilhan et al., 1999). These reports suggest that NPY is involved in the mechanisms of learning, stress, anxiety and nociception.

Low levels of NPY were found in patients who repeatedly attempted suicide (Westin et al., 1999). Alterations in the levels of NPY and NPY Y1 receptor mRNA were found in animal model of depression after treating with anti-depressant drug (Caberlotto et al., 1998a) suggesting the role of NPY in depression. These studies suggest the involvement of NPY in depression.

### 1.3.6 Effect of NPY on Seizures

NPY is considered as an endogenous anti-convulsant. Studies have revealed that NPY deficient mice showed less resistance to seizures induced by GABA antagonist (Erickson et al., 1996). NPY deficient mice exhibited uncontrollable limbic seizures induced by kainic acid which lead to 93 % of mortality in mice, these deaths were prevented by ICV NPY infusion in
these mice (Baraban et al., 1997). In addition, transgenic rats with overexpression of NPY displayed significant decrease in number of seizures and length of seizures induced by kainic acid (Vezzani et al., 2002). In in vitro models of epilepsy, infusion of NPY could successfully inhibit epileptiform activity via Y2 receptor subtype (Klapstein and Colmers, 1997) and Y5 receptor subtype potentially inhibited kainic acid induced seizures (Woldbye et al., 1997). It was also shown that mice lacking NPY Y5 receptor subtype were more susceptible to kainic acid induced seizures (Marsh et al., 1999). In epileptic human hippocampus, NPY released during profuse axonal sprouting and simultaneous up-regulation of NPY Y2 receptors may instigate the inhibition of glutamate release and consequently contribute to the seizure suppression mechanisms of NPY (Furtinger et al., 2001).

1.3.7 NPY and focal epilepsy:

1.3.7.1 NPY and focal epilepsies: In-vivo
The anticonvulsive effects of NPY have been suggested in various animal models of epilepsy. Changes in the immune-reactivity of NPY were examined in rat brain after kainic acid induced limbic seizures. The NPY levels consistently increased in the frontal cortex, hippocampus and amygdala (Marksteiner et al., 1989). Increased NPY expression was observed in neurons resistant to seizure-induced cell death (6-48 hours after i.p. Kainic acid). Using in situ hybridization histochemistry NPY mRNA expression was assessed after kainic acid injection and remarkably high hybridization signals were found in hippocampal after 8 months of kainic acid induced limbic seizures (Gruber et al., 1994). The NPY Y1 receptor expression was assessed in hippocampus after kainic acid
induced seizures, the levels of NPY Y1 receptor decreased by 80% in granule cells and concurrently increased by 75% in pyramidal neurons in the CA2 region (Kofler et al., 1997). ICV administered NPY is a potent inhibitor of kainic acid induced seizures, this study was the first to determine the seizure suppression effect of NPY which is consistent with the concept that NPY is an endogenous anticonvulsant (Woldbye et al., 1997). Receptor autoradiography with the Y2 receptor ligand and in situ hybridization of NPY Y2 receptor expression showed up-regulation of presynaptic NPY Y2 receptors in schaffer collaterals after kainic acid-induced recurrent seizures in the rat hippocampus. This up-regulation may lead to presynaptic inhibition of glutamate release from hippocampus and increased levels of NPY in mossy fibres may present endogenous seizure suppression mechanism of NPY. (Schwarzer et al., 1998). A study on expression of mRNAs for NPY and its subtypes in adult rats brains using in situ hybridization in rapid kindling model demonstrated a cell and region-specific mRNA expression for NPY and its receptor subtypes following recurrent seizures (Kopp et al., 1999). By using auto-radiographic approach it is shown that NPY Y2 and Y5 receptor binding receptors are reduced in kindling and kainic acid models of epilepsy (Bregola et al., 2000). It has been shown that seizure susceptibility in transgenic NPY knockout mice or NPY deficit mice has a significantly high mortality rate than the control rats after kainic acid injection (40 mg/kg i.p). In situ hybridization analyses in these transgenic NPY knockout mice confirmed a decrease in prepro-NPY gene expression (DePrato Primeaux et al., 2000). A constant increase of NPY and elevation of hippocampal messenger RNA were found during a spontaneous seizure in a Noda epileptic mutant rat (Jinde et al., 2002). Overexpression of endogenous NPY in rat hippocampus is associated with seizure inhibition and epileptogenesis in kindling and kainic acid models of
epilepsy (Vezzani et al., 2002). Results from all the above studies on different models of epilepsies demonstrate the anti epileptic properties of NPY but the underlying mechanism is still not completely understood and this will require further investigations in the future.

1.3.7.2 NPY and focal epilepsies: In-vitro

Pharmacological studies of NPY demonstrated the critical role in regulation of neuronal activity and neuro-modulatory effects of NPY. NPY inhibits influx Ca2+ currents, this inhibition of presynaptic Ca2+ plays a critical role in neurotransmitter release (Colmers and Bleakman, 1994, Wu and Saggau, 1997). The in vitro extracellular and whole cell patch-clamp recordings from rat cortical slices demonstrated that NPY could significantly inhibit epileptiform activity in in vitro models of epilepsy [0 Mg2+-, picrotoxin-, and stimulus-train-induced bursting (STIB)] predominantly via NPY Y2 receptors (Klapstein and Colmers, 1997). NPY inhibits synaptic excitation of CA3 pyramidal cells of the rat hippocampus via presynaptic action of Y2 receptors (McQuiston and Colmers, 1996). A study showed that NPY Y1 receptors in the hippocampus are involved in attenuating epileptic activity in kainic acid model of limbic seizures(Gariboldi et al., 1998). On examination of hippocampal function and responsiveness to kainic acid induced seizures in Y5R-deficient (Y5R2y2) model, It was demonstrated that Y5R2y2 were more prone to kainic acid seizures, suggesting significant role of NPY Y5 receptor in mediating seizures (Marsh et al., 1999). Extracellular or whole cell voltage-clamp recordings from CA3 regions in NPY Y5 receptor knock-out mice and matched wild type mice showed that, NPY and NPY agonists caused a significant reduction in excitatory postsynaptic current (EPSC) amplitudes concurrently suppressed-magnesium epileptiform burst discharge in slices from wild type mice suggesting important role of NPY in modulating excitatory synaptic transmission and inhibition of limbic seizure
activity in mouse hippocampus (Baraban, 2002). NPY Y2 receptors play a prominent role in mediating NPY induced inhibition of glutamate release in the hippocampus (Silva et al., 2001). It was shown that the antiepileptic activity of NPY is mediated predominantly by the Y1 receptor subtype in the frontal cortex and by Y2 and probably Y5 receptors in the hippocampal CA3/CA1 areas, in rat cortical and hippocampal slices in Mg2+-free medium (Bijak, 1999).

1.3.7.3 NPY and focal epilepsies: In humans

NPY is known to suppress hyper excitable activity in abnormal pathological condition in rat brain. Intracellular recordings in hippocampal slices from patients with hippocampal seizure onset showed NPY containing cells throughout the hippocampus particularly in dentate molecular layer suggesting role of NPY as a neuromodulator that may limit hyper excitability in the human dentate gyrus (Patrylo et al., 1999). In patients with Temporal lobe epilepsy (TLE), a significant increase in brain derived neurotrophic factor (BDNF) was observed in the temporal neocortex of patients. This increase was correlated significantly with levels of NPY suggesting involvement of NPY in human epileptogenesis (Takahashi et al., 1999). Receptor binding and immune studies in hippocampal specimens from patients with TLE showed increase in the NPY mRNA, up regulation of Y2 receptors and down regulation of Y1 receptors (Furtinger et al., 2001). Patients with atypical febrile convulsions (FC) showed lower NPY plasma levels compared to patients with typical FC and controls suggesting patients with inadequate NPY inhibitory activity are more susceptible to atypical FC (Lin et al., 2010).
1.3.8 NPY and generalised epilepsy:
Recent EEG studies with exogenous infusion of NPY in a genetic rat model of absence epilepsy (GAERS) showed that NPY suppresses absence seizures (Stroud et al., 2005). Another study showed NPY Y2 receptors play a significant role in mediating seizure suppression effect compared to Y1 and Y5 receptors in genetic rat models of absence epilepsy (Morris et al., 2007). Focal injections studies of NPY showed that NPY suppresses seizures when injected in cortex and showed a little but significant decrease when injected in NRT but had contrasting effects when administered in thalamus. Further, the NPY infusion in the S2 region of the somatosensory cortex showed maximal effect of seizure suppression in GAERS (van Raay et al., 2012).

1.4 Conclusion from literature review:
In summary, extensive literature suggests that, NPY is an endogenous naturally occurring neuropeptide which activates three different receptor subtypes NPY Y1, NPY Y2 and NPY Y5. NPY is widely distributed in the human CNS and mediates many physiological responses. Our interest is it’s anti-epileptic properties and ability to suppresses seizures in a various types of epilepsy and the mechanism of seizure suppression. Numerous experimental studies that have investigated this are summarized below.

In 1989, Marksteiner et al studied the changes in the immunoreactivity of NPY in rat brain and found increased levels of NPY in cortex, hippocampus and amygdala after seizures induced by kainic acid (Marksteiner et al., 1989). Increased concentrations of NPY mRNA was observed in different hippocampal regions after kainic acid induced limbic seizures (Gruber et al., 1994). Infusion of NPY in in vitro models of
epilepsy, could successfully inhibit epileptiform activity (Klapstein and Colmers, 1997). The first study to demonstrate an antiepileptic effect of NPY was done by Woldbye et al, where NPY administered into the lateral ventricle suppressed seizures induced by kainic acid (Woldbye et al., 1997). An mRNA expression study in a rapid kindling model demonstrated a neuronal and region specific mRNA expression for NPY after recurrent seizures (Kopp et al., 1999). In 2001, it was shown that up regulation of NPY receptors could be involved in the seizure suppression (Furtinger et al., 2001). An NPY knockout study showed a significantly higher mortality rate in NPY knockout mice than control mice, suggesting a critical role of NPY in mediating neuronal excitability (DePrato Primeaux et al., 2000). A consistent increase NPY expression was observed during a spontaneous seizure in a Noda epileptic mutant rat (Jinde et al., 2002). Overexpression of endogenous NPY in rat hippocampus is associated with seizure inhibition and epileptogenesis in kindling and kainic acid models of epilepsy (Vezzani et al., 2002). NPY was also shown to play an important role modulating excitatory synaptic transmission and inhibition of limbic seizure activity in mouse hippocampus (Baraban, 2002). Recent EEG studies genetic rat model of absence epilepsy (GAERS) from our laboratory showed that NPY suppresses absence seizures (Stroud et al., 2005, Morris et al., 2007, van Raay et al., 2012).

The above studies strongly indicate that NPY plays a pivotal role in suppression of seizures in various models of epilepsy. The current thesis focuses more on investigating its mechanism of seizure suppression by understanding the neuronal firing patterns in the thalamocortical circuit, which is critical for development and propagation of absence seizures.
1.5 Rationale for this study

There is an increasing amount of in vivo and in vitro evidence on the seizure suppression effects of NPY in various epilepsy models. However, the underlying neuronal mechanisms for the seizure suppression effect are still unknown. As discussed in section 1.2.6, the thalamocortical circuit plays a pivotal role in generation and propagation of absence seizures. This circuit is involved in the generation of signature spike and wave discharges and hypersynchronous rhythmic oscillatory activity in the brain during absence seizures. Electrophysiological studies investigated the cellular firing patterns of different neurons in the thalamic cortical circuit and their contribution in mechanism of seizure initiation and spread of oscillatory thalamocortical activity during a seizure. The mechanism underlying the generation of absence seizures is the pathological and inappropriate change in the thalamocortical mode from relay to oscillatory (Snead, 1995, Kostopoulos, 2001). No definitive cause for this change is currently known but it is highly plausible that it could involve a lack of inhibition within the NRT causing excessive release of GABA on thalamocortical neurons, resulting in a disruption in the delicate balance between inhibitory and excitatory neurotransmitters acting on the thalamocortical neurons (Slaght et al., 2002). Recent studies in our laboratory have also demonstrated that ICV administration of NPY clearly reduces the percentage-time spent in seizure through reducing both seizure duration and number of seizures (Stroud et al., 2005, Morris et al., 2007). Focal injections studies of NPY showed that NPY suppresses seizures when injected in cortex and showed a small but significant decrease when injected into the NRT(van Raay et al., 2012). Nevertheless, the electrophysiological effects of NPY on neuronal firing patterns and alteration in cellular patterns that underlie the mechanism of seizure suppression have not yet been described.
In this thesis, we attempt to investigate the effects of NPY on neuronal firing patterns to understand the mechanism underlying the seizure suppression effect of NPY in vivo in genetic rat model of absence epilepsy (GAERS). In this we have used in vivo electrophysiological approach to investigate the alterations in neuronal firing patterns in different regions of thalamocortical circuit, cortex, VB thalamus and NRT, which are basis for absence seizures. Using GAERS as our rat model we had an advantage of investigating the effects of NPY on spontaneously occurring seizures and as it is an in vivo study there is an advantage of studying the neuronal firing patterns during naturally occurring seizing and non-seizing periods and also assess the alteration in the neuronal firing patterns induced by NPY that may relate the seizure suppression. Thus, the data acquired in this study will enable the investigation of mechanisms related to anti-epileptic effects of NPY and also understand the underlying cellular mechanism of seizure suppression.

1.6 Research questions
The present thesis focuses on investigating and understanding the alterations in the neuronal firing properties of the thalamocortical circuit neurons that support seizure suppression effect of NPY. The following research questions will be investigated:-

1. What is the effect of ICV administration of NPY on spontaneously occurring absence seizures in GAERS?

2. How does NPY affect the neuronal firing patterns in different thalamocortical circuitry neurons when administered ICV?
3. What changes can occur to specific neuronal firing patterns that can signify the alterations of neuronal activity at network level?

4. Does focally administered NPY show similar effects on neuronal firing patterns on NRT neurons as it has when administered ICV?

5. Does NPY at amounts that suppress spontaneously occurring absence seizures also show similar effects on electrically induced seizures in GAERS?

1.7 Hypotheses

To address the above listed research questions the following hypotheses will be tested:

1. ICV administration of NPY will induce anti-epileptic effects on absence seizures in GAERS by decreasing the total and seizure frequency on EEG.

2. To assess alternations in the neuronal firing patterns in the thalamocortical circuit in vivo, electrophysiological recordings were performed under neurolept anaesthesia, ICV NPY infusions were also performed simultaneously along with EEG. NPY will induce changes in the neuronal firing properties of different thalamocortical circuitry neurons:
   a. Cortical neurons (Layer IV and V)
   b. VB thalamic neurons
c. NRT neurons

3. NPY administered ICV will induce changes in the specific neuronal firing patterns that signify network activity:
   a. Rhythmicity in the neuronal firing
   b. Synchronicity of the neuronal firing
   c. Correlation between field potentials of two different regions in the thalamocortical circuit

4. NPY was injected focally at the recording site via micro-iontophoretic process via multi-barrel electrode to investigate effect of focal NPY administration on neuronal firing patterns. Focal administration of NPY will induce similar effect on NRT neurons further confirming the local effect of NPY on NRT.

