ASSESSMENT OF THE EXPRESSION OF 5-HT RECEPTORS IN THE HIPPOCAMPUS OF PATIENTS WITH TEMPORAL LOBE EPILEPSY WITH HIPPOCAMPAL SCLEROSIS

by

HATUN ALOMAR

A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

School of Clinical and Experimental Medicine

College of Medical and Dental Sciences

University of Birmingham

September 2016

Abstract:

Temporal lobe epilepsy with hippocampal sclerosis (TLE-HS) is the most common type of pharmaco-resistant epilepsy. It is characterised by recurrent focal onset seizure that affects the medial part of the temporal lobe, in particular, the hippocampus. Patients with TLE-HS suffer from typical aura symptoms, especially epigastric aura, in addition to psychological symptoms which include dysmnestic symptoms such as memory impairment and fear. As a result of the important role of the serotonergic system in human brain physiological and pathological functions and the crucial need to identify a novel target to control seizure among this type of patient, some serotonergic excitatory (5-HT_{2A}, 5-HT_3A, 5-HT_4 and 5-HT_7) and inhibitory (5-HT_{1A}) receptors were selected to study using immunohistochemistry in the human TLE-HS hippocampus, in addition to the purinergic receptors ($P2x_7$) and the orphan G-protein (GPR61). The results indicate variable degree of upregulation of 5-HT_3A, 5-HT_{1A}, 5-HT_4, GPR61 and $P2x_7$ receptors in the sclerotic hippocampi of TLE-HS patients. Subsequent studies focused on the 5-HT_3A receptors expression; the overexpression of this excitatory receptor was established using immunohistochemistry, SDS-PAGE western blot, receptor autoradiography and radioligand binding assays that give comparable results. The upregulation of the excitatory 5-HT_3A receptor in the excitatory neurons of hippocampus may contribute to the neuronal hyperexcitability evident among TLE-HS patients. This study proposed 5-HT_3 receptors as a novel target to antagonise to control seizure in TLE-HS patients and improve their quality of life.

Dedication

This thesis is dedicated to my parents, Mr Abdulhamid Alomar and Mrs Sara Alrakan, for their love, encouragement and daily prayers that have helped me realize such an accomplishment.

Acknowledgements:

First, I offer thanks to Almighty Allah for the great blessing that enabled me to achieve such success.

I want to express my grateful acknowledgment to my supervisor, Prof Nicholas Barnes, for his supervision, generous guidance and support during my PhD studies. My deepest gratitude also goes to my co-supervisor, Dr Hardev Pall, for his help and valuable guidance.

I sincerely appreciate my colleagues in the Barnes lab, especially Dr Gillian Grafton, for their help and support.

I also want to extend my sincere thanks to Mr Ramesh Chelvarajah (Neurosurgery department), Dr Ann-Maria Gonzalez, Dr Daniel Fulton, Prof Attila Sík (Neuroscience department), Dr Santhosh Nagaraju and Dr Martyn Carey (Neuropathology department) for their expert, genuine and valuable guidance and encouragement.

In addition, I want to express my gratitude to King Saud University for my scholarship and financial support. My thanks also go to the Human Biomaterial Resource Centre (HBRC), Queen Square Brain Bank for Neurological Disorders and Edinburgh Brain Bank for their help in supplying ethically approved biological materials for my research.

I am very grateful to my parents, brothers, sisters, family and friends for their unconditional love, support and encouragement.

My deepest thanks also go to all others who supported me directly or indirectly during my PhD journey.

Contents

List of Figures

List of Tables

List of abbreviations

[^{11}C]SB207145	8-amino-7-chloro-(N-[11C]methyl-4-piperidylmethyl)-1,4-benzodioxan-5-carboxylate.
[^{11}C]WAY-100635	[^{11}C](N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide).
[^{18}F]FCWAY	[^{18}F](N-{2-[4-(2-methoxyphenyl)piperazino]}-N-(2-pyridinyl)trans-4-fluorocyclohexanecarboxamide).
[^{18}F]MPPF	[^{18}F]4-(2′-methoxyphenyl)-1-[2′-[N-(2″-pyridinyl)-p-fluorobenzamido]ethyl]-piperazine.
5-HIAA	5-Hydroxyindoleacetic acid.
5-HTP	5-hydroxytryptophan.
8-OH-DPAT	(±)-8-Hydroxy-2-(dipropylamino)tetralin hydrobromide.
ABC	Avidin-biotin complex.
AC	Adenylyl cyclase.
aCSF	Artificial cerebrospinal fluid.
AEDs	Anti-epileptic drugs.
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
APS	Ammonium persulfate.
ATL	Anterior temporal lobectomy
ATP	Adenosine triphosphate.
bid	Twice a day (bis in die)

CA	Citric acid.
cAMP	Cyclic adenosine monophosphate.
Cdc42	Cell division control protein 42.
COCs	Combined oral contraceptive.
CREB	cAMP response element-binding protein.
DAB	3, 3'-diaminobenzidine.
DPC	Diethylpyrocarbonate.
DG	Dentate gyrus.
dH_2O	Deionised water.
DNET	Dysembryoplastic neuroepithelioma.
EEG	Electroencephalogram.
EIER	Enzyme induce epitope retrieval.
ERK	Extracellular signal-regulated kinases.
ERK1/2	Extracellular signal regulated kinase 1/2.
FBS	Fetal bovine serum.
FDG	Fluorodeoxyglucose.
FFPE	Formalin-fixed paraffin-embedded.
GABA	γ-Aminobutyric acid.
GIRK	Gate inwardly rectifying the K^+ channel.
GPCRs	G-protein-coupled receptors.

GRKs	G-protein coupled receptor kinases.
HBRC	Human Biomaterial Resource Centre.
HIER	Heat induce epitope retrieval.
HS	Hippocampal sclerosis.
IL-1β	Interleukin 1β.
ILAE	International league against epilepsy.
JNKs1/2	C-Jun terminal kinases 1/2.
MAPK	Mitogen-activated protein kinases.
MDD	Major depression disorder.
MRI	Magnetic resonance imaging.
NPNC	No primary negative control.
NPNC/HC	No primary negative control counterstained with hematoxylin .
Panx1	Panexin-1.
PBS	Phosphate buffer saline.
PBTA-DAB	Polymer Biotin-Tyramide ABC - 3, 3'-diaminobenzidine.
PEI	Polyethylenimine
PET	Positron-emission tomography.
PK	Protienase K.
PKA	Protein kinase A.

PKB or AKT	Protein kinase B.
PKC	Protein kinase C.
PLA2	Phospholipase A2.
PLC	Phospholipase C.
Pls	Plasmalogens.
PMC	Post-mortem control.
PMD	Post-mortem delay.
PVDF	Polyvinyl difluoride.
RIN	RNA integrity number.
RIPA buffer	Radioimmunoprecipitation assay buffer.
rpm	Rounds per minute
RT	Room temperature
SAH	Selective amygdalohippocampectomy
SBP	Serotonin binding protein
SDS	Sodium dodecyl sulphate.
SERT	Serotonin transporter.
SIGN	Scottish intercollegiate guidelines network.
SSRIs	Selective serotonin reuptake inhibitors.
TEMED	Tetramethylethylenediamine.
TLE	Temporal lobe epilepsy.

TLE-HS	Temporal lobe epilepsy with hippocampal sclerosis.
TNFα	Tumour necrotic factor α.

CHAPTER 1

Introduction

1. Introduction

1.1. Temporal lobe epilepsy with hippocampal sclerosis

Temporal lobe epilepsy with hippocampal sclerosis (TLE-HS) is the most common form of epilepsy in humans (Engel, 1998). This disease is defined as symptomatic complex partial seizures generated from the medial part of the temporal lobe which contains the limbic structures (Engle, 1996). The hyperexcitability in this area presents in various symptoms such as epigastric aura and fear (Engle, 1996; Henkel et al., 2002; Wieser, 2004). This disease is characterised by significant histopathological changes that manifest in the form of hippocampal sclerosis (HS) (Mathern et al., 1995; Engel, 1996; Engel, 2001; Wieser, 2004; Berg, 2008; Thom, 2014; Cendes et al., 2014). In the early stages of TLE-HS, pharmacological treatment for this condition exhibits a good control of the seizure activity in this type of patient; however, surgical intervention expresses a successful control to the seizure activity in 70–90% of patients in the advanced stage of the disease when it becomes pharmaco-resistant (Engel, 2001; Wieser, 2004). On the other hand, 20–30% of surgeries failed to control seizures in this disease population (Harroud et al., 2012, Surges and Elger, 2013). These unsuccessful surgeries could be due to; progressing of underline cause of seizure, inaccurate localization or partial resection of the epileptic focus, or presence of multiple epileptic foci (Surges and Elger, 2013). Several factors have been suggested as the underlying cause of TLE-HS, which include prolonged febrile seizure, cortical malformation, cavernous angiomas, dysembryoplastic neuroepithelioma (DNET), intracranial infection and brain hypoxia (Wieser, 2004; Diehl and Duncan, 2011).

1.1.A. Clinical manifestations

Patients with TLE-HS present with pre-ictal ascending epigastric aura which is the most common type of aura affecting TLE-HS patients (Engel, 1996; Engel, 2001; Wieser, 2004; Diehl and Duncan, 2011). Additional possible symptoms are autonomic, cephalic, gustatory, psychic or perceptual auras (Engel, 2001; Diehl and Duncan, 2011). These auras present for several seconds before proceeding to complex partial seizure (Engel, 2001). When the complex partial seizure stage initiates, patients with TLE-HS begin staring, stop moving and have decreased awareness (Engel, 2001; Diehl and Duncan, 2011). This initial manifestation is followed by ictal oroalimentary automatisms that include swallowing, chewing and lip-smacking (Engel, 2001; Diehl and Duncan, 2011). Furthermore, patients could suffer from gestural automatisms such as fidgeting, undressing, fumbling, walking and running (Diehl and Duncan, 2011), in addition to unilateral upper limb automation that can present in the form of dystonic posturing of one limb contralateral to the site of the epileptic foci (Engel, 2001). The ictal phase lasts typically for 1–2 minutes (Engel, 2001). This is followed by the post-ictal phase which lasts for several minutes or hours in which TLE-HS patients express amnesia for the ictal event, disorientation, confusion, short-term memory deficit and dysphasia if the seizure affects the brain language (dominant) hemisphere (Engel, 2001; Diehl and Duncan, 2011).

In terms of electroencephalogram (EEG) examination, the inter-ictal EEG exhibits epileptiform abnormality presenting as unilateral or bilateral anterior or mid temporal spike-wave discharges (Engel, 2001; Diehl and Duncan, 2011). The ictal EEG recording of the epileptiform activity expresses alpha and theta rhythmic discharges observed in the

beginning or within 30 seconds from the onset of seizure (Risinger et al., 1989; Engel, 2001; Diehl and Duncan, 2011). In addition to EEG examination, positron emission tomography (PET), using fluorodeoxyglucose (FDG) and magnetic resonance imaging (MRI), plays an important role in diagnosing TLE-HS, which can help detect hippocampal sclerosis and any inter-ictal pathological changes in the temporal metabolism (Engel, 2001; Diehl and Duncan, 2011). Patients with TLE-HS express inter-ictal hypometabolism on FDG-PET examination (Knowlton et al., 1997; Engel, 2001). Furthermore, a reduction in *N*-acetylaspartate was also observed among this type of patients using MRI (Engel, 2001).

1.1.B. Histopathology

The hippocampal sclerosis that is associated with this disease was first observed by Bouchet and Cazauvieilh when they detect a neuronal loss in hippocampus and cerebellum in brain specimens of epileptic patients (Engel, 2001; Scharfman and Pedley, 2006 and Meencke, 2009). In 1880, Sommer generated a close observation to the hippocampus of patients with TLE-HS and link to the clinical manifestations (Engel, 2001, and Scharfman and Pedley, 2006). Sommer identified that, the hippocampal sclerosis is unilateral and the neuronal cell loss is not uniform and the most affected area is CA1. In addition, he hypothesised that hippocampal pathological changes is the cause behind hallucination and illusion in his patients under study (Mathern et al, 1995; Engel, 1996; Engel, 2001 and, Scharfman and Pedley, 2006). Two decades later Bratz identify that, the pathological changes extended over hippocampus to parahyppocampus gyrus and

amygdala and he described the CA2 hippocampal subfield “resistant zone” (Mathern et al, 1995; Engel, 1996; Engel, 2001 and, Scharfman and Pedley, 2006). Based on his observation he hypothesised that hippocampal sclerosis has an important role in epileptogenesis (Mathern et al, 1995; Engel, 1996; Engel, 2001 and, Scharfman and Pedley, 2006).

In addition to the neuronal loss in the sclerotic hippocampus, synaptic axonal reorganisation of the granular cell axons (mossy fibres) in hippocampal dentate gyrus was detected among patients with refractory epilepsy, this phenomenon is known as mossy fibre sprouting (Houser et al., 1990). The granular cells’ mossy fibres were collaterally innervating the inner molecular layer of dentate gyrus where the proximal granular cell dendrites are located (Scharfman and Pedley, 2006). As a result of that, different scientists hypothesised that, the new granular cells’ synapses play a major role in epileptogenesis in which the mossy fibres generate an excitatory circuit in dentate gyrus (Sutula et al., 1992 and Nadler, 2003). On the other hand, other scientists hypothesised that, the mossy fibres innervate some of the inhibitory interneurons, which could play an important role to reduce neuronal hyperexcitability in dentate gyrus (Scharfman and Pedley, 2006).

Another important feature that can be observed in the dentate gyrus of TLE-HS patients is granular cells dispersion (Houser et al., 1990). This dispersion presents in the form of a reduction in the number of granular cells in the dentate gyrus (Scharfman and Pedley, 2006). Different debatable hypothesis has been suggested to described the reasons behind this histopathological phenomenon. Lessberher et al., (2005), hypothesised that an abnormal migration of juvenile neuronal cells born prior seizure that led granular

dispersion. On the other hand, Frotscher et al., (2003), hypothesis that, the seizure activity in the hippocampus altered the radial glial scaffold that resulted in abnormal granular cells migration.

In the modern era, the International League Against Epilepsy (ILAE) classified the degree of hippocampal sclerosis based on the level of neuronal loss, affected hippocampal subfields and gliosis (Thom, 2014, and Cendes, 2014), this classification includes the following:

- **HS ILAE type I**

This type of HS was described as the most common type of lesion that affect 60 - 80% of the disease population (Cendes, et al., 2014). The type I HS characterised by significant neuronal loss affect all of the hippocampal field in different degree. Approximately, 80% of the neuronal cells is lost from CA1, 30 – 50% pyramidal cell loss from CA2, in addition to 30 – 90% neuronal cell loss in CA3 and 40 – 90% neuronal cells loss in CA4. Furthermore, the dentate gyrus (DG) display a variable degree (between 50 – 60%) of neuronal cell dispersion (figure 1**;** Blumcke et al., 2013, Cendes et al., 2014)

- **HS ILAE type II**

This form of HS mainly affects the CA1 region leading to pyramidal neuronal cell loss by approximately 80% (figure 1; Cendes, et al., 2014). The other hippocampal subfields may

display some neuronal loss but does not exceed 25% (Cendes, et al., 2014). Moreover, gliosis was detected in CA1, however, no significant granular cells dispersion was observed in the DG (Blumcke et al., 2013, Cendes et al., 2014). This type of HS considered as one of the rare forms of HS that affect 5 – 10% of all of TLE-HS disease population (Cendes, et al., 2014).

- **HS ILAE type III**

This form of HS affects the granular cells of DG and neuronal cells of CA4 in which the neuronal loss is approximately 30% and 40%, respectively (Cendes, et al., 2014). However, the other hippocampal subfield only shows mild to moderate neuronal cell loss (Blumcke et al., 2013, Cendes et al., 2014). Around 4 – 7% of the TLE-HS patients are suffer from type III HS (figure 1; Cendes, et al., 2014). This form of HS was also described previously among patients with limbic encephalitis (Cendes, et al., 2014).

- **Hippocampal gliosis (no-HS)**

This non-sclerotic form of histopathological changes in hippocampus was observed in approximately 20% of TLE patients (figure 1; Blumcke et al., 2013, Cendes et al., 2014). This reactive gliosis was suggested to be seizure focus based on the electrophysiology evidence and recently considered as a potent epileptogensis trigger (Cendes, et al., 2014).

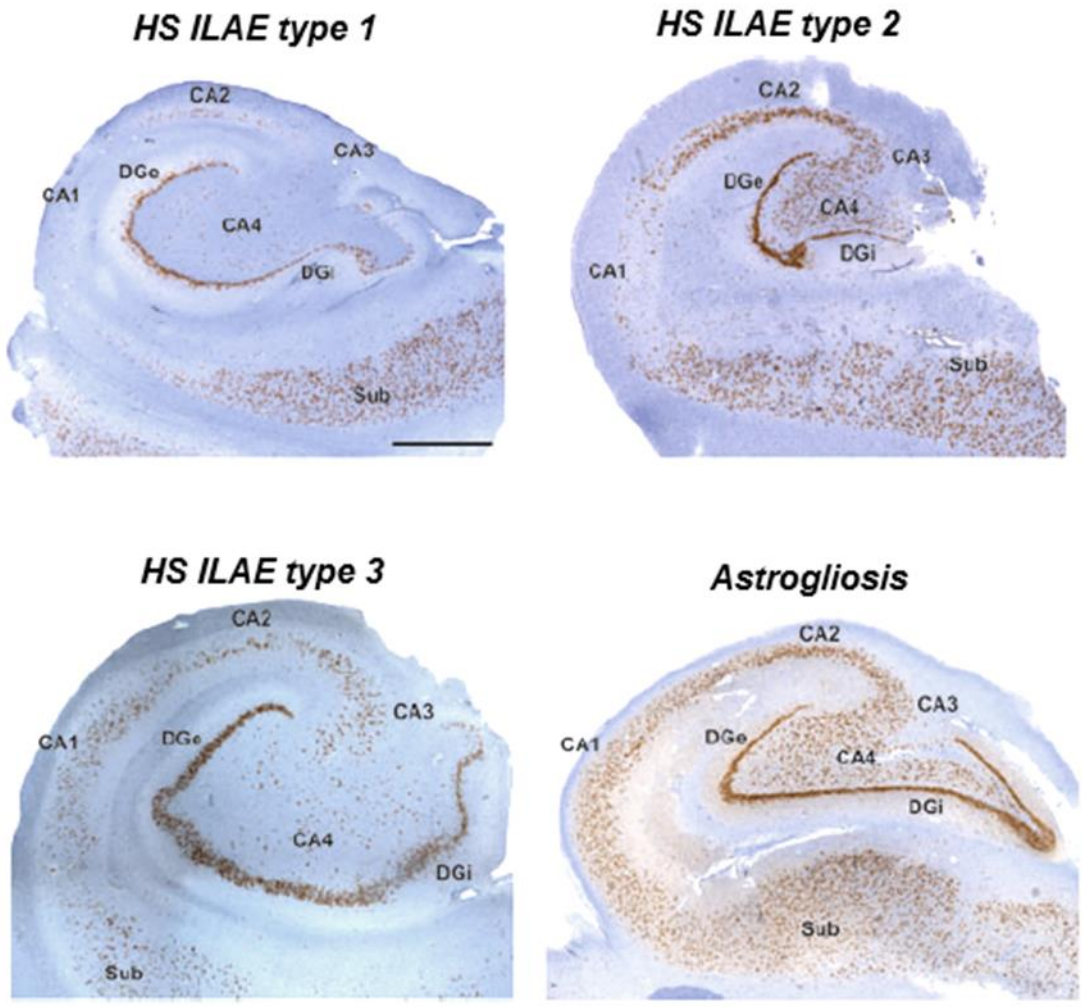

Figure 1. Histological classification of hippocampal sclerosis among TLE-HS according to ILAE.
HS ILAE type 1, neuronal cell loss in most of hippocampal subfields with preserved neurons in CA2. *HS ILAE type 2*, neuronal cell loss in CA1. *HS ILAE type 3*, neuronal cell loss affect CA4, dentate gyrus and hippocampal pyramidal neurons. *Astrogliosis*, astrogliosis with no sign of neuronal loss. NeuN immunoreactivity signals. Scale bar = 1.0 mm. This figure is adapted from Blumcke et al. (2013).

1.1.C. Pathophysiology

Even though the association between hippocampal sclerosis and seizure activity in temporal lobe epilepsy patients is confirmed, the relationship between them remains unclear. Several hypotheses were suggested to explain this relation. These hypotheses include the following:

- **The three-stage hypothesis:**

In this hypothesis, the developing TLE-HS passes through three stages. The first stage contains the initial precipitating event and includes a medical history that may have affected the brain during the early stages of childhood (Baram and Shinnar, 2001). This medical history includes meningitis, prolonged febrile seizure, encephalitis and head injuries (Scharfman and Pedley, 2006). These events are considered important in initiating hippocampal sclerosis because they are common, can cause a permanent alteration in the brain and also change its excitability (Scharfman and Pedley, 2006). This hypothetical stage remains debatable because, based on clinical observation, some patients have developed TLE-HS with no history of an initial precipitating event (Scharfman and Pedley, 2006).

The second stage is the latent period, which is the time between the initial precipitating event and the development of TLE-HS (Scharfman and Pedley, 2006). According to different experimental results generated using animal models, this is the time of the epileptogenesis process (Scharfman and Pedley, 2006). Numerous animal model studies were conducted to investigate the histo- and patho-physiological changes that occur during this period. The results expressed a wide range of abnormalities that

occurred during this time period. As an example of these alterations in the neuronal circuit, mossy fibre sprouting was observed in the animals and led to an increase in glutamatergic input (Sutula et al., 1989) and changes in the gap junctions between neurons (Nemani and Binder, 2005) and deafferentation of GABAergic interneurons (Sloviter, 1991). Regarding changes affecting voltage-gated ion channels, different ion channels (Na^{+}, K^{+} and Ca^{+2} ion channels) expressed abnormality in the distribution and cellular density and alterations affecting the channel modulators (Woo et al., 2002; Santoro and Baram, 2003) in addition to several other changes. This stage of the hypothesis is not completely agreed upon among scientists and clinicians because several TLE-HS conditions exhibit different latent period duration lengths even if they are exposed to the same initial precipitating event (Salazar et al., 1985).

The third stage is the chronic period, when the seizures become spontaneous and recurrent (Scharfman and Pedley, 2006). This stage potentially starts at the end of epileptogenesis; however, the pathophysiological changes may be continuous and affect seizure severity and frequency (Scharfman and Pedley, 2006). Several clinical studies confirm that TLE is a progressive condition in which neuronal cells loss and mossy fibre sprouting are continuously observed during the disease's progress (Mathern et al., 1995; Pitkänen and Sutula, 2002; Sutula, 2004). In addition, a direct relationship of both increased memory deficit and psychological comorbidity with the duration of the disease was observed clinically (Oyegbile et al., 2004).

- **Temporal lobe epilepsy as a genetic disorder:**

Several studies identified a relationship between TLE-HS and some genetic mutation in human and animal models. As an example of these studies is that conducted by Kasperavičiūtė et al. (2013) on TLE patients with and without hippocampal sclerosis, with medical history of febrile seizure during childhood and those had febrile seizure but not developed TLE. The results demonstrate a strong relationship between development of TLE-HS after a history of childhood febrile seizure and genetic mutation in SCN1A, which is the gene encoding brain-expressed Na^+ channel subunit and considered as a target for some AEDs (Kasperavičiūtė et al., 2013).

- **TLE-HS as neurodevelopment disorder:**

Several studies have investigated the involvement of neurodevelopmental abnormalities the in pathogenesis of TLE-HS. According to study conducted by Sloviter et al. (2004), 8% of hippocampi resected from TLE patients expressed developmental abnormalities present in form of neuronal cell dysplasia in addition to an increase in CA1 and subiculum width and in some cases introversion of the adjacent granular cells of the dentate gyrus.

1.1.D. Disease management

- **Pharmacological management**

The Scottish Intercollegiate Guidelines Network (SIGN, 2015) on the guideline of the diagnosis and management of epilepsy in adult, the following treatment strategy is recommended to control focal onset seizures:

Lamotrigine (sodium channel blocker) considered to be the first line treatment of focal seizure with better tolerating profile compared to oxycarbazepine and carbamazepine (sodium channel blockers; SIGN, 2015). Lamotrigine is also recommended to use as first line therapy especially with older patients who suffer from focal seizure due to its tolerability profile and levetiracetam is the drug of choice if the patients suffer from Alzheimer' disease in addition to focal epilepsy (SIGN, 2015). Furthermore, lamotrigine is also recommended for adulthood and young women in comparison to carbamazepine and oxycarbazepine because it has low tendency to induce hepatic enzymes and increase the metabolism of combined oral contraceptives (COCs), however, the plasma concentration of lamotrigine is significantly decreased due to glucuronidation, which is induced by COCs containing ethinyloestradiol and levonorgestrel (SIGN, 2015). Therefore, lamotrigine dose adjustment should be considered when it is used with COCs (SIGN, 2015). The effect of COCs on plasma level of lamotrigine is decreased when sodium valproate is included to the drug regimen to control seizures (SIGN, 2015).

When seizures fail to be controlled after a trial of two tolerable AEDs (as monotherapy or multiple therapy), the seizures are considered as pharmaco-resistant epilepsy (medically

intractable epilepsy) (SIGN, 2015). In this case, based on meta-analysis, levetiracetam, carbamazepine, oxycarbazepine, eslicarbazepine, perampanel, gabapentine, pregabline, lacosamide, topiramate, lamotrigine and zonisamide can be used as an adjunctive therapy to the first line treatment (SIGN, 2015). As a result of the risk of withdrawal seizure and poor tolerability, barbiturates are not considered to be used except in specialist epilepsy clinics (SIGN, 2015). In addition, some other drugs need close monitoring when they are used as adjunctive therapy to control pharmaco-resistant focal seizure, due to their sides effects (SIGN, 2015). These drugs include retigabine, which has a strong negative impact on the skin and retina (SIGN, 2015). Moreover, vigabatrine is associated with a high risk of concentric visual field deficits (SIGN, 2015).

- **Non-pharmacological management**

Surgical treatment of TLE-HS is the most effective method to control seizures in patients with refractory epilepsy (Ramey et al., 2013). Several forms of surgical approaches may be considered for resection (Ramey et al., 2013). First, standard anterior temporal lobectomy (ATL), where the hippocampus, amygdala and some of anterior temporal cortex resected en bloc (Yasargil et al., 1985, Ramey et al., 2013). Second, selective amygdalohippocampectomy (SAH), where in addition to hippocampus and amygdala, parahippocampus gyrus resected to minimise the resection of neocortex as far as possible (Yasargil et al., 1985, Ramey et al., 2013). According to some studies, there are no significant differences between these two approaches, however, other studies find that ATL is more beneficial in children and adults (Paglioli et al., 2004, Bate et al., 2007).

According to Kwan and Brodie (2000), surgical intervention may have a beneficial effect if considered soon after medical intractable epilepsy is confirmed in order to decrease the risk of disease complications. According to the randomised control clinical trial conducted by Engel et al. (2012) early surgical intervention for the recently diagnosed refractory TLE patients is associated with reduction in psychological complications. Considering the beneficial outcome of surgical treatment, this type of intervention may be associated with some complications regardless to the type of surgical approaches that is used. Some of these complications include the following; communicating hydrocephalus, depression, jaw pain, memory deficits, speech disturbance, postoperative hematoma which can lead to hemiparesis, meningitis and other neurological deficits (Ramey et al., 2013).

Given the failure of current AEDs in many patients with TLE-HS, and in order to avoid hippocampal resection where possible, there is motivation to discover a novel target to control seizure in TLE-HS patients. Due to the importance of serotonergic receptors in CNS, several serotonergic receptors were selected to be assessed in this research project. These receptors include; 5-HT_{1A}, 5-HT_{2A}, 5-HT_3A, 5-HT_4 and 5-HT_7 receptors. In addition to GPR61 and $P2x_7$ receptors were studied due to the potential relevance and the availability of suitable tools in the Barnes laboratory.

1.2. Serotonin

The monoamine serotonin (5-hydroxytryptamine, 5-HT) is one of the first discovered molecules, and it was isolated from serum by Page et al. in 1948 (Rapport et al., 1948). However, eight years previously, in 1940, Erspamer had discovered a contractile substance that is secreted by gastric mucosa, particularly the enterochromaffine cells, and which affects smooth muscles and blood vessels; a substance that was named entramine (Erspamer and Asero, 1952). In 1952, it was discovered that these two compounds were identical, and it was decided to call it serotonin (Erspamer and Asero, 1952). This ubiquitous molecule, which is known to act as a local hormone, a neuromodulator and a neurotransmitter, which is found in the CNS, as well as in other peripheral systems (Berumen et al., 2012).

1.2.A. Biosynthesis of 5-HT:

The serotonin present in the brain is synthesised in neuronal cells and represents 1–2% of the total amount of serotonin in the body (Feldman, Meyer and Quenzer, 1997). Its synthesis begins with the uptake of a dietary amino acid, tryptophan, from the bloodstream into the neuronal cells in a process called active uptake (Pytliak et al., 2011). In the neuronal cells, tryptophan is hydroxylated at the 5 position by tryptophan hydroxylase-2 (tryptophan-5’-monooxygenase; a phenotypic marker for 5-HT neurons), present in very low concentrations in neuronal cell bodies, to produce an intermediate compound called

5-hydroxytryptophan (5-HTP) (Pytliak et al., 2011). This is the rate-limiting step in 5-HT synthesis (Pytliak et al., 2011).

Immediately after synthesising 5-HTP, this intermediate molecule is transferred to the neuronal cells terminal where the majority of 5-HT synthesis occurs (Pytliak et al., 2011). Consequently, in the nerve terminal, aromatic amino acid decarboxylase acts by decarboxylating 5-HTP to form 5-HT, which is then uptaken from the cytoplasm and stored with serotonin binding protein (SBP) in the presynaptic vesicle by a vesicular monoamine transporter (Tamir and Gershon, 1990). When these neuronal cells are stimulated, 5-HT and SBP are released by exocytosis (Tamir et al., 1994).

1.2.B. Regulation of 5-HT concentration in the synaptic cleft

Three main regulatory mechanisms control the release of 5-HT and regulate its level in synapses. The first mechanism consists of autoreceptors, and two types of these regulatory receptors are found in neuronal cells; these are 5-HT_{1A} receptors, which are present in somatodendrites, and 5-HT_{1D} receptors, which are known as presynaptic autoreceptors present in the human brain (Gothert, 1990, Sharp and Hjorth, 1990). The 5-HT_{1A} soma and dendrites autoreceptors act by hyperpolarising the neuron and inhibiting its firing (Gothert, 1990, Sharp and Hjorth, 1990). This mechanism apparently acts by increasing K^+ ion conductivity in the cellular membrane and also alters the Ca^{+2} influx via voltage-gated Ca^{+2} channels (Gothert, 1980).

The second mechanism is the activation of 5-HT transporter (serotonin transporter; SERT) in the presynaptic domain of serotonergic neurons (Graham and Langer, 1992). Different hypotheses have been advanced to explain the mechanism of SERT as a carrier-mediated function. One of these is that suggested by Marcusson and Ross (1990), in which Na^+ ions bind to the extracellular domain of the SERT to facilitate 5-HT binding on its specific binding site, after which Cl^- binds to aid in translocation of these ions and molecules to the intracellular domain of the neuron (Marcusson and Ross, 1990). Following the first translocation, K^+ binds to the intracellular domain of the transporter and facilitates its outflux to the extracellular fluid (second translocation) (Marcusson and Ross, 1990).

The third regulatory mechanism is catabolism via a mitochondrial enzyme called monoamineoxydase A and B (Feldman, Meyer and Quenzer, 1997). In the presence of flavin adenine dinucleotide, this enzyme converts 5-HT into 5-Hydroxyindoleacetaldehyde, which is rapidly metabolised to the primary end product of 5-HT metabolism, 5-Hydroxyindoleacetic acid (5-HIAA), and excreted to the cerebrospinal fluid (Hildebrand et al., 1990). This mechanism, and the two previously described, is activated in a 5-HT concentration-dependent manner.

1.2.C. Localisation of the serotonergic system in the brain

In 1964, Dahlström et al. identified nine 5-HT-containing nuclei (B1-B9), described in table (1) (Dahlstroem and Fuxe, 1964). A large cluster of these cell groups is located in the

dorsal raphe nucleus of the brain; an area that contains approximately 165,000 cells (Tork, 1990). In addition, 5-HT neurons are also present in the caudal locus, the area postrema and the peri-interpeduncular nucleus (Feldman, Meyer and Quenzer, 1997).

From these brainstem structures, 5-HT neurons are divided into two main systems; the caudal and the rostral (Tork, 1990). The caudal system is located in the medulla and the pons and contains B1-B4 cell groups, in which the axons of the neurons innervate the medulla and spinal cord (Tork, 1990), while the rostral neuronal system consists of B5-B9 cell groups, which are present in the contralis superior, the raphe medianus and the raphe dorsalis (Tork, 1990). This rostral system consists of ascending neuronal projections that innervate the basal ganglia, the limbic system [including the hippocampus where they are present in high density in CA3 compared to the dentate gyrus and CA1 (Berumen et al., 2012)] and the diencephalon via the ventral ascending pathway (originating from B6-B8) (Nieuwenhuys, 1985, Feldman, Meyer and Quenzer, 1997).

The second neuronal projection is the dorsal pathway, originating from B7-B8, the neuronal axons of which innervate the superior and inferior colliculi in addition to the mesencephalic gray (Nieuwenhuys, 1985, Feldman, Meyer and Quenzer, 1997). Moreover, these ascending pathways combine in the medial forebrain bundle to form the ascending system (Nieuwenhuys, 1985, Feldman, Meyer and Quenzer, 1997). In terms of their presence in the hippocampus, particularly in the dentate gyrus (DG), these 5-HT fibres originate from the median raphe nucleus (known as the M system), and from the basket axon system in the DG (Mohler et al., 2007). They are characterised by: 1) thick,

non-varicose tract fibres; 2) large, round boutons; 3) thin and short branches and 4) extensive, repeated synapses (Molliver, 1987, Tork, 1990).

Table 1. Anatomical localization of serotonin cell groups (adapted from; Feldman, Meyer and Quenzer, 1997):

5-HT cell groups	Anatomical localisation
B1	• Raphe pallidus nucleus • Caudal ventrolateral medulla
B2	• Raphe obscurus nucleus
B3	• Raphe magnus nucleus • Rostral ventrolateral medulla • Lateral paragigantocellular reticular nucleus
B4	• Raphe obscurus nucleus, dorsolateral pary
B5	• Median raphe nucleus, caudal part
B6	• Dorsal raphe nucleus, caudal part
B7	• Dorsal raphe nucleus principal, rostral part
B8	• Median raphe nucleus, rostral main part • Caudal linear nucleus • Nucleus pontis oralis
B9	• Nucleus pontis oralis • Supralemniscal region

1.2.D. Serotonergic receptors in the hippocampus

Different 5-HT receptors are expressed in the hippocampus where they are used by 5-HT to produce its physiological and pathological effects. In humans, 5-HT receptors primarily consist of seven main types with 14 different subtypes (Nichols and Nichols, 2008). These receptors are classified into two groups. The first of these is the ligand gated ion channel, to which all 5-HT_3 receptors belong (Nichols and Nichols, 2008). The second group is G-protein-coupled receptors (GPCRs) group, to which all 5-HT receptors belong (Nichols and Nichols, 2008). The current project focuses on an assessment of the expression of some 5-HT receptors (primarily 5-HT_{1A} – 5-HT_{2A} – 5-HT_3A – 5-HT_4 – 5-HT_7 receptors) in the hippocampus of people with temporal lobe epilepsy with hippocampal sclerosis TLE-HS.

- **Ligand-gated ion channel 5-HT receptors:**
  - **5-HT_3A receptors:**

The 5-HT_3A receptor is an ionotropic ligand-gated, cation-selected ion channel receptor that is expressed pre- and post-synaptically in the CNS (Van Hooft and Yakel, 2003). These pentameric receptors consist of 5-HT3A subunits, in which the 5-HT3A is the functional subunit present in the brain (Nichols and Nichols, 2008). This subunit can form a pentameric homomeric (5-HT_3A receptor) or heteromeric 5-HT_3 receptor when combined with other 5-HT3 receptor subunits (3B, 3C, 3D and 3E subunits; Niesler et al., 2008). Activation of these receptors results in rapid calcium influx and depolarisation

(Nichols and Nichols, 2008). This mechanism mediates hippocampal neuronal hyperexcitability postsynaptically and neurotransmitters release when the presynaptic 5-HT_3 receptors activated (Barnes et al., 2009).

In terms of expression in the CNS, 5-HT_3 receptors are present in the area postrema, solitary tract nucleus and spinal trigeminal nerve nucleus (Nichols and Nichols, 2008). Furthermore, these receptors are also located in the nucleus accumbens, caudate nucleus, amygdala and putamen (Bufton et al., 1993, Nichols and Nichols, 2008). With regard to expression in the hippocampus, 5-HT_3 has been detected at relatively low levels in all areas of the hippocampal formation (Brady et al., 2007, Nichols and Nichols, 2008), with a predominant immunoreactivity detected in CA2 and CA3 pyramidal neurons (Brady et al., 2007, Berumen et al., 2012). In addition, both pyramidal neurons of CA1 and granular cells of dentate gyrus express low immunoreactivity signals (Brady et al., 2007). Expression in these areas of the CNS suggests that 5-HT_3 receptors might be involved in different physiological and pathological conditions, including higher functions of the brain, as well as mood (Nichols and Nichols, 2008) (Table 2), in addition to nausea, vomiting and depression (Rocha et al., 2007, Charnay and Leger, 2010, Matthys et al., 2011). According to Barnes (2011), 5-HT_3A receptors are overexpressed in the GABAergic and glutamatergic neurons that are present in the hilus and DG in patients with TLE-HS, respectively (Sharma et al., 2007).

Interestingly, some clinical case reports have described an association between the use of ondansetron (a relatively selective 5-HT_3 receptor antagonist that is used for the treatment and prevention of nausea and vomiting), and generalised tonic-clonic seizures

in non-epileptic individuals (Sargent et al., 1993, Sharma and Raina, 2001, George et al., 2008, Singh et al., 2009, Patel et al., 2011). Two case reports describe generalised tonic-clonic seizures in patient received ondansetron co-administered with medications that decrease seizure threshold such as imipramine, methadone and methylergonovine (Sargent et al., 1993 and Mason et al., 2007). Furthermore, seizure associated with toxic dose of granisetron (7 – 8 tablets, 10 mg) was reported in 1 years old infant (George et al., 2008). In this case, patient developed serotonin syndrome in addition to myoclonus and encephalopathy (George et al., 2008). According to Singh et al., (2009) this rare side effect is occurred during first 30 min post ondansetron intravenous administration.

- **G-protein-coupled receptors:**
    - **5-HT$_{1A}$ receptors:**

5-HT$_{1A}$ is coupled to G$_{i/0}$ protein in the hippocampus, where this receptor expresses more binding affinity for the G$_0$ than the Gi protein (Nichols and Nichols, 2008, Millan et al., 2008), and is expressed both pre- and post-synaptically (Nichols and Nichols, 2008, Millan et al., 2008, Berumen et al., 2012). Activation of 5-HT$_{1A}$ receptors leads to inhibition of the protein kinase A (PKA) and adenylyl cyclase (AC) enzymes, and decreases the presynaptic production of cyclic AMP (cAMP) (Nichols and Nichols, 2008, Millan et al., 2008). In addition, this receptor acts on G-protein gated inwardly rectifying the K^+ channel (GIRK) via postsynaptic stimulation of G$_0$ protein to mediate hyperpolarisation and inhibit neuronal firing (Nichols and Nichols, 2008, Hannon and Hoyer, 2008, Berumen et al., 2012). Furthermore, these serotonergic receptors act through activation of the G$_z$ protein, leading to inhibition of phospholipase C and activation of phospholipase A2 (PLA2) (Millan et al., 2008, Berumen et al., 2012).

The pre-synaptic expression of 5-HT$_{1A}$ receptors in the hippocampus regulates the activity of the pre-synaptic neurons and balances the synaptic concentration of 5-HT (Berumen et al., 2012). The 5-HT$_{1A}$ receptors are expressed post-synaptically in the excitatory pyramidal neurons and granule cells of the hippocampus, in addition to having an extrasynaptic localisation (Berumen et al., 2012). Moreover, the 5-HT$_{1A}$ receptor is also expressed in astrocytes, ependymal, endothelial and glial cells (Berumen et al., 2012). In terms of the expression of 5-HT$_{1A}$ in different hippocampal areas, these inhibitory

receptors are present in the DG and CA2 (Okuhara and Beck, 1998, Nichols and Nichols, 2008, Hannon and Hoyer, 2008, Berumen et al., 2012), CA1 (Nichols and Nichols, 2008) and CA3 (Burnet et al., 1995, Okuhara and Beck, 1998, Nichols and Nichols, 2008, Hannon and Hoyer, 2008).

A variety of studies have previously been conducted to assess the involvement of 5-HT_{1A} receptors in animal models of TLE-HS. For example, Lopez-Meraz (2005) found that the use of (±)-8-Hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT, a selective 5-$HT_{1A/7}$ receptor agonist) in a kainic acid-induced seizure rat model resulted in an decreased incidence of seizure and possibly increased its latency period. In an intra-hippocampal kainic acid-induced seizure rat model, this 8-OH-DPAT has been shown to delay the latency period and decrease the duration and incidence of the seizure (Gariboldi et al., 1996). Furthermore, indorenate (a 5-HT_{1A} receptor agonist) produced the same effect on the latency period of a kainic acid-induced seizure animal model (Lopez-Meraz et al., 2005). Conversely, the use of a 5-HT_{1A} receptor antagonist (WAY 100635) resulted in an increase of the seizure duration and incidence, and it increased the number of seizures in intra-hippocampal kainic acid and pilocarpine rat models, respectively (Gariboldi et al., 1996, Clinckers et al., 2004a, Clinckers et al., 2004b).

A number of researchers have attempted to understand the involvement of 5-HT_{1A} receptors in the pathogenesis of TLE-HS. Merlet and his colleagues (2004) addressed an overexpression of 5-HT_{1A} receptors in extra epileptic focus. However, Ito et al (2007) found that these receptors are under-regulated in the hippocampus of patients with TLE using PET. With regard to the extra hippocampal areas, these serotonergic receptors

showed an under-regulation in the amygdala and neocortex in TLE-HS patients that was similar to that observed in people with major depressive disorder (Drevets et al., 2000), which might explain the comorbidity of TLE-HS and depression in this type of patient.

