Exploring oxidative stress pathways in epilepsy

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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October 2018

Declaration

I hereby declare that the research reported in this thesis represents my own work carried out at the Wolfson centre for Personalised Medicine, Department of Molecular and Clinical Pharmacology, University of Liverpool, UK. This work has not been previously submitted to the University or any other institution in application for admission to a degree or other qualification except where otherwise indicated as help which is appropriately acknowledged.

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Acknowledgements

First and foremost, thanks to **Allah Almighty** for the guidance and help in giving me the strength to complete this thesis. I would like to thanks **University of Babylon College of Medicine-Iraq** for providing me scholarship to complete this PhD Journey

I would like to express my deep and sincere gratitude to my supervisor, **Dr. Graeme Sills** whose responsible supervision and constant guidance helped to develop my scientific thinking. His wide knowledge and logical way of thinking have been of great value for me. Also, I would like to show my appreciation to my co-supervisors **Professor Sir Munir Pirmohamed and Professor Anthony G. Marson** for their support and guidance during my PhD study.

I am very grateful to **Andrea Jorgensen,** Biostatistician, University of Liverpool, for her guidance regarding complex statistical analysis involved in this thesis. I would also thank my departmental faculty members who contributed to this work by technical help and scientific input especially **Dr. Dan Carr** for MDA assay and **Eunice Zhang** for Gene expression and genetic association assays. Also a word of thanks to my friend **Soban Sadiq** without him, I would never have a great encouragement and competitive atmosphere during my study. Thank you for always listening, discussing and keeping me confident and happy.

I would also like to thank my **Parents**, my brother, **Ahmed Al-Mosawi** and and sister **Dr. Asraa Al-Mosawi**, who have always supported, encouraged and believed in me, in all my present and future endeavours.

Last but not the least; I would like to thank my wife, **Nuha Hamood** and son, **Hasan** for their continuous moral support throughout my PhD. This journey cannot be completed without their support.

Reyadh Al-Mosawi

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List of abbreviations

Abbreviatio	Definitions
ΔΔCT	Delta delta cycle for threshold
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
2-dG	2-deoxuguanosine
4-HNE	4-hydroxy-2-(E)-nonenal
8-OHdG	8- hydroxydeoxyguanosine
ABCB1	ATP binding cassette subfamily B member 1
ABTS	2-2-azinobis(3-ethyllbenzothiazoline-6-sulfonic acid
ADNFLE	Autosomal dominant noctural frontal lobe epilepsy
ADP	Adenosine diphosphate
AEDs	Antiepileptic drugs
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ARE	Antioxidant response elements
ATP	adenosine triphosphate
BCA	Bicinchoninic acid assay
bZIP	Basic-region leucine zipper
C1	complex I
CAT	Catalase
CBZ	Carbamazepine
CNC	cap'n'collar
CNS	Central nervous system
CoASH	Coenzyme A
COMT	Catechol-o-methyltransrerase
COPD	Chronic obstructive pulmonary disease
CT	Computerised tomography
Cu	Copper
CVA	Cerebrovascular accident
DAG	Diacyl glycerol
DEPAC	Diethylenetriamine-penta acetic acid
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle's medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate.
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
ETC	Electron transport chain
FADH	Reduced flavin adenine dinucleotide
FBS	Fetal bovine serum
Fe	Iron
FRAP	Ferric reducing antioxidant power
GABA	Gamma-aminobutyric acid
GBP	Gabapentin
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modulatory subunit
GEFS+	Generalized epilepsy with febrile seizure plus
GLT-1	Glutamate transporter
Gpx	Glutathione peroxidase
GR	Glutathione reductase
Grx-1	Glutareduxin-1
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GTCS	Generalized tonic-clonic seizure
GWAS	Genome-wide association study
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HIV	Human immunodeficiency virus
HO-1	Haem oxygenase 1
HRP	Horseradish peroxidise
IGEs	Idiopathic generalized epilepsies
IL	Interleukin
ILAE	International League against Epilepsy
IPA	Isopropanol
IP_3	Inositol triphosphate
IsoPs	Isoprostanes
JME	Juvenile myoclonic epilepsy
KCCs	K^+-Cl^- co-transporters
KD	Ketogenic diet
Keap 1	Kelch-like ECH associated protein 1
LDH	Lactate dehydrogenase

LDS	Lithium dodecyl sulphate
LEV	Levetiracetam
LPS	Lipopolysaccharides
LTG	Lamotrigine
LVA	Low voltage activated
Maf	Masculoaponeurotic fibrosarcoma
MAO	Monoamine oxidase
MCO	Metal-catalysed oxidation
mCU	Mitochondrial calcium uniporter
MDA	Malondialdehyde
MERRF	Myoclonic epilepsy with ragged-red fibres
Mg	Milligram
MGB	Minor groove binder
mGST	Microsomal glutathione S-transferase
Ml	Millilitre
mM	Millimolar
MMP	Mitochondrial membrane potential
$mNa^{+}Ca^{2+}E$	Mitochondrial sodium calcium exchanger
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
mNICE	Mitochondrial sodium independent calcium exchanger
MPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance image
mtDNA	mitochondrial DNA
MTLE-HS	Mesial temporal lobe epilepsy with hippocampal sclerosis
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium Bromide)
NADH	Reduced nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NFE2L2	Nuclear factor, erythroid 2 like 2
Ng	Nanogram
NKCC1	Na^{+}-K^{+} co-transporters
NKCCL1	Na^{+}-K^{+}-Cl^{-} cotransporter 1
NMDA	N-methyl-D-aspartate
NQO-1	NADPH dehydrogenase
Nrf2	Nuclear factor (erythroid-derived 2) factor 2
O_2^{-}	Super oxide anion
OD	Optical density
$OH^{\bullet}$	Hydroxyl radical
$ONOO^{-}$	Peroxy nitrite
OXC	Oxcarbazepine

OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PDSs	Paroxysmal depolarization shifts
PPAR	Peroxisome proliferator–activated receptor
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SANAD	Standard and New Antiepileptic Drugs
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Status epilepticus
SEM	Standard error of mean
SNP	Single nucleotide polkymorphism
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences
SUDEP	Sudden unexpected death in epilepsy
SV2A	Synaptic vesicle protein 2A
TAS	Total antioxidant status
TBI	Traumatic brain injury
TBST	Tris buffered saline tween
TCA	Tricarboxylic acid
TGB	Tiagabine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TOS	Total oxidant status
TPM	Topiramate
Trx-1	Thioredoxin-1
VPA	Sodium valproate
WST-1	Water soluble tetrazolium 1
α-KGDH	α-ketoglutarate dehydrogenase
Ψm	Mitochondrial membrane potential

Poster presentations

1- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of commonly used AEDs on oxidative stress biomarkers in human SH-SY5Y cell line. Faculty poster day, Liverpool, 10th of June 2016.

2- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of common AEDs on oxidative stress biomarkers in human neuroblastoma cell line. Liverpool Neuroscience day, 14th of June 2016

3- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of common AEDs on oxidative stress parameters in human SH-SY5Y cell line. NWEG meeting, Liverpool, 9th of November, 2016.

4- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of selected AEDs on oxidative stress biomarkers in human SH-SY5Y cell line. ITM research day, Liverpool, 6th of July 2017.

5- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of common AEDs on oxidative stress biomarkers in human SH-SY5Y cell line. ILAE annual meeting, Leeds, 4-6th October, 2017.

6- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of CBZ, VPA, LTG, and LEV on oxidative stress biomarkers and related genes and proteins in human SH-SY5Y cell line. BPS annual meeting, London, 11-13th December, 2017.

7- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of CBZ, VPA, LTG, and LEV on oxidative stress biomarkers in human SH-SY5Y cell line. Neuroscience day, Liverpool, 15th of June 2018.

Oral presentations

1- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of common AEDs on oxidative stress biomarkers in human neuroblastoma cell line. Neuroscience day, Liverpool, 14th of June 2016

2- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of common AEDs on oxidative stress parameters in human SH-SY5Y cell line. NWEG meeting, Liverpool, 9th of November, 2016.

3- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of CBZ, VPA, LTG, and LEV on oxidative stress biomarkers and related genes and proteins in human SH-SY5Y cell line. BPS annual meeting, London, 11-13th December, 2017.

4- Al-Mosawi R, Marson A, Pirmohamed M, Sills G. Effects of CBZ, VPA, LTG, and LEV on oxidative stress biomarkers and related genes and proteins in human SH-SY5Y cell line. ECE annual meeting, Vienna, 26-30th August, 2018.

Abstract

Background: Oxidative stress is now believed to play a role in the evolution of epilepsy and antioxidants have been proposed as putative antiepileptic agents. The experimental evidence suggested that nuclear factor, erythroid 2 like 2 (Nrf2) could be a promising therapeutic target for epilepsy. The main aims of this study were to investigate the effects of common AEDs on oxidative stress biomarkers in a human SH-SY5Y cell-line, and to explore the association of common variants in the (*NFE2L2)* gene with clinical characteristics of epilepsy.

Methods: SH-SY5Y human cells were exposed to H_2O_2 (0-1000μM), LPS (0-100 μg/ml), and pilocarpine (0-100 μM) for 1, 4, and 24 hours. Cells were exposed to carbamazepine (CBZ; 0-100μM)), valproic acid (VPA; 0-1000μM) levetiracetam (LEV; 0-300μM), and lamotrigine (LTG; 0-100μM) for 1, 4, and 24hrs, with or without 100μM H_2O_2 for further 4hrs. AED effects were assessed against basal oxidative stress, determined by malondialdehyde (MDA) concentration, superoxide dismutase (SOD) activity, and reduced/oxidised glutathione (GSH/GSSG) ratio. The expression of Nrf2, haemoxygenase-1 (HO-1) and NADPH quinone oxidoreductase-1 (NQO-1) was determined using real time PCR and western blot. The genotyping of the five SNPs in *NFE2L2* gene was carried out using TaqMan® genotyping assays.

Results: MDA level was increased in a time and concentration-dependent manner up to maximum of 3-fold, following exposure to H_2O_2, LPS, and pilocarpine. However, SOD activity, GSH/GSSG ratio decreased by 5-fold, 15-fold, respectively, after exposure to H_2O_2, LPS, and pilocarpine. MDA level was elevated in a concentration-dependent-manner, up to a maximum of 13-fold, 5.5-fold, 15-fold and 9.5-fold, following exposure to CBZ, VPA, LEV and LTG, respectively. In contrast, SOD activity was reduced, again in a concentration-dependent manner, by a factor of up to 2, 2.5, 1.5 and 1.3 after incubation with CBZ, VPA, LEV and LTG, respectively. The GSH/GSSG ratio was also reduced after exposure to CBZ (up to 5.8-fold), VPA (up to 4-fold) LEV (up to 5-fold), and LTG (up to 3-fold). Further exposure to 100μM H_2O_2 amplified AEDs effects on MDA concentration, SOD activity and GSH/GSSG ratio by up to 3-fold. Expression of the Nrf2 gene was increased by 4-fold following exposure to both 100μM CBZ and 1000μM VPA, while expression of HO-1 was increased by 2.7-fold and 3-fold, respectively. NQO-1 gene expression was increased after incubation with 100μM LTG and 1000μM VPA by 2.3-fold and 2.9-fold, respectively. Additional exposure to 100μM H_2O_2 augmented AED effects on Nrf2, HO-1 and NQO-1 gene expression by up to 5-fold. Relative expression of Nrf2, HO-1 and NQO-1 proteins was increased by 1.3-fold, 1.2-fold and 1.2-fold after exposure to CBZ, VPA and LTG, respectively. There was no effect of LEV. Small additional increases (up to 10%) in protein expression were seen after further 24 hours incubation with 100μM H_2O_2. Association was observed between *NFE2L2* genotype, rs2886161, and the remission on any AED. Nonetheless, a multivariate predictive model did not show any significant contribution of *NFE2L2* SNP genotypes, demographic and clinical predictors and the response to AED treatment.

Conclusions: These findings imply that several existing AEDs have pro-oxidant rather than antioxidant effects, albeit at relatively high concentrations. This may limit their antiseizure activity and/or contribute to their adverse effect profile. There was no evidence from this pharmacogenomics investigation that common variants in the *NFE2L2* gene have any influence on the presentation of epilepsy or on its prognosis.

Chapter 1

General introduction

1.1 Historical overview

The very early deliberated depiction of epilepsy was revealed in ancient Babylonian medical texts known as *Sakikku* "All Diseases" (1067-1046 B.C.). They used special terms for all types of seizure and epilepsy such as *Antasubba* and *Miqtu*. Precise details referred to prognosis, diagnosis and treatment, and petit and grand mal, febrile seizures, ictal and post-ictal periods, auras, partial seizures and status epilepticus were fully described (Edward et al., 1990; Reynolds and Wilson, 2008).

The plate which is a part of the Babylonian sets in the British museum (figure 1.1) includes surprisingly well-explained clinical perceptions of almost all of the common types of seizure and a few rarer epilepsy syndromes which are well known nowadays.

Figure 1.1 Clay tablet 26 about epilepsy from the Neo-Babylonian collection in the British Museum, London. BM47753 (adopted from Edward et al., 1990).

The Babylonian role on the clay tablet represented miqtu “fall or epilepsy”, hayyatu “fit” and sibtu “seizure” as an extraordinary disorder that is due to infringement of the body by evil spirits (Edward et al., 1990; Reynolds and Wilson., 2008). This conception of epilepsy remained authoritative until the 5th century BC when the initial steps toward epilepsy recognition commenced. Hippocrates, in his book, On the Sacred Disease, was the first to defy the divine view of epilepsy by saying that epilepsy is a disease, just like other diseases, and has its etiological factors, hereditary malady, signs and symptoms (Magiorkinis et al., 2010).

Since then, the conception of epilepsy as a brain disorder started to develop and by the early 19th century a consensus was emerging, especially with the development of healthcare systems in Europe and the introduction of bromide as antiepileptic drug (AED) in Europe and USA, (figure 1.2) (Goldensohn et al., 1997).

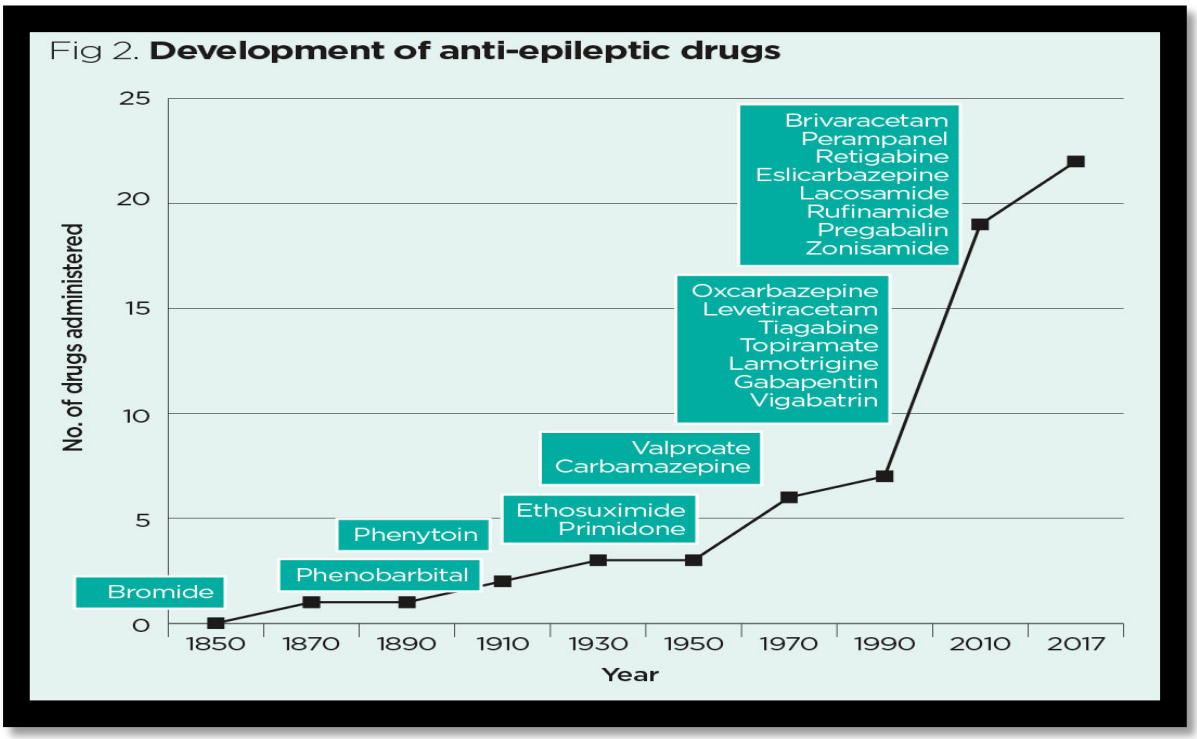


Figure 1.2 Development of antiepileptic drugs from 1850-2017 (adapted from Tittensor, 2018).

The discovery of the human electroencephalogram (EEG) in 1929 by the German neurologist Hans Berger (1873-1941) allowed noticeable progress to be achieved and confirmed the electrical principle of epilepsy which had been suggested previously by Robert Bently Todd (1809-1860) (Goldensohn et al., 1997; Magiorkinis et al., 2014). By the second half of the 20th Century, there had been a prominent progression in the diagnosis and treatment aspects of epilepsy, which was marked by increased focus on the basic mechanisms behind seizure and epilepsy. This was facilitated by evolutions in genetics, neurophysiology, molecular biology and other important techniques (Goldensohn et al., 1997). In spite of all these advances, a great challenge still exists in our knowledge regarding the mechanisms of epileptogenesis and the pathophysiology of epilepsy. A major goal of epilepsy research remains the identification of reliable prognostic and diagnostic biomarkers and of new therapeutic targets with the potential of modifying or curing the disease, rather than merely inhibiting seizures (Baulac et al., 2015).

1.2 Definitions of epilepsy and epileptic seizures

Epilepsy is a chronic brain disorder or a group of brain disorders characterised by a tendency to generate at least one epileptic seizure (Fisher et al., 2005). Epilepsy affects people worldwide and can cause long lasting disruption of cerebral structure and / or function. This brain disturbance appears clinically as several types of seizures, which are often the net result of an unstable, convoluted process (epileptogenesis) where normal cerebral structure and function are modified resulting in a permanent state which leads to the generation and propagation of abnormal electrical activity and recurrent seizures (Goldberg and Coulter, 2013). An epileptic seizure is defined as a brief episode of unpredictable involuntary movement or sensation, associated with abnormal and excessive output from a group of neurons in the brain, in the absence of aggravating

factors and that may affect part of the body (partial or focal seizure) or the entire body (generalized seizure). These episodes are occasionally accompanied by an alteration in consciousness, motor, sensory, autonomic, psychological and behavioural comorbidities (Fisher et al., 2005). In 2014, the International League against Epilepsy (ILAE) suggested a new practical definition of epilepsy to replace the previous one proposed in 2005 (Fisher et al., 2014). The updated definition represented epilepsy as a brain disease that is defined by any one of the following characteristics (Fisher et al., 2014):

1. Two unprovoked (or reflex) seizures happening more than 24 hours apart.

2. One unprovoked seizure (or reflex) seizure with a high risk of recurrence (more than 60%) which is similar to the general recurrence risk after two unprovoked seizures.

3. Diagnosis of epilepsy syndrome.

There are several significant updates in this new definition. Firstly, the new definition points to epilepsy as a disease rather than a disorder. This reflects the critical nature and sequelae of epilepsy, as the word disease notifies a more lasting disturbance of normal brain function. Secondly, the inclusion of reflex seizures, which are seizures provoked by external pathological factors (such as tumours or trauma), provided that there is abnormal and enduring liability to have seizures (Neligan et al., 2012; Fisher et al., 2014). Additionally, the new definition strongly emphasises recurrence risk after the first unprovoked seizure; patients with identifiable brain lesions, such as brain trauma, infection or tumours, have a risk of recurrence after a single seizure that is similar to those who have experienced two unprovoked seizures. This change to the definition encourages earlier diagnosis and treatment of symptomatic epilepsy, avoiding the complications of physical injury and the social consequences of seizure recurrence. Finally, the modified definition emphasises the importance of the diagnosis of epilepsy

syndromes, including those with a low risk of recurrent seizures and high rates of spontaneous remission (Fisher et al., 2014). In this regard, epilepsy is considered to be resolved if the patient is free from seizures for 10 years and off medication for at least 5 years

1.3 Epidemiology of epilepsy

Epilepsy is the most common chronic brain disease globally, and it affects people of all ages, races and social groups. Currently, around 50 million people worldwide have epilepsy and 80% of them live in developing countries (Gretchen et al., 2012). In high-income countries, the incidence of epilepsy is between 30 and 50 newly diagnosed cases per 100,000 people per year and this is doubled in low- and middle-income countries (Forsgren et al., 1996; Banerjee et al., 2009). Although the onset of epilepsy can occur at any age, it is more common in children, young people and in the elderly (Olafsson et al., 2005). The mortality rate in people with epilepsy is approximately two to three times greater than in the general population and life expectancy is shortened by two to ten years (Tomson et al., 2005; Hitiris *et al.*, 2007). The highest mortality rate occurs in those with symptomatic epilepsy and persistent neurological deficits (Kenneth et al., 2014). Sudden unexpected death in epilepsy (SUDEP) is the most common cause of death in patients with chronic epilepsy. The incidence of sudden death is reported to be 20 times greater in people with epilepsy compared with the general population (Shankar et al., 2013). SUDEP is relatively uncommon in patients with new-onset epilepsy and in those in remission from seizures, with incidence estimated at 0.1 to 0.35 cases per 1000 person-years in population-based cohorts of epilepsy patients. SUDEP is considerably more common in patients with chronic epilepsy, with an incidence of 1 to 2 cases per 1000 person-years, and highest among those with severe, refractory seizures at 3 to 9 cases per 1000 person-years (Shankar et al., 2013). SUDEP may occur at all ages but is

most often observed in those between 20 and 40 years. In most cases, SUDEP appears to be seizure-related (Tomson et al., 2005).

1.4 Classification of the epilepsies

Classification of the epilepsies is very important as it is the crucial tool used clinically to manage a patient who is presenting with seizures. It is very helpful to understand the type of seizure that a patient has, other seizures that are likely to arise in that patient, the possible precipitating factors for their seizures, approaches to management, and the likely prognosis. Moreover, classification of epilepsy is an important part in predicting the hazards of comorbidities and mortality in epilepsy patients and often gives insight into AED selection (Scheffer et al., 2017). The new 2017 ILAE classification of epilepsy is built in three levels according to seizure type, epilepsy type and syndrome and also considers aetiology (figure 1.3).

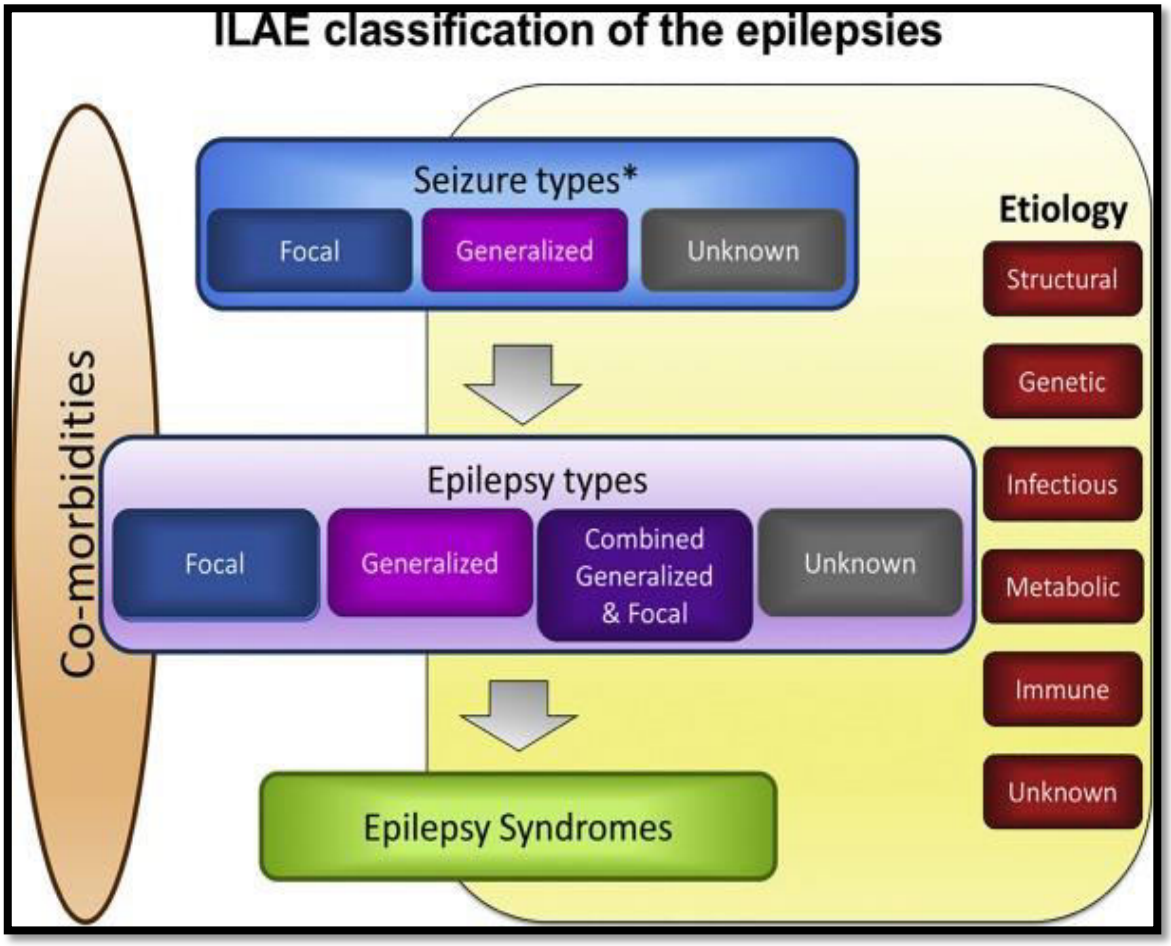


Figure 1.3 Classification of epilepsy types (adapted from Scheffer et al., 2017).

1.4.1 Classification of epileptic seizures

Older classifications (ILAE, 1981; ILAE, 1989) classified seizures based on clinical features and EEG findings. Seizures were divided into either generalised or partial. Depending on the level of retained consciousness, partial seizures were subdivided into simple and complex. With considerable progress in recognising the pathogenesis of epilepsies and seizures, modification of the 1981 and 1989 classification systems has taken place and several papers have been published suggesting a revision of the now outdated classification of seizures and epilepsies (Luders et al., 1998; Engel., 2001; Engel., 2006). The ILAE Commission on Classification and Terminology published a

new, modified classification system in 2011 in which the most novel connotation was the manner of seizure commencement (Berg et al., 2011). According to this new classification, seizures should be classified into focal, generalized, and unknown (Berg et al., 2011).

Focal (or partial) seizures refer to the electrical and clinical manifestations of seizures that arise from a specific of the brain. The clinical manifestations of a particular focal seizure depend on the part of the brain that is activated (Fisher et al., 2017). The EEG typically indicates a localised discharge over the area of onset. Focal seizures most commonly arise from the temporal lobe. Simple focal seizures are those in which consciousness is preserved. New terminology suggests that these should now be called 'focal aware' seizures. Complex focal seizures include memory loss for the clinical event and impaired responsiveness at the time of the event. New terminology suggests that these should now be called 'focal impaired awareness' seizures. Focal seizures may also evolve into secondary generalised seizures (Fisher et al., 2017).

A generalized tonic-clonic seizure (GTCS) is a seizure type that is identified by specific clinical and EEG criteria (ILAE, 1981). Clinically, this seizure type involves loss of consciousness and a phasic tonic stiffening of the limbs (either symmetrically or asymmetrically), followed by repetitive clonic jerking. The vast majority of these types of seizure are self-limiting without intervention. The observed manifestations of the seizure are correlated on the EEG with bisynchronous epileptiform activity in both cerebral hemispheres. These seizures can occur either in a primary fashion (with onset in a widespread or bilateral distribution) or in a secondary fashion (with onset in one hemisphere or region) subsequent to an initial focal seizure (Fisher et al., 2017).

Unknown seizures are those that are not readily classifiable into either focal or generalised because of a lack of clinical history or the results of investigations. This

might include seizures with EEG and/or clinical evidence to suggest that origin of the seizure is within, and which rapidly engages, a bilaterally distributed network. If the source of the seizure cannot be determined with at least 80% confidence by the clinician, EEG or other scanning technique, it should be classified as 'unknown onset'. As new information becomes available, it may be possible to change this classification to either focal or generalised (Liyanagedera et al., 2017).

1.4.2 Classification of epilepsy type

The new classification of epilepsy type is derived from the earlier 2014 definition (Fisher et al., 2014). It includes a new category of "Combined Generalised and Focal Epilepsy" in addition to the well-established and distinct generalized epilepsy and focal epilepsy classifications and also an "Unknown" category. Many types of epilepsy include multiple types of seizures and the diagnosis of epilepsy type is based on a combination of clinical history, clinical examination and EEG findings. As discussed above in relation to seizure type, the term "unknown" in this case refers to an epilepsy type which is difficult to classify into either generalized or focal because of insufficient information or uninformative EEG findings (Scheffer et al., 2017) (figure 1.3).

1.4.3 Syndromic classification of epilepsy

An epilepsy syndrome is a complex of clinical signs and symptoms that include seizure type, EEG and imaging features that determine unique epilepsy status. Age-related features such as age at onset and age at remission, provoking factors, and diurnal variation in seizures may be specified where known (Scheffer et al., 2018). Furthermore, peculiar comorbidities and aetiological and prognostic features may be identifiable for specific epilepsy syndromes. The most recent classification system (Scheffer et al., 2017) has established two major epilepsy syndrome groupings,

idiopathic generalized epilepsies (IGEs) and self-limiting focal epilepsies. The IGEs, in turn, comprise four well established epilepsy syndromes; childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy, and generalized tonic-clonic seizures alone. Likewise, several self-limiting focal epilepsy syndromes have been identified, including focal epilepsy with centrotemporal spikes plus frontal, temporal, parietal and occipital lobe epilepsies (Scheffer et al., 2017).

1.4.4 Aetiologic classification of epilepsy

The older aetiological classification of epilepsy into idiopathic, symptomatic and cryptogenic was associated with substantial contradictions and discrepancies among clinicians. The aetiological classification has been reappraised, with a focus on the causes and mechanisms of epilepsy (Shorvon, 2011). New aetiological terms were suggested by the 2017 ILAE report, as follows; genetic, structural, infectious, metabolic, immune and unknown (Scheffer et al., 2017).

Genetic aetiology: A classification of genetic epilepsy is applied when epilepsy is the result of a well-defined or presumed genetic defect (Berg et al., 2011; Moshe et al., 2015). The best known example of genetic epilepsy is Dravet syndrome in which more than 80% of patients have a mutation in the α1 subunit of the neuronal voltage-gated sodium channel (*SCN1A*) gene (Scheffer et al., 2017). In addition to Dravet syndrome, *SCN1A* gene mutations have been recognised in generalized epilepsy with febrile seizure plus (GEFS+) and in intractable childhood epilepsy with generalised tonic-clonic seizures (Mullen and Scheffer., 2009; Brunklaus et al., 2013). Other examples of gene mutations associated with epilepsy are *CHNRA4* gene mutation was associated with Autosomal dominant noctural frontal lobe epilepsy (ADNFLE) and *GABRA1* gene mutations which have been recognised in Autosomal dominant juvenile myoclonic epilepsy (JME) (Scheffer et al., 2017). A genetic contribution to epilepsy does not

preclude an environmental factor. It is well known that environmental factors contribute to seizure disorders; for instance, many epilepsy patients are likely to have a seizure with stress, illness or sleep disorders. So, a genetic factor indicates the pathogenic mutation of crucial effect in giving rise to epilepsy (Scheffer et al., 2017).

Structural aetiology: Many structural abnormalities of the brain are linked to a markedly increased risk of epilepsy development. These structural abnormalities are ordinarily visible by computerised tomography (CT) scan or magnetic resonance image (MRI). Structural aetiologies are either acquired, such as trauma, stroke and infection, or genetic, as is the case in many cerebral malformations (Scheffer et al., 2017). There are several well-defined associations with structural aetiologies within the epilepsies. These include, mesial temporal lobe seizures with hippocampal sclerosis, gelastic seizures with hypothalamic hamartoma, Rasmussen's syndrome, and hemiconvulsion-hemiplegia-epilepsy (Berg et al., 2011). Recognition of these associations is important in ensuring that brain scans from epilepsy patients are carefully examined for a particular structural lesion. This might influence decisions about brain surgery if medical therapy fails (Scheffer et al., 2017).

Metabolic aetiology: Metabolic causes allude to a well-defined metabolic disorder with manifestations or biochemical changes in the body, such as uremia, aminoacidopathies, porphyria, and pyridoxine-dependent seizures. Most of the metabolic epilepsies have genetic basis, but some may be acquired like cerebral folate deficiency seizures (Scheffer et al., 2017). It is extremely important to identify the metabolic cause of epilepsy in order to treat it effectively and prevent any intellectual impairment.

Infectious aetiology: Globally, known infectious agents are the most common causative factors in epilepsy (Singhi et al., 2011; Vezzani et al., 2016). In this case, infectious causes refer to patients with established epilepsy, rather than those experiencing

seizures as a manifestation of acute infections such as encephalitis and meningitis. Examples of infections that can give rise to epilepsy in particular regions in the world are neurocysticercosis, tuberculosis, HIV, cerebral malaria, cerebral toxoplasmosis and congenital infections such as cytomegalovirus and Zika virus. These infectious causes might sometimes have a related structural aetiology (Singhi et al., 2011).

Immune aetiology: The immunological epilepsies typically result from immune disorders in which seizures are one of several symptoms of the disease. Immune epilepsy has been identified in both children and adults and is usually associated with the evidence of auto-immune central nervous system inflammation (Vezzani et al., 2016). With the development of broad-spectrum antibody testing, accurate diagnosis of these auto-immune encephalitides, including anti-NMDA receptor encephalitis and anti-LGI1 encephalitis, is increasing (Lancaster et al., 2012). This subgroup of epilepsies has targeted treatment options with specific immunotherapy.

Unknown aetiology: There are many epilepsy patients with unknown cause in whom the epilepsy diagnosis is built on clinical and neuroimaging techniques. So, the extent of the aetiology of the epilepsy depends on the extent of the assessment available to the patient and this differs across the world in different countries and healthcare systems (Scheffer et al., 2017).

1.5 Neurobiology of seizures and epilepsy

Awareness of the basic principles of neurobiology provides a platform with which to understand the principal mechanisms behind seizures, epileptogenesis and epilepsy. The initiation and propagation of seizure activity involves some of the most fundamental aspects of nerve cell function, such as action potential generation, the electrical basis of trans membrane potential, and neuronal synchronisation.

1.5.1 Molecular mechanism of seizures

Many mechanisms can result in seizures. However, at the most basic level, it is widely accepted that seizures develop when there is a disturbance in the balance between inhibitory and excitatory neurotransmission. Any disruption of this balance that either suppresses inhibition or enhances excitation can produce seizures. In contrast, blockade of excitatory firing or enhancement of inhibitory neurons usually protects against seizures. Control of these mechanisms can occur at several different levels within the nervous system: firstly at the level of ion transport across cell membranes, secondly at the level of neuronal cell circuits and synapses, and thirdly within neuronal networks.

Electrical basis of nerve cells: The nervous system preserves ionic and chemical gradients that generate the platform for electrical activity. Neurons are not continuously active but are typically close to the threshold for firing (resting potential), meaning that action potentials, which are fundamental to central nervous system function, can be generated with relative ease. The robustness of the resting potential is however crucial to restrain excessive firing which is usually associated with seizures (McCormick et al., 1994) (figure 1.4).

In the normal state, neurons have a high intracellular concentration of potassium ions and a high extracellular concentration of sodium ions, leading to a clear transmembrane potential (McCormick et al., 1994). Any disruption of this ionic balance results in generation of an action potential, which travels down the axon and causes depolarisation of nerve terminals, neurotransmitter release, and thus cell-cell communication (Lodish et al., 2000). Essential pumps, such as sodium-potassium ATPase, are found in the cell membrane to maintain the ionic gradients. Likewise, glial cells play an important role in controlling extracellular ion concentrations, which has led scientists to consider that glial cells are as important as neurons in the generation of seizure activity (Duffy et al.,

1999; Fellin et al., 2005). Any abnormality in ion pumps, glial cells or membrane-bound ion channels, such as the voltage-gated sodium channel implicated in GEFS+, could potentially facilitate seizures (Meisler et al., 2001). Thus, the modulation of voltage-gated ion channels and the preservation of transmembrane ionic gradients provide targets for existing and new anticonvulsants.

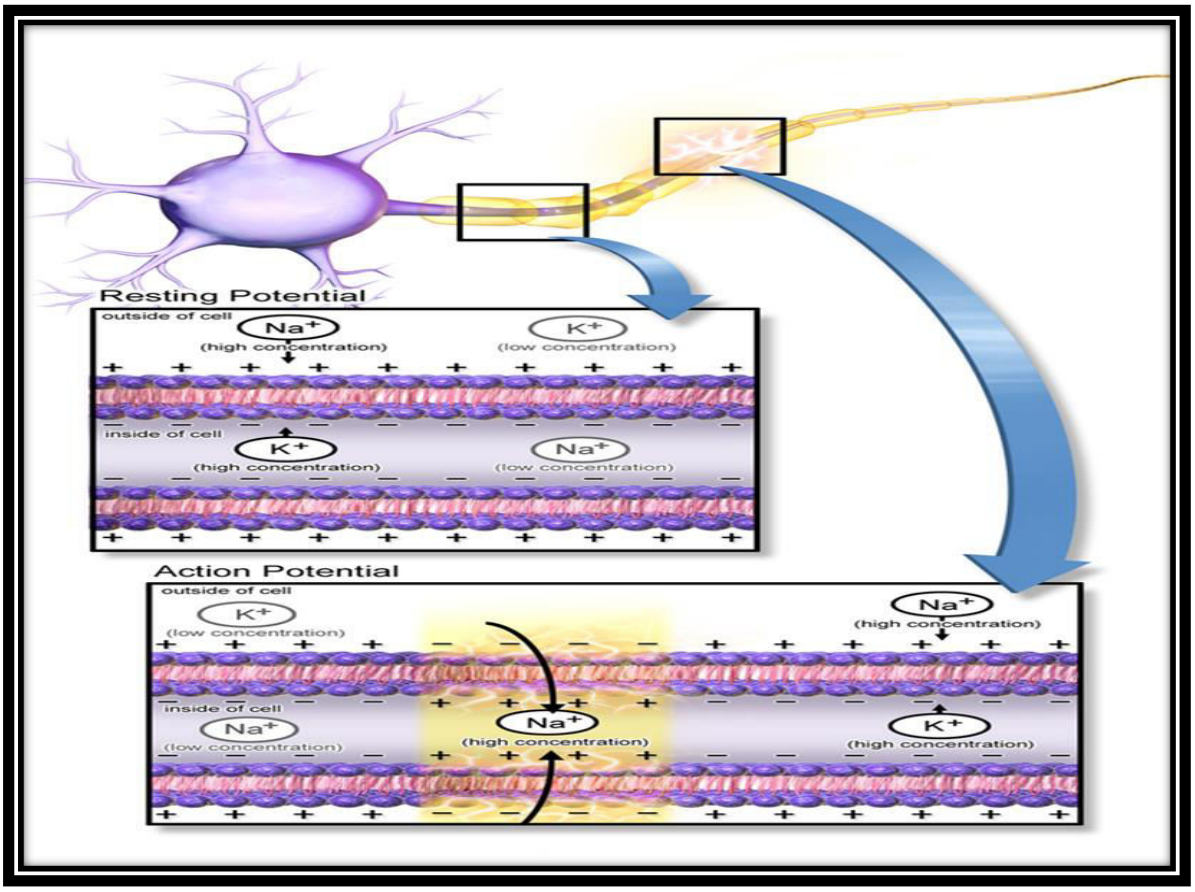


Figure 1.4: Resting potential and action potential in neuronal cells. As an action potential (nerve impulse) travels down an axon there is a change in polarity across the cell membrane. Sodium (Na^+) and potassium (K^+) gated ion channels open and close as the membrane reaches its threshold potential. Na^+ channels open at the beginning of the action potential, and Na^+ ions move into the axon, causing depolarization. Repolarization occurs when the K^+ channels open and K^+ ions move out of the axon, creating a change in polarity between the outside of the cell and the inside. The impulse travels down the axon in one direction only, to the axon terminal where it signals other neurons via release of neurotransmitter (adapted from Sanes et al., 2012).

Synaptic transmission: In epilepsy, a great deal of attention is paid to mechanisms linked to synaptic transmission because of its significant role in maintaining the balance between neuronal excitation and inhibition. The inhibitory neurotransmitter gamma-aminobutyric acid (GABA) has been explored in great detail in terms of its contribution to epilepsy, as has the excitatory neurotransmitter glutamate. Chloride (Cl^-) is the major ion that is responsible for the generation of ionic currents via $GABA_A$ receptors, with a net influx causing hyperpolarisation of the neuronal cell membrane. However, K^+-Cl^- co-transporters (KCCs), which are essential for maintaining the chloride ion gradient, are not stably expressed and may be influenced by insults to the CNS (figure 1.5). This has led to evidence that the transporter NKCCL may be an important contributor to seizures in early life. An increase in expression of NKCC1, and a corresponding decrease in KCC2 expression, leads to an increase in intracellular Cl^-, a reduction in GABAergic inhibition and greater susceptibility to seizures (Dzhala et al., 2005). Added to this, excitatory neurotransmission mediated by glutamate is not always consistent. Because glutamatergic synapses supply both glutamatergic and GABA-ergic neurons, exposure to glutamate might have a strong excitatory effect, little excitatory effect, or it may inversely raise the level of inhibition via indirect activation of GABA-ergic pathways. Thus, it can be very difficult to anticipate how modulation of either GABA-ergic or glutamatergic neurotransmission might affect seizure propagation in vivo (Scharfman, 2007).

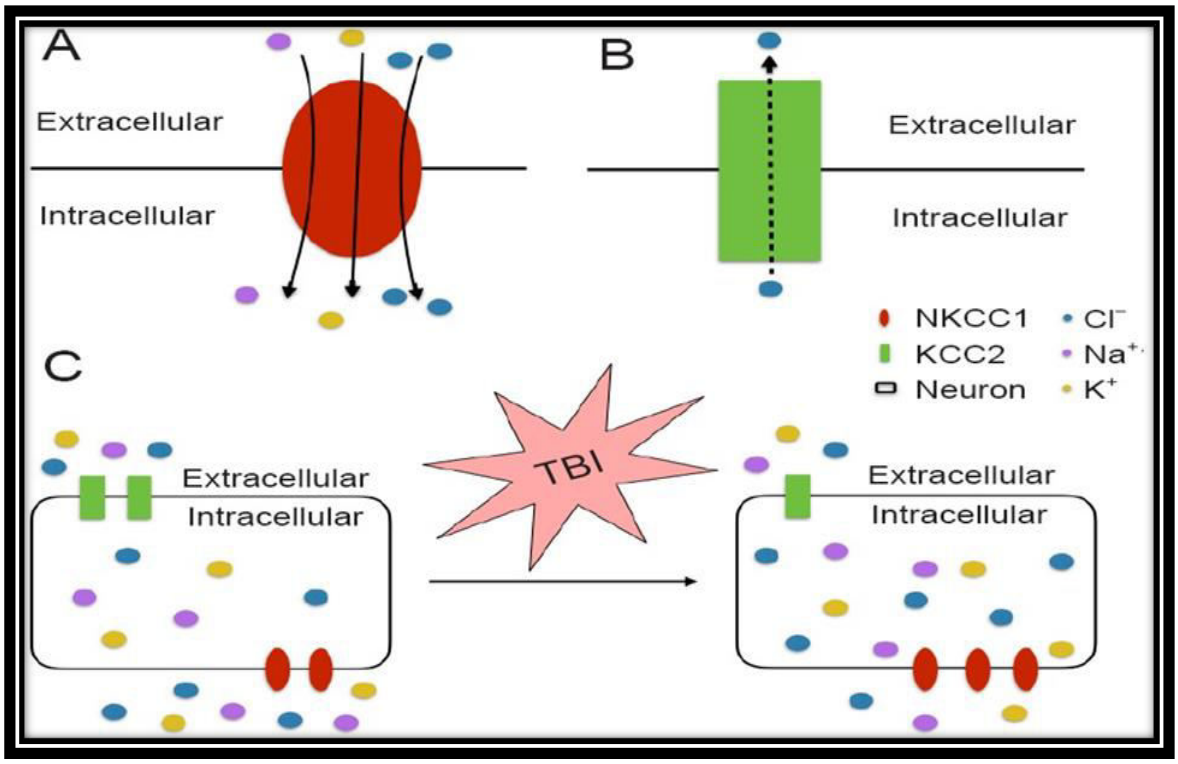


Figure 1.5: The role of NKCC1 (A) and KCC2 (B) in maintaining chloride balance. In traumatic brain injury, up-regulation of the sodium-potassium-chloride co-transporter (NKCC1) and down-regulation of the potassium-chloride co-transporter (KCC2) leads to increased intracellular concentrations of Cl^- (C). Elevated NKCC1 transporter expression is known to facilitate early post-traumatic brain injury seizures. KCC2: K^+-Cl^- cotransporter 2; NKCC1: Na^+-K^+-Cl^- cotransporter 1; TBI: traumatic brain injury (adapted from Liang et al., 2017).