5. Absence like seizures were induced in GAERS by external electrical stimulation in somatosensory cortex and effect of ICV administration of NPY is assessed on seizure inducing threshold of evoking seizures. ICV administration of NPY will induce similar effects of seizure suppression on induced seizures triggered by electrical stimulation by increasing the seizure inducing threshold to evoke seizures.

The understanding of the cellular and molecular mechanism related to electrophysiological alterations induced by NPY infusion may contribute in identifying the novel therapeutic target for seizure suppression in absence seizures.
CHAPTER 2
MATERIALS AND METHODS

2.1 Study design:
All the experiments in this study involved in vivo electrophysiology. Three different protocols of in vivo electrophysiology recordings have been performed to understand NPY induced changes in neuronal firing patterns of thalamocortical circuitry neurons in neurolept anaesthetised rats. The protocols include single and paired juxta-cellular recordings with simultaneous ICV drug administration and EEG recordings, juxta-cellular recordings with simultaneous focal administration of NPY and cortical stimulation to evoke spontaneous seizure activity combined with ICV drug administration. The data collected from these experiments was analysed before and after NPY administration and during ictal and interictal periods and results are shown in the following chapters. Detailed methodology of surgery, recordings and data analysis is explained in the current chapter.

2.2 Animals:
All experiments in this study were conducted on >12 weeks old male GAERS and non-epileptic controls (NEC) rats. Rats were acquired from breeding colonies at the animal house facility in department of Zoology, Royal Melbourne Hospital, university of Melbourne and animal house facility in Ludwig institute of Cancer research. All rats were bred and housed in standard rat boxes and kept in a temperature-controlled animal room maintained at 22-24°C with a 12hr light/dark cycle. All procedures performed in this study were approved by the University of Melbourne Animal Ethics Committee (0706287 & 1112079) and performed in accordance with the
guidelines published by the Australian National Health and Medical Research Council (NHMRC).

2.3 Anaesthesia and surgery

2.3.1 Anaesthesia:
All experiments in this study were performed under two different kinds of anaesthesia. Preparative surgeries for in vivo juxtacellular recordings were performed under general anaesthesia using a combination of ketamine (100mg/kg) and xylazine (10mg/kg) in 3:1 ratio at a dose of 0.1mg/100gm of rat. Electrophysiological recordings were performed under “neurolept anaesthesia” using a mixture of drugs \(d\)-tubocurarine (0.4 mg; Sigma Aldrich), Fentanyl (0.375 \(\mu\)g; Mayne Pharma), haloperidol (50 \(\mu\)g; Janssen Pharma) with glucose (25 mg; Sigma), which completely anaesthetises and paralyses the animal but keeps it in a pseudo-awake state or electrophysiologically active state which allows the generation of seizures (Pinault et al., 2001).

Preparative surgeries included penile vein catheterization, tracheostomy, EEG electrode implantations, ICV cannula implantation, craniotomy and duratomy.

2.3.2 Penile vein catheterization:
Dorsal penile vein catheterization is used to continuously infuse rat with neurolept anaesthetic during the electrophysiological recordings. For this procedure, the penis was retracted, and skin and connective tissue around the penile vein was removed. The distal end of the penile vein was secured tightly to stop the back flow of blood. A small incision made using a Vannas-scissors and a polyethylene tube (0.8/.04mm) was inserted into the penile vein. The position of the tubing was checked by with drawl of blood and the position of the tubing was secured with a suture.
2.3.3 Tracheotomy:

Tracheotomy was performed to enable mechanical artificial ventilation to the rats during the experiment. The artificial ventilation to the rat is facilitated using small animal ventilator (SAR-830, CWE, USA). To perform tracheostomy a small incision was made on the skin above the sternal notch and trachea was isolated by separating the muscle layers. A small incision was made between two rings of trachea and a 1 mm diameter plastic tube was inserted and secured with suture. To prevent blockage of the airway, lung secretions was aspirated with a tube connected to a 10ml syringe. The tracheal tube was then attached to the ventilator for artificial ventilation of the rat (60 breaths/minute PH$_2$O= 8-12cm). The body temperature of rat was maintained at room temperature between 36.2-37.2°C using a thermo-regulated blanket (Fine Science Tools., Inc, Germany) throughout the entire procedure. Needle ECG electrodes were placed subcutaneously, bilaterally over lateral aspects of thorax and signals from these electrodes were then conditioned and acquired by using signal conditioner (Axon instruments, CyberAmp 380). The heart rate of the animal was continuously monitored at (300-350 beats/minute) and arterial PO2 and PCO2 were continuously monitored at all times by pulse-oximeter and oxygenation was maintained >90%.

The experimental animal was then placed on a stereotaxic frame (David Kopf Instruments, USA) and neurolept anaesthesia is initiated before the end of general anaesthesia and was continuously administered at 0.5ml/hr using infusion pump (KDS 220, Kd scientific Inc, Massachusetts, USA). The rate of neurolept anaesthesia was regulated to maintain a predominantly desynchronised EEG state during recordings. To record cortical EEG, three electrodes were implanted on the rat skull.
2.3.4 EEG electrode and ICV cannula implantation:
For EEG electrodes and ICV cannula implantation, a midline incision was made on the skull between the eyes and ears. The tissue across the scalp was cleared and skull was exposed. Any bleeding was stopped using a diathermy (Medtronic Solan, USA). Three holes were drilled on the skull for recording electrodes without damaging the dura or brain (1.4 mm Easy Etch Engraver, Arlec Australia, Blackburn North, Victoria, Australia); three extradural screw electrodes were implanted in these burr holes to monitor EEG continuously. One on the right hemisphere of the brain anterior to bregma for recording cortical EEG, one hole on the left hemisphere of the brain anterior to lambda which is used as a reference and the third burr hole posterior to lambda is used as ground. The signal from these three electrodes was acquired and displayed as one single channel. Another hole was drilled (-1.5mm relative to bregma and +1mm relative to bregma) for implantation of ICV cannula (Figure 2.1). ICV cannula (22G, 5mm cut guide cannula, Bioscientific PVT LTD) was then lowered into the opening (dorsoventral -3.0-3.5mm relative to dura). Intra-ventricular location of the cannula was confirmed by withdrawing cerebrospinal fluid through the injecting line (22G injecting needle, Bioscientific PVT LTD). The injecting line is removed from guide cannula and a solid dummy line (28G, 5mm cut cannula internal, Bioscientific PVT LTD) was then inserted into the ICV guide cannula to avoid blockage. The electrodes and the ICV cannula was then secured in position using dental acrylate.
2.3.5 Electrophysiology:

Later, two stabilized craniotomies were performed at regions of interest (somatosensory cortex, VB thalamus and NRT) by drilling <0.8mm burr holes on the skull leaving a thin bone window (<200µm) above the dura. This bone window was carefully removed using fine tweezers to expose the dura. Later a small incision was made in dura to expose the brain and make it accessible for recording micropipettes (Pinault, 2005). The co-ordinates for the desired regions were made using stereotaxic rat atlas (Paxinos G, 1998).
Duratomies were covered with surgical sponge soaked in saline to avoid desiccation.

Borosilicate glass capillaries of 1mm OD x 0.58mm ID (SDR technologies) were pulled with pipette puller (P1000, Sutter instruments, USA) to a tip diameter of ≈1 micron with resistance of 15-25 mega ohms typically recorded in saline. These glass micropipettes were back filled with 1.5% N-(2 aminoethyl) biotinamide hydrochloride (Neurobiotin, Vector Laboratories, USA) (50mg of neurobiotin in 3.3ml of 1M potassium acetate) and stored at 4°C one day prior to experiment. Glass electrodes were then lowered into the brain through duratomies by using two motorized stereotaxic stepping microdrivers, one for each electrode (Omnidrive by Neurostar, Germany). These motorised microdrivers assisted slow movements of electrodes by 2µm steps through the duratomies without causing significant damage to brain tissue. After finding stable target cells from both electrodes, single neuronal firing pattern were recorded (Figure 2.2) in the regions of interest simultaneously with cortical EEG.
Figure 2.2 The Figure illustrates the dual paired juxtacellular recordings, where two glass electrodes are lowered into NRT regions where single neuronal activity is recorded. These recorded neurons are labelled at the end of the recorded and retrieved after the experiment. Pictures on the right shows two neurons that were recorded, labelled and identified after histology.
2.3.6 ICV drug infusions

Before the start of electrophysiological recordings the dummy line was replaced by an injecting line filled with 2µl of saline and 2µl drug separated by air bubble. The injecting line was connected to a 10µl syringe enabling manual administration of the drug ICV. Once stable electrophysiological recordings were attained, baseline neuronal firing patterns were recorded for 15 minutes. 2µl saline was administered (as a control) manually followed by another 15 minutes of recording. This was followed by administration of 1.5nmol NPY in 2µl of saline or saline as a control followed by another 30 minutes of recording (Figure 2.3). As used previously (Stroud et al., 2005, Morris et al., 2007, van Raay et al., 2012) a 10µl glass Hamilton syringe is connected to the injection needle using ling narrow bore line and a small air bubble is introduced to enable tracking of the injection. The injecting line and 10 µl transparent glass micro-syringe is monitored thoroughly and a slow and steady manual administration of saline and NPY is performed to ensure a successful infusion. At the end of the recordings, the recorded cell was then labelled with neurobiotin using micro iontophoresis process (Pinault, 1996). At the end of the experiments, rats were then transcardially perfused with 4% paraformaldehyde and the brains were removed for further histological studies.
This is the timeline for the ICV NPY infusions that were performed during the electrophysiological recordings under neurolept anaesthesia. After attaining stable recordings conditions, baseline neuronal recordings were performed for 15 min followed by 15 minutes of post saline and 30 minutes of post NPY recordings. The recorded neurons were than labelled juxtacellularly and rats were perfused. Rat brain was extracted and stored for further histological identification (adopted from (Gandrathi et al., 2013)).

2.3.7 Juxtacellular labelling

At the end of recording, the recorded pairs of neurons were labelled using the juxtacellular iontophoresis process described by Pinault to confirm their location (Pinault, 1996). In this process, the recorded neurons were juxtacellularly filled with 1.5 % neurobiotin iontophoretically by applying positive currents of 0.5-8 nA through the micropipette for approximately 10 minutes (Figure 2.4). After labelling, the micropipettes were withdrawn slowly, taking care not to damage the labelled cell. Following this, animals were administered a lethal dose of phenobarbital (5mL/kg, i.p. injection; Lethobarb, Virbac, Australia), perfused and brain was removed.
2.3.8 Perfusion and Histology:
An incision was made through the sternum and rib cage to expose the heart. A blunt 21G needle was pierced through the left ventricle into the ascending aorta and was connected to a peristaltic pump (Cole Parmer Instruments,
Illinois, USA). Rats were then perfused transcardially with 300 ml 0.1 mM phosphate buffered saline (PBS) to pump out the blood from the animal. This is followed by 400 ml of 4 % paraformaldehde (PFA) mixed with 0.25 % glutaraldehyde. After perfusion, rats were decapitated and brains were extracted and stored in 4% PFA at 4°C for histology.

For histological identification of the neurobiotin labelled neurons. The rat brain was cut at the posterior end fixed to the vibratome with super glue. Brain was cut into 100 μM axial sections using a vibrotome and placed in 24 well culturing plate in sequential order into each well. Brain slices were washed thrice with 0.1M PBS (10 minutes between washes). Later, 400 μl of 1:100 avidin-biotin-peroxidase complex solution (Vectastain, ABC kit; Vector Laboratories, USA) with 0.3% Triton was added to these slices and incubated overnight on a rotating stage. Next day, the brain slices were washed and 400 μl of nickel intensified 3,3 P-diarninobenzidine tetrahydrochloride activated by peroxidase (DAB; Vector Laboratories, USA) DAB solution was added in each well to reveal the neurobiotin tracer. The sections were than washed and mounted on gelatine-coated slides (6 sections per slide) allowed to dry. Finally, sections were dehydrated using ethanol and histolene and slides were cover slipped using DPX mounting media (BDH chemicals, Australia). Later, the slides were observed under the microscope for the localisation of any labelled cells.

Histological investigation in this thesis was only performed to identify and confirm the location of the recording neuron. We have not performed any investigations to morphologically identify and classify the subset of neurons in a location, which would be more helpful but we rather confined our investigation to assess and ensure the location of recorded neurons.
2.4 Focal injection studies:

For the focal injection study, a single craniotomy and durotomy was performed at the right hemisphere of the brain (A/P: 3.6 mm, M/L: 3.6 mm). A triple barrel borosilicate glass electrode was pulled to a diameter of ≈2 micron (15-30 MΩ). Only two barrels (out of three) were used in this technique; one barrel was back filled with 1.5% neurobiotin and the other barrel was filled with Saline/NPY for control and NPY investigations respectively. The electrode was then slowly lowered to desired brain region to locate a stable firing neuron. Once a stable neuronal recording is achieved, 15 mins of baseline recordings were performed followed by focal injection of saline/NPY for control and NPY studies. NPY and saline were injected focally using a microiontophoresis technique (Union-40 iontophoresis pump, kation scientific). Post NPY/control recordings were performed for 15 mins. In this technique neuroactive compounds are ejected by using small electrical impulses (Curtis DR, 1964, D.R., 1964, Singh and Maibach, 1994). The polarity and amount of current to be applied depends on the type of chemicals. We have used 200nanoAmps of ejection current (+ve) for 10 minutes to deposit NPY (0.5nM) focally on the recording neuron and a retaining current of 40 nanoAMPS was at all times of recordings (Ming and Shao, 2000). Control (saline, 165mM NaCl) was ejected focally at 80nanoAMPs for 10 mins and retaining current of 30nanoAMPS was used at all other times of recording (Haji and Takeda, 1993). All recordings were performed simultaneously with EEG and recorded cells were again labelled iontophoretically for later histological identification (Figure 2.5).
Figure 2.5 this is the experimental design for the focal injections of NPY. In this procedure triple barrel glass capillaries were used, glass electrodes were lowered onto NRT region in anaesthetized rats. One barrel filled with neurobiotin is used to record neuronal firing patterns, another barrel filled with saline/NPY were used to inject microiontophoretically at the recording site. Later the recorded neuron is labeled iontophoretically and the neurons are identified by histology procedures.
2.5 Cortical stimulation studies:

For these experiments, single craniotomy and duratomy was performed (A/P 0.2mm, M/L 4.1). A borosilicate glass capillary was pulled to form a sharp tip which was then broken gently with a paper to achieve a resistance of 2-5 mΩ. This glass capillary was backfilled with 1.5% neurobiotin and positioned in the somatosensory (S2) cortex with the stepping microdrive. The same coordinates were used for all the experiments and seizures were induced by a standard protocol to ensure minimal changes in the location of the electrode, which could alter seizure-inducing threshold for these experiments. A baseline recording was performed for 15 mins to assure stability. Baseline seizure inducing threshold was assessed by applying an electrical stimulation (7Hz) externally using and isolator stimulator. Current of 1-10milliAmps were applied by increasing 1milliamp each step until the seizure was induced and baseline seizure inducing threshold was recorded. 5 minutes after baseline recordings saline was administered ICV and seizure inducing threshold was assessed 15min, 1hour and 2 hours. Later NPY was administered ICV and the same stimulation protocol was repeated and seizure inducing threshold recorded. At the end of the experiment the whole region was labelled iontophoretically for further histological identification (Figure 2.6). Location of the electrodes and stimulation were confirmed by histological investigations.
Figure 2.6 This is the experimental design for cortical stimulations: a) where ICV cannula is implanted in lateral ventricle of left hemisphere, b) craniotomy and duratomy were performed in the right hemisphere to facilitate electrode in to S2 region. Field potentials of the S2 region were recorded along with cortical EEG. External electrical stimulation were given in S2 region to induce absence like seizures, c) this illustrates the experimental timeline for the cortical stimulations experiment. After attaining stable recording conditions baseline recordings were performed than saline was injected. Seizures were triggered after 15 minutes of saline infusion and after 15 minutes, 1 hour and 2 hours of NPY infusion and seizure triggering threshold was recorded. The
below picture is a snapshot of the recordings that displays the induction of seizure after stimulation in GAERS.