- **5-HT_{2A} receptors:**

The 5-HT_{2A} receptor is another 5-HT receptor coupled to G-protein, particularly $G_{q/11}$ (Nichols and Nichols, 2008, Millan et al., 2008 Pytliak et al., 2011). In the hippocampus, this coupling leads to hydrolysis of phosphoinositides from the cell membrane and forms the second messengers, diacylglycerol and inositol phosphate (Nichols and Nichols, 2008), which activate PLA2 and arachidonic acid in the hippocampus, leading to an increase of Ca^{+2} concentration in the cytoplasm (Millan et al., 2008, Pytliak et al., 2011). Furthermore, in the cerebral cortex, 5-HT_{2A} receptors activate protein kinase C (PKC), which also elevates intracellular Ca^{+2} concentration (Millan et al., 2008, Pytliak et al., 2011).

With regard to the expression of 5-HT_{2A} receptors in the human brain, they are distributed post-synaptically in CA1 and CA3 hippocampal pyramidal cells in the granular layer of the DG and astrocytes (Berumen et al., 2012). This expression in hippocampal fields is lower than that found in the parahippocampal gyrus and cerebral cortex (Burnet et al., 1995). The expression of 5-HT_{2A} receptors in these particular brain regions suggests that these receptors might play an important role in memory and learning (Charnay and Leger, 2010) (Table 2), which deteriorate in people with TLE-HS.

With regard to experimental studies of 5-HT_{2A} receptors in a TLE-HS animal model, ritanserin (a nonselective 5-HT_2 receptor antagonist) does not exert any effects in a kainic acid-induced seizure rat model (Velisek et al., 1994). However, there are currently no available data concerning the involvement of 5-HT_{2A} receptors in the pathogenesis of TLE-HS in humans.

- **5-HT_4 receptors:**

5-HT_4 receptors act by coupling to G_s protein, leading to activation of AC and PKA (Nichols and Nichols, 2008, Millan et al., 2008 Pytliak et al., 2011), and stimulation of cAMP (Nichols and Nichols, 2008). In CA1 pyramidal cells in the hippocampus, activation of 5-HT_4 receptors inhibits Ca^{+2} activated K^+ channels, which are responsible for slow after-hyperpolarisation (Nichols and Nichols, 2008). Moreover, activation of these receptors in CA1 is involved in long-term depression, but not long-term potentiation (Nichols and Nichols, 2008). In addition, 5-HT_4 receptors counteract the activity of 5-HT_{1A} receptors, whereby stimulation of 5-HT_4 receptors produces rapid direct stimulation of serotonergic neurons and increases their firing, leading to desensitisation of 5-HT_{1A} receptors (Berumen et al., 2012). Regarding the expression of 5-HT_4 receptors in the human brain, in situ hybridisation has identified their mRNA in the hippocampus (Berumen et al., 2012). Furthermore, this receptor type is present in the substantia nigra, the basal ganglia and the cerebral cortex (Bonaventure et al., 2000, Nichols and Nichols, 2008). According to Waeber et al. (1993) the radioligand binding activity in the hippocampus, colliculus and neocortex is lower than that found in the striatum and substantia nigra. With regard to

distribution in the hippocampus, Bonaventure et al. (2000) asserted that 5-HT_4 receptors are found in all of the hippocampal formations with the exception of the hilus. The expression of 5-HT_4 receptors in these hippocampal areas means that they play an important role in cognition (Mohler et al., 2007). In addition, they might be indirectly involved in memory formation by mediating a regulatory effect on acetylcholine release from cholinergic neurons in the hippocampus (Fink and Gothert, 2007, Pytliak et al., 2011, Berumen et al., 2012) (Table 2). Of involvement in TLE-HS, Rocha (2007) and her colleagues observed that there is an increase in the binding affinity of 5-HT_4 receptors in layers V and VI of the neocortex of people with TLE-HS. This elevation in receptor binding activity has a direct relationship with the duration of the disease (Rocha et al., 2007). However, low binding affinity was detected in layers III and IV of the neocortex in patients with a long duration of seizures or who had been on AEDs for an extended period of time (Rocha et al., 2007).

- **5-HT_7 receptors:**

In a manner similar to 5-HT_4 receptors, metabotropic 5-HT_7 receptors act by activating G_s protein, leading to activation of AC and PKA in the hippocampus (Millan et al., 2008, Berumen et al., 2012). This receptor type also activates G_{12} protein in the hippocampus, leading to the activation of Rho and cell division control protein 42 (Cdc42) (Millan et al., 2008). With regard to the level of expression of 5-HT_7 receptors, a variety of studies have been conducted on the human CNS, and the results have shown that this receptor is present in the cortex, thalamus, hippocampus (Jasper et al., 1997, Belenky and Pickard,

2001, Terron et al., 2001, Nichols and Nichols, 2008), hypothalamus (Nichols and Nichols, 2008), suprachiasmatic nucleus, amygdala and trigeminal ganglia (Jasper et al., 1997, Terron et al., 2001). The immunoreactivity of 5-HT_7 in the hippocampus has been detected in CA3, CA2 and CA1 pyramidal cell bodies, but not in interneurons (Berumen et al., 2012). This distribution in the human brain suggests the involvement of this receptor type in different physiological functions, such as mood control, neuroendocrine vegetative behaviour and learning (Berumen et al., 2012) (Table 2), as well as memory, circadian rhythms and thermal regulation (Matthys et al., 2011).

With regard to the involvement of these receptors in the neurotransmitter network, they regulate glutamate and γ-aminobutyric acid (GABA) transmission in the hippocampus (Berumen et al., 2012, Rocha, 2013). Post-synaptic 5-HT_7 receptors facilitate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor transmission between the pyramidal neurons of CA3 and CA1 (Berumen et al., 2012, Rocha, 2013, Andreeta et al., 2016), in addition to blocking the inhibitory effect of GABA (Rocha et al., 2007).

In the context of experimental studies that have been conducted to understand the role of 5-HT_7 receptors in animal models of TLE-HS, Yang (2012) found that the use of AS19 (a 5-HT_7 agonist) increased spike-wave discharge and the frequency of the spontaneous seizure, in a pilocarpine-induced seizure rat model. In contrast, the use of SB269970 (a 5-HT_7 antagonist) decreased these effects (Yang et al., 2012).

Regarding involvement in pathological conditions, a variety of studies have shown that 5-HT_7 receptor expression is altered in pain, schizophrenia, epilepsy and depression (Matthys et al., 2011). Moreover, Yang and his colleagues (2012) studied expression of

5-HT_7 receptors in the neocortex of TLE-HS patients and found an overexpression of this receptor type among this group of patients. Furthermore, the binding affinity of 5-HT_7 receptors was reduced in TLE-HS patients on long-term AEDs (Rocha et al., 2007).

Table 2. Physiological and pathological functions of 5-HT receptors in human brain (Rocha et al. 2007; Charnay et al., 2010; Barnes, 2011 and Matthys et al., 2011).

Receptor	Physiological involvement	Pathological involvement
5-HT_{1A}	Regulate 5-HT activity, learning, memory, cognition, feeding, and body thermal control.	Anxiety, schizophrenia, depression, and neurodegenerative disorders.
5-HT_{2A}	Respiratory control and mood.	Schizophrenia and anxiety.
5-HT_{3}	Mood and vomiting reflex.	Epilepsy, nausea depression, and anxiety.
5-HT_{4}	Cognition, reward and feeding.	Epilepsy, anorexia, addiction and Alzheimer.
5-HT_{7}	Cognition, mood and circadian rhythm.	Schizophrenia, depression, anxiety, and epilepsy.

1.3. The orphan G-protein, GPR61

GPCRs are considered a superfamily that acts as a transducer for the extracellular messengers and produce cellular response through a wide range of signalling pathways (Lee et al., 2001, Cikos et al., 2001, Takeda et al., 2003, Toyooka et al., 2009, Nambu et al., 2011, Martin et al., 2015, Hossain et al., 2016). Some of these GPCRs are known as orphan G-proteins which are proteins whose endogenous ligands are undiscovered (Martin et al., 2015). GPR61 is one of these orphan G-proteins that was first identified in the human brain by Lee et al. (2001) and cloned by Cikos et al. (2001). Similar to other G-proteins, GPR61 consists of seven transmembrane domains with approximately 28–31% homology to biogenic amines such as histaminergic, dopaminergic, serotonergic (in particular, 5-HT_6 receptors) and adrenergic GPCRs (Lee et al., 2001, Cikos et al., 2001).

Regarding the expression of GPR61 in human brain tissue, GPR61 mRNA is highly expressed in the hippocampus, occipital pole, amygdala, cerebral cortex, frontal lobe, temporal lobe (Cikos et al., 2001) and thalamus (Lee et al., 2001). This GPCRs mRNA is expressed at low levels in the putamen and caudate (Lee et al., 2001, Cikos et al., 2001). Expression of GPR61 in these areas may indicate that this protein may be involved in higher brain functions such as learning, memory and cognition (Cikos et al., 2001).

According to Takeda et al. (2003), GPR61 is coupled to G_s-proteins. In addition, it expresses a constitutive activity which is maintained by the GPR61 N-terminal that acts as a tether for the intramolecular ligand (Toyooka et al., 2009). Regarding the intracellular signalling of GPR61, plasmalogens (Pls) (which are cell membrane structure components in the CNS) act on GPR61 and other GPCRs (GPR1, GPR19, GPR21, GPR27) to activate

heterodimer formation among these GPCRs (Hossain et al., 2016). The formation of heterodimers lead to the phosphorylation of extracellular signal-regulated kinases (ERK) and protein kinase B (PKB or AKT) in neuronal cells (Hossain et al., 2016). This effect is evoked in GPR61, GPR1, GPR19, GPR21, GPR27 knockout cells (Hossain et al., 2016). In 2003, Takeda et al. suggested a surrogate ligand for GPR61, 5-(nonyloxy)tryptamine (a potent 5-HT_{1D} receptor agonist with moderate affinity to 5-HT1A receptors), which exhibits a low affinity GPR61 inverse agonist.

In terms of the biological role of GPR61, a physiological study conducted by Nambu et al. (2011), using GPR61 knockout mice, indicated involvement in regulating feeding behaviour. This finding is suggested to be a result of the hyperphagia and obesity observed among GPR61 knockout mice, in addition to the normal expression of GPR61 in brain regions that regulate food intake (Nambu et al., 2011). These areas include the hippocampus (regulates ingestion learning and memory), nucleus accumbens (regulates feeding behaviour motivation) and nucleus tractus solitarius (which is the response to feeding sensory input from the viscera) (Nambu et al., 2011). To our knowledge, no data are available about the protein expression of GPR61 in the human normal hippocampus or in the sclerotic hippocampus of TLE-HS patients. Therefore, it was opportunistic to study GPR61 in normal and TLE-HS diseased hippocampi.

1.4. Purinergic channel, $P2x_7$

Purinergic $P2x_7$ receptors are a subclass of the P2x non-selective ion channel family, consisting of two transmembrane domains with long extracellular loops consisting of multiple cysteine and glycosylation moieties with short intracellular N-terminals and long C-terminals in comparison to other types of P2x families (Sperlágh et al., 2006, Skaper et al., 2010, Engel et al., 2012b, Sperlágh and Illes, 2014). This receptor has a relatively low affinity to adenosine triphosphate (ATP) being activated by approximately millimolar concentrations of ATP (pathological concentrations of ATP) compared to other P2x receptor families which require low micromolar ATP to be activated (Sperlágh et al., 2006, Skaper et al., 2010, Engel et al., 2012b, Sperlágh and Illes, 2014). $P2x_7$ receptors exhibit a low tendency for desensitisation (Engel et al., 2012b). Activating $P2x_7$ receptors results in increased Ca^{+2}, Na^{+} influx and K^{+} outflux; however, prolonged activation of this receptor facilitates the opening of non-selective pores that allow large molecules (600–900 Da) to traverse the membrane (Skaper et al., 2010, Engel et al., 2012b). This later phenomenon is not well understood but there are several hypotheses to explain the formation of these pores. One is that the structural modification of the channel leads to the dilatation of the $P2x_7$ ion channel itself, similar to that observed with $P2x_2$ and $P2x_4$ (Chaumont and Khakh, 2008). According to Sun et al. (2013), both the C-terminal and the second transmembrane domain are involved in the $P2x_7$ ion channel dilatation. The second possible hypothesis is the involvement of the hemichannel protein, panexin-1 (Panx1), which acts as a pore formation protein which is activated as downstream signalling of the stimulated $P2x_7$ receptors (Skaper et al., 2010). This hypothesis is controversial. According to some scientists, Panx1 is essential for the pore formation and can be blocked using

colchicine (Marques-da-Silva et al., 2011, Suadicani et al., 2012). On the other hand, other groups of scientists have suggested that the involvement of Panx1 is not essential for pore formation because different $P2x_7$ splice variants express different pore formation characters (Xu et al., 2012, Schwarz et al., 2012).

In terms of activating signalling pathways, activating $P2x_7$ receptors can drive activation of mitogen-activated protein kinases (MAPK), caspase-1, interleukin 1β (IL-1β), phospholipase A2, phospholipase D, extracellular signal regulated kinase (ERK1/2), tumour necrotic factor α (TNFα) and nuclear factor-ƙB (Labrousse et al., 2009, Krueger et al., 2010, Csölle and Sperlagh, 2010, Girotti et al., 2013). Moreover, this receptor can activate the cAMP response element-binding protein (CREB), c-Jun terminal kinases 1/2 (JNKs1/2) and microglia, and it can control its proliferation, in addition to inhibiting glycogen synthase kinase-3 (Labrousse et al., 2009, Csölle and Sperlagh, 2010, Barberà-Cremades et al., 2012).

In the CNS, $P2x_7$ receptors play an important role in controlling glutamate, GABA and other neurotransmitters in the synaptic cleft (Sperlágh et al., 2002). $P2x_7$ acts as an autostimulatory receptor in the hippocampus by activating the release of ATP (Heinrich et al., 2012). The mechanism of this phenomenon is not fully understood; the ATP could be released by exocytosis, through Panx1 and/or connexin hemichannel proteins (Gutiérrez-Martín et al., 2011, Bennett et al., 2012, Heinrich et al., 2012).

Regarding the normal expression of $P2x_7$ receptors, Rassendren et al. (1997) and Cheewatrakoolpong et al. (2005) found that, using northern blot and qPCR, the mRNA of this receptor is present in human brain tissue. Furthermore, the immunoreactivity of

this excitatory receptor was detected in microglia using immunohistochemistry (Yiangou et al., 2006).

As to the involvement of $P2x_7$ receptors in the pathogenesis of TLE-HS, the immunoreactivity of $P2x_7$ receptors is significantly increased in the neurons, astrocytes and microglia of animal models of status epilepticus induced by pilocarpine and kainate (Kim et al., 2009, Dona et al., 2009). Moreover, stimulating $P2x_7$ receptors has been associated with an increase in the epileptic activity in the kainate animal model of status epilepticus (Engel et al., 2012a, Jimenez-Pacheco et al., 2013). However, stimulating $P2x_7$ receptors in the same animal model increased post-ictal astrogliosis and leukocyte infiltration (Kim et al., 2010, Kim et al., 2013). On the other hand, antagonizing this receptor produces a neuroprotective effect against the negative impact of epilepsy by inhibiting IL-1β release and suppressing microglia activation (Engel et al., 2012a, Jimenez-Pacheco et al., 2013). Interestingly, antagonizing $P2x_7$ receptors with A-438079, a competitive $P2x_7$ receptor antagonist, facilitates seizure activity in pilocarpine mouse models of status epilepticus (Kim and Kang, 2011). This effect results from suppressing the inhibitory effect of activated $P2x_7$ on neuronal muscarinic receptors (Kim and Kang, 2011). Regarding the expression of $P2x_7$ in brain samples of patients with TLE-HS, this purinergic receptor expresses an upregulation in the neocortex (Jimenez-Pacheco et al., 2013) and in the hippocampus of TLE-HS patients (Fernandes et al., 2009). According to Barros-Barbosa et al. (2015), activating $P2x_7$ receptors in the neocortex nerve terminals of TLE-HS patients is associated with further downregulation of GABA and glutamate uptake in comparison to post-mortem control samples. This finding indicates that the excessive release of ATP during

seizure suppresses GABA and glutamate uptake into nerve terminals (Barros-Barbosa et al., 2015).

1.5. Hypothesis

Previous research on the expression of 5-HT_3A, 5-HT_{2A}, 5-HT_4, 5-HT_7 and $P2x_7$ receptors in the human hippocampus demonstrates that these excitatory receptors are present in excitatory pyramidal and granular neurons, which exhibit neuronal hyperexcitability in TLE-HS patients. This led to the hypothesis that these excitatory receptors are upregulated in the excitatory neurons of the hippocampus and are involved in neuronal hyperexcitability and seizure generation. Antagonising these receptors introduce a better control for seizure activity in patients with TLE-HS and improve their quality of life.

Similar to the expression of the previous excitatory receptors, numerus studies on the expression of inhibitory 5-HT_{1A} receptors in the human hippocampus show the localization of this inhibitory receptors on the excitatory pyramidal and granular neurons and it is control their neuronal firing. In addition, the binding activity of 5-HT_{1A} receptors is decrease in hippocampus of patients with TLE-HS. These studies led to the following hypothesis, the expression of inhibitory 5-HT_{1A} receptors is decreased in the hippocampus of TLE-HS patients and is indirectly involved in neuronal hyperexcitability and seizure activity in such patients.

1.6. Aims

The aim of this study is to investigate the pathophysiological alteration of various relevant serotonergic receptors, P2x7 receptors and GPR61 protein associated with the most common type of epilepsy among adults (TLE-HS). These changes may play a role in pathogenesis of TLE-HS. This study may help in understanding the role of the targets receptors and protein in the mechanism underline TLE-HS. In addition, this may facilitate identification of novel targets and developed new compounds to control seizures in TLE-HS patients with less tendency to developed adverse effects. This may also help to decrease the number of patients who undergo surgical intervention.

CHAPTER 2

Materials and Methods

2. Materials and methodology

2.1. Human brain samples

The TLE-HS surgically resected hippocampal samples and some post-mortem control samples were obtained from Queen Elizabeth Hospital (Birmingham, UK) after the patients gave their informed consent which was ethically approved by the Human Biomaterial Resource Centre (HBRC, University of Birmingham, Birmingham, UK). These samples were obtained in forms of formalin-fixed paraffin-embedded (FFPE) sections or fresh and snap-frozen blocks (Table 3). In addition, other TLE-HS snap-frozen hippocampi were obtained from Queen Square Brain Bank for Neurological Disorders (University College of London, London, UK) (Table 3). Age-matched post-mortem control hippocampi were obtained from donors with no history of neurological and psychological disorders that were ethically approved by Edinburgh Brain Bank (University of Edinburgh, Edinburgh, UK). These samples were used in forms of FFPE and snap-frozen blocks. All of these institutions were accredited by the UK Human Tissue Act (HTA).

2.2. Mimicking post-mortem delay:

The surgically resected TLE-HS hippocampus was transferred from theatre to our lab in ice could oxygenated sucrose solution ($NaHCO_3$ 25, $CaCl_2$ 1, KCl 3, $MgCl_2$ 10, D-Glucose 10, sucrose 250, mM) within 30 min post resection. To mimic post-mortem delay, an artificial post-mortem delay protocol conducted using artificial cerebrospinal fluid (aCSF; $NaHCO_3$ 25, $CaCl_2$ 2, KCl 4, $MgCl_2$ 2, D-Glucose 10, NaCl 125, mM). In this protocol, the sample incubated in aCSF for 18 hour at room temperature followed by 48 hour at 4ºC then for 4 hours at room temperature before being snap frozen it for further downstream analysis.

Table 3. Demographical information of hippocampus donors.

Type	Sample	Age (years)	Gender	Medication	Post-mortem index (hr)
Frozen	TLE1	46	Male	---	---
	TLE2	33	Female	---	---
	TLE3	24	Female	---	---
	TLE4	30	Female	---	---
	TLE5	30	Female	---	---
	TLE6	37	Male	---	---
	TLE7	40	Male	---	---
	TLE8	49	Male	---	---
	PMC1	63	Male	---	41
	PMC2	47	Male	---	42
	PMC3	46	Female	---	49
	PMC4	63	Male	---	42
	PMC5	39	Female	---	43
	PMC6	38	Male	---	36

Table 3. Continue

	PMC7	53	Male	---		53
	PMC8	45	Female	---		40
	PMD1	36	Male	---		70
	PMD2	52	Male	---		70
	PMD3	30	Male	---		70
FFPE	TLE1	38	Female	Betahistine Dexamethasone Lamotrigine Movicol	Paracetamol Senna Zopiclone	---
	TLE2	18	Male	Topiramate	Zonisamide	---
	TLE3	45	Female	Chlorphenamine Codeine Phosphate Lamotrigine Lansoprazole	Oxcarbazepine Paracetamol Pregabalin Senna	---
	TLE4	31	Male	Metoclopramide Carbamazepine Codeine Phosphate Levetiracetam	Paracetamol Sodium Valproate Zopiclone	---

Table 3. Continue

FFPE	TLE5	42	Male	Chlorphenamine Citalopram Dexamethasone Lansoprazole	Levetiracetam Simvastatin Sodium Valproate	---
	TLE6	24	Female	Lamotrigine Codeine Phosphate Lactulose	Senna Lansoprazole Levetiracetam Paracetamol	---
	TLE7	33	Female	Oxcarbazepine	Pregabaline	---
	TLE8	60	Female	Carbamazepine Clobazam	Phenytoin	---
	TLE9	45	Male	Private Patient		---
	TLE10	22	Female	Oxcarbazepine Dexamethasone	Lamotrigine	---
	TLE11	30	Female	Lacosamide Carbamazepine Levetiracetam Ciprofloxacin	Probucol Levocetirizine Paracetamol	---
	PMC1	35	Female	---		44
	PMC2	20	Male	---		36
	PMC3	40	Female	---		77

Table 4. Demographic information of old samples used in receptors expression assays.

Assay	Type of sample (n)	Range of age (year)	Range of PMI (hr)
Radioligand binding assays	TLE-HS (5)	34 – 48	--
	PMC (7)	55 – 91	13 – 48
Receptor autoradiography	TLE-HS (9)	16 – 55	--
	PMC (10)	19 – 73	Up to 21

2.3. Immunohistochemistry (IHC) method development

2.3.A. Optimising antigen retrieval

In order to determine the optimal method for antigen retrievals that was compatible with different primary antibodies, various conditions were tested. The first was Heat Induced Epitope Retrieval (HIER), which used warm citric acid (CA), and the second was Enzyme-Induced Epitope Retrieval (EIER), where Protienase K (PK) was used at 37°C.

The 4 µm thickness formalin-fixed paraffin-embedded (FFPE) slides were dewaxed by incubating them in xylene overnight. The following day, they were rehydrated through a serial dilution of ethanol (100% [2X], 95%, 70%) and deionised water (dH_2O) for 10 min each at room temperature. Then, the slides that underwent antigen retrieval using HIER were incubated in boiling citric acid (10 mM, pH = 6) for 7 or 10 min before being cooled under running tap water (Table 5). On the other hand, slides that underwent EIER were incubated in 20 µg/ml PK (pH = 8) for different incubation times (5, 10, 20, and 30 min) at 37°C, followed by 10 min of incubation at room temperature, then washed with dH_2O (Table 5). After the antigen retrieval, the slides were incubated in 0.03% H_2O_2 for 30 min at room temperature to block the endogenous peroxidase activity. After that, the slides were washed with dH_2O, then phosphate buffer saline (PBS, 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH = 7.4) three times for 1 min each and incubated in permeabilization buffer (0.3% Triton X-100 in PBS, pH = 7.4) for 1 hour at room temperature. One hour later, the slides were incubated in blocking buffer containing 10% fetal bovine serum (FBS) and 0.3% Triton X-100 in PBS at room

temperature for one more hour, followed by primary antibodies (Table 6) being incubated overnight at 4°C.

The next day, the slides were washed extensively with permeabilization buffer three times for 10 min each and then covered with blocking buffer for 5 min at room temperature in order to block non-specific binding. As the next step, the slides were incubated with suitable biotinylated secondary antibodies (Table 6) for two hours at room temperature. After that, the slides were washed with permeabilization buffer and covered with Avidin-Biotin complex (ABC, ABCelite, Vector Laboratories, Peterborough, UK) for 30 min at room temperature. In order to visualise the target, staining with 0.025% diaminobenzidine (DAB, Vector Laboratories, Peterborough, UK) was used to produce a brown colour for the target. This was accomplished by incubating the slides on DAB for 6 min with 45-sec intervals between each of the two slides. Next, the slides were washed with permeabilization buffer for 1 min and then with dH_2O. Finally, the slides were dehydrated through serial dilution of ethanol and then cleaned with xylene. One drop of DePex (Sigma Aldrich, St. Louis, Missouri, US) was added to stabilise the cover-slip, and after drying the slides were visualised using a light microscope.

In order to confirm that the immunoreactivity signals are resulting from the immunological interaction between target epitopes and antibody-detection reagents complex prototypes, no primary antibody control hippocampal slides were included in the IHC experiments. In this study, the slides were subjected to the IHC staining protocol and the primary antibody was replaced with blocking buffer.

Table 5. Immunohistochemistry antigen retrieval step conditions:

IHC groups	H_2O in de- and rehydration steps	Antigen retrieval method	Incubation time I (min) / temperature	Incubation time II (min) / temperature
Citric acid / dH_2O	dH_2O / RT	10mM Citric acid pH=6	10 min / 90°C	None
Citric acid / DPC-treated H_2O	DPC-treated H_2O / RT			
Proteinase K / dH2O	dH_2O / RT	20 µg/ml Proteinase K pH=8	10 – 15 – 20 - 30 min / 37°C	10 min / RT
Proteinase K / DPC-treated H_2O	DPC-treated H_2O / RT			

RT; Room temperature.

Table 6. Immunohistochemistry and western blots antibodies concentrations:

Antibody	Type	Manufacturer	Product code	Dilution for IHC	Dilution for WB
Rabbit anti-human 5-HT_{1A} receptors	Polyclonal	Abcam	Ab79230	1:750	--
Mouse anti-human 5-HT_{2A} receptors	Monoclonal	BD PharMingen	556326	1:500	--
Rabbit anti-human 5-HT_3A receptors	Polyclonal	Abcam	Ab13897	1:600	1:750
Rabbit anti-human 5-HT_4 receptors	Polyclonal	Abcam	Ab60359	1:750	--
Rabbit anti-human 5-HT_7 receptors	Polyclonal	Abcam	Ab101913	1:750	--
Rabbit anti-human HPRT1 protein	Polyclonal	Abcam	Ab10479	--	1:2500
Mouse anti-human GFAP protein	Monoclonal	Chemicon	MAB3402	1:500	--
Mouse anti-human $P2X_7$ receptors (IgG2a)	Monoclonal	Collaborator from University of Melbourne	--	1:100	--
Goat anti-human GPR61 protein (C-terminus, GT2-135)	Polyclonal	Collaborator from University of Cambridge	--	1:6000	--
Biotinylated goat Anti-rabbit IgG (H+L)	--	Vector laboratories	BA-1000	1:500	--

Table 6. Continue.

Biotinylated horse Anti-mouse IgG (H+L)	--	Vector laboratories	BA-2000	1:500	--
Biotinylated rabbit Anti-goat IgG (H+L)	--	Vector laboratories	BA-5000	1:750	--
HRP-linked Anti-rabbit IgG (H+L)	--	Santa Cruz	SC-2004	--	1:2000

2.3.B. Optimising IHC staining for use in RNA extraction and qPCR

Due to the limited amount of samples, and in order to maximise the amount of information that could be obtained from these samples, RNA was extracted from IHC-stained slides. This was done by modifying the staining protocol and replacing dH_2O with DPC (diethylpyrocarbonate) treated water and using it to prepare buffers and solutions in addition to washing the slides (Table 5).

2.3.C. Optimised IHC protocol

The optimal conditions selected for IHC were based on the previous optimisation; 1) use HIER for 7 min for antigen retrieval for all of the sections used to study the expression of 5-HT $_{(1A-3A-4-7)}$ receptors, GFAP, $P2X_7$ receptors and GPR61. 2) Use EIER for 20 min at 37ºC then 10 min at room temperature for the sections used to study the expression of $5\text{-}HT_{2A}$ receptors.

2.3.D. Nuclear counterstain for IHC:

The nuclear counterstain was applied to the no primary control slides in order to avoid the overlap between the brown color of immunoreactivity signals and hematoxylin purple background that may affect the intensity of immunoreactivity signals. The no primary control slides were incubated in xylene to remove the mounting media and glass coverslip. The slides, then rehydrated through serial dilution of ethanol (100% [2X], 95% and 70%) followed by dH_2O for 2 min each. The slides were then incubated in hematoxylin (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 sec at room

temperature and the excess of the dye removed by dH_2O. At the end the slides were dehydrated using a gradual concentration of ethanol, then xylene for final clearing and visualized under light microscope.

2.3.E. Polymer Biotin-Tyramide ABC (PBTA) IHC staining technique

This method was conducted as described by Goto et al. (2015). First, the slides were deparaffinised and rehydrated. The sections were then placed in boiled citric acid (10mM, pH= 6) for antigen retrieval. After that, the sections were blocked with 10% FBS in a permeablising buffer for 1 hour, and then they were incubated with different concentrations (1:6000, 1:60,000, and 1:100,000) of goat anti-human GPR61 (table 6) for 48 hours at 4°C. These slides were then washed with a permeablising buffer and covered with a 10% FBS blocking buffer for 5 minutes at room temperature. Next, the slides were incubated for 30 minutes with a polymer staining solution (Histofine simple stain kit, Nichirei, Tokyo, Japan) at room temperature. The slides were then thoroughly washed with PBS and covered for 30 min with 1:50 tyramide signal amplification (TSA) biotin system (Perkin Elmer, Boston, MA, US) that had been freshly prepared according to the manufacturer's instructions. The slides were again washed with PBS and covered with ABCelite for 30 minutes at room temperature. At the end, similar to the previous protocol of IHC, the immunoreactivity signal was detected using DAB and visualised under a Nikon Eclipse E600 microscope, and pictures were taken using a Hamamatsu C4742-95 digital camera.

2.4. Receptors autoradiography

2.4.A. Tissue preparation

Frozen hippocampal blocks obtained from TLE-HS patients and PMC donors were cut into 20 µm sections in a coronal plane using cryostat under – 15 to – 19ºC and mount onto gelatin-coated slides and stored at – 80ºC until assayed.

2.4.B. [^{3}H]-*S*-zacopride autoradiography

Receptor autoradiography study of 5-HT_3 receptor was conducted by Prof Barnes and his colleague using on hippocampal sections obtained from TLE-HS and post-mortem control samples. This study was performed as described previously by Parker et al., (1996). In brief, frozen sections of the human hippocampus were allowed to equilibrate to 4ºC. The sections were incubated in Hepes/Krebs buffer (mM; Hepes, 50; glucose, 11; NaCl, 118; $NaHCO_3$, 25; KCl, 4.75; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; pH = 7.4) for 30 min at 4ºC in order to decrease the level of endogenous serotonin. Subsequently, the slides were incubated for 1 hr with 0.5 nM [^{3}H]-*S*-zacopride (78 - 83 Ci/mmol, NEN), to determine the total binding, and in the presence of 1 µM granisetron, to define the non-specific binding, at 4ºC. The slides were then washed in ice-cold Hepes/Krebs buffer followed by brief dH_2O before dried under a continuous stream of cold air. The dry slides then exposed to [^{3}H]-sensitive autoradiography film (Hyperfilm, Amersham, UK) in light resistance cassette with [^{3}H] standards (Amersham, UK) for 6 to 10 weeks. The films were developed using Kodak LX 24 developer and Kodak Unifix for 5 min each. The results were quantify using Image Analysis (MCID, Imaging Research Inc.). To enhance resolution, the slides were exposed to photographic

emulsion (K5 or G5; Ilford) that had been dried onto coverslip before developed the photographic emulsion using D19 developer (Kodak, 5 min) and AL4 fixer (Kodak, 5 min). The result was statistically analysed using unpaired t-test (Prism 5, GraphPad).

2.5. Radioligand binding assays

2.5.A. 5-HT_3 receptors radioligand binding assay

Hippocampal specimens obtained from TLE-HS patients and PMC donors were snap frozen and stored at - 80ºC. In order to prepare the radioligand binding homogenate, the tissue was defrosted in 50 mM Tris buffer (pH = 7.4) and homogenated using Polytron blender at 4ºC. After that, the tissue homogenate was pelleted by centrifugation at 48,000 x g for 10 min at 4ºC and the resulting pellet was resuspended in Tris buffer and re-centrifuged to reduce the concentration of the endogenous 5-HT. The radioligand binding homogenate was prepared by resuspending the pellet in Tris buffer to a final concentration equal to 100 mg/ml according to the original wet weight of the tissue. The binding assay was conducted as described previously by Bufton et al., (1993). Briefly, duplicate tubes contain 150 µl of 1 nM - 10 µM competing drug (ondansetron, S-zacopride and Tropisetron), or 50 mM Tris (pH = 7.4, total binding) and 100 µl of ([^{3}H]-granisetron, 0.1 – 10 nM for saturation studies, 0.8 – 1.1 nM for competition studies, NEN, 82 – 83.5 Ci/mmol, Perkin-Elmer, Buckinghamshire, UK). Ondansetron was used as competing drug in saturation study to determine the non-specific binding. To initiate the binding, 250 µl tissue homogenate was added to each tube and allowed to react for 90 min at room temperature. The reaction was terminated

by rapid filtration and washing through Whatman GF/B filters under vacuum. The remaining radioactivity on the filters assessed using liquid scintillation.

2.5.B. Single concentration 5-HT_3 receptors radioligand binding assay

In order to detect an approximate density of 5-HT_3 receptors in the hippocampus of patients with TLE-HS and postmortem control samples, and as a result of the limited weight of human tissue samples, a single-concentration radioligand binding assay was conducted using 6 nM [^{3}H]-granisetron. The concentration of radioactive granisetron was selected based on the radioligand saturation binding assay results which, at approximately this concentration, the 5-HT_3 receptors in hippocampal homogenate begin saturated.

Human hippocampal tissue was homogenized in 600 µl ice cold Tris buffer (25 mM, pH = 7.4) using sonication prior washing with Tris buffer twice at 48,000 x g for 15 min (4ºC) to minimize the level of endogenous 5-HT. The formed pellet was resuspended in a fresh Tris buffer. Protein concentration was measured using a Bradford assay (TLE-HS = 7 – 12.5 mg/ml; PMC = 2.5 – 14.5 mg/ml). The total binding activity was performed in triplicate, and the non-specific (defined by 10 µM ondansetron) was in duplicate due to the limited weight of samples. Each tube contained 100 µl Tris buffer or ondansetron, 100 µl [3H]-granisetron (approximately 6 nM, Perkin-Elmer, Buckinghamshire, UK) and 100 µl hippocampal tissue homogenate. The reaction was allowed to be at equilibrium for 1 hr at room temperature, then was terminated rapidly by filtration using a GF/B filter (Semat Technical (U K) Ltd, St Albans, UK) presoaked in 0.3% Tris/polyethylenimine (Tris/PEI) followed by washing with ice-cold Tris three

times. Finally, a 4 ml scintillation fluid (Optiphase HiSafe 2; Perkin Elmer, Waltham, Massachusetts, US) was added to each tube, and the level of radioactivity remaining on the filter paper was detected using liquid scintillation counting (Tri-carb 1500 TR Liquid Scintillation Analyser, Packard).

2.5.C. Radioligand binding assay data analysis

The saturation binding assay data was analysed using computerised interactive curve fitting program (KaleidoGraph, Synergy software, Pennsylvania, US), according to the following equation:

$$b = (Bmax \times [L]^n)/([L]^n + [K]^n)$$

Where, *b* = bound radioligand; *Bmax* = maximum binding activity at equilibrium; *L* = free molar concentration of radioligand; *n* = Hill coefficient; *K* = molar equilibrium dissociation constant (Bufton, et al., 1993).

Competing drugs affinity (Ki) calculated according to the following equation:

$$Ki = (IC_{50})/(1 + \left(\frac{[L]}{Kd}\right))$$

Where, IC_{50} = molar concentration of competing drug that decreases the specific binding by 50%; L = free molar concentration of radioligand; Kd = dissociation constant of radioligand at equilibrium (Bufton et al., 1993). The saturation binding assay data was statistically analysed using unpaired t-test and Single concentration binding assay statistically analysed using Mann Whitney U test (Prism 5, GraphPad).

2.6. Total RNA extraction, method development

2.6.A. Total RNA extraction from FFPE (stained and unstained) samples

- **Total RNA extraction using RNeasy FFPE kit**

In order to extract total RNA from 4 µm thickness FFPE hippocampal sections obtained from nine different groups (Table 7), the RNeasy FFPE kit (Qiagen, Manchester, UK) was used, based on the manufacturer's instructions. In summary, the tissue was scraped and dewaxed by using xylene, then centrifuged at 15,000 rpm for 2 min to pellet the sample. Next, the supernatant was removed and the pellet was washed with 100% ethanol and pelletted by centrifugation for 2 min at 15,000 rpm. Then, the pellet was allowed to dry at room temperature. After that, the pellet was re-suspended in 150 µl buffer PKD and 10 µl PK, then incubated for 15 min at 56°C in order to activate PK to digest the proteins.

The sample was incubated for another 15 min at 80°C in order to inactivate the PK and terminate the digestion. In the next step, the sample was cooled in ice for 3 min and then centrifuged for 15 min at 20,000 x g. Next, the supernatant was removed and the pellet was re-suspended in DNase buffer and DNase I stock (16 µl and 10 µl, respectively) and incubated at room temperature for 15 min. After that, buffer RBC (320 µl) and 100% ethanol (720 µl) were added and mixed, then the sample was passed through the RNeasy MinElute spin column and centrifuged for 15 sec at 11,000 x g, followed by 2 min of centrifugation at the same speed after adding 500 µl buffer RPE. Finally, the RNA was collected in nuclease-free water (25 µl).

- **Total RNA extraction using RNeasy FFPE kit and Poly-A-RNA Carrier**

The total RNA was extracted from FFPE samples using the RNEasy FFPE kit (Qiagen, Manchester, UK), with a modification based on the manufacturer's instructions. These modifications include using a poly-A RNA carrier (Qiagen, Manchester, UK), which was added to the FFPE tissue lysate during the extraction procedure.

Table 7. Groups of samples that used for optimising mRNA extraction from IHC stained samples based on staining steps:

Group number	Dewaxing and rehydration		Antigen retrieve				Staining
	dH_2O	DPC-H_2O	10mM Citric acid pH=6		20 µg/ml Proteinase K pH=8		Rabbit anti-human 5-HT_{1A} receptors
			In dH_2O	In DPC-H_2O	In dH_2O	In DPC-H_2O	
I*							
II	√		√				
III		√		√			
IV	√				√		
V		√				√	
VI	√		√				√
VII		√		√			√
VIII	√				√		√
IX		√				√	√

* Group I, subjected to dewaxing procedure followed by RNA extraction.

2.6.B. Total RNA extraction from frozen samples

Three different RNA extraction techniques were conducted in order to identify the most suitable method to extract a high quantity and quality of RNA from frozen brain samples. These methods included the use of Triazole, the RNeasy Mini Kit, and the Triazole/Rneasy Mini Kit. Given the limited number of available human samples, frozen rat brain samples were used.

- **Total RNA extraction using Triazole**

Approximately 5 mg rat brain tissue was homogenised with 1 ml of Triazole (Ambion Life Technologies), then incubated for 5 min at room temperature. At this point, 200 µl of chloroform were added and shaken vigorously for 15 sec, then stored at room temperature for 7 min before being centrifugated at 12,000 x g for 15 min at 4°C. The sample was divided into three layers, with the RNA present in the upper aqueous layer. This layer was transferred into RNase-free eppendorf and the RNA was precipitated by adding 500 µl of isopropanol (Sigma Aldrich, St. Louis, Missouri, US) and mixed gently, then incubated for 5 min at room temperature. After that, the RNA was pelleted by centrifugation at 12,000 x g for 8 min at 4°C. Next, the pellet was washed with 75% ethanol and centrifugated at 7500 x g for 5 min at 4°C. Finally, the pellet was allowed to dry at room temperature for 20 min and then dissolved in RNase-free water (PrimerDesign Ltd, Southampton, UK).

- **Total RNA extraction using RNeasy Mini Kit**

The RNA was extracted from approximately 5 mg rat brain using the RNeasy Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Briefly, the sample was lysated by homogenating the tissue with 350 µl buffer RLT, then centrifuged at 15,000 rpm for 3 min. After that, the supernatant was transferred into the RNase-free tube and the RNA was precipitated by adding 350 µl of 75% ethanol. Next, the sample was passed through the RNeasy MinElute spin column and centrifugated at 8000 x g for 15 sec. Then, the 700 µl buffer RW1 was added and centrifugated in the same manner as the previous sample. Then, the column was washed with buffer RPE and centrifugated for 2 min at 8000 x g. At the end, the RNA was collected in 25 µl nuclease-free water.

- **Total RNA extraction using Triazole / RNeasy Mini Kit**

This method is mimic the RNeasy Lipid Tissue kit (Qiagen), where the lysate buffer in the RNeasy Mini Kit (buffer RLT) was replaced with Triazole and chloroform. This extraction was conducted by homogenising approximately 5 mg of frozen rat brain samples with 1 ml of Triazole and incubating for 5 min at room temperature followed by chloroform for phase separation. After that, the supernatant was transferred into the RNase-free tube and the extraction procedure was conducted as previously described in the section on RNA extraction using the RNeasy Mini Kit.

2.6.C. Optimised total RNA extraction from frozen

Among these different RNA extraction methods, the most suitable way to extract RNA from brain tissue was Triazol / RNeasy mini Kit. The wet weight of each human hippocampal samples used was approximately 100 mg.