Synchronization: An excessive but otherwise isolated neuronal discharge does not lead to seizures by itself. It requires the interconnection of a synchronized neuronal network. The association between electrographic recordings at the cortical surface during seizures and paroxysmal depolarization shifts (PDSs) occurring synchronously within cortical pyramidal cells was first illustrated by Matsumoto and Ajmone-Marsan (1964). Such studies give an insight into how neurons can start to fire in harmony when conventionally they do not, and several theories have been proposed to explain the mechanisms that can result in synchronization. Many of these theories point to glutamatergic networks and the implication that the PDS is simply a "giant" excitatory postsynaptic potential which is manifest by high levels of inter-connectivity between cortical pyramidal cells that release glutamate as a neurotransmitter (Brown and

Johnston, 1984). Another possible mechanism behind synchronisation is the contribution of gap junctions on cortical neurons, which permit non-synaptic communication between neuronal cells via direct ion flow, such that these neurons can be rapidly and efficiently synchronized. Many studies suggest that even small numbers of gap junctions may have a great influence on neuronal interconnection (Roger et al., 2004).

Another mechanism of synchronization comprises, conversely, inhibition. Large numbers of GABA-ergic neurons that supply pyramidal cells of cortex have an extensive network of connections to neighbouring pyramidal cells. Thus, any discharge from a solitary neuron can synchronously hyperpolarize many other pyramidal cells. As GABA-ergic inhibition diminishes, inactive voltage-dependent currents, such as those mediated by T-type calcium channels, become activated and the result is a coordinated excitation of a group of pyramidal cells (Cobb et al., 1996). Promotion of synchronisation might result from developmental brain changes in epilepsy patients. These changes involve outgrowth of axon collaterals from excitatory neurons which use glutamate as a principal neurotransmitter. Such changes might be one of the causes of recurrent seizures, although there is debate over whether seizures can arise through augmentation of both recurrent inhibition and recurrent excitation (figure 1.6) (Sloviter et al., 2006). Nevertheless, the fact remains that development of new synaptic excitatory networks, which are insignificant or absent in normal cerebrum (Nadler et al., 2003), supports synchronization and potentially clarifies how the flexibility of the central nervous system may participate in epileptogenesis (Sutula et al., 2007).

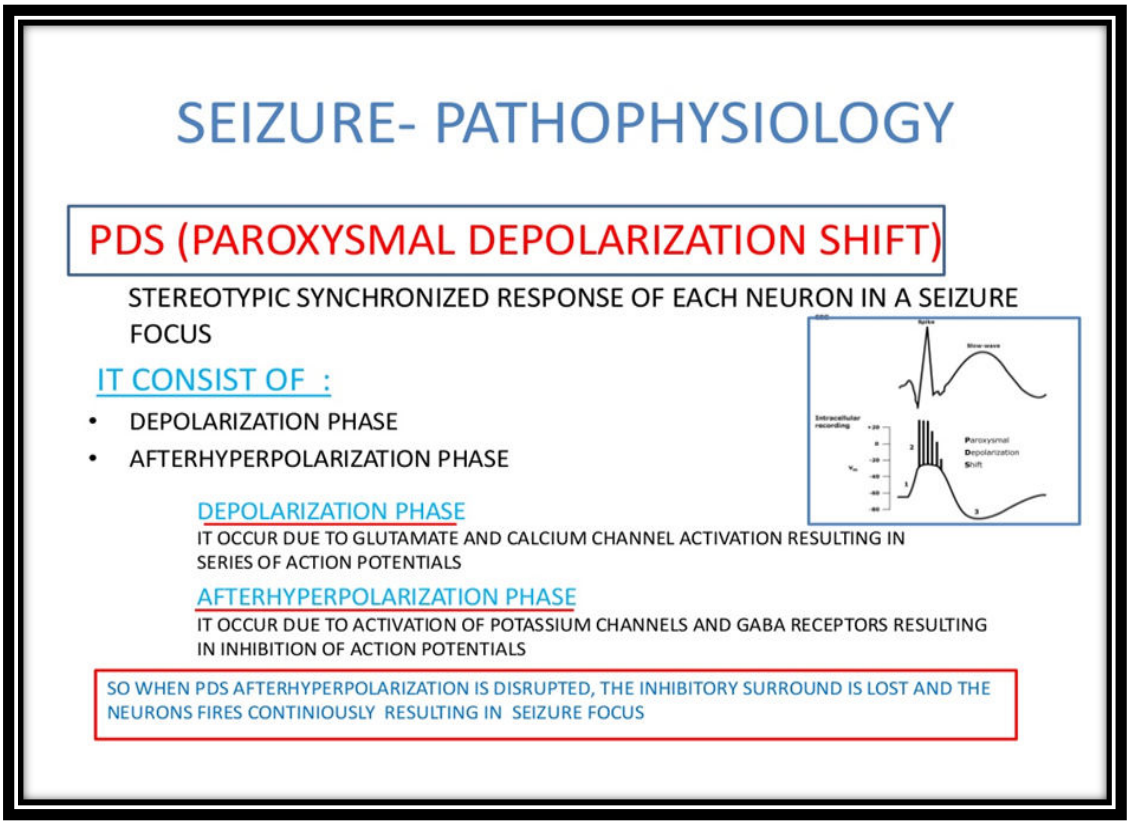


Figure 1.6: The stereotypical synchronized response of each neuron in a seizure focus. The depolarization phase occurs due to glutamate and calcium ion activation, while the repolarization phase occurs due to inhibition of action potential by activation of inhibitory GABA receptors and potassium channels (adapted from Iqbal. 2016).

1.5.2 Molecular mechanism of epilepsy

In general, the mechanisms that have been identified to explain epilepsy are either idiopathic, due to genetic causes or developmental malformations, or symptomatic (acquired) as a response to an acute insult or injury. Approximately 50% of epilepsy patients have acquired epilepsy, linked to preceding neurological insult (Delorenzo et al., 2005).

Acquired epilepsy is more prevalent than either genetic or developmental epilepsies, which mainly influence ion channels or mitochondria, respectively (Martinc et al., 2012). There are numerous genetic factors that contribute to increasing neuronal excitability in idiopathic epilepsies. Any mutation in mitochondrial respiratory chain

complexes, synapses, voltage- or ligand–gated ion channels, or neurotransmitter receptors may modify the normal excitability of the brain and lead to epileptic seizures (Martinc et al., 2012). Thus, epilepsy is multifactorial – it can emerge from genetic or acquired causes or a combination of the two. In addition, aetiological factors vary with age and geographical distribution (Martinc et al., 2012). In general, there are three major phases which comprise the development of acquired epilepsy: the acute phase (or insult), the latent phase (or epileptogenesis), and the chronic phase (or recurrent epilepsy) (Pitkanen and Sutula, 2002).

The acute phase – injury: The process of epileptogenesis in acquired epilepsy can be initiated by a several types of cerebral lesions and these aetiologies may vary with age. Such lesions may themselves be caused by infections, trauma, tumours, childhood febrile convulsions, stroke, hypoxia, status epilepticus and neurodegenerative disorders. All of these insults, injuries and illnesses increase the occurrence of acquired epilepsy. Brain trauma, cerebrovascular accident (CVA) and status epilepticus are the most three significant aetiological factors in acquired epilepsy (Delorenzo et al., 2005). Despite significant heterogeneity in the nature of these precipitating events, they are associated with a common mechanism for generating cerebral injury, i.e. excitotoxicity caused by an excess in extracellular glutamate concentrations. This leads to sustained stimulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, recruitment of N-methyl-D-asparate (NMDA) receptors, a massive increase in intracellular calcium (Ca^{2+}) concentration, and activation of Ca^{2+} signalling pathways resulting in neuronal death (Choi, 1988; Michaels and Rothman, 1990).

The latent phase – epileptogenesis: Epileptogenesis refers to the transition processes involved in the development of the epileptic condition following an initial cerebral insult. These processes might take years or even decades after the initial event and

almost certainly involves a host of molecular and cellular changes (Patel et al., 2004; Pitkanen et al., 2009). Additionally, epilepsy is ordinarily not a stagnant condition, but develops through the lifespan (Scharfman et al., 2007).

Despite huge advancement in the understanding of neuroscience in the last few decades, the precise pathophysiological mechanisms of epileptogenesis are still not known (Guerrini et al., 2003). Neuronal cell damage, as a response to initial insult, has received considerable attention as the primary aetiological factor that may result in epilepsy. However, the actual role of cell damage in epileptogenesis is still a matter of debate. It can be either a cause or a consequence of seizures or both, supported by the fact that surgical resection of the damaged hippocampus can alleviate the symptoms of temporal lobe epilepsy (Shin and McNamara., 1994).

Initial brain injury results in a cascade of physiological, structural, and biochemical changes, including disturbance of the blood-brain barrier, alteration in brain blood flow, raised intracranial pressure, cerebral haemorrhage, inflammation and necrosis (Willmore et al., 2009). In traumatic epilepsy, for instance, it has been proposed that following intracerebral haemorrhage there is a localised breakdown of red blood cells and release of haemoglobin (Hb) and iron ions (Fe^{2+} and Fe^{3+}) into brain parenchyma, which results result in generation of reactive oxygen species (ROS) (Willmore et al 2009). Increased ROS production, consequently, stimulates a series of pathological changes that can lead to increased neuronal hyperexcitability or to neuronal cell death (Gupta et al., 2006). Recently, a great deal of attention has been paid to the effect of oxidative stress on neuronal cell death as a common pathogenic process that can contribute to epileptogenesis and to the development and progression of spontaneous recurrent seizures (Chuang et al., 2010).

Additionally, many convulsant chemicals and methods had been used as an initial precipitating injury in many animal models of epileptogenesis, through administration of convulsant chemicals such as pilocarpine or kainic acid (Ben-Aft et al., 1980; Cavalheiro et al., 1982; Turski et al., 1983) or by sustained electrical stimulation of the hippocampus and other limbic regions (McIntyre et al., 1982; Brandt et al., 2003). Neuronal damage secondary to SE is the result of neuronal hyper-excitability that is characterised by an increased frequency of discharges leading to depolarization of membrane potential, an excessive generation of free radicals due to high metabolic rate, and a considerable increase in extracellular glutamate (Sherwin et al., 1999). Together, these variables lead to an increase in intracellular Ca2+ concentration that can precipitate neuronal cell damage and death. Multiple experimental studies have shown an association between SE, neuronal cell damage and an increase in extracellular glutamate (Pena and Tapia, 1999; Ueda et al., 2002). This is supported by animal model studies in which selective inhibition of glutamate receptors during SE is neuroprotective (Ebert et al., 2002). Likewise, Ca^{2+} concentrations remain elevated during the chronic epilepsy phase, contributing to the sporadic generation of recurrent seizures. High Ca^{2+} concentrations also lead to alterations in the functionality of inhibitory GABAA receptors, changes in gene transcription and protein expression, and neurogenesis (Morris et al., 1999; Blair et al., 2004). Thus, glutamate excitotoxicity and increased Ca^{2+} level, plus other pathophysiological effects, play a significant role in development of spontaneous recurrent seizures.

The chronic phase – recurrent epilepsy: As shown by epidemiological studies, several types of brain insult can lead to epileptogenesis (Herman. 2002). In chronic phase, which is the recurrent seizures phase, there is a failure of repair enzymes such as complex I, aconitase, and reduced / oxidised glutathione (GSH/GSSG) redox status

which may further exacerbate oxidative damage by continuous O_2^- and H_2O_2 liberation during the chronic phase of epileptogenesis (Rowley and Patel. 2013). Subsequently these molecular changes might contribute to the cellular damage and chronic epilepsy (figure 1.7).

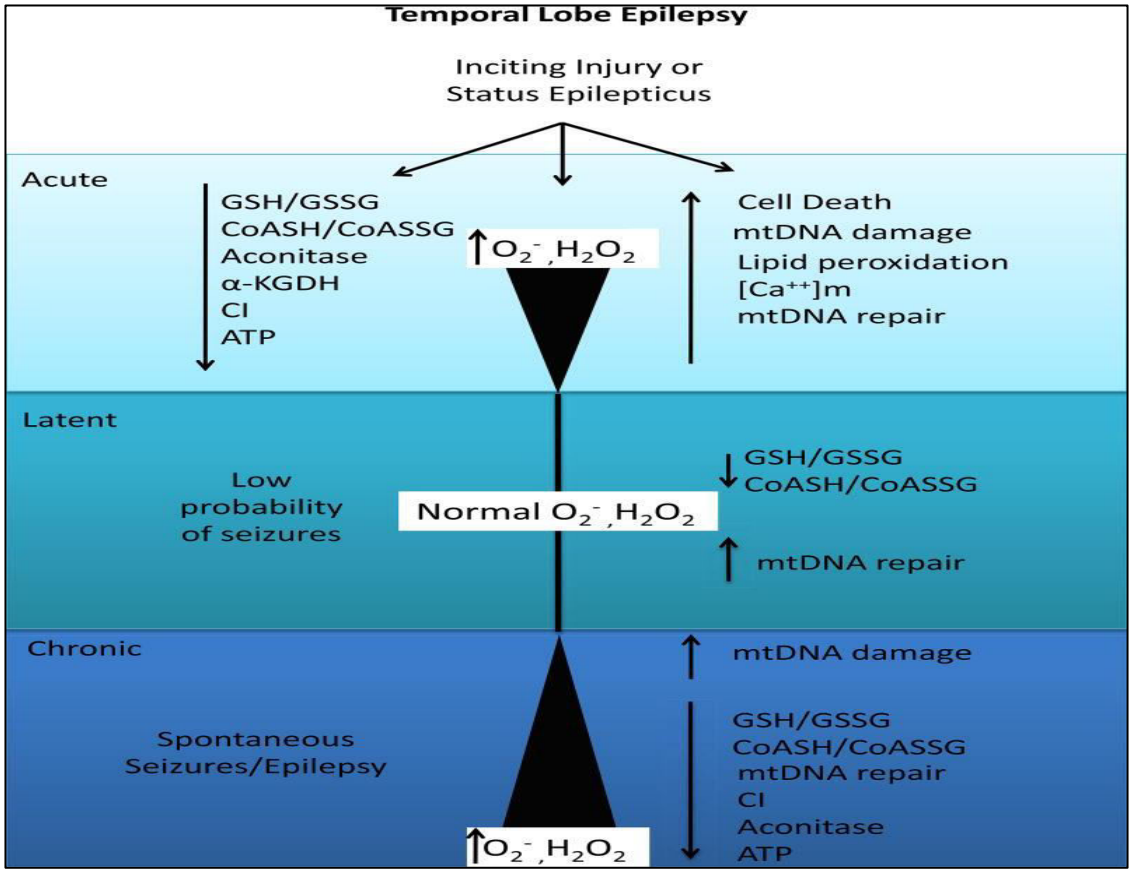


Figure 1.7: Mitochondrial oxidative stress during the acute, latent and chronic phases of epileptogenesis. During the acute phase, shortly following an inciting injury increased production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals via Fenton chemistry and oxidative damage to mitochondrial targets and altered redox status occur. ROS production returns to control values during the latent period during which seizure probability is low; however mitochondrial and cellular redox environment remains persistently altered. ROS production and oxidative damage returns during the chronic phase accompanied by spontaneous recurrent seizures. Failure of repair enzymes may further exacerbate oxidative damage during the chronic phase of epileptogenesis. GSH=glutathione; GSSG=glutathione disulfide; α-KGDH= α-ketoglutarate dehydrogenase; CI= complex I; ATP= adenosine triphosphate; mtDNA= mitochondrial DNA; [Ca++]m= mitochondrial calcium concentration; ROS=reactive oxygen species (adopted from Rowley and Patel. 2013).

1.6 The role of mitochondrial dysfunction in epilepsy

1.6.1 Mitochondrial structure

Mitochondria are subcellular organelles with inner and outer membranes, separated by the inter-membranous matrix, that are responsible for many important functions in almost all eukaryotic cells (Koehler et al., 2004). The outer membrane pores are permeable to specific small molecules. However, large molecular weight proteins require a specific signalling sequence to be transported across this membrane (Benz et al., 1994). The inner membrane contains ion channels and transporter systems and is highly selective for solutes and ions. It has a high protein to phospholipid ratio and is extensively folded, which increases its surface area. Furthermore, the inner membrane hosts enzymes responsible for ATP synthesis and the electron transport chain (ETC) and generates the mitochondrial membrane potential (MMP). Mitochondrial DNA (mtDNA) is a highly specific, unique and independent genome which is inherited maternally (Leonard & Schapira, 2000). However, most mitochondrial proteins are encoded by nuclear DNA (nDNA) (DiMauro & Schon., 2003). Due to its hypermobility, mtDNA can be directed on the basis of metabolic demands (Saxton and Hallenbeck. 2005), which increase with epileptic seizures.

1.6.2 Mitochondrial function in CNS

In the CNS, the mitochondrion is the major subcellular organelle of neuronal cells. Its primary function is to provide cells with energy via the metabolic processes of the electron transport chain (ETC) and tricarboxylic acid (TCA) cycle. Mitochondria have multiple functions; primarily they provide cells with energy by generation of adenosine triphosphate (ATP) via the process of oxidative phosphorylation (OXPHOS) through the synchronous action of five enzyme complexes (I–V) found in the inner membrane of

mitochondria. Mitochondria are also involved in; (i) the liberation of ROS which play important role in cellular signalling, (ii) maintenance of pre- and post-synaptic calcium concentrations which play an important role in the turnover of free radicals, (iii) regulation of programmed cell death through pro-apoptotic factors, and (iv) synthesis of metabolites, lipids and amino acids involved in neurotransmission and oxidation of fatty acids (Schapira, 2002). The generation of ATP by OXPHOS starts by passing of electrons from Reduced nicotinamide adenine dinucleotide (NADH) and Reduced flavin adenine dinucleotide ($FADH_2$)to complex I and II respectively, then subsequently to complex III and IV via cytochrome C and coenzyme Q10, and then to molecular oxygen to produce water. Complexes I to IV act as proton pumps, producing an electrochemical gradient across the inner membrane of mitochondria that is exploited by complex V to generate ATP, ADP and inorganic phosphate (Enrique and Kelvin, 2000). The majority of ATP generated by the mitochondria in excitable cells is used to drive Na^+/K^+ ATPase which maintains the membrane potential and prevents hyperexcitability. The ETC phosphorylates ADP and produces ATP by the expeditious transport of electrons. However, leakage of electrons from this process can react with molecular oxygen to form superoxide radicals ($O_2^{\cdot-}$), predominantly at complex I and III (Muller et al., 2004). Furthermore, the action of monoamine oxidase on the outer membrane is associated with reduction of two electrons of oxygen (O_2) to form hydrogen peroxide (H_2O_2) (Hauptmann et al., 1996) (figure 1.8). The steady state level of superoxide ion ($O^{2\cdot-}$) in the mitochondria relies on the rate of ($O^{2\cdot-}$) generation by the ETC and the level of mitochondrial antioxidant manganese superoxide dismutase (MnSOD) enzyme. Intrinsically, mitochondria are the main source of intracellular reactive oxygen species (ROS) with estimates ranging from 0.1 to 4% of total oxygen utilization dedicated to $O^{2\cdot-}$ production (Boveris, 1984).

All of the mitochondrial functions discussed above are crucial for the maintenance of normal brain activity, and any dysfunction in one or more of these could potentially result in neuronal hyperexcitability and epileptogenesis.

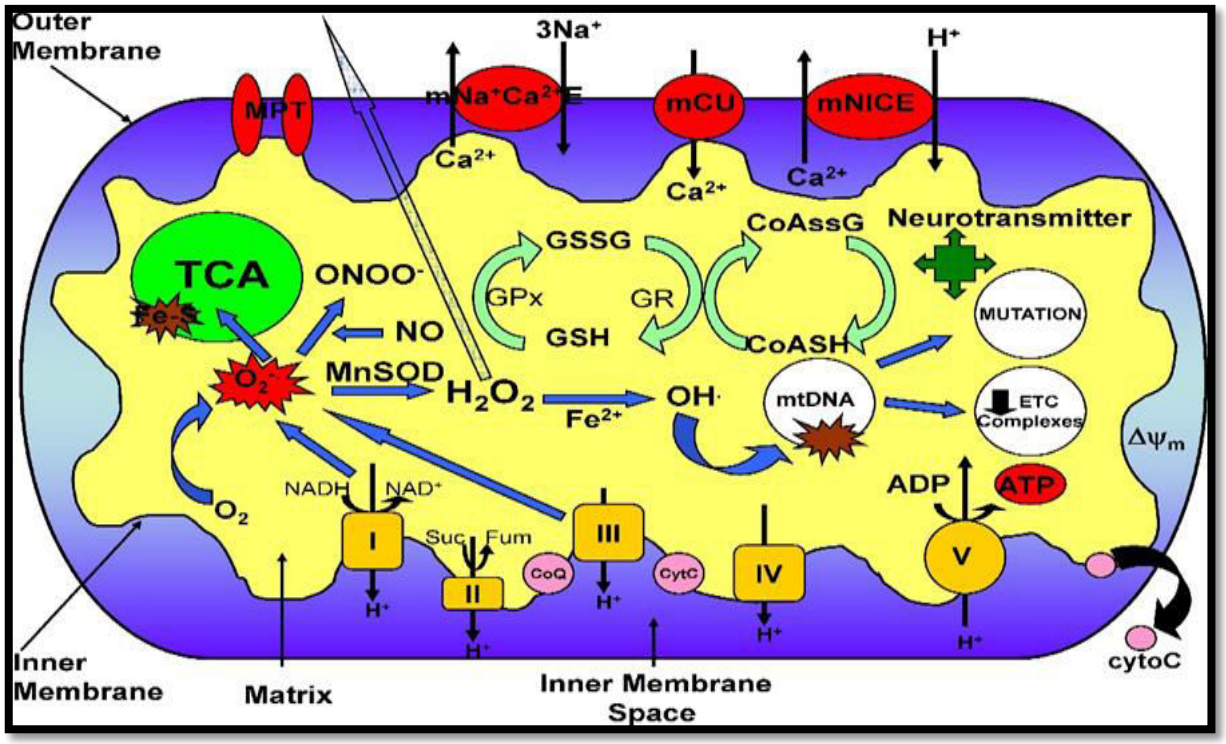


Figure 1.8: Mitochondrial function and neuronal excitability. Superoxide anion ($O_2^{\bullet-}$) production by complexes I and III of the ETC leads to the production of ONOO− in a reaction with NO, and H_2O_2 through dismutation by the antioxidant MnSOD. H_2O_2 is membrane permeable and able to diffuse out of the mitochondria causing widespread oxidative damage. Excessive $O_2^{\bullet-}$ production also damages Fe–S containing enzymes involved in the TCA cycle, such as aconitase. Hydroxyl radicals ($OH^{\bullet}$) can be formed from H_2O_2 through Fenton chemistry and lead to further oxidative damage of macromolecules such as ETC complexes and mtDNA. Oxidative damage to mtDNA can lead to increased mutation rates and a decrease in ETC subunit expression encoded by the mitochondrial genome. Alterations in the redox status of GSH/GSSG and CoASH/CoASSG can cause an inability to protect against the deleterious effects of ROS. Modification of neurotransmitter biosynthesis within the mitochondria can affect levels of neuronal excitability/inhibition. Oxidative damage to these targets can result in increased neuronal excitability resulting from decreased mitochondrial membrane potential and ATP levels affecting the Na^+/K^+ ATPase and the release of cyto C leading to apoptosis. $mNa^+Ca^{2+}E$ = mitochondrial sodium calcium exchanger; mCU = mitochondrial calcium uniporter; mNICE = mitochondrial sodium independent calcium exchanger; MPTP = mitochondrial permeability transition pore; GSH = glutathione; GSSG = glutathione disulfide; CoASH = coenzyme A; CoASSG = coenzyme A glutathione disulfide; GR = glutathione reductase; GPx = glutathione peroxidase; cyto C = cytochrome C; ψm = mitochondrial membrane potential (adopted from Waldbaum and Patel, 2010)

1.6.3 Mitochondrial dysfunction

Primary mitochondrial dysfunction: In general terms, primary mitochondrial disorders are those that are caused by genetic variations, affecting the structural composition and function of the ETC. These disorders disrupt mitochondrial OXPHOS, with the phosphorylation of ADP to ATP significantly affected. The ETC is a unique metabolic pathway and the only pathway under the control of both nuclear (nDNA) and mitochondrial (mtDNA) genomes. As such, mitochondrial disorders can be transmitted via mitochondrial genetics or Mendelian genetics, with the former affected by critical considerations such as mitotic segregation, heteroplasmy, maternal inheritance, and threshold effect (Dimauro and Davidzon. 2005).

nDNA-based primary mitochondrial diseases are complex and mostly inherited in an autosomal recessive pattern, although x-linked and dominant inheritance patterns have also been described (Carrozo et al., 1998). Some primary mitochondrial disorders, such as Leigh syndrome, are characterised by multigenic mutations, while others including Alpers-Huttenlocher syndrome (POLG) and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) are linked to mtDNA depletion and deletions.

Nuclear gene mutations are increasingly recognised as the main cause of paediatric mitochondrial disorders (Dimauro and Davidzon. 2005), largely because of the predominance of mitochondrial proteins that are synthesized by nDNA (~850 genes) in comparison to mtDNA (~13 genes) (Cotter et al., 2004).

Nuclear genes involved in mitochondrial disease encode proteins which are structural subunits of mitochondrial enzyme cofactors, complexes, translation factors, assembly factors, mtDNA maintenance factors, and factors that are critical for the cleavage and incorporation of this essential organelle (Alston et al., 2017). However, the causative

genetic defect remains to be identified in most patients with suspected mitochondrial disease. For instance, the genetic basis of complex I defect, which is the largest and the most significant ETC disorder, and coenzyme Q10 deficiency are still ambiguous in 50% of patients (Quinzii et al., 2006).

Primary mitochondrial dysfunction arising from mtDNA is purely maternally inherited. This inheritance follows the threshold-effect which reflects the attribution of normal to mutant mtDNA in the cells where mitochondrial dysfunction occurs (Vu et al., 2002). For example, Leber hereditary optic neuropathy and neuropathy, ataxia and retinitis pigmentosa (NARP) are caused by mtDNA mutations in cells of the nervous system and retina, whereas myoclonic epilepsy with ragged-red fibres (MERRF) results from mutations in mitochondrial tRNA.

All of the mitochondrial-related diseases discussed above are associated with mutations in mtDNA that occur prior to conception, are hereditary, and result in primary mitochondrial dysfunction. If these mutations occur after conception, they are considered acquired, for instance due to environmental factors or oxidative stress, and result in secondary mitochondrial dysfunction (Niyazov et al., 2016).

Secondary mitochondrial dysfunction: Secondary mitochondrial dysfunction includes any abnormal mitochondrial function other than those considered primary. It does not include inborn genetic disorders which control OXPHOS and which usually happen after conception (Niyazov et al., 2016). Instead, secondary mitochondrial dysfunction results from the influence of external mechanisms such as environmental or pharmacological toxins that can damage the mtDNA (DiMauro et al., 2008).

The effects of pathological processes on the mitochondrial function, as shown in many acquired or inherited disorders (i.e. non-primary mitochondrial dysfunction disorders),

can weaken the capability of the mitochondrion to produce ATP and attenuate mitochondrial dynamics such as fission and fusion that are highly involved in mitochondrial disorders (Juan et al., 2017),. Mechanisms underlying mitochondria-related disease states have predominantly focused on nDNA, mtDNA damage, and ROS generation (Pieczenik and Neustadt 2007).

The brain is commonly involved in mitochondrial diseases because of its high energy demand (Rahman, 2012). Mitochondria in brain are responsible for supplying ATP for neurotransmission, regulation of pre- and post-synaptic calcium (Ca^{2+}) concentrations, and ROS signalling (Wei et al., 2001). The pathophysiological mechanisms that underlie mitochondrial-induced injury are primarily driven by ROS produced by mitochondria themselves (Wei et al., 2001).

1.6.4 Mitochondrial oxidative stress and epileptogenesis

Oxidative stress arises from an imbalance between pro-oxidants and anti-oxidants, in favour of the former. Free radicals (either ROS or RNS) are a group of atoms/molecules that have one or more unpaired electron in their outer orbital, giving them a very strong ability to interact with other biomolecules by accepting or donating an outer shield electron to saturate their orbital (Halliwell and Gutteridge, 2007). ROS are products of normal, aerobic mitochondrial respiration, constantly produced as unpaired electrons are released from complexes I and III of the mitochondrial ETC (Muller et al., 2004).

It is estimated that nearly up to 2% of all oxygen molecules used by cells is converted to ROS, mainly superoxide anion ($O_2^{\bullet-}$) which is the primary ROS generated by ETC complex I and III (Boveris and Chance., 1973). $O_2^{\bullet-}$ is converted into H_2O_2 by spontaneous dismutation via superoxide dismutase (SOD). Consequently, H_2O_2 is broken down into H_2O and O_2 by glutathione peroxidase and catalase. If this step does

not happen, either due to lack of antioxidant enzymes or excessive liberation of H_2O_2, the Fenton reaction can occur via the interaction of H_2O_2 with divalent cations, such as iron (Fe^{2+}) and copper (Cu^{2+}) to release a highly reactive hydroxyl radical ($OH^{\bullet -}$) which readily oxidizes proteins, lipids and DNA (Figure 1.9).

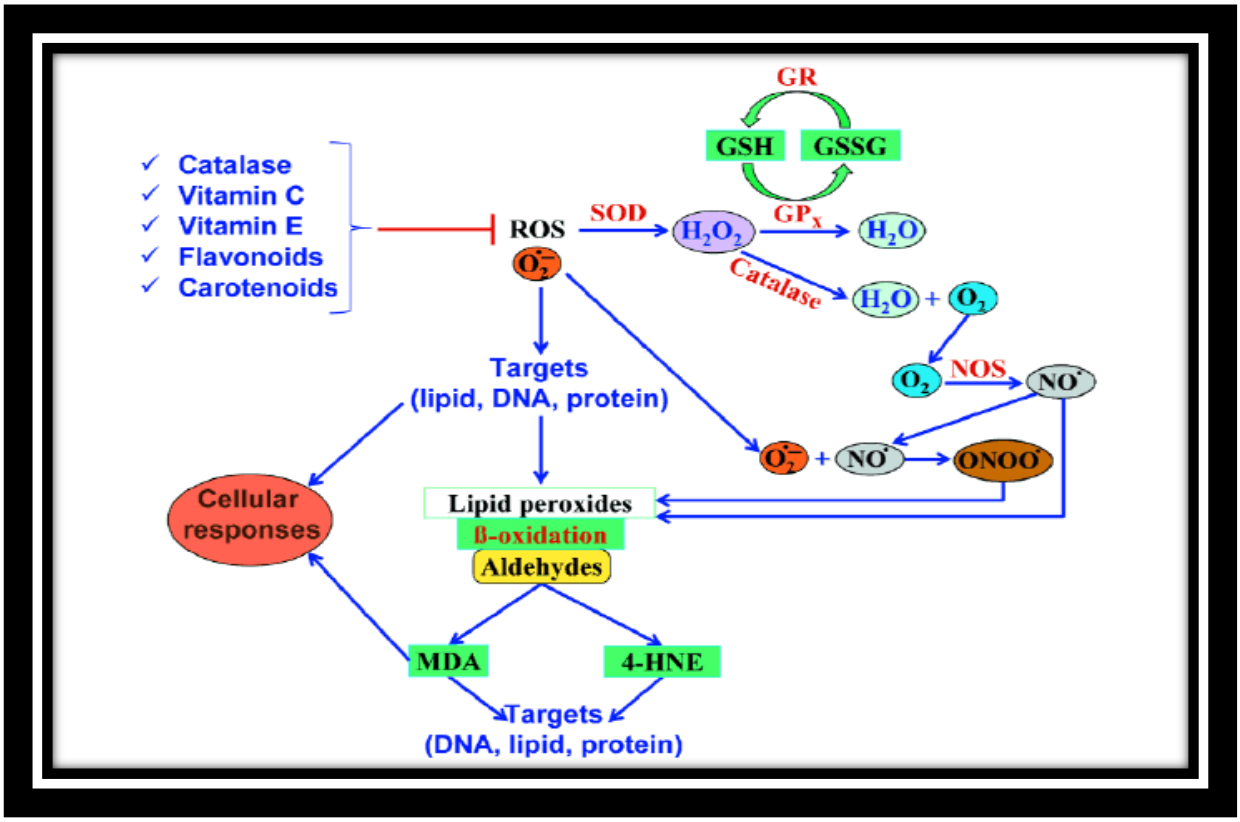


Figure 1.9: Oxidative stress mechanism. Superoxide radical anion ($O_2^{\bullet -}$) reacts with cellular lipids, leading to the formation of lipid peroxides that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Other cellular molecules, such as DNA and proteins, may also may be modified by $O_2^{\bullet -}$, however peroxidation of lipids is more intense because it has chain reaction properties. Nitric oxide synthase (NOS) produces nitric oxide (NO), which reacts with $O_2^{\bullet -}$ to produce peroxynitrite ($ONOOO^{\bullet}$). $ONOOO^{\bullet}$ also participates in formation of lipid peroxides. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion $O_2^{\bullet -}$ into less noxious hydrogen peroxide (H_2O_2), which is further degraded by glutathione peroxidase (GPx) and cooperating glutathione reductase (GR). The reduction of H_2O_2 into water by GPx is accompanied by the conversion of glutathione from its reduced form (GSH) into its oxidized form (GSSG). GR restores the pool of GSH by reduction of GSSG, by means of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Another enzyme - catalase - also participates in H_2O_2 neutralization, releasing oxygen (O_2). Antioxidants, such as vitamins C and E, flavonoids and carotenoids, counteract reactive oxygen species (ROS) generation. (adopted from Kwiecien et al., 2013).

ROS are temporary, unstable and highly reactive to cellular components, making their direct quantification very difficult. Instead of this, the measurement of ROS has been done through the estimation of indirect markers of oxidative damage such as malondialdehyde (MDA) which is the by-product of lipid peroxidation, free radical antioxidant enzymes activity (i.e. SOD, catalase), glutathione (reduced/oxidized ratio), and transcription factor Nrf2 and its related genes (Patel., 2004).

Recently, mitochondrial dysfunction (primary and secondary) in CNS has gained substantial attention as a possible cause of epileptic seizures and has been described in humans (Kunz et al., 2000; Lee et al., 2008) and in many experimental models (Chuang et al., 2004; Sleven et al., 2006). It is proposed that oxidative stress influences a number of metabolic processes might result in neuronal hyper-excitability and potentially be an source of active epilepsy. Likewise, several mechanisms have been suggested to contribute to the latent period of epileptogenesis, including derangement in mitochondrial redox status resulting in irreversible oxidation, mtDNA destruction, mitochondrial respiration failure, and a drop in ATP production. In turn, decreased ATP production leads to a decrease ATP-dependent Na^+/K^+ ATPase activity and in the expression of glutamate transporters and glutamate synthetase and may be an additional factor linked to increased neuronal excitability (Fighera et al., 2006).

1.6.5 Oxidative damage to cellular macromolecules

Most of the data that supports the influence of mitochondrial oxidative stress in the epilepsies has come from studies of animal models in which recurrent seizures are induced by exposure to chemoconvulsant agents, such as kainic acid and pilocarpine, or via the kindling method in which repeated electrical stimulation of the limbic system gives rise to spontaneous seizures. Both approaches result in an increase in ROS production in the brain, while the hippocampal slice culture model has also been shown

to be suitable for assessing macromolecular (i.e. protein, lipid and nuclear) changes associated with oxidative stress (Sashindranath et al., 2011).

Protein oxidation: Oxidative damage can alter the structure, function, and activity of structural proteins and key enzymes (Stadtman et al., 2001). The brain is highly susceptible to oxidative damage due to its large quantity of mitochondria, high oxygen consumption, lack of some antioxidant enzymes, poor repair mechanisms, and richness in polyunsaturated fatty acids (Halliwell et al., 1992).

Mitochondrial complex I (CI), alpha-ketoglutarate dehydrogenase (α-KGDH), and aconitase are the most common proteins that are affected, either by post-translational modifications or inhibition by ROS during the epileptogenic period (Rowley and Patel. 2013). Mitochondrial aconitase is a TCA cycle enzyme that catalyses the interconversion of citrate to isocitrate via cis-aconitate during the second stage of the TCA cycle. It contains an iron-sulphur (Fe-S) cluster which facilitates the elimination of the hydroxyl (OH) group (Dupuy et al., 2006) and is highly liable to oxidation by $O^{\bullet-}$ and other related free radicals. Quantification of endogenous aconitase activity can indicate both the $O^{\bullet-}$ level and the extent of protein oxidative damage (Gardner et al., 1997). A consequence of oxidative aconitase inhibition is the release of iron and formation of H_2O^2 and consequently $OH^{\bullet-}$, leading to further oxidative damage. This vicious cycle can be stopped with iron-chelating agents that provide neuroprotection in the kainate experimental model (Jarrett et al., 2008).

CI plays an important role in the initiation of the electron cascade in the mitochondrial ETC. A recent study showed an oxidative stress-induced post-translational modification, mediated by carbonylation, of CI in experimental TLE (Ryan et al., 2012). Mass spectrophotometric analysis recognised a specific type of carbonylation called metal-catalysed oxidation (MCO) that is typically produced by oxidative stress in the

presence of highly abundant metals such as iron. Involvement of this process in kainate-induced TLE models is supported by previous reports that demonstrated an increase in the Fenton reaction, H_2O_2 and iron, and the neuroprotective effects of HBED, which is an iron chelator (Jarrett et al., 2008). Other authors have shown that CI is both a target and source of $O^{\bullet-}$, leading to further mitochondrial damage and further production of ROS (Kudin et al., 2002; Murphy et al., 2009).

Membrane proteins are also susceptible to oxidative damage and to changes in local ATP concentrations. Several such proteins, including glutamine synthetase, glutamate transporters and Na^+/K^+ ATPase, are also known to be associated with neuronal hyperexcitability (Rowley and Patel, 2013). GLT-1 and GLAST (glutamate transporters) are regulated by extracellular ATP in the hippocampus, are involved in maintaining extracellular glutamate concentrations (Perego et al., 2000), and oxidative stress can impair their transport activity (Trotti et al., 1998). Decreased GLT-1 and GLAST expression coincides with mitochondrial oxidative stress and increased susceptibility to experimental seizures (Liang and Patel, 2004) and has also been demonstrated in human epilepsy patients (Meldrum et al., 1999).

Lipid peroxidation: Lipids are a well-known target for oxidative damage during epileptogenesis as a consequence of the initial insult. Lipid peroxidation is initiated by abstraction of hydrogen from polyunsaturated fatty acids (PUFA) by $OH^{\bullet-}$ to form PUFA radicals, which are highly unstable and react eagerly with molecular oxygen to create peroxyl-PUFA radicals. These radicals are also unstable and react strongly with free PUFA to generate more PUFA radicals and lipid peroxides in chain reaction. Termination of this chain reaction can be effected by endogenous antioxidants, such as vitamin E, SOD, and peroxidase (figure 1.8) (Madhura et al., 2015). Malondialdehyde (MDA), 4-hydroxy-2-(E)-nonenal (4-HNE), F2-isoprostanes (IsoPs) and isofurans

(isoPs) are by-products of PUFA peroxidation and major indicators of lipid peroxidation. MDA is a ubiquitous compound produced in considerable quantity in mammalian cells as a result of PUFA peroxidation and also prostaglandin and thromboxane biosynthesis (Madhura et al., 2015). It can react with structural proteins to form a variety of adducts, and with DNA to form mutagenic compounds. Furthermore, MDA itself has been reported to be toxic and carcinogenic (Hemminki et al., 1994). In experimental epilepsy models involving the administration of chemoconvulsants, MDA acts as a marker of ongoing lipid peroxidation in the cerebral cortex, hippocampus and other brain regions within 4 hours of an initial insult, and for up to 24 hours thereafter (Waldbaum and Patel, 2010). This suggests that lipid peroxidation occurs as a result of seizures and that it may also be involved in the early stages of epileptogenesis.

Oxidative mtDNA damage: mtDNA is highly susceptible to oxidative damage for two reasons. First, mtDNA does not have histones which play an important role in chromatin regulation and in keeping DNA strands compact. Second, it lies close to the inner mitochondrial membrane where the ROS are liberated (Rowley and Patel, 2013). It has been shown that increased ROS generation can result in mtDNA damage and potentially a decrease in the efficiency of the ETC (Esposito et al., 1999). Several studies have focused on oxidative damage to mtDNA following experimental seizures. One month after pilocarpine treatment, there is a significant decrease in mtDNA copy number in CA1 and CA3 regions of the hippocampus (Kudin et al., 2002). There is also an increase in mtDNA damage after kainate administration, with a corresponding increase in mitochondrial 8- hydroxyl-2-deoxyguanosine (mt-8-OHdG), a by-product of DNA oxidation, relative to a total 2-deoxuguanosine (2-dG) (Jarrett et al., 2008). Additionally, endogenous DNA repair systems are activated by kainate-induced

seizures and epileptogenesis, which suggests a constant adaptive restoration and response to oxidative insult (Rowley and Patel, 2013).

1.7 Seizure-induced changes in endogenous antioxidant defence and redox status

1.7.1 Seizure-induced changes in antioxidant mechanism

The endogenous antioxidant defence is a complex integrated system that protects cells against the destructive and deleterious pro-oxidant effects of ROS. When this physiological antioxidant system fails to get rid of ROS efficiently, cellular oxidative damage occurs and accumulates, especially in the mitochondria (James and Murphy, 2002).

SOD is an endogenous antioxidant enzyme that has been shown to neutralize $O^{\bullet-}$ and protect cells against programmed death (Greenlund et al., 1995). SOD is found in three forms; SOD1 (CuZnSOD) localized in cytoplasm, SOD2 (MnSOD) localized in mitochondria, and SOD3 (ECSOD), localized in the extracellular matrix. Under normal physiological conditions, $O^{\bullet-}$ undergoes dismutation by SOD to H_2O_2 which is further broken down to O_2 and H_2O by CAT and glutathione peroxidase (GPx), with GSH as a co-factor. However, if this process is not controlled adequately, excessive $O^{\bullet-}$ release can result in the formation of more deleterious oxidants, such as $OH^{\bullet-}$, which can cause further oxidative destruction and cell death (Waldbaum and Patel, 2011).

Seizures are associated with a change in the antioxidant defence mechanism. However, it is still controversial if the endogenous antioxidant level is increased or decreased. In the pilocarpine model, SOD activity in the hippocampus has been shown to decrease at 24 hours and during the chronic phase of epilepsy, whereas the activity of GPx is increased (Bellissimo et al., 2001). However, it has also been reported that the activities of CAT, GPx and SOD were increased at two hours after pilocarpine induction of status

epilepticus (Tejada et al., 2007). Other authors demonstrated that hippocampal CAT activity, but not that of SOD, was reduced at 24 hours after pilocarpine, proposing that the hippocampus does not use SOD as a key antioxidant but more likely CAT and/or GSH instead (Freitas et al., 2005). Similar findings have been reported by Erakovic et al (2000), who showed a decrease in total brain antioxidant levels 48 hours after acute administration of kainate. In contrast, however, Bruce and Baudry (1995) reported an increase in CAT and SOD activity in both hippocampus and piriform cortex at 48 hours and again at five days after kainate. Considering the wide discrepancies in results obtained from different seizure models at different times, the issue of whether there is endogenous antioxidant compensation in response to seizure activity remains to be settled.

1.7.2 Seizure-induced changes in Nrf2 regulated-redox status

Nrf2 is increasingly recognized as an important redox-sensitive transcription factor that regulates anti-oxidant protection and cellular integrity in many tissues (Baird, 2011). All biological cells have an intrinsic ability to combat ROS, which is dynamically under the control of Nrf2, the master regulator of the phase II cellular antioxidant response (Nguyen et al., 2003).

Recently, it has been shown that Nrf2 could have an anti-inflammatory action, improve mitochondrial function, enhance xenobiotic detoxification, and influence autophagy. Therefore, modulating Nrf2 might be expected to be helpful in the prevention and treatment of dozens of common chronic disorders such as neurodegenerative diseases, chronic lung diseases, chronic renal diseases, multiple sclerosis, type 2 diabetes, and epilepsy (Martin et al., 2015).

Physiologically, Nrf2 is rendered inactive in the cytoplasm through binding to Kelch-like ECH associated protein 1 (Keap1) (Howden et al., 2013). Any oxidative stress induced elevation in ROS oxidizes cysteine residues within the Keap1 active site, preventing Nrf2 attachment. This encourages cytoplasmic accumulation of Nrf2, which then translocates to the nucleus where it first complexes with musculoaponeurotic fibrosarcoma (Maf) proteins and then binds to antioxidant response elements (AREs) in the promoter region of selected genes. ARE activation results in transcription of Nrf2-related genes, such as those encoding NADPH dehydrogenase (NQO-1), haem oxygenase 1 (HO-1), glutareduxin-1 (Grx-1), and thioredoxin-1 (Trx-1) (Kasper et al., 2009) (Figure 1.9). Enzymes responsible for ROS neutralization, such as SOD and CAT, and those involved in GSH biosynthesis and regeneration are also under transcriptional control by Nrf2. GSH is the most important non-enzymatic intracellular defence against ROS in most organs including the brain and alongside glutathione disulfide (GSSG), can also be considered one of the most important markers oxidative stress in biological systems (Patel et al., 1995). Glutathione peroxidase (Gpx) catalyses the degradation of H_2O_2 via oxidation of GSH to GSSG, with GSSG then recycled to GSH via the action of glutathione reductase (GR) in the presence of NADPH (Patel et al., 1995). In this respect, the activity of GR is crucial to maintaining GSH levels and keeping GSSG at low concentrations.