2.6 Data acquisition and analysis

EEG signals were acquired from the rat brain, the recorded data was then digitised at a sampling rate of 20 kHz (high-pass filtering) per channel (Digidata 1440; Axon Instruments, USA) processed at band passes of 0.1-600Hz for EEG (Clampex 10.0, Molecular Devices).

Neuronal signals recorded with glass electrodes were recorded with a Neurodata dual channel recording amplifier (Cygnus technology Inc). The data were processed with band passes of 0-6000 Hz (Cyberamp, Axon Instruments, USA) for neuronal activity. The signals acquired were conditioned using a programmable signal conditioner (Axon instruments, CyberAmp 380).

Along with EEG and Neuronal recordings, field potentials were also recorded for some experiments in this thesis. Unlike neuronal recordings and EEG, field potentials is the activity of group of cells in a region. This is similar to micro EEG and these are recorded by using blunt glass electrodes around 2-5Mohm resistance. The field potentials are recorded and acquired similarly as neuronal recordings using the same filters and programmable signal conditioner.

To quantify single neuronal firing patterns, data was analysed using Clampfit software (pClamp, Molecular Devices, California, USA) displaying multiple channels of data, i.e. paired single cell recordings, 2 EEG traces and 1 ECG trace. Spike events were semi-automatically detected using a threshold detection protocol. A low-pass filtering of 20Hz was necessary and applied to the single cell recording traces.
The recordings were arranged into standardized 15 minute time intervals – baseline, following saline injection and following drug injection. These were then subdivided into ictal, interictal and pre-ictal depending on the SWD’s appearing on the recorded EEG. Periods with prominent SWDs on EEG are defined as ictal episodes. Periods occurring between two ictal episodes or periods without any SWDs are considered as interictal episodes. The pre-ictal period is defined as the period just before an ictal episode, generally five seconds before ictal period is considered as interictal period.

To assess neuronal firing properties, several firing parameters were derived from single neural firing patterns. Different parameters were defined to classify neural firing during ictal and interictal periods. Five parameters were used for interictal firing properties: (1) Mean action potential (AP) firing frequency (MAPF) – the average action potential firing frequency of the neuron during the recorded period; (2) Percentage of APs in burst (PAPB) – this gives us the proportional of action potential firing in bursts in a recording. Bursts in the recording were identified as the group of action potentials with interspike interval less than 6ms; (3) Mean number of APs per burst (MAPB) – this parameter describes the average number of action potentials in burst in a recording; (4) Maximum number of APs per burst (MxAPB) – describes the maximum number of action potentials recorded in a burst; (5) Intraburst frequency (IBF) – defined as frequency at which action potentials within the burst. The same parameters were used to quantify the firing patterns of thalamic and cortical neurons.

These interictal firing patterns are better described with an illustration of interictal neuronal firing patterns in the below figure 2.6.1:

1) Mean action potential firing frequency (MAPF) for the whole stretch of recording below is $\approx 11$
2) Percentage of APs in burst (PAPB) ≈ 27%
3) Mean number of APs per burst (MAPB) ≈ 3
4) Maximum number of APs per burst (MxAPB) Maximum number of APs per burst (MxAPB) ≈ 6
5) Intraburst frequency (IBF) is frequency measure within the burst

Ictal periods were parameterized by: (1) AP firing proportion per EEG spike (APFP) – defined as the proportion of action potential firing per EEG spike; (2) Percentage of burst with EEG spike (PB) describes the proportion of action potential firing in burst within EEG spikes; (3) Mean number of AP’s per EEG spike (MAP) gives the average number of action potentials firing per EEG spike; and (4) Intraburst frequency gives the frequency of action potentials firing in burst (IBF).

In the below exemplary figure (Fig 2.6.2) of an NRT neuron firing during seizure, the ictal neuronal firing parameters are described as follows:

1) AP firing proportion per EEG spike (APFP) ≈ 1 as each EEG spike (in SWDs) has an correlated action potential firing.
2) Percentage of burst with EEG spike (PB) is 100% as each EEG spike is characterized by a burst of action potentials.

3) Mean number of APs per EEG spike (MAP) ≈ 7 i.e. an average number of actions potentials per burst

4) Intraburst frequency is the frequency within the burst.

Fig 2.6.2 NRT neuron firing during seizure

Four parameters were defined quantify drug effects on cortical EEG. These were: (1) Total length of seizures (TLS) in the recording; (2) Mean duration of seizure (MDS) – defined as the average duration of the seizure length; (3) Cycle frequency (CF); and (4) Proportion of recording time of seizures (PTS).

Autocorrelation and cross-correlation analyses were performed to analyse the rhythmicity of single neuronal firing, and synchronicity of firing between two neurons, respectively. Rhythmicity is defined as the periodicity in neuronal firing patterns of spontaneously firing neuronal activity. Lomb periodogram of the autocorrelation function was used to access the periodicity of the signals (Press et al., 1992). We defined synchronization as the presence of simultaneous activity in the two signals. Wave form correlation was also performed to assess the correlation between waveform signals (field potentials) from different regions in the brain.
2.6.1 Autocorrelation analysis:

The rhythmicity of the spontaneous neuronal (i.e. significant periodicity observed in the neuronal firing) was assessed using the method developed by Kaneoke and Vitek, 1996. In this method an autocorrelation function is created by inverse Fourier transform of the power spectrum and significance of the peaks in the autocorrelation function is determined by using the power spectrum as described in (Press et al., 1992) for a frequency range from 0 to 50Hz (Figure 2.7). Using this method, multiple frequencies in the spontaneous neuronal firing patterns can be assessed.

Juxtacellular neuronal firing data was divided into three different phases: ictal period (seizing period), interictal period (non-seizing period) and pre-ictal period (transition period from non-seizing period to seizing period) and rhythmicity in neuronal firing was analysed before and after drug administration.
Figure 2.7 Showing results from Kanoke’s autocorrelation program which helps in detecting and quantifying the rhythmicity in neuronal firing. These Figures here show an interspike interval histogram, an autocorrelogram and spectrum of autocorrelogram which detects significant rhythmicity in neuronal firing and also provides the frequency at which neuronal firing is Rhythmic. The Figure below show an exemplary recording of a rhythmic and non-rhythmic neuronal firing (adopted from (Gandrathi et al., 2013)).
2.6.2 Cross correlation analysis:

To assess the inter-dependence between two simultaneously firing neurons cross correlation was used. Time stamps of the spontaneously firing neuronal signals were collected and a cross-correlogram was constructed using a custom program written in Matlab (1 milli second bin width and maximum of 500 lags). If two spike trains in the same recording are independent and neuronal firing is randomly correlated with each other, the cross-correlogram for that spike train will be flat. On the other hand, a correlated spike train and a peak in the cross-correlogram would suggest that the neurons have a common input or are synaptically connected.

We used the synchronization index (SI), developed by (Wiegner and Wierzbicka, 1987) to quantify the height of the peak in the correlogram, giving a measure of synchronicity between the two input spike trains. The SI values range between 0.0 (not synchronous) to 1.0 (100% synchronous). To access the statistical significance of SI, a critical value for the index is calculated based on the notion of extra counts beyond ‘expected counts’, which corrected for random fluctuations of the SI (Figure 2.8).

SI of ictal, interictal and pre-ictal phases were compared before and after and ICV drug administration to examine the effect of the drug on synchronization between two simultaneously firing neurons.
Cross correlation is the robust method to understand the synchronicity in the neuronal firing and network behaviour. This Figure here shows cross correlograms constructed using timestamps of the neuronal firing before and after ICV drug administration. Figures on the right showing is a recording of typical NRT neurons firing recorded simultaneously with EEG during ictal period (adopted from (Gandrathi et al., 2013)).

### 2.6.3 Waveform Correlation

Waveform correlation (spike 2, version 5.2.1) is used to understand the correlation between waveforms recorded from two different regions in the brain (Blumenfeld et al., 2007). The signals of interest were imported into Spike 2 software (CED, UK) and processed to produce a cross correlogram. The peak of the cross correlogram close to zero is calculated and the result of waveform correlation is scaled from -1 (correlated but inverted), through 0 (uncorrelated), to +1 (full correlation).

Waveform correlations were assessed for cortical EEG and field potentials recorded from NRT region during ictal, interictal and pre-ictal phases were compared before and after and ICV drug administration to examine the effect
of the drug on correlation between two waveforms recorded from two different regions (Figure 2.9)

**Figure 2.9** Waveform correlations were performed to understand the network dynamics of the structures involved in path physiology of the disorder. Waveform correlations show the correlation between the waveform of the recordings performed in different brain regions and this Figure shows characteristic correlations between two waveforms during ictal and interictal periods.
CHAPTER 3

EFFECTS OF ICV ADMINISTERED NPY ON EEG AND NEURONAL FIRING PATTERNS IN THALAMOCORTICAL CIRCUIT IN GENETIC RAT MODEL OF ABSENCE EPILEPSY.

3.1 Introduction

Absence (petit mal) seizures are a common seizure type in patients with generalized epilepsy. Absence seizures are associated with symmetrical and bilaterally synchronous 3Hz spike and wave discharges in the EEG (Gibbs FA, 1935, Meeren et al., 2002). Absence episodes start and end abruptly on a normal EEG background (Porter, 1993) with brief loss of consciousness (Meeren et al., 2002). An absence seizure usually lasts 10-30 seconds and can occur during quiet wakefulness with sudden arrest of ongoing behaviour. Absence epilepsy generally occurs between the ages of 2-13, peaking at 6 years of age and females are twice more likely to have absence-like seizures than males.

There are inadequate satisfactory therapeutic options available for patients with absence epilepsy and many patients are unable to tolerate or are unresponsive to currently available drugs: Ethosuximide, trimethadione, valproate and benzodiazepines (Mattson et al., 1985, Micheletti et al., 1985, Loiseau and Jallon, 1995, Liu et al., 2006). Valproate and ethosuximide cause adverse effects in 40% and are ineffective in 20% of patients (Kwan and Brodie, 2000, Murphy and Delanty, 2000) and benzodiazepines are highly sedative. Drugs used effectively against focal epilepsies such as carbamazepine are either ineffective or aggravating if used to treat absence seizures(Liu et al., 2006). These facts show the necessity of developing new
agents for treating absence epilepsy. Neuropeptide associated mechanisms may represent novel targets for seizure suppression, particularly neuropeptide Y (NPY).

NPY is a 36 amino acid peptide discovered in 1982 in the pig brain by Tatemoto (Tatemoto, 1982b). NPY is abundantly found in many brain regions (Allen et al., 1983, Chronwall et al., 1985, de Quidt and Emson, 1986) and is widely expressed in the human CNS (Morris, 1989).

NPY is a member of the pancreatic polypeptide (PP) family with six subunits Y1, Y2, Y3, Y4, Y5, and y6. It acts by binding to six G-protein coupled heptahelical receptor subtypes (Stroud et al., 2005), inhibiting adenylate cyclase and thus decrease intracellular calcium levels (Michel et al., 1998, Berglund et al., 2003). NPY plays a critical role in regulating the neuronal excitability. In vivo studies in animal models of acquired focal epilepsy revealed the evidence for involvement of NPY in seizure suppression (Marksteiner et al., 1989, Gruber et al., 1994, Erickson et al., 1996, Kofler et al., 1997, Woldbye et al., 1997, Kopp et al., 1999, DePrato Primeaux et al., 2000, Vezzani et al., 2002) and the evidence for involvement of NPY is also shown in in vitro studies (Greber et al., 1994b, Marsh et al., 1999, Baraban, 2002) and also in humans studies (Patrylo et al., 1999, Takahashi et al., 1999, Furtinger et al., 2001).

The seizures in generalized epilepsies, especially in absence epilepsy, involve particular neurophysiological circuitry that mediates hyper synchronous oscillatory thalamocortical activity. The best documented example of this is absence seizures. Recent studies have shown NPY reduces absence-like seizures in genetic rat model of absence epilepsy (GAERS) (Stroud et al.,
A recent study shows that NPY receptor subtype Y2 plays a crucial role in mediating seizure suppression effect of NPY in hippocampus (El Bahh et al., 2005). Additionally an intracerebro-vascular injection study with NPY and subtypes (Y1, Y2 and Y5) on GAERS showed that the Y2 subtype is more involved in anti-seizure activity compared to other two subtypes compared to Y1 and Y5 (Morris et al., 2007).

Understanding the cellular and network dynamics in the brain during epileptic activity and its changes with anti-epileptic drugs interference would help us identify in greater detail the possible pathophysiology of these neurological disorders. Many techniques have been developed in recent years to understand the properties of cell, neuronal networks and changes occurring in the brain. Juxtacellular recording (Pinault, 1996) is one such technique where, sharp electrodes filled with neurobiotin were used to obtain extracellular recordings and then labelling was performed by applying a train of current pulses i.e. juxtacellular iontophoretic labelling. This technique permits high resolution single cell electrophysiological recordings in anaesthetized animals (Pinault, 1996, 2003, Pinault and O'Brien, 2005, Zheng et al., 2012). Pinault has reported the properties and cellular mechanisms of thalamocortical cells and reticular thalamic cells in GAERS (Pinault, 2003, Pinault and O'Brien, 2005). The staining technique assists in localisation and morphological characterisation of recorded neurons (Pinault, 1996).