2.6.D. RNA quantity, purity, and integrity assessment

The RNA's quantity and purity was assessed by using the Nanodrop™ 1000 Spectrophotometer (Thermoscientific, Wilmington, US) in which a 2 µl RNA sample was used. The RNA was considered pure RNA if the 260/280 absorption ratio was close to 2, according to the manufacturer's instructions.

In terms of the RNA's integrity, the samples were assessed by using the Agilent 2100 Bioanalyser System (Agilent Technologies, Berkshire, UK), with a 1 µl (5 ng/µl) sample used for the very low RNA concentration (< 5 ng/µl). A vacuum concentrator was used to concentrate the sample and then it was diluted into the recommended concentration using RNA-free water. Based on the manufacturer's instructions, the RNA integrity number (RIN) was in the range of 1 to 10, with the high number representing the higher integrated RNA sample. This number represents the ratio between 28S/18S ribosomal RNA.

2.7. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

To evaluate the change in the level of expression of mRNA in TLE-HS samples compared to PMC, qRT-PCR was conducted in duplex using RNA extracted from the hippocampal samples due to the limited amount of extracted materials available. This technique was performed using TaqMan Style (hydrolysis probes) primers synthesised by PrimerDesign Ltd (Southampton, UK) (Table 8). These primers synthesised close to 5' end. The Precision™ OneStep 2x qRT-PCR MasterMix with low ROX was obtained from the same company and the qRT-PCR was performed according to the manufacturer's instructions (Table 9). The housekeeping gens were selected based on the comparative study conducted by Maurer-Morelli (et al., 2012). The target genes were relatively quantified and statistically analysed using REST2009 (Qiagen, Limburg, Netherlands). This software calculates the relative quantification of the interest gene based on the efficiency of PCR reaction and the deviation of the crossing point of both sample and control group using Pair Wise Fixed Reallocation Randomisation Test© (Pfaffl et al., 2002).

Table 8. RT-qPCR primers and probes sequences, *A*: Human. *B*: Rat.

A

Target gene symbol	Type of gene	Primer Sequences (position)	Probe Sequence (position)
5-HT_{1A}	GI	*Sense primer:* CCGAGAGAGGAAGACAGTGAA (1587) *Anti-sense primer:* GCAGAAGGGCAGAACAAGAG (1686)	CACCTTCATCCTCTGCTGGCTGCC (1629)
5-HT_{2A}	GI	*Sense primer:* ATGCCAAGACAACAGATAATGACT (1295) *Anti-sense primer:* GCAACTAGCCTATCACACACAG (1419)	CACCTTTTCATTCACTCCGTCGCTATTGTCT (1395)
5-HT_{3}A	GI	*Sense primer:* CAGGGAATGCCAGGGACAT (1656) *Anti-sense primer:* GCTTGTGATTGCTGAGATGAATT (1739)	AAGTCCCGTGCCCTGTTTCCAATGC (1563)
5-HT_{4}	GI	*Sense primer:* GGGCATTGAAATCCACTCACT (9) *Anti-sense primer:* GCACCACCTTCTCCACTGA (111)	CCGAAACCCTCCTCAGAACTCACATTAGCAT (91)
5-HT_{7}	GI	*Sense primer:* TTCTCTCCGTCTGGCTTCTC (851) *Anti-sense primer:* GCACACCTTATCATTTACATTCT (936)	CCGCCTCCATCACCTTACCTCCACTCTT (872)

$P2X_7$	GI	*Sense primer:* CTTGAAGTTAAAGACTCCTGCTAAAA (14) *Anti-sense primer:* GTGACAGCCTCCCTCCCT (142)	CCACAGCAAGCCCCCTCCCAGTAAC (94)
GPR61	GI	*Sense primer:* CTTTCGAATCCCAGGCCAGA (1916) *Anti-sense primer:* GCAGGACGGAGGTAGCTG (2014)	AGCAGCAACTCACCAGCGACATCATC (1963)
HPRT1	RG	*Sense primer:* TTAGTGTTTCAGTAATGTTGACT (1301) *Anti-sense primer:* ATTTTTGGGAATTTATTGATTTGCAT (1411)	CCAGTGAATCTTTGTCAGCAGTTCCCTTT (1354)
ENO2	RG	*Sense primer:* CTTGGCTTACCTGACCTCTTG (1669) *Anti-sense primer:* ATCCTCATTCACATTTCTAGTTTCTG (1799)	CTCGCCCTCCTTTCTGTGCCCTACTCA (1699)

B

Target gene symbol	Type of gene	Accession number	Sequence length (bp)
18S	RG	NR_046237	98

Table 9. qRT-PCR amplification thermocycler programmes, according to the Precision™ OneStep 2x qRT-PCR MasterMix manufacturer instructions

Step	Temperature (°C)	Time	Note
Reverse transcription	55	10 min	Steps 3 and 4 repeated for 40 cycles.
Polymerase enzyme activation	95	8 min	
Denaturation	95	10 sec	
Data collection	60	60 sec	

2.8. SDS-PAGE western blot

2.8.A. Gel preparation

- **Preparation of separating gels**

The gel was prepared a day in advance of the western blot experiment in an acrylamide percentage equal to 12 (molecular weight range 10 - 70 kDa), based on the target proteins sizes (Table 10). The following components were added in the Falcon tube to prepare two gels on the same sequence: 3.35 ml dH_2O, 2.5 ml 1.5 M Tris (pH = 8.8), 100 µl 10% SDS (sodium dodecyl sulphate), 4 ml acrylamide, 50 µl 10% APS (ammonium persulfate), and 10 µl TEMED (tetramethylethylenediamine). Then, the gel was loaded in the glass casting plate and 200 µl dH_2O saturated butanol was loaded over to push the gel down and maintain the uniformity of the gel level in each part of the cast. Then, the gel was kept at room temperature for 15 min.

Table 10. Target proteins molecular weights:

Target Protein	Gene Symbols	Molecular weight (kDa)	Reference
5-HT_3A	HTR3A	55	Phosphosite.org A. (n. d.)
HPRT1	HPRT1	25	Phosphosite.org B. (n. d.)

- **Preparation of stacking gels**

In order to prepare two stacking gels, the following were added in the falcon tube on the same sequence: 3.05 ml dH_2O, 1.25 ml 0.5 M Tris (pH = 6.8), 50 µl 10% SDS, 670 µl acrylamide, 25 µl 10% APS, and 5 µl TEMED. After that, a well-forming comb was placed then incubated for 20-30 min until the gel was gelated. Finally, the gels were wrapped with cling film and stored in the refrigerator until use.

2.8.B. Protein extraction

The tissue was harvested with 100 µl Tris buffer (25 mM) by using a sonicator at full power for 1-2 seconds. Then, 5 µl tissue homogenate was used to measure the protein concentration. A 50 µl tissue homogenate was added to an equal volume of the RIPA buffer (radioimmunoprecipitation assay buffer) and mixed well. After that, the laysate was mixed with the sample buffer (0.5 M Tris/HCl pH=6.8, 12% [w/v] SDS, 25% [v/v] glycerol, 5 mM EDTA, and a small amount of bromophenol blue) that contained 10% β-mercaptoethanol. Then, the sample was placed in boiling water for 10 min to denature the protein before chilling on ice.

2.8.C. Gel electrophoresis and western blot

In order to perform gel electrophoresis, the electrophoresis tank was prepared and filled with electrophoresis running a buffer that contained 25 mM Tris/HCl pH = 8.3, 192 mM glycine, and 1% (w/v) SDS. After that, the protein molecular weight markers (Color Prestained Protein Standard, Biolab, Ipswich, US) were loaded and protein

samples were loaded and electrophoresis was run at a 35 mA constant current, 400 v, for 90 min. Later, the gel was removed and allowed to equilibrate in transfer buffer (25 mM Tris/HCl pH = 8.3, 150 mM glycine, 20% [v/v] methanol, and 0.1% [w/v] SDS) for 15 min at room temperature. After that, the gel blot was transferred to a PVDF (polyvinyl difluoride) membrane (Millipore, Immobilon, Watford, UK) using a semi-dry transfer blotter, which ran under a 1.2 mA/cm2 constant current for 1 hour.

When the transfer was finished, the PVDF membrane was blocked in blocking buffer (10% non-fat milk in washing buffer [10 mM Tris/HCl (pH = 7.5), 100 mM NaCl, and 1% (v/v) Tween 20]) for 2 hours at room temperature. The membrane was then incubated with primary antibody diluted in blocking buffer (Table 6) are incubated overnight on an orbital shaker.

The next day, the primary antibody was removed and the membrane was washed extensively with washing buffer, before incubation with HRP-linked secondary antibody (Table 5) for two hours at room temperature. Later, and after removing the secondary antibody, the membrane was washed and covered with ECL solution (Biological Industries, Kibbutz Beit-Haemek, Israel) for 5 min at room temperature. The membrane was placed in the film cassette and exposed to the Hyperfilm (Hyperfilm MP, Amersham) before being developed using an ECOMAX™ X-ray Processor.

2.9. Protein concentration measurement, Bradford protein assay

A 5 µl of tissue homogenate was used to measure the protein concentration in 1:10 and 1:100 tissue dilutions prepared in 25 mM Tris/HCl (pH = 7.4). For each sample, two cuvettes were used in which 1 ml of Bradford reagent (Sigma Aldrich, St. Louis,

Missouri, US) was added per cuvette and mixed with 20 µl of each tissue dilute, separately. A third cuvette was used as a blank wherein the tissue homogenate was replaced with 20 µl 25 mM Tris/HCl. Five min later, after allowing the dye to reach equilibrium, the sample concentration was measured using a spectrophotometer (Biophotometer Plus, Eppendorf).

2.10. Data analysis and statistical tests

The data were analysed by Mann-Whitney U for two groups comparison and Kruskal-Wallis test followed by Dunn multiple comparison for multiple groups after normalisation test was preformed using IBM SBSS statistics 23 software in which the level of significance is $p \leq 0.05$.

CHAPTER 3

Results

3. Results

3.1. Experimental research development

3.1.A. Immunohistochemistry experimental development

As a result of the limited amount and availability of the human hippocampus, and in order to maximise the amount of information that can be obtained from it, two different antigen retrieval techniques (HIER [10mM CA, pH= 6] and EIER [20 µg/ml PK, pH= 8]) were used in order to examine the possibility of using the same IHC-stained sections for other downstream applications such as RT-qPCR.

- **Suitability of antigen retrieval techniques**

Heat-induced antigen retrieval was tested on TLE-HS FFPE human hippocampal samples using several primary antibodies, and the results show that the HIER technique is suitable because it gave a clear immunoreactivity signal with the primary antibodies that had been tested.

The EIER technique was selected for the process in order to prevent the possible negative effects of the high temperatures used in the HIER technique on the total amount of RNA extracted from the FFPE sections. This technique was optimised with a rabbit anti-human 5-HT_{1A} antibody at 37°C for different incubation periods (10, 15, 20, and 30 minutes) to give a result that was close to the HIER technique without detaching the sections from the slides before the compatibility of this technique with the other primary antibodies had been tested.

The results of the experiment show that there is a relationship between the incubation time in the PK and the quality of the immunoreactivity signal of the 5-HT_{1A} receptors. However, this relationship was associated with a higher risk of detaching the sections from the slides. As a result, the 20-minute incubation time of the slides in PK at 37°C was selected as the optimum incubation time (Figure 2). This incubation period produced a reasonable immunoreactivity signal compared to the HIER technique, with a low risk of tissue detachment from the slides (Figure 3). In terms of signal quality and background intensity, the EIER technique was associated with a relatively low immunoreactivity signal and a slightly higher background when compared to HIER (Figure 3).

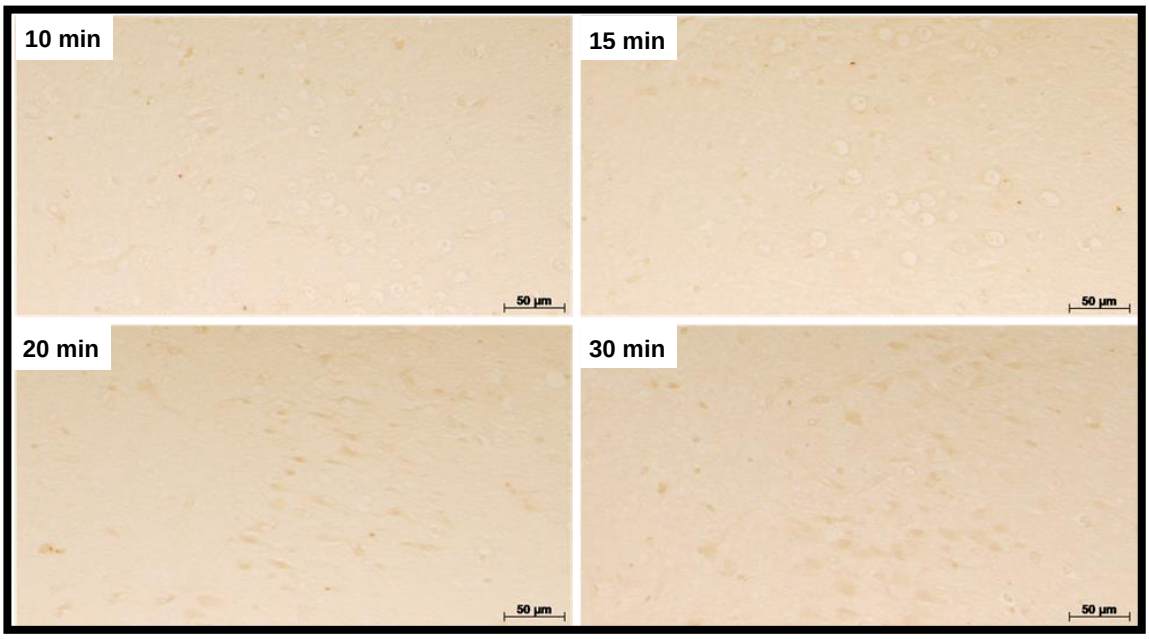

Figure 2. Effect of proteinase K incubation times on immunohistochemistry staining of 5-HT_{1A} receptors in the dentate gyrus of human TLE-HS samples.
10 minutes (no staining signal); 15 minutes (no staining signal); 20 minutes (moderate staining signal); 30 minutes (moderate staining signal); scale bar = 50 µm.

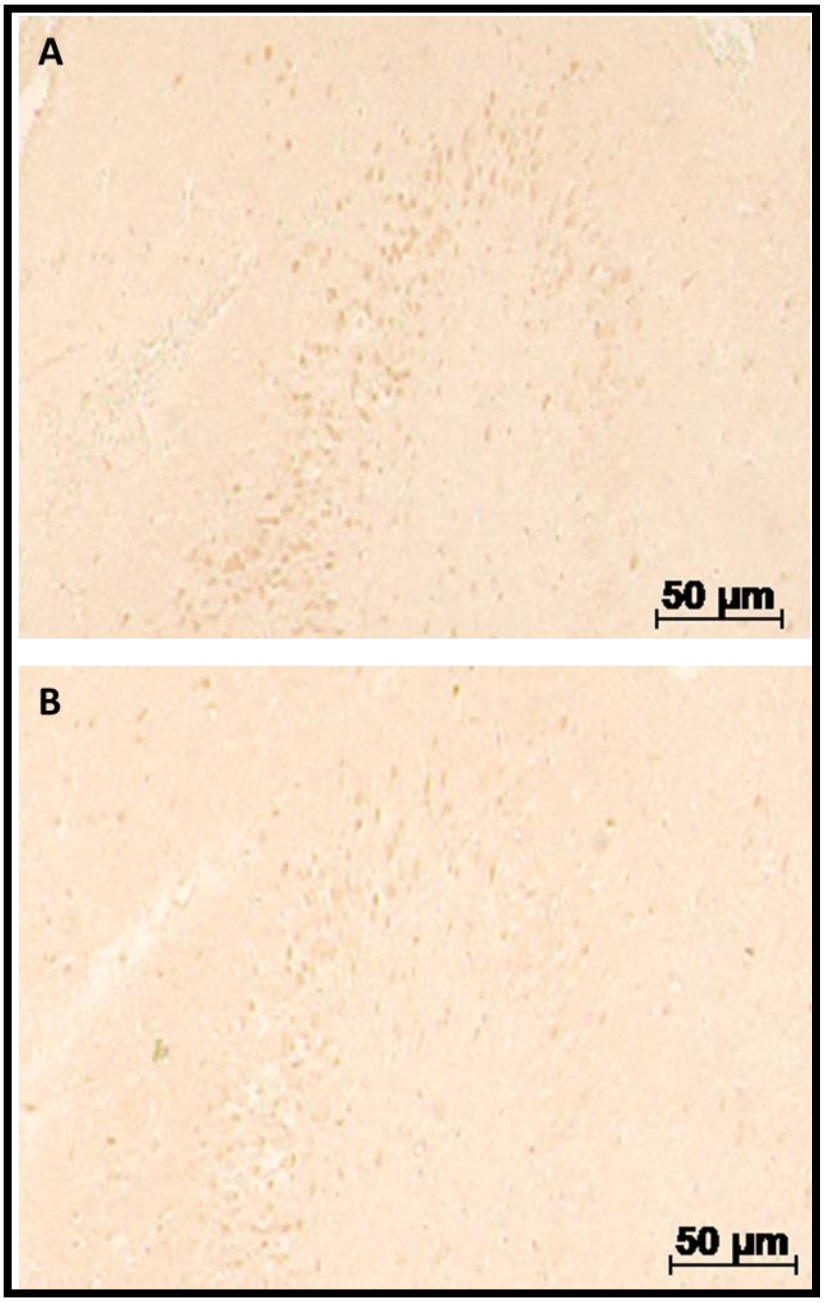

Figure 3. Effect of heat-induced epitope retrieval (boiled citric acid/dH_2O, 7 minutes) and enzyme-induced epitope retrieval (proteinase K/dH_2O) on immunohistochemistry staining of 5-HT_{1A} receptors in the dentate gyrus of human TLE-HS samples.
(A) boiled citric acid/dH_2O (7 minutes, dark); (B) proteinase K/dH_2O (37°C, 20 minutes, moderate); scale bar = 50 µm.

- **Compatibility of EIER with target protein primary antibodies:**

Regarding the compatibility of the optimised protocol of the EIER technique with different anti-human (5-HT_{1A} – 5-HT_{2A} – 5-HT_3A – 5-HT_4 – 5-HT_7) primary antibodies, the results, shown in Figure 4, confirmed a strong specific 5-HT_{2A} immunoreactivity signal in the hilum of the TLE-HS hippocampal section. On the other hand, a moderate specific 5-HT_{1A} immunoreactivity signal was detected in the DG. Furthermore, 5-HT_4 and 5-HT_7 receptor-immunoreactivity signals were detected with relatively low and very low level, respectively, however, no 5-HT_3A signal was detected using this antigen retrieval protocol (Figure 4).

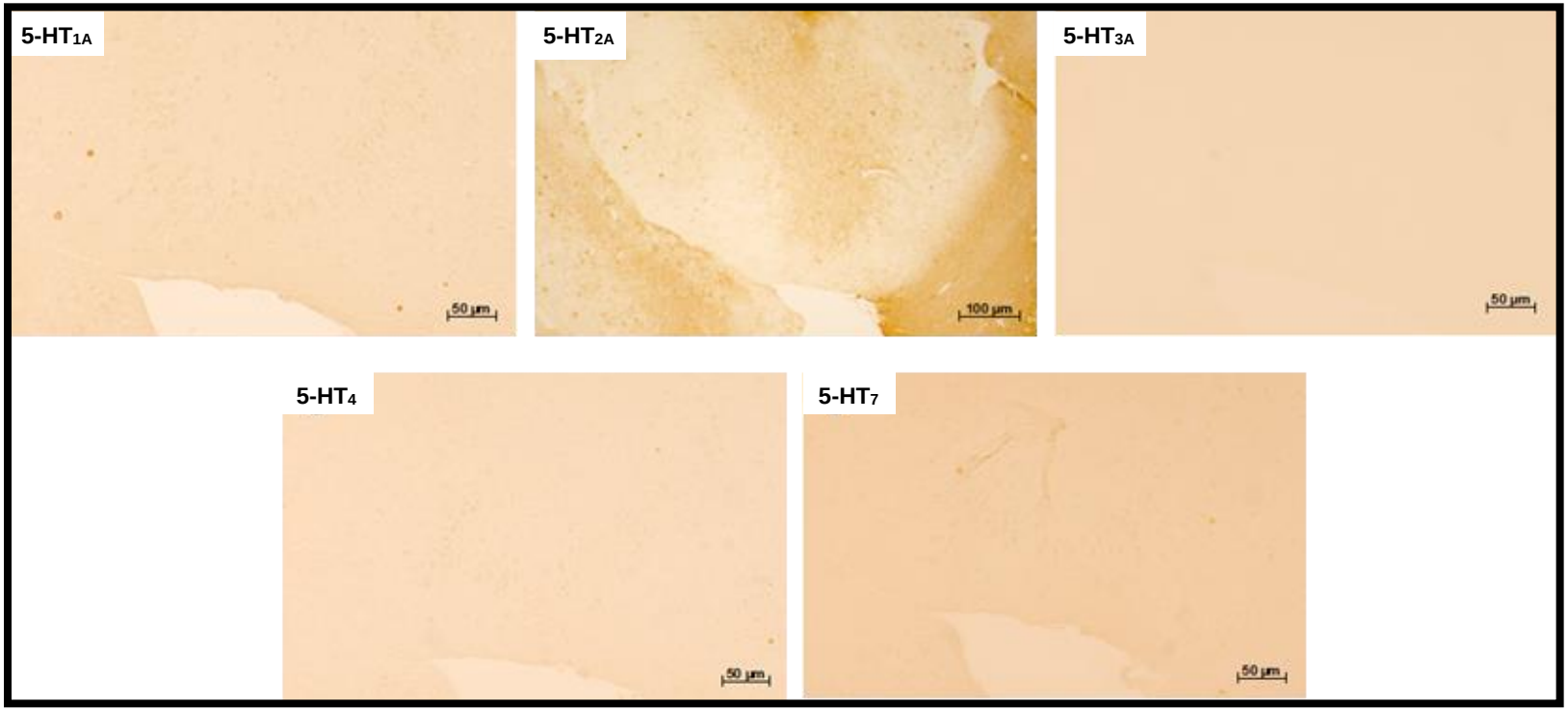

Figure 4. Suitability of the proteinase K antigen retrieval protocol for immunohistochemistry with different primary antibody staining in the dentate gyrus of human TLE-HS samples.
5-HT_{1A} antibody (moderate staining signals); 5-HT_{2A} antibody (good staining signals); 5-HT_3A antibody (no signals), 5-HT_4 antibody (low staining signals); 5-HT_7 antibody (very low staining signals); scale bar = 50 and 100 for 5-HT_{2A} staining.

- **Effect of using dH_2O and DPC-treated dH_2O immunoreactivity signals:**

In order to decrease the risk of RNA degradation and increase the yield of total RNA extracted from the FFPE sample, the IHC staining buffers and solutions were prepared using DPC-treated H_2O and processed under nuclease-free conditions. Figure 5 shows that replacing dH_2O with DPC-treated H_2O has some impact on the quality of the 5-HT_{1A} immunoreactivity signal depending on the antigen retrieval method that was used. DPC-treated H_2O significantly decreases the background and increases the intensity of the specific 5-HT_{1A} immunoreactivity signal in the section that was treated with PK for antigen retrieval, compared to the dH_2O when it was used to prepare the staining solutions (Figure 5). On the other hand, DPC-treated H_2O significantly decreased the immunoreactivity signal of 5-HT_{1A} and slightly improved the background in the section that was subjected to the HIER technique, compared to using dH_2O to prepare staining solutions (Figure 5).

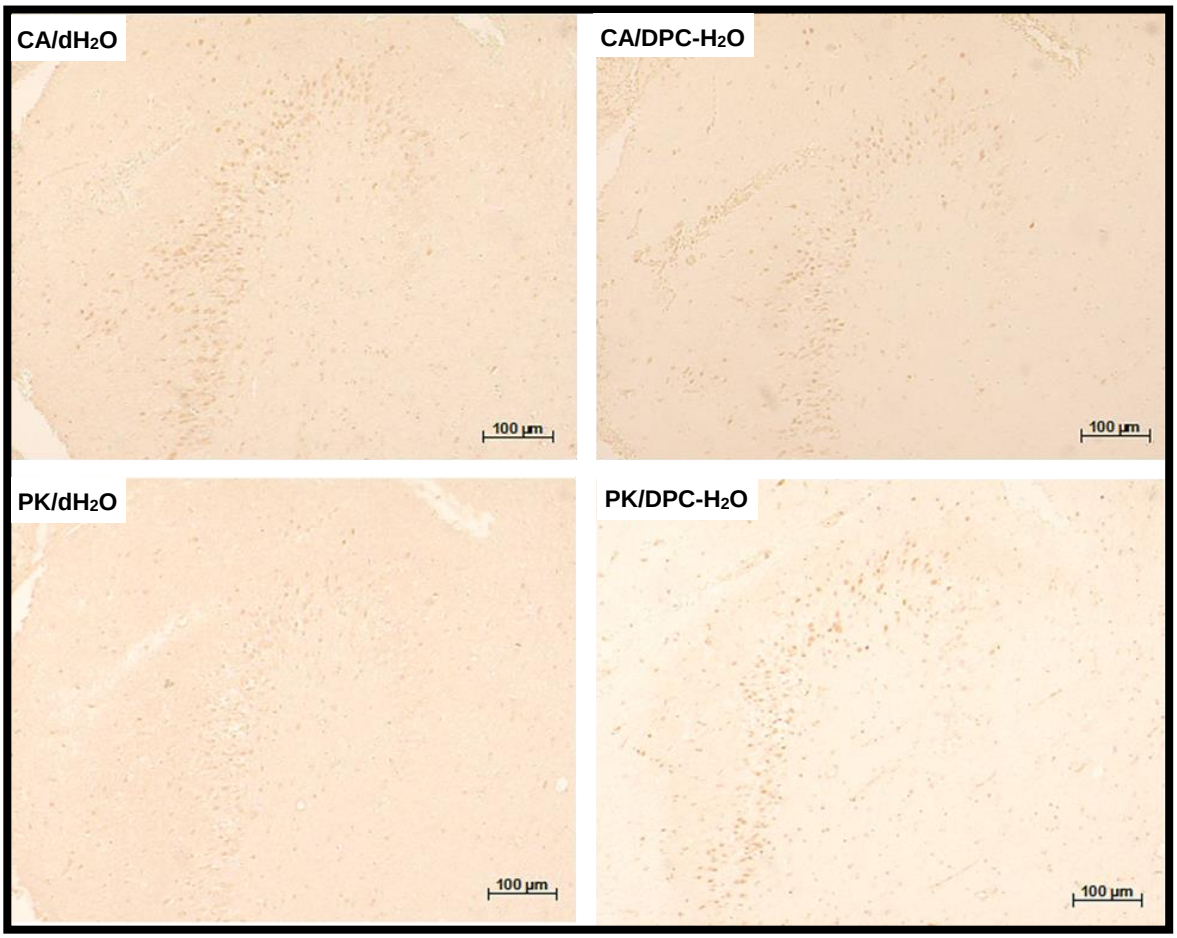

Figure 5. The effect of using dH_2O or DPC-treated H_2O with citric acid and proteinase K on the immunohistochemistry staining quality of 5-HT_{1A} receptor-staining in the dentate gyrus of human TLE-HS samples.
CA= citric acid; *PK*= proteinase K; *DPC-H_2O* = DPC-treated H_2O; scale bar = 100 µm.

- **Polymer Biotin-Tyramide ABC (PBTA) IHC staining technique**

Because there was only a limited amount available of the goat anti-human GPR61 antibody (generated by a collaborator from the University of Cambridge), this staining method was conducted in order to increase the sensitivity of immunohistochemistry primary antibodies to the target antigen. The results (Figure 6) show that the sensitivity of the signal was increased for approximately 10 times than when DAB only used to enhance the immunoreactivity signal. However, the resolution was clearly decreased (figure 6).

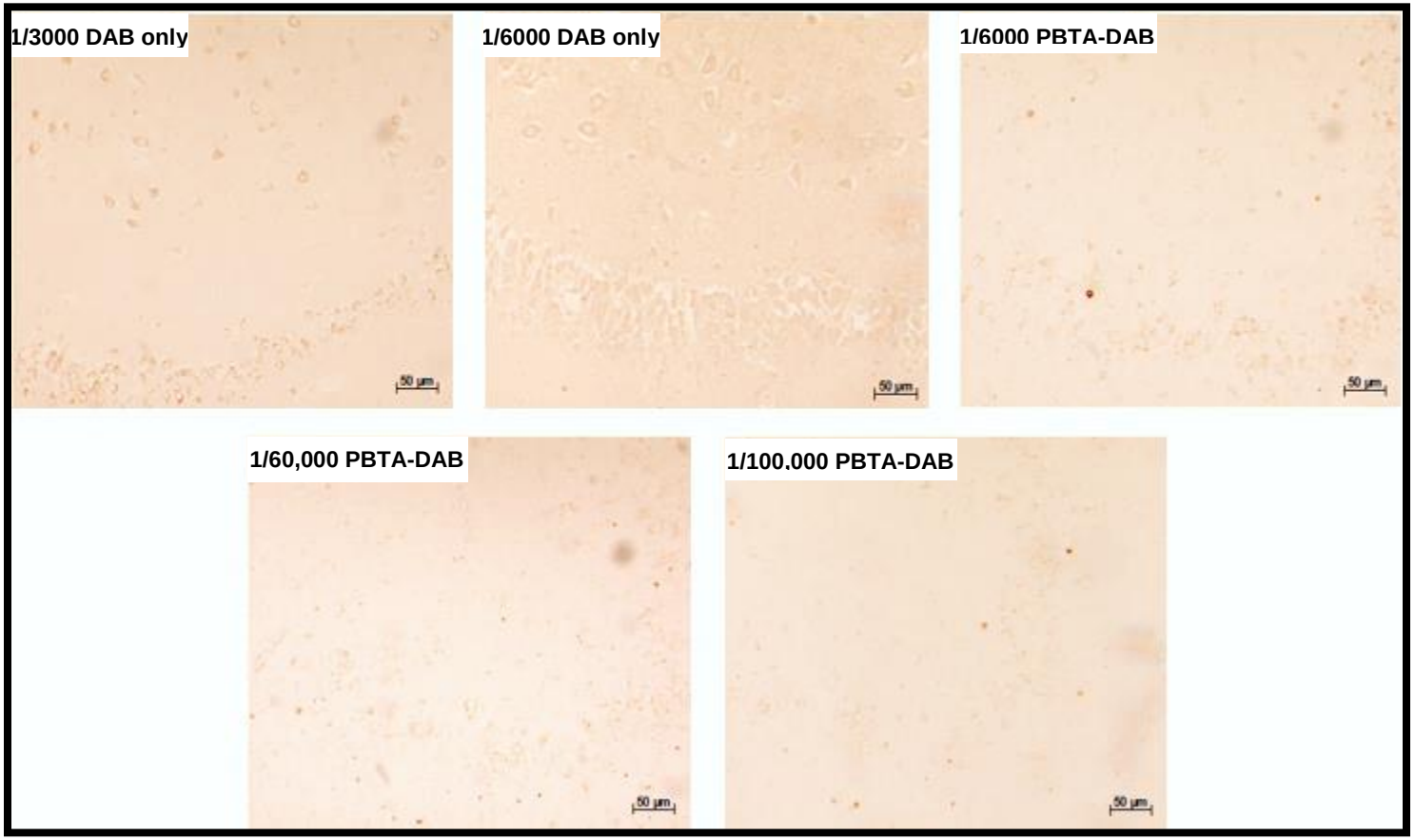

Figure 6. Effect of DAB and PBTA-DAB staining techniques on the sensitivity of goat anti-human GPR61 antibody to recognise the target antigen on human TLE-HS hippocampal samples. Scale bar 50 µm.

3.1.B. Total RNA extraction

- **Total RNA extraction from FFPE sections**

In order to examine the most suitable method to maximise the RNA yield from the samples, the total RNA was extracted from FFPE TLE-HS human hippocampal sections using two different methods: the RNEasy FFPE kit and the RNEasy FFPE kit with poly-A RNA carrier. In Figure 7, the result does not demonstrate any significant differences between the total RNA yields from the FFPE sections when using the RNEasy FFPE kit, either with or without using the poly-A RNA carrier (p= 0.45). Furthermore, the addition of poly-A RNA carrier did not show any significant improvement on the RNA integrity number, which was kept under 3, with and without the carrier (p= 0.239, Figure 8). In terms of the effect of using poly-A RNA carrier on the RT-qPCR Ct value of the housekeeping gene HPRT1, the result does not show any significant differences between the Ct values of HPRT1 in the sample that was extracted with or without using the carrier (p= 0.974, Figure 9).

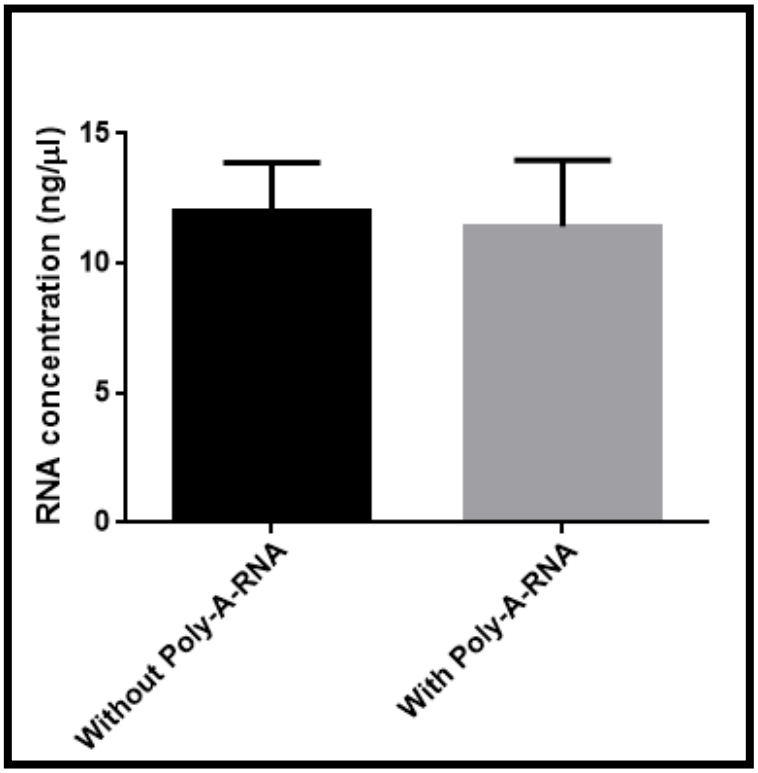

Figure 7. Effects of using poly-A RNA carrier on the RNA yield that was extracted from FFPE human TLE-HS hippocampal samples.
without poly-A RNA, n= 11; with poly-A RNA, n= 11; *p*= 0.45.

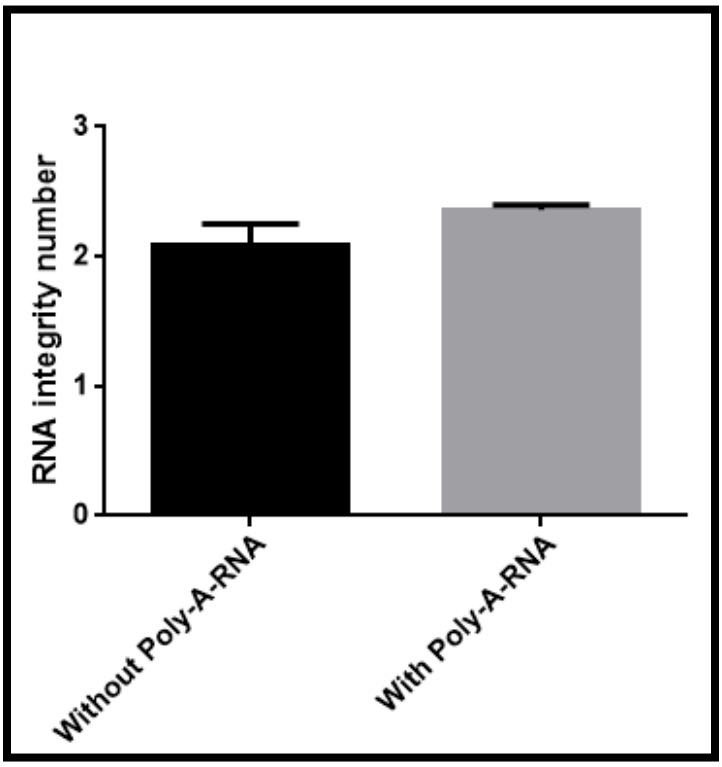

Figure 8. Effects of using poly-A RNA carrier on the RNA integrity (RIN) on the RNA that was extracted from FFPE human TLE-HS hippocampal samples.
Without poly-A RNA, n= 11; with poly-A-RNA, n= 11; *p*= 0.239.

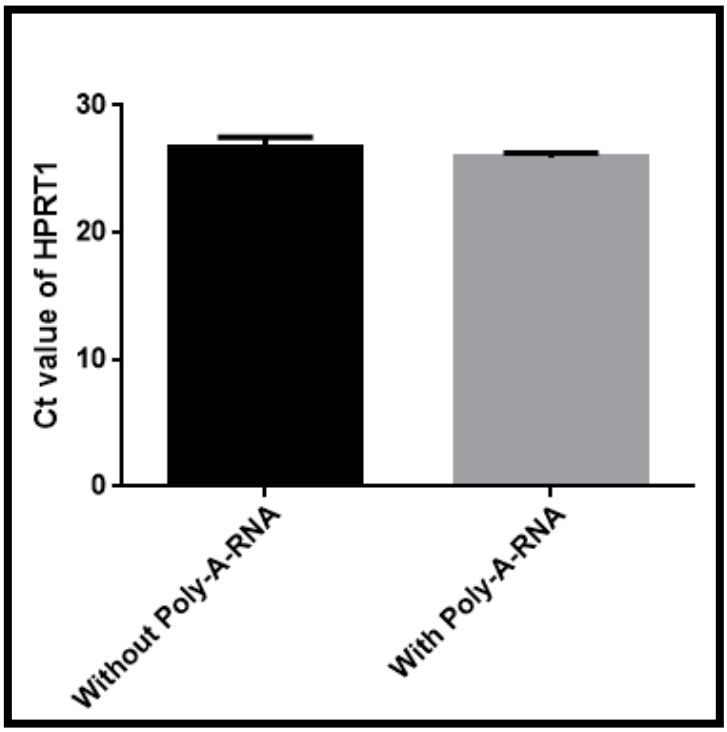

Figure 9. Effect of using poly-A RNA carrier on the HPRT1 RT-qPCR Ct value of the RNA that was extracted from FFPE human TLE-HS hippocampal samples. Without poly-A RNA, n= 11; with poly-A RNA, n= 11; *p*= 0.974.

- **Effect of IHC staining protocols on total RNA extraction**

Regarding the total RNA quantity that was extracted from IHC-stained and non-stained FFPE TLE-HS human hippocampal samples, Figure 10 demonstrates that, among all of the 9 different groups that were previously described in table 6, the non-stained group that was subjected to PK/dH_2O was associated with a significantly higher total RNA yield when compared to the rest of the groups (p= 0.02, Figure 10). Moreover, when compared to the total RNA yield from non-stained, antigen retrieval only and IHC-stained sections, the results show that a significantly higher RNA yield was obtained from the sections that were subjected to antigen retrieval without staining (regardless of the type of antigen retrieval method), compared to the other groups (p= 0.002, Figure 10). In terms of the differences in the yields between the non-stained antigen retrieval only (CA or PK) and IHC-stained sections that were subjected to antigen retrieval using CA or PK, the results show that sections which were treated with antigen retrieval solutions without staining were associated with higher total RNA yields (boiled CA, p= 0.016 and PK, p= 0.025, Figure 10).

Regarding the effect of using dH_2O or DPC-treated H_2O on the total RNA yield, the results demonstrate that there are no significant differences among both groups (p= 0.885), or between the type of water among the CA and PK groups (p= 0.378 and 0.378, respectively). In addition, there were no significant differences between the RNA yield of the sections that were exposed to a relatively high temperature (boiled CA) or that were exposed to 37°C (PK) (p= 0.094) (Figure 10).

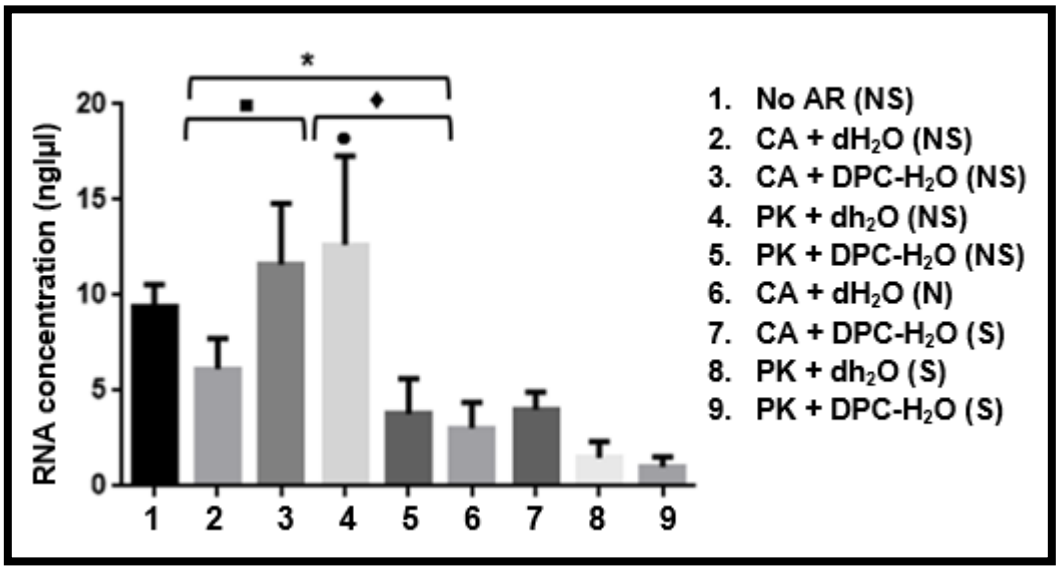

Figure 10. The effect of using dH_2O or DPC-treated H_2O with citric acid and proteinase K on the RNA yield extracted from immunohistochemistry staining sections.

n= 3, $p \leq 0.05$; star p= 0.002; square p= 0.016; diamond p= 0.025; circle p= 0.02. *AR*= antigen retrieval; *CA*= citric acid; *PK*= proteinase K; *DPC*= DPC-treated H_2O. *NS*= non-stained*; S*= stained.