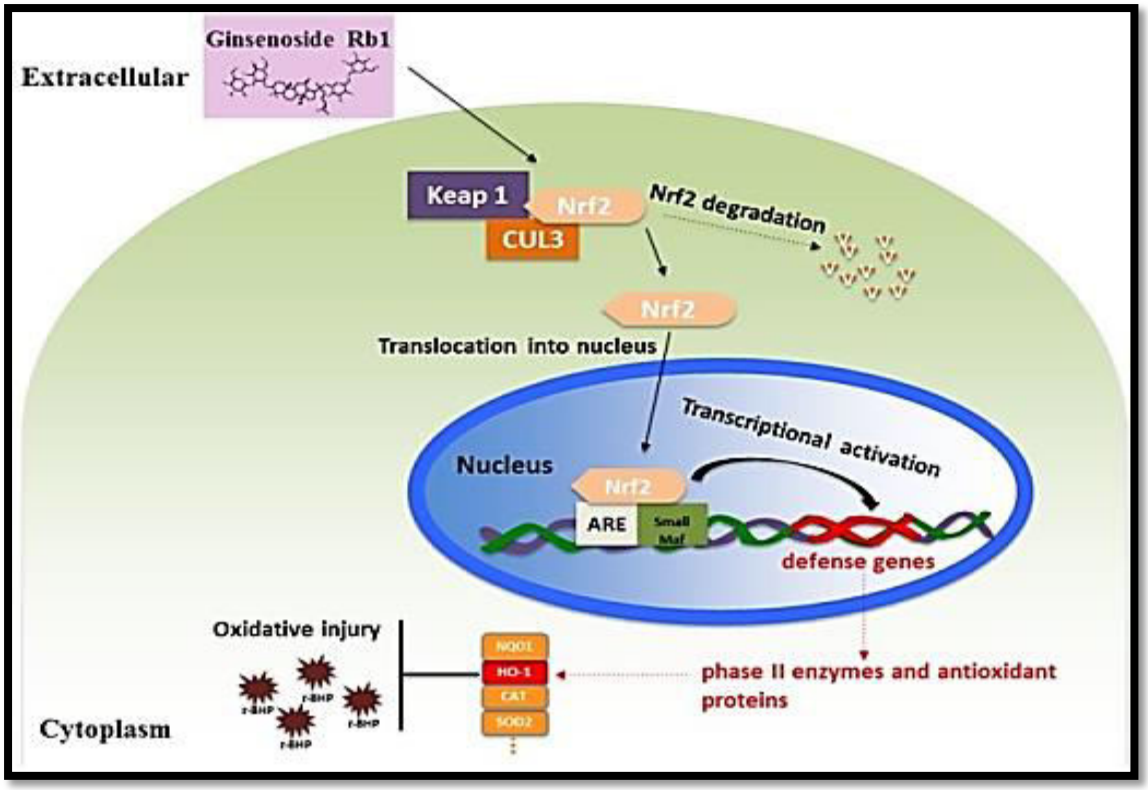


Figure 1.10: The mechanism underlying the response of the Nrf2-Keap-1 signalling pathway to oxidative stress. Under homeostatic conditions, Nrf2 signalling is repressed by Kelch-like ECH-associated protein 1 (Keap1). Nrf2 is bound to Keap1 and sequestered in the cytoplasm, where it is susceptible to degradation by ubiquitination. Under stress conditions, Nrf2 is released and translocates into the nucleus, where it initiates a series of anti-oxidant responses. It first forms a complex with a group of small musculoaponeurotic fibrosarcoma (Maf) proteins and then binds to antioxidant response elements (AREs), which are a cis-acting enhancers in the promoter region of selected genes, that leads to the expression of downstream antioxidants genes, such as HO-1 and NQO-1(adapted from Ye et al., 2016),

Recent studies have revealed that the Nrf2-ARE pathway is an important target in protecting brain from oxidative and inflammatory damage induced by ischemic stroke (Shih et al., 2005a; Zhao et al., 2007) and kainate toxicity (Kraft et al., 2006). Seizures are a major cause of oxidative stress and recent evidence suggests that the expression of Nrf2 and some of its downstream target enzymes (i.e. NQO-1 and HO-1) is significantly increased in rat hippocampus after rapid kindling (Wang et al., 2016). This suggests that

activation of the Nrf2-ARE pathway might play an important role at the early stages of epilepsy. Targeting this pathway might be of benefit in treating seizures associated with oxidative stress or in preventing the development of epilepsy following insults that are known to cause oxidative stress (Wang et al., 2016).

1.8. Potential therapeutic approaches to targeting metabolic pathways and ROS in epilepsy

It is evident that oxidative damage and ROS play a role in the pathogenesis and progression of epilepsy, and contribute to seizure-induced neuronal damage (Rowley and Patel, 2013). There are, however, two significant issues associated with therapeutic targeting of mitochondrial metabolic pathways; (1) the ubiquitous role of mitochondrial function in cell signalling, and (2) redundancies within and adaptive control of metabolic agitation. Nevertheless, the efficacy of the KD suggests that targeting mitochondrial oxidative stress may be useful in controlling established seizures, and perhaps also in delaying or preventing epileptogenesis, by switching mitochondrial fuel utilization (Rowley and Patel, 2013).

More than a dozen new AEDs have been licensed in the past three decades but one third of epilepsy patients remain refractory to conventional therapy. The aim of traditional AED treatment has been to decrease neuronal hyperexcitability and thus control the incidence of epileptic seizures (Waldbaum and Patel, 2009). The hyperexcitability associated with seizures causes an excessive Ca^{2+} uptake which has been shown to increase mitochondrial ROS, inhibit ATP production, activate the MPTP, and promote cytochrome C release, resulting in cell death (Sullivan et al., 2005). Several licensed AEDs have selective effects on Ca^{2+} flux, which might directly impact the downstream involvement of mitochondria following seizures, and all AEDs limit neuronal hyperexcitability and therefore potentially reduce the involvement of mitochondria and

alleviate their potential to cause oxidative damage and cell death. However, this remains speculative and the extent to which any current AED can impact on mitochondrial oxidative stress, either directly or indirectly, is currently unclear.

1.8.1 Antiepileptic drugs and oxidative stress

There are currently 25 drugs licensed for the treatment of epilepsy in the UK but these are effective in only 60-70% of patients, irrespective of whether they are used in monotherapy or in combination. Although AED monotherapy is preferred, polytherapy can occasionally be successful where individual drugs have failed but is frequently associated with enhanced risk of neurological side effects. Thus, there is an urgent need for new, better-tolerated AEDs that are effective against refractory epilepsy (Brigo et al., 2012).

In terms of mechanism of action, AEDs essentially restore the balance between neuronal excitation and inhibition. Three major classes of mechanism are recognised: modulation of voltage-gated ion channels; enhancement of gamma-aminobutyric acid (GABA)-mediated inhibitory neurotransmission; and attenuation of glutamate-mediated excitatory neurotransmission. The principal pharmacological targets of currently available AEDs are highlighted in Table 1.1 (Schmidt and Schachter, 2014).

Table.1.1 List of established AEDs, year of introduction, and primary mechanism(s) of action. Data is from Schmidt and Schachter (2014).

AED	Year	Presumed mechanism of action
Potassium bromide	1857	GABA potentiation?
Phenobarbital	1912	GABA potentiation
Phenytoin	1938	Na^+ channel blockade
Primidone	1954	GABA potentiation
Ethosuximide	1958	T-type Ca^{2+} channel blockade
Diazepam	1963	GABA potentiation
Carbamazepine	1964	Na^+ channel blockade
Valproate	1967	Multiple mechanisms: GABA potentiation, glutamate (NMDA) inhibition, Na^+ channel and T-type Ca^{2+} channel blockade
Clonazepam	1968	GABA potentiation
Clobazam	1975	GABA potentiation
Vigabatrin	1989	GABA potentiation
Lamotrigine	1990	Na^+ channel blockade
Oxcarbazepine	1990	Na^+ channel blockade
Gabapentin	1993	Ca^{2+} channel blockade ($\alpha_2\lambda$ subunit)
Topiramate	1995	Multiple mechanisms: GABA potentiation, glutamate (AMPA) inhibition, Na^+ channel and Ca^{2+} channel blockade
Levetiracetam	2000	SV2A modulation
Zonisamide	2000	Na^+ and T-type Ca^{2+} channel blockade
Stiripentol	2002	GABA potentiation
Pregabalin	2004	Ca^{2+} channel blockade ($\alpha_2\lambda$ subunit)
Rufinamide	2004	Na^+ channel blockade
Lacosamide	2008	Na^+ channel blockade (enhanced slow inactivation)
Eslicarbazepine	2009	Na^+ channel blockade (enhanced slow inactivation)
Perampanel	2012	Glutamate (AMPA) inhibition

As discussed above, seizures are associated with alterations in the balance between intracellular pro-oxidants and antioxidants and there is some evidence to suggest that AEDs can, at least in part, further impair the endogenous antioxidant system (Martinc et al., 2012). This would suggest that AED treatment can reduce the ability of neuronal cells to counteract oxidative damage, either by inhibiting specific antioxidants, increasing ROS production, or a combination of the two. However, the precise mechanisms by which this might occur remains debateable and may vary from AED to AED.

1.8.2 Effect of commonly used AEDs on oxidative stress markers in experimental studies

An *in vitro* study of oxidative stress markers in rat primary astrocyte cultures has shown that a broad range of AEDs, including carbamazepine (CBZ), gabapentine (GBP), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), topiramate (TPM) and tiagabine (TGB), can increase intracellular levels of MDA, ROS and nitric oxide, and decrease concentrations of GSH, suggesting that AEDs, as a class, have pro-oxidant effects (Pavone et al., 2003). In addition, VPA has been shown to increase ROS level TBARs, HO-1, and NQO-1 expression in rats (Abdalla et al., 2016) and oxcarbazepine (OXC) reported to cause neuronal swelling and cell damage due to increased ROS production in isolated hippocampal neurons (Araujo et al., 2004).

Turning to *in vivo* models, CBZ has been shown to increase MDA concentrations and decrease GSH levels and SOD activity in the kindled rat model of epilepsy whereas LTG was interestingly without effect (Arora et al., 2009). Treatment of mice with CBZ and phenytoin caused a significant increase in the expression of transcription factors that specifically recognise oxidative stress genes, such as those encoding GSTM1 and heat shock proteins, and which are related to Keap1-Nrf2-ARE signalling pathway (Lu, 2017). Similarly, CBZ, phenytoin, felbamate, and VPA have been shown to elevate Nrf2-related gene expression in rat liver (Leone et al., 2007).

1.8.3 Effect of commonly used AEDs on oxidative stress markers in clinical studies

Most of the studies of oxidative stress in patients with epilepsy have been performed on peripheral tissues. However, ROS may originate from different sources in the body and peripheral samples might not precisely reflect oxidative insults in the brain.

Nevertheless, there is evidence that experimental seizures induced by direct injection of kainate into rat brain can cause an increase in MDA concentrations both in the CNS and in blood (Singh et al., 1990; Bruce and Baundry, 1995). The reverse has also been shown, with peripheral ROS generation causing oxidative damage in CNS (Aytan et al., 2008).

In terms of drug studies, increased levels of MDA, a by-product of lipid peroxidation, have been demonstrated in the serum of epilepsy patients treated with phenytoin (Liu et al., 1998). Likewise, it has been reported that several AEDs, including phenytoin and topiramate, are able to weaken the OXPHOS process when assessed by respiratory chain complexes (II-IV), enzymatic activity, and mitochondrial ATP synthesis (Benz, 1994).

Valproic acid (VPA) is one of the most widely prescribed AEDs and is primarily used to treat generalized epilepsy but is also effective in focal epilepsy, bipolar disorder, neuropathic pain and in migraine (Rossi, 2013). VPA is a broad spectrum AED and its main mechanism of action is believed to be the potentiation of GABAergic activity. It also has effects on voltage-gated Na^+ channels and on low voltage activated (LVA) Ca^{2+} channels (Johannessen, 2000). VPA has been shown to exacerbate oxidative stress by reducing TAC and enhancing total oxidant status (TOS) (Hamed et al., 2004; Schulpis et al., 2006). Furthermore, Gpx (Niketic et al., 1995; Yuksil et al., 2001), GR (Sobaniec et al., 2006), and serum selenium (Graf et al., 1998), all of which are known endogenous, enzymatic and non-enzymatic antioxidant molecules, have been reported to be decreased in the serum of adults and children treated with VPA. Similarly, increased serum levels of MDA (Yuksil et al., 2000; Schulpis et al., 2006) and 15F-2t-isoP (Michoulas et al., 2006), both of which are by-products of lipid peroxidation, and

8-OHdG (Schulpis et al., 2006), which is a nucleic acid marker, are increased in patients with epilepsy treated with VPA.

CBZ is a commonly used AED, indicated for focal and generalised tonic-clonic seizures. It is a voltage-gated Na^+ channel blocker, which is considered as the most common target among AEDs and is responsible for the upstroke of the action potential in neurons and other excitable cells (Brodie et al., 2011). The effect of CBZ on endogenous pro- and anti-oxidant mechanisms in epilepsy patients is controversial. CBZ has been shown to increase MDA levels in adults and children with epilepsy (Hamed et al., 2004; Aycicek and Iscan, 2007) and also levels of 8-OHdG (Varoglu et al., 2010). Moreover, CBZ decreases TAC (Hamed et al., 2004), GSH in plasma (Ohno et al., 2000), and Gpx and CAT activities in erythrocytes (Niketic et al., 1995). However, there have also been several studies that failed to identify any oxidative effects of CBZ (Hamed et al., 2004) and some that reported an anti-oxidant action (Liu et al., 1998).

LEV is a relatively new, broad spectrum AED which is clinically effective against partial and generalized seizures (Oliveira et al., 2004). The mechanisms of action of LEV remain unclear, although some reports suggest that its cellular effects are unrelated to conventional AED mechanisms of action (Oliveira et al., 2007). Elegant studies have shown that synaptic vesicle protein 2A (SV2A) is the binding site for LEV in the brain (Laxmikant and Deshpande. 2014). SV2 proteins are believed to act as transporters of common constituents of the vesicles, such as Ca^{2+} or ATP. As such, modulation of SV2 proteins could protect against seizures through effects on synaptic release of Ca^{2+} (Deshpande and Delorenzo, 2014). However, there is also evidence that LEV may act as a histone deacetylase inhibitor, which might confer anti-inflammatory and antioxidant properties (Safar et al., 2010). This hypothesis is potentially discredited by a study

which showed an increase in serum levels of 15F-2-isoprostane, a marker of oxidative damage, following initiation of LEV treatment in epilepsy patients, suggesting that it might actually induce oxidative stress (Ozden et al., 2010).

LTG is another of the new generation of AEDs which is predominantly used in the treatment of focal epilepsy seizures, seizure associated with Lennox-Gastaut syndrome, and bipolar affective disorders (Schiller and Krivoy. 2009). It is thought to act by reducing the excitability of individual neurons and inhibiting the release of glutamate, an excitatory neurotransmitter, via inhibition of voltage-sensitive sodium channels (Schiller and Krivoy. 2009).

1.9 Aims

Oxidative stress and oxidative damage to neuronal cells is increasingly recognised as a potential cause and/or consequence of epileptic seizures and may be an attractive target for development of much needed new treatments for epilepsy. However, it is important to first understand the effect of conventional AEDs on endogenous pro- and anti-oxidant systems to better predict the likely outcome of manipulating these systems in an effort to alleviate the hyperexcitability associated with seizures and epilepsy.

As such, this thesis describes a series of experiments that aimed to characterise the effects of the four most commonly prescribed drugs for epilepsy, namely CBZ, VPA, LTG and LEV, in terms of their effects on oxidative stress markers in mammalian cells. Much of the work was performed using the human immortalised neuroblastoma cell line SH-SY5Y, which is a widely used preparation in experimental neuroscience and neuropharmacology. Oxidative stress was induced by a number of approaches, including H_2O_2, pilocarpine and lipopolysaccharide (LPS), and was assessed by conventional biochemical assays for MDA concentration, SOD activity and GSH/GSSG

concentration ratio and also by the expression of Nrf2 and its downstream targets NQO-1 and HO-1 at both mRNA and protein level. A final series of experiments took advantage of local access to an extensive collection of DNA samples from people with epilepsy and explored common variants in the Nrf2 gene and their association with clinical characteristics of epilepsy and its response to AED treatment.

Specific aims relating to individual results chapters were as follows:

Chapter 2: Effect of H_2O_2, lipopolysaccharide, and pilocarpine on oxidative stress markers in SH-SY5Y cell line

1- Investigate the effects of different concentrations of LPS, H_2O_2 and pilocarpine on MDA levels as a marker of lipid peroxidation, on the reduced/oxidized glutathione (GSH/GSSG) ratio as a marker of the antioxidant defence system, and on superoxide dismutase (Cu-Zn-SOD) activity as a marker of antioxidant enzyme activity in SH-SY5Y cell line.

2- Explore the optimum conditions (inducer, concentration, time) for induction of oxidative stress in SH-SY5Y cells to be employed in further investigations.

3- Choose between differentiated and undifferentiated SH-SY5Y cells for use in further investigations.

Chapter 3: Effects of commonly used antiepileptic drugs on markers of oxidative stress in a human neuroblastoma cell line

1- Explore the effects of CBZ, VPA, LEV and LTG on MDA concentration, as a marker of lipid peroxidation, in the SH-SY5Y cell line under basal conditions and following induction of oxidative stress by H_2O_2.

2- Explore the effects of CBZ, VPA, LEV and LTG on SOD activity, as a marker of antioxidant enzyme activity, in the SH-SY5Y cell line under basal conditions and following induction of oxidative stress by H_2O_2.

3- Explore the effects of CBZ, VPA, LEV and LTG on the reduced/oxidized glutathione (GSH/GSSG) ratio, as a marker of the antioxidant defence system, in the SH-SY5Y cell line under basal conditions and following induction of oxidative stress by H_2O_2.

Chapter 4: Effects of oxidative stress inducers and selected AEDs on Nrf2, HO-1 and NQO-1 gene expression in SH-SY5Y cells

1- Investigate the effects of LPS, H_2O_2 and pilocarpine on Nrf2, HO-1, and NQO-1 gene expression as a marker of phase II detoxification antioxidant response in the SH-SY5Y neuroblastoma cell line.

2- Choose optimum conditions (inducer, concentration of inducer, duration of exposure) for induction of oxidative stress in SH-SY5Y cells to be employed in further investigations.

3- Explore the effects of carbamazepine (CBZ), valproic acid (VPA), levetiracetam (LEV), and lamotrigine (LTG) on the expression of Nrf2, HO-1 and NQO-1 genes in the SH-SY5Y cell line.

Chapter 5: Effects of selected AEDs on Nrf2, HO-1, and NQO-1 protein expression in SH-SY5Y cells

The present study aimed to investigate the effects of CBZ, VPA, LTG and LEV on the expression of Nrf2, HO-1 and NQO-1 proteins as a marker of phase II detoxification antioxidant response in the SH-SY5Y cell line.

Chapter 6: Association between *NFE2L2* gene variants and treatment outcome in people with epilepsy

To assess the association between AED treatment outcome (remission on any AED) and genotype of selected SNPs in the *NFE2L2* gene, demographic variables and clinical characteristics in people with epilepsy.

Chapter 2

Effect of H_2O_2, lipopolysaccharide, and pilocarpine on oxidative stress markers in SH-SY5Y cell line

2.1 Introduction

Oxidative stress is a condition under which an imbalance exists between the factors that promote oxidation, such ROS and RNS, and antioxidant defences, including GSH, Nrf2, which is a master regulator of intracellular redox status, antioxidant enzymes such as SOD and catalase (CAT), and dietary antioxidants (Jones, 2006). ROS including superoxide anion O^{-}, hydrogen peroxide H_2O_2, singlet oxygen $O^{\cdot}$, and hydroxyl radical OH^{-} in low or moderate levels have several beneficial physiological functions such as tissue repair, killing pathogens and cancer cells, and wound healing. However, disruption of normal redox balance by excessive generation of ROS or RNS, and/or impairment of enzymatic and non-enzymatic antioxidant defence systems might result in several systemic diseases, such as peptic ulcer, inflammatory bowel disease, pulmonary fibrosis, lung cancer, Parkinson's disease, and Alzheimer dementia, (Park et al., 2009; Bhattacharyya et al., 2014).

The role of oxidative insult in neurological disorders, such as epilepsy is of major interest since cells in the brain are particularly susceptible to damaging effects of ROS and RNS. Moreover, antioxidant protection mechanisms in the CNS, such as CAT, Gpx, vitamin E, and GSH, are relatively poor (Shivakumar et al., 1991). This is important because the brain is rich in mitochondria, is characterised by high aerobic metabolic activity and a high ratio of membrane to cytoplasmic volume, and consumes 20% of total body oxygen despite accounting for only 2% of total body mass (Mahadik et al., 1996). The brain is also rich in iron, and oxidative damage releases iron ions capable of catalysing the Fenton reaction which results in further cellular damage (Halliwell, 1992)

Free radicals can react with the lipid bilayer of the cell membrane leading to peroxidation of polyunsaturated fatty acids and release of toxic aldehyde by-products, such as MDA, which can bind covalently to proteins leading to enzyme inactivation and

disturbances of cellular function (Choi et al., 1993; Patel et al., 2004; Bhattacharyya et al., 2014). ROS may trigger nucleic acid damage; they can break DNA strands and modify nitrogenous bases resulting in cellular dysfunction and/or cell death (Facchinetti et al., 1998). RNA is most liable to oxidative damage, as it is single stranded, less protected by antioxidants, and not protected by hydrogen bonding. RNA damage may result in protein alterations and/or abnormalities in gene expression (Nunomura et al., 2007). Mitochondrial DNA (mtDNA) is also highly vulnerable to oxidative stress because of poor repair mechanisms, a lack of protection by histones, and its proximity to the site of ROS generation. mtDNA disruption leads to mitochondrial damage and disturbed cellular function (Nunomura et al., 2007). Oxidative insults may alter primary, secondary, and tertiary structure of proteins leading to functional impairment after exposure to free radicals (Rowley and Patel, 2013).

Antioxidant mechanisms, such as SOD enzyme and GSH-scavenging reactions, provide a primary defence against ROS, and the activities of these enzymes and detoxifying agents are known to decrease under ROS-induced oxidative stress (Michiels et al., 1994). GSH is a scavenger of free radicals, including OH^- and O^- species, whereas SOD is responsible for the dismutation of two O^- molecules to form H_2O_2 and O_2 (Halliwell and Gutteridge, 2007).

Oxidative stress can be induced in living systems (such as cell cultures) by administration of several chemicals, including H_2O_2 (Coyle et al., 2006), pilocarpine (Santos et al., 2011), and LPS (Noworyta et al., 2013) and as a toxic effect of many drugs, such as paracetamol (Stirnimann et al., 2010), diclofenac (Hickey et al., 2001; Boelsterli, 2003), and cisplatin (Pabla and Dong, 2003).H_2O_2 is one of the most important ROS generated through oxidative stress. It is a by-product of NADPH oxidase, SOD activity, and catecholamine's oxidation that produces highly reactive

hydroxyl radicals, such as OH^-, via the Fenton reaction (Forman et al., 2007). It has been reported that H_2O_2 induced depletion of antioxidant mechanisms in SH-SY5Y cells, which are a cell line widely used as *in vitro* model in neurological disorders (Nirmaladevi et al., 2014). These cells are human in origin, derived from neuroblastoma cell source, and possess several chemical, physiological, and functional properties of neuronal cells (Borland et al., 2008).

LPS is a major constituent glycolipid component of the outer cell wall of Gram-negative bacteria, covering up to 75% of its outer surface (Zha et al., 2007). Intraperitoneal LPS injection induces inflammation and oxidative stress in male Swiss albino mice (Ruiz-Miyazawa et al., 2015). It also induces neurodegeneration through modulation of the intrinsic apoptotic cascade in the cortex and hippocampus of Swiss albino mice, resulting in elevated levels of lipid peroxidation with subsequent depletion in the antioxidant status (Santhana et al., 2015).

Pilocarpine is a muscarinic agonist drug, targeting mainly M_1 receptors (expressed in CNS and exocrine glands), and M_3 receptors (expressed in endocrine and exocrine glands, lungs, pancreas, and CNS). The major medical uses are for dry mouth, particularly in Sjogrens syndrome, after head and neck radiation which causes dry mouth as a side effect, and in chronic open and close angle glaucoma (Katzung et al., 2012). Common side effects are excessive secretions, including salivation, sweating and bronchial mucous secretion, plus bronchospasm and bradycardia (Katzung et al., 2012).

It has been suggested that pilocarpine induced seizures were associated with increased lipid peroxidation and nitrite formation which could be responsible for the GABAergic and glutamatergic receptor changes during SE (Freitas et al., 2004). It has been found that there was an accumulation of free radicals in rat hippocampus after seizure induction by pilocarpine (Tome et al., 2010). This finding suggested that seizures

induced by pilocarpine have a large influence on brain oxidative stress which is closely related to the mechanism of evolution and/or maintenance of epilepsy focus produced by pilocarpine (Tome et al., 2010).

2.1.1 Aims and objectives

The present study was planned to:

1- Investigate the effects of different concentrations of LPS, H_2O_2 and pilocarpine on MDA levels as a marker of lipid peroxidation, on the GSH/GSSG ratio as a marker of the antioxidant defence system, and on Cu-Zn-SOD activity as a marker of antioxidant enzyme activity in SH-SY5Y cell line.

2- Explore the optimum conditions (inducer, concentration, time) for induction of oxidative stress in SH-SY5Y cells to be employed in further investigations.

3- Choose between differentiated and undifferentiated SH-SY5Y cells for use in further investigations.

2.2 Materials and Methods

2.2.1 Chemicals

Dulbecco's modified eagle's medium (DMEM) W/O L-glutamine, trypsin / EDTA solution 0.25%, fetal bovine serum (FBS), L-glutamine, phosphate-buffered saline (PBS), bicinchoninic acid assay (BCA) protein assay kit and penicillin-Streptomycin Solution were purchased from Thermo Fisher Scientific, Loughborough, UK. Premix water soluble tetrazolium 1 (WST-1) cell proliferation assay system was purchased from Abcam, Cambridge, UK. Pilocarpine hydrochloride, lipopolysaccharides (LPs) from *Escherichia coli*, 1X butylated hydroxytoluene (BHT), malondialdehyde tetrabutylammonium salt, oxidised glutathione (GSSG), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), sulfosalicylic acid, metaphosphoric acid, triethanolamine, 2-vinylpyridine, sodium phosphate, diethylenetriamine-penta acetic acid (DEPAC), catalase, xanthine oxidase, bovine Cu/Zn SOD, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), hypoxanthine, radio-immune precipitation assay (RIPA) buffer, sodium dodecyl sulphate (SDS), glutathione reductase from Baker's Yeast, SH-SY5Y cell line from the European Collection of Authenticated Cells Culture (ECACC), retinoic acid (RA), and reduced glutathione (GSH) were purchased from Sigma-Aldrich Co., Dorset, UK.

2.2.2 Cell Culture

SH-SY5Y cells were stored frozen in liquid nitrogen before culturing. Cells were grown as a monolayer in T-75 flasks that contained 15 mL of DMEM supplemented with 1% penicillin/streptomycin (pen/strep), 1% L-glutamine and 10% FBS. Cultured cells were placed in an incubator chamber containing humidified 95% air and 5% CO_2 at 37^{o}C. For passage, 80-90 % confluent cells were washed twice with pre-warmed 15 ml of PBS.

Then, the cells were detached with 5.0 mL trypsin-EDTA, 0.25 % solution. After that, 5 ml of culture medium was added and spinned at 250 x g for 5 minutes. The pellet was re-suspended in 5 mL of culture medium and 10 µl was taken for cell counting by using Countess^{tm} Automated Cell Counter. This procedure was performed by mixing 10µl of cell suspension with 10µl trypan blue dye in 500µl Eppendorf. Thereafter 10µl of mixture was loaded into a Countess Cell chamber slide that was then inserted into the slide inlet on the instrument. After approximately 30 seconds, live, dead, and total cells and viability percentage was displayed in the screen and then the number of cells needed was calculated according to the initial seeding densities in different culture vessels (table 2.1).

Table 2.1 Surface areas, number of cells, and recommended working volumes for different cell culture vessels. Approximate confluent growth is 1.25 X 10^5 cells / cm^2.Initial plating density is 10^4 cells / cm^2.Trypsinization volume is 0.05ml / cm^2. Working volume is 0.2 ml / cm^2.

Cell Culture Vessels	Growth area (cm^2)	Initial No. of cells plated.	Approximate No. of Cells when confluent	Working volume(ml)	Trypsinization (ml)
Multi-well plates					
6-well plates	9.4	9.4 X 10^4	1.175 X 10^6	2	0.5
12-well plates	3.8	3.8 X 10^4	4.75 X10^5	1	0.2
24-well plates	1.88	1.88 X 10^4	2.35 X 10^5	0.5	0.1
96-well plates	0.43	0.4 X 10^4	0.5 X 10 ^{5}	0.1	0.025
Flasks					
T-25	25	2.5 X 10^5	3.1 X 10^6	6	1.5
T-75	75	7.5 X 10^5	9.4 X 10^6	15	5
T-175	175	17.5 X 10^5	2.2 X 10^7	45	10
Dishes					
100mm X 20mm	57	5.7 X 10^5	7 X 10^6	12.5	3

2.2.3 Cells differentiation

Undifferentiated SH-SY5Y cells were grown in T-75 flasks until 80-90 % confluent. Cells were then plated in 12-well plates at a density of $4x10^4$ cells/well and 10µM of all-trans retinoic acid was added to the culture medium. The cells were then incubated for 3-5 days, with the medium replaced every 48 hours, to induce differentiation into a neuronal phenotype (Cheung et al., 2009).

2.2.4 Induction of oxidative stress

SH-SY5Y cells were seeded in 12-well plates at a density of $4x10^4$ cells / well in 1 ml culture medium and placed in an incubator containing humidified 95% air and 5% CO_2 at 37^oC for 2-3 days until 80-90 % confluent. Thereafter, the existing medium was aspirated and the cells treated with H_2O_2 (0, 10, 30, 100, 300 and 1000 µM), pilocarpine (0, 1, 3, 10, 30 and 100 µM) or LPS (0, 1, 3, 10, 30 and 100 µg/ml) in serum free medium (DMEM with 1% pen/strep and 1% L-glutamine, without FBS) for 1, 4 or 24 hours.

2.2.5 Lipid peroxidation assay

SH-SY5Y cell lysates was assayed for lipid peroxidation by a modified thiobarbituric reactive substance (TBARS) method for measuring MDA which forms a colorimetric product after reaction with TBA in acidic media (Devasagayam et al., 2003). The cells were washed twice with PBS after aspiration of growth media and then scraped in 1ml PBS containing 5mM BHT to prevent oxidation. MDA standards were prepared in a range of 1.25 to 10 nM in PBS containing 5mM BHT. A 100 µl aliquot of both MDA standards and unknown samples was mixed with 100 µl of 10% SDS and 250 µl of 70 mM TBA reagent (pH is 3) in a 1.5 ml Eppendorf and incubated at 95^oC for 60 minutes. Then, after cooling on ice, a 200 µl aliquot from both MDA standards and samples was

transferred (in duplicate) to a 96 well-plate, shaken briefly, and then read at 535 nm at room temperature in a microplate reader.

2.2.6 GSH/GSSG assay

Intracellular GSH and GSSG level was measured using a modification of the method of Tietze (Rahman et al., 2007). Confluent cells in 12 well plates were washed twice with 1 ml of pre-warmed PBS and then scraped cells in 1 ml of pre-warmed PBS. After that, cells were centrifuged at 1000 x g for 5 minutes, the supernatant was discarded and the pellet was re-suspended in 500 µl ice-cold extraction buffer (0.1% Triton-X and 0.6% sulfosalicylic acid) in 0.1 M potassium phosphate buffer containing 5mM EDTA (KPE buffer). GSH standards were prepared in a range of 1.65 to 26.4 nM in KPE buffer. A 20 µl aliquot of both standards and unknown samples was transferred (in duplicate) to a 96 well-plate. Then, 100 µl of 5mM DTNB and 0.5 units of glutathione reductase were added to each well. Finally, a 60 µl aliquot of 2.4 mM NADPH was added to each well immediately prior to the measurement of absorbance at 414 nm using microplate reader at 0, 30, 60, 90, and 120 seconds.

For the GSSG assay, 20 µl of the samples and standards were treated with 2µl of 95% 2-vinylpyridine, and mixed rapidly to derivatize GSH. The reaction was allowed to take place in a fume hood for one hour. Then, 6µl of 98% triethanolamine was added to the side of the tube and the solution mixed vigorously. Absorbance was read at room temperature at 414 nm in a microplate reader on one occasion only. The GSH/GSSG ratio was calculated from the equation:

GSH/GSSG ratio = ([GSH nmol/mg] – [GSSG nmol/mg]) / [GSSG nmol/mg].

2.2.7 SOD assay

SOD activity was determined according to method of Peskin & Winterbourn (Peskin et al., 2000). After aspiration of growth medium and washing twice with 1 ml pre-warmed PBS, SH-SY5Y cells were scraped with 1 ml PBS and transferred to 2 ml Eppendorf tubes. After spinning down at 2500 xg at 4^0C for 5 minutes, supernatants were transferred to clean tubes and placed on ice. SOD standards ranging from 0.625 to 10U/ml were then prepared in PBS. A reaction mixture was prepared immediately before use, containing sodium phosphate, 50mM, pH 8.0, 0.1 mM diethylenetriamine-pentaacetic acid (DEPAC), and 0.1 mM hypoxanthine (HOX), 4.5mU/ml xanthine oxidase, 100 µl of catalase (4000U/ml), and 100 µl of water soluble tetrazolium (WST-1). A 5 µl aliquot from both standards and samples was added to a 96-well plate, 200 µl reaction mixture was added to each well, and the absorbance was read immediately at 450 nm at room temperature and again every 5 minutes for 20 minutes.

2.2.8 Protein quantitation

Protein in cell lysates was quantified by BCA Protein Assay Kit (https://www.thermofisher.com/order/catalog/product/23225). In this protocol, the following formula was used to determine the final volume of working reagent (WR) required for the assay: total volume WR required (µl) = (number of standards + number of unknowns) × number of technical replicates × 80. WR was then prepared by combining 50 parts of BCA reagent A with 1 part of BCA reagent B (both reagents provided with the BCA Protein Assay Kit). Thereafter, 10µl of each standard and unknown sample was pipetted in duplicate onto a 96 well plate, 80µl of WR was added to each well, and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered with aluminium foil and incubated at 37°C, 5% CO_2 for 30

minutes. After allowing the plate to cool to room temperature, the absorbance was measured at 595 nm using microplate reader.

2.2.9 Analysis of cell viability using MTT assay

The MTT assay was performed as described previously by Abate and colleagues (Abate et al., 1998). The MTT assay is based on the conversion of the tetrazolium salt MTT into a coloured and water-soluble formazan. Inherent dehydrogenase enzyme activity of viable cells reduces the yellow MTT solution to a purple colour at 37^{o}C. The amount of formazan produced is directly proportional to the number of living cells and provides a good indication of cell viability. Briefly, cells were seeded onto 96-well plates ($0.4x10^4$ cells/well) and cultured for 24-48 hours until they reached 80-90% confluence. The media was then aspirated, and the cells were treated with various concentrations (see above) of H_2O_2, pilocarpine or LPS in serum free medium for 1, 4 or 24 hours. Thereafter, 20 µl of 5 mg/mL MTT stock was added to each well. After 4 hours of incubation, the existing media was removed, and 100 µl of 0.3% DMSO was added to each well. After 10 min, the 96-well plate was placed in a microplate reader (at room temperature) and the absorbance at 595nm was measured.

2.2.10 Statistical analysis

All experiments were performed using two technical replicates (i.e. all analyses performed in duplicate) and three biological replicates (i.e. n=3 per group). Results were reported as mean ± standard deviation (SD). Statistical comparisons were undertaken using either one way or two ways ANOVA (with a Dunnett correction for multiple comparisons) using Stats Direct software version 2.7.9. Differences were considered significant at $p \leq 0.05$.

2.3 Results

2.3.1 Effect of H_2O_2, pilocarpine, and LPS on cell viability

This study investigated the effect of H_2O_2, pilocarpine, and LPS on SH-SY5Y cell viability in order to identify a concentration of each oxidative stress inducer that would not be highly cytotoxic. Identification of such a concentration was important for the study, in order that the expected oxidative stress was not simply attributable to cell death.

Cells exposed to H_2O_2 (0-1000μM) for 1, 4 and 24 hours showed a decrease in viability in a time- and concentration-dependent manner (figure 2.1). At 1 hour, viability was maximally reduced to 66.4% of control following exposure to 1000μM H_2O_2 and was statistically significant at all concentrations above 10μM ($p<0.05$ at 30μM and 100μM; $p<0.001$ at 300μM and 1000μM). At 4 hours, the maximal reduction was 54.6% of control with 1000μM H_2O_2 and was again significant at all concentrations above 10μM ($p<0.05$ at 30μM and 100μM; $p<0.001$ at 300μM and 1000μM). After exposure for 24 hours, cell viability was reduced to 33.2% of control with 1000μM H_2O_2 and was once more statistically significant at all concentrations above 10μM (all $p<0.001$).

Cells exposed to pilocarpine (0-1000μM) for 1, 4 and 24 hours also showed a decrease in viability in a time- and concentration-dependent manner (figure 2.2). At 1 hour, viability was maximally reduced to 72.8% of control following exposure to 1000μM pilocarpine but the reductions in viability failed to reach statistical significance at any concentration. At 4 hours, the maximal reduction was 70.9% of control with 1000μM pilocarpine, which was the only concentration at which a statistically significant difference from control was observed ($p<0.05$). After exposure for 24 hours, cell viability was reduced to 46.7% of control with 1000μM pilocarpine, an effect that was statistically significant at all concentrations above 10μM (all $p<0.05$).

Cells exposed to LPS (0-100µg/ml) for 1, 4 and 24 hours similarly showed a decrease in viability in a time- and concentration-dependent manner (figure 2.3). At 1 hour, viability was maximally reduced to 82.2% of control following exposure to 100µg/ml LPS, which was the only concentration at which a statistically significant difference from control was observed ($p<0.05$). At 4 hours, the maximal reduction was 76.0% of control with 100µg/ml LPS, which was again the only concentration that achieved statistical significance ($p<0.05$). After exposure for 24 hours, cell viability was reduced to 57.5% of control with 100µg/ml LPS, and was significant at all concentrations above 1µg/ml ($p<0.05$ at 3µg/ml and 10µg/ml; $p<0.001$ at 30µg/ml and 100µg/ml).

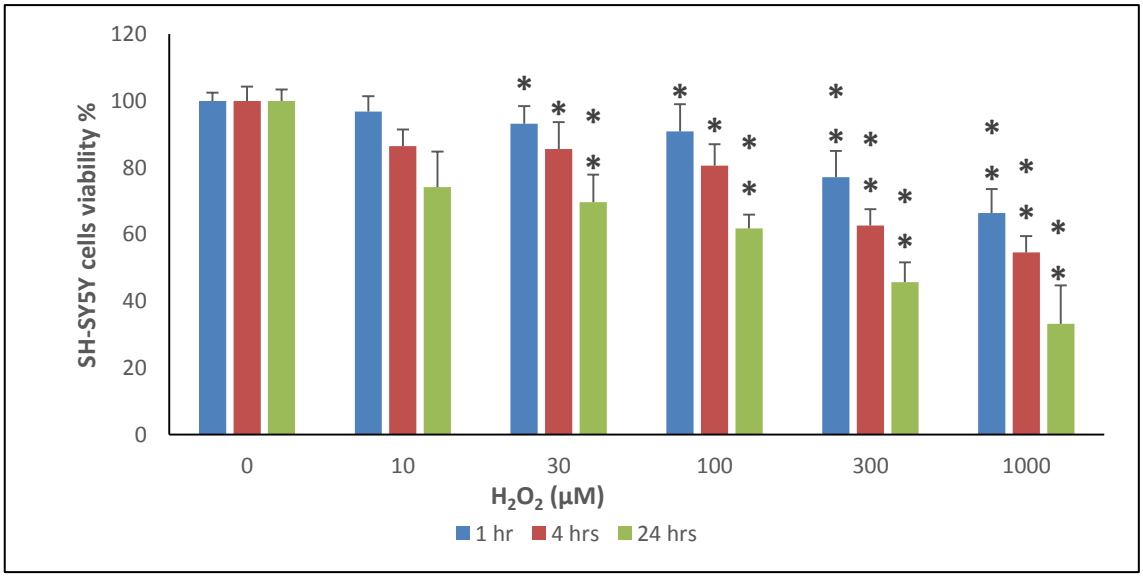


Figure 2.1. Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0µM) value in each data series. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control within each data series (*$p<0.05$) and (**$p<0.001$).

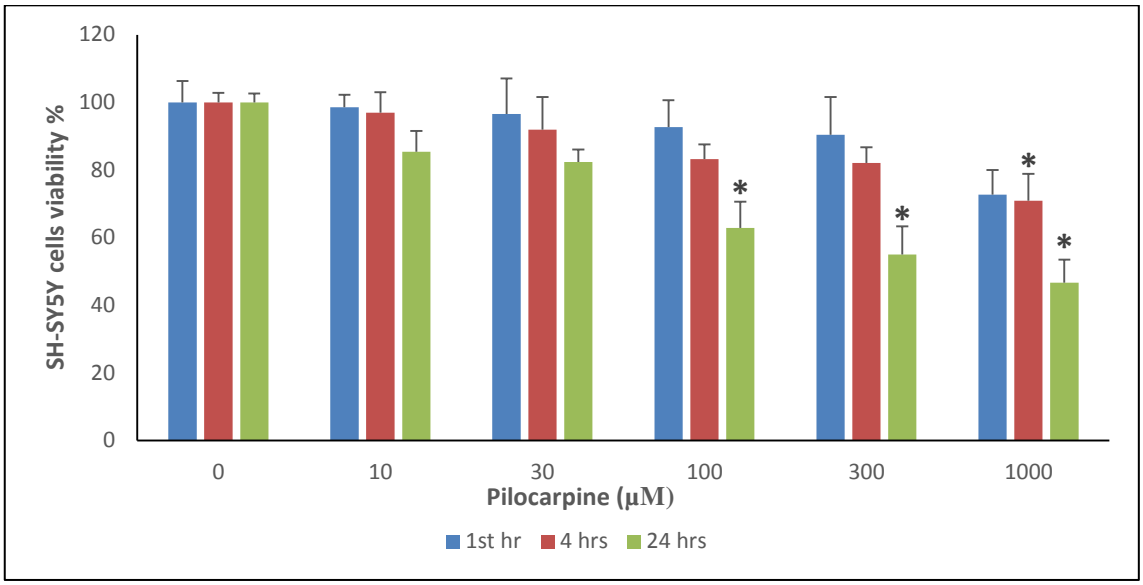


Figure 2.2. Effect of 1, 4 and 24 hour exposure to pilocarpine (0-1000µM) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0µM) value in each data series. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control within each data series (*p<0.05) and (**p<0.001).

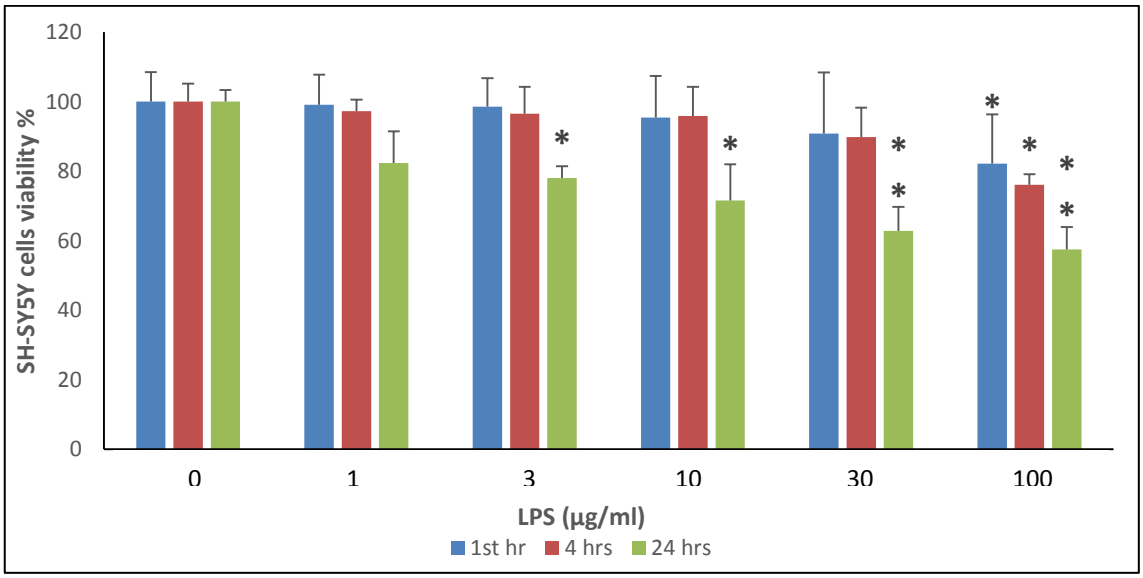


Figure 2.3. Effect of 1, 4 and 24 hour exposure to LPS (0-100µg/ml) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0µg) value in each data series. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control within each data series (*p<0.05) and (**p<0.001).

2.3.2 Effect of H_2O_2 on MDA concentration in SH-SY5Y cells

SH-SY5Y cells showed no significant changes in MDA concentration after exposure to 10-100 µM and 10-30 µM H_2O_2 for 1 and 4 hours, respectively. However, a significant increase in MDA concentration ($p<0.05$) at 100, 300 and 1000 µM H_2O_2 concentration after 4 hours exposure. Furthermore, 10-1000 µM H_2O_2 showed a significant increase in MDA concentration after 24 hours exposure in comparison to control (0µM) value (figure 2.4).