In the present study for this chapter we have used GAERS and NEC rats. GAERS is a widely accepted genetic model for absence seizures, exhibiting spontaneous and non-convulsive absence-like seizures. They display 5-9 Hz SWDs on EEG and exhibit similar pathophysiological, behavioural,
pharmacological, aetiological characteristics to human absence seizures (Danober et al., 1998).

From the previous studies we know that NPY suppresses seizures. Understanding the underlying cellular and network changes involved in the suppression of seizures will provide more knowledge on pathophysiology of absence seizure and suggest mechanisms of action of NPY. In this study we performed in vivo electrophysiological single and paired neuronal recordings in thalamocortical circuit concurrently with ICV NPY infusions and assess changes in the neuronal firing patterns that may contribute to the seizure suppression effect of NPY.

3.2 Materials and Methods
The data showed in the present chapter involves EEG electrode implantation, ICV cannula implantation and in vivo electrophysiology. Experiments were conducted on 12-15 weeks old male GAERS (n=22) and NEC (n=6) rats. Animals housing and all experimental procedures were performed in accordance with the requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.2.1 Anaesthesia
Detailed descriptions of types of anaesthesia used in these experiments are explained in methods (Chapter 2.3). Briefly, all surgical procedures were performed under general anaesthesia (ketamine/xylazine in 3:1 ratio; 0.1ml/100gm) and electrophysiological recordings were performed under neurolept anaesthesia (Figure 3.1) (d-tubocurarine (2mg/1ml of water), Fentanyl (0.2ml), Haloperidol (0.2ml) with glucose) which completely anaesthetises and paralyses the animal it in a pseudo-awake state, allowing the rats to seize spontaneously.
3.2.2 Surgery

The detailed description of surgical procedures involved in this study is given in the methods (chapter 2.3). Briefly, the rats were anaesthetised with an intraperitoneal injection of ketamine/xylazine (3:1). Once adequately anaesthetised, penile vein catheterization and tracheotomy were performed and rat was connected to mechanical ventilator for artificial ventilation (2.3.1, 2.3.2). The rats were then mounted on stereotaxic frame (David Kopf instruments, Tujunga, CA, USA). Neurolept anaesthesia was initiated before the end of general anaesthesia, and was administered continuously with micro injection pump at 0.5ml/hr. An Active EEG electrode was epidurally implanted on the remote cortex for cortical Electroencephalogram (EEG) (2.3.3). For the ICV infusions, an ICV cannula was implanted on the left hemisphere (A/P: 1mm, M/L: -1.5 mm and -3.0 to -3.5mm deep relative to dura). Placement of cannula was confirmed by withdrawing cerebrospinal fluid through the ICV injecting line (2.3.3) (Figure2.1).

Heart rate, body temperature and oxygen were continuously monitored and maintained at physiological limits (PO2>90%, Heart rate 200-350 beats/minute, temperature-37°C) at all times during the experiment

3.2.3 Electrophysiology:

The surgeries and recording protocols have been described in methods (chapter 2.4). Briefly, Two craniotomies and duratomies were performed on the right hemisphere of the skull (A/P: 2.8 mm, M/L: 4.8 with -12.5 ° and (A/P: 3.6 mm, M/L: 3.6 mm) for paired electrode recordings. Borosilicate glass micropipettes filled with 1.5% were lowered into the desired brain region using motorized stepping microdrive with 2µm steps and at relatively low speed of 0.04mm/sec. Paired juxtacellular single neuronal recordings
were performed from target neuronal cells from thalamocortical regions simultaneously with EEG and ECG (Figure 2.2).

3.2.4 ICV drug infusions:

The ICV drug infusion protocol is detailed in methods (chapter 2.5). Briefly, juxtacellular single neuronal firing was recorded with the intervention of saline and NPY at different time points. Baseline neuronal was performed for the first 15 mins followed by ICV administration of 2µl saline. 15 minutes of post-saline juxtacellular recordings were performed. This was followed by ICV administration of 2µl of 1.5nmol NPY 30 minutes of post NPY recording were performed (Figure 2.3). The recorded cell was then labelled and for further histological investigations.

3.2.5 Juxtacellular labelling and Histology:

At the end of the ICV drug infusions, the recorded cells were iontophoretically labelled for further histological investigations. The labelling and histology procedures are described in methods (chapter 2.6, 2.7). Briefly, the recorded cell was juxtacellularly filled with neurobiotin by applying positive currents (0.5-8 nA). Rats were perfused and brain is removed. The whole brain was sectioned into 100µm sections and stained with DAB solution. The stained brain slices were mounted on glass slides, cover slipped and left to dry overnight. The sections were then observed under bright field inverted microscope (Olympus BX 51) under 20 x magnifications to discover the labelled cells (Figure 2.2 and 2.4).
Figure 3.1 Figure illustrating the experimental time line for paired juxta cellular recordings. Procedures under grey area are preparative surgeries performed under general anaesthesia (a combination of Ketamine/xylazine), and electrophysiological recordings, ICV NPY infusions and juxtacellular labelling performed under neurolept anaesthesia are shaded in black. At the end of the experiment rats are perfused and brains are extracted for further histological identification.

3.3 Data acquisition and analysis:

The data acquisition and analysis is done using Clampex and Clampfit software (pClamp, Molecular Devices). More information on data acquisition and analysis is provided in the methods (chapter 2.10). Briefly, Data was recorded at a sampling rate of 20,000 KHz/channel and processed at band passes of 0.1-600Hz for EEG and 0-6000Hz for neuronal activity (Clampex 10.0, Molecular Devices). To quantify single neuronal firing patterns, data was analysed using Clampfit software (pClamp, Molecular Devices, California, USA).
The recordings were arranged into standardized 15 minute time intervals – baseline, following saline injection and following drug injection. These were then subdivided into ictal and interictal periods. To assess EEG and neuronal firing patterns, several parameters have been determined. More detailed information of these parameters is described in methods Chapter (2.10.1, 2.10.2, 2.10.3)

3.4 Statistical analysis:
Statistical analysis was performed using the Graph Pad Prism software (GraphPad Software, San Diego California USA). One way ANOVA test was performed to determine the significance levels of variables between the treatments followed by planned analysis to compare significance between two individual treatments. All data were expressed as mean ± standard error of mean. P values less than 0.05 were considered to be significant.

3.5 Results:
3.5.1 Effect of NPY on seizures:
The percentage of recording time spent in seizures and total length of seizures in the EEG recording were calculated before and after the ICV administration of NPY. Highly significant decrease was found in Total length in seizures (Baseline=131.41 ± 23.77 seconds, post NPY= 95.83 ± 20.48 seconds, P=0.0001, n=11) and percentage of time spent in seizures (Baseline=15.91 ± 6.08, post NPY= 9.08 ± 4.12 seconds, P=0.0001, n=11) after ICV NPY administration proving the anti-epileptic property of NPY (Figure3.2).

Other parameters were also assessed to determine NPY induced changes on EEG such as mean length of seizures recorded and cycle frequency of seizures. No significant change was observed in cycle frequency of seizures (P=0.92, n=11) and Mean length of the seizures (P=0.10, n=11).
Figure 3.2 Cortical EEG of GAERS was analysed before and after administration of NPY. ICV administration of NPY significantly suppresses seizures in GAERS. This above graphs illustrates the seizure suppression effect of NPY by significantly decreasing the total length of seizures and percentage of time spent in seizures and the below picture depicts the suppression of seizures after
the administration of NPY. The below two graphs illustrates the results of the other two parameters - EEG seizure duration and cycle frequency were not affected by ICV administration of NPY

fig 3.2.1

<table>
<thead>
<tr>
<th>Mean Length of the Seizures</th>
<th>Cyclic Frequency of the Seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Saline</td>
</tr>
<tr>
<td>7.5</td>
<td>4.7</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>12.5</td>
<td>6.2</td>
</tr>
</tbody>
</table>

P value=0.10 (>0.05)
Not Significant

P value=0.82 (>0.05)
Not significant

3.5.2 Effect of NPY on interictal Neuronal firing patterns

A significant increase was found in the mean firing frequency of the NRT cells after administration of NPY (Baseline=12.32 ± 4.71, post NPY= 21.74 ±8.09, P=0.008, n=12). A significant difference was observed in the percentage difference of neuronal firing patterns between baseline and saline. No significant change was observed in all other interictal parameters, Percentage of burst firing (P=0.14, n=12), Mean number of action potentials (P=0.46, n=12), Maximum number of action potentials (P=0.11, n=12), interictal intra burst frequency (0.62, n=12) and ictal firing patterns. No significant difference was observed between baseline and saline findings contributing to the fact that this increase observed in the neuronal firing frequency is not an artefact of ICV infusion procedure of saline injections but an effect of NPY infusions.

None of the parameters of thalamic and cortical cells were found significant during ictal and interictal periods. Though a trend was observed in some parameters none of them were statistically significant (Figure 3.3).
**Figure 3.3** Neuronal firing patterns of the single neurons were recorded and compared before and after the administration of NPY during seizing and non-seizing periods. These are the main findings of my study:

a) A significant increase in the interictal neuronal firing patterns in NRT cell

b) A significant difference is seen in the percentage difference in neuronal firing patterns between baseline and post NPY neuronal firing

c) The below figure illustrates the increase in firing frequency of NRT neuron before ICV administration of NPY which is around 12Hz and the firing frequency
frequency of the NRT neuron has significantly increased after NPY administration d) the graph below is the control data performed using saline infusions to show the change in neuronal firing patterns is sole effect of NPY but not due to ICV procedure or any other artifact.

**Fig 3.3.1** Illustration of burst of action potentials during an EEG spike at a high resolution showing a burst of 6-8 action potentials firing with each EEG spike

**Fig 3.3.2** Illustration of burst of action potentials during an EEG spike at a low-medium resolution showing a burst of 6-8 action potentials firing with each EEG spike
Fig 3.4.1

**interictal % burst of nrt cells**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Saline</th>
<th>Npy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.76 (&gt;0.05)</td>
<td>Not significant</td>
<td></td>
</tr>
</tbody>
</table>

Fig 3.4.2

**Interictal mean AP/Burst of nrt cells**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Saline</th>
<th>Npy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.61 (&gt;0.05)</td>
<td>Not significant</td>
<td></td>
</tr>
</tbody>
</table>
Fig 3.4.3

**Interictal maximum number of AP/Burst in nrt cells**

P value-0.30 (>0.05)
Not significant

Fig 3.4.4

**Interictal intra burst frequency of nrt cells**

P value-0.61 (>0.05)
Significant

Fig3.4 The above graphs show the results of other interictal firing patterns of the NRT neurons that does not show any significant changes with ICV administration of NPY.
P value – 0.05 (not significant)

P value – 0.05 (not significant)
Fig 3.5.3

The above graphs show the results of other ictal firing patterns of the NRT neurons that do not show any significant changes with ICV administration of NPY.

P value – 0.07 (not significant)

Fig 3.5.4

The above graphs show the results of other ictal firing patterns of the NRT neurons that do not show any significant changes with ICV administration of NPY.

P value – 0.076 (not significant)
<table>
<thead>
<tr>
<th>NRT interictal neuronal firing patterns</th>
<th>ICV administered NPY induced changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPF</td>
<td>Increased significantly</td>
</tr>
<tr>
<td>% Burst</td>
<td>No significant difference</td>
</tr>
<tr>
<td>MAPB</td>
<td>No significant difference</td>
</tr>
<tr>
<td>MxAPB</td>
<td>No significant difference</td>
</tr>
<tr>
<td>IBF</td>
<td>No significant difference</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NRT ictal neuronal firing patterns</th>
<th>ICV administered NPY induced changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>APFP</td>
<td>No significant difference</td>
</tr>
<tr>
<td>PB</td>
<td>No significant difference</td>
</tr>
<tr>
<td>MAP</td>
<td>No significant difference</td>
</tr>
<tr>
<td>IBF</td>
<td>No significant difference</td>
</tr>
</tbody>
</table>
3.5.3 Effect of NPY on the Rhythmicity and synchronization in neuronal firing of NRT cells

Rhythmicity of the neuronal firing was quantified applying spectral analysis to the autocorrelogram, termed the Lomb periodogram. This method will detect the multiple frequencies and also signifies the frequency at which neuronal firing is rhythmic. In this study, the proportion of cells firing significantly in rhythmic mode were identified and number of cells firing rhythmically were compared before and after NPY administration in three different phases (Ictal, pre-ictal, interictal). We have not assessed the changes in the frequency before and after NPY infusions. No significant change was observed in proportion of rhythmic cells before and after the ICV administration of NPY during ictal, pre-ictal and interictal periods (Figure 3.6).

![Graphs showing spectral analysis and autocorrelogram](image)

**Figure 3.6** Data was recorded from 12 NRT cells and analysed. Kaneoke’s autocorrelation program was used to detect rhythmicity in the spike train. a) This program uses Interspike intervals of the spike train to plot a autocorrelogram and Lomb periodogram. This method enables to detect
the presence of multiple frequencies in the spike train and assess significances of frequencies detected. b) Out of 12 nrt cells, proportion of rhythmically firing neurons showed a decreasing trend after NPY infusion but results were not significant. c) Below is the illustration of neuronal recording showing transition from non-rhythmic tonic firing in interictal period to rhythmic neuronal firing.

To access the synchrony in the neuronal firing cross correlation analysis was performed using the timestamps of spike events and synchronization index. Time stamps of the spike events were used to plot cross correlograms and synchronization index was calculated for each cross correlogram. To signify the synchronicity in neuronal firing a critical index is also calculated using peak values and off peak values in the cross correlogram. All the bin values in the peaks present in correlogram are considered as peak values and values on non-peak areas/troughs are classified as off peak values. Wiegner et al, has given some clear calculations and formulas to determine these values and calculate critical index (Wiegner and Wierzbicka, 1987). SI of the cross correlogram for the neuronal recordings were compared before and after NPY administration during interictal, pre-ictal and ictal phases of a seizure. No significant change was observed in synchrony of the neuronal firing in NRT cells before and after NPY in three different Phases (Figure 3.7).
Figure 3.7 As an approximation of the network behavior, we investigated the changes in the synchronicity of neuronal firing. 8 pairs of NRT neurons were recorded and analysed, time stamps of the recordings were used to plot cross correlograms and synchronization index was calculated for each cross correlogram to signify the synchronicity in neuronal firing. SI showed a decreasing trend during ictal period after NPY administration but was not significant. Here are exemplary diagram of paired neuronal recording along with cortical EEG during seizure, here you can see two neurons firing synchronously with each other along with the EEG spike.

3.5.4 Effect of ICV administered NPY on the waveform correlation between cortical EEG and local field potentials of NRT

During a seizure cortex, thalamus and NRT are engaged in a highly rhythmic oscillatory mode. To assess the effect NPY on correlation between networks, we have performed cross correlation between local field potentials of two different regions in thalamocortical circuit. As local field potentials signifies the summation of neuronal activity of several cells in particular region, performing correlation of field potentials will determine alterations in correlation between networks. Waveform correlation analysis in Spike 2
software (Cambridge electronic design, Cambridge, England) measures the similarity in waveforms with same sample interval. Cross correlation between local field potentials of cortex and NRT was conducted and cross correlograms were constructed. Observation of a peak near time displacement zero suggested a correlated and synchronous signal (Blumenfeld et al., 2007). A significant decrease in the correlation of field potentials was found between cortex and NRT with ICV NPY drug administration (n=14, p=0.01) (Figure 3.8).