- **Total RNA extraction from frozen samples**

As a result of the difficulties that were associated with extracting total RNA from fatty tissue due to the high fatty acid and triglyceride contents (Hemmrich, et al., 2009, Cirera, 2013), and because of the limited amount of fresh and frozen human hippocampal tissue that was used in this project, different RNA extraction techniques were used to optimise the most suitable extraction method that would produce a higher RNA yield and lower protein contamination. In this optimisation procedure, an equal amount of rat brain homogenate (approximately 5 mg) was used. The results in Figure 11 show clear difference in the RNA yield eluted in a constant total volume (25 µl DPC-treated H_2O), based on the method of extraction. Compared to the RNEasy mini kit, Triazole was associated with a RNA yield (Figure 11) that was six times higher. Interestingly, when combining Triazole and the RNEasy mini kit, the yield was increased 2.3 times higher than when RNA was extracted by Triazole alone, and 14 times higher when compared to the RNEasy mini kit alone (Figure 11). Moreover, combining Triazole with the RNEasy mini kit produces purer RNA (less protein contamination), compared to the other methods (Figure 12).

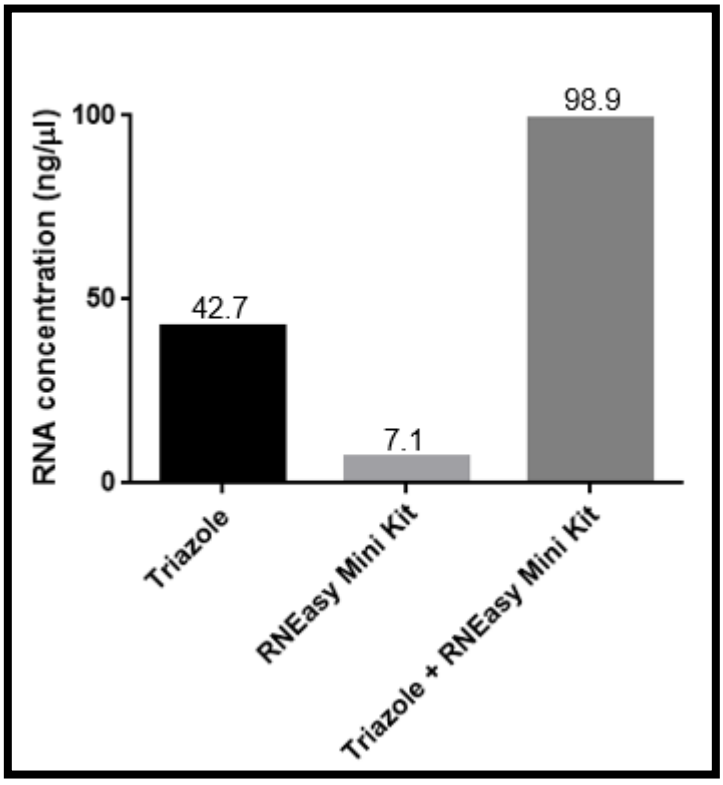

Figure 11. The effects of different RNA extraction techniques on the RNA yield from 5.2 mg frozen rat brain homogenate.

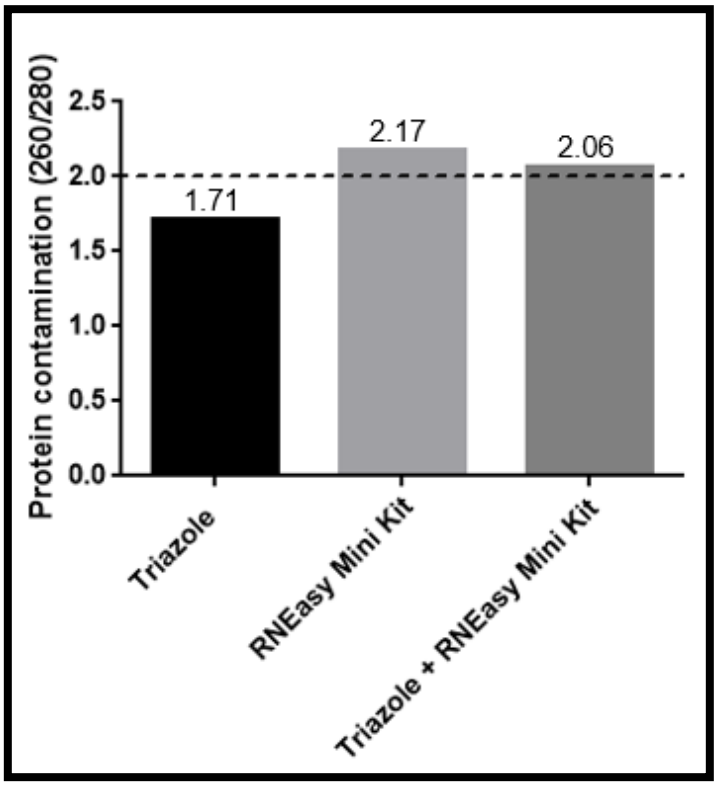

Figure 12. The effects of different RNA extraction techniques on protein contamination of the extracted RNA from 5.2 mg of frozen rat brain homogenate. 260/280 ≈2 conceder as protein-free RNA.

3.2. Receptors and protein expression in TLE-HS

3.2.A. Ligand gated ion channel, 5-HT_3 receptors

- **5-HT_3A receptors**

  - **Immunohistochemistry of 5-HT_3A receptors expression**

The study of the immunoreactivity of serotonergic ion channel 5-HT3A subunits in the hippocampus of TLE-HS patients, compared to post-mortem control samples, revealed an upregulation of 5-HT3A subunit among disease samples (Figure 13). This overexpression affects the preserved neurons in the dentate gyrus and CA2 (Figure 13). Furthermore, a relative increase was found in the remaining neurons of CA3 in TLE-HS patients (Figure 13). However, no immunoreactivity signal was reliably detected in CA1 (Figure 13).

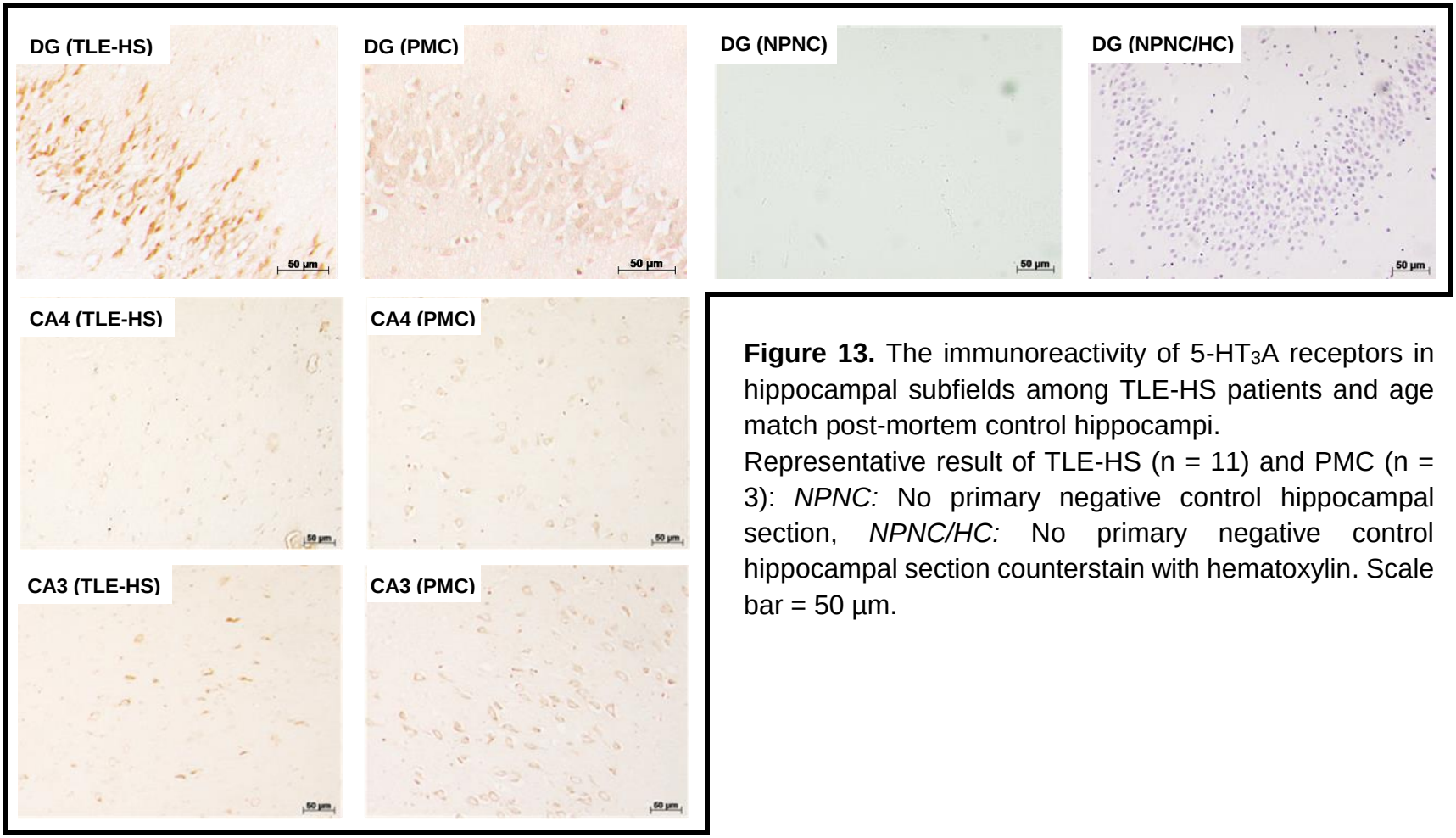

Figure 13. The immunoreactivity of 5-HT_3A receptors in hippocampal subfields among TLE-HS patients and age match post-mortem control hippocampi.
Representative result of TLE-HS (n = 11) and PMC (n = 3): *NPNC:* No primary negative control hippocampal section, *NPNC/HC:* No primary negative control hippocampal section counterstain with hematoxylin. Scale bar = 50 µm.

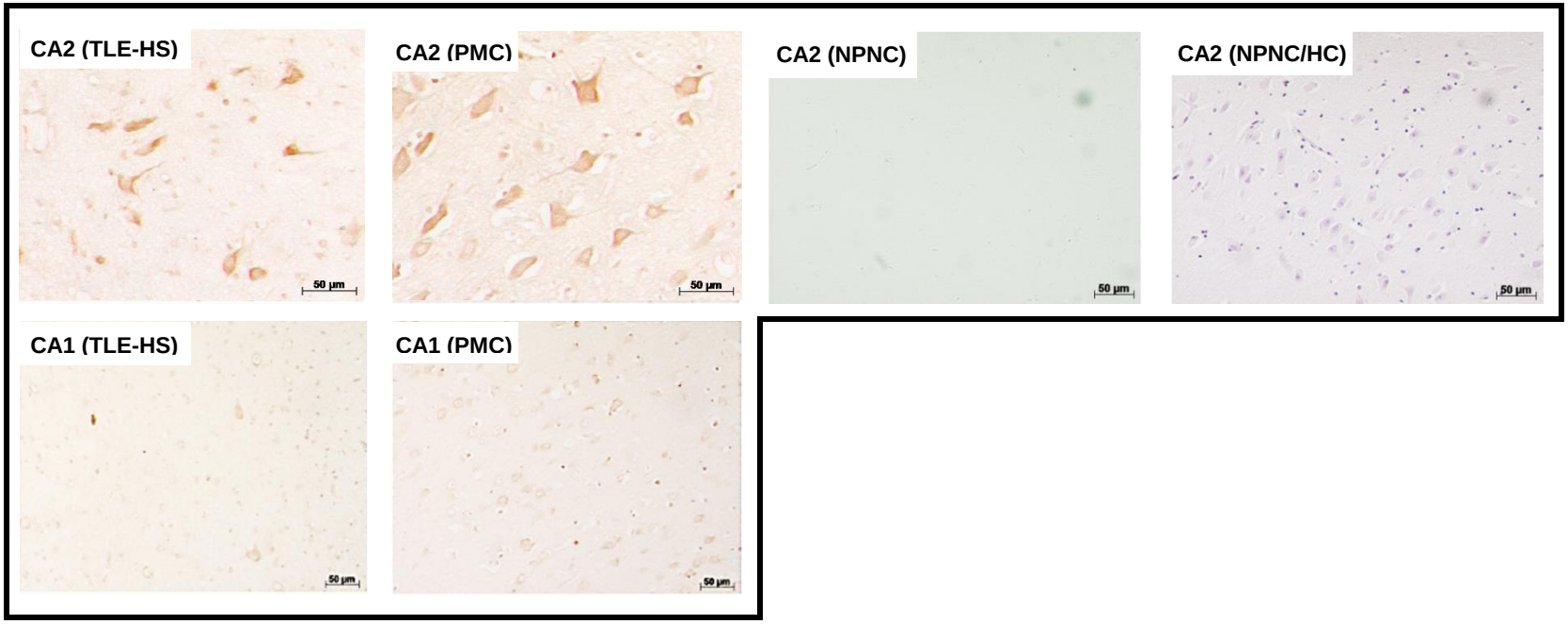

Figure 13. *continue.*

- **SDS-PAGE western blot of 5-HT_3A receptors density**

Immunoreactivity of 5-HT3A subunits in the hippocampal lysate of the TLE-HS patients, compared to that in the post-mortem controls, expressed an upregulation in TLE-HS tissue lysate as assessed by SDS-PAGE western blot (Figure 14). In addition, this upregulation was observed in the hippocampal lysate of the TLE-HS samples that were subjected to artificial post-mortem delay protocol in comparison to that in the post-mortem controls' hippocampal lysate (Figure 14).

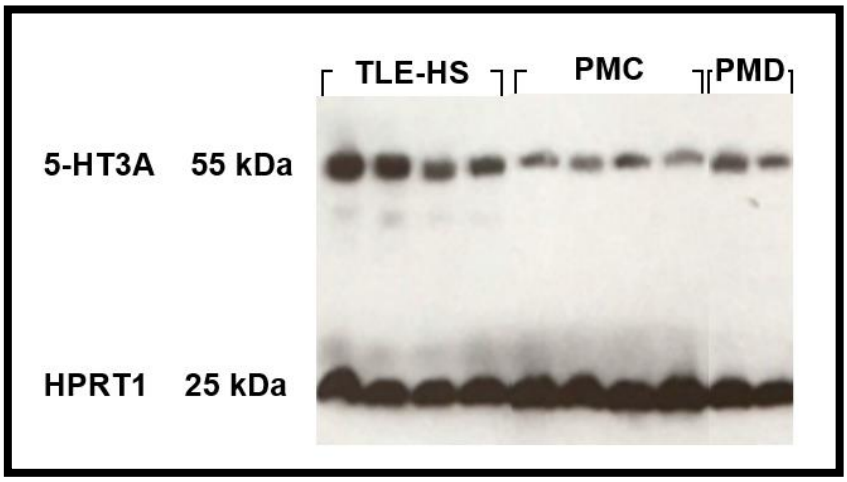

Figure 14. SDS-PAGE Western Blot of 5-HT_3A receptors immunoreactivity in hippocampal homogenates from patients with TLE-HS and control donors.
Also presented are results from hippocampal homogenates from patients with TLE-HS that were subject to an artificial post-mortem delay (PMD) prior to processing.
Data from a single experiment is presented, which is representative of five different experiments. TLE-HS (n= 8), PMC (n=8) and PMD (n=3). Detection of HPRT1 immunoreactivity was used as a loading control.

- **[^{3}H]-S-zacopride autoradiography**

Autoradiography using [^{3}H]-*S*-zacopride reveal heterogeneity in the level of 5-HT_3 receptors expression within hippocampal subfields between TLE-HS and PMC samples (table 10, figure 15). The specific binding activity of [^{3}H]-*S*-zacopride (non-specific binding defined by 1 µM granisetron) reflects the density of 5-HT_3 receptors in the hippocampus. The result expressed a significant increase in the receptor density in some hippocampal areas particularly CA2 (p = 0.0002) and in the molecular layer of the dentate gyrus (p = 0.0025) among TLE-HS in comparison to PMC samples. The experiments and results quantification were previously done by Professor Nicholas Barnes.

Table 11. Specific binding activity of [^{3}H]-*S*-zacopride in the hippocampus of TLE-HS patients (n = 9) and PMC samples (n = 10).
Non-specific binding define by 1 µM granisetron, mean ± SEM, $p \leq 0.05$. Data generated by Prof Barnes.

Hippocampal subfields	**TLE-HS**	**PMC**	***p* value**
DG (granular layer)	7.2 ± 0.9	7.3 ± 1.2	0.9486
DG (molecular layer)	5.6 ± 0.7	2.6 ± 0.5	0.0025*
CA1	2.4 ± 0.6	1.4 ± 0.3	0.1423
CA2	10.3 ± 1.1	4.7 ± 0.5	0.0002*
CA3	2.1 ± 0.5	3.2 ± 0.5	0.1393

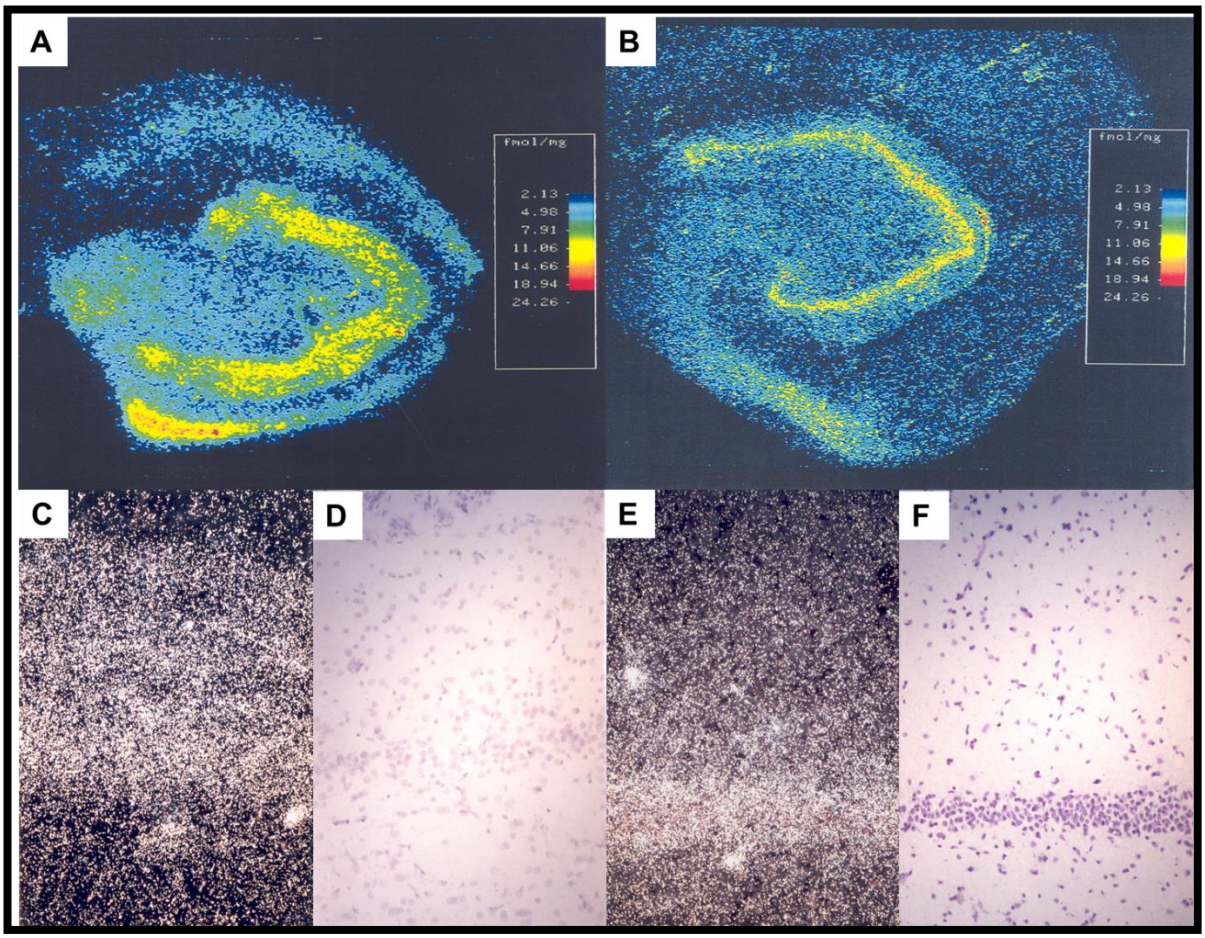

Figure 15. Expression of 5-HT_3 receptors in the hippocampus of TLE-HS patients (n = 9) and PMC donor (n = 10).
Pseudo-colour of [^{3}H]-*S*-zacopride binding activity in the hippocampus of TLE-HS patients (A) and PMC donors (B). C, E; Coverslip dark-field autoradiogram of DG from TLE-HS (C) and PMC (E) samples. D, F; histological staining of DG from TLE-HS (D) and PMC (F) samples. Data generated by Prof Barnrs.

- **Radioligand binding assay**

The saturation studies of 5-HT_3 receptors using [^{3}H]-granisetron (0.1 – 10.0 nM) labelling hippocampal tissues homogenate express higher specific binding activity (in presence of 10 µM ondansetron) detected in tissue homogenate of TLE-HS in comparison with PMC (table 12, figure 16A). This data generated by Prof Barnes.

Single concentration radioligand binding assay of the 5-HT_3 receptor was conducted using 6 nM [^{3}H]-granisetron. The result indicates a higher binding level of [^{3}H]-granisetron detected in TLE-HS in comparison with postmortem control samples (8.9 ± 1.32, 3.93 ± 4.9 fmol/mg, respectively, p = 0.0019, mean ± SEM, figure 17).

The competition studies of [^{3}H]-granisetron (0.8 – 1.1 nM) in presence of some selected structurally distinct 5-HT_3 receptor ligands display a consistent pharmacological profile of the radiolabelled granisetron in TLE-HS compared with PMC tissues homogenate for competing drugs under study (table 13). These results previously created by Professor Nicholas Barnes.

Table 12. Specific binding activity of [^{3}H]-granisetron in the hippocampus homogenate of TLE-HS patients and PMC samples.
Non-specific binding define by 10 µM ondansetron, mean ± SEM, $p \leq 0.05$. Data generated by Prof Barnes.

Sample (n)	Bmax (fmol/mg)	Kd	Hill Coefficient
TLE-HS (5)	126 ± 10	1.4 ± 0.29	1.01 ± 0.07
PMC (7)	59 ± 8	1.89 ± 0.8	1.05 ± 0.06
p Value	0.0064*	0.596	0.9573

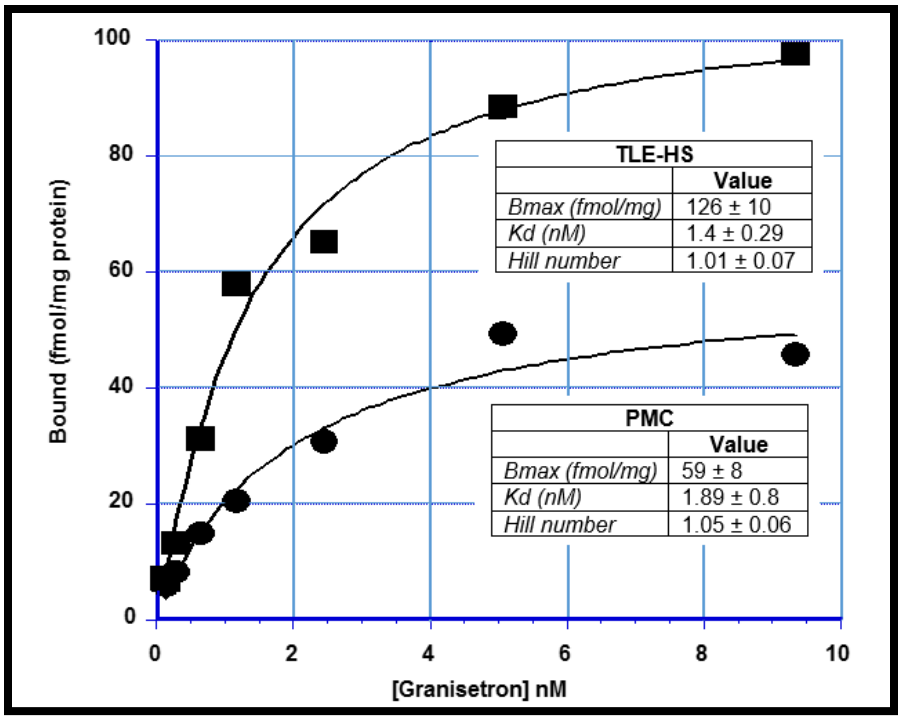

Figure 16. [^{3}H]-Granisetron saturation binding assays of 5-HT$_3$ receptors within hippocampal homogenate from patients with TLE-HS (n = 5) and PMC (n = 7) samples.

The non-specific binding defined by ondansetron (10 µM). Bmax of TLE-HS = 126 ± 10 fmol/mg and PMC 59 ± 8 fmol/mg, p = 0.0064, mean ± SEM, $p \leq 0.05$. Data present from single experiment repeated three times. *Squares* = TLE-HS; *Circles* = PMC. Data generated by Prof Barnes.

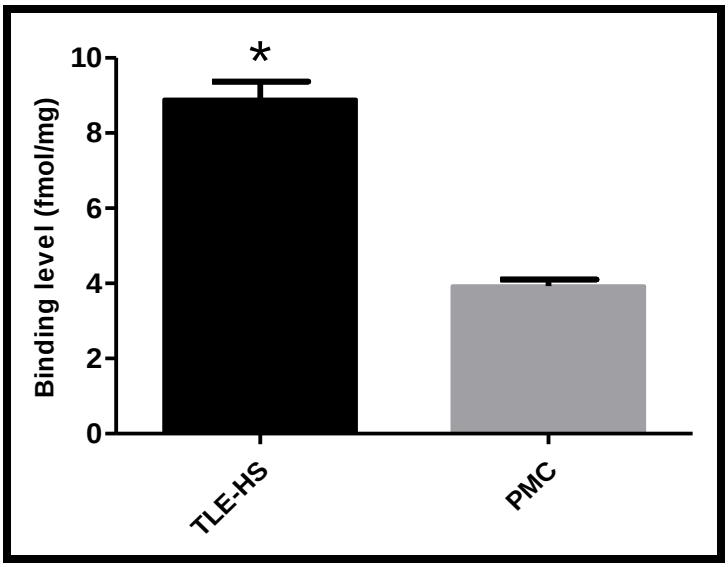

Figure 17. Single concentration [^{3}H]-granisetron binding assay of 5-HT$_3$ receptors within hippocampal homogenate from patients with TLE-HS (n = 8) and PMC (n = 8) samples.

The non-specific binding defined by ondansetron (10 µM), Binding level TLE-HS = 8.9 ± 1.32 fmol/mg and PMC 3.93 ± 4.9 fmol/mg, $p = 0.0019$, mean ± SEM, $p \leq 0.05$. Data present from single experiment repeated three times.

Table 13. Pharmacological binding studies of [^{3}H]-granisetron in tissue homogenate of TLE-HS (n = 1) and PMC (n = 1) samples.
Values represent the mean of pKi value from three independent experiments (SEM was less than 2%).

Competing drug	pKi	
	TLE-HS	PMC
(S)-zacopride	8.97	8.93
Granisetron	8.20	8.27
Tropisetron	8.07	8.13
Ondansetron	7.90	7.93
5-HT	6.73	6.80

○ RT-qPCR of 5-HT$_3$A receptors expression

In terms of the alteration in the level of expression of the 5-HT3A receptors' subunit mRNA in the hippocampus of patients with TLE-HS, the RT-qPCR results showed that there were no significant differences between the disease and the control group samples; $p = 0.342$ and $p = 0.882$ were found, respectively, when HPRT1 and ENO2 gene expression were used to normalise the results (Figure 18). This result is inconsistence with 5-HT$_3$ receptors' protein studies previously done in this project.

On the other hand, when the RT-qPCR results for the 5-HT3A receptors' subunit mRNA in the TLE-HS samples that were subjected to a 70 hour post-mortem delay protocol (PMD) were compared to the results for the post-mortem delay samples and TLE-HS samples that were not subjected to artificial post-mortem delay protocol, it was revealed that there was a significant upregulation (approximately 13 folds) of 5-HT$_3$A receptors mRNA among PMD samples. The level of significance was $p = 0.0007$ and 0.001 for the PMD samples in comparison to the post-mortem control mRNA when using different housekeeping genes, HPRT1 and ENO2, respectively (Figure 19). When comparing the PMD to the TLE-HS samples, the result showed a significant overexpression (approximately 10–12 folds) of 5-HT3A subunit mRNA in PMD samples (Figure 20). Using the HPRT1 gene as a housekeeping gene, the $p = 0.02$ and $p = 0.008$ values for the statistical evaluation of the RT-qPCR experiment normalised using the ENO2 gene (Figure 20). Interestingly, the Ct values of the housekeeping genes, but not target gene, were increased in the PMD group compared to other groups by approximately 2 cycles for both the HPRT1 and the ENO2 genes.

In order to confirm the RT-qPCR results and to assess the risk of fluorescence channel bleed with the RT-qPCR fluorescence detection instrument, a RT-qPCR experiment was run in monoplex using biological and technical duplicates for 5-HT3A receptors subunit, HPRT1 and ENO2 genes, and the result was compared to the duplex RT-qPCR reaction. The comparison revealed some increase in the Ct value of 5-HT3A subunit mRNA by approximately 1–2 cycles in both the disease and the post-mortem control samples in the monoplex experiment compared to that in the duplex experiment (Table 14).

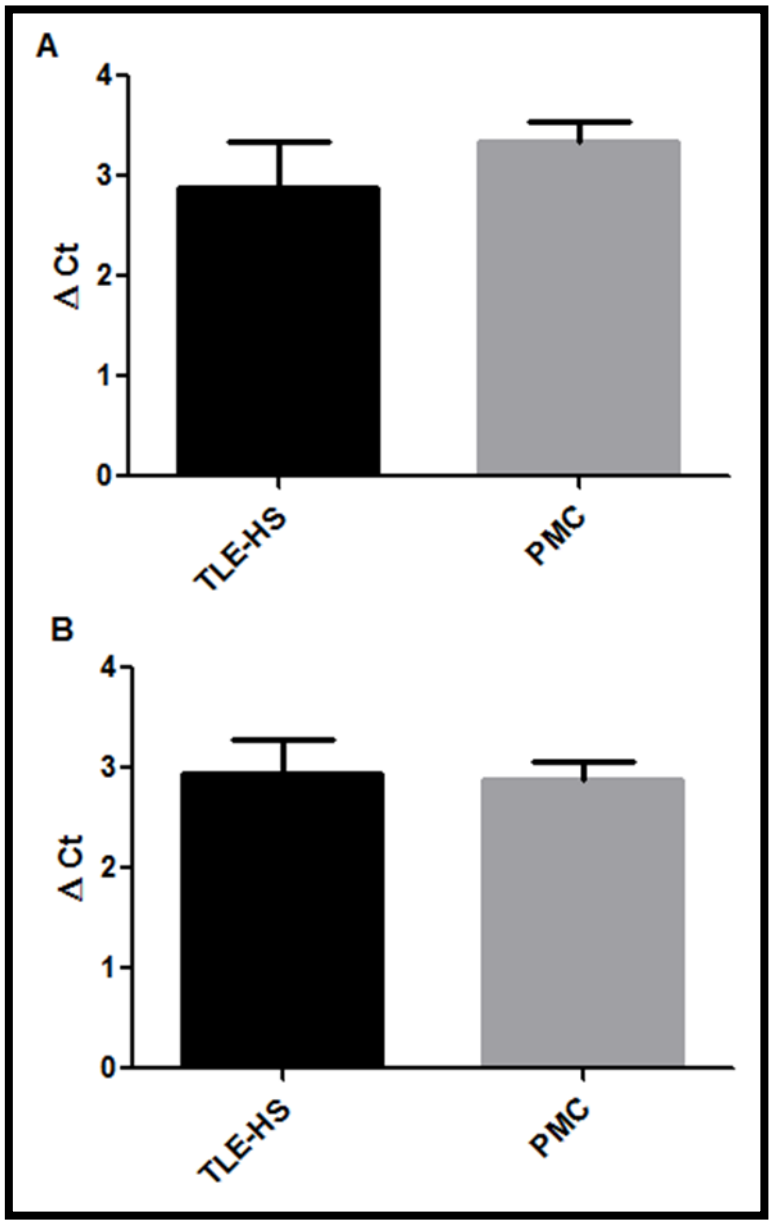

Figure 18. 5-HT$_3$A receptors subunit gene expression in hippocampus of patients with TLE-HS.

A) 5-HT$_3$A receptors gene expression normalised with the expression of HPRT1 gene expression (housekeeping gene), TLE-HS n = 7, PMC n = 9, mean ± SEM, p = 0.342. B) 5-HT$_3$A receptors gene expression normalised with the expression of ENO2 gene expression (housekeeping gene), TLE-HS n = 7, PMC n = 10, mean ± SEM, p = 0.882.

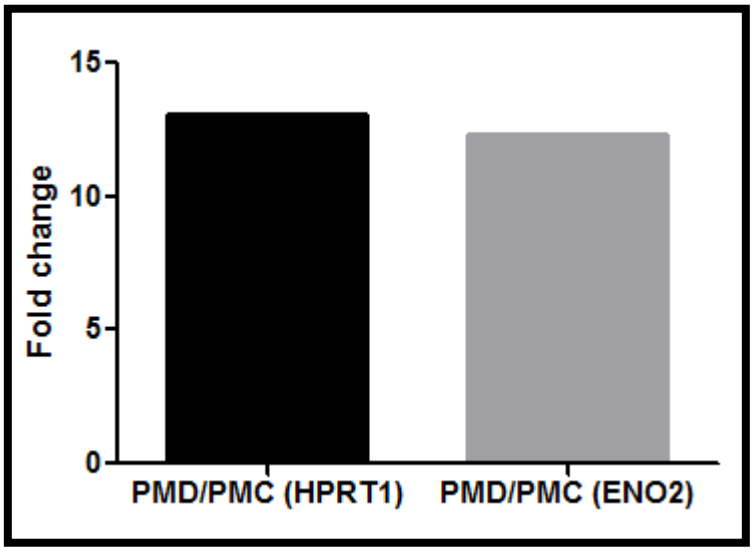

Figure 19. 5-HT$_3$A receptors subunit gene expression in hippocampus of patients with TLE-HS subjected to approximate 70 hours' artificial post-mortem delay protocol in comparison to post-mortem control.
5-HT$_3$A receptors gene expression normalised with the expression of HPRT1 gene expression, (PMD n = 4, PMC n = 9, p = 0.0007) and normalised with the expression of ENO2 gene expression, PMD n = 4, PMC n = 10, p = 0.001.

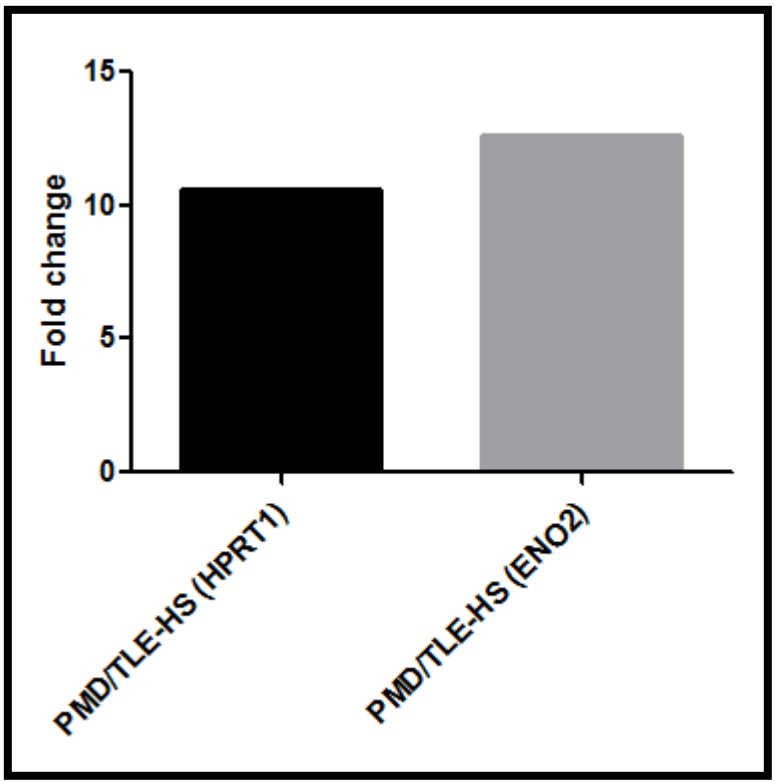

Figure 20. 5-HT$_3$A receptors subunit gene expression in hippocampus of patients with TLE-HS subjected to approximate 70 hours' artificial post-mortem delay protocol in comparison with TLE-HS samples.
5-HT$_3$A receptors gene expression normalised with the expression of HPRT1 gene expression, (PMD n = 4, TLE-HS n = 5, p = 0.02) and normalised with the expression of ENO2 gene expression, PMD n = 4, TLE-HS n = 5, p = 0.008.

Table 14. Assessment of the qPCR fluorescence channels blood-through risk between target gene (5-HT$_{3A}$, FAM-labeled ≈ 520 nm) and housekeeping gene (HPRT1 and ENO2, VIC-labeled ≈ 550 nm).

Sample	Monoplex experiment						Duplex experiment			
	5-HT$_{3A}$		HPRT1		ENO2		5-HT$_{3A}$	HPRT1	5-HT$_{3A}$	ENO2
	Ct	Average Ct	Ct	Average Ct	Ct	Average Ct	Ct	Ct	Ct	Ct
TLE-HS 1	26.53682	26.62444	22.23825	22.81327	23.33466	23.28309	25.37846	23.65804	25.55085	23.62633
	26.71207		23.3883		23.23152					
TLE-HS 2	27.64462	27.63965	23.05686	23.25472	23.46519	23.57245	26.08395	23.40858	26.40475	23.50788
	27.63467		23.45258		23.67971					
PMC 1	27.76908	27.79566	22.22435	22.1971	23.06866	23.02981	27.16982	22.76707	26.33833	23.38214
	27.82224		22.16986		22.99095					
PMC 2	28.20498	28.42317	23.05642	23.04241	23.94097	23.9424	25.68957	23.04241	26.28687	23.9662
	28.64137		23.0284		23.94383					

3.2.B. Serotonergic G-protein coupled receptors

- **5-HT$_{1A}$ receptors**

  - **Immunohistochemistry of 5-HT$_{1A}$ receptors expression**

The expression of the inhibitory metabotropic 5-HT$_{1A}$ receptors in the hippocampus of patients with temporal lobe TLE-HS was assessed by studying the immunoreactivity of these receptors. The result indicated that 5-HT$_{1A}$ receptor expression was increased in the hippocampi of patients with TLE-HS compared to age-matched post-mortem control hippocampi. This alteration in the level of expression affected predominantly the DG and CA2 (Figure 21) of the hippocampus where the neurons were preserved among TLE-HS patients. Moreover, in the area where the disease has a relatively strong negative impact on the viability of hippocampal neurons (CA4, CA3 and CA1), the result expressed relatively slight to moderate upregulation of 5-HT$_{1A}$ receptors (Figure 21).

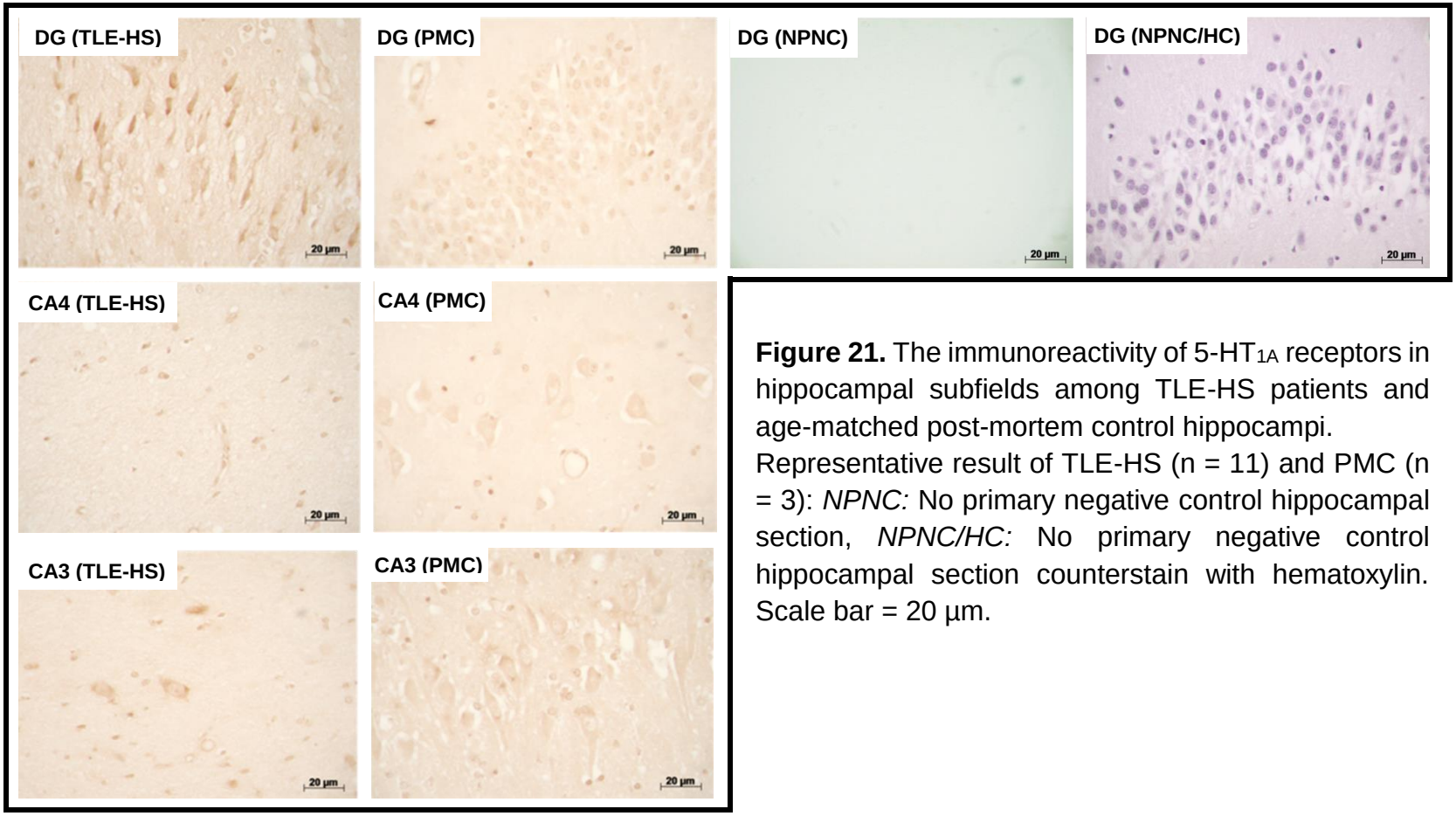

Figure 21. The immunoreactivity of 5-HT$_{1A}$ receptors in hippocampal subfields among TLE-HS patients and age-matched post-mortem control hippocampi. Representative result of TLE-HS (n = 11) and PMC (n = 3): *NPNC:* No primary negative control hippocampal section, *NPNC/HC:* No primary negative control hippocampal section counterstain with hematoxylin. Scale bar = 20 µm.