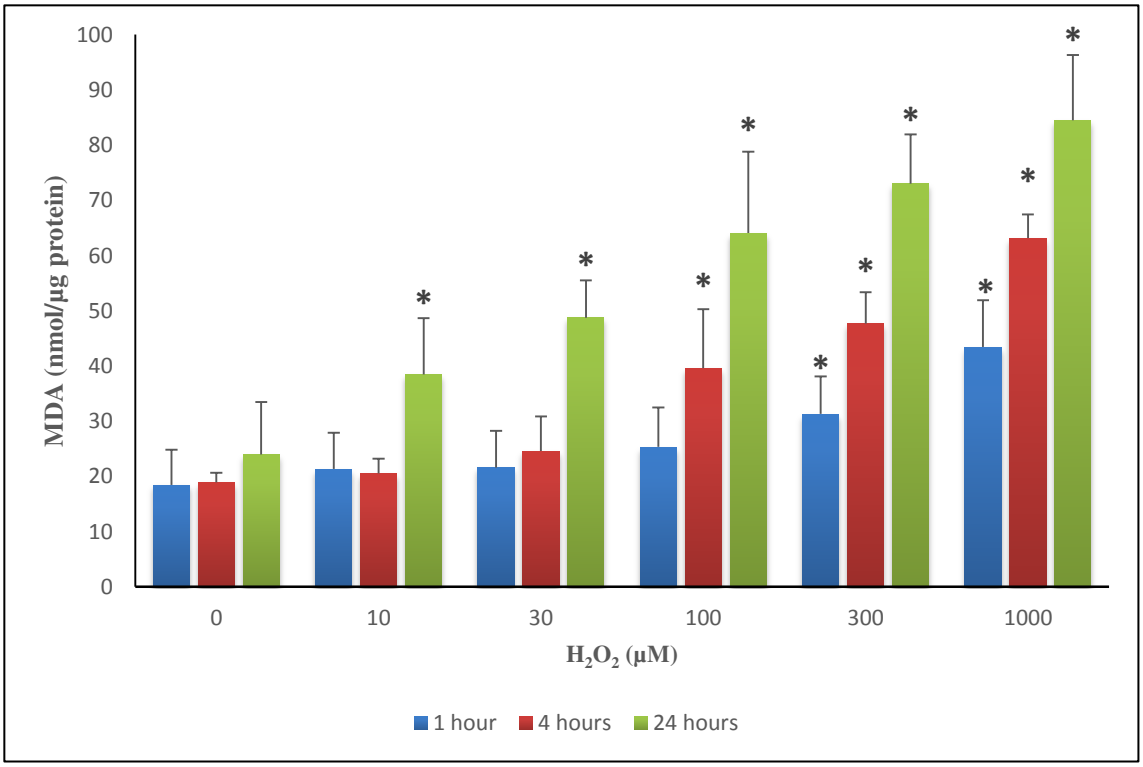


Figure 2.4 Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration in nmols/µg protein and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.3 Effect of pilocarpine on MDA concentration in SH-SY5Y cells

Pilocarpine (0-100uM) was without statistically significant effect on MDA concentration in SH-SY5Y cells following exposure for 1 hour and 4 hours (figure 2.5). In contrast, 24 hour exposure to pilocarpine was associated with a concentration-related increase in MDA concentration, which was significantly different from the control (0µM) value at 10µM and above ($p<0.05$).

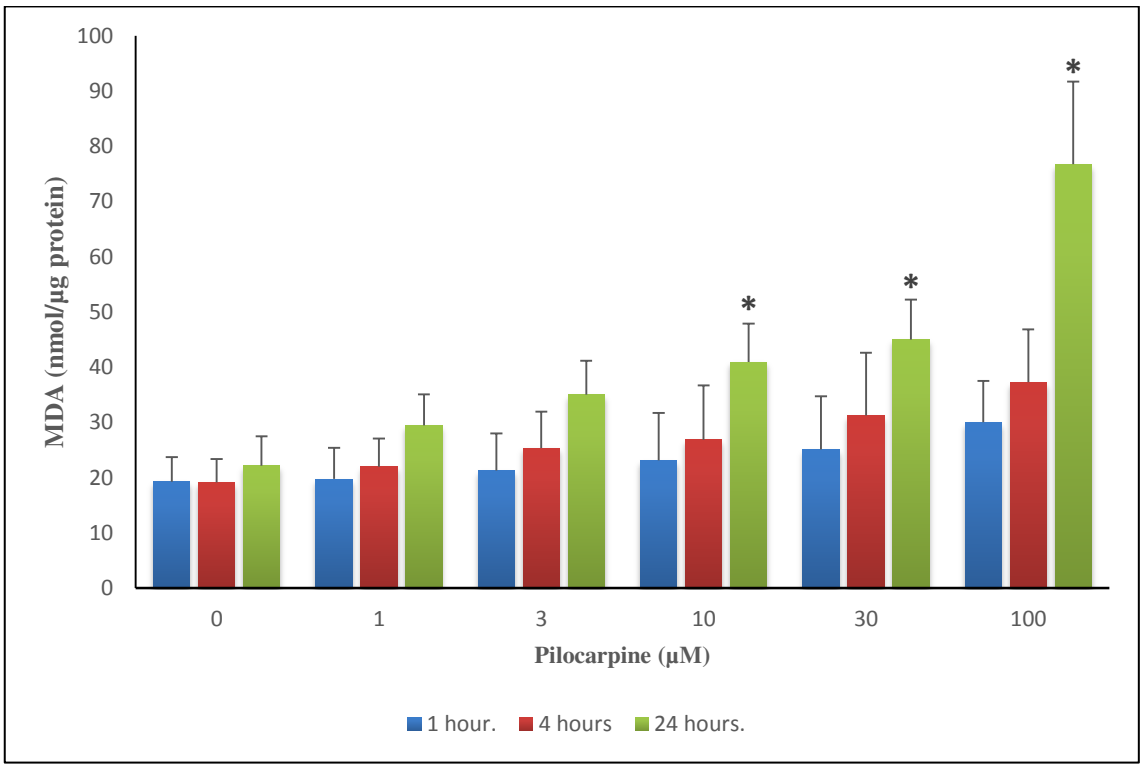


Figure 2.5 Effect of 1, 4 and 24 hour exposure to pilocarpine (0-100µM) on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration in nmols/µg protein and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.4 Effect of LPS on MDA concentration in SH-SY5Y cells

LPS (0-30uM) was without statistically significant effect on MDA concentration in SH-SY5Y cells following exposure for 1, 4, and 24 hours (figure 2.6). In contrast, 4 and 24 hour exposure to LPS was associated with a concentration-related increase in MDA concentration, which was significantly different from the control (0µM) value at 100µM ($p<0.05$).

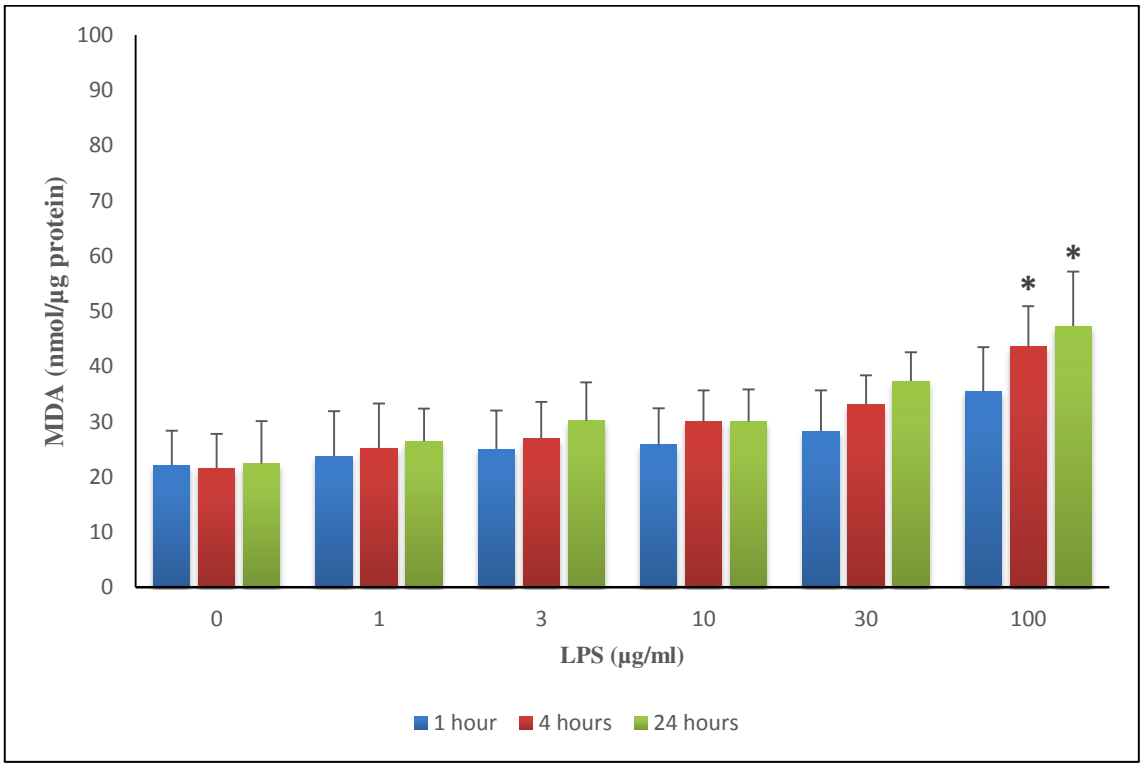


Figure 2.6 Effect of 1, 4 and 24 hour exposure to LPS (0-100µg/ml) on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration in nmols/µg protein and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µg/ml) within each data series (*$p<0.05$).

2.3.5 Effect of H_2O_2 on SOD activity in SH-SY5Y cells

H_2O_2 (0-30uM) was without statistically significant effect on SOD activity in SH-SY5Y cells following exposure for 1, 4, and 24 hours (figure 2.7). In contrast, 1,4, and 24 hours exposure to H_2O_2 was associated with a concentration-related increase in MDA concentration, which was significantly different from the control (0µM) value at 100µM and above ($p<0.05$).

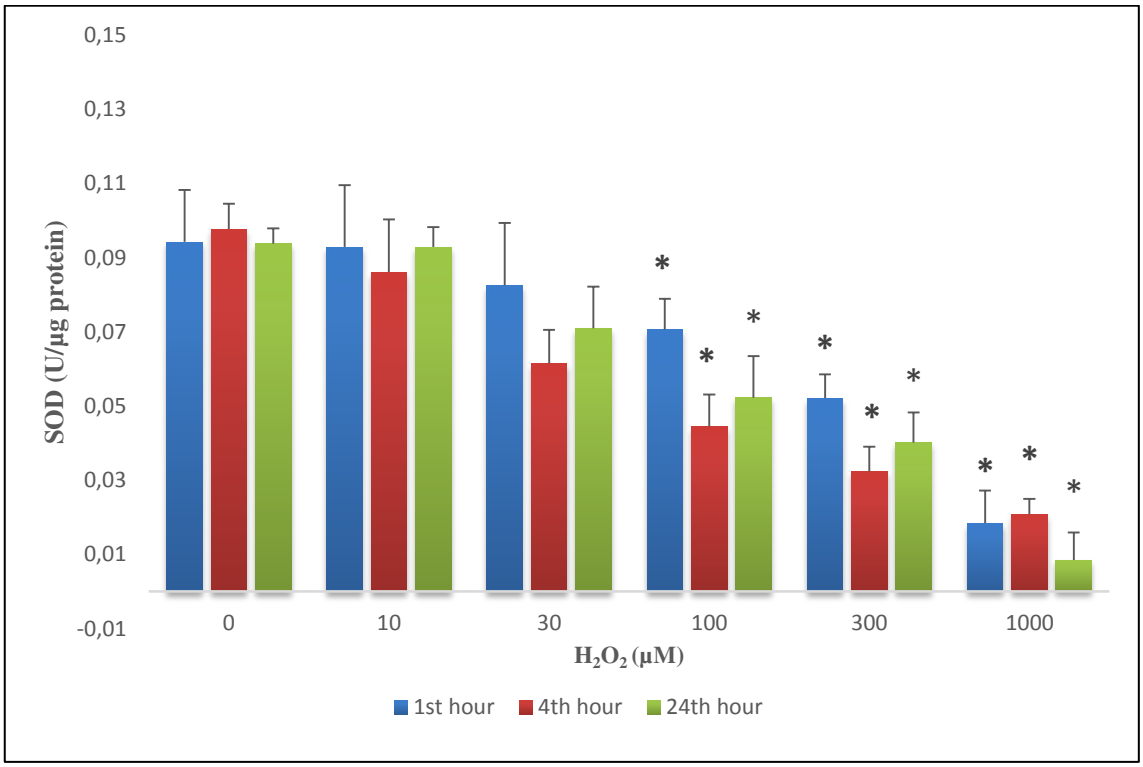


Figure 2.7 Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on superoxide dismutase (SOD) activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity in U/µg protein and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.6 Effect of pilocarpine on SOD activity in SH-SY5Y cells.

Pilocarpine (0-100uM) was without statistically significant effect on SOD activity in SH-SY5Y cells following exposure for 1 hour and 4 hours (figure 2.8). Likewise, 24 hours exposure to pilocarpine (0-10µM) was without statistically significant effect on SOD activity following exposure for 24 hours. In contrast, 24 hour exposure to pilocarpine was associated with a concentration-related increase in MDA concentration, which was significantly different from the control (0µM) value at 30 and 100 µM ($p<0.05$).

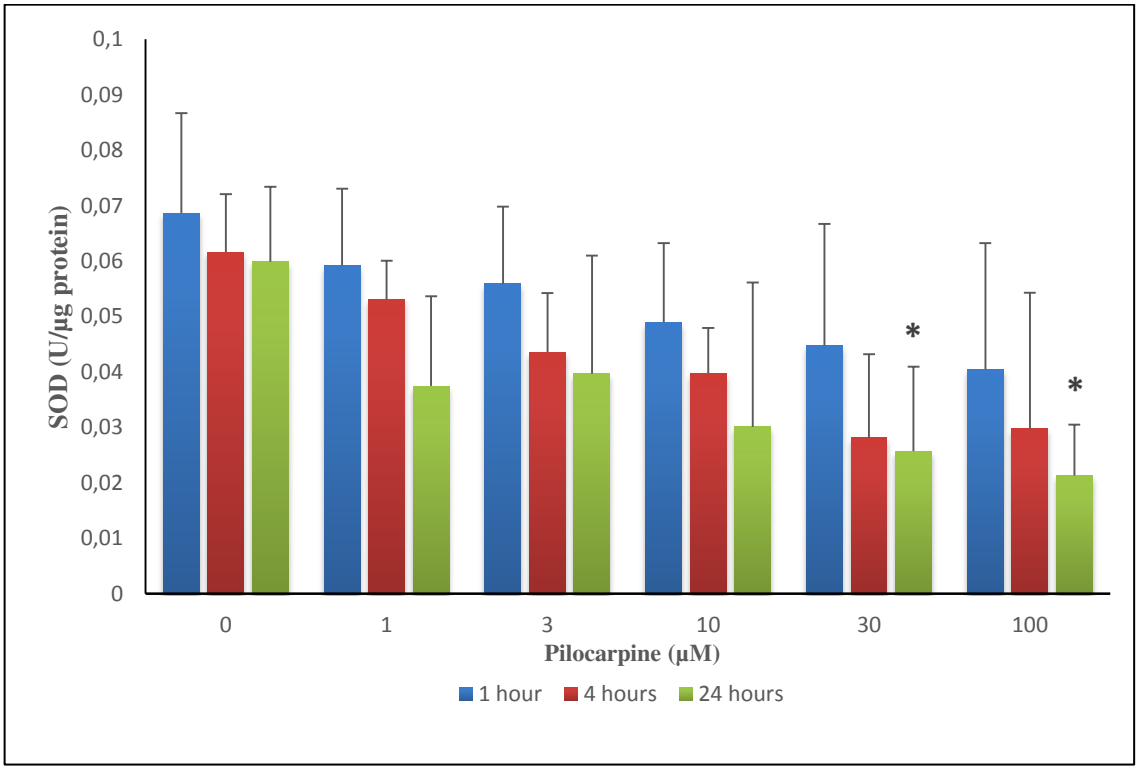


Figure 2.8 Effect of 1, 4 and 24 hour exposure to pilocarpine (0-100µM) on superoxide dismutase (SOD) activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity in U/µg protein and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.7 Effect of LPS on SOD activity in SH-SY5Y cells.

LPS (0-30uM) was without statistically significant effect on SOD activity in SH-SY5Y cells following exposure for 1, 4, and 24 hours (figure 2.9). In contrast, 4 and 24 hour exposure to LPS was associated with a concentration-related increase in SOD activity, which was significantly different from the control (0µg/ml) value at 100µM ($p<0.05$).

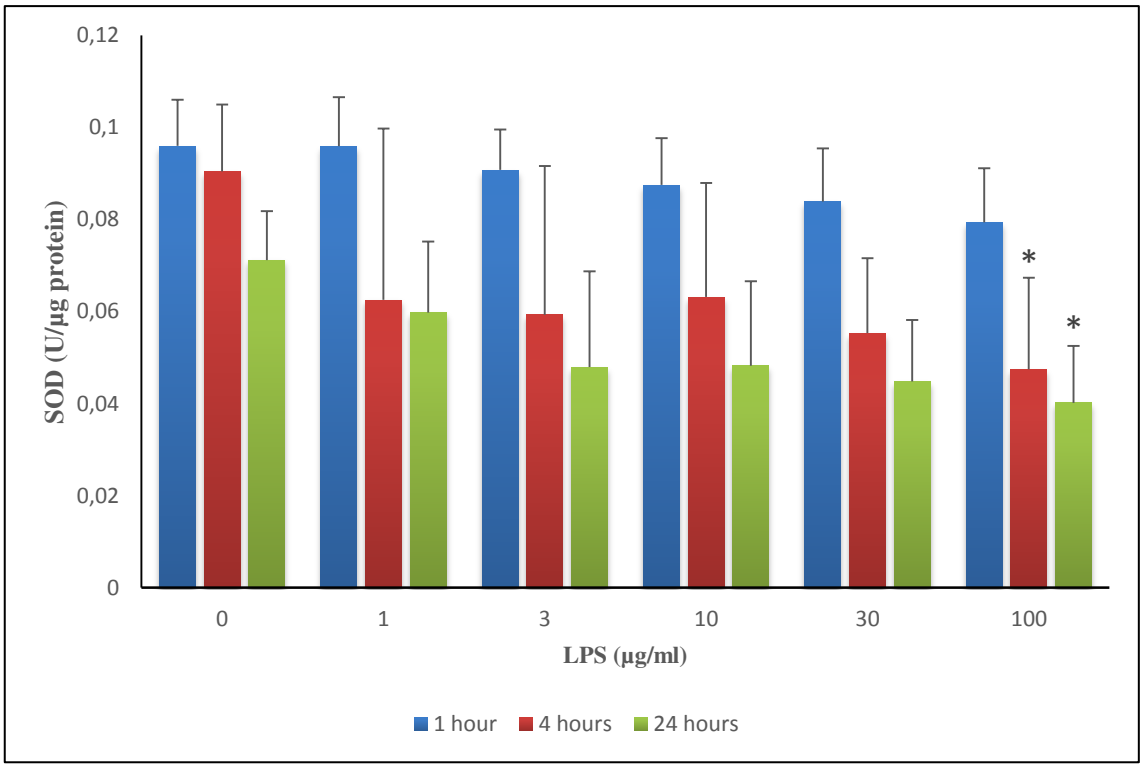


Figure 2.9 Effect of 1, 4 and 24 hour exposure to LPS (0-100µg/ml) on superoxide dismutase (SOD) activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity in U/µg protein and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.8 Effect of H_2O_2 on GSH/GSSG ratio in SH-SY5Y cells.

H_2O_2 (0-30uM) was without statistically significant effect on GSH/GSSG ratio in SH-SY5Y cells following exposure for 1hour and 4 hours (figure 2.10). In contrast, 1,4, and 24 hours exposure to H_2O_2 was associated with a concentration-related increase in GSH/GSSG ratio, which was significantly different from the control (0µM) value at 30µM and above and 10 µM after exposure for 24 hours ($p<0.05$).

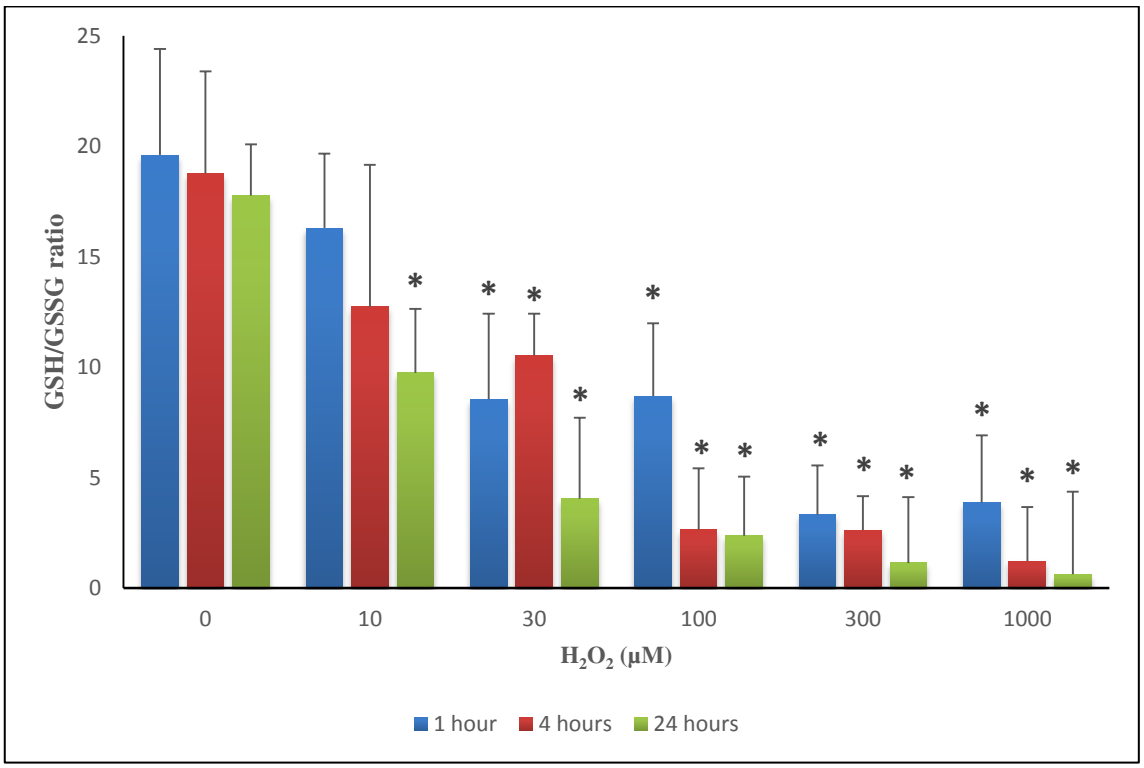


Figure 2.10 Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on reduced to oxidised glutathione (GSH/GSSG) ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) (GSH/GSSG) ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.9 Effect of pilocarpine on GSH/GSSG ratio in SH-SY5Y cells.

Pilocarpine (0-10uM) was without statistically significant effect on GSH/GSSG ratio in SH-SY5Y cells following exposure for 1hour and 4 hours (figure 2.11). In contrast, 1,4, and 24 hours exposure to pilocarpine was associated with a concentration-related increase in GSH/GSSG ratio, which was significantly different from the control (0µM) value at 3µM and above ($p<0.05$).

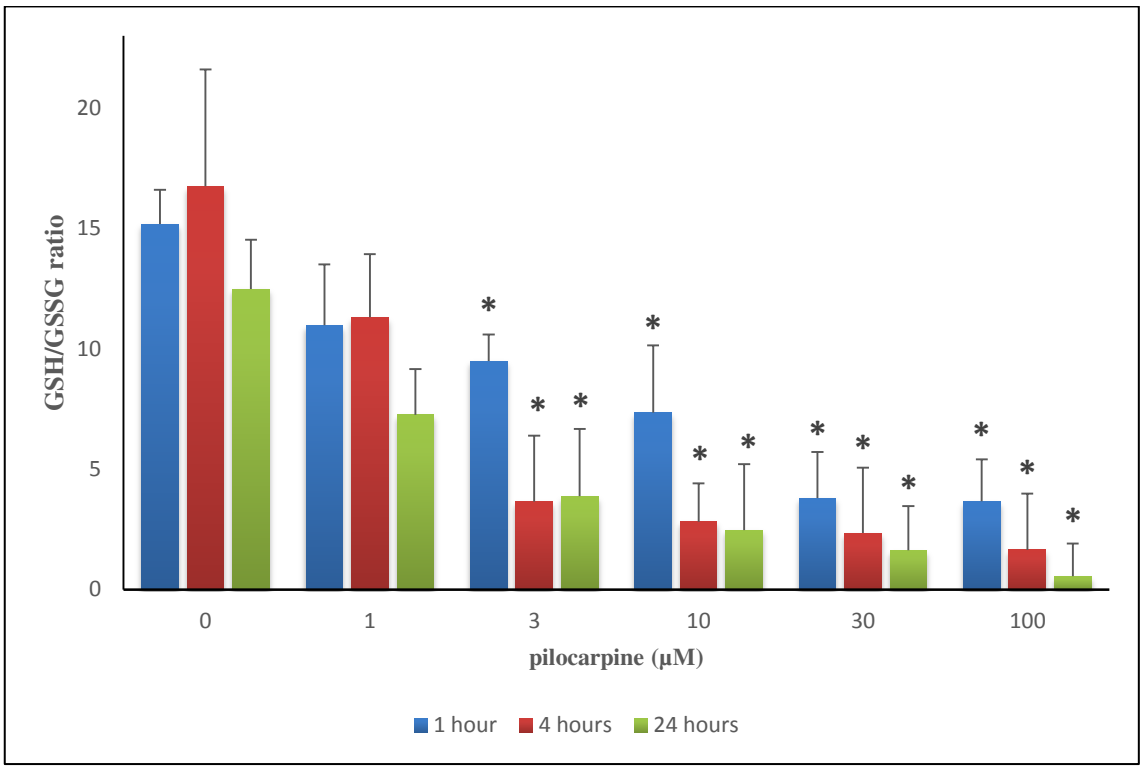


Figure 2.11 Effect of 1, 4 and 24 hour exposure to pilocarpine (0-100µM) on reduced to oxidised glutathione (GSH/GSSG) ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) (GSH/GSSG) ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.10 Effect of LPS on GSH/GSSG ratio in SH-SY5Y cells.

LPS (0-10ug/ml) was without statistically significant effect on GSH/GSSG ratio in SH-SY5Y cells following exposure for 1hour and 4 hours (figure 2.12). In contrast, 1,4, and 24 hours exposure to LPS was associated with a concentration-related increase in GSH/GSSG ratio, which was significantly different from the control (0µM) value at 3µg/ml and above ($p<0.05$).

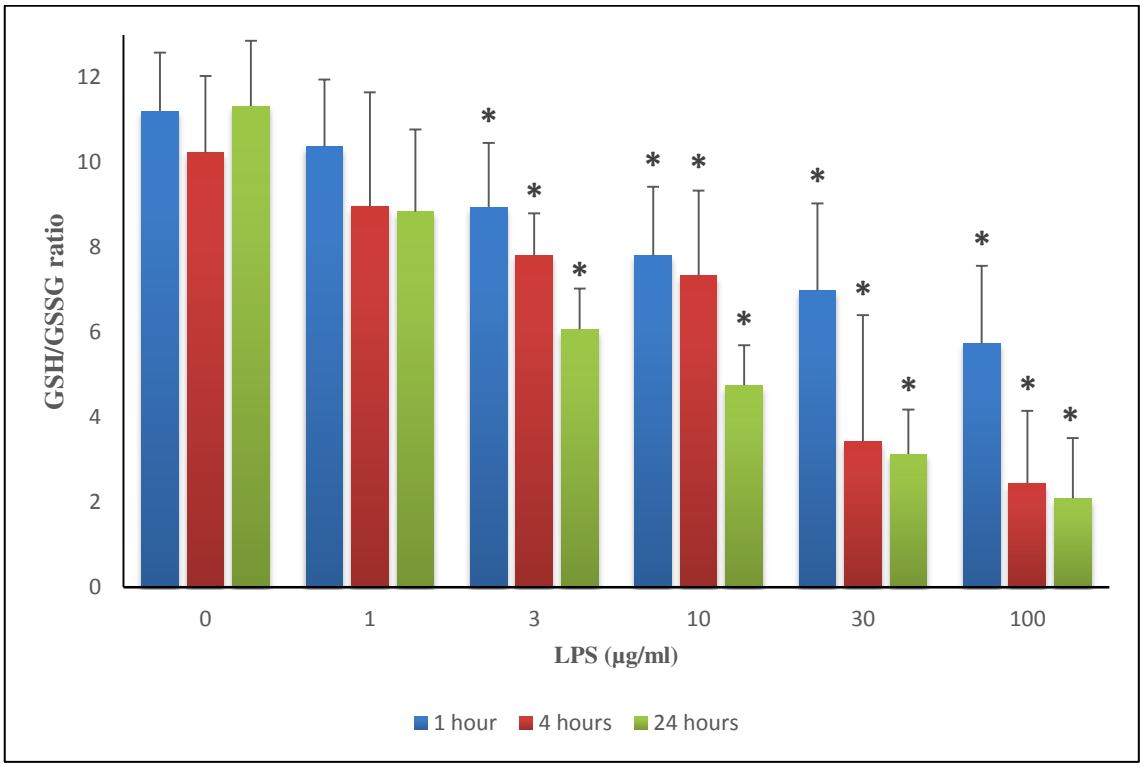


Figure 2.12 Effect of 1, 4 and 24 hour exposure to LPS (0-100µg/ml) on reduced to oxidised glutathione (GSH/GSSG) ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) (GSH/GSSG) ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

2.3.11 Effect of RA on MDA concentration in 100 µM H_2O_2 treated SH-SY5Y cells

Exposure to 100µM H_2O_2 for 1 hour was associated with a statistically significant ($p<0.05$) increase in MDA concentration in both differentiated and undifferentiated SH-SY5Y cells. However, there were no significant differences between differentiated and undifferentiated cells in terms of both basal (un-induced) MDA concentration and following induction with 100µM H_2O_2 (figure 2.13).

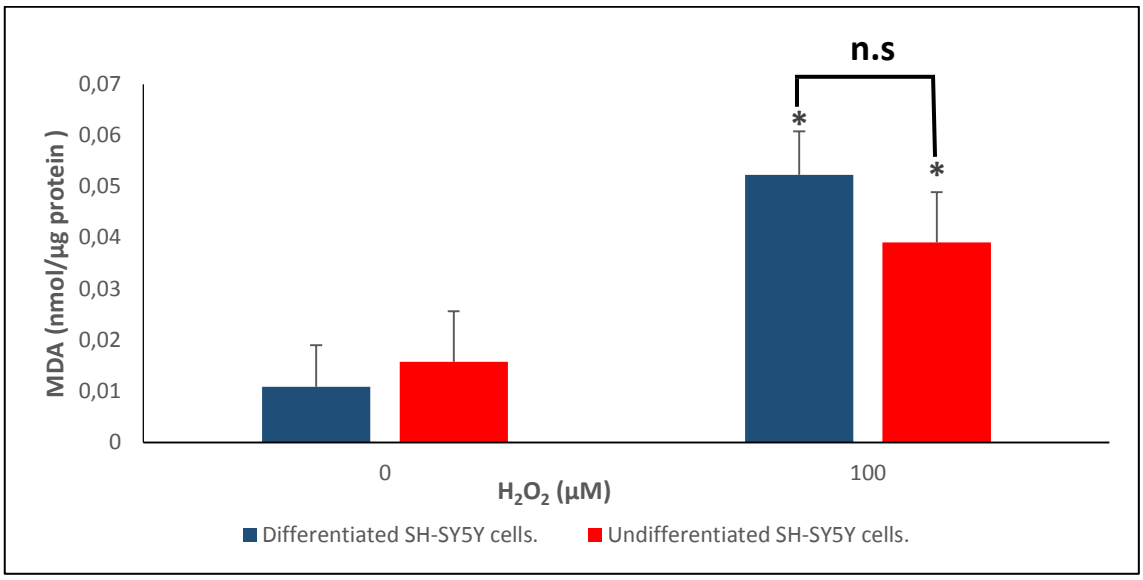


Figure 2.13 Effect of differentiation with 10µM retinoic acid on MDA concentration in SH-SY5Y cells under basal conditions and following induction of oxidative stress with exposure to 100µM H_2O_2 for 1 hour. Results are expressed as mean (±SEM) MDA concentration in nmol/µg protein. (n=4 per group) and statistical significance (*$p<0.05$) was determined by 2-way ANOVA test. n.s: no significant difference.*p: different H_2O_2 concentrations vs control (0µM).

2.3.12 Effect of RA on SOD activity in 100 µM H_2O_2 treated SH-SY5Y cells

Exposure to 100µM H_2O_2 for 1 hour was associated with a statistically significant ($p<0.05$) decrease in SOD activity in both differentiated and undifferentiated SH-SY5Y cells. However, there were no significant differences between differentiated and undifferentiated cells in terms of both basal (un-induced) SOD activity and following induction with 100µM H_2O_2 (figure 2.14).

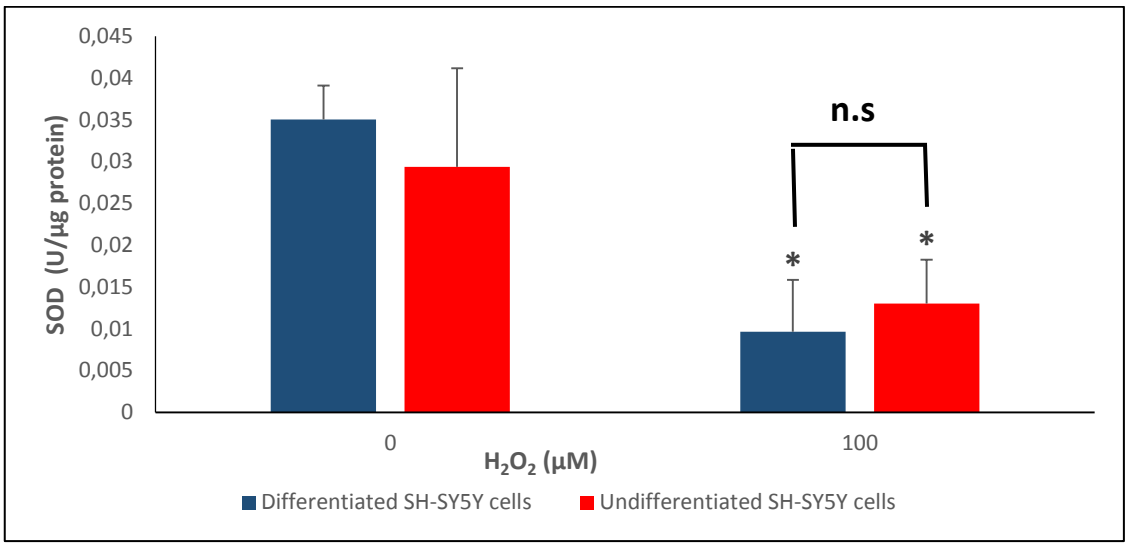


Figure 2.14 Effect of differentiation with 10µM retinoic acid on SOD activity in SH-SY5Y cells under basal conditions and following induction of oxidative stress with exposure to 100µM H_2O_2 for 1 hour. Results are expressed as mean (±SEM) SOD activity in U/µg protein. (n=4 per group) and statistical significance (*$p<0.05$) was determined by 2-way ANOVA test. n.s: no significant difference.*p: different H_2O_2 concentrations vs control (0µM).

2.3.13 Effect of RA on GSH / GSSG ratio in 100 µM H_2O_2 treated SH-SY5Y cells

Exposure to 100µM H_2O_2 for 1 hour was associated with a statistically significant ($p<0.05$) decrease in GSH/GSSG ratio in both differentiated and undifferentiated SH-SY5Y cells. However, there were no significant differences between differentiated and undifferentiated cells in terms of both basal (un-induced) GSH/GSSG ratio and following induction with 100µM H_2O_2 (figure 2.15).

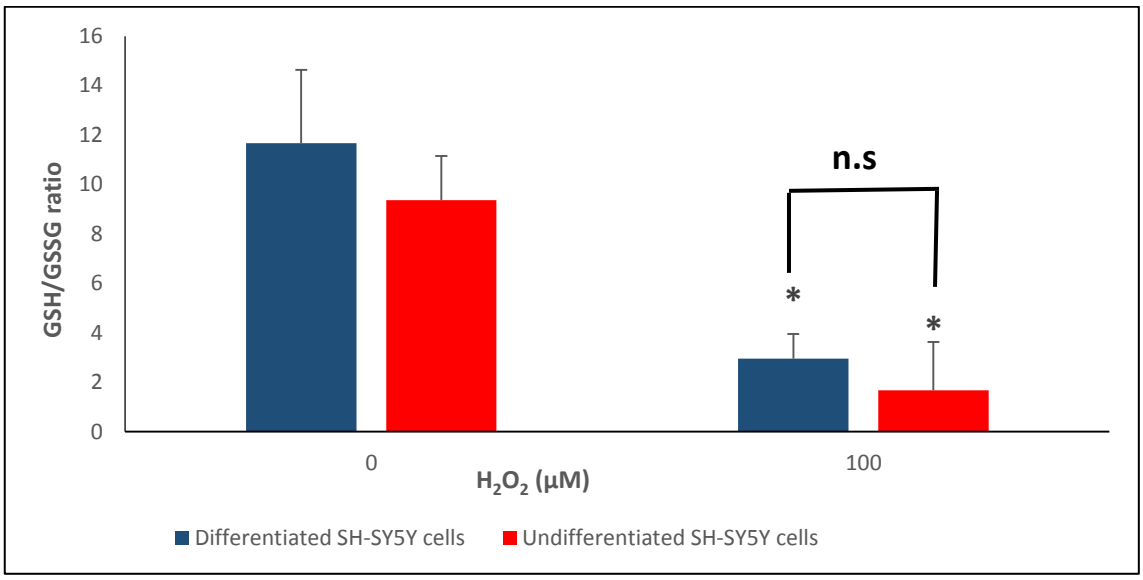


Figure 2.15 Effect of differentiation with 10µM retinoic acid on GSH/GSSG ratio in SH-SY5Y cells under basal conditions and following induction of oxidative stress with exposure to 100µM H_2O_2 for 1 hour. Results are expressed as mean (±SEM) GSH/GSSG ratio. (n=4 per group) and statistical significance (*$p<0.05$) was determined by 2-way ANOVA test. n.s: no significant difference.*p: different H_2O_2 concentrations vs control (0µM).

2.4 Discussion

There is a growing body of evidence suggesting that oxidative stress is potentially neurotoxic (Sgaravatti et al., 2009). This study has shown that1-100 µM pilocarpine produced oxidative stress and decreased SH-SY5Y cells viability, especially at higher concentrations, and that 10-1000µM H_2O_2 and 1-100 µg/ml LPS also induced oxidative insult and cell death in a time and concentration dependent manner.

Many studies have demonstrated that oxidative stress is a major cause of cell death in different kinds of human diseases including neurological disorders. Reactive oxygen species such as H_2O_2, O^- and OH^- instantly destroy biological molecules, such as lipids, proteins and nucleic acids, which can ultimately lead to apoptotic cell death (Bayani et al., 2009). Thus, removal of excess reactive oxygen species or suppression of their generation by endogenous and/or exogenous antioxidants may be effective in preventing oxidative stress and subsequent cell death. In normal basal conditions, there is a steady state balance between the production of ROS and their neutralization by the cellular antioxidant system.

The SH-SY5Y neuroblastoma cell line is widely used as an *in vitro* model for the study of different neurological disorders (Agholme et al., 2010; Kovalevich and Langford. 2013; Krishna et al., 2014). This study showed a non-significant ($p<0.05$) change in MDA concentration, SOD activity and GSH/GSSG ratio between differentiated and undifferentiated SH-SY5Y cells after induction of oxidative stress by 100 µM H_2O_2. Thus, undifferentiated SH-SY5Y cells are sensitive to oxidative damage and are a suitable *in vitro* system for these experiments. Furthermore, it was found that cellular differentiation has a number of issues that might impact on the interpretation of experimental results, such as proteomic changes, cellular proliferation, apoptosis, and transcription (Cheung et al., 2009). These authors also suggested that undifferentiated

SH-SY5Y cells are more appropriate for studying neurotoxicity and neuroprotection in experimental neurological disease research. In addition, it is reported that in studies assessing cell stresses, including oxidative stress and metabolic stress, differentiation may be undesirable, due to the potential for artefacts in the proteomic data from the differentiation process, (Hermann A. 2014).

H_2O_2 is one of the most important non-radical ROS produced via metabolism in living cells. It is the by-product of enzymatic activity and catecholamine's oxidation and if not metabolised, might generate highly toxic hydroxyl radicals through the Fenton reaction (Cho et al., 2009). H_2O_2 has been extensively used as an inducer of oxidative stress in *in vitro* models (Satoh et al., 1996). Moreover, the exposure of undifferentiated SH-SY5Y cells to H_2O_2 results in an imbalance in energy metabolism because of the harmful effects of hydroxyl and peroxyl radicals on membrane lipids and proteins (Satoh et al., 1996).

The current study confirmed that cells exposure to H_2O_2 resulted in a time- and concentration-dependent decrease in viability (figure 2.1). This result was consistent with previous data reporting a concentration-dependent decrease in cell viability after exposure to different concentrations of H_2O_2 with maintaining cell viability above 80% even after 100 µM H_2O_2 exposure for 24 hours when using SH-SY5Y cells as an *in vitro* model (Webster et al., 2003; Gu et al., 2013; Kang et al., 2014).

Undifferentiated SH-SY5Y cells exposure to H_2O_2 in high concentrations revealed a decrease in antioxidant mechanisms (i.e. SOD activity and GSH/GSSG ratio) and an increase in MDA concentration, and therefore lipid peroxidation, which reflects the strong pro-oxidant activity of H_2O_2. These results link to a recently published papers reported an increase in MDA concentration and a decrease in GSH, GPx and SOD and CAT activity in various cell lines after exposure to different concentrations of H_2O_2 (Wang et al., 2017; Xiaolong. 2017). In addition, previous reports suggested that

exogenous added H_2O_2 induced intracellular ROS generation and affected accompanying cellular metabolic responses (Gough and Cotter. 2011; Park et al., 2015). The strong pro-oxidant activity of H_2O_2 has been suggested to be due to its direct damaging effect, as it is a highly diffusible ROS that can cross the plasma membrane (Forman. 2007). Treatment with H_2O_2 has been shown to produce more O^- by activation of NADPH oxidase and subsequently the formation of peroxunitrite (i.e. $ONOO^-$) through superoxide scavenging of nitric oxide (Beckman et al., 1990; Pryor and Squadrito. 1995). On the other hand, H_2O_2 induces lipid peroxidation, which leads to lactate dehydrogenase (LDH) release and ultimately cell death by necrosis. Moreover, LDH can be involved in membrane and protein damage (Garcimartin et al., 2014).

LPS has occasionally been used as an experimental inducer of oxidative stress, although it is more commonly employed to induce inflammatory or autoimmune responses. In the current study, LPS showed a time-dependent decrease in cell viability and an increase in oxidative damage in SH-SY5Y cells. These findings are consistent with recently published data reporting an increase in MDA concentration and a decrease in GSH level and SOD activity in different experimental animals and cell line after exposure to various concentrations of LPS (Pourganji et al., 2014; Gou et al., 2015; Frimmel et al., 2016). It has been found that LPS-induced brain inflammation is accompanied by neuronal and glial cell activation resulting in the release of neurotoxic factors such as inflammatory cytokines and free radicals (Noworyta et al., 2013). Moreover, cellular exposure to LPS may trigger the release of inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), which might in turn result in oxidative stress and inflammation (Figure 16) (Choudhury et al., 2015; Ruiz-Miyazawa. 2015). To the best of our knowledge, no previous reports have used the SH-SY5Y cell line as an *in vitro* model to assess oxidative stress markers after exposure to LPS. However, several studies have evaluated the neuroinflammatory effect of LPS on SH-SY5Y cells.

Neuroinflammation can contribute to neuronal dysfunction and the development of several neurodegenerative diseases, including Parkinson's diseases, Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis, at least in part by triggering ROS generation and engaging the same TLR pathway that is involved in oxidative stress (Glass et al., 2010).

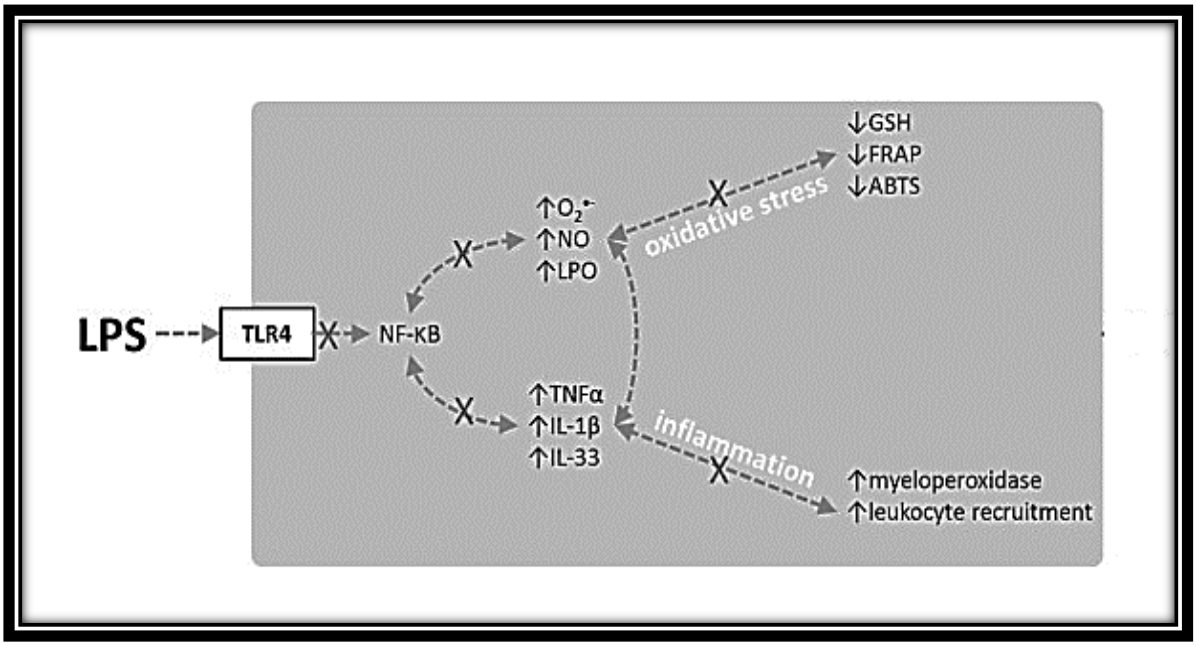


Figure 2.16 LPS-induced inflammation and oxidative stress. LPS activates toll-like receptor 4 (TLR4), which leads to activation of nuclear factor kappa B (NF-κB) and consequent production of inflammatory cytokines (tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β), interleukin 33 (IL-33)), superoxide anion (O2−), nitric oxide (NO) and lipid peroxidation (LPO), and diminished levels of reduced glutathione (GSH), ferric reducing antioxidant power (FRAP) and to scavenge 2-2-azinobis(3-ethyllbenzothiazoline-6-sulfonic acid (ABTS) free radicals. Adapted from (Ruiz-Miyazawa. 2015).