Fig.3.8

_Cx-NRT ictal waveform correlation_

Figure 3.8 Waveform correlations were performed to understand the network dynamics of the structures involved in path physiology of the disorder. Waveform correlations show the correlation between the waveform of the recordings performed in different brain regions. Waveform correlation between cortical EEG and field potential of NRT region decreased significantly after NPY infusion. The Figure on the top right shows the waveform correlation between two regions
during ictal period. Below here is a visual example of correlation in field potentials of NRT neuron and cortical EEG during ictal period.

3.6 Discussion:
Pathological oscillations within reciprocal connections of thalamocortical circuits involving VB, NRT and somatosensory cortex, are responsible for generation and initiation of absence seizures both in humans and rats. The NRT is a critical structure in the thalamocortical circuit. The rhythmic hyper synchronous oscillatory thalamocortical activity that characterizes absence seizures is synchronized by the NRT (Steriade et al., 1986, Buzsaki et al., 1990, Steriade et al., 1993, Contreras and Steriade, 1995, Snead et al., 1999, Noebels et al., 2010). NRT is mainly composed of GABAergic neurons and provides recurrent inhibitory synapses to the thalamic relay neurons (Kim et al., 1997, Pinault et al., 2001, Pinault, 2003) and receives glutaminergic axon collaterals from both thalamocortical and corticothalamic neurons (Ohara and Lieberman, 1985, Pinault and Deschenes, 1998).

GABA is a major inhibitory neurotransmitter in vertebrate central nervous system, blockade of GABA results in uncontrolled neuronal excitability in neuronal circuits. GABA exhibits inhibitory actions via iontophoretic GABA_A receptors, activation of these receptors triggers opening of a chloride ion-selective channel and selectively conducts Cl\(^{-}\) resulting increased chloride conductance causing hyperpolarization in the neuron (Meldrum, 1989, Treiman, 2001). Nevertheless, there are several reports of excitatory GABA_A receptors. The excitatory nature of GABA_A is a result of increased intracellular concentration of Cl\(^{-}\) ions either during development of the nervous system or in certain cell populations (Cherubini et al., 1991, Ben-Ari et al., 2007, Li and Xu, 2008). The NRT neurons regulate the rhythmic oscillatory
thalamocortical activity via GABA-mediated hyperpolarization of VB thalamus neurons, thereby de-inactivating low threshold T –type calcium (Ca2+) channels, which results in high frequency action potential bursts(Snead, 1995, Snead et al., 1999, Zhang and Jones, 2004).

In the current chapter, we showed that ICV administration of NPY suppressed absence seizures in a genetic rat model of absence epilepsy by exhibiting reduction in total length of seizures and percentage of time spent in seizures under neurolept analgesia. In addition, we also demonstrated that NPY mediated reduction in the seizure levels (absence) i.e decrease in total length of seizures and percentage of time spent in seizures were associated with increased firing rate of the NRT neurons interictally in these rats. A study on rat neocortex tissue showed NPY reduces evoked IPSP amplitude in GABAergic interneurons which might lead to an increased firing rate. Additionally, they also found an increase in IPSP amplitude in pyramidal cells, this NPY mediated potentiation of inhibition might be derived from increased excitability of interneurons by NPY (Bacci et al., 2002). Results of the current studies indicate that the effects of NPY on neuronal firing patterns were predominantly in NRT region in the thalamocortical circuit, as other structures of the thalamocortical circuit did not have any significant change in neuronal firing patterns, further confirming the critical role of NRT in suppressing seizures. Previous studies have looked at the firing patterns of NRT neurons in vivo in GAERS but this is the first study to look at the changes in the neuronal firing patterns of the NRT neurons with application of NPY. The neuronal recordings were only performed and assessed in thalamocortical circuit, which is critical for generation of absence seizures. Understanding the changes in this centrally based circuit responsible for SWDs is more important to understand the underlying mechanism of absence
seizures. Given the complexity of the circuit and limitations of the method, it is difficult to formulate a strong mechanistic hypothesis at this stage. To briefly explore some possible mechanisms basing on the results of this chapter: Increased NRT firing frequency following ICV administration of NPY could be because of increased inhibition of GABA receptors with in the NRT. This increase in firing frequency of NRT neurons may contribute to seizure suppression by increased deposition of inhibitory GABA in thalamus and causing decreased GABA mediated hyperpolarization of the thalamocortical neurons there by maintaining the T type calcium channels (Ca^{2+}) in the inactivated state and inhibiting oscillatory thalamocortical activity. During a seizure, the thalamocortical circuit goes into hyper-synchronous state and we found a decrease in waveform correlation of field potentials in cortex and NRT, this may also be due to increased firing rate of NRT cells that might be disrupting the hyper-synchronous rhythm that characterizes absence seizures. A previous study showed that enhanced GABA activity in VB thalamus by injection of GABA transaminase inhibitor, γ-vinyl GABA, aggravated seizures in GAERS (Liu et al., 1991) but this technique is unlikely to be directly comparable with the increase in GABA-ergic transmission proposed here. Another argument is that NPY may be acting on specifically on interneurons present in cortex and NRT (Kuruba et al., 2011). Further experiments with agents selective for NPY receptor subtypes, and assessing changes in the firing of specific interneurons will provide more information on the possible mechanisms of seizure suppression.
Further, NPY injections led to a significant reduction in the correlation of waveform activity between the NRT and cortical region suggesting an effect on the overall thalamocortical network. As this occurs during the ictal period, it may suggest a separate mechanism from the increased firing rate interictally. We have analysed auto correlation and cross correlation with custom made programs that provide a critical point or threshold to assess the significance in autocorrelation and cross correlation. All statistical analysis was performed by using widely accepted parameters for this kind of data.
Further experiments with agents selective for NPY receptor subtypes, and assessing changes in the firing of specific interneurons will provide more information on the possible mechanisms of seizure suppression.

Previous studies from our laboratory have shown that ICV administration of NPY has seizure suppressive effects (Stroud et al., 2005) that were mediated through Y2 receptors (Morris et al., 2007, van Raay et al., 2012). The cellular or molecular mechanism in suppression of seizures is poorly understood. Reports from previous studies indicate that Y2 receptor is critical to mediate the anti-epileptic actions of NPY (Colmers et al., 1991, Greber et al., 1994b, Schwarzer et al., 1998, El Bahh et al., 2005). A study in rat brain slices reported that NPY produced inhibitory effects on NRT and VB neurons mostly via activation of GIRK channels coupled to NPY Y1 receptor (Sun et al., 2001a). However, this study was performed in brain slices and on stimulated spike responses. The neuronal and network dynamics are different in live and intact brain compared to brain slices. Brain slices has limitations, brain slices are not ideal to investigate neuronal excitability and pathological oscillations of absence seizures, which arise from spontaneous hypersynchronous thalamocortical oscillations because of massive disconnections involved in the preparation and lack of spontaneous firing in neurons. We suspect that this is not the mechanism underlying our observation as increase in K\(^+\) current would likely reduce spontaneous firing and the studies in vivo strongly support predominant Y2 effect. Nevertheless, Sun et al demonstrated that repetitive burst stimulation of the NRT induces NPY release that inhibits the NRT by activating the Y1 coupled GIRK channels and this endogenous, oscillation induced NPY release acts to suppress the oscillations (Sun et al., 2003). In the same paper it is shown that NPY Y2 receptor activation (by application of NPY 3–36) decreased spontaneous and evoked GABA release
(Sun et al., 2001a, Sun et al., 2001b) in NRT. Overall, these studies on brain slices may not be discordant with the current study, given the large differences in preparation and even possibly complementary effects. Unfortunately, NPY Y2 receptor antagonists were not available at that time to investigate the effect of endogenous NPY Y2 activation on oscillations in thalamic slices. The authors of these studies agree to the fact that NPY Y2 and NPY Y5 receptor subtypes may also be involved in the modulation of neuronal excitability and GABA release (Sun et al., 2001a). The previous results from our lab indicate that NPY mediates its anti-epileptic effects primarily through Y2 receptor subtype (Morris et al., 2007, van Raay et al., 2012). In vitro patch clamping with specific NPY receptor subtype agonists and antagonists will be extremely useful to clarify these mechanisms.

NPY receptor subtypes Y1 and Y5 are primarily situated on somata and dendrites of the neurons. These receptors act to inhibit their activity via activation of G-protein-activated inwardly rectifying potassium channels (GIRK). NPY Y2 receptors are predominantly located on nerve terminals and act to inhibit GABA and glutamate release via inhibition of voltage-gated calcium channels (Sun et al., 2001a, El Bahh et al., 2005). Possibly, NPY might be acting post-synaptically via Y1 or Y5 receptor enhancing GABAergic inhibition with in the NRT or cortex (decreasing the hyperpolarizing output to VB), or pre-synaptically via Y2 receptor on the NRT neurons projection on to VB, inhibiting GABA release and thus reducing the hyperpolarization in these neurons that promote oscillatory rhythmic activity during absence seizures (Stroud et al., 2005, Morris et al., 2007).

In addition to anti-epileptic activity, NPY mediates other physiological responses. NPY is anxiolytic, appetite stimulant, and shows cardiovascular
effects (Zini et al., 1984, Cabrele et al., 2000b). Nevertheless, the appetite stimulating effects of NPY are primarily mediated by Y1 and Y5 receptors (Gerald et al., 1996, Cabrele et al., 2000a, Chaffer and Morris, 2002). Therefore, activating the NPY Y2 receptor could be a novel and effective therapeutic option for treating absence seizures without inducing unwanted side effects. Most importantly, NPY has a strong seizure suppressant effect at very low concentrations than other drugs and difficult to compare as dose-response study in not performed in this study. Other first line drugs for absence seizures such as valproate and ethosuximide are effective but can cause adverse effects in 40% of patients and are ineffective in 20% of patients (Kwan and Brodie, 2000, Murphy and Delanty, 2000) and benzodiazepines are highly sedative. ICV and focal administration studies of NPY Y2 agonists and antagonists might provide more information on the mechanism of action of NPY.

It would be more interesting to study effect of NPY at network level. One of the limitations of this juxtacellular cellular recording is the sample size, we could record from a maximum of two neurons at a time to interpret the changes at network level. This may be the reason we do not have any result in oscillation index and synchronization index to assess the changes at network level. Multi electrode or Tetrode technology can be adopted to investigate and understand network dynamics and properties of one region. Tetrode technology is one of the novel techniques developed in neurophysiology, with which one can record and analyse neuronal activity of more than two neurons at a time.

In conclusion, the results of this study demonstrate for the first time that exogenous NPY suppresses seizures concurrently increasing the mean firing frequency of the NRT cells interictally in a model of genetic absence seizures. A significant decrease was found in waveform correlation between NRT and
cortex suggesting the effect of NPY at network level. This decrease was observed waveform correlation was observed during seizures. It is possible that this may be attributed to the increased firing rate of NRT neurons observed through disruption of hyper-synchronous rhythm seen in absence seizures. Further investigations using focal injections are required to delineate the site and mechanism action of NPY. Endogenous NPY release in the NRT may play a critical role in decreasing the number of seizures and length in GAERS. Moreover, NPY related mechanisms could be used to develop novel anti-seizure therapies for absence seizures and other genetic generalized epilepsy syndromes.
CHAPTER 4

EFFECTS OF NPY FOCAL INFUSIONS ON SINGLE NEURONAL FIRING PATTERNS IN RETICULAR THALAMIC NUCLUES CELLS IN A GENETIC RAT MODEL OF ABSENCE EPILEPSY (GAERS).

4.1 Introduction

Rhythmic neuronal activity in the thalamocortical circuit vary from moderately synchronous oscillations during sleep to hyper-synchronous oscillations that characterize SWDs associated with absence epilepsy (Huntsman et al., 1999). The NRT is considered to be a critical primarily inhibitory component of the thalamocortical circuit important in generating the normal thalamocortical rhythmicity of sleep wave and plays a critical role in the maintenance and progression of abnormal rhythms associated with absence seizures (Danober et al., 1998, Pinault and O’Brien, 2005). The NRT consists of a large number of GABAergic cells (Houser et al., 1980, Pinault, 2004). The pathological rhythmic oscillatory activity during absence seizures is synchronized by these NRT neurons, critically mediated by low threshold calcium spikes generated in a rhythmic fashion (Steriade and Deschenes, 1984a).

Recent studies showed that NPY suppressed absence seizures in GAERS model of absence epilepsy (Stroud et al., 2005, Morris et al., 2007), mainly mediated through the NPY Y2 receptor subtype (Morris et al., 2007). Another study from our lab investigated the site within the thalamocortical circuit that effectively suppress seizures on EEG in GAERS upon NPY infusions. The seizure suppression effect on seizures in freely moving GAERS were assessed by focal microinjections of NPY into main regions of thalamocortical circuit:
the primary somatosensory cortex (S1), the secondary somatosensory cortex (S2), the ventrobasal thalamus (VB) and the NRT. Focal infusions of NPY into primary somatosensory cortex (S1), the secondary somatosensory cortex (S2) resulted in a significant decrease of time spent in seizure over the 90-min EEG recording in a dose dependent manner and a significant but small decrease in average seizure duration with NPY (0.5nmol) administration into the NRT (van Raay et al., 2012). The results of the previous chapter showing ICV administration of NPY significantly suppresses seizures by a decrease in total length of seizure, and percentage of time spent in seizures, complementing these studies. Results of neuronal firing patterns showed increased firing frequency of NRT neurons in the interictal period at the cellular level and also decreasing the waveform correlation between cortex and NRT at the network level confirming the critical role of the NRT in absence seizures. These results were obtained using ICV administration of NPY. With the limitations in the technique and complexity of the thalamocortical circuit, region specific effect and mechanistic hypotheses for seizure suppression effect remain tentative. A suggestive mechanism (increased firing rate) for seizure suppression had been identified in the previous chapter, it was important to try to assess the direct effect of NPY on single neurons to attempt to differentiate direct effects on NRT neurons from possible indirect effects, for example cortical inputs. It was hypothesized that focally administered NPY would have a direct effect on NRT neuronal firing. Clarification of single neuron effects would also contribute to an explanatory framework for the potent anti-seizure activity of NPY.

To better understand the regional specific changes and to examine whether the increase in neuronal firing frequency is a specifically NRT related mechanism that supplements the mechanism of seizure suppression by NPY, the effects of focal administered NPY on neuronal firing patterns of NRT
were studied. This is the first study where NPY is administered micro-iontophoretically onto the NRT invivo to assess the changes in the neuronal firing patterns upon direct administration of NPY.