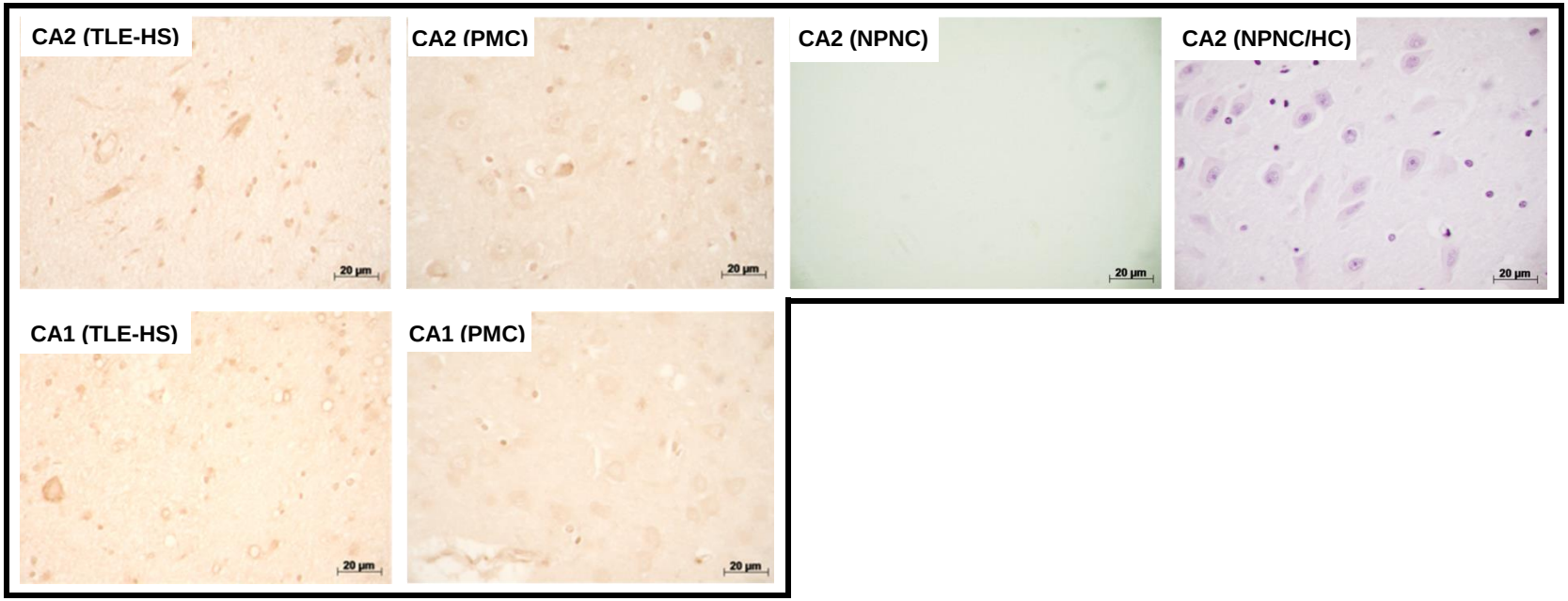

Figure 21. *continue.*

○ RT-qPCR of 5-HT$_{1A}$ receptors expression

In order to study the effect of TLE-HS on the expression level of 5-HT$_{1A}$ receptors' mRNA, the duplex RT-qPCR technique was used. This technique was selected due to the limited amount of tissue available and the nature of this type of sample to avoid any negative effects of the cDNA synthesis procedure on the quality and quantity of the sample. The result of the 5-HT$_{1A}$ mRNA expression among TLE-HS patients did not reveal any significant differences compared to the age-matched post-mortem control hippocampal mRNA (p = 0.185, Figure 22). This result is inconsistence with 5-HT$_{1A}$ receptors' protein expression assessed using immunohistochemistry technique. In the RT-qPCR study, one stable housekeeping gene (HPRT1) was used due to the interaction that was detected between 5-HT$_{1A}$ receptors and ENO2 primers, which resulted in undetected signals from both target genes.

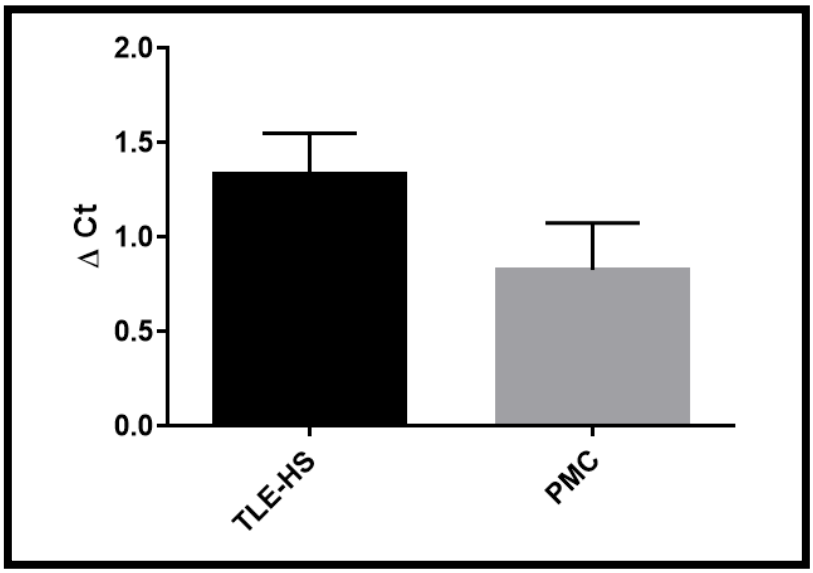

Figure 22. 5-HT_{1A} receptors' gene expression in hippocampi of patients with TLE-HS.
HPRT1 was used as a housekeeping gene (TLE-HS n = 6, PMC n = 9, *p* = 0.185, mean ± SEM).

- **5-HT$_{2A}$ receptors**

  - **Immunohistochemistry of 5-HT$_{2A}$ receptors expression**

The immunoreactivity of the $G_{q/11}$ coupled serotonergic receptors (5-HT$_{2A}$) was studied in TLE-HS patients in comparison to post-mortem control hippocampi using immunohistochemistry. The results showed that there was a different expression pattern of 5-HT$_{2A}$ receptors in different areas of hippocampus of TLE-HS patients compared to the post-mortem control samples (Figure 23). The reduced expression affects astrocyte cell bodies and processes especially within CA4 (Figure 24) and CA3, in addition to apparent neuronal fibres in the hippocampus (Figures 23). Furthermore, 5-HT$_{2A}$ receptors were expressed in the regions prove to astrocytosis cells that were observed in all of the hippocampal subfields in TLE-HS patients in comparison to post-mortem control. In addition, the 5-HT$_{2A}$ receptor positive astrocytes present in the molecular layer of the dentate gyrus (Figures 23).

To phenotype the cells that expressed 5-HT$_{2A}$ receptors, the immunoreactivity of glial fibrillary acidic protein (GFAP), a mature astrocyte biomarker, was studied on the adjacent sections of those immunologically stained slides to detect the expression of 5-HT$_{2A}$ receptors. The results revealed that the immunoreactivity of both 5-HT$_{2A}$ receptors and GFAP filaments were approximately co-localised on hippocampal astrocytes; however, the level of expression of GFAP in the astrocyte cell bodies of the disease samples was less than in the control samples (Figure 25).

Interestingly, the astocytes in the CA4 region of TLE-HS samples show relatively high 5-HT_{2A} receptor expression compared to that in the molecular layer of the dentate gyrus (Figure 25). On the other hand, GFAP is highly expressed in the molecular layer of the dentate gyrus in comparison to CA4 among TLE-HS patients.

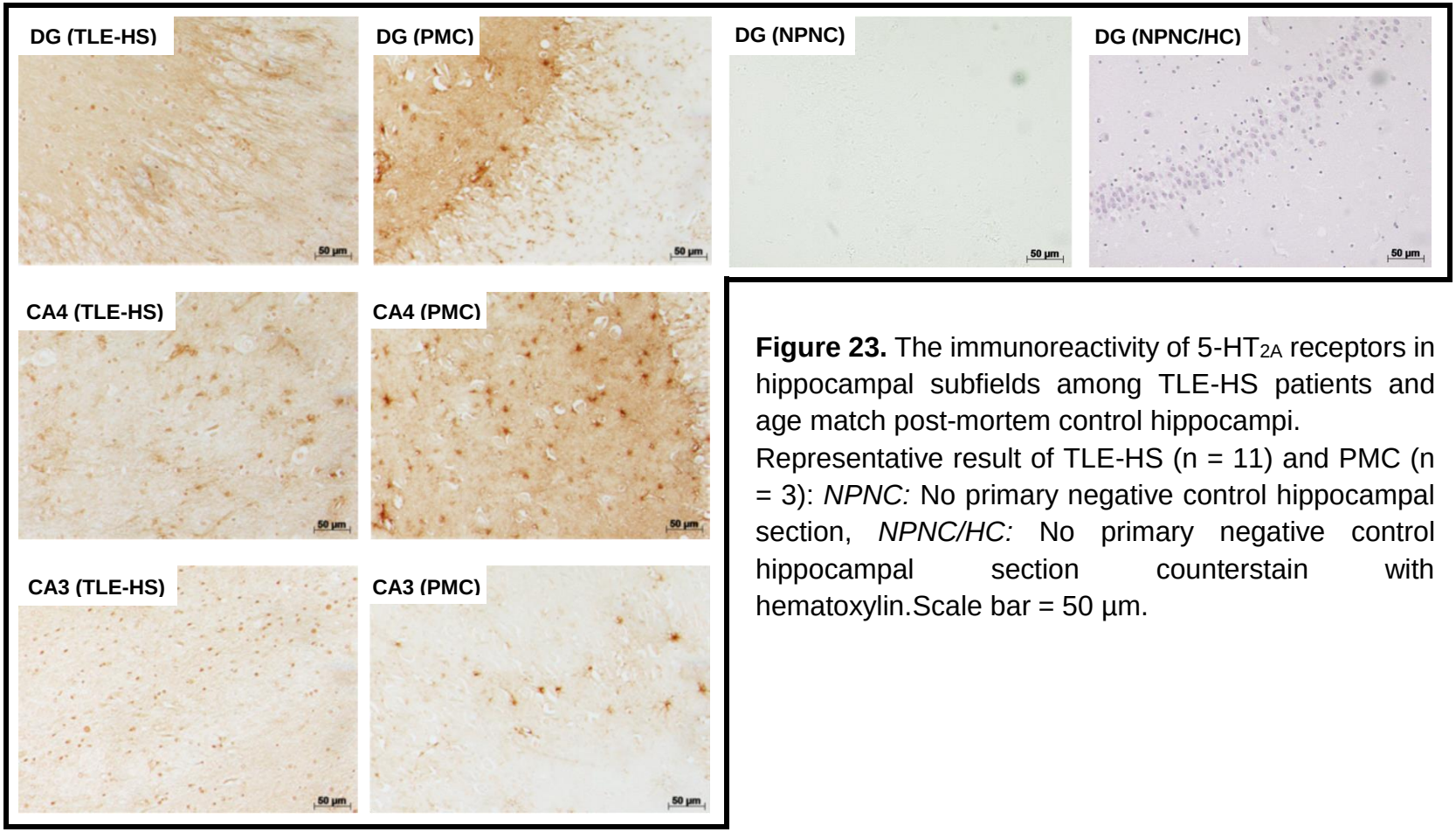

Figure 23. The immunoreactivity of 5-HT$_{2A}$ receptors in hippocampal subfields among TLE-HS patients and age match post-mortem control hippocampi.
Representative result of TLE-HS (n = 11) and PMC (n = 3): *NPNC:* No primary negative control hippocampal section, *NPNC/HC:* No primary negative control hippocampal section counterstain with hematoxylin.Scale bar = 50 µm.

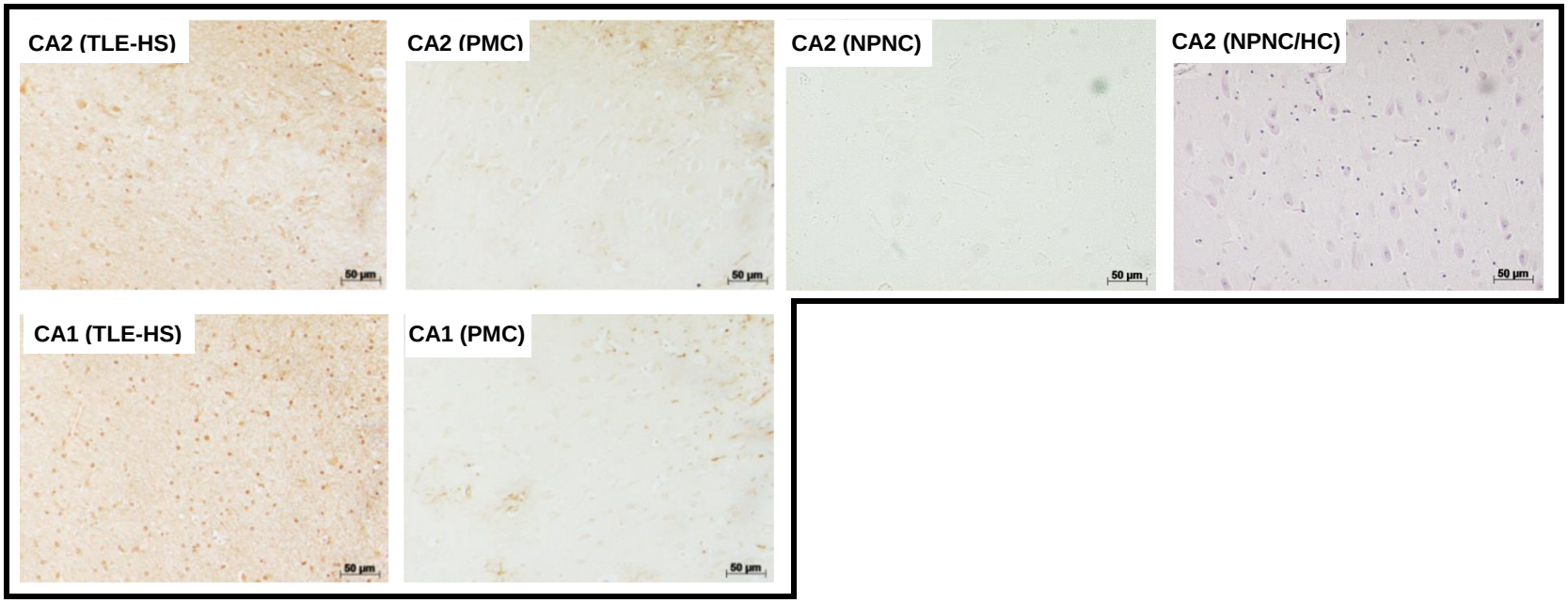

Figure 23. continue.

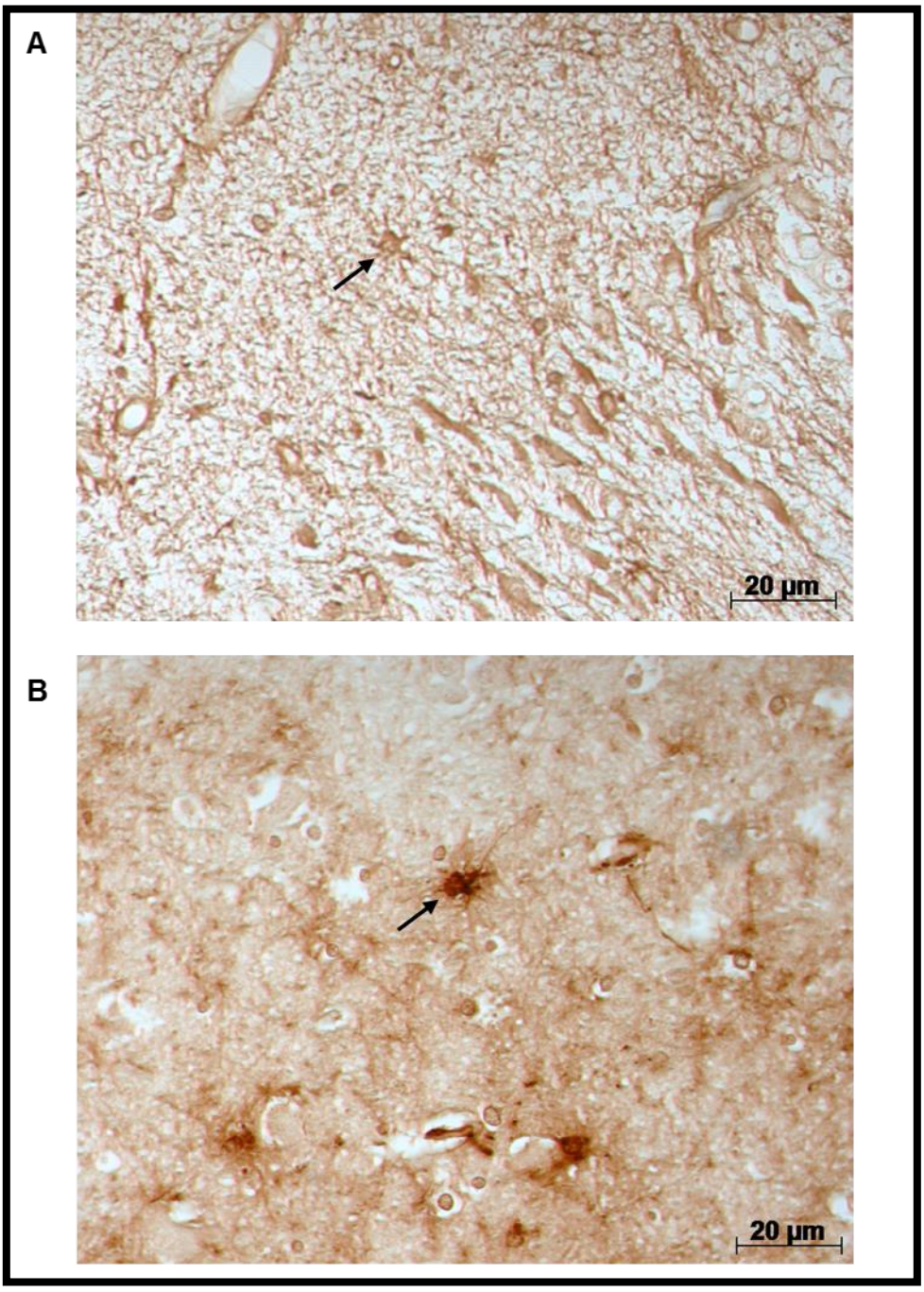

Figure 24. Immunoreactivity of 5-HT_{2A} receptors in astrocytes of human hippocampus CA4.
A) TLE-HS hippocampal sample, B) post-mortem control hippocampus. Scale bar: 20 µm.

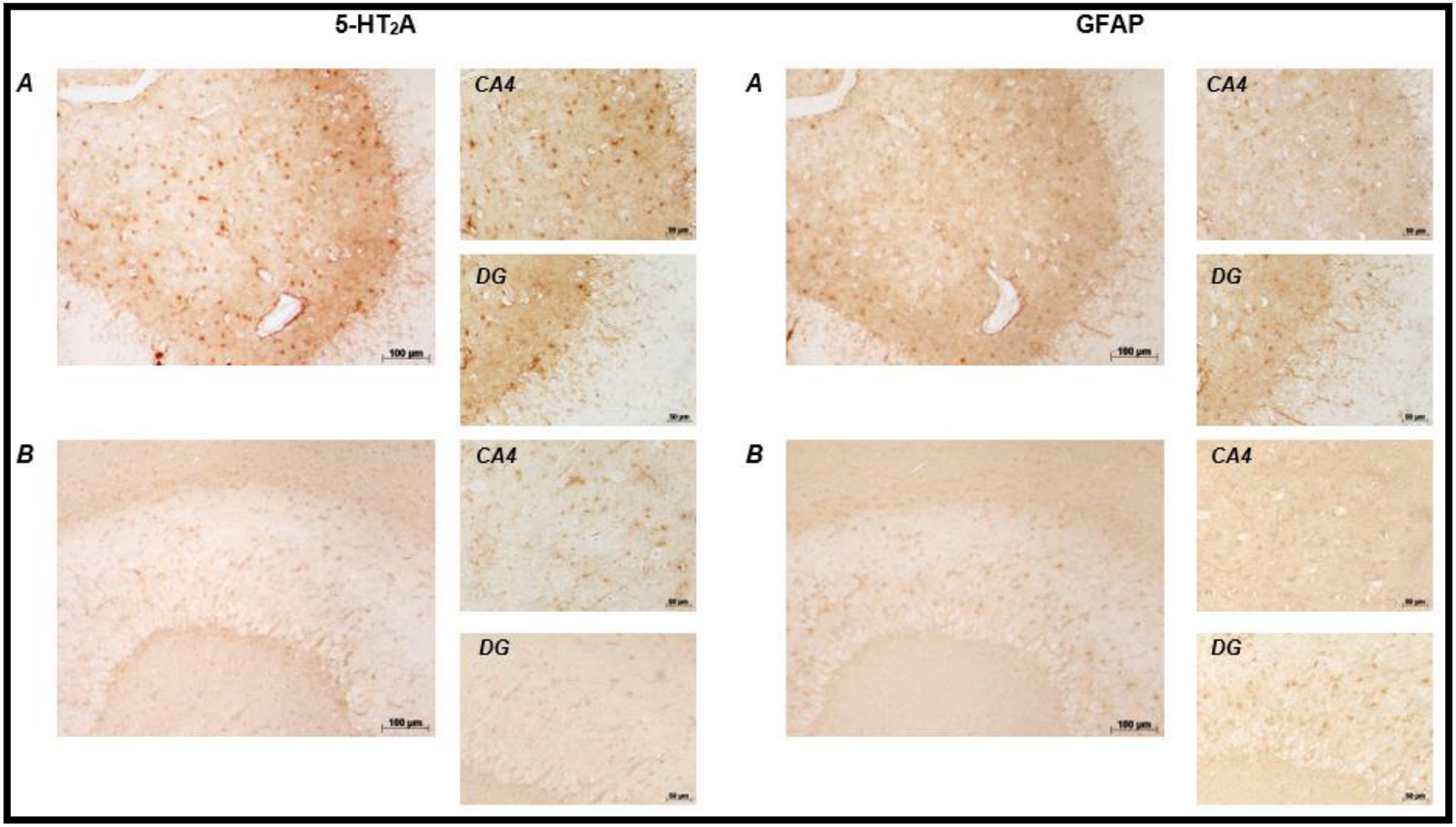

Figure 25. Immunoreactivity of 5-HT_{2A} receptors and GFAP astrocytes biomarker in the adjacent sections in CA4 and dentate gyrus of human hippocampus.
A) post-mortem control hippocampus, B) TLE-HS hippocampal sections, *Right*: 5-HT_{2A} immunoreactivity, *Left*: GFAP immunoreactivity. Scale bar: 50 µm and 100 µm. DG: dentate gyrus.

○ RT-qPCR of 5-HT$_{2A}$ receptors expression

The expression of the mRNA of the 5-HT$_{2A}$ receptors, a RT-qPCR experiment was conducted that did not reveal a change in expression from the TLE-HS disease on the mRNA of the receptors; $p = 0.404$ and $p = 0.75$ were found when the level of expression was normalised using HPRT1 and ENO2 gene expression, respectively (Figure 26). The results were inconsistence to immunoreactivity signals of 5-HT$_{2A}$ receptors.

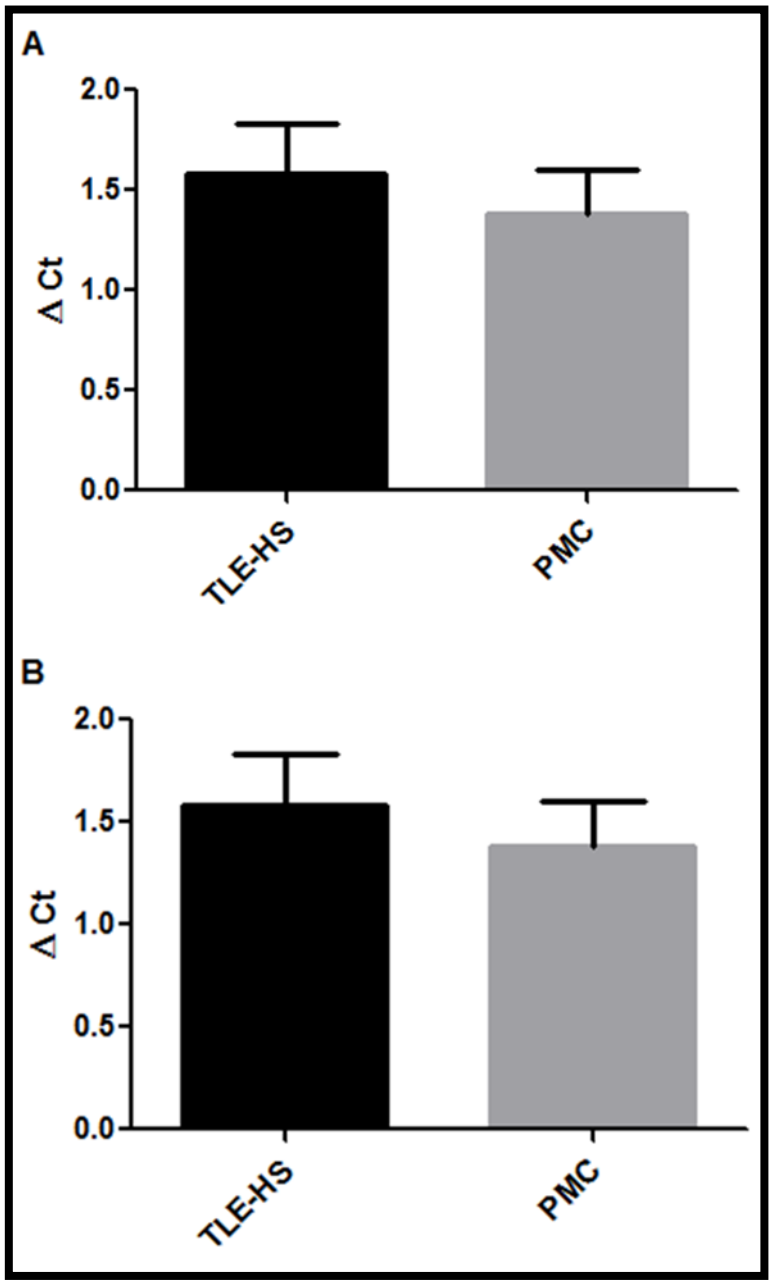

Figure 26. 5-HT_{2A} receptors subunit gene expression in hippocampus of patients with TLE-HS.

A) 5-HT_{2A} receptors gene expression normalised with the expression of HPRT1 gene expression (housekeeping gene), TLE-HS n = 6, PMC n = 8, mean ± SEM, *p*= 0.404. B) 5-HT_{2A} receptors gene expression normalised with the expression of ENO2 gene expression (housekeeping gene), TLE-HS n = 6, PMC n = 9, mean ± SEM, *p* = 0.750.

- **5-HT$_4$ receptors**

  - **Immunohistochemistry of 5-HT$_4$ receptors expression**

In order to study the involvement of the Gs protein coupled receptors (5-HT$_4$) in the pathogenesis of TLE-HS, the immunoreactivity of these receptors was examined in the hippocampi of TLE-HS patients compared to post-mortem control samples. The results revealed an apparent increased expression of these excitatory receptors in disease group samples compared to the post-mortem control hippocampi (Figure 27). This increase was detected in dentate gyrus and CA2 of the TLE-HS diseased hippocampi, where the neurons are relatively preserved from the negative effect of disease in the hippocampus (Figure 27). Whereas, no differences in immunoreactivity were observed in the other hippocampal subfields of TLE-HS samples.

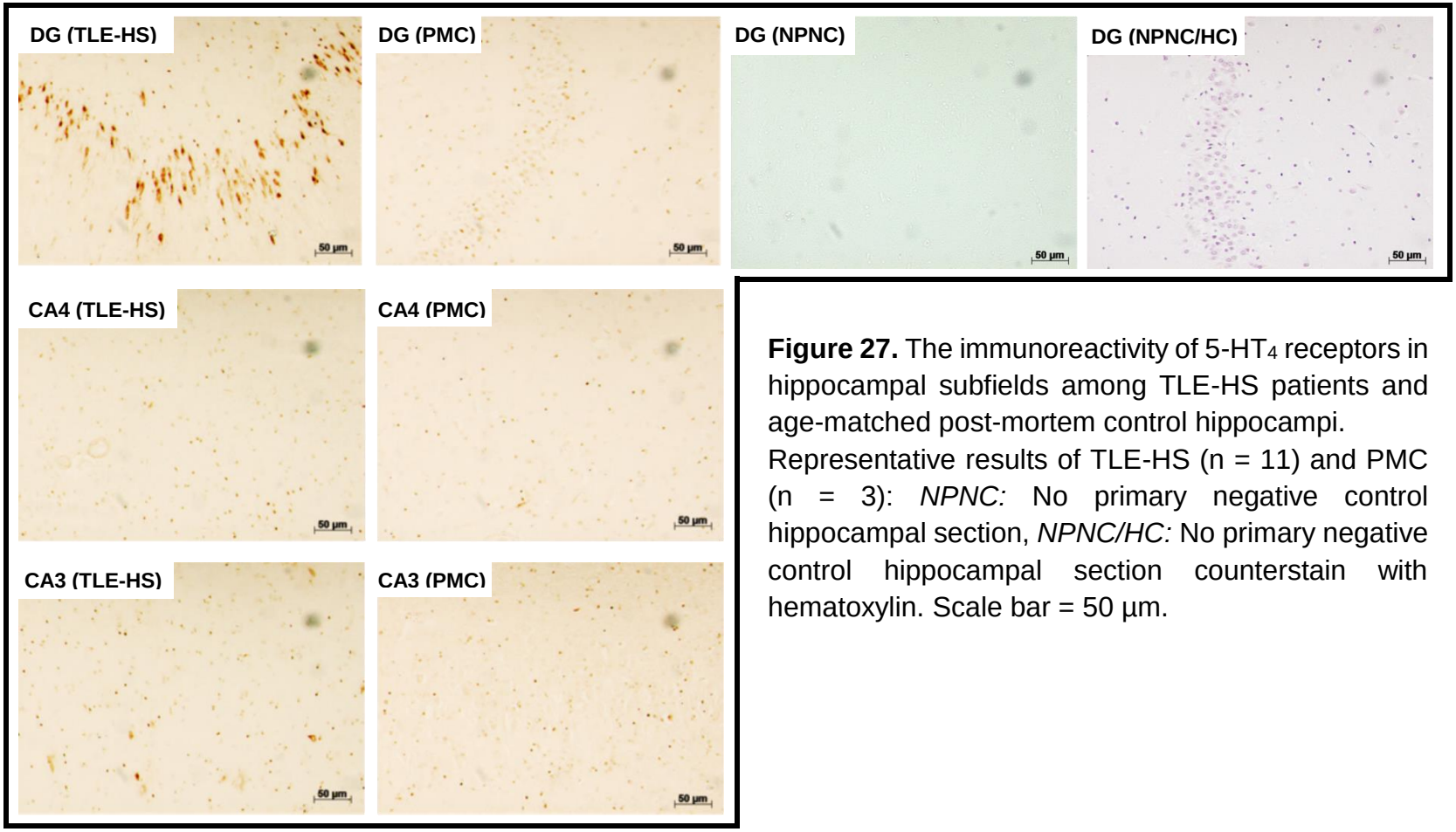

Figure 27. The immunoreactivity of 5-HT$_4$ receptors in hippocampal subfields among TLE-HS patients and age-matched post-mortem control hippocampi. Representative results of TLE-HS (n = 11) and PMC (n = 3): *NPNC:* No primary negative control hippocampal section, *NPNC/HC:* No primary negative control hippocampal section counterstain with hematoxylin. Scale bar = 50 µm.

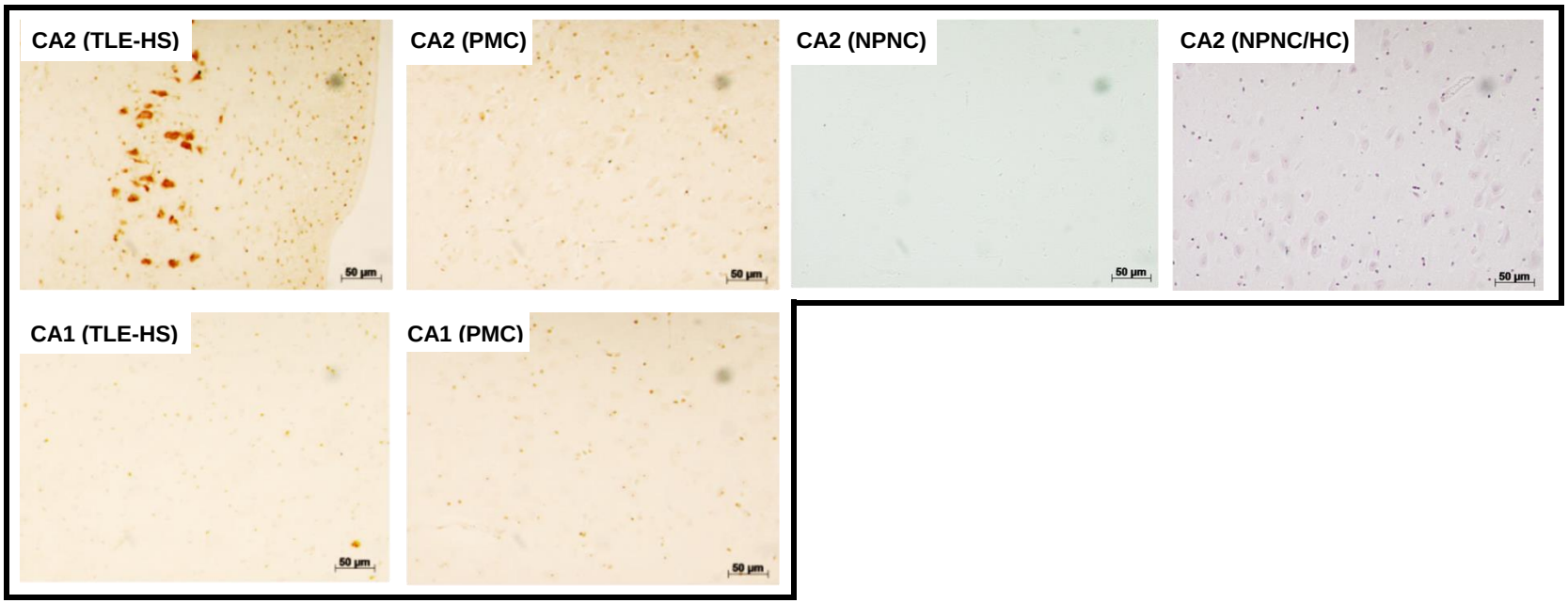

Figure 27. *Continue*.

○ RT-qPCR of 5-HT$_4$ receptors expression

The effect of the disease on the 5-HT$_4$ receptors' mRNA, the result does not reveal any significant differences between the disease and post-mortem control hippocampal samples (Figure 28). The same result was observed when two different housekeeping genes (HPRT1, ENO2) were used to normalise the data (p= 0.281 and p= 0.650, respectively, Figure 28). This expression of 5-HT4 receptors' mRNA is inconsistence with receptors= protein expression studied using immunohistochemistry technique.

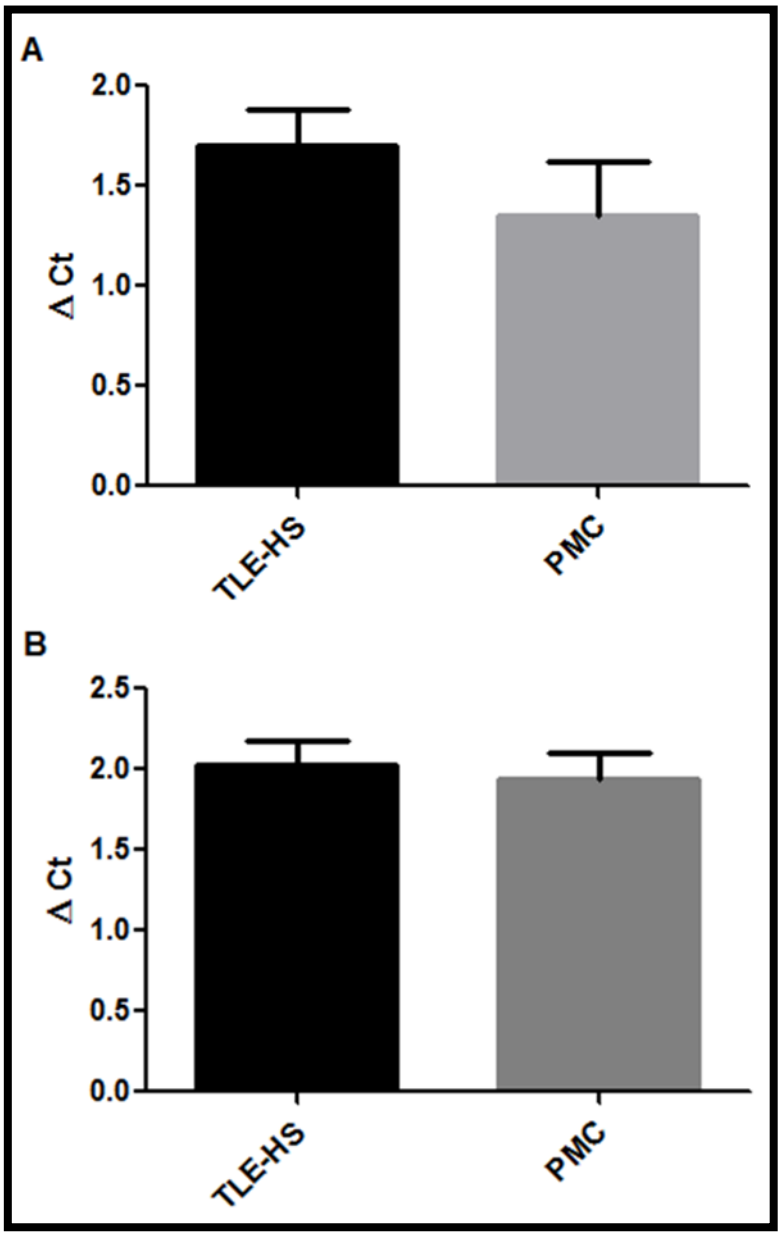

Figure 28. 5-HT$_4$ receptors' gene expression in hippocampi of patients with TLE-HS.

A) 5-HT$_4$ receptors gene expression normalised with the expression of HPRT1 gene expression (housekeeping gene), TLE-HS n = 4, PMC n = 4, p= 0.281, mean ± SEM.

B) 5-HT$_4$ receptors gene expression normalised with the expression of ENO2 gene expression (housekeeping gene), TLE-HS n = 6, PMC n = 8, p= 0.650, mean ± SEM.

- **5-HT$_7$ receptors**

Immunoreactivity studies of the 5-HT$_7$ receptors were conducted using TLE-HS and post-mortem control samples. The results generated did not indicate well-defined immunoreactivity signals in either the diseased or the control samples (some non-specific staining was observed). This data is shown in Appendix A (Figure 37).

3.2.C. Orphan G-protein, GPR61

The orphan G protein (GPR61) immunoreactivity results indicate that this protein is normally present in the neuronal cell membrane in the form of small immunoreactive dots (Figure 29). These signals are highly expressed in CA1, CA2 and CA3 of post-mortem control hippocampi (Figure 30). In addition, this orphan G-protein is moderately expressed in granular cells of the dentate gyrus and CA4 (Figure 30).

In terms of the alteration in the expression of GPR61 protein among diseased samples, the results revealed that there is a relatively moderate upregulation of GPR61 in the dentate gyrus, CA2 and in the remaining neurons of the CA3 in the TLE-HS hippocampi in comparison to the post-mortem control samples (Figure 31). Moreover, this protein expressed a slight upregulation in CA1 of the TLE-HS samples (Figure 31). Inconsistency, the gene expression of GPR61 mRNA did not reveal significant differences between the diseased and post-mortem control samples, $p = 0.231$ (Figure 32).

An interesting observation was made in one of the TLE-HS surgically resected hippocampi. In this sample, unknown neuronal inclusion bodies positively reacted with the GPR61 protein antibodies present in the CA1 neuronal cells of the hippocampal subfield (Figure 33).