The current results showed an increase in lipid peroxidation by-product (i.e. MDA) and a decrease in SOD activity and GSH/GSSG ratio in a time- and concentration-dependent manner after exposure to different concentrations of pilocarpine. Pilocarpine is widely used to induce seizures-related oxidative stress in several experimental models (Freitas et al., 2004; Tome et al., 2010; Hermann. 2014). The results reported here are

agreed with previous studies which reported that treatment of various experimental animals with different pilocarpine concentrations induced lipid peroxidation, decrease GSH level and SOD activity in addition to increased nitric oxide, TNF-α, and IL-6 which play an important role in inflammation and oxidative stress as well (Freitas et al., 2004; Santos et al., 2011; Kabel. 2014; Noor et al., 2015). Moreover, it has been reported that pilocarpine binding to M1 activates the phospholipase C and therefore produces diacylglycerol (DG) and inositol triphosphate (IP3), which results in alteration in Ca^{++} and K^{+} current and increases the excitability of the brain. The high concentration of Ca^{++} promotes the high release of glutamate, which induces the status epilepticus (SE). The tissue excitability and/or SE increase the utilization rate of catecholamine's which are degraded by MAO and COMT and, during these processes, free radicals can be formed. These free radicals are also generated during glucose metabolism and mitochondrial transport chain, which is over activated during SE. In addition, the SOD presented a decreased activity during seizures, associated to an increased MDA concentration and tissue damage in the hippocampus of epileptic animals (Scorza, F. A. et al., 2009).

2.4.1 Conclusions

The present study revealed that H_2O_2, LPS and pilocarpine can attenuate basal antioxidant mechanisms and decrease cell viability in a time and concentration-dependent manner in human SH-SY5Y cells. In addition, this study demonstrated that 100 µM H_2O_2 was the ideal pro-oxidant because of efficient induction of oxidative stress with minimum time exposure (i.e. 4 hours) and minimum cell death (less than 20%) in comparison to other suggested H_2O_2, LPS, and pilocarpine concentrations used in this experiment. Moreover, in terms of oxidative stress indicators, no significant

changes have been found between differentiated and undifferentiated SH-SY5Y cells after exposure to 100µM H_2O_2. So, according to these findings and others from previous reports mentioned many issues and undesirability in using differentiated SH-SY5Y cells in these kinds of experiments, undifferentiated cells were chosen for subsequent investigations.

Chapter 3

Effects of commonly used antiepileptic drugs on markers of oxidative stress in a human neuroblastoma cell line

3.1 Introduction

Oxidative stress is an imbalance between pro-oxidants and anti-oxidants in favourite of pro-oxidants. Free radicals are involved in pathogenesis of various diseases including atherosclerosis, diabetes, stroke, inflammatory bowel diseases and lung cancer (Sundstrom et al., 1984; Plachta et al., 1992). Awareness of the important role of oxidative damage by ROS and/or RNS in seizure disorders has arisen in the last two decades (Cengis et al., 2000; Ercegovac et al., 2010).

The brain is particularly susceptible to oxidative damage because of high oxygen consumption, production of large amounts of ROS and RNS, susceptibility to lipid peroxidation, richness in iron content, and lack of important antioxidant enzymes (i.e. catalase) (Shin et al., 2011; Naziroglu 2012). Therefore, any pathological brain condition requires an increase in energy consumption which might eventually make the brain most sensitive to oxidative insult. (Naziroglu 2007; Ozmen et al., 2007; Uttara et al., 2009). However, the role of oxidative stress in epilepsies has only recently started to be recognized (Ashrafi et al., 2007). Still, the question of whether oxidative stress is a cause or a consequence of seizures remains unanswered.

It has been reported that increased generation of free radicals or reduced activity of anti-oxidative defence mechanisms can cause some forms of epilepsy and, in addition, increase the risk of seizure recurrence (Ashrafi et al., 2007). Moreover, free radicals may also exacerbate some forms of seizures (Menon et al., 2012) and there are several experimental and clinical studies revealing that seizures can result directly from free radical production and oxidative damage to proteins, lipids and DNA molecules (Wagner et al., 2004). In addition, animal studies in which exogenous antioxidants were used together with AEDs showed a decrease in oxidative insults and a reduction in seizure frequency (Willmore et al., 1981; Patsoukis et al., 2004). The sophisticated

mechanism of epileptogenesis remains elusive but neuronal cell death mediated by oxidative damage is one of the important factors that might affect the evolution of epilepsy through mitochondrial dysfunction (Menon et al., 2012).

The pro-oxidant/antioxidant balance in epilepsy is not only modulated by seizures intrinsically, but also by AEDs (Verrotti 2002; Johannesson et al., 2009). Most of the commonly used AEDs, including CBZ and VPA, are metabolized to generate reactive metabolites with the capability of covalent binding to macromolecules, such as proteins, lipids or other vital biomolecules, and thus provoking systemic toxicity (Yuksil et al., 2000; Hamed et al., 2004). Furthermore, it has been found that long-term use of AEDs, such as CBZ, VPA or phenobarbital (PB), increases free radical formation and causes oxidative damage to vital biomolecules within neuronal cells (Maertens et al., 1995; Cengis et al., 2000). However, it remains uncertain whether the use of AEDs is associated with a significant pro-oxidant effect, since major antioxidant enzyme activities, including SOD and Gpx, have been reported to be increased, unchanged or decreased following AED exposure (Cengis et al., 2000; Yuksil et al., 2000; Hamed et al., 2004).

CBZ, VPA, LEV and LTG are amongst the most commonly prescribed AEDs in epilepsy (Chadwick et al., 2008; Johannesson et al., 2009). These drugs are considered the first-line and the most effective AEDs for common types of seizure (Marson et al., 2007). As with other conventional anticonvulsants, they reduce neuronal excitability through effects on ion channels and synaptic function (Stafstorm et al., 2009).

Studies exploring the effect of AEDs on pro-oxidant/antioxidant balance have, so far, mainly focused on VPA and CBZ and their effect on lipid peroxidation and enzymatic antioxidant defence mechanisms (Martinez-Ballestros et al., 2004; Sobaniec et al., 2006). The extent of protein oxidative damage with AEDs has not been fully evaluated,

while DNA oxidative damage has only been examined in children receiving VPA therapy and recently, in one study, on adult patients receiving VPA, CBZ and LEV (Schulpis et al., 2006; Varoglu et al., 2010). It is suggested that VPA impairs the liver function resulting in free radicals production in children and VPA, LEV, and CBZ increased oxidative stress in adults after two months of treatment. Furthermore, data on LTG effects on oxidative stress in patients with epilepsy are very limited. Unfortunately, at this time, little is known about the clinical pro-oxidant or antioxidant effects of AEDs since the presence of seizure activity is associated with changes in the intracellular levels of antioxidants and pro-oxidants, potentially confounding the results of drug studies (Martinc et al., 2012).

3.1.1 Aims and objectives

The aims of the present study were to:

1- Explore the effects of CBZ, VPA, LEV and LTG on MDA concentration, as a marker of lipid peroxidation, in the SH-SY5Y cell line under basal conditions and following induction of oxidative stress by H_2O_2.

2- Explore the effects of CBZ, VPA, LEV and LTG on SOD activity, as a marker of antioxidant enzyme activity, in the SH-SY5Y cell line under basal conditions and following induction of oxidative stress by H_2O_2.

3- Explore the effects of CBZ, VPA, LEV and LTG on the GSH/GSSG ratio, as a marker of the antioxidant defence system, in the SH-SY5Y cell line under basal conditions and following induction of oxidative stress by H_2O_2.

3.2. Materials and Methods

3.2.1 Chemicals

Dimethylsulfide (DMSO), CBZ, LEV, LTG and VPA were purchased from Thermo Fisher Scientific, Loughborough, UK. Other chemicals were sourced as described in chapter 2, section 2.2.1.

3.2.2 Cell culture

SH-SY5Y cells were cultured as described in chapter 2, section 2.2.2.

3.2.3 AED effects on cell viability

SH-SY5Y cells were seeded in 96-well plates at a density of 0.4 x 10^4 cells / well in 0.1 ml culture medium (see section 2.2.2) and placed in an incubator containing humidified 95% air and 5% CO_2 at 37^{o}C for 2-3 days until they reached 80-90% confluence. Cells were then treated with CBZ (0, 1, 3, 10, 30 and 100 µM), VPA (0, 10, 30, 100, 300 and 1000 µM), LTG (0, 1, 3, 10, 30 and 100 µM) or LEV (0, 3, 10, 30, 100 and 300 µM) for 1, 4 or 24 hours and an MTT assay was performed as described in chapter 2, section 2.2.9.

3.2.4 AED effects on oxidative stress

SH-SY5Y cells were seeded in 24-well plates at a density of 1.88 x 10^4 cells / well in 0.5 ml culture medium (see section 2.2.2) and placed in an incubator containing humidified 95% air and 5% CO_2 at 37^{o}C for 2-3 days until they reached 80-90% confluence.

In the first series of experiments, cells were treated with either CBZ (0, 10, 30, 100, 300 and 1000µM), VPA (0, 10, 30, 100, 300 and 1000µM), LTG (0, 1, 3, 10, 30 and

100μM) or LEV (0, 3, 10, 30, 100 and 300μM) for 24 hours. VPA and LTG were dissolved in serum-free medium (SFM; see chapter 2, section 2.2.4) whereas CBZ and LEV were dissolved in SFM containing 0.2% DMSO. Thereafter, cells were exposed to 100 μM H_2O_2 for 4 hours to induce oxidative stress, with an equivalent number of wells left untreated as non-oxidative stress controls (working volumes; see-chapter2-table2.1). Existing culture medium was then aspirated; cells washed three times with 0.5ml PBS, and scraped in 1 ml PBS prior to analysis of oxidative stress markers.

In a second series of experiments, cells were initially treated with H_2O_2 (0, 10, 30, 100, 300 and 1000 μM) in SFM for 4 hours. Thereafter, cells were exposed to single concentrations of either CBZ (10 μM), VPA (100 μM), LEV (30 μM) or LTG (10 μM) for a further 4 hours, with an equivalent number of wells left untreated in each case as non-AED controls. Existing culture medium was then aspirated; cells washed three times with 1ml PBS, and scraped in 1 ml PBS prior to analysis of oxidative stress markers.

3.2.5 Protein quantitation

Protein in cell lysates was quantified by BCA Protein Assay Kit as described in chapter 2, section 2.2.8.

3.2.6 Oxidative stress markers

MDA concentration, GSH/GSSG ratio, and SOD activity were determined as described in chapter 2, sections 2.2.5, 2.2.6 and 2.2.7, respectively.

3.2.7 Statistical analysis

All experiments were performed using two technical replicates (i.e. analyses of all samples performed in duplicate) and three biological replicates (i.e. n=3 per group).

Results were reported as mean ± standard deviation (SD). Statistical comparisons were undertaken using either one way or two way ANOVA as appropriate, and with a Dunnett correction for multiple comparisons, using Stats Direct software version 2.7.9. Differences were considered significant at $p \leq 0.05$.

3.3 Results

3.3.1 Effect of CBZ, VPA, LEV and LTG on cell viability

Cells exposed to CBZ (0-100 μM) for 1, 4 and 24 hours showed a decrease in viability in a time- and concentration-dependent manner (figure 3.1). At 1 hour, viability was maximally reduced to 77.6% of control following exposure to 100 μM CBZ and was statistically significant at the same concentration ($p<0.05$ at 100 μM). At 4 hours, the maximal reduction was 69.7% of control with 100 μM CBZ and was significant at 10 μM CBZ and above (all $p<0.05$). After exposure for 24 hours, cell viability was reduced to 64.4% of control with 100 μM CBZ and was statistically significant at all concentrations tested ($p<0.05$ at 1 to 30 μM CBZ; $p<0.001$ at 100 μM CBZ).

Cells exposed to VPA (0-1000 μM) for 1, 4 and 24 hours also showed a decrease in viability in a time- and concentration-dependent manner (figure 3.2). At 1 hour, viability was maximally reduced to 76.2% of control following exposure to 1000μM VPA and was statistically significant at 300μM VPA and above ($p<0.05$). At 4 hours, the maximal reduction was 61.9% of control with 1000μM VPA and was statistically significant at 30μM VPA and above ($p<0.05$). After exposure for 24 hours, cell viability was reduced to 57.1% of control with 1000μM VPA and was statistically significant at all concentrations tested ($p<0.05$ at 10 to 300 μM VPA; $p<0.001$ at 1000 μM VPA).

Cells exposed to LEV (0-300 μM) for 1, 4 and 24 hours similarly showed a decrease in viability in a time- and concentration-dependent manner (figure 3.3). At 1 hour, viability was maximally reduced to 87.0% of control following exposure to 300 μM LEV but was not significantly different from control at any concentration ($p>0.05$). At 4 hours, the maximal reduction was 78.0% of control with 300 μM LEV, which was statistically significant at 3 μM LEV and above ($p<0.05$). After exposure for 24 hours, cell viability was reduced to 64.0% of control with 300 μM LEV, and was significant at concentrations of 10μM LEV and above ($p<0.05$ at 10 to 100 μM LEV; $p<0.001$ at 300 μM LEV).

Cells exposed to LTG (0-100 μM) for 1, 4 and 24 hours showed a decrease in viability in a time- and concentration-dependent manner (figure 3.4). At 1 hour, viability was maximally reduced to 85.0% of control following exposure to 100 μM LTG but was not significantly different from control at any concentration ($p>0.05$). At 4 hours, the maximal reduction was 82.0% of control with 100 μM LTG and was statistically significant at the same concentration ($p<0.05$ at 100 μM LTG). After exposure for 24 hours, cell viability was reduced to 74.0% of control with 100 μM LTG and was statistically significant at concentrations of 3μM and above (all $p<0.05$).

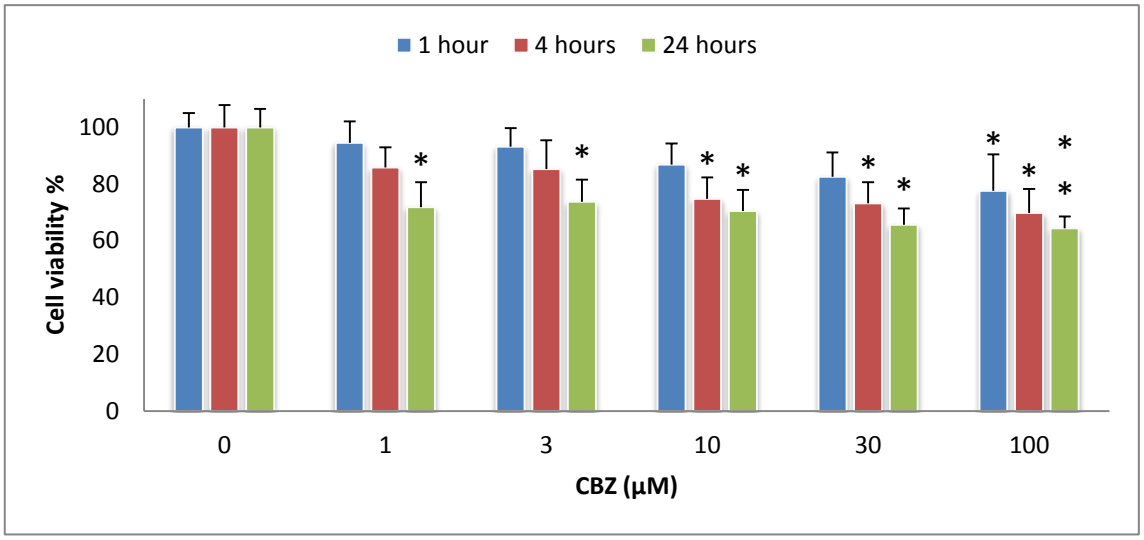


Figure 3.1 Effect of 1, 4 and 24 hour exposure to CBZ (0-100 µM) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0 µM) value in each data series. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series

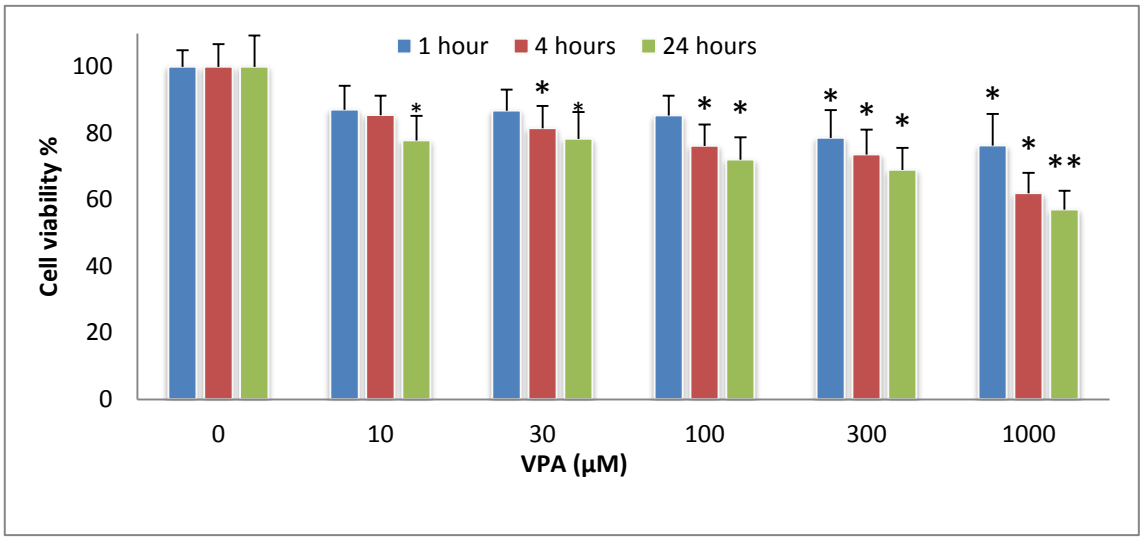


Figure 3.2 Effect of 1, 4 and 24 hour exposure to VPA (0-1000 µM) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0 µM) value in each data series. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

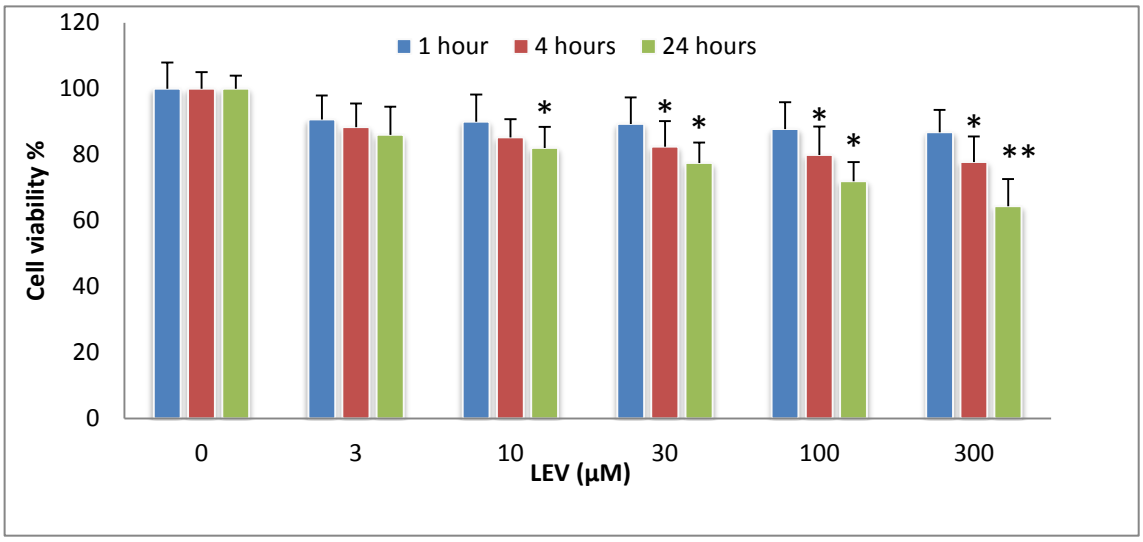


Figure 3.3 Effect of 1, 4 and 24 hour exposure to LEV (0-300 µM) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0 µM) value in in each data series. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

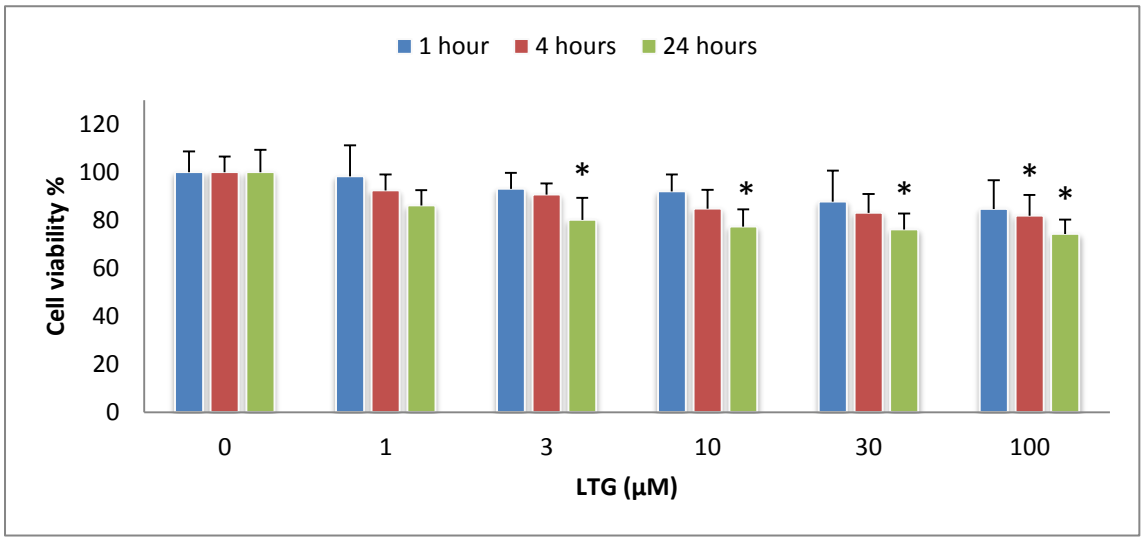


Figure 3.4 Effect of 1, 4 and 24 hour exposure to LTG (0-100 µM) on the viability of SH-SY5Y cells as determined by MTT assay. Results (n=3) are expressed as the mean (± SD) percentage of the mean control (i.e. 0 µM) value in in each data series. Statistical significance was determined by one- -way ANOVA with Dunnett correction for multiple comparisons (*p<0.05) with control (0µM) within each data series.

3.3.2 Effect of 1 hour exposure to CBZ, VPA, LEV, and LTG on basal and induced MDA concentrations in SH-SY5Y cells

One hour exposure to CBZ (0-100 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was statistically significant at concentrations of 30 and 100 μM CBZ ($p<0.001$). Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on MDA concentrations, with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.05$ at 1 and 3 μM CBZ; $p<0.001$ at 10 to 100 μM CBZ; figure 3.5A).

One hour exposure to VPA (0-1000 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 100 μM VPA and above ($p<0.05$ at 100 μM VPA; $p<0.001$ at 300 and 1000 μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on MDA concentrations but the pattern of statistical significance was unchanged ($p<0.05$ at 100 μM VPA; $p<0.001$ at 300 and 1000 μM VPA; figure 3.5B).

One hour exposure to LEV (0-300 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was statistically significant at concentrations of 30 μM LEV and above ($p<0.05$ at 30 μM LEV; $p<0.001$ at 100 and 300 μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on MDA concentrations, with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above (all $p<0.001$; figure 3.5C).

One hour exposure to LTG (0-100 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30 and 100 μM LTG (both $p<0.001$). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on MDA concentrations, with statistical significance observed at LTG pre-treatment concentrations of 3 μM and above ($p<0.05$ at 3 and 10 μM LTG; $p<0.001$ at 30 and 100 μM LTG; figure 3.5D).

3.3.3 Effect of 4 hours exposure to CBZ, VPA, LEV, and LTG on basal and induced MDA concentrations in SH-SY5Y cells

Four hours exposure to CBZ (0-100 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was statistically significant at concentrations of 30 and 100 μM CBZ ($p<0.001$). Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on MDA concentrations, with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.05$ at 10μM CBZ; $p<0.001$ at 30 to 100 μM CBZ; figure 3.6A).

Four hours exposure to VPA (0-1000 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 100 μM VPA and above ($p<0.05$ at 100 to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on MDA concentrations but the pattern of statistical significance was unchanged ($p<0.05$ at 100 μM VPA; $p<0.001$ at 300 and 1000 μM VPA; figure 3.6B).

Four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was statistically significant at concentrations of 30 μM LEV and above ($p<0.05$ at 10 to 30μM LEV; $p<0.001$ at 100 and 300 μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on MDA concentrations, with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.05$ at 10 to 30μM LEV; $p<0.001$ at 100 and 300 μM LEV figure 3.6C).

Four hours exposure to LTG (0-100 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30 and above ($p<0.05$ at 10μM LTG; $p<0.001$ at 30 and 100 μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on MDA concentrations, with statistical significance observed at LTG pre-treatment concentrations of 10μM and above ($p<0.05$ at 10μM LTG; $p<0.001$ at 30 and 100 μM LTG; figure 3.6D).

3.3.4 Effect of 24 hours exposure to CBZ, VPA, LEV, and LTG on basal and induced MDA concentrations in SH-SY5Y cells

Twenty four hours exposure to CBZ (0-100 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10 and 100 μM CBZ ($p<0.05$ at 10μM CBZ; $p<0.001$ at 30μM and 100μM CBZ). Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on MDA concentrations, with significant differences from control observed at all CBZ pre-treatment concentrations ($P<0.05$ at 10μM CBZ; $p<0.001$ at 300μM and 1000μM CBZ; figure 3.7A).

Twenty four hours exposure to VPA (0-1000 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 100 μM VPA and above ($p<0.05$ at 100μMVPA; $p<0.001$ at 300μM and 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on MDA concentrations but the pattern of statistical significance was unchanged ($p<0.05$ at 100 μM VPA; $p<0.001$ at 300 and 1000 μM VPA; figure 3.7B).

Twenty four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was statistically significant at concentrations of 30 μM LEV and above ($p<0.001$ at 30μM and 300 μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on MDA concentrations, with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.001$ at 30μM and 300 μM LEV figure 3.7C).

Twenty four hours exposure to LTG (0-100 μM) was associated with a concentration-dependent increase in MDA levels in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30 and above ($p<0.05$ at 3μM and10μM LTG; $p<0.001$ at 30μM and 100 μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on MDA concentrations, with statistical significance observed at LTG pre-treatment concentrations of 10μM and above ($p<0.05$ at 3μM and 10μM LTG; $p<0.001$ at 30μM and 100 μM LTG; figure 3.7D).

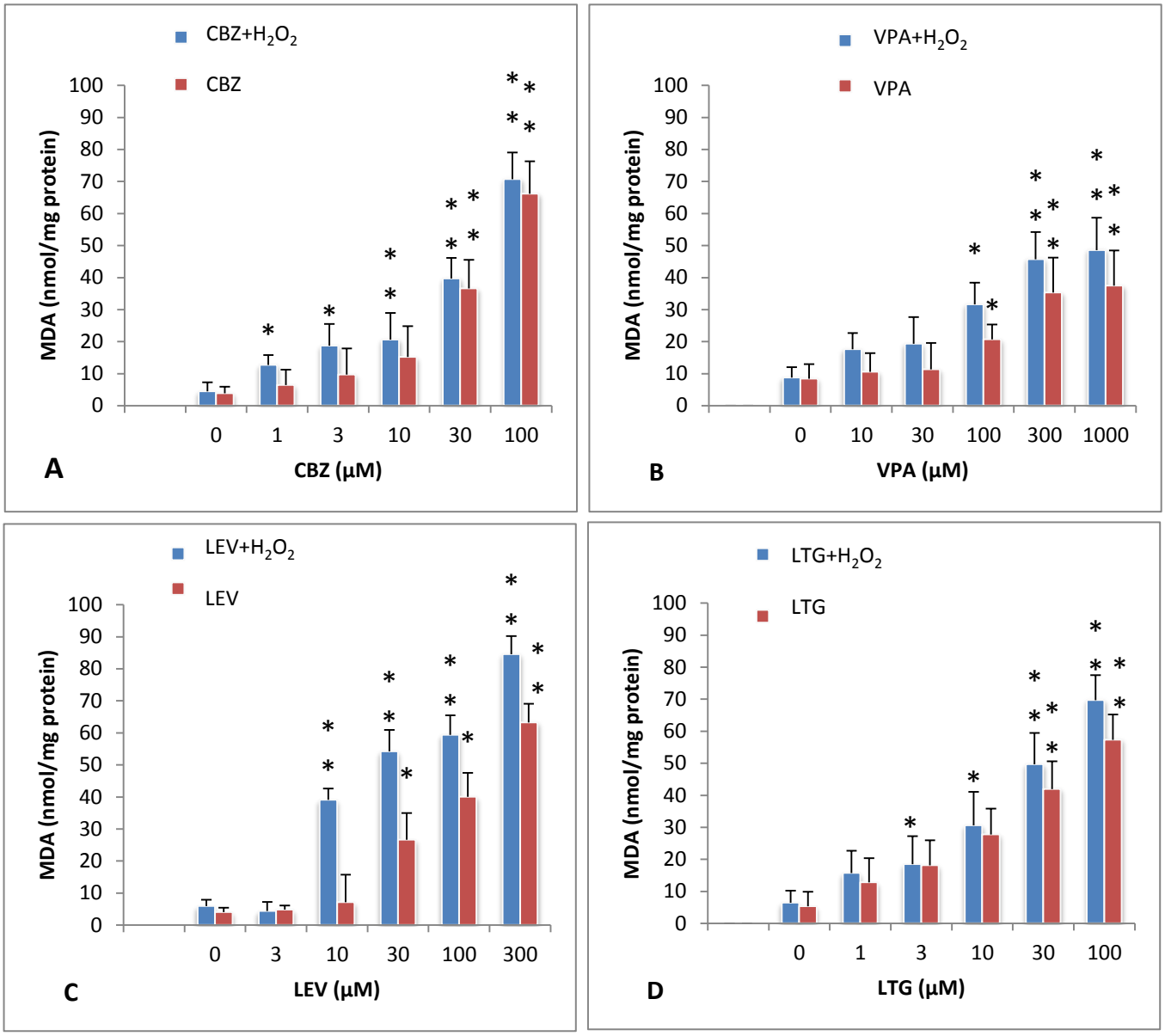


Figure 3.5 Effect of 1 hour exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration (nmol/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

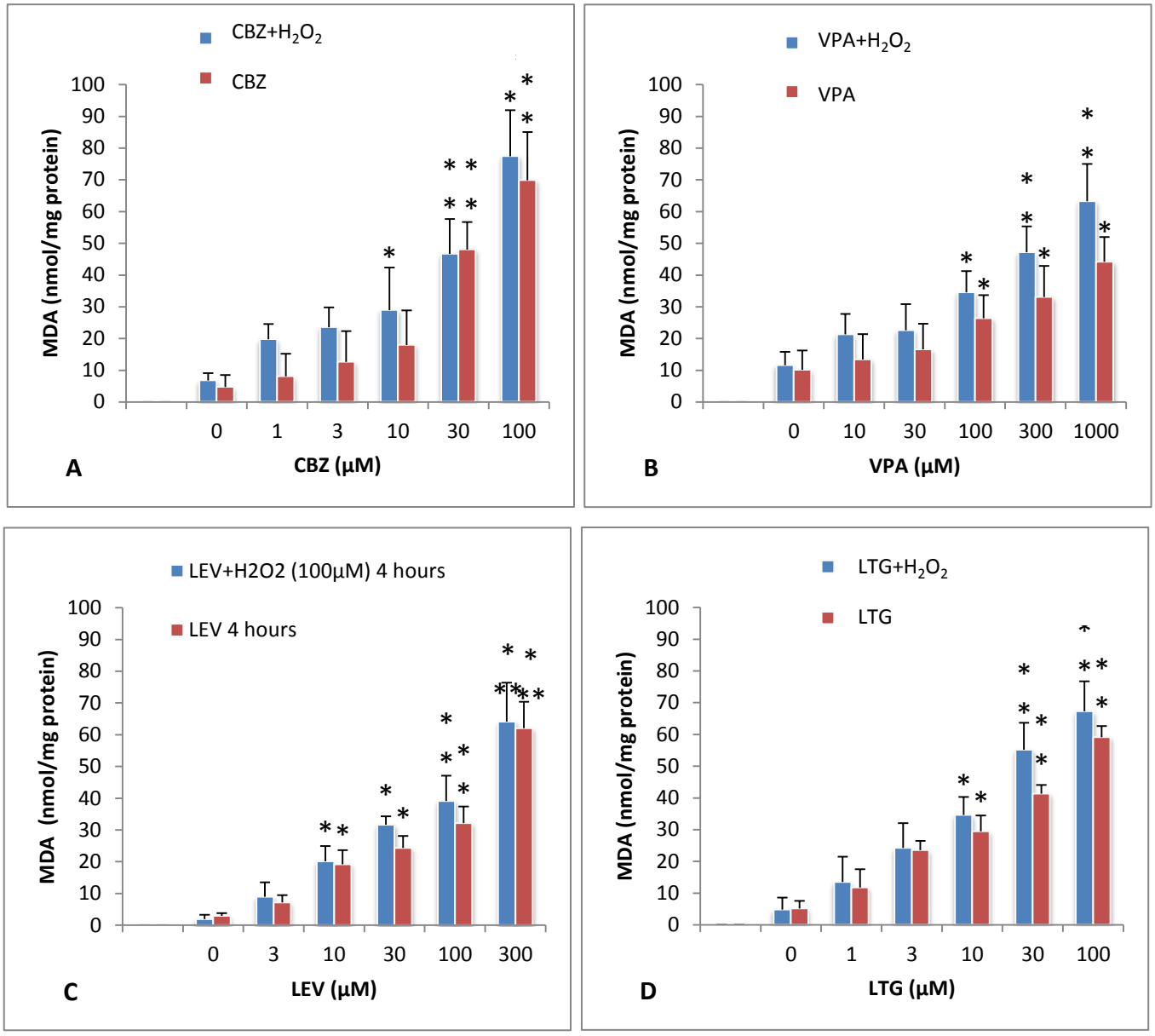


Figure 3.6 Effect of 4 hours exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration (nmol/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

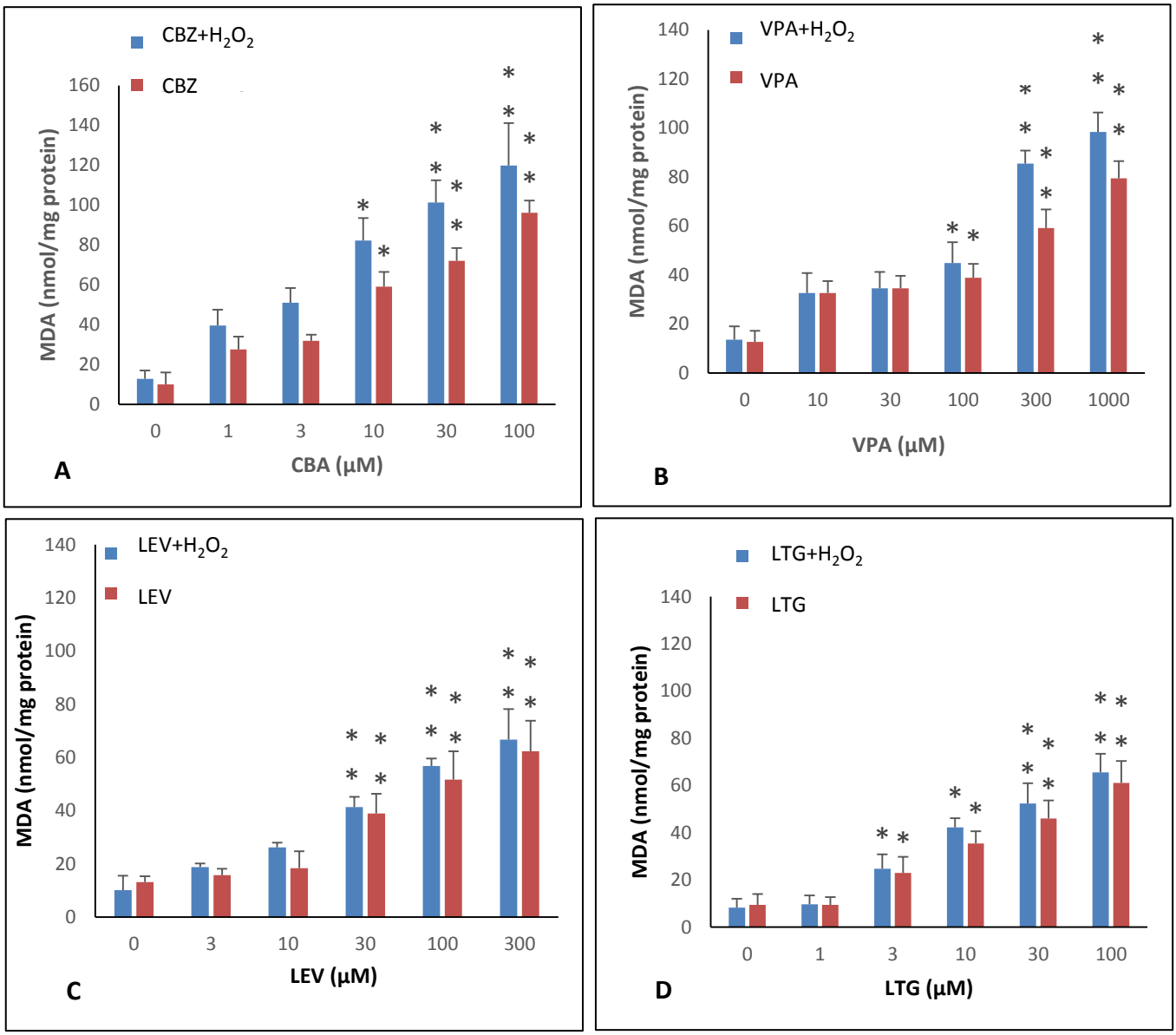


Figure 3.7 Effect of 24 hours exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration (nmol/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

3.3.5 Effect of 1 hour exposure to CBZ, VPA, LEV, and LTG on basal and induced SOD activity in SH-SY5Y cells

One hour exposure to CBZ (0-100 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10 and 100 μM CBZ ($p<0.05$ at 10μM to 100μM CBZ). Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on SOD activity with significant differences from control observed at all CBZ pre-treatment concentrations ($P<0.01$ at 10μM to 100μM CBZ; figure 3.8A).

One hour exposure to VPA (0-1000 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 100 μM VPA and above ($p<0.01$ at 100μM to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on SOD activity but the pattern of statistical significance was unchanged ($p<0.01$ at 100 μM to 1000 μM VPA; figure 3.8B).

One four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was statistically significant at concentrations of 30 μM LEV and above ($p<0.05$ at 30μM and 100 μM LEV; $p<0.01$ at 300 μM VPA). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on SOD activity, with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.05$ at 30μM and 100μM LEV; $p<0.01$ at 300μM LEV; figure 3.8C).

One hour exposure to LTG (0-100 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30μM LTG and above ($p<0.05$ at 30μM and100μM

LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on SOD activity, with statistical significance observed at LTG pre-treatment concentrations of 30μM and above ($p<0.05$ at 30μM and 100μM LTG; figure 3.8D).

3.3.6 Effect of 4 hours exposure to CBZ, VPA, LEV, and LTG on basal and induced SOD activity in SH-SY5Y cells

Four hours exposure to CBZ (0-100 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was not statistically significant at all concentrations CBZ. Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on SOD activity with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.05$ at 10μM to 100μM CBZ; figure 3.9A).

Four hours exposure to VPA (0-1000 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 300 μM VPA and above ($p<0.05$ at 300μM to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on SOD activity but the pattern of statistical significance was unchanged ($p<0.05$ at 300 μM to 1000 μM VPA; figure 3.9B).

Four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10 μM LEV and above ($p<0.05$ at 10μM to 300 μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on SOD activity, with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.05$ at 30μM to 300μM LEV; figure 3.9C).

Four hours exposure to LTG (0-100 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30μM LTG and above ($p<0.05$ at 10μM to 100μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on SOD activity, with statistical significance observed at LTG pre-treatment concentrations of 10μM and above ($p<0.05$ at 10μM to 100μM LTG; figure 3.9D).

3.3.7 Effect of 24 hours exposure to CBZ, VPA, LEV, and LTG on basal and induced SOD activity in SH-SY5Y cells

Twenty four hours exposure to CBZ (0-100 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30μM CBZ and above($p<0.05$ at 30μM and 100μM CBZ) . Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on SOD activity with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.05$ at 1μM to 100μM CBZ; figure 3.10A).

Twenty four hours exposure to VPA (0-1000 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30 μM VPA and above ($p<0.01$ at 30μM to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on SOD activity but the pattern of statistical significance was unchanged ($p<0.01$ at 30 μM to 1000 μM VPA; figure 3.10B).

Twenty four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10 μM LEV and above ($p<0.01$ at 10μM to 300μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced

its action on SOD activity, with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.01$ at 10μM to 300μM LEV; figure 3.10C).

Twenty four hours exposure to LTG (0-100 μM) was associated with a concentration-dependent decrease in SOD activity in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30μM LTG and above ($p<0.05$ at 30μM and 100μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on SOD activity, with statistical significance observed at LTG pre-treatment concentrations of 30μM and above ($p<0.05$ at 30μM and 100μM LTG; figure 3.10D).