4.2 Methods

The data presented in this chapter derives from single cell juxtacellular electrophysiology recordings before and after the focal administration of NPY. Focal administration of NPY was achieved with micro iontophoresis and is complimented by the usage of multi-barrel electrodes. All the experiments were conducted on male GAERS aged >12weeks.

4.2.1: Anaesthesia and surgery

For detailed information on animals, anaesthesia and preparative surgeries like penile vein catheterization and tracheotomy please refer to sections 2.2, 2.3.1, 2.3.2, 2.3.3. Briefly, rats were anaesthetized with ketamine/xylazine (3:1 ratio) at 0.1ml /100gm of rat. Penile vein catheterization and tracheostomy were performed, and the rat was then placed in a stereotaxic frame and connected to a mechanical ventilator for artificial ventilation. Neurolept anaesthesia was initiated before the end of general anaesthesia (Figure 3.1).

4.2.2 EEG electrode implantation

EEG electrodes were implanted to monitor EEG of the rat continuously though out the experiment. For detailed description of EEG electrode implantation please refer to section 2.3.4. Briefly, three active electrodes were implanted on rat skull to record EEG (Figure 2.1). Continuous monitoring of EEG will offer information about the physiological state of brain during the experiment.
4.2.3 Electrophysiology

The basic surgery and recordings protocols for electrophysiology are described in detail in section 2.4. For this particular study few changes have been made to the basic protocol which is described in section 2.8. Briefly, a double barrel borosilicate glass electrode was pulled to a diameter of ≈2 micron (15-30 MΩ). One barrel was back filled with 1.5% neurobiotin and other barrel is filled with saline or NPY for control and NPY investigations respectively. The electrode was then slowly lowered to NRT to locate a stable firing neuron (Figure 4.1). Once a stable neuronal recording was achieved, 15 mins of baseline recordings were performed followed by focal injection of saline/NPY for control and NPY studies. Post NPY/control recordings were performed for 15 mins (Figure 4.2). NPY (0.5mM) and saline were injected focally using a microiontophoresis technique (Union-40 iontophoresis pump, kation scientific) (Hicks et al., 1993, Ouyang and Wang, 2000, van Raay et al., 2012) (Figure 4.1). 200nA of cationic current was used to microiontophoresse NPY and a 40nA of anionic current was applied as a holding current for NPY. These iontophoretic currents had a minimal effect on the quality of recordings. The electrical interference between micro-iontophoretic unit and the recording unit caused a decrease in the amplitude of the spike by 20% and an increase in baseline noise levels in baseline but this did not alter the stability of the recordings. Moreover we never lost a neuron or recording because of this interference. All recordings were performed simultaneously with EEG and recorded cells were again labelled iontophoretically for later histological identification (Figure 4.1).
Figure 4.1 The above Figure illustrates the focal administration protocol: multiple barrel electrodes were used in this procedure, here one barrel is filled with neurobiotin to record neuronal activity and another barrel is filled with NPY/Saline and is infused iontophoretically on the recording neuron. Alterations in the neuronal activity were assessed after the micro-iontophoretic administration of NPY.

Figure 4.2 Timeline for the electrophysiological recordings performed under neurolept anaesthesia for focal infusion experiments. In this procedure multi-barrel electrodes are lowered into brain region and neuronal activity is recorded. In stable neuronal conditions, 15 minutes of baseline recordings were performed, NPY is infused locally via micro-iontophoresis and 15 minutes of post NPY recordings were performed.
4.2.4 Juxtacellular labelling and histology

The labelling histology protocols have been described in sections 2.6 and 2.7. Briefly, at the end of the experiment, recorded neurons were labelled by passing multiple depolarising currents through the neurobiotin-filled recording electrode. The rat was then perfused via the left ventricle and the brain was retained for histology. Histology was performed on the retained brain sections to identify the cell location microscopically referring to stereotaxic atlas (Paxinos G, 1998) (Figure 2.4).

4.2.5 Data acquisition and analysis:

Detailed information on data acquisition and analysis has been described in section 2.10. In this chapter we have analysed the neuronal firing patterns of NRT neurons interictally using the parameters described in the section 2.10. Briefly, five parameters were used to quantify interictal firing patterns (1) Mean AP firing frequency (MAPF), the average action potential firing frequency, (2) Percentage of AP’s in burst (PAPB), the proportional of action potential firing in bursts in a recording. Bursts were identified as a group of action potentials with interspike interval less than 6ms (3) Mean number of AP’s per burst (MAPB). (4) Maximum number of APs per burst (MxAPB). (5) Intraburst frequency (IBF) is defined as the frequency at which action potentials within the burst.

Relevant comparative t-test was used to assess significant differences in neuronal firing patterns in response to individual treatments (saline/NPY). To assess significance for this kind of data, a paired and non-parametric T-test (Wilcoxon matched pair test) is performed to assess significance between two individual treatments.
4.3 Results:

In this chapter, the changes in neuronal firing patterns of the NRT neurons were assessed before and after focal administration of NPY. Similar to our early findings with ICV NPY infusions, interictal mean firing frequency was significantly increased after the focal administration of NPY (n=8, p=0.003) (Figure 4.3). A significant decrease was also found in intra burst frequency after focal NPY administration (n=8, p=0.01) as well as with saline control experiments when compared with baseline intraburst frequency (n=9, P=0.003) but this change was not significant when compared between saline and NPY infusions. To assess the change in firing patterns between saline and NPY infusion, a percentage difference of neuronal firing patterns between treatment (NPY or saline) and baseline was calculated and compared between saline and NPY focal infusions. A significant increase in the percentage change of mean firing frequency was observed after NPY infusions (n=9, p=0.0002) (Figure 4.3). No change was found in all other parameters. Control saline experiments did not alter any firing patterns when compared with NPY.
Figure 4.3 This picture illustrates the results of focal NPY injections on the single neuronal firing patterns. NPY was injected focally on the recorded neurons microiontophoretically and is found to significantly increase the firing frequency of NRT cells interictally (n=9, P=0.003). The percentage difference between treatment and baseline was calculated and compared to assess the change in firing patterns between saline and NPY focal infusions. A significant increase in the percentage change of mean firing frequency was found in NPY infusions (n=9, p=0.0002).

Firing rate before focal administration of NPY

Firing rate after focal administration of NPY
**Figure 4.4** This is an example recording from focal infusion studies showing the firing frequency of NRT neurons before and after focal administration of NPY, a strong increase in the neuronal firing frequency after focal NPY administration.

**Fig 4.5**

<table>
<thead>
<tr>
<th>NRT interictal neuronal firing patterns</th>
<th>Focal NPY induced Changes</th>
</tr>
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<tbody>
<tr>
<td>MAPF</td>
<td>Increased significantly</td>
</tr>
<tr>
<td>% Burst</td>
<td>No significant difference</td>
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<tr>
<td>MAPB</td>
<td>No significant difference</td>
</tr>
<tr>
<td>MxAPB</td>
<td>No significant difference</td>
</tr>
<tr>
<td>IBF</td>
<td>No significant difference</td>
</tr>
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</table>

Figure 4.5 The above table shows the results of all the interictal parameters of neuronal firing patterns observed in this chapter.

**4.4 Discussion:**

The thalamocortical network involving somatosensory cortex, VB thalamus (VB) and NRT are responsible for generation and progression of seizures absence seizures (Danover et al., 1998). The effects of ICV administration of NPY on cortical EEG, neuronal firing patterns of thalamocortical circuit neurons, rhythmicity in the neuronal firing patterns of NRT neurons, synchronicity of the firing in NRT neurons and waveform correlation between cortex and NRT were described in the previous chapter. In the present chapter effects of focally administered NPY on neuronal firing patterns of NRT neurons have been investigated. Neuronal firing patterns between treatments
(saline/NPY) and baseline were compared. A significant increase in the interictal neuronal firing frequency was found in NRT neurons after the focal administration of NPY. We also found a significant decrease in intraburst frequency of the interictal NRT neuronal firing after NRT firing. A similar effect was found with saline infusions which were later found insignificant when compared between saline and NPY. Control experiments with local saline administration had no effect on neuronal firing patterns of NRT neurons. All recordings were performed in the NRT region: the location and morphology of these neurons were not compared to analyze the relative change in effect for each neuron. The relative change in firing frequency after NPY infusions could depend on the independent properties of the cells that were recorded. In the present chapter we replicated the findings of the previous chapter of an increase in the neuronal firing frequency of NRT after ICV NPY administration. The mechanism of NPY effect via ICV application in the previous chapter is further clarified by local application of NPY using multiple barrel pipettes. Even though these two studies cannot be compared in terms of doses and the method of drug infusions these observations strongly suggest a direct effect of NPY on NRT neurons rather than an indirect effect on other thalamocortical structures as could occur following ICV NPY injection.

Possible mechanisms for increased NRT firing frequency following focal administration of NPY could be because of increased inhibition of GABA receptors with in the NRT. The increased firing rate of NRT cells may be disrupting the hyper-synchronous rhythm that characterizes absence seizures. Another argument is that NPY may be acting on specifically on interneurons (Bacci et al., 2002, Kuruba et al., 2011). NPY receptor subtypes Y1, Y2, and Y5 have all been demonstrated to be present in thalamocortical circuit (Dumont et al., 1993, Sun et al., 2001a) and mediate NPY responses. The
anti-epileptic effect of NPY on the pathological oscillatory activity underlying absence epilepsy is mainly mediated via the Y2 receptor (Morris et al., 2007). In contrast a number of studies have reported the role of different NPY receptor subtypes in suppression of other form of epilepsy (Reibel et al., 2001). NPY specific receptor subtypes Y1 and Y5 primarily located in postsynaptic position on somata and dendrites of neurons may act directly to inhibit their activity by activation of G-protein-activated inwardly rectifying potassium (GIRK) channels. On the other hand NPY Y2 receptors are located at presynaptic positions on nerve terminals of neurons and act to inhibit GABA and glutamate release via inhibition of voltage-gated calcium channels (Sun et al., 2001a, El Bahh et al., 2005). Possibly, NPY might be acting postsynaptically via Y1 or Y5 receptor enhancing GABAergic inhibition within the NRT, or pre-synaptically via Y2 receptor on the NRT neurons projection on to VB (Stroud et al., 2005, Morris et al., 2007). Further experiments with NPY receptor subtype Y1, Y2 and Y5 agonists and antagonists in vivo will be extremely useful in clarifying these mechanisms.

Although the effects of NPY on single neuronal firing properties could plausibly account for seizure suppression, it is of great interest to see if we could identify evidence of altered interaction between different populations of neurons in this complex network. Because of the small sample size (n= 9) autocorrelations and cross correlation were not performed as in the previous chapter. For this kind of analysis a larger sample size is required to achieve significant results. Multi electrode or tetrode technology is the best alternative to record and analyse from higher population of neurons to understand the effect of focally administered NPY at network level.

The results from this suggest NRT as the critical structure in seizure initiation and progression and NPY acts on this structure for effective seizure suppression. Further investigation using agonists at specific receptor subtypes
Y2 and Y5 on NRT could reveal more information on the molecular reasons explaining the antiepileptic effects of NPY. The NRT could possibly become a target region in the brain for anti epileptic drug delivery for effective seizure suppression.

The advantage of the technique used in this chapter is that we can investigate the effect of focally administered drugs on the single neuron firing patterns in vivo. Conversely, there are some limitations too 1) The present study is limited to a single treatment at a time, we could only deliver either control or NPY at one time given that only one microiontophoresis unit was available. 2) Neuro-active compounds with high ejection current (<200nAmp) cannot be used with this type of micro-iontophoretic pump. 3) Sample size is another limitation of this study, with this current technique we can record and analyse changes in neuronal firing patterns of a single neuron. 4) Quality of the recordings is at considerable risk with electrical interphase between recording and iontophoresis unit which decreases the amplitude of the spike train and increases the noise levels in baseline. This problem is also accountable for single treatment at a time in this study. This problem can be overcome by finding relatively more stable firing neurons with higher amplitude (eg: 2mV) and proper grounding of the equipment.

Overall, data from this chapter confirmed that interictal neuronal firing is increased with focal administration of NPY in NRT neurons suggesting a direct effect of NPY on NRT neurons.
CHAPTER 5

EFFECT OF ICV ADMINISTERED NPY ON SEIZURE INDUCING THRESHOLD UPON EXTERNAL ELECTRICAL STIMULATION IN S2 REGION IN GAERS.

5.1 Introduction

The thalamocortical circuit involving NRT, VB thalamus and cerebral cortex is critical for generation and progression of absence seizures (Danobe et al., 1998) (Figure 1.4 and 1.6). The neuronal mechanism underlying the generation and progression of SWDs associated with absence seizures has been the basis for many in vitro and in vivo studies (McCormick and Contreras, 2001, Pinault and O'Brien, 2005). This gave rise to several hypotheses that are widely debated, with opposing views about whether the cortex, the thalamus or both play a pivotal role in generation of absence seizures (Pinault and O'Brien, 2005).

Genetic rat model of absence epilepsy from Strasbourg (GAERS) (Vergnes et al., 1987) and Wistar Albino Glaxo/Rijswijk rats (WAG/Rij) (Coenen et al., 1992b) are two genetic rat models of absence epilepsy that exhibit spontaneous and generalized absence-like seizures characterized by SWDs on the EEG (Figure 5a, b). Recent studies using these genetic models demonstrated that absence related SWDs initiate from a specific region “focus”, residing in the somatosensory cortex. In freely moving WAG/Rij rats using multi-site field recordings, Meeren et al in 2002 demonstrated that seizures were initiated in the S1 of somatosensory region and then generalized to other regions of cortex and thalamus. (Meeren et al., 2002). Studies by Pinault have revealed that layer VI neurons of S1 region play a critical role in proliferation of SWD (Pinault, 2003, Pinault et al., 2006). Recently an in vitro
study in brain slices from thalamocortical region in GAERS proved that the SWDs were initiated in the deeper cortical layers before scattering further to other cortical and thalamus regions (Adams et al., 2011).

However, recent studies from our group have also demonstrated the critical role of secondary somatosensory cortex (S2) in seizure initiation in GAERS (van Raay et al., 2012, Zheng et al., 2012). From the results of previous chapter and other studies from our lab we know that ICV administered NPY suppresses spontaneous seizures in GAERS (Stroud et al., 2005, Morris et al., 2007, van Raay et al., 2012). In addition to these results, it is also demonstrated that absence related SWDs could be triggered by external stimulation in somatosensory cortex in GAERS (Zheng et al., 2012). It is also shown in WAG/Rij rats that SWDs like after discharges were observed in somatosensory cortex (Luttjohann et al., 2011). In this chapter, we assessed the seizure induction threshold (amount of stimulation required to induce absence seizures) to trigger a seizure in GAERS in S2 region and the changes in seizure induction threshold in response to ICV administered NPY. By this approach we aim to investigate the effect of NPY on seizure initiation and propagation in the thalamocortical circuit. In previous studies it has been shown that NPY suppresses spontaneous seizures in GAERS; here, we hypothesise that ICV NPY will affect externally induced seizures by altering the seizure induction threshold

5.2 Methods

The data presented in this chapter is derived from cortical stimulations performed in the S2 region. External cortical stimulations were performed to induce seizure like activity and the inducting electrical threshold was calculated and compared before and after ICV administration of NPY. All the experiments were conducted on male GAERS aged >12 weeks.
5.2.1: Anaesthesia and surgery

For detailed information on animals, anaesthesia and preparative surgeries please refer to section 2.2, 2.3.1, 2.3.2, 2.3.3. Briefly, rats were anaesthetized with ketamine/xylazine (3:1 ratio) at 0.1ml /100gm of rat. Penile vein catheterization and tracheostomy were performed. The rat was then placed in a stereotaxic frame and connected to a mechanical ventilator for artificial ventilation. Neurolept anaesthesia was started before the general anaesthesia was completed.