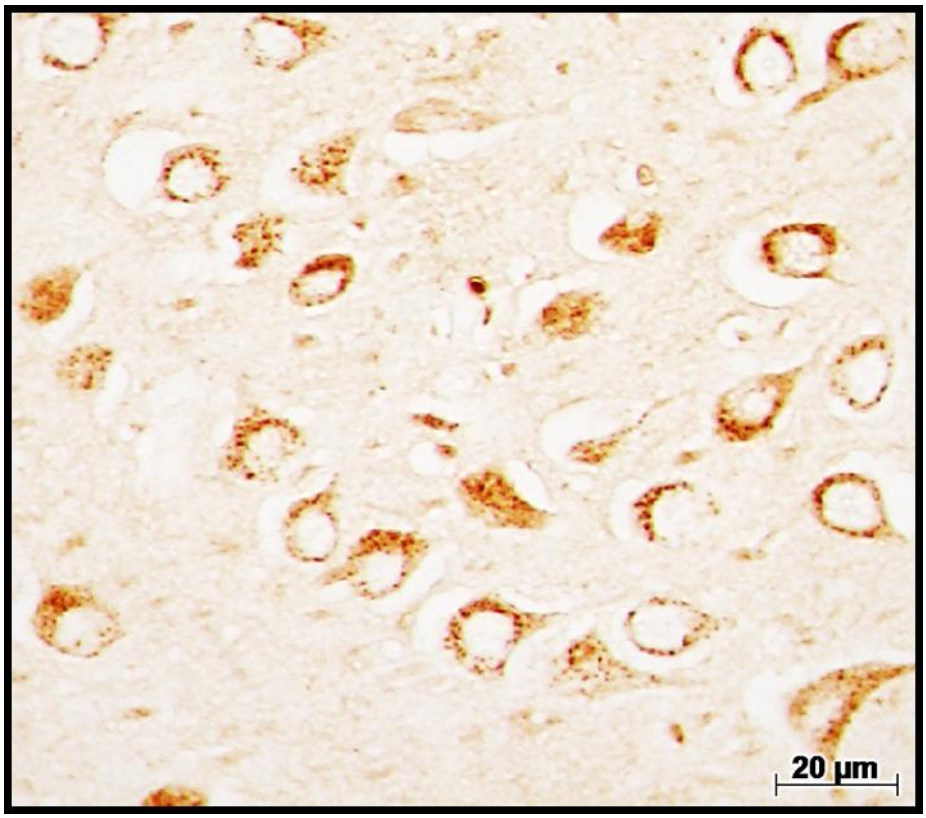

Figure 29. Localisation of GPR61 protein, present as small brown immunoreactive dots, in the cell membrane of pyramidal neurons of normal hippocampal CA1.
n = 3, scale bar = 20 µm.

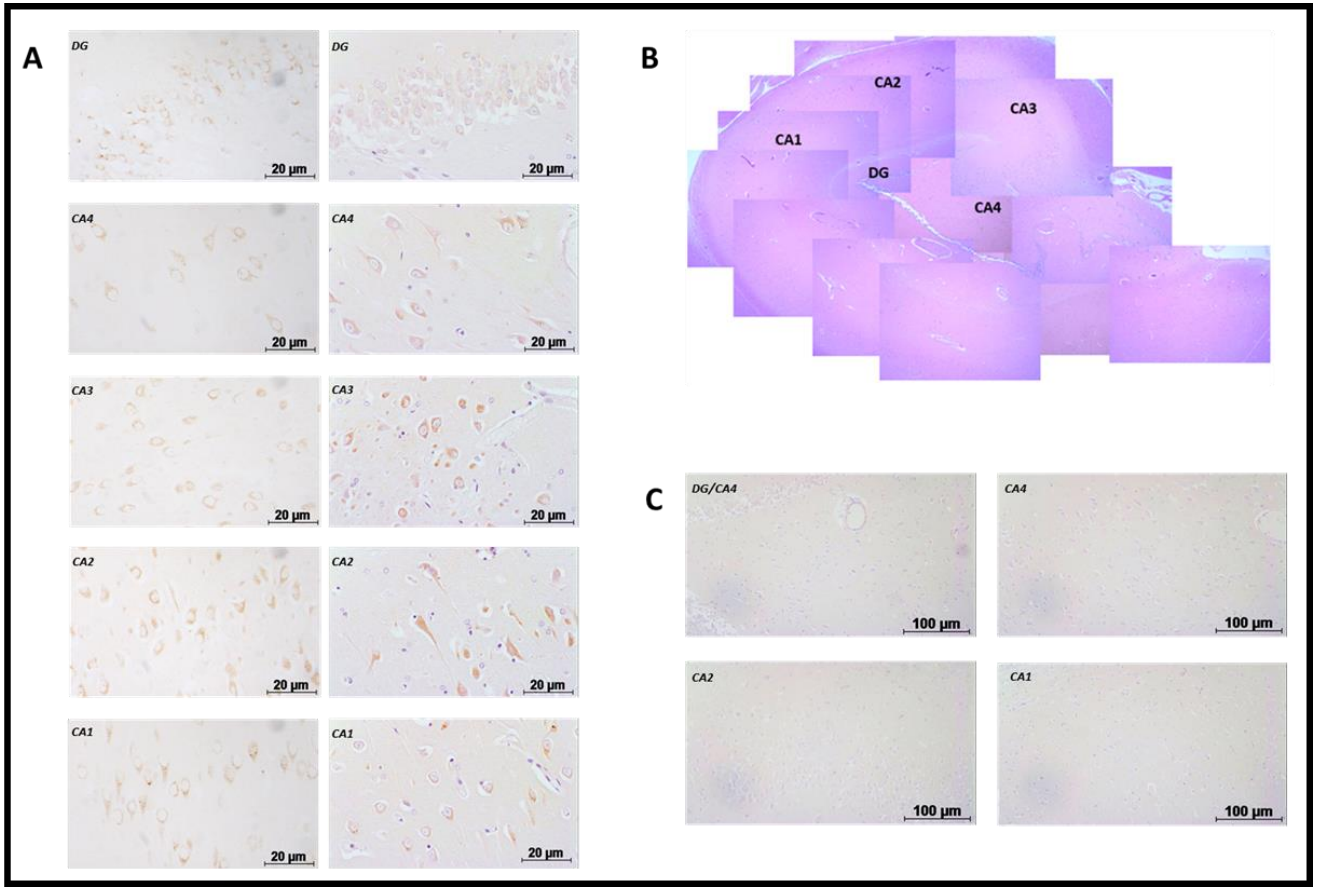

Figure 30. The immunoreactivity of GPR61 protein in hippocampal subfields in normal post-mortem control hippocampi, PMC (n = 3, representative result of 5 different experiments).

A) GPR61 protein immunoreactivity. *Left;* pre-hematoxylin counterstain. *Right;* post-hematoxylin counterstain. *B)* Histological staining and the localisation of the selected immunoreactivity signals. *C)* No primary negative control counterstained with hematoxylin. *High signal;* CA3, CA2 and CA1, Moderate signa*l;* Dentate gyrus, *Low signal;* CA4 (assessed based on pre-hematoxylin counterstain GPR61 protein immunoreactivity signals). Scale bars = 20, 100 µm.

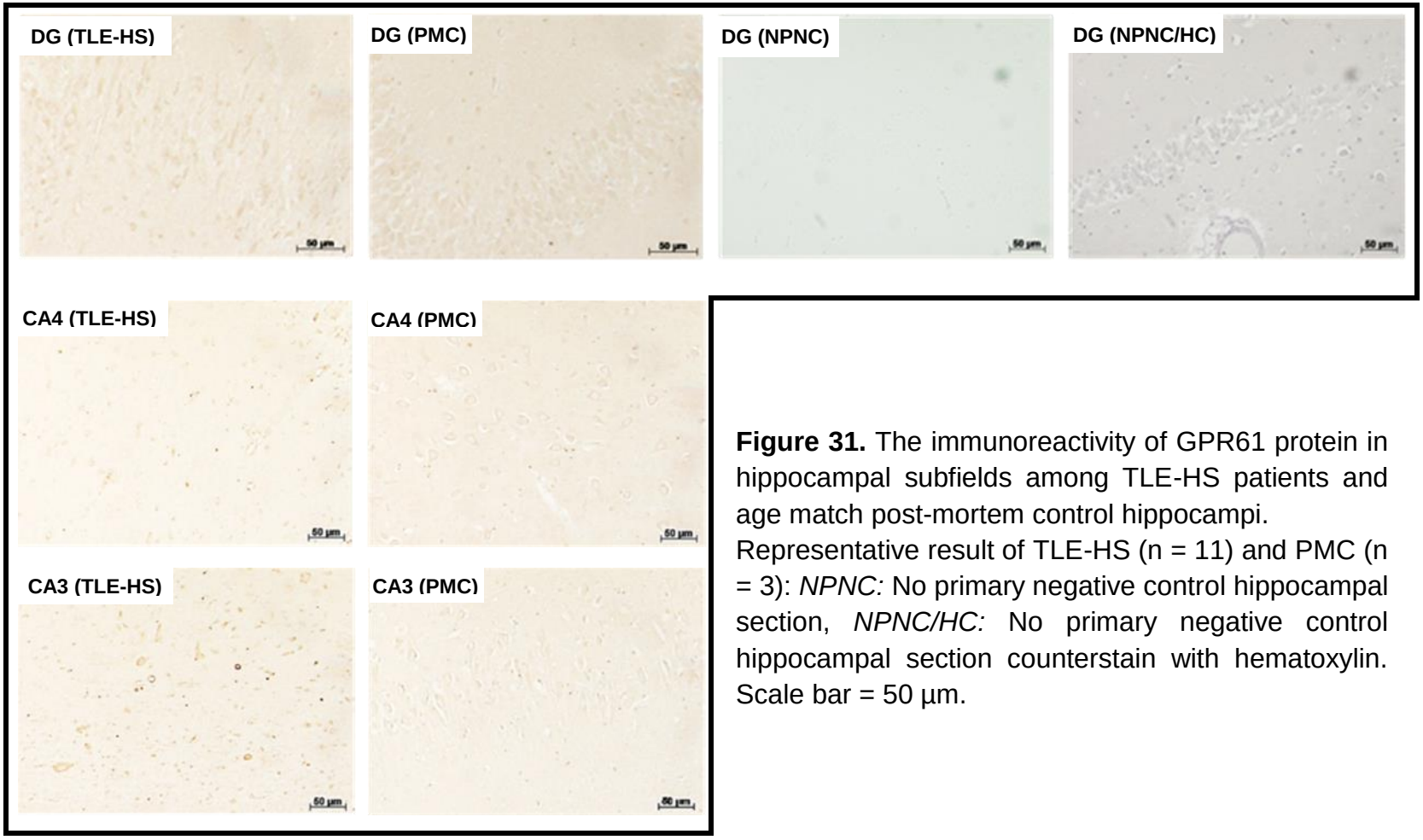

Figure 31. The immunoreactivity of GPR61 protein in hippocampal subfields among TLE-HS patients and age match post-mortem control hippocampi. Representative result of TLE-HS (n = 11) and PMC (n = 3): *NPNC:* No primary negative control hippocampal section, *NPNC/HC:* No primary negative control hippocampal section counterstain with hematoxylin. Scale bar = 50 µm.

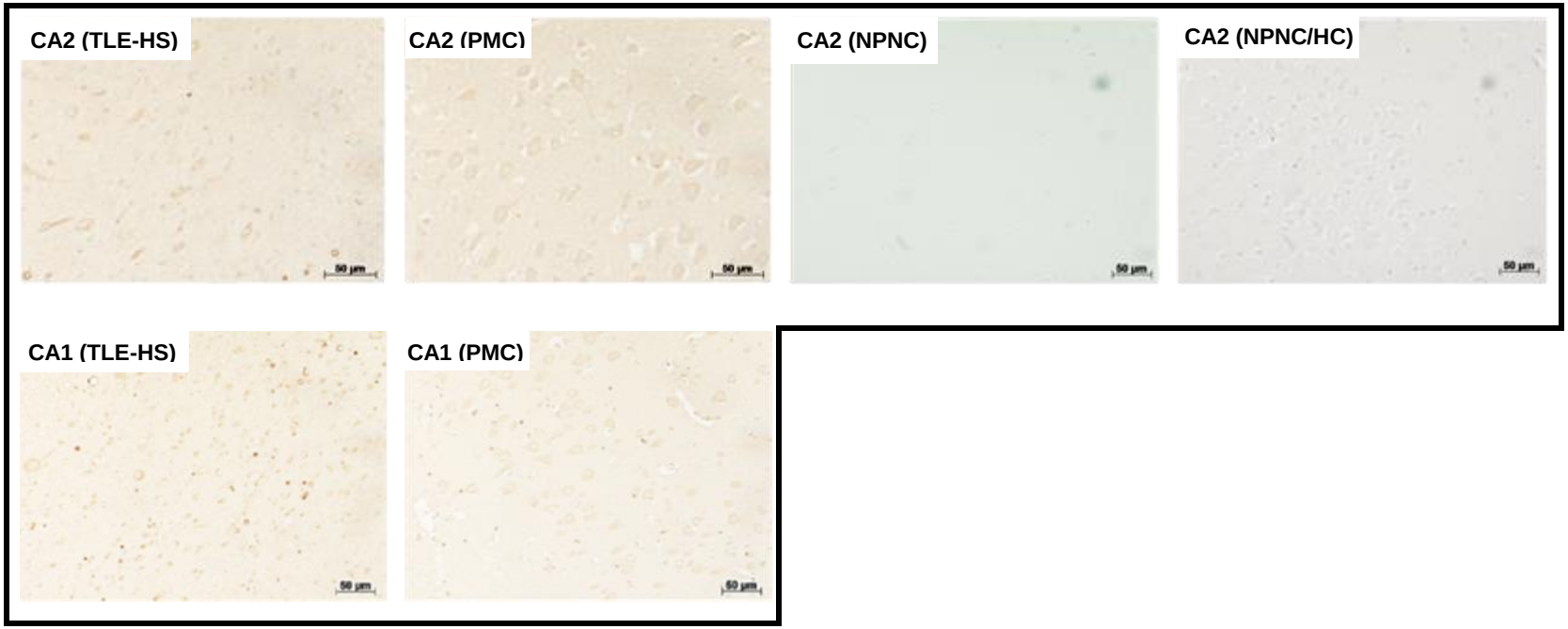

Figure 31. *continuous.*

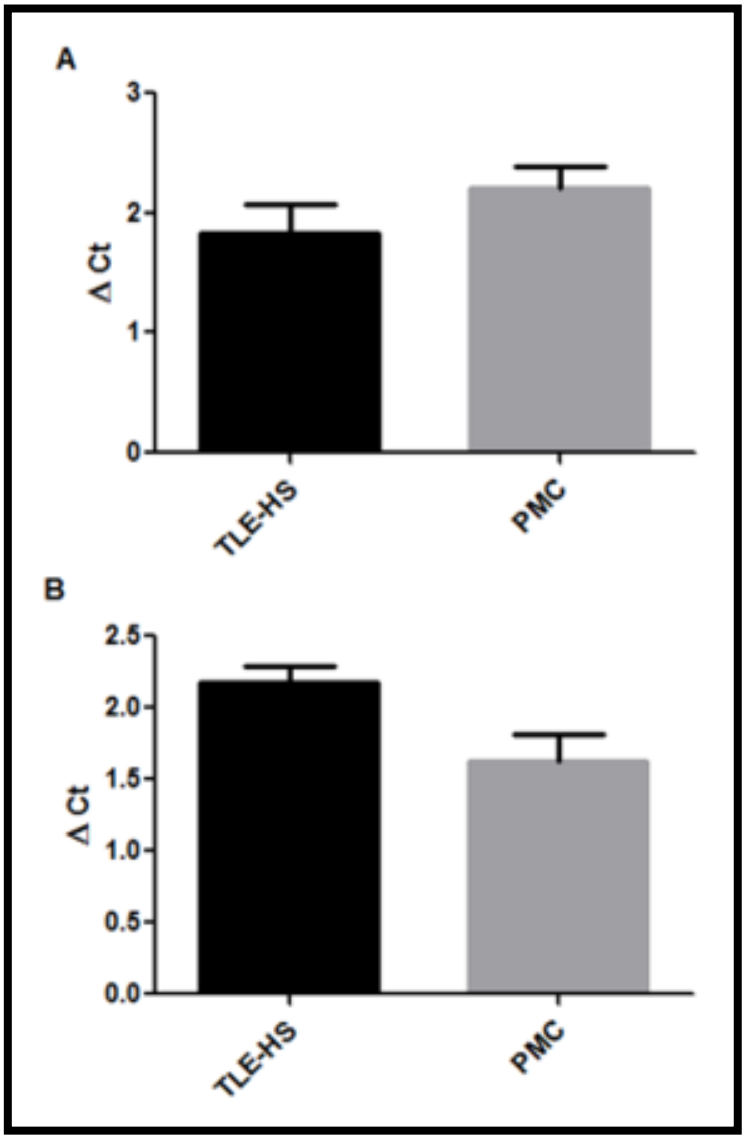

Figure 32. GPR61 protein gene expression in hippocampus of patients with TLE-HS.

A) GPR61 protein gene expression normalised with the expression of HPRT1 gene expression (housekeeping gene), TLE-HS n = 6, PMC n = 9, mean ± SEM, p= 0.231.

B) GPR61 protein gene expression normalised with the expression of ENO2 gene expression (housekeeping gene), TLE-HS n = 4, PMC n = 9, mean ± SEM, p= 0.092.

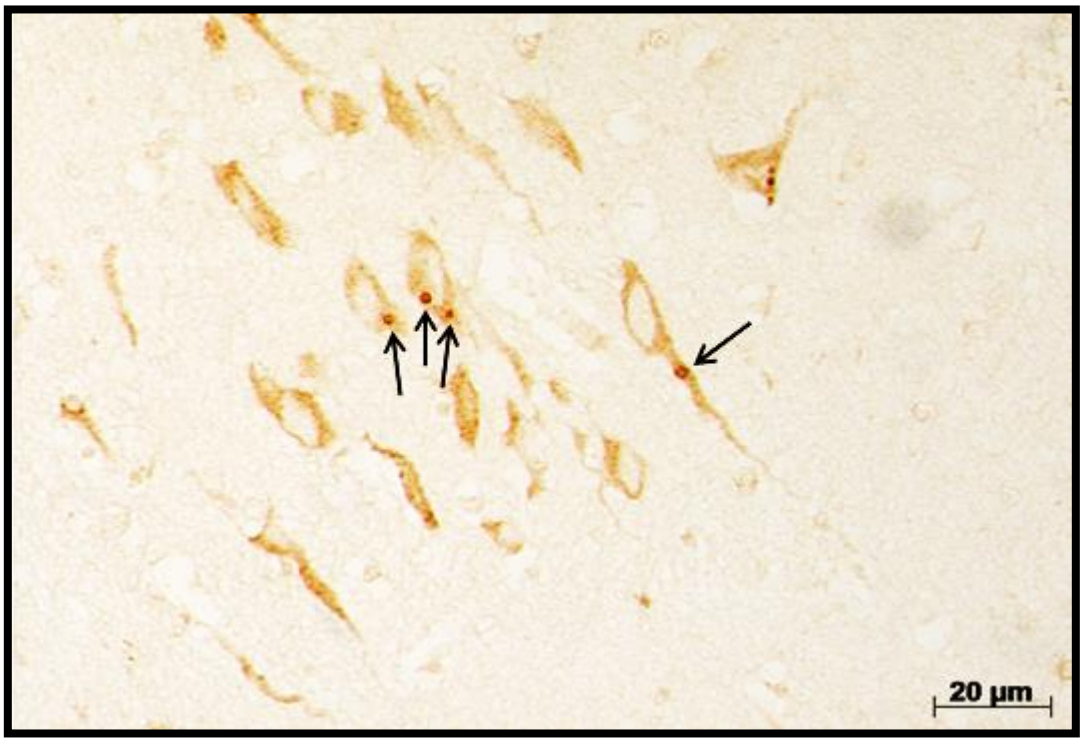

Figure 33. GPR61 protein positive neuronal inclusion bodies in neuronal cells in CA1 in one of TLE-HS hippocampal samples.
Representative result of immunohistochemistry staining of GPR61 from two adjacent sections.

3.2.D. Purinergic ion channel, $P2x_7$ receptors

The study of the excitatory ligand gated purinergic ion channel immunoreactivity indicated an upregulation of $P2x_7$ receptors in the dentate gyrus and CA2, where the neurons were preserved from the disease's negative effect on neuronal cells viability in comparison to the post-mortem control hippocampi (Figure 34). Furthermore, the immunoreactivity signal showed a slight overexpression of P2x7 in CA3 and CA1 with respect to neuronal loss in the diseased samples (Figure 34).

Regarding the $P2x_7$ receptors' gene expression, the results (inconsistence to protein expression) do not show any significant change in the TLE-HS patients in comparison to the age-matched post-mortem control hippocampi, $p = 0.644$, (Figure 35).

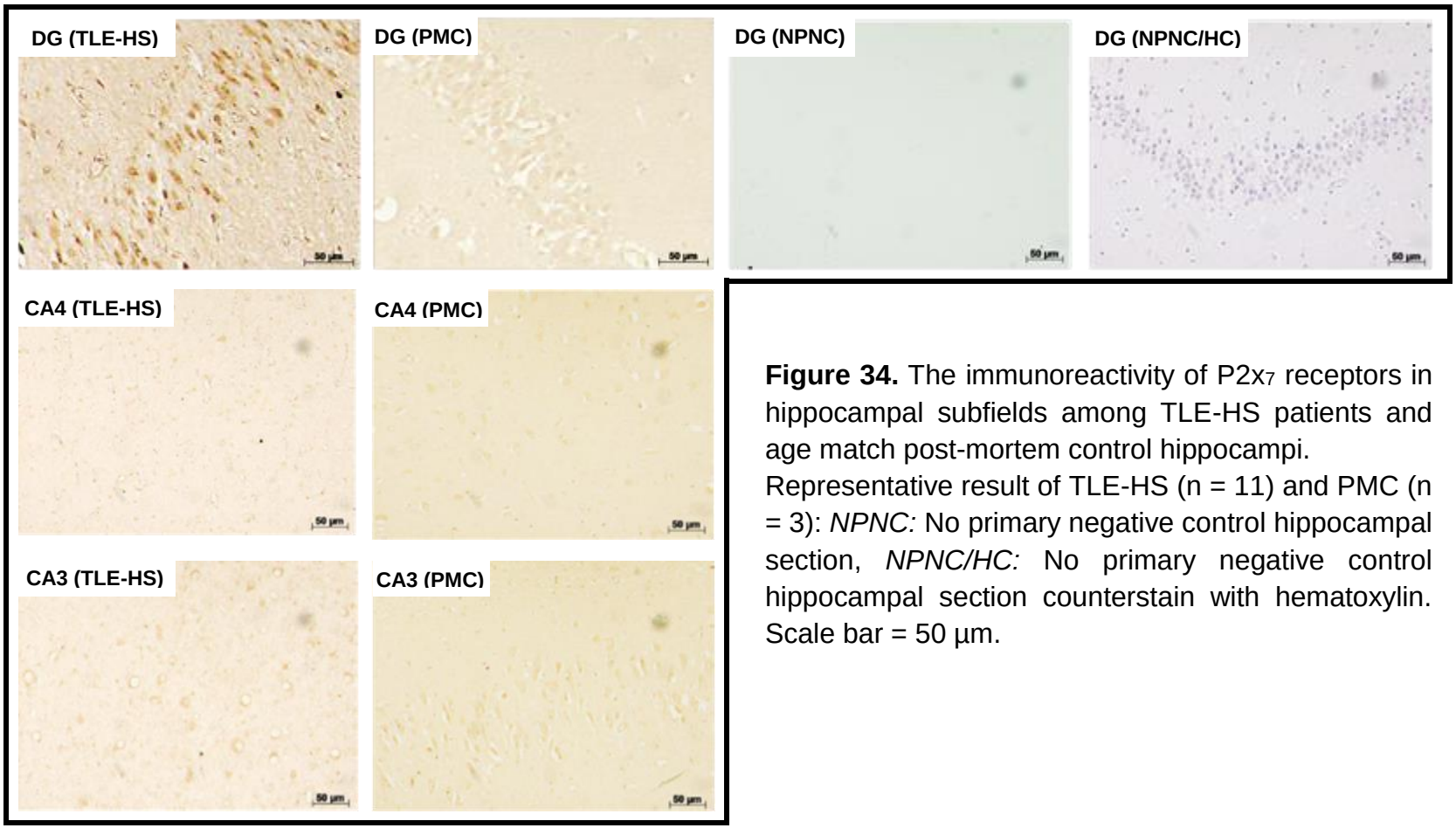

Figure 34. The immunoreactivity of P2x$_7$ receptors in hippocampal subfields among TLE-HS patients and age match post-mortem control hippocampi.
Representative result of TLE-HS (n = 11) and PMC (n = 3): *NPNC:* No primary negative control hippocampal section, *NPNC/HC:* No primary negative control hippocampal section counterstain with hematoxylin. Scale bar = 50 µm.

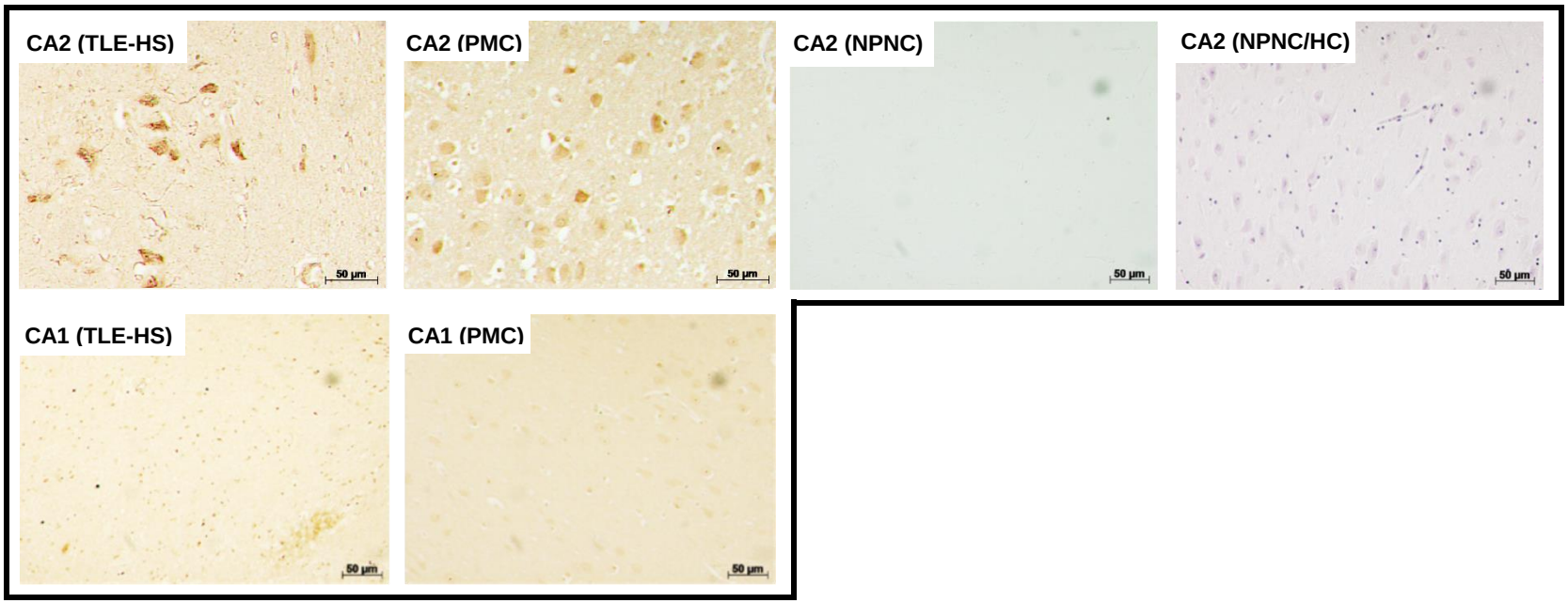

Figure 34. *Continue*.

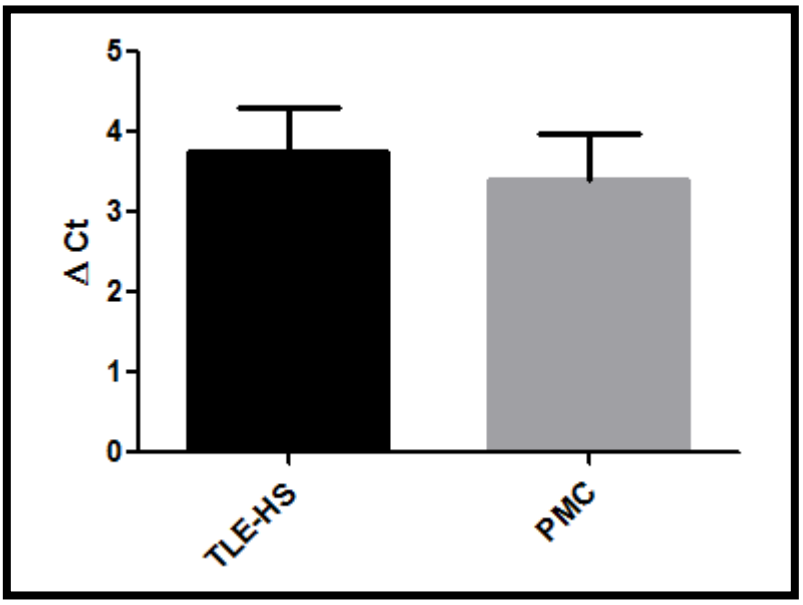

Figure 35. P2x$_7$ receptors gene expression in hippocampus of patients with TLE-HS.
The P2x$_7$ receptors gene expression normalised with the expression of ENO2 gene expression (housekeeping gene), TLE-HS n = 4, PMC n = 3, mean ± SEM, *p*= 0.644.

CHAPTER 4

Discussion

4. Discussion

4.1. Experimental optimization

4.1.A. Immunohistochemistry

Due to the limited number of human hippocampal samples, the IHC optimisation processes were conducted to maximise the amount of information that could be obtained from each sample. Thus the most suitable IHC staining protocol to give a specific immunoreactivity signal without affecting the RNA samples, which could then be used to study the effect of this disease on the expression of target receptors and protein mRNA. This also provided a direct correlation between the expression of the target proteins and the mRNA level. The results of the optimisation process with the different antigen retrieval techniques demonstrate the suitability of using HIER – for de-masking the antigen on the human hippocampal samples – to study the expression of the target protein using the selected antibodies (Table 5) and giving a strong, specific signal to each antibody.

Regarding the use of the optimised EIER protocol, this resulted in a strong, specific immunoreactivity signal when used to recover the antigens to allow detection of 5-HT_{2A} receptor. This indicated that EIER can be employed to study the expression of 5-HT_{2A} receptors using this antibody. Furthermore, a reasonably moderate signal of 5-HT_{1A} receptor was detected using this method. For this reason, and because of the limited amount of the mouse anti-human 5-HT_{2A} antibody – which is no longer synthesised – the rabbit anti-human 5-HT_{1A} antibody was used for the downstream experiments optimisation.

This enzyme protocol was associated with some effects on the immunoreactivity signal of the tested antibody (5-HT_{1A}). These included a reduction in the IHC signal and a relative reduction in the background; however, when this experiment was run under nuclease-free conditions, the immunoreactivity signal increased and the background decreased. This suggests that EIER could produce an improved result when the protocol is conducted under nuclease-free conditions.

Regarding the increase of the sensitivity of the GPR61 immunoreactivity signal using the PBTA-DAB technique, the result showed an increase in GPR61 antibody sensitivity, with a negative effect on the resolution of the signal. The loss of the signal resolution limited the use of this technique to localise the GPR61 protein in brain tissue samples using this antibody (goat anti-human GPR61 antibody, C-terminus, GT2-135). Nevertheless, this staining involved a combined technique including polymer and DAB, and it could be used to enhance the sensitivity of low-sensitivity primary antibodies.

4.1.B. Total RNA extraction

- **FFPE hippocampal samples**

In terms of extracting RNA from FFPE hippocampal samples, the use of the poly-A-RNA carrier failed to have a positive impact on the yield or integrity of the extracted RNA. In addition, no improvement was detected in the Ct value of the RT-qPCR of the housekeeping gene (HPRT1). These results could be due to the severe level of degradation of the extracted RNA, which made the detection of differences is difficult. The degradation and low yield may have been due to the following: a) the tissue fixation method (FFPE), b) the type of tissue (adipose tissue), which adds more challenges for extracting RNA, c) the thickness of the sections (4 µm), or d) the storage conditions (room temperature). These factors can accelerate the rate of degradation of cellular RNA.

Regarding to the effect of IHC staining protocols on the RNA yield, the antigen retrieval step was associated with unexpected effect on the RNA yield; this yield was significantly increased, especially when PK/dH2O was used for antigen retrieval without staining. It seems to be essential to perform this step before extracting RNA from FFPE brain samples. Interestingly, the staining process that followed the antigen retrieval caused a strong negative effect on the RNA yield from the FFPE stained slides. This effect on the RNA yield may explain the harmful effect of IHC staining chemicals on the cellular RNA.

There were no differences in the effect of using DPC-treated H2O on RNA yield under nuclease-free conditions compared to when the RNA was extracted regardless of nuclease-free conditions and solutions. This may be due to the sever degradation level of

RNA that affected by the fixation method (FFPE), tissue thickness and storage time and condition.

- **Frozen brain samples**

To maximise the amount of mRNA extracted from the limited—and thus highly precious—number of human hippocampal samples, alternate RNA extraction methods were examined using equal amounts of rat brain homogenate. Results indicated that Triazole acts as a powerful solubilising agent, capable of dissolving the plasma membrane of brain tissue samples containing high concentrations of fatty acids and triglycerides (Cirera, 2013; Hemmrich, et al., 2009). Thus, Triazole causes an increase in the amount of RNA extracted from a sample. Disadvantages of Triazole compound use involve the risk of protein contamination and the presence of a negative safety profile. However, use of an RNEasy mini column to clean the aqueous layer of Triazole-containing RNA produced a high yield and pure RNA sample. Therefore, a protocol was developed involving the combined use of Triazole and RNEasy mini kits to extract RNA from human hippocampal samples.

The work on the extracted RNA from FFPE samples had been stopped due to the arriving of human hippocampus frozen samples (TLE-HS and PMC).

4.2. Receptors and protein expression in human TLE-HS hippocampal samples

The present studies investigated potential druggable targets within hippocampus that can be used to control seizures in TLE-HS patients. These studies focused on the involvement of some excitatory and inhibitory serotonergic receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_3A, 5-HT_4 and 5-HT_7) in TLE-HS with more emphasis on the clinically important ionotropic excitatory receptor (the 5-HT_3 receptor). In addition, some interesting non-serotonergic targets (GPR61 and P2x_7), which are poorly understood in this type of epilepsy were also investigated since suitable antibodies were available in the laboratory due to other projects. The laboratory investigation of these targets revealed the following;

4.2.A. Ligand gated ion channel, 5-HT_3 receptors

- **5-HT_3A receptors**

Ionotropic excitatory serotonergic 5-HT_3 receptors were studied due to their important physiological and pathological role in the human brain. This study is complementary to the work done previously in Professor Nicholas Barnes' laboratory. The 5-HT3A receptor subunit is the critical subunit to convey function of the 5-HT_3 receptor. The 5-HT3A receptor subunit can form a pentameric homomeric 5-HT_3A receptor, or can be combined with other 5-HT3 receptor subunits (3B, 3C, 3D and 3E subunits) to form a heteromeric 5-HT_3 receptor (Niesler et al., 2008). The best studied heteromeric 5-HT_3 receptors are the 5-HT_3AB receptors, that are pharmacologically identical to 5-HT_3A receptors, yet have greater single channel conductance and are relatively less permeable to Ca^{2+} (Davies et

al., 1999). The immunoreactivity of 5-HT3A receptor subunits in hippocampal samples obtained from TLE-HS patients was examined using immunohistochemistry techniques and compared with hippocampal samples from post-mortem 'controls' (ie individuals who had died without a neurological or psychiatric disorder). There was a clear upregulation in the 5-HT3A-immunoreactivity within the resected hippocampi from patients with TLE-HS. This overexpression primarily affected granular cells of the dentate gyrus and pyramidal neurons of the CA2 field, as well as the CA3 field. This clear change in levels of immunoreactivity was unlikely to simply represent a difference in the 'post-mortem' delay (for the control tissue the period of time from death until the hippocampal tissue was cryopreserved, whereas for the resected hippocampi the time from surgical resection to cryopreserving the tissue) since the difference in immunoreactivity was also evident after subjecting the resected hippocampal tissue from patients with TLE-HS to an artificial post-mortem delay to better mimic the delay necessitated to obtain the tissue from the control donors. This result likely excludes the possible negative biological effect of the post-mortem condition on protein expression. This finding is parallel to the data generated by Professor Barnes, where a 5-HT_3 receptor expresses an upregulation in the molecular layer of the dentate gyrus, as a result of pathological granular cell migration and mossy fiber sprouting from dentate granular layer, and the CA2 of the hippocampus of TLE-HS patients using 5-HT_3 receptor autoradiography. Moreover, this increase in receptor binding activity appears due to an increase in 5-HT_3 receptor density rather than an increase in the affinity of the radioligand ([^{3}H]-granisetron) for the receptor in diseased samples, which were examined using saturation binding studies and followed up with

single concentration radioligand binding assays to assess the levels of 5-HT_3 receptor binding in the hippocampal tissue from controls and patients with TLE-HS.

Within the hippocampus, the excitatory 5-HT_3 receptor is normally expressed in pyramidal neurons (Brady et al., 2007) and GABAergic interneurons (Tecott et al., 1993; Morales and Bloom, 1997), and it is detected at a very low level in dentatal granular cells (Brady et al., 2007). This receptor modulates neurotransmitters' (especially glutamate and GABA) release in the hippocampus (Pehrson and Sanchez, 2014). The activation of this receptor increases the cellular excitability and firing of neuronal cells (Nicholas and Nicholas, 2008). In the hippocampus, excitatory pyramidal neurons integrate hippocampal information and transfer its outputs to different brain regions (Graves et al., 2012). These principle neurons received tens of thousands of stimulatory and a few thousand inhibitory synaptic neurotransmitters (Megias et al., 2001). Similar to the pyramidal neurons, fully developed dentate granular cells received an excitatory input that was predominantly glutamate and produced a synaptic glutamatergic response on the neuronal cells of the hippocampal CA3 (Gomez-Lira et al., 2005). This response was preserved even after the transient upregulation of GABA that results from continuous neuronal excitability during a seizure (Gomez-Lira et al., 2005).

The consistent findings of upregulated 5-HT_3 receptors in the hippocampus of TLE-HS patients in comparison to control samples strongly suggest that the upregulation of the excitatory 5-HT_3 receptors in the excitatory pyramidal and granular neurons may contribute to the seizure activity in the sclerotic hippocampus of TLE-HS patients. Therefore, the 5-HT_3 receptor could be proposed as a potential novel target to antagonise

in order to control seizures in this type of epileptic patient. There are a number of 5-HT3 receptor antagonists marketed for clinical conditions (e.g. cancer chemotherapy-induced emesis, post-operative nausea and vomiting; Garrett et al., 2003), which may allow the testing of such drugs in an attempt to broaden their therapeutic utility. Indeed, there are already a number of 'off-label' uses of 5-HT3 receptor antagonists, such as to relieve chronic fatigue syndrome and fibromyalgia (Tolk et al., 2004).

Normally with such a potential identified, a logical next step would be to assess the action of the potential therapeutic within an animal model. Unfortunately, there are no good animal models of TLE-HS – since in the human condition patients tend to be refractory to drug treatment, hence the need for surgical intervention – yet in proposed animal models a number of anti-epileptic drugs display efficacy, hence deviating from the human clinical condition. According to Sloviter (2008) chemo-induced SE in rodents (e.g. the lithium-pilocarpine rat model) is associated with high mortality rates and the surviving animal exhibits less severe seizure activity. Moreover, a variable degree of histological damage in the hippocampus (more often limited damage) is observed among the surviving animals; however, extrahippocampal damages are more pronounced in this type of animal model (Sloviter, 2008). The significant extrahippocampal changes and the limited hippocampal damage indicate that extrahippocampal neurons could be the source of the continuous SE activity (Sloviter, 2008). Different to chemo-induced SE, electric shock-induced SE is not associated with a high mortality rate or extrahippocampal damages. Nevertheless, interpreting the electrophysiological recording of an animal brain is more difficult as a result of the presence of the implanted electrodes, which are themselves

considered a brain injury (Sloviter and Bumanglag, 2013). A further complication arises due to the cellular expression of the 5-HT_3 receptor within the rodent hippocampus. Thus within the rodent hippocampus, 5-HT_3 receptor expression is restricted to GABAergic interneurons (Tecott et al., 1993; Morales and Bloom, 1997), whereas in human hippocampus, 5-HT_3 receptor expression is also evident in the excitatory glutamate neurons (pyramidal neurons and dentate gyrus neurons; Brady et al., 2007; present studies). Indeed, throughout the forebrain of rodents, the pattern of 5-HT_3 receptor expression differs to humans (Barnes et al., 1989; Parker et al., 1996). Therefore, this further negates the benefit of testing the action of a 5-HT_3 receptor antagonist in a rodent model of TLE-HS.

In spite of the issues associated with animal models, the effect of a 5-HT_3 receptor antagonist was studied on the lithium-pilocarpine rat model. The result revealed that ondansetron (a 5-HT_3 antagonist) protects animals from seizures (Kasture and Kasture, 1999). Moreover, in comparison to phenytoin (an anticonvulsant drug acting as a voltage-gated sodium channel blocker), ondansetron alone or in combination with phenytoin exhibits higher anticonvulsant activity on the electric shock-induced SE rat model (Balakrishnan et al., 2000).

Despite these interesting results, the extrapolation of these data from animal models to patients with TLE-HS is questioned due to the differences in the expression pattern of the 5-HT_3 receptor between human and rodents' hippocampi (Tecott et al., 1993; Morales and Bloom, 1997; Brady et al., 2007), in addition to the limitations of the animal models (Sloviter, 2008; Sloviter and Bumanglag, 2013).

In vitro electrophysiological studies of the effect of antagonising 5-HT_3 receptors on the seizure activity of resected hippocampi from TLE-HS patients may assess the possibility of using such medications to control seizures among TLE-HS patients. However, such type of studies associated with some limitations that affect the interpretation of the results. These limitations are including; 1) absence of neuorotransmitters and modulators inputs and outputs received from and to the resected hippocampus from other brain areas, 2) risk of neuronal ischemia and hypoxia on the resected hippocampus due to lack of blood circulation, 3) neurotrauma as a result of surgical resection and slicing procedure, 4) short lifespan of sliced neuronal block (Xiong and Xia, 2014). Therefore, the results of this type of study could be questioned. However, in this research project, electrophysiological recordings of hippocampal tissue resected from patients with TLE-HS were attempted (5 patients, data not present). However, despite indications of neuronal electrical activity being recorded, no clear and consistent results were obtained, potentially due to some of the issues raised above. Nevertheless, the positive results of present project concerning the upregulation of the excitatory 5-HT_3 receptor by surviving excitatory hippocampal neurons within the hippocampus of patients with TLE-HS (Table 15), along with the availability of marketed safe and selective 5-HT_3 receptor antagonists (e.g. granisetron), affords the opportunity to assess the potential therapeutic utility of 5-HT_3 receptor antagonists to reduce epileptic episodes of patients with TLE-HS. According to the pharmacological study of different 5-HT_3 receptor antagonists presented in this project, the result reveals a compatible affinity for the target receptor between TLE-HS and PMC samples for the drugs investigated in this study. Such data facilitates the selection of the drug candidate for use in a clinical study of patients with TLE-HS.