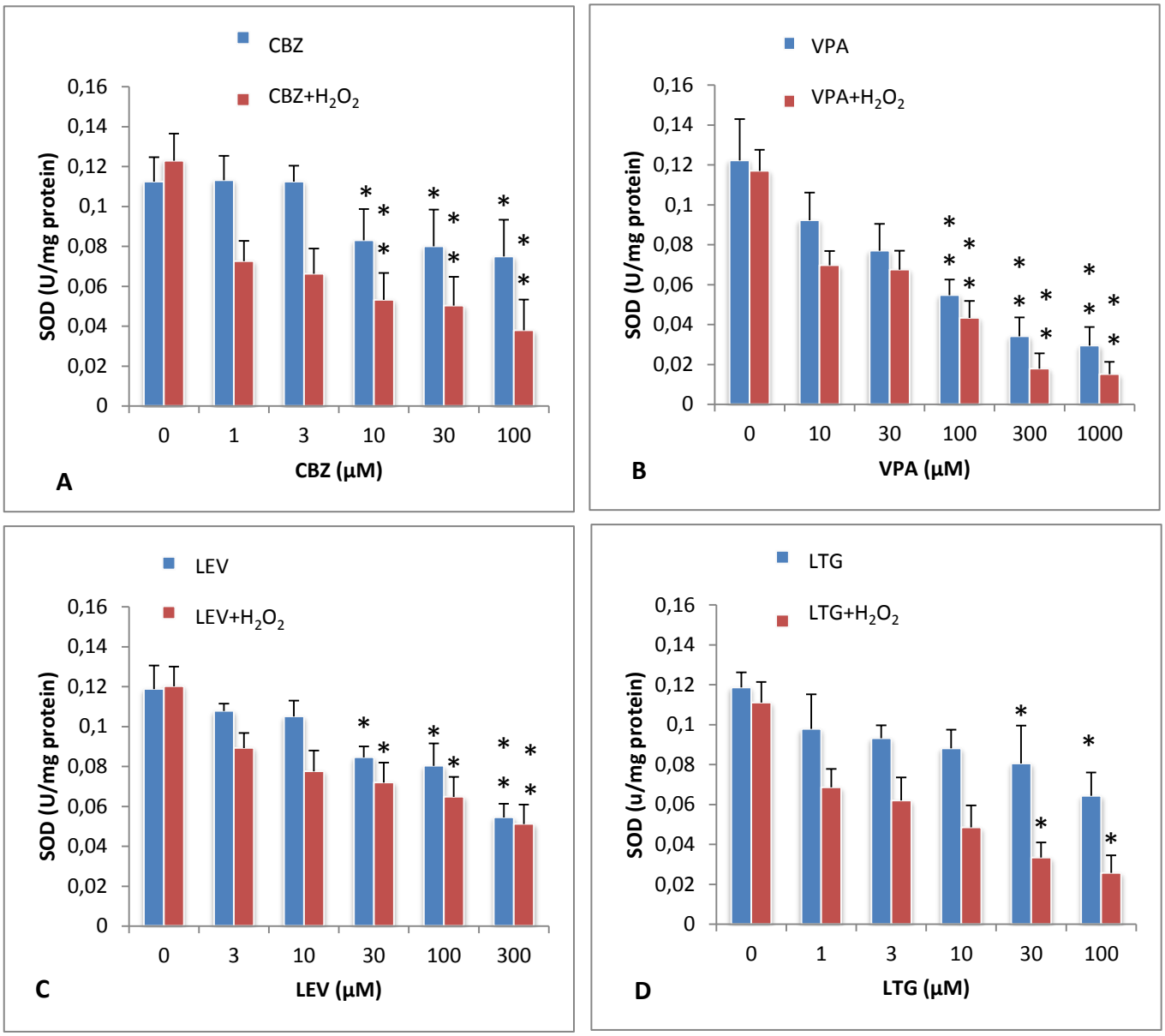


Figure 3.8 Effect of 1 hour exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on SOD activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity (U/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

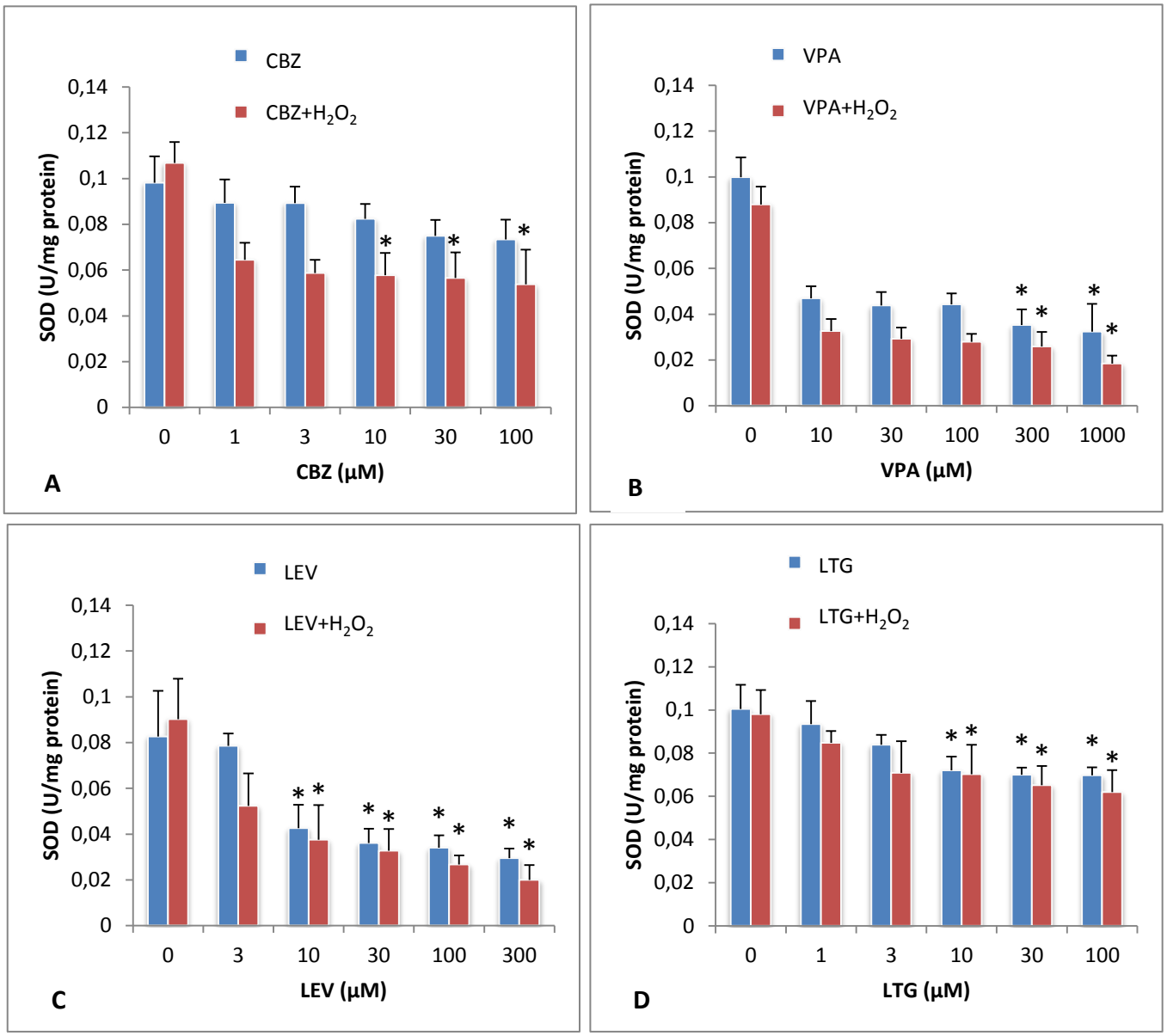


Figure 3.9 Effect of 4 hours exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on SOD activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity (U/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

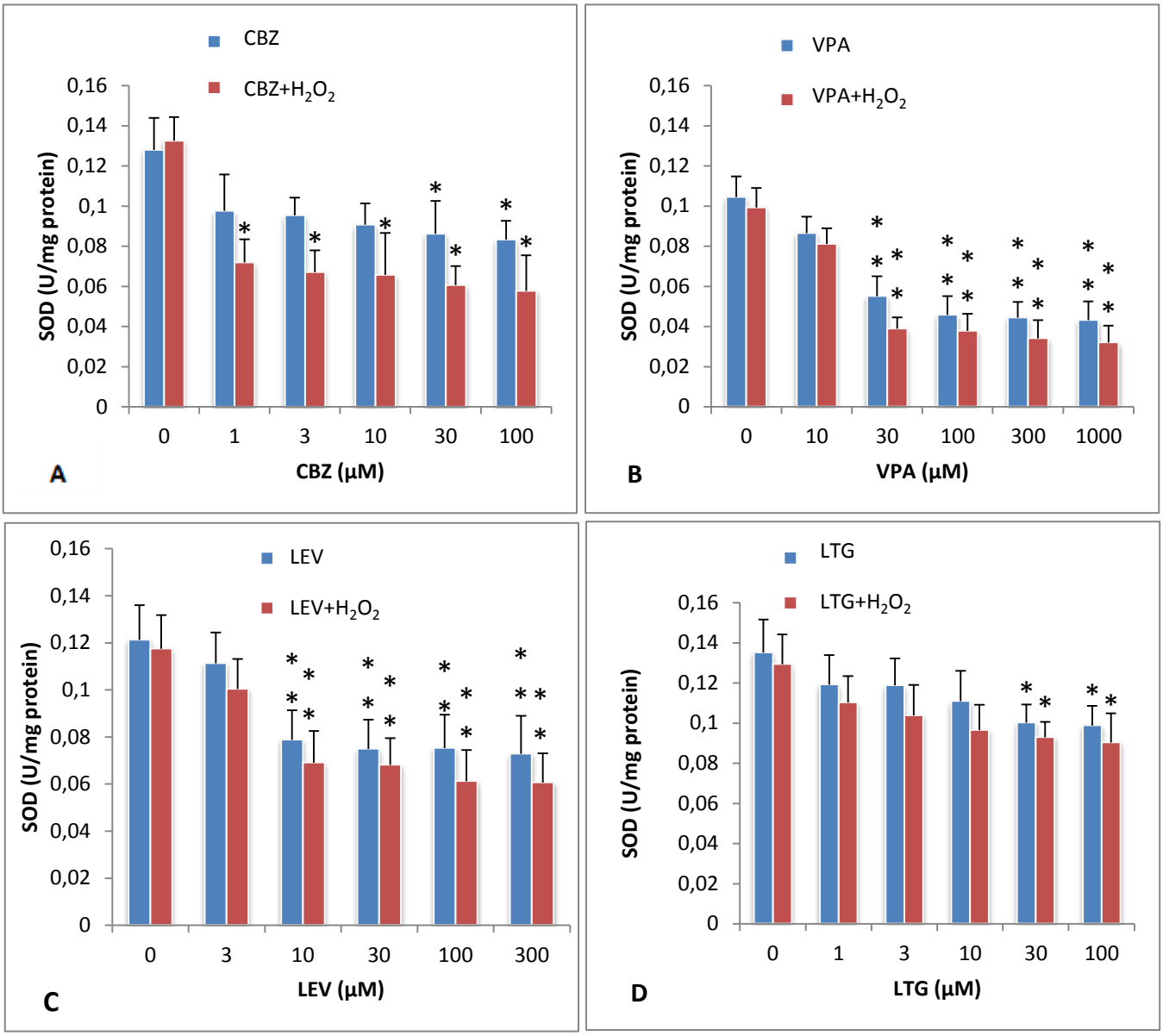


Figure 3.10 Effect of 24 hours exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on SOD activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity (U/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

3.3.8 Effect of 1 hour exposure to CBZ, VPA, LEV, and LTG on basal and induced GSH/GSSG ratio in SH-SY5Y cells

One hour exposure to CBZ (0-100 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 1μM CBZ and above($p<0.01$ at 1μM to 100μM CBZ) . Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on GSH/GSSG ratio with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.01$ at 1μM to 100μM CBZ; figure 3.11A).

One hour exposure to VPA (0-1000 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 100 μM VPA and above ($p<0.05$ at 100μM to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on GSH/GSSG ratio but the pattern of statistical significance was unchanged ($p<0.05$ at 10μM VPA; $p<0.01$ at 30μM to 1000 μM VPA; figure 3.11B).

One hour exposure to LEV (0-300 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10 μM LEV and above ($p<0.05$ at 10μM and 30μM LEV; $p<0.01$ at 100μM and 300μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on GSH/GSSG ratio with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.05$ at 10μM ; $p<0.01$ at 30μM to 300μM LEV; figure 3.11C).

One hour exposure to LTG (0-100 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different

from control at concentrations of 30μM LTG and above ($p<0.05$ at 30μM; $p<0.01$ at 100μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on GSH/GSSG ratio, with statistical significance observed at LTG pre-treatment concentrations of 30μM and above ($p<0.05$ at 30μM; $p<0.01$ at 100μM LTG; figure 3.11D).

3.3.9 Effect of CBZ, VPA, LEV, and LTG and H_2O_2 on GSH/GSSG ratio after 4 hours exposure in SH-SY5Y cells

Four hours exposure to CBZ (0-100 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 1μM CBZ and above($p<0.05$ at 30μM and 100μM CBZ) . Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on GSH/GSSG ratio with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.05$ at 1μM to 100μM CBZ; figure 3.12A).

Four hours exposure to VPA (0-1000 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 30 μM VPA and above ($p<0.01$ at 30μM to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2 for 4 hours appeared to augment its effect on GSH/GSSG ratio but the pattern of statistical significance was unchanged ($p<0.01$ at 10μM to 1000 μM VPA; figure 3.12B).

Four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10 μM LEV and above ($p<0.01$ at 10μM to 300μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced

its action on GSH/GSSG ratio with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.01$ at 10μM to 300μM LEV; figure 3.12C).

Four hours exposure to LTG (0-100 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 10μM LTG and above ($p<0.05$ at 10μM; $p<0.01$ at 30μM and 100μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on GSH/GSSG ratio, with statistical significance observed at LTG pre-treatment concentrations of 10μM and above ($p<0.05$ at 10μM; $p<0.01$ at 30μM and 100μM LTG; figure 3.12D).

3.3.10 Effect of CBZ, VPA, LEV, and LTG and H_2O_2 on GSH/GSSG ratio after 24 hours exposure in SH-SY5Y cells

Twenty four hours exposure to CBZ (0-100 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 1μM CBZ and above($p<0.01$ at 1μM to 100μM CBZ) . Subsequent exposure of CBZ-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on GSH/GSSG ratio with significant differences from control observed at all CBZ pre-treatment concentrations ($p<0.01$ at 1μM to 100μM CBZ; figure 3.13A).

Twenty four hours exposure to VPA (0-1000 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 10 μM VPA and above ($p<0.01$ at 10μM to 1000μM VPA). Subsequent exposure of VPA-treated cells to 100 μM H_2O_2

for 4 hours appeared to augment its effect on GSH/GSSG ratio but the pattern of statistical significance was unchanged ($p<0.01$ at 10μM to 1000 μM VPA; figure 3.13B).

Twenty four hours exposure to LEV (0-300 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was statistically significant at concentrations of 10μM LEV and above ($p<0.05$ at 10μM and 30μM LEV; $p<0.01$ at 100μM and 300μM LEV). Subsequent exposure of LEV-treated cells to 100 μM H_2O_2 for 4 hours enhanced its action on GSH/GSSG ratio with significant differences from control observed at LEV pre-treatment concentrations of 10 μM and above ($p<0.05$ at 3μM LTG; $p<0.01$ at 10μM to 300μM LEV; figure 3.13C).

Twenty four hours exposure to LTG (0-100 μM) was associated with a concentration-dependent decrease in GSH/GSSG ratio in SH-SY5Y cells, an effect that was significantly different from control at concentrations of 10μM LTG and above ($p<0.01$ at 10μM to100μM LTG). Subsequent exposure of LTG-treated cells to 100 μM H_2O_2 for 4 hours enhanced its effect on GSH/GSSG ratio, with statistical significance observed at LTG pre-treatment concentrations of 1μM and above ($p<0.01$ at 1μM to 100μM LTG; figure 3.13D).

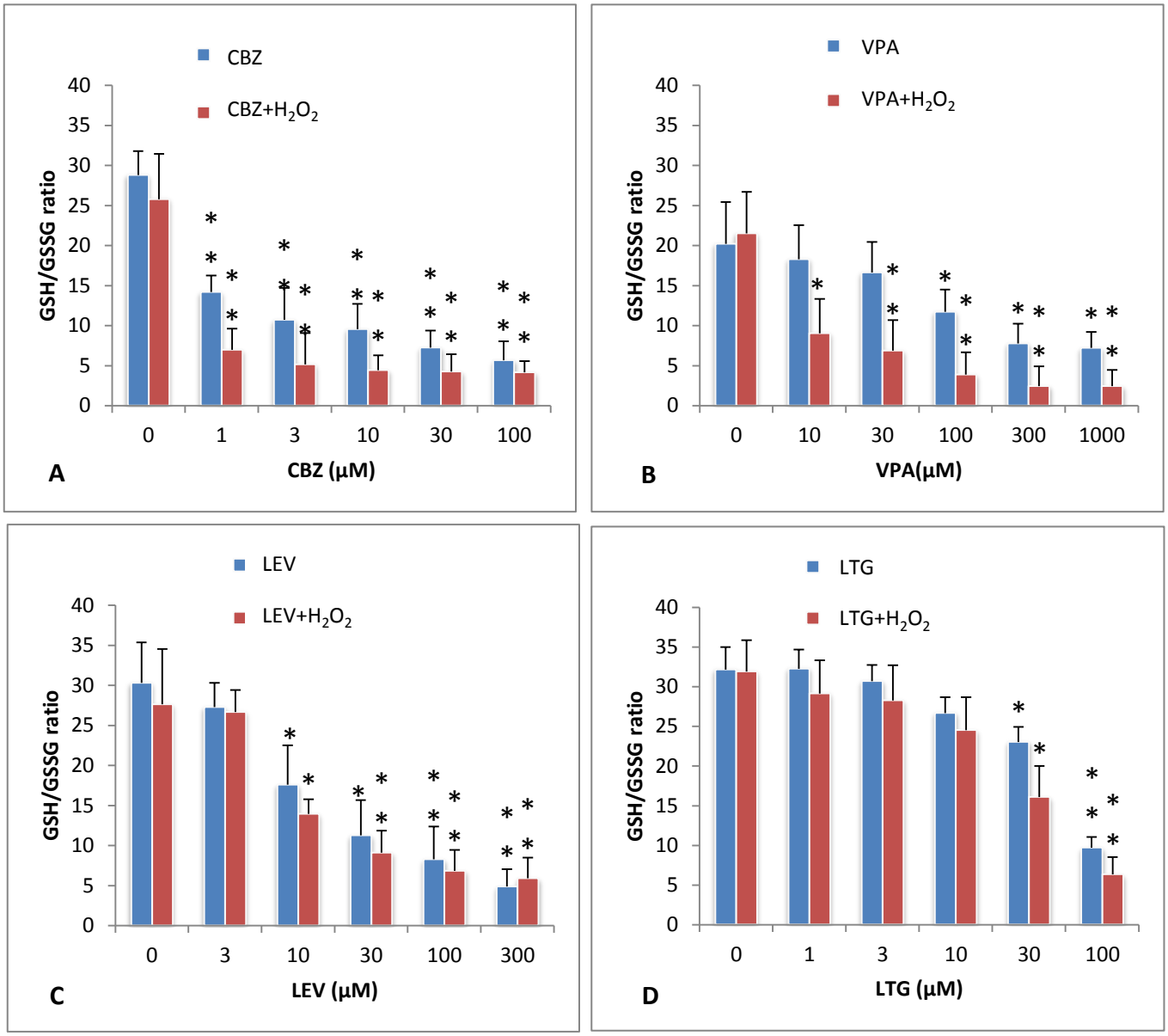


Figure 3.11 Effect of 1 hour exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on GSH/GSSG ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) GSH/GSSG ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

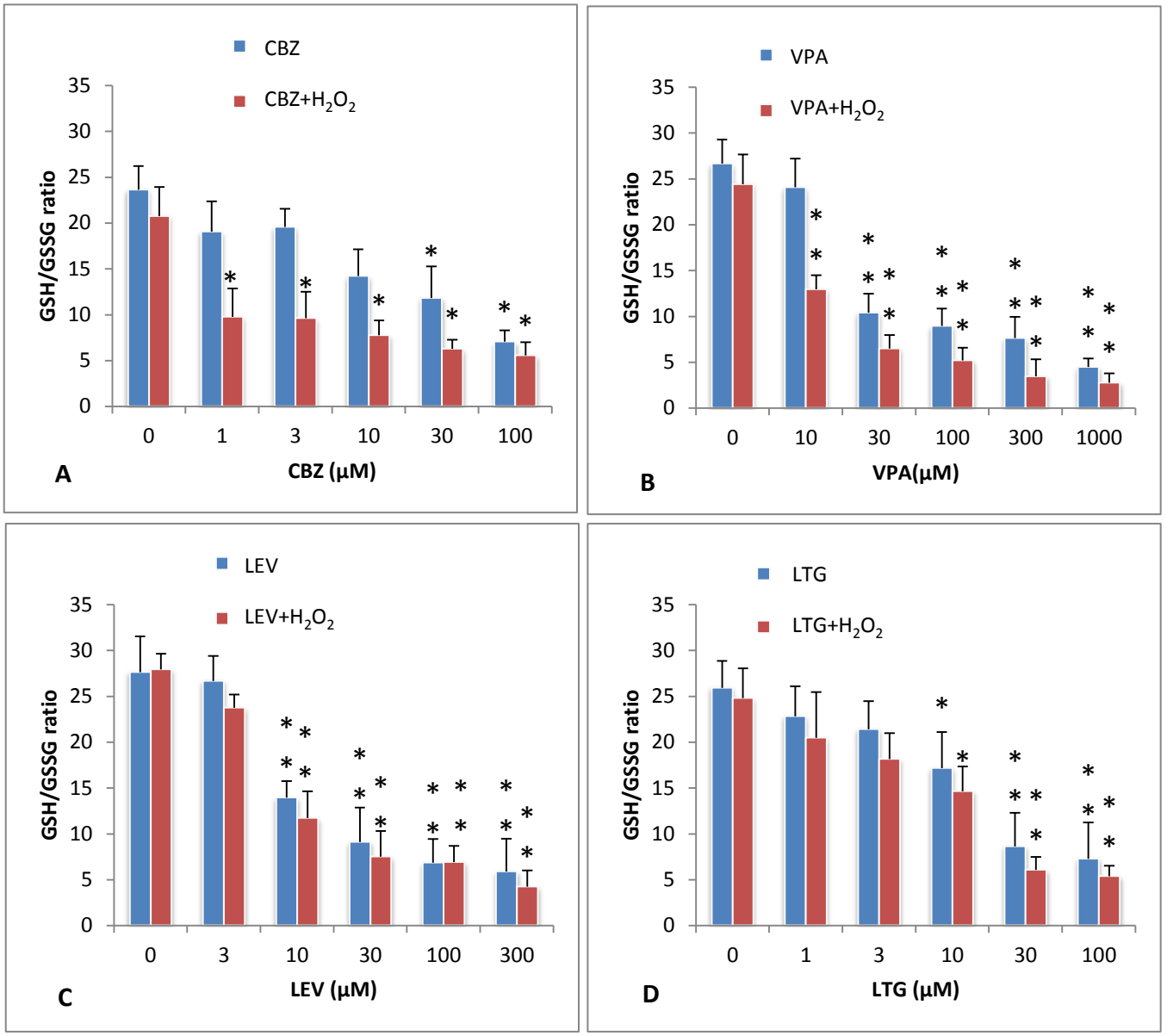


Figure 3.12 Effect of 4 hours exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on GSH/GSSG ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) GSH/GSSG ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

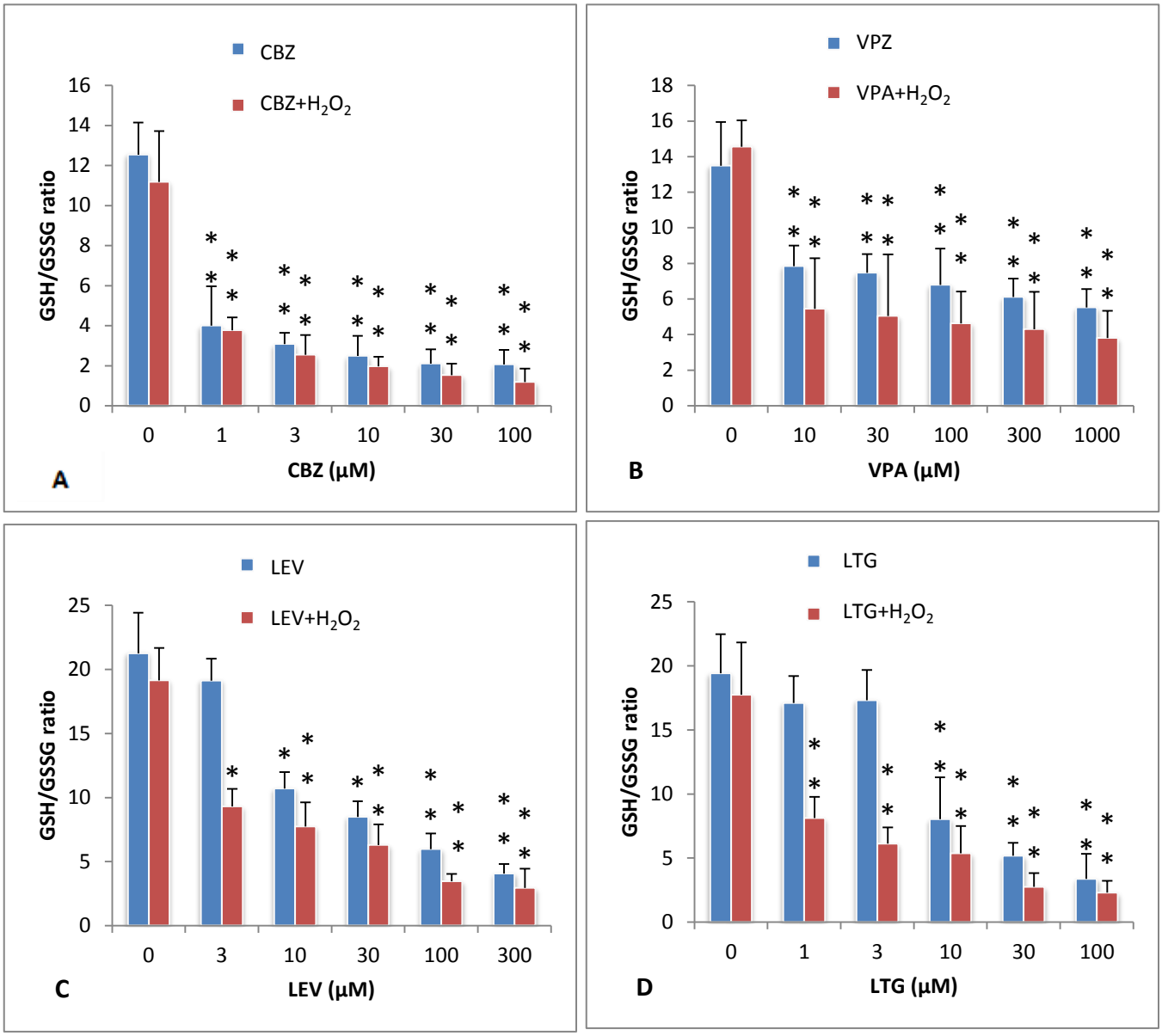


Figure 3.13 Effect of 24 hours exposure to (A) carbamazepine (CBZ; 0-100 µM), (B) sodium valproate (VPA; 0-1000 µM), (C) levetiracetam (LEV; 0-300 µM) and (D) lamotrigine (LTG; 0-100 µM), with and without subsequent exposure to 100 µM H_2O_2 for 4 hours, on GSH/GSSG ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) GSH/GSSG ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

3.3.11 Effect of selected AEDs on MDA concentrations in SH-SY5Y cells following 4-hour pre-treatment with H_2O_2

Four hour exposure of SH-SY5Y cells to H_2O_2 (0-1000 μM) was associated with a concentration-related increase in MDA levels that was significantly different from control at 100μM H_2O_2 and above ($p<0.05$ at 100 μM H_2O_2; $p<0.001$ at 300 and 1000 μM H_2O_2; figure 3.14). Subsequent 1 hour exposures to either 10 μM CBZ or 100 μM VPA enhanced the effect of H_2O_2, with statistical significance observed in the CBZ group at H_2O_2 concentrations of 30 μM and above ($p<0.05$ at 30 and 100 μM H_2O_2; $p<0.001$ at 300 and 1000 μM H_2O_2; figure 3.14) and, likewise, in the VPA group at H_2O_2 concentrations of 30 μM and above ($p<0.05$ at 30 μM H_2O_2; $p<0.001$ at 100 to 1000 μM H_2O_2; figure 3.14).

Four hour exposure of SH-SY5Y cells to H_2O_2 (0-1000 μM) was associated with a concentration-related increase in MDA levels that was significantly different from control at 100μM H_2O_2 and above ($p<0.05$ at 100 and 300 μM H_2O_2; $p<0.001$ at 1000 μM H_2O_2; figure 3.15). Subsequent 1 hour exposures to either 30 μM LEV or 10 μM LTG enhanced the effect of H_2O_2, with statistical significance observed in the LEV group at H_2O_2 concentrations of 30 μM and above ($p<0.05$ at 30 to 300 μM H_2O_2; $p<0.001$ at 1000 μM H_2O_2; figure 3.15) and, likewise, in the LTG group at H_2O_2 concentrations of 30 μM and above ($p<0.05$ at 30 μM H_2O_2; $p<0.001$ at 100 to 1000 μM H_2O_2; figure 3.15).

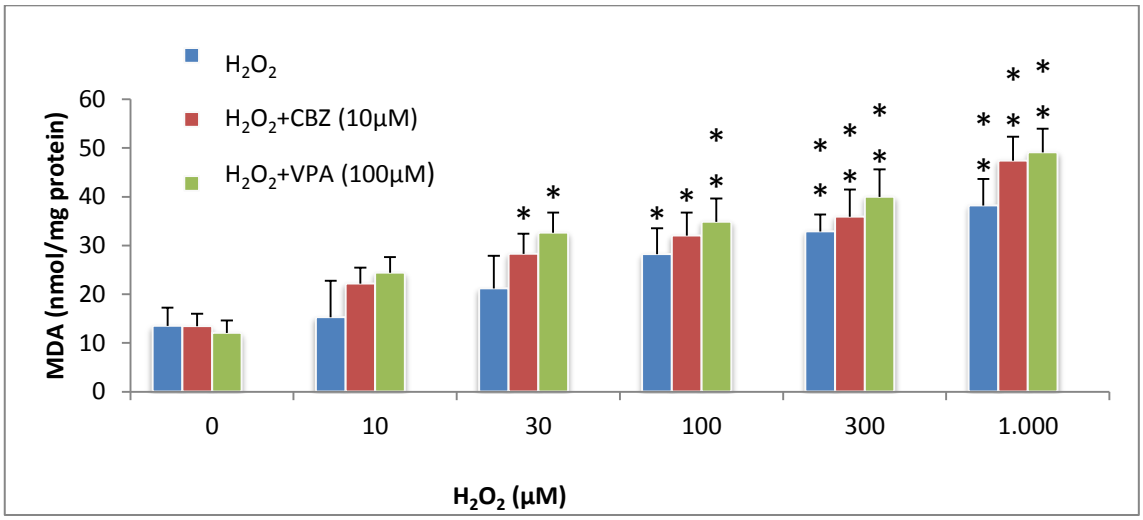


Figure 3.14 Effect of 4 hour exposure to 0-1000 µM H_2O_2, with or without subsequent 1 hour exposures to either 10 µM carbamazepine (CBZ; red bars) or 100 µM sodium valproate (VPA; green bars), on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentration (in nmol/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

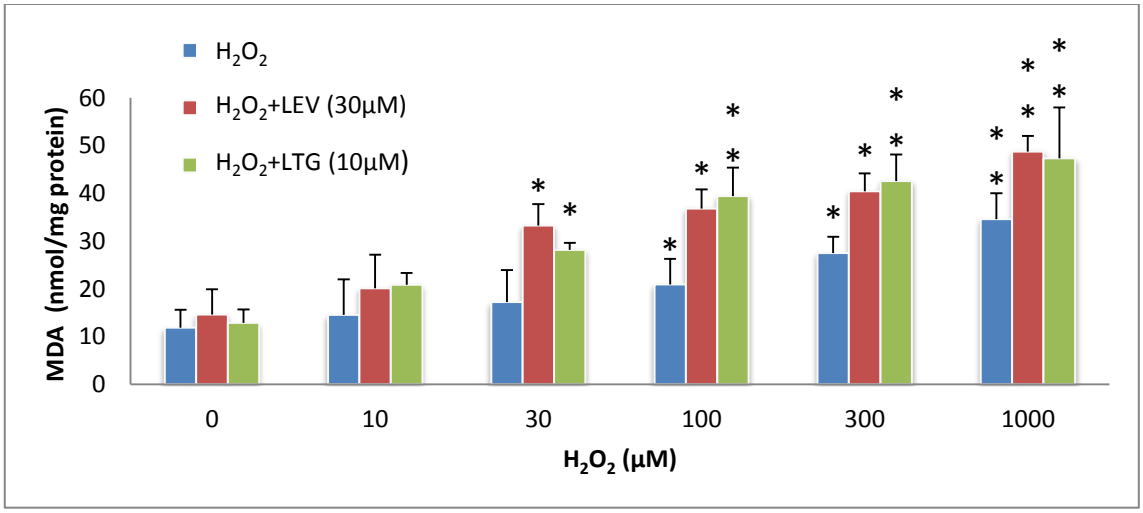


Figure 3.15 Effect of 4 hour exposure to 0-1000 µM H_2O_2, with or without subsequent 1 hour exposures to either 30 µM levetiracetam (LEV; red bars) or 10 µM lamotrigine (LTG; green bars), on malondialdehyde (MDA) concentration in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) MDA concentrations (in nmol/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

3.3.12 Effect of H_2O_2 and fixed concentrations of CBZ and VPA on SOD activity after 4 hours exposure in SH-SY5Y cells

Four hour exposure of SH-SY5Y cells to H_2O_2 (0-1000 μM) was associated with a concentration-related decrease in SOD activity that was significantly different from control at 100μM H_2O_2 and above ($p<0.05$ at 100 μM H_2O_2; $p<0.001$ at 300 and 1000 μM H_2O_2; figure 3.16). Subsequent 1 hour exposures to either 10 μM CBZ or 100 μM VPA enhanced the effect of H_2O_2, with statistical significance observed in the CBZ group at H_2O_2 concentrations of 30 μM and above ($p<0.001$ at 10 to 1000 μM H_2O_2; figure 3.16) and, likewise, in the VPA group at H_2O_2 concentrations of 100 μM and above ($p<0.001$ at 100 to 1000 μM H_2O_2; figure 3.16).

Four hour exposure of SH-SY5Y cells to H_2O_2 (0-1000 μM) was associated with a concentration-related decrease in SOD activity that was significantly different from control at 100μM H_2O_2 and above ($p<0.05$ at 100 and 300 μM H_2O_2; $p<0.001$ at 1000 μM H_2O_2; figure 3.17). Subsequent 1 hour exposures to either 30 μM LTG or 300 μM LEV enhanced the effect of H_2O_2, with statistical significance observed in the LEV group at H_2O_2 concentrations of 300 μM and above ($p<0.001$ at 300μM and above H_2O_2; figure 3.17) and, likewise, in the LTG group at H_2O_2 concentrations of 30 μM and above ($p<0.05$ at 30 to 100 μM H_2O_2; $p<0.001$ at 300 to 1000 μM H_2O_2; figure 3.17).

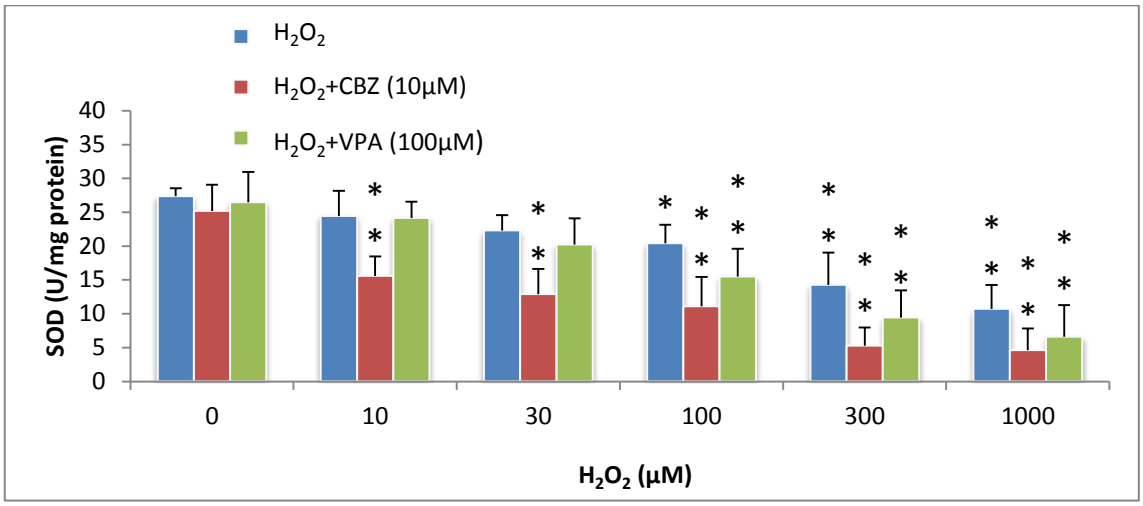


Figure 3.16 Effect of 4 hours exposure to 0-1000 µM H_2O_2, with or without subsequent 1 hour exposures to either 10 µM carbamazepine (CBZ; red bars) or 100 µM sodium valproate (VPA; green bars), on SOD activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity (U/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

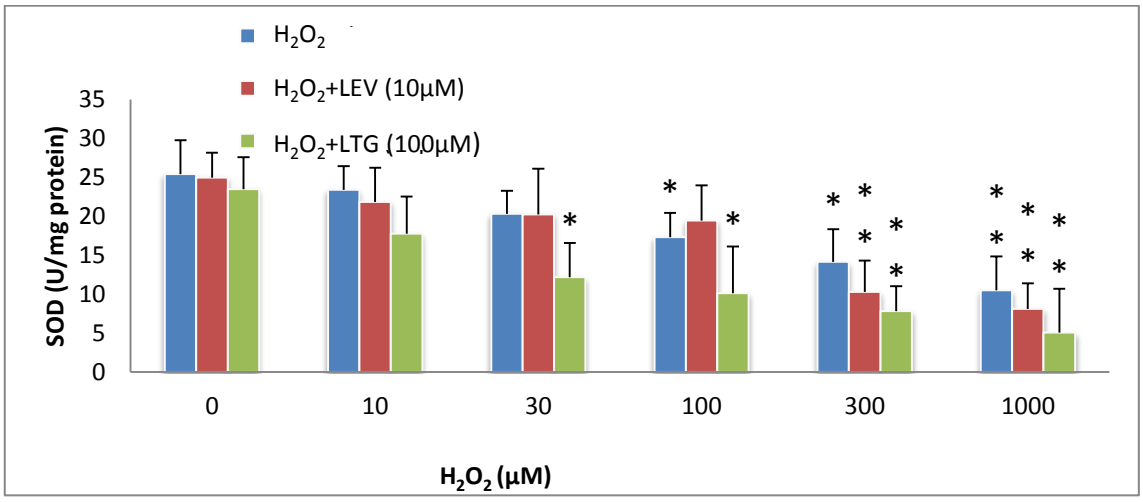


Figure 3.17 Effect of 4 hour exposure to 0-1000 µM H_2O_2, with or without subsequent 1 hour exposures to either 30 µM levetiracetam (LEV; red bars) or 10 µM lamotrigine (LTG; green bars), on SOD activity in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) SOD activity (U/mg protein) and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

3.3.13 Effect of H_2O_2 and fixed concentrations of CBZ and VPA on GSH/GSSG ratio after 4 hours exposure in SH-SY5Y cells

Four hour exposure of SH-SY5Y cells to H_2O_2 (0-1000 μM) was associated with a concentration-related decrease in GSH/GSSG ratio that was significantly different from control at 100μM H_2O_2 and above ($p<0.05$ at 10 μM H_2O_2; $p<0.001$ at 30 and 1000 μM H_2O_2; figure 3.18). Subsequent 1 hour exposures to either 10 μM CBZ or 100 μM VPA enhanced the effect of H_2O_2, with statistical significance observed in the CBZ group at H_2O_2 concentrations of 10 μM and above ($p<0.001$ at 10 to 1000 μM H_2O_2; figure 3.18) and, likewise, in the VPA group at H_2O_2 concentrations of 100 μM and above ($p<0.001$ at 10 to 1000 μM H_2O_2; figure 3.18).

Four hour exposure of SH-SY5Y cells to H_2O_2 (0-1000 μM) was associated with a concentration-related decrease in GSH/GSSG ratio that was significantly different from control at 100μM H_2O_2 and above ($p<0.05$ at 100 and 300 μM H_2O_2; $p<0.001$ at 1000 μM H_2O_2; figure 3.19). Subsequent 1 hour exposures to either 30 μM LTG or 300 μM LEV enhanced the effect of H_2O_2, with statistical significance observed in the LEV group at H_2O_2 concentrations of 10 μM and above ($p<0.001$ at 10μM and above H_2O_2; figure 3.19) and, likewise, in the LTG group at H_2O_2 concentrations of 30 μM and above ($p<0.001$ at 10 to 1000 μM H_2O_2; figure 3.19).

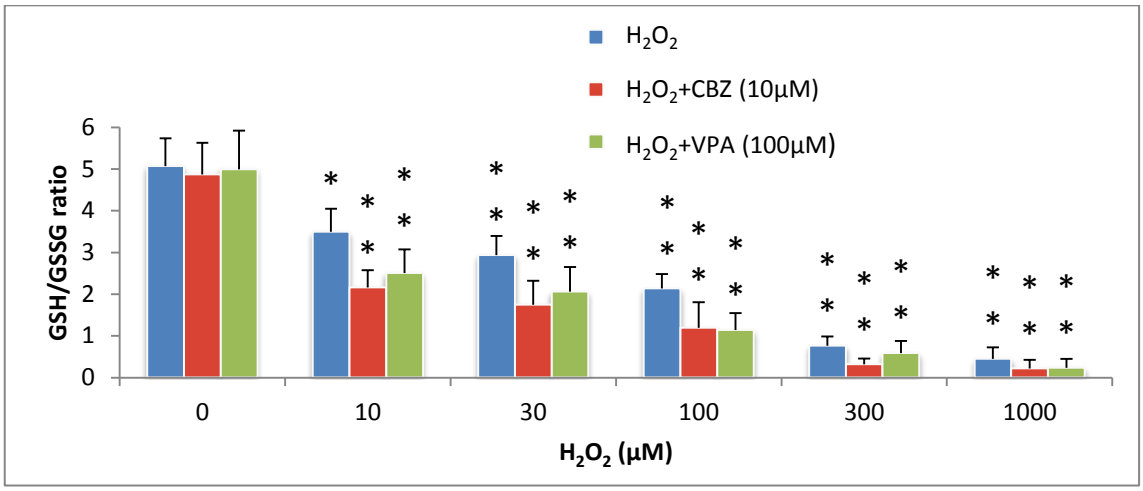


Figure 3.18 Effect of 4 hours exposure to 0-1000 µM H_2O_2, with or without subsequent 1 hour exposures to either 10 µM carbamazepine (CBZ; red bars) or 100 µM sodium valproate (VPA; green bars), on GSH/GSSG ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) GSH/GSSG ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

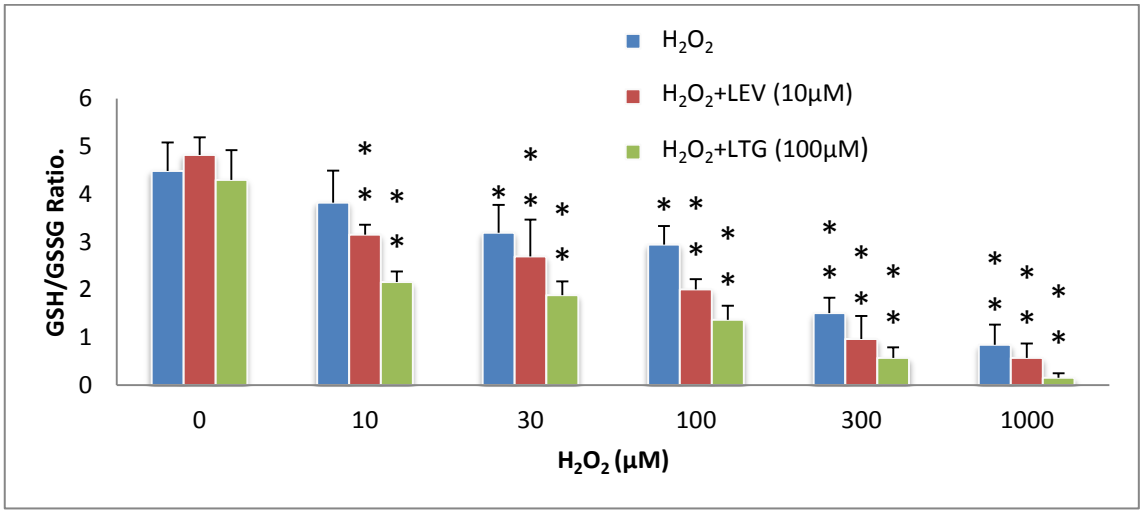


Figure 3.19 Effect of 4 hour exposure to 0-1000 µM H_2O_2, with or without subsequent 1 hour exposures to either 30 µM levetiracetam (LEV; red bars) or 10 µM lamotrigine (LTG; green bars), on GSH/GSSG ratio in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) GSH/GSSG ratio and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons (*p<0.05; **p<0.001) with control (0µM) within each data series.

3.4 Discussion

Oxidative damage to the brain can be initiated by exposure to specific chemicals and is associated with a number of CNS diseases. It has been reported that the increase in ROS and/or RNS or any drop in basal antioxidant activity may increase the frequency of seizures (Willmore et al., 1981; Jesberger et al., 1991). An increase in free radicals may similarly cause neuronal damage as a result of membrane lipid peroxidation, decreased GSH levels and a reduction in the expression and/or activity of antioxidant enzymes.

The current study revealed that incubation of SH-SY5Y cells with different concentrations of CBZ, VPA, LEV and LTG for 1, 4, and 24 hours had a negative effect on cell viability and on the pro-oxidant/antioxidant balance. Subsequent addition of 100 μM H_2O_2 for 4 hours worsened this oxidative damage. Moreover, exposure of SH-SY5Y cells to various concentrations of H_2O_2 caused expected oxidative stress changes which were exacerbated following subsequent exposure to fixed concentrations of the selected AEDs.

Oxidative stress has been defined as an imbalance of oxidantsand antioxidants in favor of the oxidants, potentially leading tocell damage (van der Schalie et al., 2004; Li et al., 2010a). In order to cope with the oxidative damage, organisms have evolved multiple systems of antioxidant defense. Endogenous enzymatic and nonenzymatic antioxidants are essential for the conversion of ROS to harmless substances and for maintenance of cellular metabolism and function (Zhang et al., 2008). The brainis a target for different stressors because of its high sensitiveness to stress-induced degenerative conditions (Li et al., 2010).

Oxidative stress has been suggested as a possible mechanism implicated in the pathogenesis of epilepsy (Chang and Yu, 2010). Various clinical and experimental studies have reported that homeostasis of trace elements (i.e. zinc and copper), membrane lipid peroxidation, and antioxidants are critical for brain function, and are directly or indirectly involved in the pathophysiology of neuronal excitability, neuronal excitotoxicity, seizure recurrence and in the resistance of seizures to treatment with commonly used AEDs (Hamed et al., 2004). In addition, AEDs themselves can alter the homeostasis of trace elements and critically increase by-products of MDA at the expense of protective antioxidant mechanisms, which might lead to an increase in the resistance to drugs, seizure recurrence and unwanted side effects of treatment (Hamed et al., 2004).

Potential benefits of antioxidants, used as add-on therapy, were extensively studied in different *in vitro* or *in vivo* animal models of seizures and epilepsy and in patients with epilepsy (Martinc et al., 2014). It is found that endogenous antioxidants (α-lipoic acid, melatonin, and ubiquinone) play an important role in decreasing nitrite content and lipid peroxidation level, while increased SOD, CAT, and GPx activities, direct scavenging of hydroxyl and other free radicals, by inhibiting NO synthase (Martinc et al., 2014). Further support for the role of free radicals in seizures came from the successful use of exogenously administered antioxidants, as vitamin C and vitamin E, to protect the brain against seizure-induced damage (Ueda et al., 1997; Murashima 1998; Barros et al., 2007; Santos et al., 2009).