5.2.2 EEG electrode and ICV cannula implantation

EEG electrodes were implanted to monitor EEG of the rat continuously throughout the experiment. For detailed description of EEG electrode implantation please refer to section 2.3.4 (Figure 2.1). Briefly, three active electrodes were implanted on rat skull to record EEG. Continuous monitoring of EEG provided information about the physiological state of brain during the experiment. An ICV cannula was also implanted for central administration of NPY, detailed description of the procedure is provided in section 2.3.4. Briefly, an ICV cannula was implanted on the left hemisphere (A/P: 1mm, M/L: -1.5 mm and -3.0 to -3.5mm deep relative to dura). The position of cannula was assured by withdrawal of cerebrospinal fluid through the ICV injecting line.

5.2.3 Cortical stimulations and seizure induction

Absence-like seizures were induced by external electrical stimulation in the cortical regions such as in motor cortex, insular cortex S1 and S2 region. Seizure inducing threshold was recorded in S2 region before and after ICV administration of NPY, detailed information about the procedure is provided in methods (section 2.5). Briefly, for this experiment a single craniotomy and
duratomy was performed (A/P 0.2mm, M/L 4.1). Glass electrodes with a resistance of 2-5 mΩ were backfilled with 1.5% neurobiotin and were used for stimulation and recording from S2 region of somatosensory cortex (S2). In this method, a 2-second monopolar stimulus train (1 millisec square pulse, 7 Hz) of 7-Hz electrical stimulation was given to S2 region in GAERS to induce SWDs in GAERS rats (n=8) as described and published in Zheng et al (Zheng et al., 2012). A baseline recording was performed for 15 mins, followed by ICV saline infusions. After few minutes of saline infusion, current of 1-10milliAmps were applied by increasing 1milliamp each step until the seizure was induced and seizure induction threshold was recorded. Thirty minutes after seizure induction NPY was administered ICV and seizure-inducing threshold was assessed at 15min, 1hour and 2 hour time intervals (Figure 5.1). At the end of the experiment the whole region was labelled iontophoretically for further histological identification.

Figure 5.1 This Figure show the three main structures of thalamocortical circuit involved in the generation of absence seizures. In this procedure, blunt glass electrodes were lowered into S2 region of the cortex and external electrical stimulation were applied to induce an absence like
seizure. On right is an exemplary picture of cortical stimulation that induces seizures similar to spontaneous absence seizures in GAERS.

5.2.4 Juxtacellular labelling and histology

The labelling histology protocols have been described in sections 2.6 and 2.7. Briefly, at the end of the experiment, recorded neurons were labelled iontophoretically, rats were perfused and brain was retained for histology (Figure 2.4). Histology was performed on the retained brain sections to identify the cell location microscopically referring to stereotaxic atlas (Paxinos G, 1998).

5.2.5 Data acquisition and analysis:

Detailed information on data acquisition has been described in section 2.10. Briefly, seizure induction threshold in S2 region is assessed before and after the ICV administration of NPY. Non-parametric and repeated measure Anova was performed to analyse the statistical difference between the treatments. Relevant comparative t-test was also performed to assess significance between two individual treatment time lines.

5.3 Results:

Taking advantage of method to induce seizures by external stimulation in cortical regions from recent studies (Zheng et al., 2012). We have successfully triggered absence like seizures by external electrical stimulation in motor cortex, insular cortex, S1 and S2 region, S2 being more hyper-excitabile and epileptogenic, requiring less intensity of electrical stimulation to induce seizure. The threshold for induction of absence like seizures in S2 was measured. The seizure induction threshold in the somatosensory cortex was assessed before and after the ICV administration of NPY. Seizure induction threshold for seizure induction before NPY administration was 1.20 ± 0.51 mAmp (n= 8) which was significantly increased to 3.05 ± 0.68 mAmps 15
minutes after ICV NPY infusion (n=8, p=0.02). The seizure triggering threshold showed an increasing trend after NPY infusion (2.00 ± 0.44 1 hour after ICV NPY administration and 1.70 ± 0.50 2 hours after ICV NPY administration, n=7) but this did not reach significance (Figure 5.2). No difference was observed with control saline/saline treatment.

![Figure 5.2](image)

**Figure 5.2** the above graph illustrates the results of ICV administration of NPY on the seizure inducing threshold in the S2 region. ICV administration of NPY increased the seizure induction threshold significantly 15 minutes after NPY infusion; an increasing trend was observed in seizure induction threshold after 1 hour and 2 hours after NPY infusion but it did not reach significance.
Control experiments with saline did not show any significant changes to the seizure-inducing threshold.

5.4 Discussion:
A recent study from our lab demonstrated that SWDs could be induced by 7 Hz electrical stimuli in the cerebral cortex (Zheng et al., 2012). These stimulations could reliably induce absence-like SWDs in S1, S2, or IC (insular cortex) of somatosensory cortex in GAERS. However, the required amplitude of electrical stimulation to induce seizure varied for each experiment and also for the region of cortex where the seizure was triggered. The seizure inducing threshold was significantly lower in S2 region (0.5 to 2 mAmps) than other cortical regions in GAERS, suggesting that the S2 cortical region may have more pivotal role in generation of seizures than the S1 region (Zheng et al., 2012). External stimulations in S2 regions were performed irrespective of the layers and the regions of stimulation were labelled iontophoretically at the end of the recordings. The location of the recording was later confirmed by histology. These evoked SWDs were similar in morphology and frequency to typically occurring absence related spontaneous SWDs. Zheng et al, has already described that these evoked seizures were accompanied by similar behavioural characteristics such as immobility and head nodding (Zheng et al., 2012). However, the evoked seizures can be differentiated from spontaneous seizures as they longer than normal, sometimes lasting for more than 30 seconds and also the evoked seizure starts as soon as the stimulation ends as shown in figure 5.1. The seizure must be induced at least 2 times with the same stimulation parameters to confirm that the seizure is induced due to external stimulation.

It is significant that SWDs can only be induced when rats were quite awake manifesting a desynchronised EEG. This state could be achieved by anesthetizing the rat with neurolept anaesthesia, which keeps the rats in
pseudo-active state allowing this method to be implicated on anaesthetized animals. In contrast, identical stimulations in the NEC rats could not induce SWDs (Zheng et al., 2012).

Previous studies with ICV drug infusions decreased spontaneous absence seizures after 90 min of drug infusion in a dose dependent manner (Stroud et al., 2005, Morris et al., 2007) and also showed a similar prolonged effect on spontaneous seizures by focal NPY administration (van Raay et al., 2012). However, these prolonged effects were observed in spontaneous seizures unlike in this study where we evoked seizures and used a standard dose of 1.5nmol. Given the fact that we could reliably induce absence like seizures with external electrical stimulation and also that NPY suppresses naturally occurring spontaneous seizures in GAERS, we investigated the seizure suppression effect of NPY on induced seizures by evoking absence-like seizures. We stimulated the S2 region of cortex to initiate seizures that manifests similar hyper-resonant oscillatory thalamocortical activity allowing us to investigate the alterations in this induced resonant activity in response to NPY. In this chapter, we revealed that seizure induction threshold to trigger SWDs was significantly increased after ICV NPY administration in GAERS.

As discussed in previous chapters, because of complexity of the network and limitation of methodology a strong mechanistic hypothesis cannot be formulated at this stage. In the previous chapter we demonstrated that the seizure suppression effect of NPY is concomitant with increase in firing frequency in NRT neurons interictally and a decrease in waveform correlation between local field potentials of cortex and NRT.

From the above results, possible mechanism for escalation in the seizure induction threshold might be enhanced GABAergic inhibition within NRT instigating increased firing rate of NRT cells that might be disrupting the
hyper-synchronous rhythm and resonant oscillations in epileptic thalamocortical circuit that characterizes absence seizures. This disruption in NRT might restrict the generalization and spread of SWDs in the whole brain, as the NRT is suggested to promote the propagation and synchronization of SWDs (Kim et al., 1995, Cox et al., 1996). Another factor that facilitates the thalamocortical circuit to express self-synchronising hyper excitable SWDs is the ability of NRT and relay neurons to fire in phasic Ca\(^{2+}\) dependent burst of action potentials mediated by T-type calcium channels. NPY would suppress release of the inhibitory neurotransmitter GABA from NRT neurons projecting to the thalamocortical neurons. This would lead to reduced GABA facilitated hyperpolarization of the thalamocortical neurons, thus retaining the Ca\(^{2+}\) T-channels in a de-inactivated state and preventing oscillatory hyper-synchronous thalamocortical activity. Furthermore, previous studies have confirmed that NPY receptors are located on the axon terminals of NRT neurons projection to VB (Sun et al., 2001a), where they inhibit neurotransmitter release by modulating the activity of voltage gated Ca\(^{2+}\) channels (Sun et al., 2001b). Another alternative possibility is that NPY may be acting on specifically on interneurons present in cortex and NRT (Bacci et al., 2002, Kuruba et al., 2011). Another study from our lab performed focal injections of NPY in motor cortex, S1 and S2 cortical regions and found greater seizure suppression effect in S2 suggesting this region is more critical for seizure generation (van Raay et al., 2012). These results suggest that here might be other cortical related mechanisms involved in the increase of seizure induction threshold, which can be only clarified with focal NPY administration experiments. Further \textit{in vivo} ICV/focal NPY injection experiments using specific receptor subtypes NPY Y1, Y2 and Y5 and more specifically on interneurons involved in the thalamocortical circuit and similar stimulation experiments with ICV/focal NPY administration in other regions.
of the circuit might reveal more relevant information on underlying mechanisms of NPY seizure suppression effect.

The results from this study suggest that NPY can not only suppress spontaneous seizures, but also is effects against induced seizures, which implies that NPY strongly suppresses seizures. An increase in seizure induction threshold may be related to the increase in interictal NRT firing that we found in previous chapters but another important consideration is that a probable focus has been identified in the somatosensory cortex. Therefore it is possible that ICV administered NPY also has effects more generally in the thalamocortical circuit and perhaps has an interneuron specific effect. This suppression could most possibly be mediated by affecting the initiation of seizure or could be inhibiting these seizures to go into hyper synchronous pathological oscillatory mode.
The advantages of this study are that: it allows us to investigate the effects of NPY on seizure induction in live animal models, it is already known that electrical activity similar to SWDs has been observed in slice preparations but an intact thalamocortical circuit is essential for expression of the seizure (Gloor P, 1990, Vergnes and Marescaux, 1992) This method can be improved with tetrode/multi-electrode technology to understand the dynamics of the neuronal networks during pathological epileptic oscillations and their response to antiepileptic drugs in different epileptic models. The only drawback of this experiment is complexity in the methodology and restricted to animals that are not freely moving.
CHAPTER 6

General discussion and conclusion:

6.1 Key findings of this study:

Absence epilepsy is the most common type of generalized epilepsy, characterized by symmetrical and bilaterally synchronous 3 Hz spike and wave discharges (SWDs) in the electroencephalogram (EEG). The thalamocortical circuit involving somatosensory cortex, VB thalamus and NRT plays a critical role in generation of signature SWDs that characterizes absence seizures. One third of the patients with absence seizures do not have adequate seizure control with presently existing anti-epileptic drugs. This increased the desire for better therapeutic agents to treat patients with absence epilepsy. NPY related mechanisms may represent a novel therapeutic approach for treating generalized epilepsies.

NPY is a naturally occurring neurotransmitter, found abundantly in many brain regions and modulates many physiological responses such as sleep, appetite, memory and stress etc. NPY more importantly is also shown to mediate seizure suppression in different models of epilepsy. The underlying neuronal mechanisms for the seizure suppression by NPY are still unknown.

The aim of the research conducted in this thesis was to investigate the effects of NPY on single neuronal firing patterns of the thalamocortical circuitry neurons to understand the underlying cellular mechanism of seizure suppression in genetic model of absence seizure (GAERS) and the effect of NPY on network dynamics. In this study we have investigated the effects of NPY on EEG, single neuronal firing patterns and other properties that signify the hyper-synchronous oscillation in the circuit.

For experiments in this research, genetic absence epilepsy rats from
Strasbourg (GAERS) were used. This is a widely accepted rat model for absence epilepsy with similar pathophysiological and pharmacological characteristics as human absence seizures. Juxtacellular electrophysiology recordings were performed in rats in vivo under neurolept analgesia to examine alterations in neuronal firing in the thalamocortical regions during interictal and ictal periods that could be associated with seizure suppression and alterations in the seizure threshold.

Electrophysiology recording under neurolept anaesthesia is well-established technique and has been previously used in many studies to understand the neural firing patterns of several types of neurons. In current thesis, typical absence related SWDs are more related to a state of awakeness. In GAERS rats, the characteristic SWDs and 5-9 Hz oscillations arise from a relatively desynchronized EEG activity (Pinault et al., 2006). Neurolept anaesthesia maintains the rats in the similar state of immobile wakefulness or drowsiness (Pinault et al., 2001, Pinault et al., 2006). Such experimental conditions are suitable for recording SWDS at similar frequency to those of freely moving rats. It has also been shown that cellular and network mechanisms underlying 5-9 Hz oscillations during neurolept anaesthesia are similar to those in non anesthetised intact brain (Pinault et al., 2006).

The key findings from the current project were:

6.1.1. Effect of NPY on seizures:

These findings of this chapter indicate that we have successfully replicated our previous studies findings demonstrating seizure suppression after NPY infusion both ICV and focally. In the current study, NPY had a strong seizure suppressant effect. A significant reduction was found in the
total length of seizures and percentage of time spent in seizures after ICV NPY administration.

6.1.2. Effect of NPY on interictal Neuronal firing patterns

This is a novel and key finding of the current study. ICV administration of NPY led to a significant increase in the neuronal action potential firing frequency in the NRT region concurrently supressing seizures. This may account for the possible mechanism of seizure suppression and also suggest that NRT could be the critical structure in thalamocortical circuit involved in the absence seizures.