The suggested plan for the phase II clinical trial is to start with a small number of clinically diagnosed TLE-HS patients (n = 10), to enter a phase IIa pilot study. The proposed candidate drug to use in this study is granisetron. The rationale for this selection is that clinical grade generic granisetron is available in the UK (Palliativedrugs.com, 2008) and the drug is considered a better 5-HT_3 receptor antagonist to use over ondansetron due to greater selectivity for the 5-HT_3 receptor. Furthermore, to our knowledge, granisetron has at least a lower tendency to cause seizures in its own right (a rare side effect of some 5-HT_3 receptors antagonists e.g. ondansetron). Granisetron was reported to be possibly involved in a few case reports of seizures regarding pediatric patients receiving polypharmacy, including some drugs that have the known tendency for seizure activity, such as opioids, such that a specific role for granisetron is difficult to assess. However, because of this potential for 5-HT_3 receptor antagonists to cause seizures, it is recommended that a carefully monitored dose ranging 'safety' study in patients with TLE-HS is first initiated.

Under close clinical monitoring, a group of five TLE-HS patients will receive low dose granisetron (0.25 mg bid) in addition to standard treatment. If this dose of granisetron is well tolerated for a week, the dose would be elevated to 0.5 mg bid for a further week. In the absence of concerns over the granisetron medication, the dose would then be elevated to 1.0 mg bid for a further week. The plasma concentrations of granisetron will be monitored during this safety trial. Whilst the primary endpoint is to assess safety, the use of seizure diaries and with additional information from carers, any potential efficacy would also be assessed.

If the initial safety trial reached a primary end point of no adverse safety profile, then a subsequent more extensive study aimed at assessing efficacy as a primary endpoint would be warranted. This latter study (phase IIb) would take the form of a double blind placebo-controlled cross over study of granisetron (1.0 mg bd) in patients with TLE-HS for three months with a further three months following treatment crossover. Seizure incidence and any patient-reported side effects would be assessed by a patient diary. In addition, plasma concentrations of granisetron would be recorded periodically along with a standard set of physiological and biochemical assessments designed to monitor potential side-effects resulted. The primary endpoints of this study will be a reduction in seizure frequency and/or severity. By utilizing patients that were pre-selected for surgical intervention a clear diagnosis and results from a battery of tests would allow some degree of harmonization in the patients in terms of their TLE-HS, which may facilitate positive results from a smaller cohort.

Table 15. Assessment of the 5-HT_3 receptors expression in the hippocampus of patients with TLE-HS using different experimental techniques:

Assessment technique	5-HT_3 receptors expression level in TLE-HS patients
Homogenate radioligand binding assay of [^{3}H]-granisetron	Increase
Receptors autoradiography of [^{3}H]-*S*-zacopride	Increase
Immunohistochemistry	Increase
SDS-PAGE western blot	Increase
RT-qPCR	No change

The positive results obtaining concerning the upregulated expression of 5-HT_3 receptors in the hippocampus of patients with TLE-HS focussed attention upon the potential dysfunction of the 5-HT system in TLE-HS. Hence, a broader assessment of 5-HT function in TLE-HS was initiated. This included assessing the expression of other 5-HT receptor subtypes as detailed below.

4.2.B. Serotonergic G-protein coupled receptors

- **5-HT_{1A} receptors**

Whilst the 5-HT_3 receptor is a LGIC, all the other 5-HT receptor subtypes are G-protein coupled receptors (GPCRs). The first 5-HT GPCR investigated in the present study was the Gi-protein coupled 5-HT_{1A} receptor. Further rationale for study of the 5-HT_{1A} receptor in TLE-HS is the well-recognised comorbidity between epilepsy and depression in some epileptic patients. The comorbidity has been reported to be present in 11% of the patients with epilepsy (Lothe et al., 2008). This percentage increases to around 60% among patients with recurrent seizures. Depression is frequently observed among patients with TLE-HS, especially when TLE-HS affected the left hippocampus (Altshuler et al., 1990; Quiske et al.; 2000; Jones et al., 2005). Hence the well-recognised role of the 5-HT_{1A} receptor with respect to depression adds rationale to the study of this receptor in TLE-HS.

The results of this project revealed an upregulation of 5-HT_{1A} receptors among TLE-HS hippocampal samples compared to the age-matched control hippocampi. The pathological change in the expression of these inhibitory receptors' expression might be

a physiological compensatory mechanism to adapt the hyper-excitable neuronal activity in the hippocampus (or indeed vice versa). Interestingly, no significant alteration in the receptor mRNA was observed in the disease samples compared to the control hippocampi.

To the best of our knowledge, there is no scientific data about the correlation between the 5-HT_{1A} receptor density at the molecular level and the binding activity in the hippocampi of patients with TLE-HS in comparison to age-matched post-mortem control samples, except those conducted using positron-emission tomography (PET). These studies were conducted using either high affinity 5-HT_{1A} receptors' antagonists such as [^{18}F]FCWAY (Toczek et al., 2003, Giovacchini et al., 2005, Theodore et al., 2007) and [^{11}C]WAY-100635 (Savic et al., 2004, Parsey et al., 2006 and Assem-Hilger et al., 2010), or a low affinity ligand, e.g., [^{18}F]MPPF (Lothe et al., 2008), which has a close affinity to that of 5-HT in order to allow the endogenous 5-HT to bind to the 5-HT_{1A} receptors, (table 16). According to Savic (2004) and Assem-Hilger (2010), the ligands' binding activity was significantly lower among TLE-HS patients free from major depression disorder (MDD) using high affinity 5-HT_{1A} receptors' ligands. Moreover, data generated by Hasler et al. (2007) in antidepressant-naive patients with TLE-HS comorbid with MDD (TLE-HS/MDD) show a significant reduction in 5-HT_{1A} receptors binding activity. The comparable findings were observed among antidepressant-naïve MDD patients (Hirvonen et al., 2008). These results may support the hypothesis that there is a contribution from TLE-HS and MDD that underlies the pathological mechanisms (Hasler et al., 2007). Although, the TLE-HS samples in this project are obtained from anti-depressant naïve donor (except one),

however, no apparent difference in receptors immunoreactivity was observed among anti-depressant naïve and the one whose exposed to anti-depressant medication. Based on these data, the upregulation of the 5-HT_{1A} receptors that observed in our study requires the need for further *in vitro* laboratory investigation to the binding activity of this inhibitory receptor using diseased and control human hippocampal homogenate. Saturation radioligand binding assays with relevant tissue homogenates would help address the pathological alteration in the 5-HT_{1A} receptors affinity to the radio-labeled ligand and the receptor density in TLE-HS and PMC samples. Such a study would allow a better understanding of the involvement of this inhibitory receptor in TLE-HS.

In contrast to these finding, results were observed in antidepressant-naïve TLE-HS comorbid with MDD (Lothe et al., 2008) and in MDD patients (Parsey et al., 2006) by using low and high affinity 5-HT_{1A} receptor's antagonist, respectively, expressed an increase in the receptors binding activity. These results are in parallel to the inverse relationship between chronic treatment using serotonergic anti-depressant agents and the binding activity of 5-HT_{1A} receptors (Gray et al., 2013). The results of the upregulation of 5-HT_{1A} receptors' immunoreactivity in TLE-HS hippocampal sections, compared with age-matched post-mortem controls identified in this study, can give a possible explanation of the higher binding activity of different 5-HT_{1A} receptors' antagonists that were observed in these PET studies of the antidepressant-naive TLE-HS/MDD and MDD patients.

One of the pathological effects that may result from the upregulation of the 5-HT_{1A} receptors in the hippocampus of a TLE-HS patient is memory impairment, which was clinically observed in some of the TLE-HS patients. Thus, the overexpression of the

postsynaptic 5-HT_{1A} receptors can result in hyperpolarisation and suppress glutamate release, leading to inhibited neuronal activity and memory impairment (King et al., 2008).

In terms of functionality of the 5-HT_{1A} receptors, data generated by Cuellar-Herrera (et al., 2014) using 8-OH-DPAT-stimulated [^{35}S]-GTPγS binding assay on TLE-HS and PMC tissue homogenate, show a significant increase in 5-HT_{1A} receptors functionality among TLE-HS compared to age match control hippocampi. The result of this study associated with significant diversity in the 5-HT_{1A} functionality between TLE-HS patients. In order to minimise this inter-individual variability, the number of samples needs to be increased.

Hence whilst an interesting finding that finds some support from previous studies using different research techniques, further laboratory studies are needed to better understand the upregulation of 5-HT_{1A} receptors in the hippocampi of TLE-HS patients. This can be done using several methods, such as polyacrylamide gel electrophoresis (SDS-PAGE) Western blot in the presence of TLE-HS samples subjected to artificial post-mortem protocol to exclude the molecular effect of post-mortem delay upon protein expression.

Table 16. Studies on 5-HT_{1A} receptors in TLE and MDD patients

Result	Type of patients	Technique used for assess (ligand used)	Study	Comment
Decrease 5-HT_{1A} receptors binding activity	TLE	PET ([^{11}C]WAY-100635)	Savic et al. (2004)	High affinity 5-HT_{1A} radioligand
	TLE	PET ([^{11}C]WAY-100635)	Assem-Hilger et al. (2010)	
	TLE	PET ([^{18}F]FCWAY)	Toczek et al. (2003)	
	TLE	PET ([^{18}F]FCWAY)	Giovacchini et al. (2005)	
	TLE	PET ([^{18}F]FCWAY)	Theodore et al. (2007)	
	TLE/MDD (AD naïve)	PET ([^{11}C]WAY-100635)	Hasler et al. (2007)	
	MDD (AD naïve)	PET ([^{11}C]WAY-100635)	Hirvonen et al. (2008)	
Increase 5-HT_{1A} receptors binding activity	TLE/MDD (AD naïve)	PET ([^{18}F]MPPF)	Lothe et al. (2008)	Low affinity 5-HT_{1A} radioligand
	MDD (AD naïve)	PET ([^{11}C]WAY-100635)	Parsey et al. (2006)	High affinity 5-HT_{1A} radioligand
Increase 5-HT_{1A} receptors protein expression	TLE (AD naïve)	Immunohistochemistry	Result of present study	Highly selective rabbit anti-human 5-HT_{1A} receptors antibody

- **5-HT$_{2A}$ receptors**

The second serotonergic G-protein was selected to study due to its important role in crucial brain functions such as memory development, was the expression of $G_{q/11}$ coupled excitatory 5-HT receptors, the 5-HT$_{2A}$ receptor. An immunohistochemistry study of 5-HT$_{2A}$ receptor immunoreactivity in the hippocampi of patients with TLE-HS revealed that, in comparison with post-mortem control hippocampi, this excitatory receptor was downregulated within what displayed the morphology of astrocytes within the hippocampus. This reduction in expression appeared to affect all expressed astrocytes across the entirety of the TLE-HS hippocampal subfields.

This apparent downregulation of 5-HT$_{2A}$ receptors may have appeared as the result of a compensatory mechanism to decrease the activity of hyperactive neurons in the hippocampus by modulating glutamate release (Guiard and Di Giovanni, 2015). The downregulation might also result from continuous psychological stress, contributing to the reduction of 5-HT$_{2A}$ receptor density in hippocampal astrocytes (Egger, 2007, Dwivedi et al., 2005). Moreover, 5-HT$_{2A}$ receptor downregulation may manifest to improve the depressant-like behaviour associated with epilepsy (Sibille et al., 1997, Guiard and Di Giovanni, 2015). However, no data has been published regarding the cellular levels of 5-HT$_{2A}$ receptor expression in the hippocampi of patients suffering from TLE-HS comorbid with depression or the levels of depression-free TLE-HS patients in comparison to post-mortem control samples. A possible explanation for the reduction of 5-HT$_{2A}$ receptor density in TLE-HS patients is that this reduction may amount to a hippocampal physiological compensation strategy to improve memory impairment. However, the

downregulated 5-HT_{2A} receptors might result from diffused immunoreactivity signals associated with an increase in the number of astrocyte processes. Considering the astrocytosis of TLE-HS samples, the overall 5-HT_{2A} receptor expression level was higher than in post-mortem control samples. In order to better evaluate the level of expression of 5-HT_{2A} receptors in the astrocyte with respect to the increased number of cellular processes, a quantitative 5-HT_{2A} immunofluorescence assay needs to be conducted using TLE-HS and PMC hippocampal sections. This study facilitates understanding the effect of TLE-HS on the 5-HT_{2A} receptor expression in astrocytes and assess the possibility of 5-HT2A receptors immunoreactivity signal diffusion through the increased number of astrocyte process. Such laboratory investigation helps to evaluate the possibility of targeting this receptor to control seizures among TLE-HS patients.

In order to confirm the expression of 5-HT_{2A} receptors in the astrocytes of human hippocampi, the immunoreactivity of GFAP proteins was used as a phenotypic marker in sections adjacent to those stained for 5-HT_{2A} receptor immunoreactivity. The result displayed an approximate co-localisation of 5-HT_{2A} receptors and GFAP proteins in hippocampal astrocytes. In agreement with previous data generated by Proper et al., (2000), GFAP immunoreactivity signals were significantly decreased in the CA4 cell bodies of patients with TLE-HS hippocampi, as compared with post-mortem controls. However, GFAP immunoreactivity signals were spread within the CA4 as a result of the significant increase in astrocyte processes (Proper et al., 2000). Moreover, in contrast to the astrocyte cell bodies of the CA4, the astrocyte cell bodies of the dentate gyrus molecular layer expressed higher immunoreactivity signals (Proper et al., 2000).

Interestingly, 5-HT_{2A} receptor immunoreactivity signals followed patterns similar to those of GFAP expression in the dentate gyrus and CA4 of TLE-HS hippocampi. This finding parallels those observed in patients suffering from frontotemporal dementia, cerebral infarction, Alzheimer's disease, Creutzfeldt-Jakob disease, hypertensive encephalopathy, and Huntington's disease (Wu et al., 1999).

Regarding the effect of TLE-HS upon expression of 5-HT_{2A} receptor mRNA, the RT-qPCR result did not reveal any significant difference between diseased and control samples. The discrepancy between protein and mRNA expression may be a result of the disease negatively affecting the level of expression of 5-HT_{2A} mRNA, leading to a decrease in the levels of 5-HT_{2A} mRNA per cell. As a result of astrocytosis associated with TLE-HS, the downregulation of 5-HT_{2A} mRNA was difficult to detect without conducting single cell RT-qPCR using an equal number of cells from TLE-HS and post-mortem control samples.

Further laboratory investigation is required to confirm findings and to assess the potential of antagonising 5-HT_{2A} receptors to control seizures in TLE-HS patients. This can be accomplished using 5-HT_{2A} receptor autoradiography and SDS-PAGE western blot to confirm the effect of TLE-HS on the expression of 5-HT_{2A} receptors in patients. Moreover, in order to evaluate the level of expression of 5-HT_{2A} receptors in the astrocyte with respect to the increased number of cellular processes, a quantitative 5-HT_{2A} immunofluorescence assay need to be conducted using TLE-HS and PMC hippocampal sections. This study facilitates understanding the effect of TLE-HS on the 5-HT_{2A} receptor expression in astrocytes and assess the possibility of 5-HT_{2A} receptors immunoreactivity signal diffusion through the increased number of astrocyte process. Such laboratory

investigation helps to evaluate the possibility of targeting this receptor to control seizures among TLE-HS patients. Additionally, such methods can be used to study the effect of 5-HT_{2A} antagonists on the electrophysiological activity of hippocampi resected from TLE-HS patients. The effect of 5-HT_{2A} antagonists on attempts to elevate the chemo-induced (pentetrazol) seizure threshold was studied using juvenile C57BL/6 mice treated with ketanserin (selective 5-HT_{2A} receptor antagonists) (Puzerey et al., 2014). Dual immunofluorescent staining of 5-HT_{2A} receptors and GFAP proteins in hippocampi resected from TLE-HS patients need to be conducted and compared to post-mortem controls in order to assess the co-localisation and distribution patterns of these proteins in hippocampal astrocytes. In addition, study the possible pathways in which 5-HT_{2A}/GFAP proteins may intracellularly interact in order to understand the involvement of 5-HT_{2A} receptor in GFAP expression and astrocytes activation.

- **5-HT$_4$ receptors**

Given the well-recognised important role of 5-HT$_4$ receptors in the hippocampus, particularly associated with memory processes, and the evident colocalisation of 5-HT$_4$ receptors with inhibitory 5-HT$_{1A}$ receptors in hippocampal neurones (Roychowdhury, et al., 1994), the protein expression of the excitatory 5-HT$_4$ receptors among TLE-HS was selected to be the next GPCR assessed in this study in comparison to post-mortem control samples.

The results revealed an overexpression of the 5-HT$_4$ receptors in the dentate gyrus and CA2 of patients with TLE-HS. This upregulation of these excitatory 5-HT$_4$ receptors, like the increase in excitatory 5-HT$_3$ receptor expression identified in the present study, is consistent with an increase in the over neuronal activity within the hippocampi of patients with TLE-HS. This indicates that this upregulation of 5-HT$_4$ receptors could also play an important role in neuronal hyper-excitability among patients with TLE-HS. Based on that premise, antagonising the 5-HT$_4$ receptors in TLE-HS patients may also help in managing this type of neuronal disease. Unfortunately, there are no marketed 5-HT$_4$ receptor antagonists that could be assessed in a similarly designed clinical trial detailed earlier for assessment of a 5-HT$_3$ receptor antagonist. It may be relevant, however, that some 5-HT$_4$ receptor agonists are marketed to promote gastric motility (e.g. the selective agonists prucalopride, cisapride and tegaserod [although the latter two have been withdrawn due to cardiac side-effects], and the non-selective agonist, metoclopramide; Quigley, 2015). However, it would be interesting to mine clinical data of patients with TLE-HS that have received any of these drugs to determine if treatment altered the frequency of their

seizures or not. Whilst such a meta-analysis of clinical data was outside the scope of the present thesis, such a project is worthy of consideration.

On the other hand, this overexpression of 5-HT_4 receptors may manifest as a result of a compensatory mechanism to memory impairment that was clinically observed in some of the TLE-HS patients (Hermann et al., 1997). Surprisingly, according to Haahr et al. (2013), there is an inverted relationship between the 5-HT_4 receptors' binding activity in the hippocampus and episodic memory in young healthy volunteers using PET. In this study 5-HT_4 receptors' binding potential was decrease during episodic memory examination task in young healthy volunteers using highly selective 5-HT_4 ligand, [^{11}C]SB207145, (Haahr, et al., 2013).

Furthermore, using the same 5-HT_4 receptor ligand ([^{11}C]SB207145) in a PET study of the level of expression of 5-HT_4 receptors in healthy human volunteers and the level of 5-HT tone, the results showed that increased synaptic 5-HT levels in the human brain are associated with a reduction in 5-HT_4 binding activity (Haahr et al., 2014). As a result of the insensitivity of [^{11}C]SB207145 radioactive ligand to the endogenous level of serotonin in the neuronal synapses (Marner et al., 2010), the possibility of pharmacological competition between endogenous serotonin and [^{11}C]SB207145 binding activity is low. Accordingly, the result of this PET study may reflect a reduction in the 5-HT_4 receptors' expression after chronic treatment with selective serotonin reuptake inhibitors (SSRIs) for three weeks, which can cause a chronic increase in the synaptic concentration of 5-HT in the brain (Haahr et al., 2014). Therefore, the overexpression of 5-HT_4 receptors in the hippocampus may result from a reduction in the synaptic concentration of 5-HT. This

hypothesis could be investigated further by measuring the 5-HT concentration in the hippocampi of patients undergoing surgical resection by *in vivo* microdialysis.

Moreover, the 5-HT_4 receptor upregulation in TLE-HS patients could be consequences of serotonergic neurons denervation that present in the form of neuronal cell loss phenomena in TLE-HS hippocampi. According to Compan et al., (1996), the 5-HT_4 receptor was upregulated in rat hippocampus (especially CA2) after exposed the animal to chemo-induced neuronal cell lesion using 5,7-dihydroxytryptamine injected into rat raphe nuclei.

To the best of our knowledge, there is no available data about the expression or binding activity of 5-HT_4 receptors in TLE-HS patients. Therefore, in order to confirm these results, further laboratory and clinical investigations using SDS-PAGE Western blot using selective anti-human 5-HT_4 receptors antibody (to our knowledge, it is not available up to present time), and PET need to be conducted to compare with age-matched controls. This should be done to understand the role of these excitatory receptors in TLE-HS disease. Moreover, organotypic electrophysiological studies need to be conducted to examine the effect of antagonising these overexpressed excitatory receptors on the epileptic activity of the surgically resected epileptic hippocampi.

- **5-HT$_7$ receptors**

Studies of 5-HT$_7$ receptors were conducted to understand the effect of TLE-HS disease on the expression levels of another Gs-coupled excitatory 5-HT receptors and to investigate the possibility of targeting those receptors to control seizures in TLE-HS patients as a number of 5-HT$_7$ receptor antagonists are already available in the clinic e.g. the anti-psychotic agent, risperidone. In the present study, however, no consistent immunoreactivity signals were generated using the antibody available to label the 5-HT$_7$ receptor (Table 6), which prevents confident discussion of 5-HT$_7$ receptor expression (Appendix 1). Since the 5-HT$_7$ receptor remains a logical target to investigate, it is suggested that this receptor should be investigated using alternate anti-human 5-HT$_7$ receptor antibodies, which may perform better in the immunolabelling of the human 5-HT$_7$ receptors.

4.2.C. Orphan G-protein, GPR61

In addition to the serotonergic receptors, some interesting non-serotonergic proteins were selected to be assessed in the present study due to the opportune availability of useful antibodies in the Barnes laboratory. These studies also showed whether there was impact of TLE-HS upon other receptor systems. One of the proteins investigated was the orphan G-protein (GPR61). Relevance to study this receptor also arises due to its homologous genetic sequence and inclusion of biogenic amine receptors—specifically, 5-HT_6 receptors (Toyooka et al., 2009). Also considered was the expression of mRNA within the CNS, particularly in the hippocampus, caudate, putamen (Cikos et al., 2001, Lee et al., 2001) and thalamus of the human brain (Lee et al., 2001). My study aimed to evaluate the level of expression of GPR61 protein in the human hippocampal subfield and to examine the level of expression of this protein in the pathogenesis of TLE-HS. Results of protein immunoreactivity revealed for the first time that GPR61 is widely expressed in human hippocampal subfields, to varying degrees. Thus, GPR61 immunoreactivity was strongly detected in CA1, CA2, and CA3, with more moderate signals detected in CA4 and DG. The expression of the GPR61 protein in these areas may reflect its crucial role in higher brain functions (such as cognition, learning, and memory). The small immunoreactive blots appearing in the neuronal cell membrane may indicate that GPR61 proteins present in a small vesicle or bind with other proteins in the cell membrane. However, there is no published data regarding the localisation of GPR61 protein in human hippocampal subfields or neuronal cells.

Regarding the impact of TLE-HS on GPR61 protein expression, it is clear that the disease has a pathological impact on the expression of the orphan G-protein in human hippocampal subfields. These effects include a moderate increase in the protein immunoreactivity in the granular cells of the dentate gyrus and the survival neurons of the CA3; moreover, a slight increase in the survival neurons of CA1 and preserved neurons of CA2 was shown. This finding may indicate the overexpression of GPR61 protein receptor partners—which, until the writing of this project, was scientifically undiscovered.

Conversely, the level of GPR61 mRNA expression did not reveal any significant difference between TLE-HS and post-mortem control samples. This might be due either to TLE-HS's lack of negative impact on GPR61 mRNA, or due to neurodegeneration phenomena of TLE-HS in hippocampal neurons that masks differences in the level of expression of mRNA compared to protein.

Numerous laboratory studies conducted by multiple scientists aim to understand physiological functions, uncover downstream cellular mechanism pathways, reveal receptor partners, and discover a highly selective ligand to activate the orphan G-protein. To confirm results of protein expression in normal and TLE-HS human hippocampi, further laboratory investigations of resected human hippocampal samples should be conducted by for instance using SDS-PAGE western blot to evaluate the level of expression of GPR61 in each hippocampal comparison. Moreover, it would be very revealing to perform RT-qPCR evaluations using resected hippocampal subfields or even more revealing single cell RT-qPCR samples to relatively quantify the level of GPR61 mRNA changes to diseased samples, as compared with post-mortem control samples. To determine the

normal expression of GPR61 mRNA in human hippocampus subfields, in situ hybridisation must be used. Additionally, the impact of GPR61 proteins on higher brain functions should be investigated using GPR61 knockout mice.

To assess the observation that GPR61 protein positive neuronal inclusion bodies were detected in one TLE-HS patient, neuropathological consultation was obtained from two consultant neuropathologists; Dr. Martyn Carey and Dr. Santhosh Nagaraju of Queen Elizabeth Hospital, Birmingham, United Kingdom. The consultants did not recognise these neuronal inclusion bodies in this type of neurological disease. Based on the relevant literature, neuronal inclusion bodies can be histopathologically observed in certain neurodegenerative diseases, such as frontotemporal dementia, where the inclusion bodies are ubiquitin positive (Forno et al., 2002, Mochizuki et al., 2004). The medical history of this patient did not reveal any neurological or psychological disease other than TLE-HS. Therefore, further laboratory and clinical investigations must be conducted to better understand the nature of these inclusions.

4.2.D. Purinergic ion channel, $P2x_7$ receptors

The other non-serotonergic receptor investigated in this study is the excitatory ionotropic non-selective purinergic cation channel, the $P2x_7$ receptor. Similar to the other receptors studied in this project, expression of this receptor was studied using immunohistochemistry. Results revealed that the receptor was expressed at a relatively high level in the dentate gyrus and CA2 of hippocampi resected from TLE-HS patients, when compared to post-mortem control hippocampi. The upregulation of $P2x_7$ in TLE-HS samples, particularly in the dentate gyrus, is comparable to findings by Doná et al., (2009) where $P2x_7$ receptor immunoreactivity was studied in the pilocarpine-induced TLE-HS rat model during the chronic status epilepticus stage of the disease. This comparable finding between the species adds significance to the data and may suggest a role for this inotropic receptor in the excitotoxicity and neurodegeneration associated with TLE-HS, perhaps resulting in an increase in glutamate release and the facilitation of an influx of Ca^{2+} to the intracellular environment (Vianna et al., 2002). Using pilocarpine rat modals at the acute, latent, and chronic stages of status epilepticus (Doná et al., 2009), it was determined that the CA3 and CA1 are most affected. There is a lack of published data regarding the expression of $P2x_7$ in human temporal lobe structures, with the exception of data generated by Jimenez-Pacheco et al. (2013), where the level of $P2x_7$ expression was assessed in resected neocortex samples of TLE-HS patients using SDS-PAGE western blot. In this study, the level of expression of $P2x_7$ was significantly higher among TLE-HS patients, consistent with the findings in the present study.

Similar to my previous discussion of the antagonism of the excitatory 5-HT_3 receptor being potentially able to reduce seizure activity in patients with TLE-HS, antagonism of the $P2x_7$ receptor may also find therapeutic utility. Indeed, at the level of animal models, this hypothesis has already been examined using the $P2x_7$ receptor antagonists, A-438079, in animal models of status epilepsy (Jimenez-Pacheco et al., 2013). In both studies, the $P2x_7$ receptor antagonists displayed an anticonvulsive effect on seizure severity in the subject animals and was found to reduce the risk of neuronal death when using A-438079 (Jimenez-Pacheco et al., 2013). No published data was found regarding the use of $P2x_7$ antagonists in clinical and experimental studies using TLE-HS patients or surgically resected TLE-HS hippocampi.

Further laboratory investigation is warranted to confirm the upregulation of the excitatory $P2x_7$ receptors among TLE-HS patients, which is consistent with the hyperexcitability of neuronal cells in this disease condition. Such studies could use SDS-PAGE western blot and receptor autoradiography in order to assess the level and location of protein expression. In addition, electrophysiological studies of $P2x_7$ antagonists could be conducted to assess the anticonvulsive and neuroprotective properties of $P2x_7$ receptor antagonists in patients with TLE-HS. These studies will facilitate introducing $P2x_7$ as a potential target to control seizures in patients with TLE-HS.

4.2.E. Inconsistency between protein level of 5-HT_{1A}, 5-HT_3A, 5-HT_4 and $P2x_7$ receptors, and their mRNA in disease samples

A clear and consistent finding in the present thesis was the discrepancy between the differences in protein expression or mRNA level of 5-HT_3A, 5-HT_{1A}, 5-HT_4, $P2x_7$ receptors and GPR61 protein when comparing tissues from patients with TLE-HS or 'controls'. First, on the cellular protein expression level, the alteration in the protein expression level (especially 5-HT_3A receptors) in TLE-HS hippocampal samples is due to the impact of disease on the protein level rather than the effect of post-mortem on the stability of protein and mRNA in hippocampal tissue. This finding is supported by the following observations in the expression of 5-HT_3A receptors. A) data generated from hippocampal tissue lysate obtained from TLE-HS, PMC and TLE-HS, subjected to an artificial post-mortem delay protocol, samples on the 5-HT_3A receptors expression compared to the constant level of housekeeping protein still reveals an upregulation in the receptor expression among TLE-HS and those subjected to artificial post-mortem delay protocol. B) the expression pattern of 5-HT_3A receptors in the dentate gyrus of TLE-HS resected hippocampi follows the histopathological changes (granular cells migration and sprouting of mossy fibres) that are associated with the disease.

Second, on the level of tissue, the neurodegenerative effect of TLE-HS disease on the hippocampal neuronal cells viability. Thus the considerable reduction in the number of neurons in TLE-HS hippocampus compared to PMC sample may mask the changes in the level of mRNA expression and increase the difficulty in assessing this change among TLE-HS hippocampi in comparison to PMC samples. Further studies need to be

conducted to investigate this hypothesis. These studies can be performed by using different methods such as single cell RT-qPCR to quantify the level of mRNA in TLE-HS patients and controls using the same number of neurons from the same area or by using *in situ* hybridisation for localisation and semi-quantification of mRNA on a single cell basis.

To exclude the post-mortem effect on the level of expression of mRNA, RT-qPCR was conducted to compare levels of expression in TLE-HS and post-mortem control samples to TLE-HS samples subjected to artificial post-mortem protocols. Due to the limited amount of total RNA, 5-HT_3A receptor subunit genes were selected as a representative sample of target genes. Results indicated that the artificial post-mortem protocol affects the expression of the housekeeping genes (HPRT1 and ENO2), but not the target gene (5-HT_3A), which was somewhat unexpected. Hence, study of a broader range of housekeeping genes may be warranted and hence allow a better understanding of the relative levels of receptor specific mRNA between tissues from patients with TLE-HS and relevant controls.

Furthermore, technical issues associated with RT-qPCR (technical and biological differences, fluorescence-bleed) were assessed using 5-HT_3A receptors, HPRT1, and ENO2 genes as a representative sample. Results did not indicate any significance (technical or biological) differences between disease and control samples. This result excludes the risk of pipetting error and the presence of significant biological differences between individuals in diseased and control groups. Conversely, some risk of fluorescence-bleed was observed when the RT-qPCR was conducted in duplex experimental protocols. However, this bleed did not significantly contribute to mask

differences between diseased and control samples. Therefore, the ideal method of detecting differences between TLE-HS and post-mortem controls involves the use of single-cell RT-qPCR to quantify the fold changes with respect to the number of neurons taken from diseased and post-mortem control samples. Additionally, *in situ* hybridisation techniques can be used to semi-quantify differences and uncover the anatomical distribution of targeted hippocampal genes.

4.2.F. General discussion

The present study was conducted to obtain a better understanding of the impact of TLE-HS on the expression level of multiple serotonergic and non-serotonergic receptors and proteins in an attempt to discover a novel target to reduce the seizures of patients with TLE-HS. The results of the present study demonstrate upregulations in the expression of the following proteins; 5-HT_3A, 5-HT_{1A}, 5-HT_4 and P2x_7 receptors in addition to the GPR61 receptor in the hippocampus of patients with TLE-HS (see summary Figure 36). The confirmed overexpression of the excitatory 5-HT_3 receptors in the hippocampus of patients with TLE-HS is consistence to the neuronal hyperexcitability. Therefore, we propose antagonizing this receptor with drugs that are already marketed may offer symtomatic relief for patients with TLE-HS and hence may improve their quality of life without the need for potentially debilitating neurosurgery. This suggest that this hypothesis is evaluated in a Phase II trial, which may forward evidence to expand the clinical indications for which 5-HT_3 receptor antagonists provide benefit.

In addition, the overexpression of inhibitory 5-HT_{1A} receptors could be a compensatory mechanism to adapt the hyperexcitable neurons due to disease. Moreover, this increase in 5-HT_{1A} receptors expression may play a role in the underline mechanism of memory defect that is clinically observed in some patients with TLE-HS. Regarding the excitatory 5-HT_4 and P2x_7 receptors, the upregulation of these receptors could also play an important role in the underline mechanism of seizure activity in TLE=HS disease. The overexpression of 5-HT_4 receptors could also be a compensatory mechanism for memory impairment in some patients with TLE-HS patients or as a result of the reduction in the

synaptic concentration of serotonin. Therefore, antagonizing this receptor may also help in seizure control and improve memory among TLE-HS patients. $P2x_7$ overexpression may be involved in the neuronal excitotoxicity and neurodegeneration that is associated with the disease. Hence, antagonizing these excitatory receptors may introduce $5\text{-}HT_4$ and $P2x_7$ as additional novel targets to benefit patients with TLE-HS.

Regarding the orphan G-protein (GPR61), this protein expresses a variable degree of protein upregulation among hippocampus of TLE-HS patients. The biological function of this protein is poorly understood. The present study also investigates the impact of disease on $5\text{-}HT_{2A}$ receptors expression, however, no clear results were obtained.

In contrast to the protein expression of the receptors understudy, the mRNA level did not reveal any significant differences between disease and control samples. This inconsistency in the result could be due to the neurodegenerative effect of the disease in hippocampus neurons.

In summary, the main finding from this project is the forwarding of a scientific rationale based on experimental evidence that $5\text{-}HT_3$ receptor antagonists may offer a novel pharmacological strategy to reduce the seizures of patients with TLE-HS and hence improve their quality of life. Although with less evidence that with respect to the $5\text{-}HT_3$ receptor antagonists, the present study also forwards some support to investigate the potential therapeutic actions of antagonists of $5\text{-}HT_4$ receptors and $P2x_7$ receptors to control seizures in patients with TLE-HS.

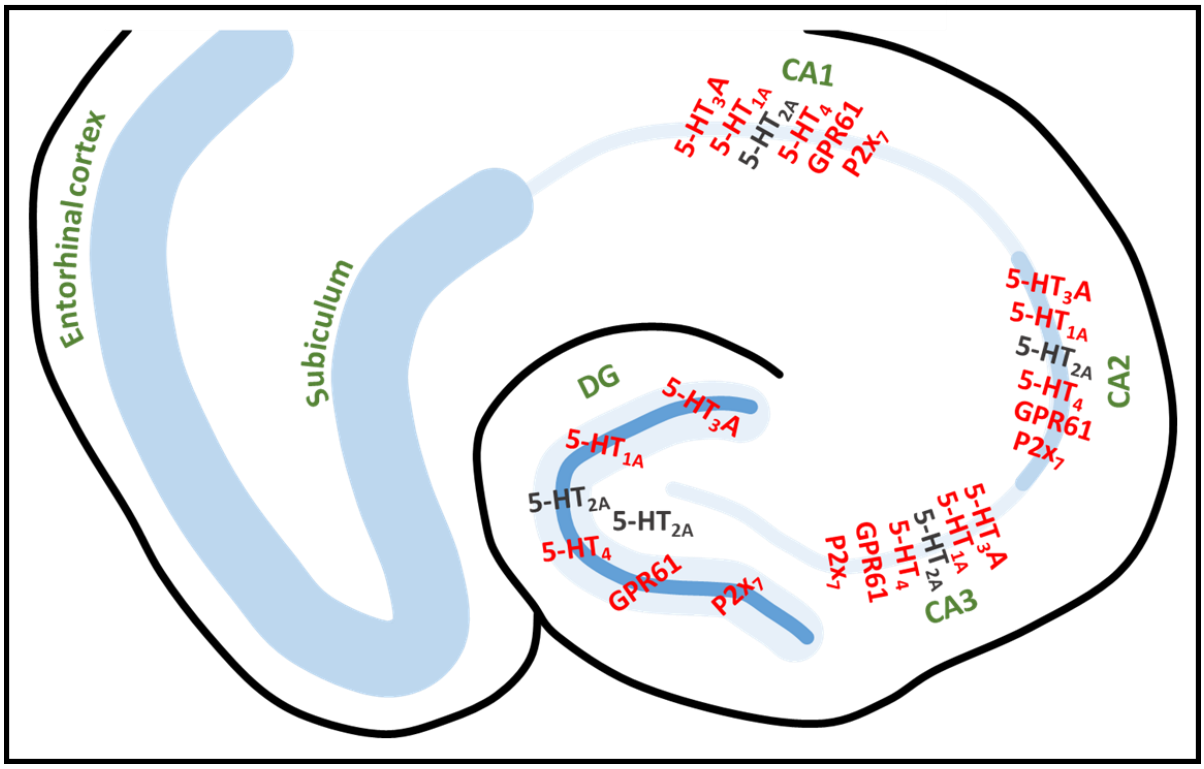

Figure 36. Schematic representation of the changes in various receptors expressed in the hippocampus of patients with TLE-HS compared with control donors.
The naming of a receptor in red indicates a higher level of expression in TLE-HS whereas the naming of a receptor in grey indicates inconclusive results.

CHAPTER 5

Conclusion

5. Conclusion

Temporal lobe epilepsy with hippocampal sclerosis is the most common type of epilepsy affecting adults. This disease is characterised by recurrent partial seizures affecting the medial part of the temporal lobe, in particular the hippocampus. Seizure in this neurological disorder can be controlled using various types of AEDs in the early stages of the disease; however, these medications are failing to control seizures during the advanced stages of TLE-HS. As a result of this pharmaco-resistance, surgical intervention (hippocampectomy or amygdalohippocampectomy) is the last chance to control seizures in these patients. In order to control seizures to improve TLE-HS patients' quality of life and to avoid the negative effects of surgical intervention on patients' memory and cognition, the identification of novel targets is crucial in controlling seizures and decreasing the risk of surgical intervention. Therefore, this study was conducted to assess the possible involvement of select serotonergic receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_3A, 5-HT_4 and 5-HT_7) and other selected interesting targets (GPR61 protein and $P2x_7$ receptors) in order to identify a potential novel targets to evaluate drug action. The results of these screening present various alteration in targets expression in hippocampus of TLE-HS patients. These results propose possible novel target (the priority from the presence study is to investigate antagonising 5-HT_3 receptors) that could be used alone or as an adjunctive therapy with AEDs to control seizures and potentially also improve memory deficit in TLE-HS patients. It may be precedent to perform further laboratory investigation to confirm those finding and to physiologically assess targeting these receptors in TLE-HS resected hippocampi.

APPENDICES

6. Appendices

6.1. Appendix A – 5-HT$_7$ receptors immunoreactivity.

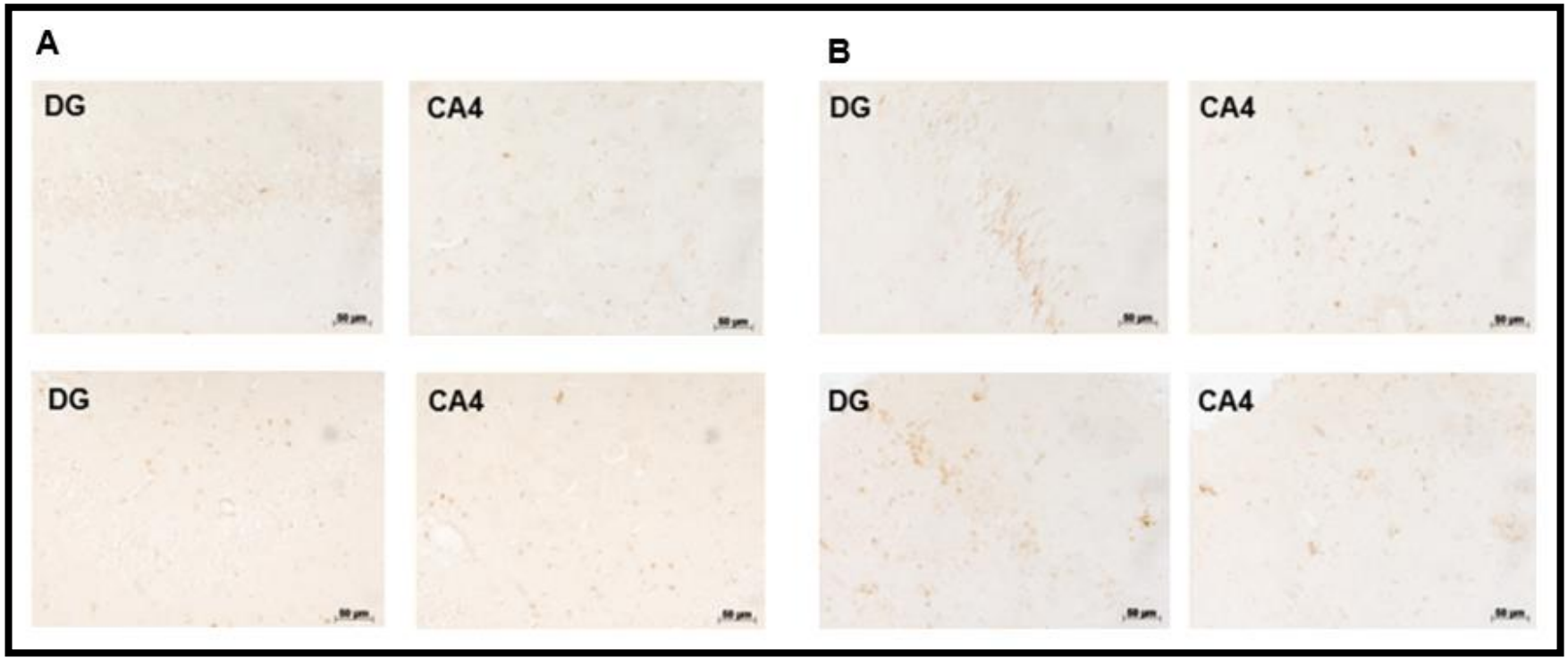

Figure 37. Intermittent and non-specific immunoreactivity signals of 5-HT$_7$ receptors antibody.
A) Dentate gyrus and CA4 of two different PMC samples, *Top* = PMC (1), *Bottom* = PMC (2). *B)* Dentate gyrus and CA4 of two TLE-HS samples, *Top* = TLE-HS (1), *Bottom* = TLE-HS (2). Scale bar = 50 µm.