The current study confirmed that exposure of cells to different concentrations of selected AEDs resulted in a time and concentration-dependent decrease in viability. Therapeutic concentrations which are considered to be 15-50µM for CBZ (Ching, 2016), 350-700µM for VPA (Brunton et al., 2011), 15-40µM for LTG (Wolf, 1992) and

45-180μM for LEV (Martinc et al., 2012) were included in this study. The average undifferentiated SH-SY5Y cells viability was 65-70% at the selected AEDs therapeutic concentrations. Comparison of the findings with those of other studies confirms that CBZ (2.5μM, 5μM, and 10μM) and valproic acid (1.25mM, 2.5mM, and 5mM) exposure to SW480 colon cancer cell lines for 24, 48, and 72 days decreased cells viability in a time-and-concentration dependent manner and less than 40% at their highest concentrations (Akbarzadeh et al., 2016). This also accords with earlier observations, which showed that CBZ decreased astrocyte cell viability by 50% at 850μM after 48 hours exposure (Pavone and Cardile. 2003). These results are in agreement with (Stockhausen et al., 2005) findings which showed that SH-SY5Y cells exposure to 1-4mM VPA resulted in decteasing cells viability by less than 50% at its highest concentration.

Another important finding was that LEV and LTG decreased the undifferentiated SH-SY5Y cells viability in a-time and-concentration dependent manner. These results did not match those observed in earlier studies which indicated a slight decrease, i.e. about 10%, in astrocyte cells viability after exposure to 300μM LEV and 100μM for 48 hours (Pavone and Cardile, 2003). A possible explanation for this might be that astrocyte cells are more resistant to apoptotic damage than the undifferentiated SH-SY5Y cells (Yasmeen et al., 2015). To the best of our knowledge, there was no previous data about the LTG and LEV viability test done on SH-SY5Y cells.

This study also showed that exposure of undifferentiated SH-SY5Y cells to different concentrations of CBZ, VPA, LEV and LTG for 1, 4 and 24 hours was associated with oxidative stress changes in terms of increased MDA concentration, decreased SOD activity, and decreased GSH/GSSG ratio in a time-and-concentration dependent-manner. These changes were exacerbated by further exposure to H_2O_2 for 4 hours.

These findings corroborate the ideas of (Li et al., 2010), who suggested the antioxidant defense system in brain homogenates was affected under the oxidative stress induced by CBZ. In antioxidant enzymes, SOD is always considered as the first line of defense against ROS due to the inhibitory effects on oxyradical formation (Firat et al., 2009). They found that SOD activities were strongly inhibited along with increased CBZ concentration and prolonged incubation period, which could be due to the flux of superoxide radicals, resulting in H_2O_2 increase in the cell (Zhang et al., 2008). These results reflect those of (Hamed et al., 2004; Shin et al., 2011) who also found that in CBZ treated patients there was a significant increase in MDA level and a decrease in total antioxidant activity. This study supports evidence from previous observations indicate that VPA increases plasma and hepatic levels of 15-F2-IsoP, TBARs level, and lipid hydroperoxide , a marker for oxidative stress, in rats (Vincent et al., 2005). Similarly, in another study it has been suggested that patients on VPA therapy may be at risk of developing oxidative stress resulting in DNA oxidation as shown by the remarkably high 8- hydroxydeoxyguanosine (8-OHdG) levels, as a marker of oxidative stress, evaluated in their sera (Schulpis et al., 2006). In another study, these results are in agreement with (Cengis et al., 2000) findings which evaluated the effects of VPA and CBZ therapy on erythrocyte GSH, GSH-Px, SOD, and lipid peroxidation in epileptic children. They found that GSH levels were reduced and may result in an increase of organic hydroperoxides (Cengiz et al., 2000).

The pro-oxidant effects of the newer AEDs, LEV and LTG, are less widely reported. To the best of our knowledge, there is just one previous report that investigated the effect of LEV on oxidative stress markers. LEV was shown to cause oxidative stress in epilepsy patients by increasing urinary 15F-2t-isoprostane level which is a highly reliable marker of oxidative damage (Ozden et al., 2010). Likewise, these results

supports evidence from previous observations by (Varoglu et al., 2010) which suggested an increase in serum 8-OHdG by 3 fold with LEV, and by 4 fold with CBZ and VPA treatment for 2 months. In respect of LTG, there is again, to the best of our knowledge, just one report that assessed the effect of LTG on oxidative insults. It was found that LTG increased MDA concentration 2-fold and decreased catalase activity 3-fold in comparison to control, using a rat liver tissue model (Poorrostami A. et al., 2014). The mechanism behind LTG-induced liver injury is not clearly explained. Some reports have suggested this may be caused by an immune reaction to the arene oxide metabolite of LTG, leading to an inflammatory process and oxidative stress (Mecarelli et al., 2005). Another important finding was that incubation of SH-SY5Y cells with fixed concentrations of AEDs exacerbated the expected oxidative stress changes H_2O_2. These results support previous findings in present study which proved the pro-oxidant behaveour of the selected AEDs.

The methods have been used in this experiment are considered reliable as they have been used by many researchers previously. SH-SY5Y cells are widely used as a suitable model to investigate neurononal disorders and to induce oxidative stress (Akki et al., 2018). Nevertheless, the findings of the current study are limited because of low n-numbers, lack of independent validation of findings, and potentially by the use of cell lines at a variable passage number, particularly with respect to comparison between these results and those in previous chapters. It is well known that passage number has an impact on growth rates and on physiological and chemical characteristics of immortalised cell lines. It should also be recognized that a weakness of *in vitro* cell cultures is that they are studied in isolation and in the absence of their normal environment, which includes interactions with other cell types that might be critical to the process being tested. In such cases, *in vivo* testing is often preferred because it is

better suited for observing the overall effects of experiment conditions or an intervention on a living subject.

3.4.1 Conclusions

In conclusion, the current study revealed negative effects of the selected AEDs on the pro-oxidant / anti-oxidant balance which were both time- and concentration-dependent and which were excacerbated by subsequent exposure of cells to H_2O_2. The reverse was also true, in that the expected expected oxidative stress observed with H_2O_2 was boosted, rather than attenuated, by subsequent exposure to AEDs. This pro-oxidant behaviour of AEDs that was observed throughout these experiments was unexpected but may be related to covalent binding of their toxic metabolites, resulting in cell stress and death.

Chapter 4

Effects of oxidative stress inducers and selected AEDs on Nrf2, HO-1 and NQO-1 gene expression in SH-SY5Y cells

4.1 Introduction

Oxidative damage and mitochondrial dysfunction are currently accepted as key hallmarks of classic inflammatory, degenerative, systemic, and neuronal diseases (Floyd and Carney, 1992; Valko et al., 2006). ROS perform diverse physiological functions and play important roles in host defence, cell signalling, regulation of gene expression, and cell differentiation. ROS levels are tightly regulated in the CNS because the brain is particularly sensitive to oxidative stress (Halliwell and Gutteridge, 1985; Valko et al., 2004). When CNS ROS levels increase as a result of pathological conditions, the cellular antioxidant capacity is overwhelmed and this results in oxidative stress and damage to essential cellular structures (Wood-Kaczmar et al., 2006; Henchcliffe and Beal., 2008). To counteract the detrimental effects of ROS and to restore the delicate redox balance in the CNS, cells are equipped with endogenous antioxidant defence mechanisms that comprise several antioxidant enzymes (Aparicio et al., 2015).

Antioxidants can be divided into direct and indirect varieties depending on the mechanism by which protection against oxidative stress is delivered (Aparicio et al., 2015). Direct antioxidants modify reactive oxygen and nitrogen radicals by donating hydrogen ions or electrons, while indirect anti-oxidants exert effects via upregulation of phase II detoxification and anti-oxidant enzymes through activation of Nrf2 (Nguyen et al., 2003; Nguyen et al., 2004).

Nrf2 has been described as the master regulator of the anti-oxidant response. Under homeostatic conditions, Nrf2 transcription is repressed by its negative regulator, Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al, 2003). Following exposure to ROS, Nrf2 dissociates from cytosolic Keap1 and translocates to the nucleus. It subsequently binds to antioxidant response elements (ARE) in the promoter region of hundreds of genes that are involved in antioxidant protection and detoxification, including those

encoding heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO-1), microsomal glutathione S-transferase (mGST), glutathione peroxidase (Gpx), and superoxide dismutase (SOD) (Li et al., 1992; Long et al., 2000).

Many studies have demonstrated that activation of the Nrf2/ARE pathway occurs in response to an excess of ROS such as H_2O_2 (Purdom-Dickinson et al., 2007), inflammation and infection through activation of Toll-like receptors (TLRs) by LPS (Vijayan et al., 2011; Birgisdottir et al., 2013), and activation of M1 neuronal receptors by cholinomimetic compounds such as carbachol and pilocarpine (Espada et al., 2009).

Further evidence suggests that the Nrf2-Keap1 system contributes to protection against various disorders, such as cancer, liver toxicity and inflammation (Fainstein 2007), and has relevance in the pathophysiology of various neurological conditions, such as Alzheimer's disease (Yehiely et al., 1997), Parkinson's disease (Clements et al., 2006), multiple sclerosis (Schreibelt et al., 2007), amyotrophic lateral sclerosis (Pehar et al., 2005), Huntington's disease (Van Roon-Mom et al., 2008), and epilepsy (Kraft et al., 2006).

Currently, it is believed that sustained neuronal hyperactivity and seizures can lead to neuronal injury and death as a result of underlying biochemical mechanisms, including the formation of excessive ROS (Mariani et al., 2005), and subsequently induced abnormal structural alterations to cellular proteins, membrane lipids, DNA, and RNA.

A strong line of evidence supports the hypothesis that oxidative stress is an underlying mechanism in the initiation and progression of epilepsy and also contributes to neuronal degeneration in the epileptic focus (Shin et al., 2011). Nrf2 and its related gene cascade, which includes as HO-1 and NQO-1, have been suggested as neuroprotective therapeutic strategies for the treatment of epilepsy (Aparicio et al., 2015).

The aim of treatment of epilepsy is to control seizures with the most appropriate AED without causing any significant side effects. The commonly used AEDs such as CBZ, VPA, LTG, and LEV are intended to provide symptomatic relief by inhibiting seizure generation by targeting neuronal hyper-excitability. However, it is unclear whether they are able to modulate or prevent the underlying epileptogenic process in high risk patients. Currently, there is no available treatment known to inhibit the process of epileptogenesis (Choi 1993; Aparicio et al, 2015).

Until now, the majority of research exploring the relationship between AEDs and oxidative stress has been either clinical or has involved *in vivo* models of epilepsy (Cengiz et al., 2000; Arora et al., 2010). One of the few *in vitro* studies to be published used rat primary astrocyte cell cultures which have higher concentration of reduced glutathione and higher levels of antioxidant enzymes than cells from a neuronal lineage (Cardile et al., 2000).

4.1.1 Aims and objectives

The present study aimed to:

1- Investigate the effects of LPS, H_2O_2 and pilocarpine on Nrf2, HO-1, and NQO-1 gene expression as a marker of phase II detoxification antioxidant response in the SH-SY5Y neuroblastoma cell line.

2- Choose optimum conditions (inducer, concentration of inducer, duration of exposure) for induction of oxidative stress in SH-SY5Y cells to be employed in further investigations.

3- Explore the effects of carbamazepine (CBZ), valproic acid (VPA), levetiracetam (LEV), and lamotrigine (LTG) on the expression of Nrf2, HO-1 and NQO-1 genes in the SH-SY5Y cell line.

4.2 Materials and methods

4.2.1 Chemicals

Reverse transcription kit, trizole reagent solution for RNA extraction, FAM labelled Nrf2, HO-1, NQO-1, and VIC labelled GAPDH Taqman gene expression assays, Taqman gene expression master mix, isopropanol (IPA), chloroform, and ethyl alcohol were purchased from Thermo Fisher Scientific, Loughborough, UK. Other chemicals were sourced as described in chapter 2, section 2.2.1 and chapter 3, section 3.2.1.

4.2.2 Cell culture

SH-SY5Y cells were cultured as described in chapter 2, section 2.2.2.

4.2.3 Induction of oxidative stress

SH-SY5Y cells were seeded in 12-well plates at a density of 4 x 10^4 cells / well in 1ml culture medium and placed in an incubator containing humidified 95% air and 5% CO_2 at 37^{o}C for 3 days until they reached 80-90% confluence. Thereafter, the existing medium was aspirated and the cells treated with H_2O_2 (0, 10, 30, 100, 300 and 1000µM), pilocarpine (0, 1, 3, 10, 30 and 100µM) or LPS (0, 1, 3, 10, 30 and 100µg/ml) in serum free medium for 1, 4 or 24 hours. In each case, activation of the phase II detoxification antioxidant response was determined by fold-changes in the expression of Nrf2, HO-1 and NQO-1 genes by reverse-transcription polymerase chain reaction (RT-PCR), as described below.

4.2.4 Cell treatment with AEDs

SH-SY5Y cells were seeded in 12-well plates at a seeding density of 4 x 10^4 cells / well in 1 ml culture medium and placed in an incubator containing humidified 95% air and 5% CO_2 at 37^{o}C for 2-3 days until they reached 80-90% confluence. Then, cells were

treated with either CBZ (0, 10, 30, 100, 300 and 1000µM), VPA (0, 10, 30, 100, 300 and 1000µM), LTG (0, 1, 3, 10, 30 and 100µM) or LEV (0, 3, 10, 30, 100 and 300µM) for 24 hours. VPA and LTG were dissolved in serum-free medium (SFM; see chapter 3, section 3.2.3) whereas CBZ and LEV were dissolved in SFM containing 0.2% DMSO. A further batch of SH-SY5Y cells was treated with the same AEDs at the same range of concentrations but was subsequently exposed to 100µM H_2O_2 for an additional 4 hours. Following treatment with AEDs (with or without additional exposure to H_2O_2), total mRNA was isolated and the expression of Nrf2, HO-1 and NQO-1 quantified by RT-PCR.

4.2.5 Isolation of mRNA

After aspirating the medium, SH-SY5Y cells were washed twice with 1ml PBS. Cells were lysed by adding 500µl of trizole reagent to each well. The lysates were transferred to autoclaved Eppendorf tubes, mixed with 100µl of 100% chloroform, shaken vigorously for 15 seconds to obtain a homogenised mixture, and then incubated at room temperature for 10 minutes. Thereafter, lysates were centrifuged at 14000xg for 15 minutes at 4°C which resulted in separation into three distinct phases; a lower, red-coloured phenol-chloroform phase, an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). The aqueous phase containing RNA was transferred carefully to a fresh Eppendorf tube and the remaining solution discarded. Thereafter, 250µl of 100% isopropanol was added to each tube and samples were mixed by vortexing, incubated at room temperature for 10 minutes, and centrifuged at 14000xgfor 10 minutes at 4°C. This caused the RNA to precipitate in the form of a white pellet at the bottom of the tube. The supernatant was carefully aspirated without disturbing pellet, 100µl of 75% ethanol was added and all samples again centrifuged at 14000 xg for 10 minutes at 4°C. Thereafter, the supernatant was again discarded

without disturbing the resulting pellet. The RNA pellet was then air-dried for 15 minutes to allow evaporation of any remaining ethanol, 15µl of RNAase/DNAase free water was added, and samples were mixed gently by pipetting up and down. The quality and quantity of extracted RNA were determined using a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, TX, USA). All samples were stored at -20°C until required for reverse transcription.

4.2.6 Reverse transcription of total RNA

A volume equivalent to 2µg of total RNA for each sample was added to reverse transcription reagents as shown in table 2.1.

Component	Volume in 20µl reaction	Final concentration
DEPC-treated water	Up to 20µl	-
10X RT buffer	2.0µl	1X
25mM MgCl2	1.4µl	1.75mM
10mM dNTP mix (2.5mM each)	4.0µl	0.5mM each
RNase inhibitor (20U/µL)	1.0µl	1.0U/µl
MultiScribe™ RT (50U/µL)	1.0µl	2.5U/µl
Random hexamers (50µM)	1.0µl	2.5µM
Template RNA	Dependent on concentration	2µg per reaction

Table 4.1 Reverse transcription reaction components. DEPC= diethyl pyrocarbonate, RT=reverse transcriptase, MgCl2=magnesium chloride, dNTP= deoxyribonucleotide triphosphate.

Samples were mixed gently and then placed on the heating block of a 96-well Veriti thermal cycler (Applied Biosystems, Warrington, UK). The thermal cycler was programmed to the following reaction cycling parameters; 25^{oC} for 10 mins, 37^{oC} for 30 mins, 95oC for 5 mins, and soak at 4oC indefinitely. On completion of the reaction cycle, samples were again mixed by vortex and briefly centrifuged at high speed for a few seconds. Thereafter, the quality and quantity of the resulting cDNA was determined

using a Nanodrop 8000 Spectrophotometer and cDNA normalised to a concentration of 20ng/µl by dilution with RNAase/DNAase free water.

4.2.7 Gene expression by real-time PCR

Gene expression analysis was carried out using a 7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA). Taqman gene expression assays (FAM-labelled target gene; VIC-labelled endogenous control) and Taqman gene expression master mix were used to perform duplex real-time PCR reactions to assess the levels of mRNA of target genes relative to mRNA levels of GAPDH (housekeeping gene). Composition of the real-time PCR reaction mix is described in Table 2.3. All reactions were performed in a 384-well PCR plate (Thermo Fisher Scientific, TX, USA) using the cycling parameters outlined in in section 4.2.6 above. After cycling, plates were sealed, mixed by vortex for 2 minutes, centrifuged at 2000xg for 5 minutes at 4^0C, and finally loaded into the Taqman Real Time PCR machine. mRNA expression was calculated using the comparative CT method according to the manufacturer's protocol. Fold-changes in expression of target genes relative to the housekeeping gene was reported as 2^(-ΔΔCT).

Table 4.2 Reaction components for real-time PCR

For each sample	Volume per reaction (µl)
TaqMan Gene Expression Assay (target) (20X FAM dye-labelled)	**1**
TaqMan Gene Expression Assay (housekeeping) (20X VIC dye-labelled, primer-limited)	**1**
TaqMan Gene Expression Master Mix (2X)	**10**
RNAse-free water	**4**
cDNA (20ng/µl)	**4**
Total	**20**

4.2.8 Statistical analysis

All experiments were performed using two technical replicates (i.e. analyses of all samples performed in duplicate) and four biological replicates (i.e. n=4 per group). Results were reported as the mean (± SD) 2^(-ΔΔCT) Statistical comparisons were undertaken using either one way or two way ANOVA as appropriate, and with a Dunnett correction for multiple comparisons, using Stats Direct software version 2.7.9. Differences were considered statistically significant at $p \leq 0.05$.

4.3 Results

4.3.1 Effect of H_2O_2, pilocarpine, and LPS on selected gene expression in SH-SY5Y cells

Cells exposed to H_2O_2 (0-1000μM) for 1, 4 and 24 hours showed an increase in gene expression in a time- and concentration dependent-manner. At 1 hour, Nrf2 expression was maximally increased by 7.2 fold in comparison to that of GAPDH (housekeeping gene) following exposure to 1000μM H_2O_2 and was statistically significant ($p<0.001$) at H_2O_2 concentrations of both 300μM and 1000μM in comparison to control (0μM; figure 4.1). At 4 and 24 hours, the maximal increases in Nrf2 expression were 11.9-fold and 15.2-fold, respectively, after exposure to 1000μM H_2O_2 and were significant ($p<0.001$) at all H_2O_2 concentrations above 30μM when compared with control (0μM; figure 4.1). At 1, 4 and 24 hours, HO-1 expression was maximally increased to 3.7-fold, 5.7-fold, and 12.6-fold, relative to the expression of GAPDH, following exposure to 1000μM H_2O_2 and was statistically significant ($p<0.05$) at H_2O_2 concentrations of 300μM and 1000μM compared to control (0μM; figure 4.2). At 1 hour, NQO-1 expression was maximally increased to 3.2-fold relative to GAPDH following exposure to 1000μM H_2O_2 and was statistically significant ($p<0.05$) at 1000μM H_2O_2 when compared to

control (0μM; figure 4.3). At 4 and 24 hours, the maximal increases in NQO-1 expression were 2.9-fold and 16.1-fold, respectively, after 1000μM H_2O_2 exposure and was statistically significant at all concentrations above 100μM ($p<0.05$ at 300μM H_2O_2; $p<0.001$ at 1000μM H_2O_2) in comparison to NQO-1 expression in control (0μM) samples.

Cells exposed to pilocarpine (0-100μM) for 1, 4 and 24 hours also showed time- and concentration-dependent increases in gene expression. At 1, 4, and 24 hours, Nrf2 expression was increased by a factor of 1.4, 1.2, and 1.9, respectively, following exposure to 100μM pilocarpine but the elevation in expression was only statistically significant ($p<0.05$) in those cells exposed to 100μM pilocarpine for 24 hours when compared to the corresponding controls (0μM; figure 4.4). At 1 hour exposure to the highest concentration of pilocarpine (i.e. 100μM), HO-1 expression relative to that of GAPDH was unchanged (1.0-fold), while at 4 and 24 hours, expression was elevated by a factor of 1.5 and 2.2, respectively (figure 4.5). When compared to control samples (0μM), the effects of pilocarpine on HO-1 expression were only statistically significant ($p<0.05$) following 24 hour exposure to pilocarpine concentrations of 30μM and 100μM (figure 4.5). At 1, 4 and 24 hours, NQO-1 expression relative to that of GAPDH was increased by 1.9-fold, 2.0-fold and 2.2-fold, respectively, after exposure to 100μM pilocarpine, achieving statistical significance ($p<0.05$) following 4 and 24 hour exposures to 100μM pilocarpine only (figure 4.6).

Cells exposed to LPS (0-100μg/ml) for 1, 4 and 24 hours again showed an increase in gene expression that was time- and concentration-dependent. At 1 hour, Nrf2 expression was increased up to 2.3-fold in relation to GAPDH expression but failed to reach statistical significance at any concentration of LPS in comparison to control (0μM)

value (figure 4.7). At 4 hours, there was a 4.8-fold increase in Nrf2 expression at the highest LPS concentration (100µg/ml) with significant ($p<0.05$) differences from control (0µM) observed at LPS concentrations above 10µM (figure 4.7). At 24 hours, the maximal increase in Nrf2 expression relative to GAPDH was 13.9-fold after 100µM LPS exposure and was statistically significant ($p<0.05$) at all LPS concentrations (3-100µg/ml) in comparison to control (0µM; figure 4.7). For HO-1, gene expression was maximally increased by a factor 5.8 at 1 hour, 11.7 at 4 hours, and 13.6 at 24 hours after exposure to 100µg/ml LPS and was significantly ($p<0.05$) different from control (0µM) at LPS concentrations above 10µg/ml (figure 4.8). Likewise, at 1, 4 and 24 hours NQO-1 expression was increased to 2.3-fold, 2.6-fold and 4.1-fold relative to GAPDH, respectively, following 100µg/ml LPS exposure and was statistically significant ($p<0.05$) at all concentrations above 10µg/ml (figure 4.9).

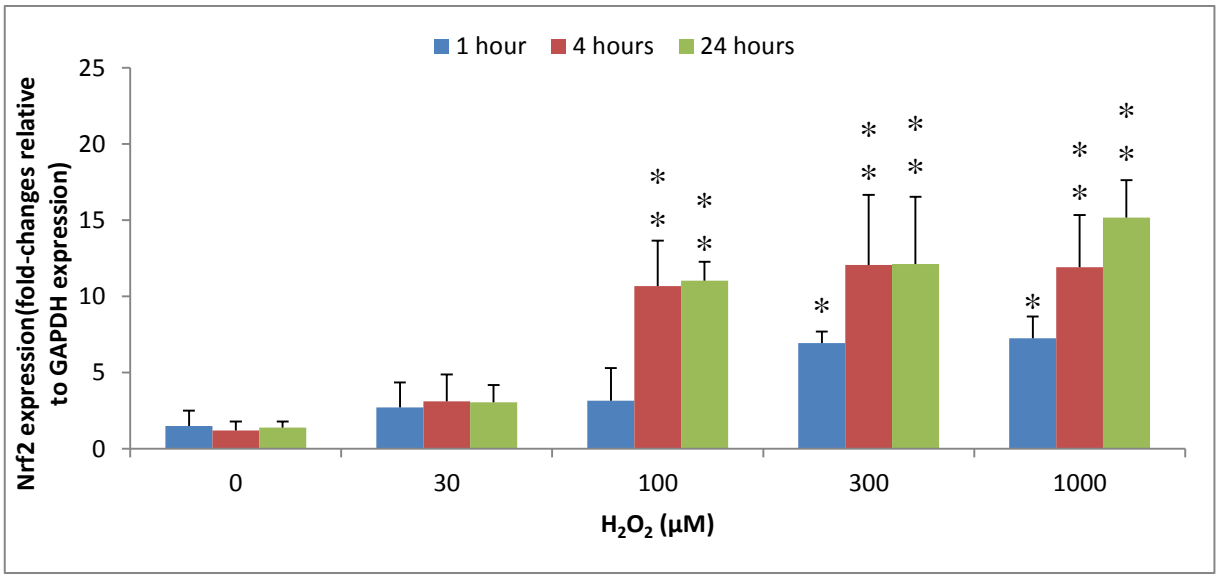


Figure 4.1 Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on Nrf2 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in Nrf2 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$; **$p<0.001$).

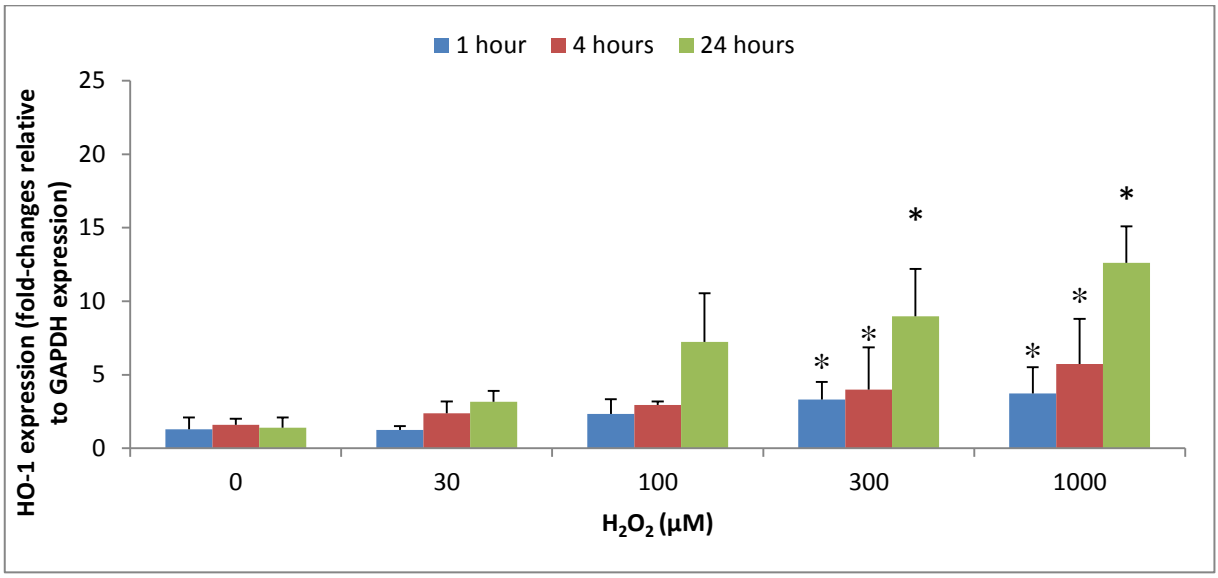


Figure 4.2 Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on the HO-1 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD HOX- fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

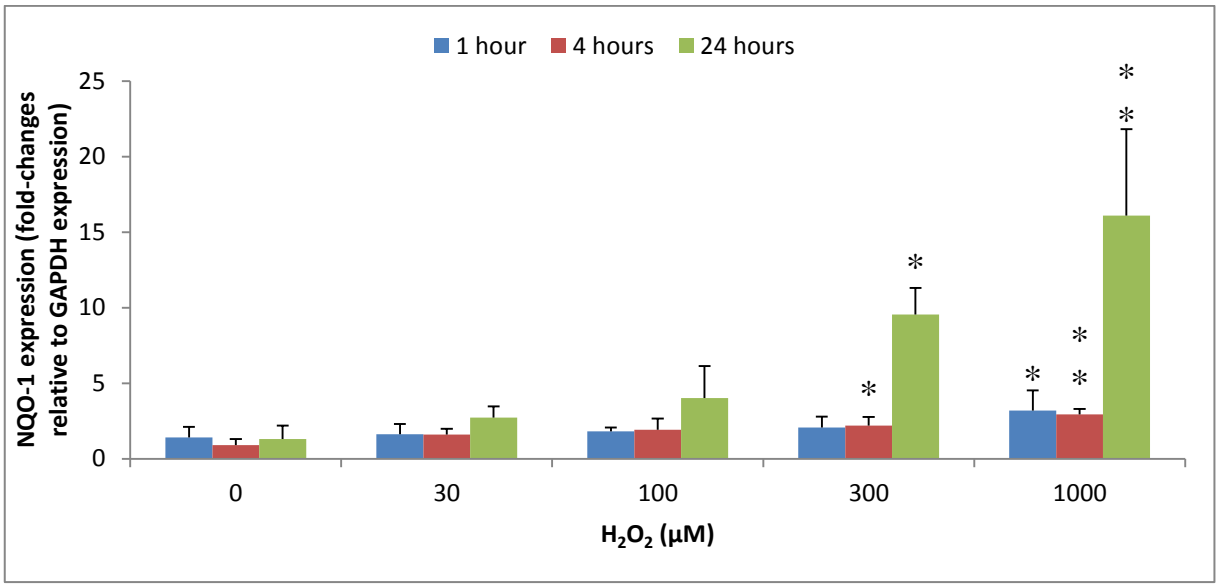


Figure 4.3 Effect of 1, 4 and 24 hour exposure to H_2O_2 (0-1000µM) on the NQO-1 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD NQO-1 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

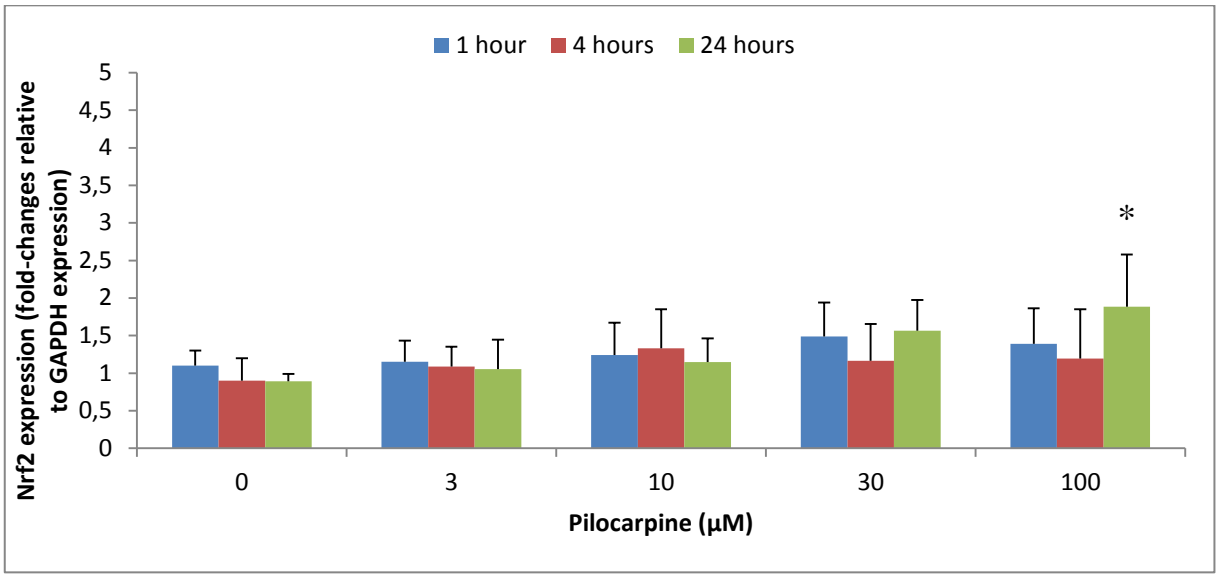


Figure 4.4 Effect of 1, 4 and 24 hour exposure to pilocarpine (0-100µM) on the Nrf2 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD Nrf2 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

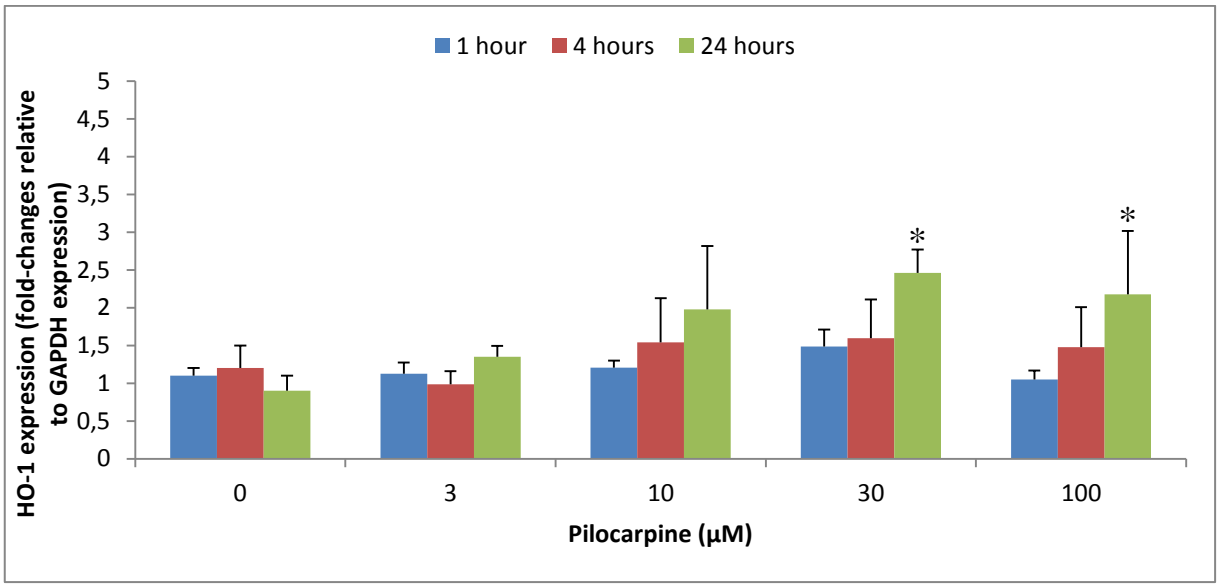


Figure 4.5 Effect of 1, 4 and 24 hour exposure to pilocarpine (0-100µM) on the HO-1 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD HO-1 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

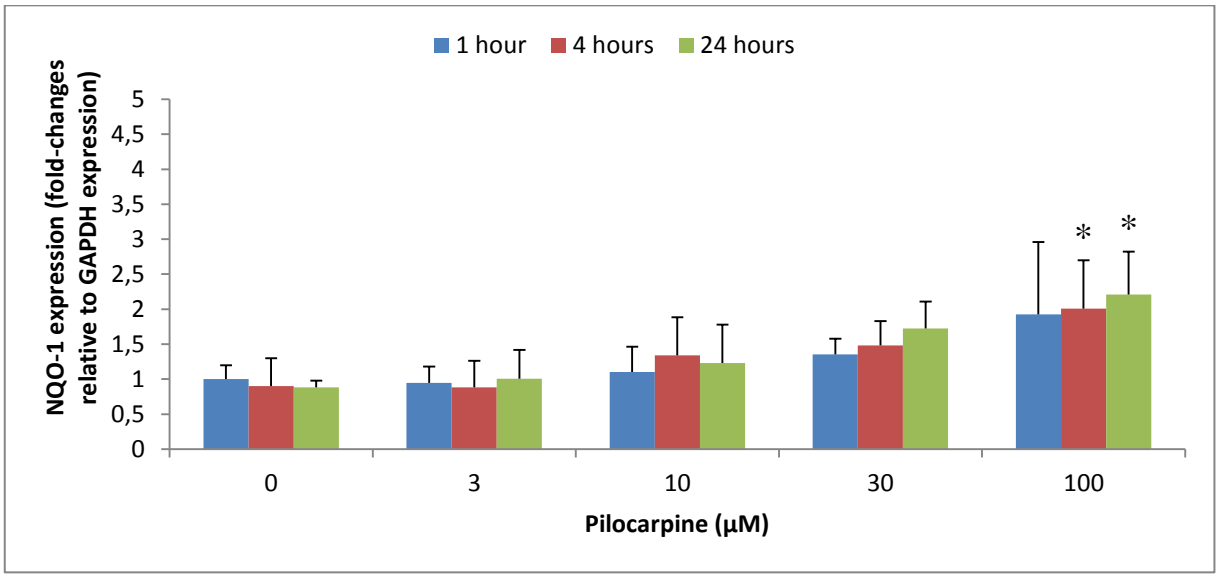


Figure 4.6 Effect of 1, 4 and 24 hour exposure to pilocarpine (0-100µM) on the NQO-1 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD NQO-1 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

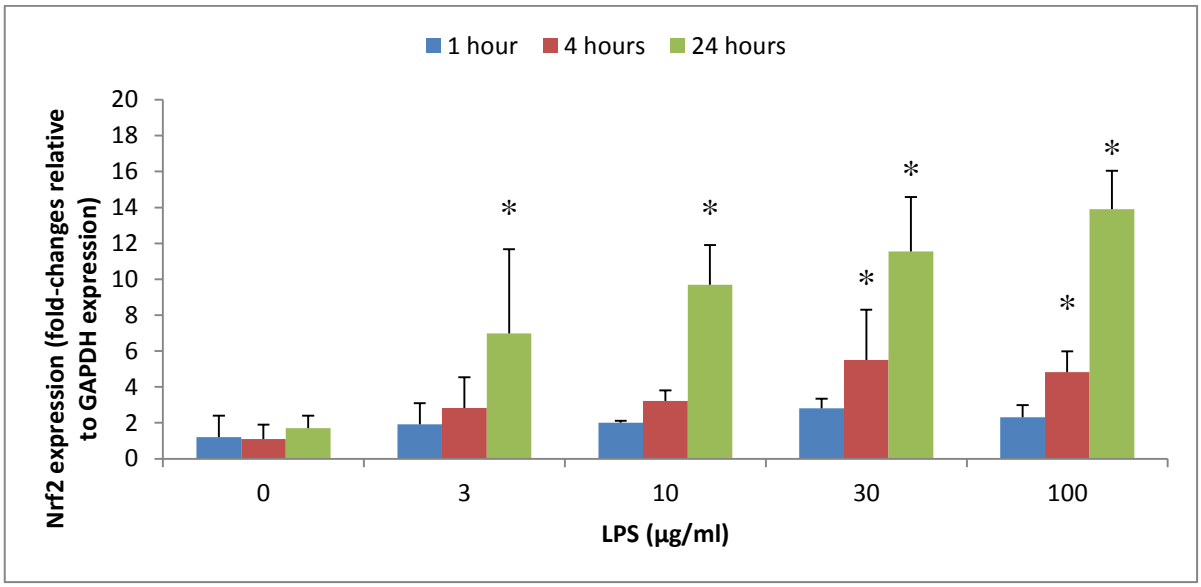


Figure 4.7 Effect of 1, 4 and 24 hour exposure to LPS (0-100µM) on the Nrf2 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD Nrf2 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

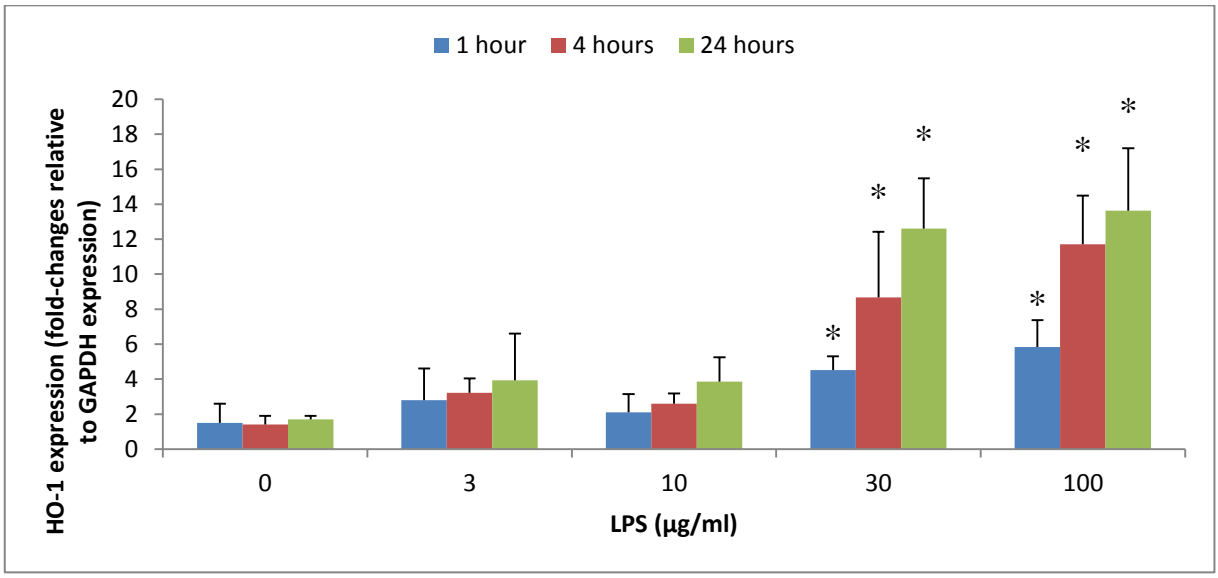


Figure 4.8 Effect of 1, 4 and 24 hour exposure to LPS (0-100µM) on the HO-1 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD HO-1 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

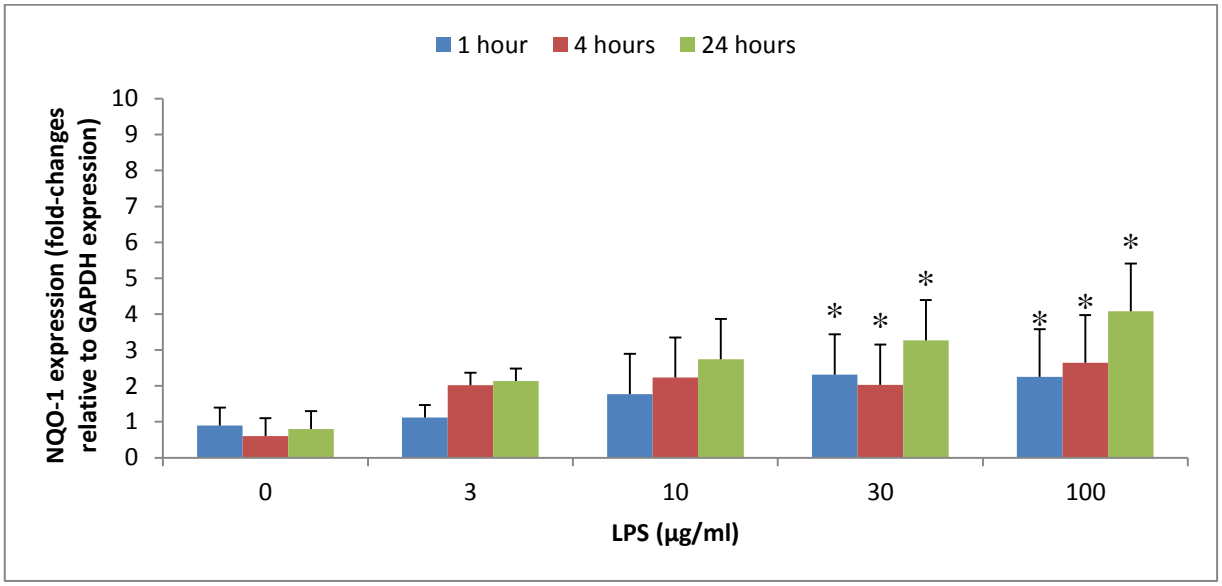


Figure 4.9 Effect of 1, 4 and 24 hour exposure to LPS (0-100µM) on the NQO-1 fold changes in SH-SY5Y cells. Results (n=4) are expressed as the mean ± SD NQO-1 fold changes and statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05), (**p<0.001).

4.3.2 Effect of carbamazepine on basal and H_2O_2-induced gene expression in SH-SY5Y cells

There were no statistically significant changes in Nrf2 expression in SH-SY5Y cells after treatment with 1-30μM CBZ for 24 hours, either with or without exposure to 100μM H_2O_2 for a further 4 hours (figure 4.10). However, 24 hour treatment with 100μM CBZ was associated with a significant ($p<0.001$) increase in both basal (4-fold) and H_2O_2-stimulated (5.3-fold) Nrf2 expression, relative to that of GAPDH, when compared with their respective controls (figure 4.10).

Treatment with CBZ concentrations up to 10μM for 24 hours was without significant effect on HO-1 expression in SH-SY5Y cells, either with or without exposure to 100μM H_2O_2 for a further 4 hours (figure 4.11). However, higher concentrations of CBZ were associated with statistically significant increases in relative HO-1 expression (2.2-fold at 30μM, $p<0.05$; 2.7-fold at 100μM, $p<0.001$) in comparison to control samples (0μM) (figure 4.11). The effect of CBZ was augmented by subsequent exposure to 100μM H_2O_2 for 4 hours, with a maximum increase in HO-1 expression of 3.3-fold, but was only significantly different from the corresponding control at 100μM CBZ ($p<0.05$; figure 4.11).

There was no statistically significant change in NQO-1 expression, relative to that of GAPDH, in SH-SY5Y cells following 24 hour treatment with 1-30μM CBZ, either with or without exposure to 100μM H_2O_2 for a further 4 hours (figure 4.12). NQO-1 expression was, however, significantly increased in comparison to control (0μM) after treatment with 100μM CBZ (2.1-fold, $p<0.05$) and was also increased in cells that were additionally exposed to 100μM H_2O_2 (2.8-fold, $p<0.05$) (figure 4.12).