6.1.3. Effect of NPY on the waveform correlation between cortical EEG and local field potentials of NRT.

This is another novel finding from this study. ICV administered NPY decreased the waveform correlation between cortical EEG and local field potential recorded from the NRT region. These results compliment the precious results suggesting that the decrease in correlation could be because of changes in neuronal firing patterns demonstrated in NRT. These data also suggest that, NRT could be critical structure involved in seizures and that NPY could be acting more at a network level.

6.1.4. Effects of Focal administration of NPY on neuronal firing pattern in NRT region:

This is again a new key finding. Significant changes in the neuronal firing patterns in NRT were observed after ICV NPY infusions. We found similar results after focal administration of NPY, whereby interictal mean firing frequency was significantly increased after NRT administration of NPY. The difference in percentage of neuronal firing frequency between NPY and baseline group was assessed. A significant increase in percentage change of
mean firing frequency was found after NPY infusions. These results again suggest that the NRT could be the target region in the thalamocortical circuit, where NPY acts to suppress seizures.

6.1.5. Effects of NPY on Seizure induction threshold:

Previous studies showed that NPY suppresses spontaneous absence seizures in GAERS. This study showed that ICV administered NPY suppresses seizures evoked by external stimulation. Most importantly we investigated the changes in the threshold to induce a seizure following a cortical stimulation. The seizure inducing threshold doubled after ICV administration of NPY. The maximal effect was seen during the initial recording period, i.e. 15 minutes after NPY infusion as compared to the observations made during later time points. NPY injected ICV may have effects throughout the thalamocortical circuit and perhaps has an interneuron specific effect.

6.2 Integration and interpretation of results:

A large number of studies have described the effects of NPY on seizure suppression Previous studies on focal seizures suggest that NPY is a potent anti-convulsant, when considering the effect of endogenous NPY deficiency (Erickson et al., 1996, DePrato Primeaux et al., 2000, Richichi et al., 2004, Woldbye et al., 2005) and NPY administration on seizures (Colmers and Bleakman, 1994, Greber et al., 1994a, Woldbye et al., 1996, Klapstein and Colmers, 1997, Woldbye et al., 1997, Woldbye, 1998, Vezzani et al., 2000, Reibel et al., 2001, Silva et al., 2001, El Bahh et al., 2002, Husum et al., 2002, Reibel et al., 2003, Woldbye and Kokaia, 2004). Studies from our lab suggested the anti-epileptic role of NPY in generalized seizures by
suppressing absence seizures in a genetic rat model of absence epilepsy (Stroud et al., 2005, Morris et al., 2007, van Raay et al., 2012).

Despite a large number of studies suggesting a role of NPY on seizure suppression the mechanism underlying the seizure suppression is still highly debated. In the present research, we were able to show similar seizure suppression effects of NPY administration in GAERS, when they were anesthetized under a neurolept cocktail. We also demonstrated that NPY mediated seizure suppression was associated with an increased interictal firing frequency of neurons in the NRT in these rats. This escalation in NRT firing frequency following ICV NPY infusion could be possibly due to an increased inhibition of GABA receptors within the NRT and contribute to seizure suppression by increased deposition of inhibitory GABA in thalamus decreasing GABA mediated hyperpolarization of the thalamocortical neurons. Given the complexity of the network and limitations of the NPY infusion method, there could be many other mechanisms associated with the increase in firing frequency and seizure suppression. To get away from these limitations, we used NPY focal injections to study the effects on neuronal firing patterns of NRT, which showed similar results to ICV administration of NPY by increasing the interictal firing frequency of NRT neurons. This confirmed the direct effect of NPY on NRT and not a network-dependent effect.

During a seizure, structures involved in thalamocortical circuit engage themselves in a hyper-synchronous oscillatory rhythmic state. In this thesis, we have also demonstrated a decrease in waveform correlation in local field potentials between the cortex and NRT during seizures after ICV NPY administration. Increase in seizure induction threshold may be related to the increase in interictal NRT firing and these changes in the neuronal firing
frequency or increased firing rate of NRT cells might be disrupting the hyper-synchronous rhythm that characterizes absence seizures. These results suggested an effect of NPY on overall thalamocortical circuit, possibly acting on interneurons present in the network but the results from focal injection of neuronal firing patterns in NRT further confirmed the direct effect of NPY on the NRT.

Zheng et al from our lab recently demonstrated that absence related SWD’s were first identified in S2 region before spreading to other regions of the brain (Zheng et al., 2012), it was also demonstrated that absence like SWDs could be induced in cortical regions by 7 Hz electrical stimuli in the cortical regions with a lower intensity in S2, suggesting its critical role in generation of seizures compared to other cortical regions IC and S1 (Zheng et al., 2012). In this thesis, we have adapted the same technique to induce seizures in S2 region, which is considered as focus for seizure generation and investigated the effects of NPY on hyper resonance of the thalamocortical circuit. In this thesis, we showed an increase in the seizure induction threshold to induce a SWD after ICV NPY administration. As discussed in previous chapters, because of complexity of the network and limitation in methodology a strong mechanistic hypothesis cannot be formulated at this stage. Basing on the results from previous chapters in this thesis, mechanism for escalation in the seizure induction threshold might be enhanced GABAergic inhibition within NRT instigating increased firing rate of NRT cells that might be disrupting the hyper-synchronous resonant oscillations that signifies absence seizures, as NRT is postulated to promote the spread and synchronization of SWDs (Kim et al., 1995, Cox et al., 1996), this disruption in NRT might restrict the generalization and spread of SWDs in the whole brain.
However, the cellular and molecular mechanism underlying NPY related suppression of seizures is still unknown and the specific receptor subtype mediating the seizure suppression effect is highly debated. Reports showed that NPY receptor subtypes Y1 (Sun et al., 2001a, Sun et al., 2001b, Sun et al., 2003), NPY Y2 (Greber et al., 1994b, McQuiston and Colmers, 1996, Schwarzer et al., 1998, Furtinger et al., 2001, Morris et al., 2007, van Raay et al., 2012) and NPY Y5 (Woldbye et al., 1997, Marsh et al., 1998, Morris et al., 2007) may be mediating seizure suppression effect by NPY in different models and types of epilepsies. Previous studies from our lab in freely moving animals indicate that NPY primarily mediates its anti-epileptic effects via Y2 receptor subtype (Morris et al., 2007, van Raay et al., 2012). Further research with NPY specific receptor subtypes especially Y2 will clarify the possible mechanisms underlying the seizure suppression effect of NPY.
Figure 6.1 Above is the schematic diagram of neuron illustrating the locations of Y1, Y2 and Y5 receptors for better understanding of the possible mechanism.

- Y1 receptors predominantly located post-synaptically on somata and dendrites of the gabaergic NPY neurons and glutamergic neurons in cortex and VB
- Y5 receptors predominantly located post synaptically
- Y5 receptors are located pre-synaptically
Fig 6.2

Possible mechanism of action for seizure suppression and concurrent increase in NRT neuronal firing

\[ \text{NRT} \]

\[ \text{Increased inhibition of GABA with in NRT} \]

\[ \text{Increased NRT firing, which causes increased inhibitory GABA in thalamus resulting in decreased hyperpolarization in TC circuit} \]

\[ \text{Increased NRT firing may disrupt the hyper-synchronous rhythm and resonant oscillations in thalamocortical circuit thus restricting the generalization} \]

Another possibility is that NPY could be primarily acting on interneurons present in the circuit but focal injection studies suggest that NRT could be the key structure involved in seizure suppression effect of NPY

**Fig 6.2** The above figure illustrates the possible mechanism for seizure suppression, increase in the neuronal firing patterns in response to NPY administration ICV and focally and also possible mechanism for increase in seizure induction threshold and decrease in waveform correlation
6.3 Limitations of this study and experimental considerations:

Longitudinal studies in humans have shown an association of adverse prenatal and postnatal experiences on the development of neurological disorders later in life (Lupien et al., 2009). The mechanisms related to these effects are difficult to study in humans. Furthermore, some studies have also argued an opposite cause-and-effect interpretation of this association, i.e. that children with pre-existing brain abnormalities are at higher risk of abuse. Therefore, studies in animal models including rodents and primates have provided a rich framework for hypothesizing the changes in neurodevelopment and behavioural outcomes, and the related mechanisms.

6.3.1 Limitations in ICV NPY infusion study:

Despite having a rat model, these experiments cannot be carried out in freely moving rats because of the experimental complications and surgeries. The alterations in neuronal firing patterns induced by ICV and focally administered NPY have been observed in rats under neurolept analgesia. Neurolept anaesthesia is a mixture of drugs that can set the brain in a state that electrophysiologically corresponds to immobile wakefulness, altering between desynchronized and synchronized states that allow generation of seizures similar to spontaneous epileptic seizures (Pinault et al., 2001). However, neurolept analgesia itself is known to promote certain pathological thalamic oscillations associated with absence-like seizures (Warter et al., 1988, Inoue et al., 1994). All the recordings were conducted in rats during predominantly desynchronized EEG conditions by titrating the levels of analgesia as well as by monitoring and controlling critical physiological
parameters.

Another methodological consideration in ICV infusion studies is maintaining the stability of the paired neuronal recording for longer periods is always an issue. We have previously reviewed this concern in methods section 2.4.2 in detail to achieve paired stable neuronal recordings.

6.3.2 Limitations in focal injection study:

Yet again this study is also restricted to anaesthetized animals. Maintaining the quality and stability of the recording is always an issue with triple barrel electrodes, which is addressed in the methods section 2.4 and 4.2.3.

6.3.3 Limitations in cortical stimulations study:

The cortical stimulations study has an experimental complication where seizures can be triggered only when rats were in a state of quiet wakefulness accompanied by desynchronized EEG. This state could be achieved by anesthetizing the rat with neurolept anesthesia, which keeps the rats in pseudo-active state allowing this method to be carried out on anaesthetized animals (Zheng et al., 2012).

All the experiments in these studies were performed under neurolept anaesthesia. Therefore all comparison was made between observations in rats under neurolept analgesia.

NPY is believed to suppress absence seizures predominantly by two specific receptor subtypes Y2 and Y5 (Morris et al., 2007, van Raay et al., 2012). Further ICV and focal infusion experiments in GAERS rats are required to investigate and understand the underlying mechanisms of seizure suppression at molecular level. Recordings from chronically-implanted tetrodes in freely moving conditions is one possibility that has further advantage, as it is
possible to sample a large population of neurons from a particular region, which was not possible in the experimental set up of the current study.

6.4 Future research directions:

6.4.1 Investigating the particular receptor subtype with predominant seizure suppression effect (ICV and focal administration)

The current study provided important information on changes in the neuronal firing patterns in NRT associated with the seizure suppression and also about the possible mechanisms of seizure suppression. It is important to investigate the most possible mechanism and find a particular receptor subtype with predominant seizure suppressing effect. Investigating the effects of ICV administered Y2 and Y5 receptor agonists will provide extended knowledge on the specific receptor subtype principally suppressing seizures and its mechanism of action.

6.4.2 Investigating the effect of NPY and its subtypes at network level

This current study is limited to paired neuronal recordings. Much more knowledge could be gained if we could sample multiple neurons from a region at a time. Neuronal recordings with tetrodes will overcome the disadvantage of limited sample size as in the current study, since a large population of neurons can be recorded simultaneously and can be sorted and analysed individually. Tetrodes can be used to record from multiple units (max 4 units per tetrode) and more than one tetrode can be used in experiments. Thus using the novel tetrode system recordings can be made from 32-128 units at a time in one experiment, from different regions of the brain. By combining ICV infusion technique with Tetrode recording techniques we can analyze changes in the neuronal properties in a large sample size and from different regions at a time. In addition to these
advantages, it will also facilitate recordings in freely moving rats. This combined technique would also enable assessment of correlations between two different regions at a time using large sample sizes and help us understand the network dynamics and also the mechanism of seizure suppression at a network level. In addition to these advantages, it will also facilitate recordings in freely moving rats.

6.4.3 To investigate the effect of NPY and its subtypes on neuronal firing patterns of freely moving rats

This study provided insight on possible mechanisms related to seizure suppression and alteration in the neuronal firing patterns in thalamocortical circuit neurons during ictal and interictal periods in controlled conditions under neurolept analgesia. As discussed previously, it cannot be certain that the observed findings are not specific to the experimental conditions. Hence future experiments with a similar study design should be conducted in freely moving conditions. Neuronal recordings with tetrodes chronically implanted in freely moving rats have proved useful in understanding hippocampal cell firing during interictal (Zhou et al., 2007), ictal (Bower and Buckmaster, 2008, Cymerblit-Sabba and Schiller, 2010)(Bower and Buckmaster, 2008, Cymerblit-Sabba and Schiller, 2010, 2012) and postictal states in rodent models. Thus, similar experiments in GAERS could assist in overcoming the limitations of the current study. In addition to these advantages, recordings in freely moving rats would also allow precise monitoring of the electrophysiological alterations in response to NPY and its receptor subtypes.

6.5 Other new therapeutic options:

Until now, ethosuximide and valproate remain the principal anti-absence drugs. However, these drugs do not show adequate seizure control in some
patients and are not well tolerated in some patients as they cause unwanted side effects (Panayiotopoulos, 2001). Thus recent research is focused on developing drugs that are more effective and show minimal side effects.

Several experimental drugs are under development for treating absence seizures and have also shown efficacy in animal absence epilepsy models:

1) Brivaracetam (BRV), a novel synaptic vesicle glycoprotein 2A ligand (SV2A) demonstrated anti-absence activity in GAERS (Bialer et al., 2013).

2) Rapamycin is an mTOR (mammalian target of rapamycin) inhibitor, that predominantly exhibits modulatory activity on cerebral inflammation has displayed anti-epileptic effects in WAG/Rij rats. In addition to antiepileptic effects it also showed sustained anti-epileptogenic effect (Russo et al., 2013).

3) The GABA B receptor antagonists are also found to exhibit effective antiepileptic properties in animal models of absence seizures (Han et al., 2010).

4) Metabotropic glutamate (mGlu) receptors, which are located at the synapse of thalamocortical neurons and underlie development of absence related SWDs are presently undergoing clinical evaluation after exhibiting encouraging results in animal models of absence epilepsy (Ngomba et al., 2011).

5) Two new compounds, Z941 and Z944 that specifically block T-type calcium channels displayed significant and promising anti-absence activity in GAERS (Tringham et al., 2012).

6.6 Final conclusion:

The findings in the current study provide electrophysiological insight into the possible mechanisms underlying the seizure suppression effect of NPY in GAERS. It also strengthens the argument that the S2 region might be focus
for origin of SWDs and NRT as the key target structure of thalamocortical circuit by which NPY suppresses seizures. These findings are associative in nature and further studies are required to ascertain if alterations in neuronal firing patterns are induced by specific NPY receptor subtypes to understand more about the seizure suppressive effects at cellular and network level. The clinical relevance of this study is that it potentially uncovers the possibility of ICV and focal drug delivery systems, administering NPY or other anti-epileptic neuro-active agents via mechanical or viral infusion methods to control seizures in patients with generalized epilepsies.
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