6.2. Appendix B – Publications Arising from Research

Alomar H., Sheilabi M., Princivalle A., Massoura A., Chelvarajah R., Pall H., Barnes N.M. Impact of temporal lobe epilepsy with hippocampal sclerosis upon expression of 5-HT_3 receptors: potential novel antiepileptic target. 498.09. 2015 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2015. Online.

Alomar H., Sheilabi M., Princivalle A., Massoura A., Chelvarajah R., Pall H., Barnes N.M. Overexpression of 5-HT_{3A} Receptors in Patients with Temporal Lobe Epilepsy with Hippocampal Sclerosis. Poster presentation at Pharmacology 2015, British Pharmacology Society, London December 2015.

REFERENCES

7. References

Altshuler, L., Devinsky, O., Post, R., & Theodore, W. 1990. Depression, anxiety, and temporal lobe epilepsy. Laterality of focus and symptoms. Arch Neurol, 47: 284–8.

Andreetta, F., Carboni, L., Grafton, G., Jeggo, R., Whyment, A. D., Van Den Top, M., Hoyer, D., Spanswick, D. & Barnes, N. M. 2016. Hippocampal 5-HT7 receptors signal phosphorylation of the GluA1 subunit to facilitate AMPA receptor mediated-neurotransmission in vitro and in vivo. Br J Pharmacol, 173, 1438-51.

Assem-Hilger, E., Lanzenberger, R., Savli, M., Wadsak, W., Mitterhauser, M., Mien, L., Stögmann, E., Baumgartner, C., Kletter, K., & Asenbaum, S. 2010. Central serotonin 1A receptor binding in temporal lobe epilepsy: a [carbonyl-(11)C]WAY-100635 PET study. Epilepsy Behav, 19(3): 467-473.

Balakrishnan, S., Bhargava, V. K. & Pandhi, P. 2000. Anticonvulsant Profile of Ondansetron in Rats. Epilepsy & Behavior, 1, 22-26.

Barberà-Cremades, M., Baroja-Mazo, A., Gomez, A. I., Machado, F., Di Virgilio, F. & Pelegrín, P. 2012. P2X7 receptor-stimulation causes fever via PGE2 and IL-1β release. The FASEB Journal, 26, 2951-2962.

Barnes, J. M., Barnes, N. M., Costall, B., Ironside, J. W. & Naylor, R. J. 1989. Identification and Characterisation of 5-Hydroxytryptamine3 Recognition Sites in Human Brain Tissue. Journal of Neurochemistry, 53, 1787-1793.

Barnes, N. M. 2011. Novel epilepsy treatment, US Patent 0092535.

Barnes, N. M., Hales, T. G., Lummis, S. C. & Peters, J. A. 2009. The 5-HT3 receptor--the relationship between structure and function. Neuropharmacology, 56, 273-84.

Barros-Barbosa, A. R., Fonseca, A. L., Guerra-Gomes, S., Ferreirinha, F., Santos, A., Rangel, R., Lobo, M. G., Correia-De-Sá, P. & Cordeiro, J. M. 2015. Up-regulation of P2X7 receptor–mediated inhibition of GABA uptake by nerve terminals of the human epileptic neocortex. Epilepsia.

Belenky, M. A. & Pickard, G. E. 2001. Subcellular distribution of 5-HT(1B) and 5-HT(7) receptors in the mouse suprachiasmatic nucleus. J Comp Neurol, 432, 371-88.

Bennett, M. V., Garré, J. M., Orellana, J. A., Bukauskas, F. F., Nedergaard, M., Giaume, C. & Sáez, J. C. 2012. Connexin and pannexin hemichannels in inflammatory responses of glia and neurons. Brain research, 1487, 3-15.

Berumen, L. C., Rodriguez, A., Miledi, R. & Garcia-Alcocer, G. 2012. Serotonin receptors in hippocampus. ScientificWorldJournal, 2012, 823493.

Blumcke, I., Thom, M., Aronica, E., Armstrong, D. D., Bartolomei, F., Bernasconi, A., Bernasconi, N., Bien, C. G., Cendes, F., Coras, R., Cross, J. H., Jacques, T. S., Kahane, P., Mathern, G. W., Miyata, H., Moshe, S. L., Oz, B., Ozkara, C., Perucca, E., Sisodiya, S., Wiebe, S. & Spreafico, R. 2013. International consensus classification of hippocampal sclerosis in temporal lobe epilepsy: a Task Force report from the ILAE Commission on Diagnostic Methods. Epilepsia, 54, 1315-29.

Bonaventure, P., Hall, H., Gommeren, W., Cras, P., Langlois, X., Jurzak, M. & Leysen, J. E. 2000. Mapping of serotonin 5-HT(4) receptor mRNA and ligand binding sites in the post-mortem human brain. Synapse, 36, 35-46.

Brady, C. A., Dover, T. J., Massoura, A. N., Princivalle, A. P., Hope, A. G. & Barnes, N. M. 2007. Identification of 5-HT3A and 5-HT3B receptor subunits in human hippocampus. Neuropharmacology, 52, 1284-90.

Bufton, K. E., Steward, L. J., Barber, P. C. & Barnes, N. M. 1993. Distribution and characterization of the [3H]granisetron-labelled 5-HT3 receptor in the human forebrain. Neuropharmacology, 32, 1325-31.

Burnet, P. W., Eastwood, S. L., Lacey, K. & Harrison, P. J. 1995. The distribution of 5-HT1A and 5-HT2A receptor mRNA in human brain. Brain Res, 676, 157-68.

Cendes, F., Sakamoto, A. C., Spreafico, R., Bingaman, W. & Becker, A. J. 2014. Epilepsies associated with hippocampal sclerosis. Acta Neuropathol, 128, 21-37.

Charnay, Y. & Leger, L. 2010. Brain serotonergic circuitries. Dialogues Clin Neurosci, 12, 471-87.

Chaumont, S. & Khakh, B. S. 2008. Patch–clamp coordinated spectroscopy shows P2X2 receptor permeability dynamics require cytosolic domain rearrangements but not Panx-1 channels. Proceedings of the National Academy of Sciences, 105, 12063-12068.

Cheewatrakoolpong, B., Gilchrest, H., Anthes, J. C. & Greenfeder, S. 2005. Identification and characterization of splice variants of the human P2X 7 ATP channel. Biochemical and biophysical research communications, 332, 17-27.

Cikos, S., Gregor, P. & Koppel, J. 2001. Cloning of a novel biogenic amine receptor-like G protein-coupled receptor expressed in human brain. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1521, 66-72.

Cirera, S., 2013. Highly efficient method for isolation of total RNA from adipose tissue. BMC Res Notes, 6:472.

Clinckers, R., Smolders, I., Meurs, A., Ebinger, G. & Michotte, Y. 2004a. Anticonvulsant action of GBR-12909 and citalopram against acute experimentally induced limbic seizures. Neuropharmacology, 47, 1053-61.

Clinckers, R., Smolders, I., Meurs, A., Ebinger, G. & Michotte, Y. 2004b. Anticonvulsant action of hippocampal dopamine and serotonin is independently mediated by D and 5-HT receptors. J Neurochem, 89, 834-43.

Compan, V., Daszuta, A., Salin, P., Sebben, M., Bockaert, J., Dumuis, A., (1996). Lesion study of the distribution of serotonin 5-HT4 receptors in rat basal ganglia and hippocampus. Eur J Neurosci, 8(12): 2591-8.

Csölle, C. & Sperlagh, B. 2010. Peripheral origin of IL-1β production in the rodent hippocampus under in vivo systemic bacterial lipopolysaccharide (LPS) challenge and its regulation by P2X 7 receptors. Journal of neuroimmunology, 219, 38-46.

Cuellar-Herrera, M., Velasco, A., Velasco, F., Trejo, D., Alonso-Vanegas, M., Nuche-Bricaire, A., Vázquez-Barrón, D., Guevara-Guzmán, R., & Rocha, L. 2014. Alterations of 5-HT1A receptor-induced G-protein functional activation and relationship to memory deficits in patients with pharmacoresistant temporal lobe epilepsy. Epilepsy Res, 108(10): 1853-1863.

Dahlstroem, A. & Fuxe, K. 1964. Evidence for the Existence of Monoamine-Containing Neurons in the Central Nervous System. I. Demonstration of Monoamines in the Cell Bodies of Brain Stem Neurons. Acta Physiol Scand Suppl, SUPPL 232:1-55.

Davies, P. A., Pistis, M., Hanna, M. C., Peters, J. A., Lambert, J. J., Hales, T. G. & Kirkness, E. F. 1999. The 5-HT3B subunit is a major determinant of serotonin-receptor function. Nature, 397, 359-63.

Delom, F., Fessart, D., (2011). Role of Phosphorylation in the Control of Clathrin-Mediated Internalization of GPCR. International Journal of Cell Biology, 2011: 246954.

Dona, F., Ulrich, H., Persike, D. S., Conceicao, I. M., Blini, J. P., Cavalheiro, E. A. & Fernandes, M. J. 2009. Alteration of purinergic P2X4 and P2X7 receptor expression in rats with temporal-lobe epilepsy induced by pilocarpine. Epilepsy Res, 83, 157-67.

Drevets, W. C., Frank, E., Price, J. C., Kupfer, D. J., Greer, P. J. & Mathis, C. 2000. Serotonin type-1A receptor imaging in depression. Nucl Med Biol, 27, 499-507.

Dwivedi, Y., Mondal, A., Payappagoudar, G., & Rizavi, H. 2005. Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated stress in rat brain: role in learned helplessness behavior. Neuropharmacology, 48(2):204-14.

Eggers, A. 2007. Temporal lobe epilepsy is a disease of faulty neuronal resonators rather than oscillators, and all seizures are provoked, usually by stress. Med Hypotheses, 69(6): 1284-9.

Engel, J. 1996. Introduction to temporal lobe epilepsy. Epilepsy research, 26, 141-150

Engel, T., Gomez-Villafuertes, R., Tanaka, K., Mesuret, G., Sanz-Rodriguez, A., Garcia-Huerta, P., Miras-Portugal, M. T., Henshall, D. C. & Diaz-Hernandez, M. 2012a. Seizure suppression and neuroprotection by targeting the purinergic P2X7 receptor during status epilepticus in mice. The FASEB Journal, 26, 1616-1628.

Engel, T., Jimenez-Pacheco, A., Miras-Portugal, M. T., Diaz-Hernandez, M. & Henshall, D. C. 2012b. P2X7 receptor in epilepsy; role in pathophysiology and potential targeting for seizure control.

Erspamer, V. & Asero, B. 1952. Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. Nature, 169, 800-1.

Eugene, E., Cluzeaud, F., Cifuentes-Diaz C., Clemenceau, S., Baulac, M., Poncer, Jc., & Miles, R. 2014. An organotypic brain slice preparation from adult patients with temporal lobe epilepsy. J Neurosci Methods, 235: 234–244

Feldman, R. S., Meyer, J. S. & Quenzer, L. F., 1997. Principles of Neuropsychopharmacology. Massachusetts: Sinauer Associates, Inc.

Fernandes, M. S., Speciali, D. S., Blini, J., Canzian, M., Cavalheiro, E. A., Ulrich, H., Carrete Jr, H., Centeno, R. S. & Yacubian, E. Purinergic P2 receptors are up-regulated In the hippocampus of patients with temporal lobe epilepsy associated with hippocampal sclerosis. Epilepsia, 2009. WILEY-BLACKWELL PUBLISHING, INC COMMERCE PLACE, 350 MAIN ST, MALDEN 02148, MA USA, 78-78.

Fink, K. B. & Gothert, M. 2007. 5-HT receptor regulation of neurotransmitter release. Pharmacol Rev, 59, 360-417.

Forno, L., Langston, J., Herrick, M., Wilson, J., & Murayama, S. 2002. Ubiquitin-positive neuronal and tau 2-positive glial inclusions in frontotemporal dementia of motor neuron type. Acta Neuropathol. 103(6): 599-606.

Gariboldi, M., Tutka, P., Samanin, R. & Vezzani, A. 1996. Stimulation of 5-HT1A receptors in the dorsal hippocampus and inhibition of limbic seizures induced by kainic acid in rats. Br J Pharmacol, 119, 813-8.

Garrett, K., Tsuruta, K., Walker, S., Jackson, S. & Sweat, M. 2003. Managing Nausea and Vomiting: Current Strategies. Critical Care Nurse, 23, 31-50.

George, M., Al-Duaij, N., O'donnell, K. A. & Shannon, M. W. 2008. Obtundation and seizure following ondansetron overdose in an infant. Clin Toxicol (Phila), 46, 1064-6.

Giovacchini G, Toczek Mt, Bonwetsch R, Bagic A, Lang L, Fraser C, Reeves-Tyer, P., Herscovitch, P., Eckelman, W., Carson, R., & Theodore, W. 2005. 5-HT 1A receptors are reduced in temporal lobe epilepsy after partial-volume correction. J Nucl Med, 46: 1128-1135.

Girotti, P. A., Misawa, R., Palombit, K., Mendes, C. E., Bittencourt, J. C. & Castelucci, P. 2013. Differential effects of undernourishment on the differentiation and maturation of rat enteric neurons. Cell and Tissue Research, 353, 367-380.

Gomez-Lira, G., Lamas, M., Romo-Parra, H. & Gutierrez, R. 2005. Programmed and induced phenotype of the hippocampal granule cells. J Neurosci, 25, 6939-46.

Gothert, M. 1980. Serotonin-receptor-mediated modulation of Ca2+-dependent 5-hydroxytryptamine release from neurones of the rat brain cortex. Naunyn Schmiedebergs Arch Pharmacol, 314, 223-30.

Gothert, M. 1990. Presynaptic serotonin receptors in the central nervous system. Ann N Y Acad Sci, 604, 102-12.

Goto, S., Morigaki, R., Okita, S., Nagahiro, S., & Kaji, R., 2015. Development of a highly sensitive immunohistochemical method to detect neurochemical molecules in formalin-fixed and paraffin-embedded tissues from autopsied human brains. Front Neuroanat, 9.

Graham, D. & Langer, S. Z. 1992. Advances in sodium-ion coupled biogenic amine transporters. Life Sci, 51, 631-45.

Graves, Austin R., Moore, Shannon J., Bloss, Erik B., Mensh, Brett D., Kath, William L. & Spruston, N. 2012. Hippocampal Pyramidal Neurons Comprise Two Distinct Cell Types that Are Countermodulated by Metabotropic Receptors. Neuron, 76, 776-789.

Gray, N., Milak, M., Delorenzo, C., Ogden, R., Huang, Y., Mann, J., & Parsey, R. 2013. Antidepressant Treatment Reduces Serotonin-1A Autoreceptor Binding in Major Depressive Disorder. Biol Psychiatry, 74(1): 26–31.

Guiard, B., & Di Giovanni G. 2015. Central serotonin-2A (5-HT2A) receptor dysfunction in depression and epilepsy: the missing link?. Front Pharmacol, 17;6:46.

Gutiérrez-Martín, Y., Bustillo, D., Gómez-Villafuertes, R., Sánchez-Nogueiro, J., Torregrosa-Hetland, C., Binz, T., Gutiérrez, L. M., Miras-Portugal, M. T. & Artalejo, A. R. 2011. P2X7 receptors trigger ATP exocytosis and modify secretory vesicle dynamics in neuroblastoma cells. Journal of Biological Chemistry, 286, 11370-11381.

Haahr, M., Fisher, P., Holst, K., Madsen, K., Jensen, C., Marner, L., Lehel, S., Baaré, W., Knudsen, G., & Hasselbalch, S. 2013. The 5-HT4 receptor levels in hippocampus correlates inversely with memory test performance in humans. Hum Brain Mapp, 34(11): 3066-3074

Haahr, M., Fisher, P., Jensen, C., Frokjaer, V., Mahon, B., Madsen, K., Baaré, W., Lehel, S., Norremolle, A., Rabiner, E., & Knudsen, G. 2014. Central 5-HT4 receptor binding as biomarker of serotonergic tonus in humans: a [11C]SB207145 PET study. Mol Psychiatry, 19(4): 427-432

Hannon, J. & Hoyer, D. 2008. Molecular biology of 5-HT receptors. Behav Brain Res, 195, 198-213.

Harroud, A., Bouthillier, A., Weil, A. G. & Nguyen, D. K. 2012. Temporal Lobe Epilepsy Surgery Failures: A Review. Epilepsy Research and Treatment, 2012, 10.

Hasler, G., Bonwetsch, R., Giovacchini, G., Toczek, M., Bagic, A., Luckenbaugh, D., Drevets, W., & Theodore, W. 2007. 5-HT1A receptor binding in temporal lobe epilepsy patients with and without major depression. Biol Psychiatry, 62(11), 1258–1264.

Heinrich, A., Andó, R. D., Túri, G., Rózsa, B. & Sperlágh, B. 2012. K+ depolarization evokes ATP, adenosine and glutamate release from glia in rat hippocampus: a microelectrode biosensor study. British Journal of Pharmacology, 167, 1003-1020.

Hemmrich, K., Denecke, B., Paul, N., Hoffmeister, D., & Pallua, N., 2010. RNA isolation from adipose tissue: an optimized procedure for high RNA yield and integrity. Labmedicine, 41 (2): 104-106

Hermann, B., Seidenberg, M., Schoenfeld, J., & Davies, K. 1997. Neuropsychological characteristics of the syndrome of mesial temporal lobe epilepsy. Arch Neurol, 54(4): 369-76.

Hildebrand, J., Bourgeois, F., Buyse, M., Przedborski, S. & Goldman, S. 1990. Reproducibility of monoamine metabolite measurements in human cerebrospinal fluid. Acta Neurol Scand, 81, 427-30.

Hirvonen, J., Karlsson, H., Kajander, J., Lepola, A., Markkula, J., Rasi-Hakala, H., Någren, K., Salminen, J., & Hietala, J. 2008. Decreased brain serotonin 5-HT1A receptor availability in medication-naive patients with major depressive disorder: an in-vivo imaging study using PET and [carbonyl-11C]WAY-100635. Int J Neuropsychopharmacol, 11(4): 465-476.

Hossain, M. S., Mineno, K. & Katafuchi, T. 2016. Neuronal Orphan G-Protein Coupled Receptor Proteins Mediate Plasmalogens-Induced Activation of ERK and Akt Signaling. PloS one, 11, e0150846.

Ito, S., Suhara, T., Ito, H., Yasuno, F., Ichimiya, T., Takano, A., Maehara, T., Matsuura, M. & Okubo, Y. 2007. Changes in central 5-HT(1A) receptor binding in mesial temporal epilepsy measured by positron emission tomography with [(11)C]WAY100635. Epilepsy Res, 73(1), 111-118

Jain, S., Agarwal, N., Mediratta, P., & Sharma, K. 2012. Evaluation of anticonvulsant and nootropic effect of ondansetron in mice. Hum Exp Toxicol, 31(9): 905-12.

Jasper, J. R., Kosaka, A., To, Z. P., Chang, D. J. & Eglen, R. M. 1997. Cloning, expression and pharmacology of a truncated splice variant of the human 5-HT7 receptor (h5-HT7b). Br J Pharmacol, 122, 126-32.

Jimenez-Pacheco, A., Mesuret, G., Sanz-Rodriguez, A., Tanaka, K., Mooney, C., Conroy, R., Miras-Portugal, M.T., Diaz-Hernandez, M., Henshall, D.C., & Engel, T. 2013. Increased neocortical expression of the P2X7 receptor after status epilepticus and anticonvulsant effect of P2X7 receptor antagonist A-438079. Epilepsia, 54, 1551-1561.

Jones, J., Hermann, B., Barry, J., Gilliam, F., Kanner, A., & Meador, K. 2005. Clinical assessment of axis I psychiatric morbidity in chronic epilepsy: a multicenter investigation. J Neuropsychiatry Clin Neurosci, 17: 172–9.

Kasture, S., & Kasture, V. 1999. Effect of some serotonergic agents on lithium-pilocarpine model of status epilepticus in rats. Indian J Pharmacol, 31: 370–372.

Kim, J.-E. & Kang, T.-C. 2011. The P2X7 receptor–pannexin-1 complex decreases muscarinic acetylcholine receptor–mediated seizure susceptibility in mice. The Journal of Clinical Investigation, 121, 2037-2047.

Kim, J.-E., Kim, D.-S., Jin Ryu, H., Il Kim, W., Kim, M.-J., Won Kim, D., Young Choi, S. & Kang, T.-C. 2013. The effect of P2X7 receptor activation on nuclear factor-kappa B phosphorylation induced by status epilepticus in the rat hippocampus. Hippocampus, 23, 500-514.

Kim, J.-E., Kwak, S.-E., Jo, S.-M. & Kang, T.-C. 2009. Blockade of P2X receptor prevents astroglial death in the dentate gyrus following pilocarpine-induced status epilepticus. Neurological research, 31, 982-988.

Kim, J.-E., Ryu, H. J., Yeo, S.-I. & Kang, T.-C. 2010. P2X7 receptor regulates leukocyte infiltrations in rat frontoparietal cortex following status epilepticus. J Neuroinflammation, 7, 65-70.

King, M., Marsden, C., & Fone, K. 2008. A role of the 5-HT(1A), 5-HT4 and 5-HT6 receptors in learning and memory. Trends Pharmacol Sci, 29(9): 482-4892.

Krueger, J. M., Taishi, P., De, A., Davis, C. J., Winters, B. D., Clinton, J., Szentirmai, É. & Zielinski, M. R. 2010. ATP and the purine type 2 X7 receptor affect sleep. Journal of Applied Physiology, 109, 1318-1327.

Labrousse, V. F., Costes, L., Aubert, A., Darnaudéry, M., Ferreira, G., Amédée, T. & Layé, S. 2009. Impaired Interleukin-1β and c-Fos Expression in the Hippocampus Is Associated with a Spatial Memory Deficit in P2X 7 Receptor-Deficient Mice. PLoS One, 4, e6006.

Lee, D. K., George, S. R., Cheng, R., Nguyen, T., Liu, Y., Brown, M., Lynch, K. R. & O'dowd, B. F. 2001. Identification of four novel human G protein-coupled receptors expressed in the brain. Brain Res Mol Brain Res, 86, 13-22.

Li, X., DiFiglia, M., (2012). The recycling endosome and its role in neurological disorders. Prog Neurobiol, 97(2): 127-41.

Licht, C., Marcussen, A., Wegener, G., Overstreet, D., Aznar, S., & Knudsen, G. 2009. The brain 5-HT4 receptor binding is down-regulated in the Flinders Sensitive Line depression model and in response to paroxetine administration. J Neurochem, 109(5): 1363-1374.

Lopez-Meraz, M. L., Gonzalez-Trujano, M. E., Neri-Bazan, L., Hong, E. & Rocha, L. L. 2005. 5-HT1A receptor agonists modify epileptic seizures in three experimental models in rats. Neuropharmacology, 49, 367-75.

Lothe, A., Didelot, A., Hammers, A., Costes, N., Saoud, M., Gilliam, F., & Ryvlin, P. 2008. Comorbidity between temporal lobe epilepsy and depression: a [18F]MPPF PETstudy. Brain, 2765–2782.

Marcusson, J. O. & Ross, S. B. 1990. Binding of some antidepressants to the 5-hydroxytryptamine transporter in brain and platelets. Psychopharmacology (Berl), 102, 145-55.

Marner, L., Gillings, N., Madsen, K., Erritzoe, D., Baare, W. F., Svarer, C., Hasselbalch, S. G. & Knudsen, G. M. 2010. Brain imaging of serotonin 4 receptors in humans with [11C]SB207145-PET. Neuroimage, 50, 855-61.

Marques-Da-Silva, C., Chaves, M. M., Castro, N. G., Coutinho-Silva, R. & Guimaraes, M. Z. P. 2011. Colchicine inhibits cationic dye uptake induced by ATP in P2X2 and P2X7 receptor-expressing cells: implications for its therapeutic action. British Journal of Pharmacology, 163, 912-926.

Martin, A. L., Steurer, M. A. & Aronstam, R. S. 2015. Constitutive activity among orphan class-A G protein coupled receptors. PloS one, 10, e0138463.

Mason, C. & Munnur, U. 2007. Generalized seizure following ondansetron administration during cesarean section. Anesthesiology, 106, 105.

Mathern, G., Pretorius, J., Mendoza, D., Lozada, A., Leite, J., Chimelli, L., Fried, I., Sakamoto, A., Assirati, J., & Adelson, P. 1998. Increased hippocampal AMPA and NMDA receptor subunit immunoreactivity in temporal lobe epilepsy patients. J Neuropathol Exp Neurol, 57(6):615-34.

Matthys, A., Haegeman, G., Van Craenenbroeck, K. & Vanhoenacker, P. 2011. Role of the 5-HT7 receptor in the central nervous system: from current status to future perspectives. Mol Neurobiol, 43, 228-53.

Maurer-Morelli, C., De Vasconcellos, J., Reis-Pinto, F., Rocha Cde, S., Domingues, R., Yasuda, C., Tedeschi, H., De Oliveira, E., Cendes, F., & Lopes-Cendes, I. 2012. A comparison between different reference genes for expression studies in human hippocampal tissue. J Neurosci Methods, 208(1): 44-7.

McMahon, H., Boucrot, E., (2011). Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol, 12(8): 517-33.

Meencke, H.J. 2009. Clinical neuropathology of the epilepsies in the 100 years of the ILAE (1909–2009). Epilepsia 50(3):8–16

Megias, M., Emri, Z., Freund, T. F. & Gulyas, A. I. 2001. Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. Neuroscience, 102, 527-40.

Merlet, I., Ryvlin, P., Costes, N., Dufournel, D., Isnard, J., Faillenot, I., Ostrowsky, K., Lavenne, F., Le Bars, D., & Mauguiere, F. 2004. Statistical parametric mapping of 5-HT1A receptor binding in temporal lobe epilepsy with hippocampal ictal onset on intracranial EEG. Neuroimage, 22(2), 886-896.

Millan, M. J., Marin, P., Bockaert, J. & Mannoury La Cour, C. 2008. Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. Trends Pharmacol Sci, 29, 454-64.

Mochizuki, A., Komatsuzaki, Y., Iwamoto, H., & Shoji, S. 2004. Frontotemporal dementia with ubiquitinated neuronal inclusions presenting with primary lateral sclerosis and parkinsonism: clinicopathological report of an autopsy case. Acta Neuropathol, 107(4): 377-380.

Mohler, E. G., Shacham, S., Noiman, S., Lezoualc'h, F., Robert, S., Gastineau, M., Rutkowski, J., Marantz, Y., Dumuis, A., Bockaert, J., Gold, P. E. & Ragozzino, M. E. 2007. VRX-03011, a novel 5-HT4 agonist, enhances memory and hippocampal acetylcholine efflux. Neuropharmacology, 53, 563-73.

Molliver, M. E. 1987. Serotonergic neuronal systems: what their anatomic organization tells us about function. J Clin Psychopharmacol, 7, 3S-23S.

Monk, S. A., Williams, J. M., Hope, A. G. & Barnes, N. M., 2004. Identification and importance of N-glycosylation of the human 5-hydroxytryptamine3A receptor subunit. Biochem Pharmacol, 68 (9):1787-1796.

Morales, M. & Bloom, F. E. 1997. The 5-HT3 receptor is present in different subpopulations of GABAergic neurons in the rat telencephalon. J Neurosci, 17, 3157-67.

Nambu, H., Fukushima, M., Hikichi, H., Inoue, T., Nagano, N., Tahara, Y., Nambu, T., Ito, J., Ogawa, Y. & Ozaki, S. 2011. Characterization of metabolic phenotypes of mice lacking GPR61, an orphan G-protein coupled receptor. Life sciences, 89, 765-772.

Nichols, D. E. & Nichols, C. D. 2008. Serotonin receptors. Chem Rev, 108, 1614-41.

Niesler, B., Kapeller, J., Hammer, C. & Rappold, G. 2008. Serotonin type 3 receptor genes: HTR3A, B, C, D, E. Pharmacogenomics, 9, 501-4.

Nieuwenhuys, R. 1985. Chemoarchitecture of the Brain. 1st ed. Berlin: Springer-Verlag Berlin Heidelberg

Okuhara, D. Y. & Beck, S. G. 1998. Corticosteroids alter 5-hydroxytryptamine1A receptor-effector pathway in hippocampal subfield CA3 pyramidal cells. J Pharmacol Exp Ther, 284, 1227-33.

Palliativedrugs.com, (2008). © palliativedrugs.com, [online] Available at: http://www.palliativedrugs.com/news/october/tropisetron-navoban-to-be-discontinued-in-uk.html. [Accessed 15 Jul. 2016].

Parker, R. M. C., Barnes, J. M., Ge, J., Barber, P. C. & Barnes, N. M. 1996. Autoradiographic distribution of [3H]-(S)-zacopride-labelled 5-HT3 receptors in human brain. Journal of the Neurological Sciences, 144, 119-127.

Parsey, R., Oquendo, M., Ogden, R., Olvet, D., Simpson, N., Huang, Y., Heertum, R., Arango, V., & Mann, J. 2006. Altered serotonin 1A binding in major depression: a [carbonyl-C-11]WAY100635 positron emission tomography study. Biol Psychiatry, 59: 106–13

Patel, A., Mittal, S., Manchanda, S. & Puliyel, J. M. 2011. Ondansetron-induced dystonia, hypoglycemia, and seizures in a child. Ann Pharmacother, 45, e7.

Pehouron, A. L. & Sanchez, C. 2014. Serotonergic modulation of glutamate neurotransmission as a strategy for treating depression and cognitive dysfunction. CNS Spectr, 19, 121-33.

Pfaffl, M., Horgan, G., & Dempfle, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res, 30(9): 36.

Phosphosite.org A. (n. d.) 5-HT(3A) (human). [online] Available from: <http://www.phosphosite.org/proteinAction.do?id=5291&showAllSites=true> [Accessed 17 May 2015].

Phosphosite.org B. (n. d.) HPRT1 (human). [online] Available from: < http://www.phosphosite.org/proteinAction.action?id=2473283&showAllSites=true > [Accessed 17 May 2015].

Proper, E., Oestreicher, A., Jansen, G., Veelen, C., Van Rijen, P., Gispen, W., & De Graan, P. 2000. Immunohistochemical characterization of mossy fibre sprouting in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. Brain, 123 (1): 19-30

Puzerey, P., Decker, M., & Galán, R. 2014. Elevated serotonergic signaling amplifies synaptic noise and facilitates the emergence of epileptiform network oscillations. J Neurophysiol, 15;112(10): 2357-73.

Pytliak, M., Vargova, V., Mechirova, V. & Felsoci, M. 2011. Serotonin receptors - from molecular biology to clinical applications. Physiol Res, 60, 15-25.

Quigley, E. M. M. 2015. Prokinetics in the Management of Functional Gastrointestinal Disorders. Journal of Neurogastroenterology and Motility, 21, 330-336.

Quiske, A., Helmstaedter, C., Lux, S., & Elger, C. 2000. Depression in patients with temporal lobe epilepsy is related to mesial temporal sclerosis. Epilepsy Res, 39: 121–5.

Rapport, M. M., Green, A. A. & Page, I. H. 1948. Crystalline Serotonin. Science, 108, 329-30.

Rassendren, F., Buell, G. N., Virginio, C., Collo, G., North, R. A. & Surprenant, A. 1997. The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. J Biol Chem, 272, 5482-6.

Reutens, A., Begley, C., (2002). Endophilin-1: a multifunctional protein. Int J Biochem Cell Biol, 34(10): 1173-7.

Rocha, L. 2013. Interaction between electrical modulation of the brain and pharmacotherapy to control pharmacoresistant epilepsy. Pharmacol Ther, 138, 211-28.

Rocha, L., Lorigados-Pedre, L., Orozco-Suarez, S., Morales-Chacon, L., Alonso-Vanegas, M., Garcia-Maeso, I., Villeda-Hernandez, J., Osorio-Rico, L., Estupinan, B. & Quintana, C. 2007. Autoradiography reveals selective changes in serotonin binding in neocortex of patients with temporal lobe epilepsy. Prog Neuropsychopharmacol Biol Psychiatry, 31, 1208-18.

Roychowdhury, S., Haas, H., Anderson, E. G., (1994). 5-HT1A and 5-HT4 receptor colocalization on hippocampal pyramidal cells. Neuropharmacology, 33(3-4): 551-7.

Sargent, A. I., Deppe, S. A. & Chan, F. A. 1993. Seizure associated with ondansetron. Clin Pharm, 12, 613-5.

Savic, I., Lindström, P., Gulyás, B., Halldin, C., Andrée, B., & Farde, L. 2004. Limbic reductions of 5-HT1A receptor binding in human temporal lobe epilepsy. Neurology, 62(8): 1343-1351.

Schwarz, N., Drouot, L., Nicke, A., Fliegert, R., Boyer, O., Guse, A. H., Haag, F., Adriouch, S. & Koch-Nolte, F. 2012. Alternative splicing of the N-terminal cytosolic and transmembrane domains of P2X7 controls gating of the ion channel by ADP-ribosylation. PloS one, 7, e41269.

Scottish Intercollegiate Guidelines Network (SIGN). Diagnosis and management of epilepsy in adults. Edinburgh: SIGN; 2015. (SIGN publication no. 143). [May 2015]. Available from URL: http://www.sign.ac.uk

Semenova, T., & Ticku, M. 1992. Effects of 5-HT receptor antagonists on seizure susceptibility and locomotor activity in DBA/2 mice. Brain Res, 588: 229–236.

Sharma, A. & Raina, V. 2001. Generalised seizures following ondansetron. Ann Oncol, 12, 131-2.

Sharma, A. K., Reams, R. Y., Jordan, W. H., Miller, M. A., Thacker, H. L. & Snyder, P. W. 2007. Mesial temporal lobe epilepsy: pathogenesis, induced rodent models and lesions. Toxicol Pathol, 35, 984-99.

Sharp, T. & Hjorth, S. 1990. Application of brain microdialysis to study the pharmacology of the 5-HT1A autoreceptor. J Neurosci Methods, 34, 83-90.

Singh, N. N., Rai, A., Selhorst, J. B. & Acharya, J. N. 2009. Ondansetron and seizures. Epilepsia, 50, 2663-6.

Skaper, S. D., Debetto, P. & Giusti, P. 2010. The P2X7 purinergic receptor: from physiology to neurological disorders. The FASEB Journal, 24, 337-345.

Sloviter, R. S. & Bumanglag, A. V. 2013. Defining "epileptogenesis" and identifying "antiepileptogenic targets" in animal models of acquired temporal lobe epilepsy is not as simple as it might seem. Neuropharmacology, 69, 3-15.

Sloviter, R. S. 2008. Hippocampal epileptogenesis in animal models of mesial temporal lobe epilepsy with hippocampal sclerosis: the importance of the "latent period" and other concepts. Epilepsia, 49 Suppl 9, 85-92.

Sperlágh, B. & Illes, P. 2014. P2X7 receptor: an emerging target in central nervous system diseases. Trends in pharmacological sciences, 35, 537-547.

Sperlágh, B., Köfalvi, A., Deuchars, J., Atkinson, L., J. Milligan, C., Buckley, N. J. & Vizi, E. S. 2002. Involvement of P2X7 receptors in the regulation of neurotransmitter release in the rat hippocampus. Journal of Neurochemistry, 81, 1196-1211.

Sperlágh, B., Vizi, E. S., Wirkner, K. & Illes, P. 2006. P2X 7 receptors in the nervous system. Progress in neurobiology, 78, 327-346.

Suadicani, S. O., Iglesias, R., Wang, J., Dahl, G., Spray, D. C. & Scemes, E. 2012. ATP signaling is deficient in cultured pannexin1-null mouse astrocytes. Glia, 60, 1106-1116.

Sun, C., Heid, M. E., Keyel, P. A. & Salter, R. D. 2013. The second transmembrane domain of P2X7 contributes to dilated pore formation. PLoS One, 8, e61886.

Surges, R. & Elger, C. E. 2013. Reoperation after failed resective epilepsy surgery. Seizure - European Journal of Epilepsy, 22, 493-501.

Swain, T., & Mohanty, M. 1999. Antiepileptic action of ondansetron. Indian J Pharmacol, 31: 157.

Takeda, S., Yamamoto, A., Okada, T., Matsumura, E., Nose, E., Kogure, K., Kojima, S. & Haga, T. 2003. Identification of surrogate ligands for orphan G protein-coupled receptors. Life sciences, 74, 367-377.

Tamir, H. & Gershon, M. D. 1990. Serotonin-storing secretory vesicles. Ann N Y Acad Sci, 600, 53-66; discussion 67.

Tamir, H., Liu, K. P., Hsiung, S., Adlersberg, M. & Gershon, M. D. 1994. Serotonin binding protein: synthesis, secretion, and recycling. J Neurochem, 63, 97-107.

Tecott, L. H., Maricq, A. V. & Julius, D. 1993. Nervous system distribution of the serotonin 5-HT3 receptor mRNA. Proc Natl Acad Sci U S A, 90, 1430-4.

Terron, J. A., Bouchelet, I. & Hamel, E. 2001. 5-HT7 receptor mRNA expression in human trigeminal ganglia. Neurosci Lett, 302, 9-12.

Theodore, W., Hasler, G., Giovacchini, G., Kelley, K., Reeves-Tyer, P., Herscovitch, P., & Drevets, W. 2007. Reduced hippocampal 5HT1A PET receptor binding and depression in temporal lobe epilepsy. Epilepsia, 48(8): 1526-1530.

Toczek, M., Carson, R., Lang, L., Ma, Y., Spanaki, M., Der, M., Fazilat, S., Kopylev, L., Herscovitch, P., Eckelman, W., & Theodore, W. 2003. PET imaging of 5-HT1A receptor binding in patients with temporal lobe epilepsy. Neurology, 60(5): 749-756.

Tolk, J., Kohnen, R. & Muller, W. 2004. Intravenous treatment of fibromyalgia with the 5-HT3 receptor antagonist tropisetron in a rheumatological practice. Scand J Rheumatol Suppl, 119, 72-5.

Tork, I. 1990. Anatomy of the serotonergic system. Ann N Y Acad Sci, 600, 9-34; discussion 34-5.

Toyooka, M., Tujii, T. & Takeda, S. 2009. The N-terminal domain of GPR61, an orphan G-protein-coupled receptor, is essential for its constitutive activity. Journal of neuroscience research, 87, 1329-1333.

Van Hooft, J. A. & Yakel, J. L. 2003. 5-HT3 receptors in the CNS: 3B or not 3B? Trends Pharmacol Sci, 24, 157-60.

Velisek, L., Bohacenkova, L., Capkova, M. & Mares, P. 1994. Clonidine, but not ritanserin, suppresses kainic acid-induced automatisms in developing rats. Physiol Behav, 55, 879-84.

Vianna, E., Ferreira, A.T., Naffah-Mazzacoratti, M., Sanabria, E., Funke, M., Cavalheiro, E., & Fernandes, M. 2002. Evidence that ATP participates in the pathophysiology of pilocarpine-induced temporal lobe epilepsy: fluorometric, immunohistochemical, and Western blot studies. Epilepsia, 43, 227-229.

Vidal, R., Valdizán, E., Mostany, R., Pazos, A., & Castro, E. 2009. Long-term treatment with fluoxetine induces desensitization of 5-HT4 receptor-dependent signalling and functionality in rat brain. J Neurochem, 110(3): 1120-1127.

Vidal, R., Valdizan, E., Vilaró, M., Pazos, A., & Castro, E. 2010. Reduced signal transduction by 5-HT4 receptors after long-term venlafaxine treatment in rats. Br. J. Pharmacol, 161(3): 695–706.

Waeber, C., Sebben, M., Grossman, C., Javoy-Agid, F., Bockaert, J. & Dumuis, A. 1993. [3H]-GR113808 labels 5-HT4 receptors in the human and guinea-pig brain. Neuroreport, 4(11), 1239-1242.

Wu, C., Singh, S., Dias, P., Kumar, S., & Mann, D. 1999. Activated astrocytes display increased 5-HT2A receptor expression in pathological states. Exp Neurol, 158(2): 529-33.

Xiong, H. and Xia, J. (2014). Preparation and Use of Rodent Hippocampal Slices. In: H. Xiong and H. Gendelman, ed., Current Laboratory Methods in Neuroscience Research. New York: Springer Science, 95 - 103.

Xu, X. J., Boumechache, M., Robinson, L. E., Marschall, V., Gorecki, D. C., Masin, M. & Murrell-Lagnado, R. D. 2012. Splice variants of the P2X7 receptor reveal differential agonist dependence and functional coupling with pannexin-1. Journal of Cell Science, 125, 3776-3789.

Yang, Z., Liu, X., Yin, Y., Sun, S. & Deng, X. 2012. Involvement of 5-HT(7) receptors in the pathogenesis of temporal lobe epilepsy. Eur J Pharmacol, 685, 52-8.

Yiangou, Y., Facer, P., Durrenberger, P., Chessell, I. P., Naylor, A., Bountra, C., Banati, R. R. & Anand, P. 2006. COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. BMC Neurol, 6, 12.

Yu, Y., Cao, D., Xu, H., Sun, M., Huang, Z., Yung, W., Lu, N., & Huang, Y. 2014. 5-HT3A receptors are required in long-term depression and AMPA receptor internalization. Neuroscience, 278: 105-12.