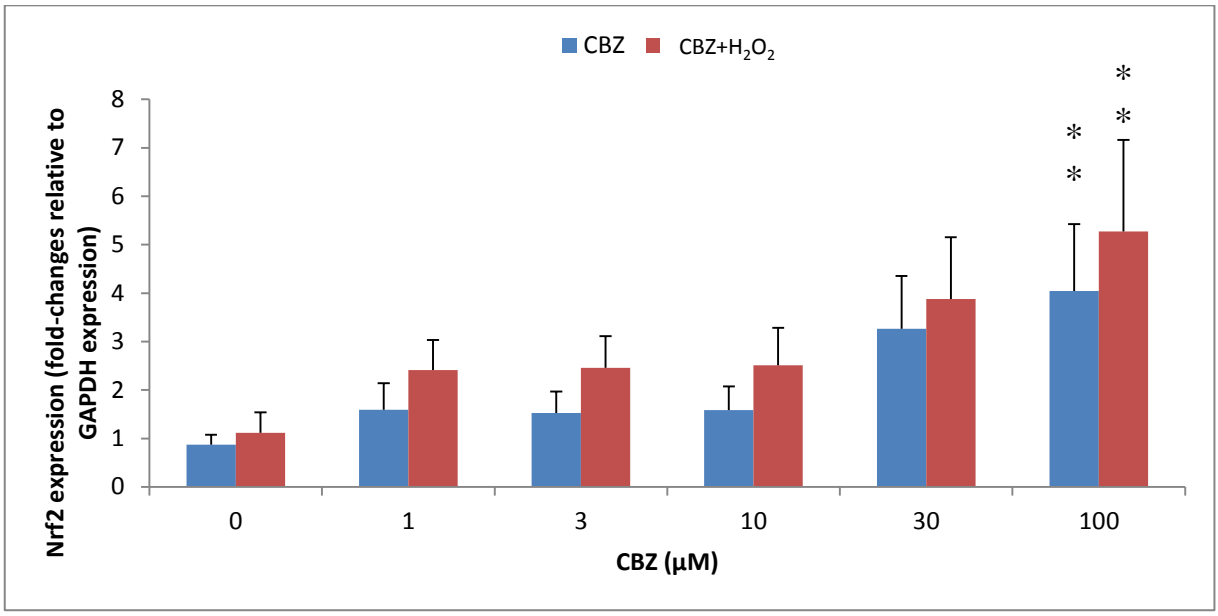


Figure 4.10 Effect of 24 hour treatment with CBZ (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on Nrf2 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in Nrf2 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (**p<0.001).

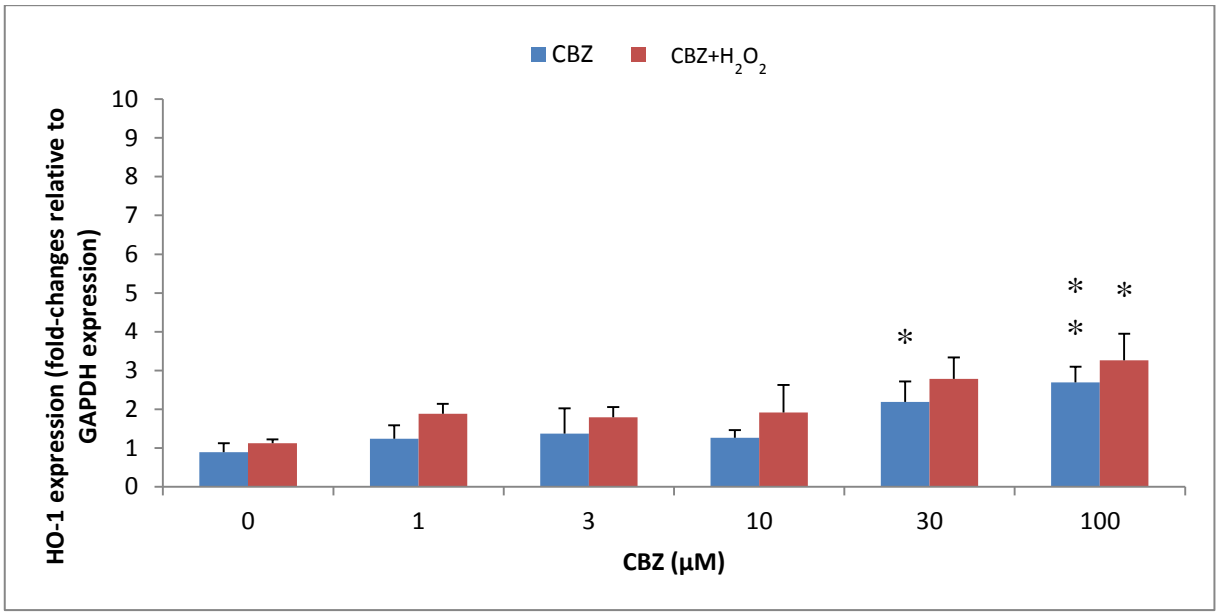


Figure 4.11 Effect of 24 hour treatment with CBZ (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on HO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in HO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (**p<0.001; *p<0.05).

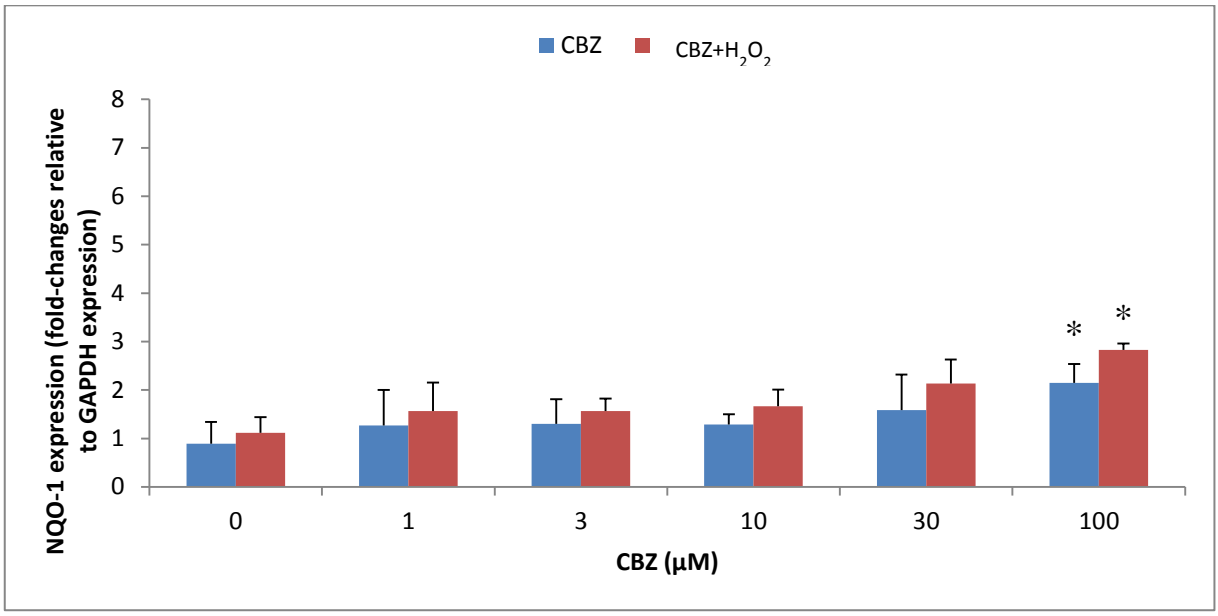


Figure 4.12 Effect of 24 hour treatment with CBZ (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on NQO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in NQO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*$p<0.05$).

4.3.3 Effect of valproic acid on basal and H_2O_2-induced gene expression in SH-SY5Y cells

There were no significant changes in Nrf2 expression, relative to that of GAPDH, following treatment of SH-SY5Y cells with VPA at concentrations up to 300µM for 24 hours, except where 300µM VPA was followed by a 4 hour exposure to 100µM H_2O_2, which resulted in a 2.2-fold increase in expression that was statistically different from control ($p<0.05$; figure 4.13). Nrf2 expression was also significantly increased following treatment with 1000µM VPA, by a factor of 3. ($p<0.05$) following VPA alone and by a factor of 4.02 ($p<0.001$) when cells were additionally exposed to 100µM H_2O_2 (figure 4.13).

VPA (10-300µM) was without statistically significant effect on HO-1 expression, relative to GAPDH, in SH-SY5Y cells following 24 hour treatment, with or without subsequent exposure to 100µM H_2O_2 for 4 hours (figure 14). In contrast, 1000µM VPA was associated with a increase in HO-1 expression, both alone (2.9-fold) and following additional exposure to 100µM H_2O_2 for 4 hours (3.6-fold), which was significantly ($p<0.001$) different from the corresponding control (0µM) in both cases (figure 14).

SH-SY5Y cells showed no significant changes in the expression of NQO-1, relative to GAPDH, after treatment with 10-1000µM VPA for 24 hours (figure 15). However, when VPA treatment was followed with a further 4 hour exposure to 100µM H_2O_2 there was a significant increase in NQO-1 expression at 300µM VPA (2.3-fold; $p<0.05$) and also at 1000µM VPA (2.9-fold; $p<0.001$) in comparison to relative gene expression in control (0µM) samples (figure 15).

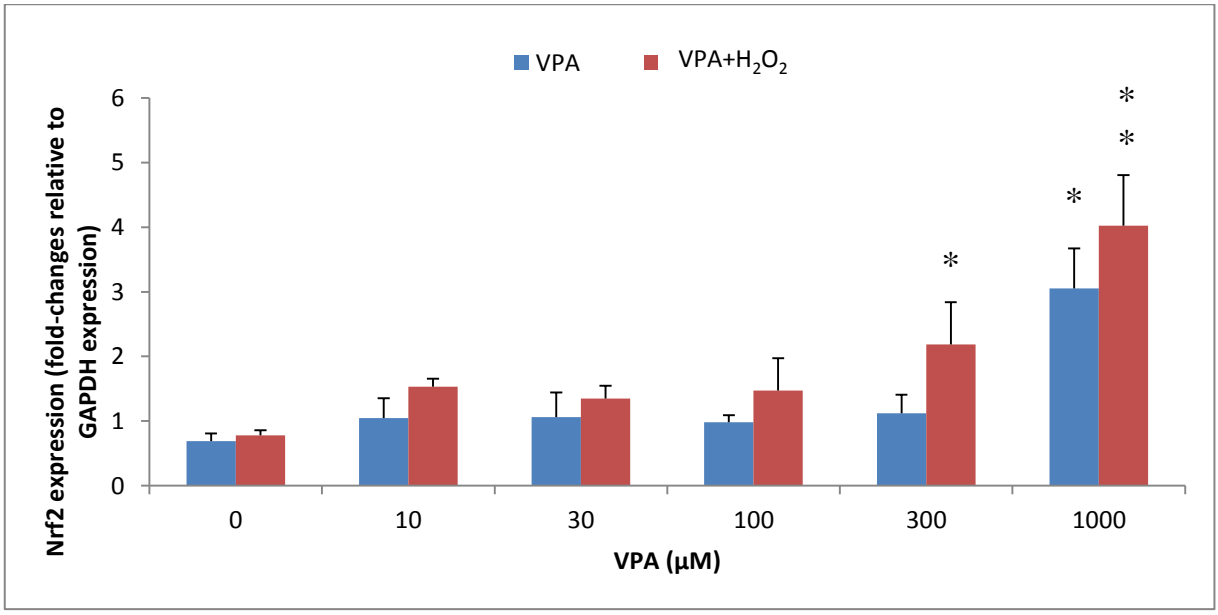


Figure 4.13 Effect of 24 hour treatment with VPA (0-1000µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on Nrf2 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in Nrf2 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (**$p<0.001$; *$p<0.05$).

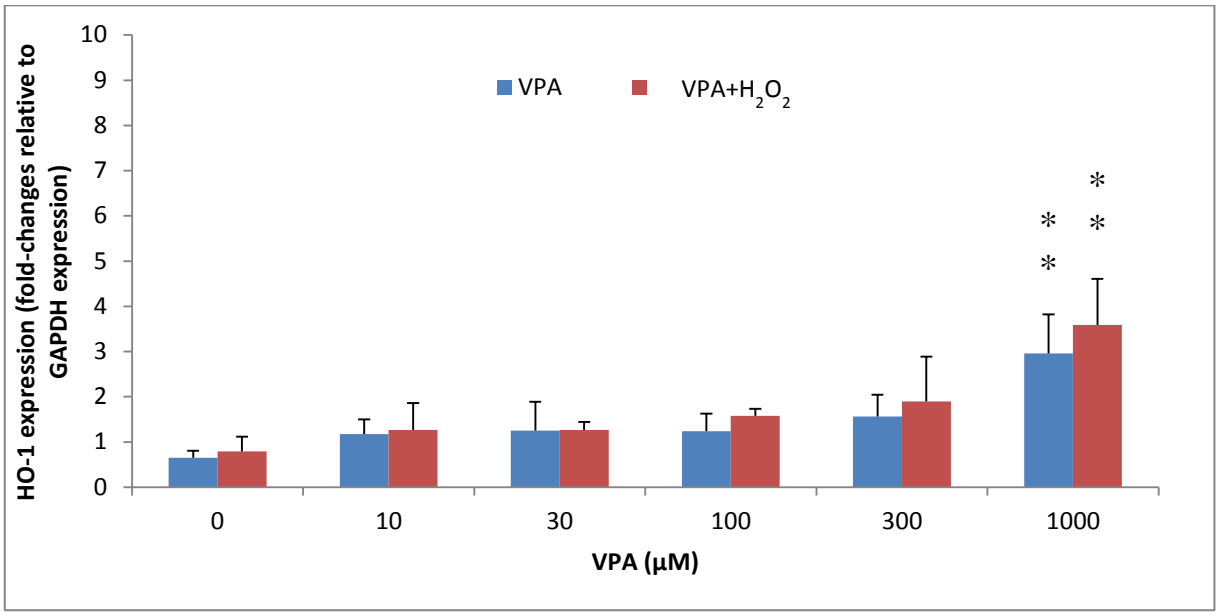


Figure 4.14 Effect of 24 hour treatment with VPA (0-1000µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on HO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in HO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (**p<0.001).

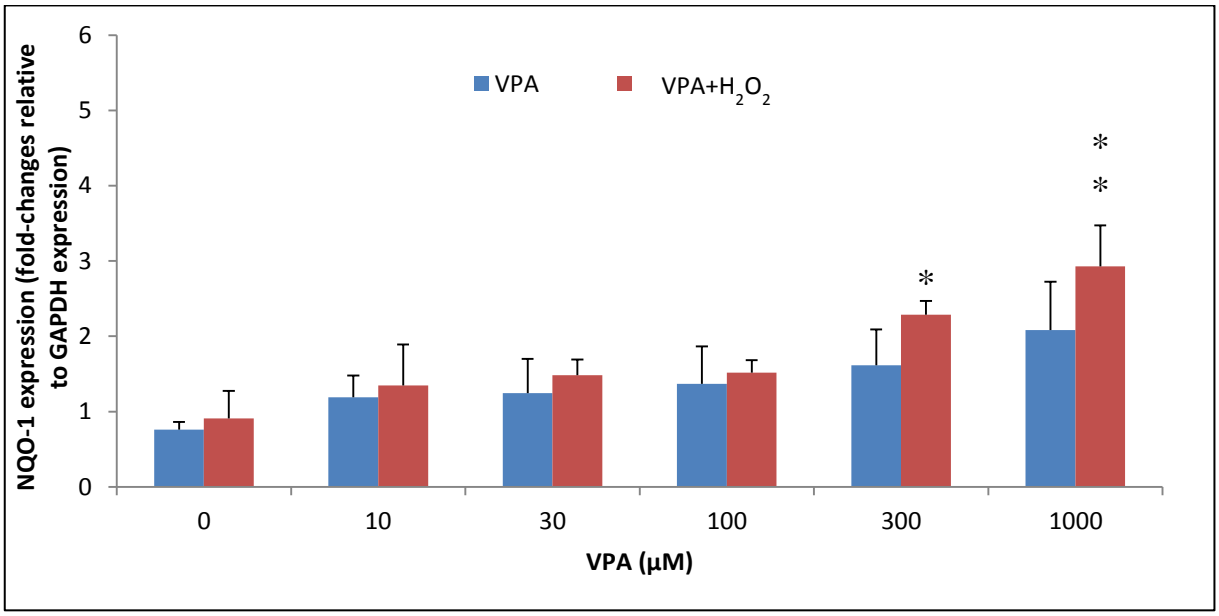


Figure 4.15 Effect of 24 hour treatment with VPA (0-1000µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on NQO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in NQO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (**p<0.001; *p<0.05).

4.3.4 Effect of lamotrigine on basal and H_2O_2-induced gene expression in SH-SY5Y cells

For the most part, there was no significant change in the expression of Nrf2, relative to that of GAPDH, after treatment of SH-SY5Y cells with 1-100μM LTG for 24 hours, with or without additional exposure to 100μM H_2O_2 for 4 hours (figure 4.16). However, when cells were treated with 100μM LTG for 24 hours, followed by 100μM H_2O_2 for 4 hours, there was a modest but nonetheless statistically significant increase (6.3-fold; $p<0.05$) in the relative expression of Nrf2 when compared to expression in control cells (figure 4.16).

SH-SY5Y cells again showed almost no significant changes in HO-1 expression, relative to GAPDH, after treatment with 1-100μM LTG for 24 hours, with or without additional exposure to 100μM H_2O_2 for 4 hours (figure 4.17). The only exception was a statistically significant ($p<0.05$) increase (2.3-fold) in the expression of HO-1 following combined treatment with 100μM LTG for 24 hours followed by 100μM H_2O_2 for 4 hours (figure 17).

The expression of NQO-1, relative to the housekeeping gene GAPDH, was significantly increased, with a maximal increase of 2.9-fold, following combined treatment with LTG (1-100μM) for 24 hours followed by 100μM H_2O_2 for 4 hours ($p<0.05$ at 1μM, 3μM and 10μM LTG; $p<0.001$ at 30μM and 100μM LTG) (figure 4.18). In addition, there was a significant effect of 100μM LTG alone, with a 2.3-fold increase in NQO-1 expression that was statistically ($p<0.001$) different from expression observed in the corresponding control cells (figure 4.18).

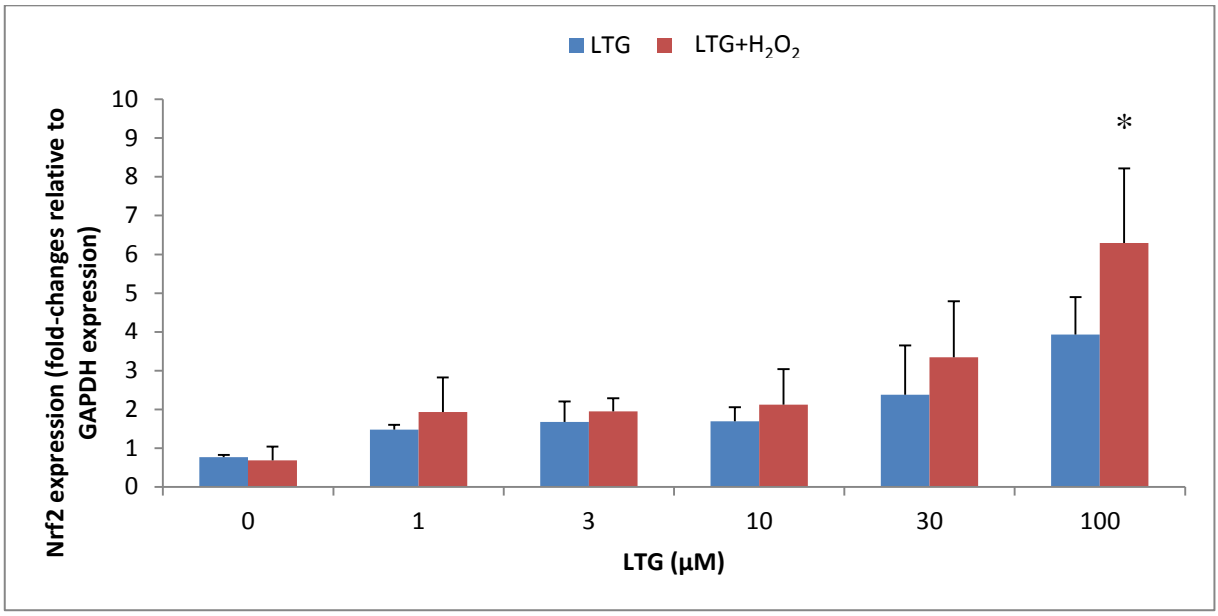


Figure 4.16 Effect of 24 hour treatment with LTG (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on Nrf2 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in Nrf2 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05).

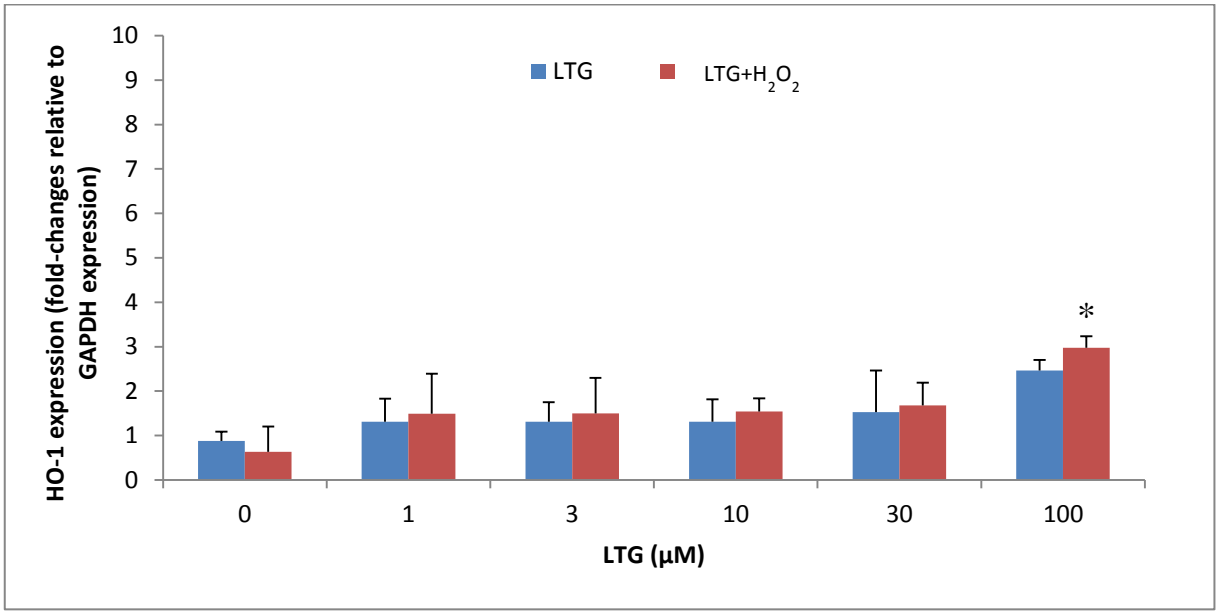


Figure 4.17 Effect of 24 hour treatment with LTG (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on HO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in HO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05).

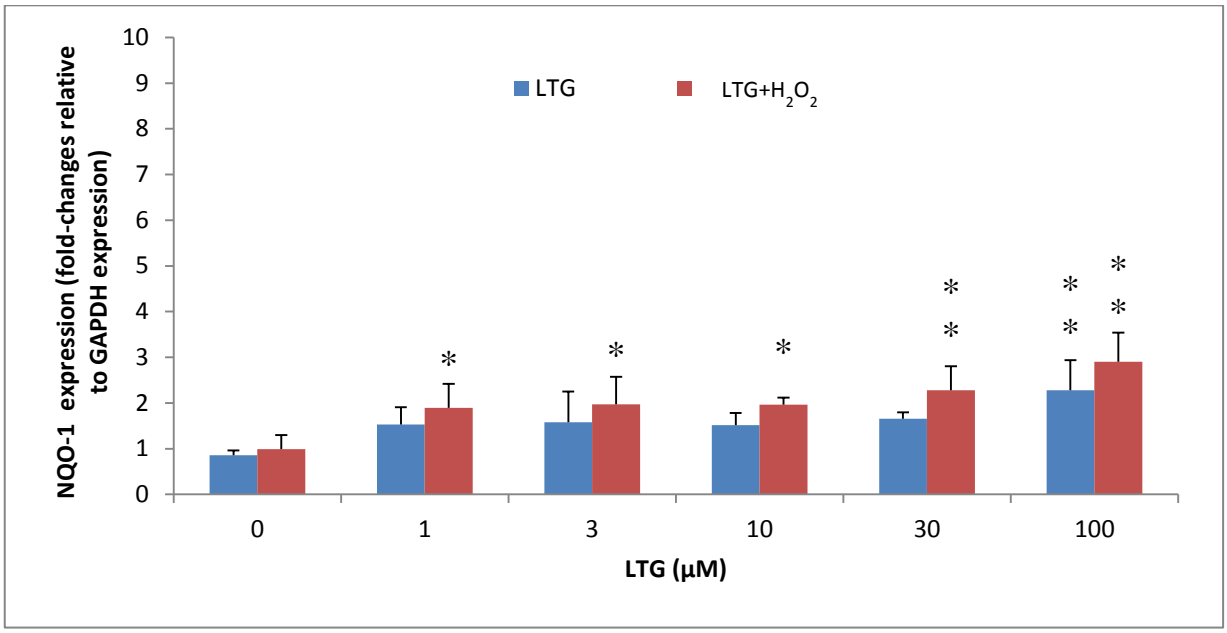


Figure 4.18 Effect of 24 hour treatment with LTG (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on NQO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in NQO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (**p<0.001; *p<0.05).

4.3.5 Effect of levetiracetam on basal and H_2O_2-induced gene expression in SH-SY5Y cells

SH-SY5Y cells showed no significant changes in Nrf2 expression, relative to that of GAPDH, following exposure to 1-300µM LEV for 24 hours, with or without further exposure to 100µM H_2O_2 for 4 hours (figure 4.19).

Likewise, there was no significant change in HO-1 expression, relative to GAPDH, after exposure to 1-300µM LEV for 24 hours, with or without further exposure to 100µM H_2O_2 for 4 hours (figure 4.20).

Finally, the expression of NQO-1 was not significantly altered, compared to control (0µM), by treatment with 1-300µM LEV for 24 hours, with or without further exposure to 100µM H_2O_2 for 4 hours (figure 4.21).

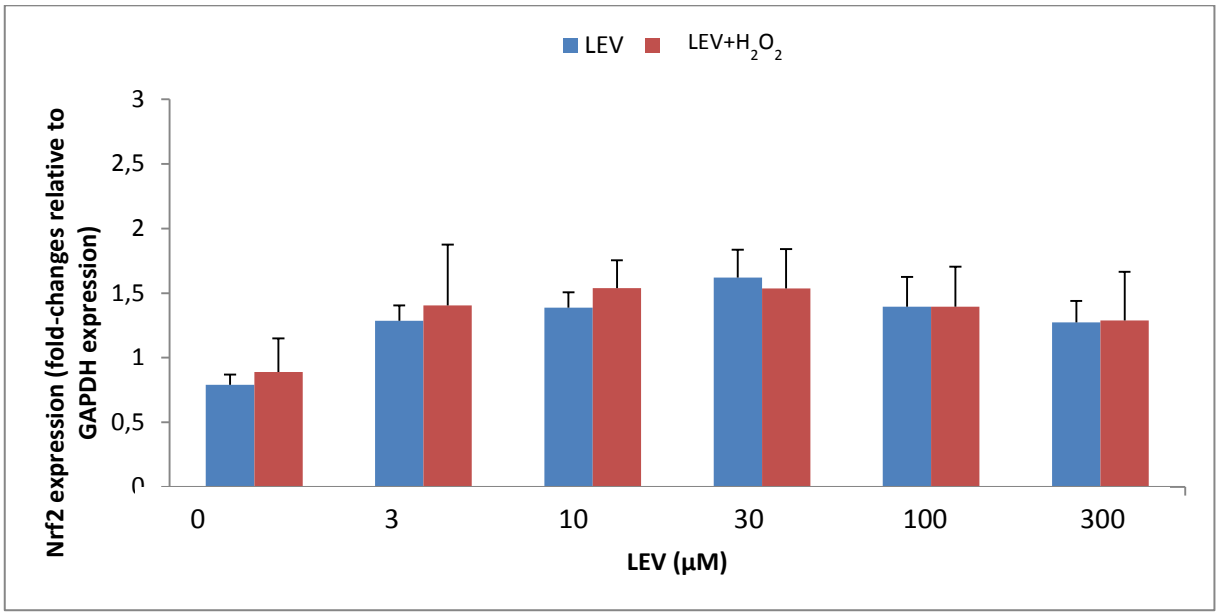


Figure 4.19 Effect of 24 hour treatment with LEV (0-300µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on Nrf2 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in Nrf2 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series.

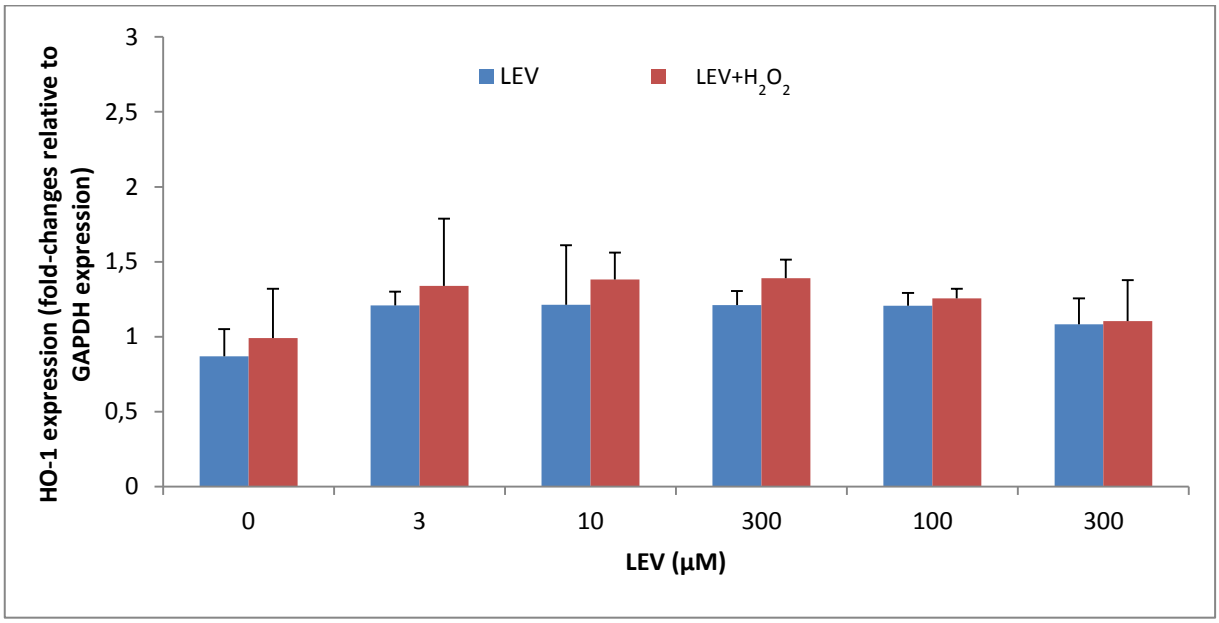


Figure 4.20 Effect of 24 hour treatment with LEV (0-300µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on HO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in HO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series.

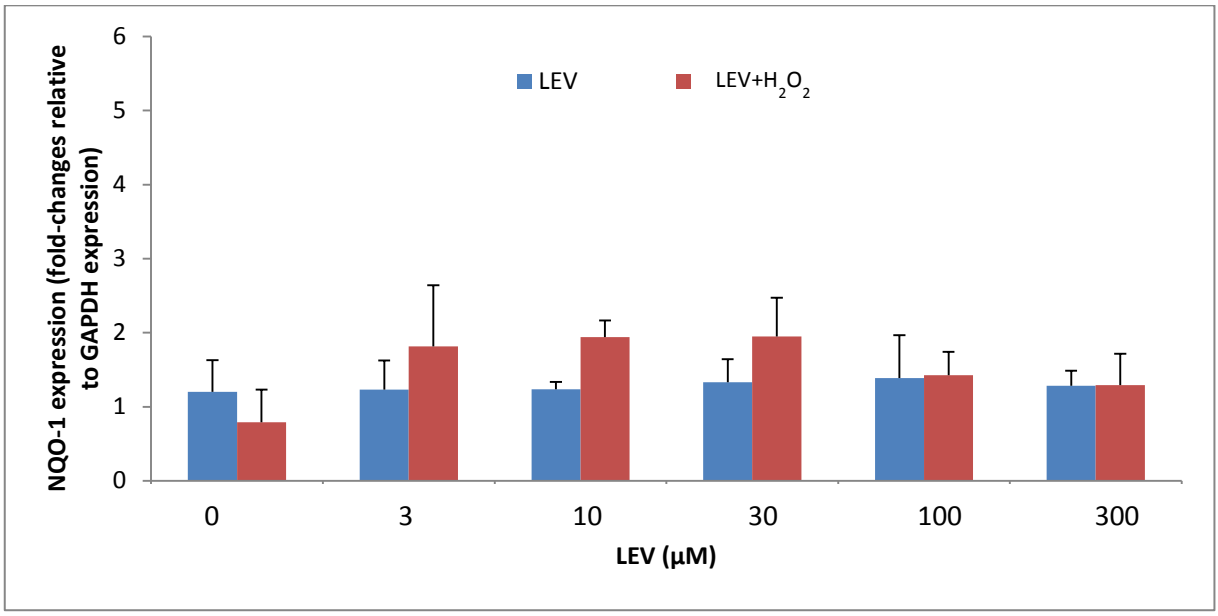


Figure 4.21 Effect of 24 hour treatment with LEV (0-300µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on NQO-1 expression in SH-SY5Y cells. Results (n=4) are expressed as the mean (± SD) fold-change in NQO-1 expression, relative to GAPDH expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series.

4.4 Discussion

Oxidative stress and mitochondrial dysfunction are considered as contributing factors to various neurological disorders. Recently, there has been increasing evidence supporting the association between oxidative stress and the process of epileptogenesis (Martinc et al., 2012). Nrf2 and its related genes, HO-1 and NQO-1, have been suggested to play a protective role in a number of neurodegenerative disorders and cancers by virtue of their antioxidant action (Lee and Johnson, 2013). The experimental evidence suggests that Nrf2 could be a promising therapeutic target for chronic neurological diseases such as epilepsy (Patel, 2015). The present study was designed to determine the effects of selected oxidative stress inducers and commonly used AEDs on Nrf2 and its related gene expression.

The current study found that exposure of SH-SY5Y cells to different concentrations of H_2O_2, LPS, and pilocarpine positively affected expression of the Nrf2 gene, and those encoding HO-1 and NQO-1, in a time and concentration dependent manner. Another important finding was that incubation of SH-SY5Y cells with different concentrations of CBZ, VPA and LTG, with or without further exposure to H_2O_2, resulted in an increase in the expression of Nrf2 and its downstream genes in a time and concentration dependent-manner. This effect was invariably most prominent at the highest AED concentrations. Interestingly, another commonly used AED, LEV, had no effect on either basal or H_2O_2-induced, Nrf2-related gene expression.

In the cell, regulation of Nrf2 levels and its activity occurs at several levels, including transcription, translation, degradation, translocation, and post-translational modifications (Perez-Leal et al., 2017). One of the most important mechanisms determining the increase of Nrf2 protein levels involves a decreased rate of Nrf2 degradation by Keap-1 protein.

In the current study, H_2O_2 was found to induce the expression of Nrf2 and its related genes, HO-1 and NQO-1. These findings are consistent with previously published data which reported an up-regulation in Nrf2 expression after H_2O_2 exposure (Fourquet et al., 2010).

It is known that H_2O_2 can be metabolized to several ROS, including hydroxyl radicals, which are considered to be amongst the most cytotoxic of free radicals. An excess of un-eliminated H_2O_2 and its metabolites can oxidize virtually all types of macromolecules: carbohydrates, nucleic acids, lipids and proteins (Yin et al., 2015). The increased levels of ROS overwhelm the antioxidant defences and lead to a state of oxidative stress. Nrf2 is a master regulator of oxidative stress which contributes to the environmental stress response by regulating the expression of genes for antioxidant and detoxification enzymes (Vomund et al., 2017). In its inactivated state, Nrf2 is sequestered in the cytosol via Keap1 protein and is quickly degraded. Modification of Keap1 is necessary for the accumulation of free Nrf2 in the cytoplasm, which then translocates into the nucleus (Kobayashi et al., 2004). Under conditions of oxidative stress and an excess of ROS, the oxidation or conjugation of key cysteine residues in Keap1 increases. The net effect of Keap1 modification is a reduction in its affinity for Nrf2, which prevents poly-ubiquitination and proteasomal degradation of Nrf2 in the cytosol. This allows accumulated Nrf2 to translocate into the nucleus and binded to AREs, leading to the regulated transcription of its target genes (Kobayashi et al., 2004). Therefore, the degradation of Keap1 is logically associated with up-regulation of Nrf2 as other reports have described (Kobayashi et al., 2004) (figure 4.22).

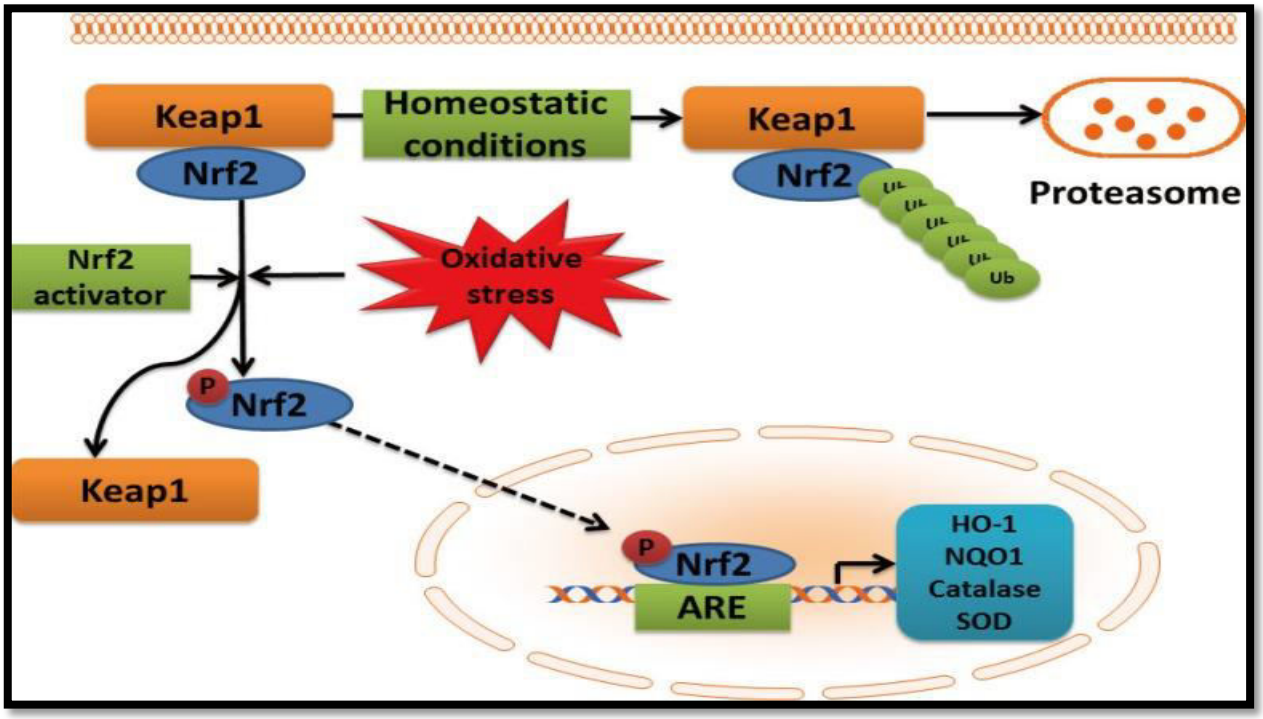


Figure 4.22: Schematic diagram of the nuclear factor erythroid 2-like 2 (Nrf2) / antioxidant response element (ARE) pathway and its activation by oxidative stress. Under normal conditions, Nrf2 is constitutively bound to Kelch-like ECH associated protein 1 (Keap1) protein in the cytoplasm. Keap1 inhibits Nrf2 signalling by promoting Nrf2 ubiquitination (ub) and subsequent degradation through proteasomal pathway. Mild oxidative stress and Nrf2 activators cause dissociation of the Nrf2-Keap1 complex, leading to phosphorylation (P) of Nrf2 and its nuclear translocation. In the nucleus, Nrf2 promotes transcriptional activation of gene encoding antioxidant enzymes haem oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), catalase, and superoxide dismutase (SOD) (adapted from Chen et al., 2015).

In this study, pilocarpine was found to induce Nrf2 and the expression of its related genes. Pilocarpine is a non-selective agonist at muscarinic receptors (M1, M2 and M4). Previous reports based on pharmacological data have suggested the existence of at least M1, M2 and M3 muscarinic receptor subtypes in SH-SY5Y cells (Adem et al., 1987; Lambert et al., 1989; Mei et al., 1989). Furthermore, previous reports have shown that those ROS may also participate as second messengers in the regulation of ARE-driven genes by disrupting the Nrf2/Keap1 interaction (Mangelus et al., 2001; Nguyen et al., 2003).

A possible explanation for the link between M1 receptors and activation of the antioxidant response might be that pilocarpine binding to the M1 receptor activates phospholipase C and therefore produces diacylglycerol (DG) and inositol triphosphate (IP3), which in turn result in alteration of Ca^{2+} and K^{+} currents and increased excitability of brain cells (Segal, 1988). The high concentration of Ca^{2+} promotes the release of glutamate. Glutamate promotes the entrance of Na^{+} and Ca^{2+} into the cell and, as a consequence, removes the Mg^{2+} block of N-methyl-D-aspartate (NMDA) receptors, and allows the entrance of more Ca^{2+} into the cell, which can lead to excitotoxicity and cell death (Li and Wang. 2016). Tissue excitability increases the utilization rate of monoamines (Cavalheiro et al. 1994). After binding to their own receptors, these monoamines are degraded by monoamine oxidase (MAO) and catechol-o-methyltransrerase (COMT) enzymes and, during these processes, free radicals can be formed. These free radicals are also freed during glucose metabolism and the mitochondrial transport chain. In the absence of oxidant injury, Nrf2 interacts with the Keap1 protein, and this association promotes Nrf2 degradation through the ubiquitin-proteasome pathway by Keap-1 protein. On the other hand, excessive production of free radicals promotes the modification of Nrf2/Keap1 complexes resulting in stabilization of the Nrf2 protein, and its translocation to the nucleus, where it induces transcription of phase II genes such as HO-1 and NQO-1 (Tong et al., 2006). In the current study, LPS was also found to induce the expression of Nrf2 and its related genes. LPS is the endotoxic component of the Gram-negative bacterial cell wall that may activate an acute inflammatory response (Mai et al., 2013). The main mechanism of action of LPS is the activation of TLRs and NF-κB and of a variety of pro-inflammatory genes and oxidative damage (Vaisid et al., 2013). Neurons themselves, including SH-SY5Y cells, are capable of expressing TLRs (Vaisid et al., 2013; Kim et al., 2015). ROS are central to a host of pathologies including inflammation and infection (Reddy et al., 2006) and

numerous studies have shown that ROS can regulate NF-κB activation (Mirochnitchenko and Inouye 1996; Asehnoune et al., 2004; Powers et al., 2006). TLR2 activation by LPS has been shown to cause activation of the Nrf2 signalling pathway and negatively regulates inflammatory responses (Yin and Cao, 2015). Inflammation can induce the expression of Nrf2 downstream genes (Rushworth and MacEwan, 2008), and the Nrf2 pathway cross-talks with NF-κB and other signalling pathways to repress the inflammatory response (Yang et al., 2014). The key event that leads to Nrf2 pathway activation appears to be TLR receptor stimulation by LPS, which results in ROS release, with the Keap-1 protein undergoing oxidation and the formation of an intramolecular disulfide bond (Hu et al., 2011). This conformational change in Keap1 leads to the release of Nrf2 and its subsequent nuclear translocation and activation of antioxidant genes (Yamamoto et al., 2008).

Contrary to expectations, this study found a pro-oxidant effect of several AEDs, with or without further exposure to H_2O_2, on the expression of Nrf2 and its related genes. CBZ, VPA, and LTG were found to increase Nrf2 expression, and that of HO-1 and NQO-1, particularly at higher concentrations. Lower AED concentrations, including therapeutic concentrations which are considered to be 15-50µM for CBZ (Ching, 2016), 350-700µM for VPA (Brunton et al., 2011) and 15-40µM for LTG (Wolf, 1992), had little or no effect on gene expression. It is encouraging to compare these results with those of Pavone and Cardile (2003) who found that there was a similar time- and concentration-dependent decrease in cell viability and an increase in oxidative stress parameters, including total ROS, lactate dehydrogenase, TBARS, and nitric oxide (NO), when rat astrocyte cultures were exposed to AEDs. These observations are also consistent with the results previously described in chapter 2 of this thesis. In those studies, CBZ, VPA, and LTG were found to have a pro-oxidant effect particularly at higher concentrations

and so one can speculate that this pro-oxidant effect leads to an increase in the expression of Nrf2 and its downstream target genes as an attempt to provide protection against cytotoxic damage. As mentioned above, oxidative stress and cell damage are important inducers of Nrf2 either by causing damage to cellular components or by direct prevention of degradation by the Keap-1 protein. This is the most likely explanation for the results observed in this chapter. However, another possible explanation for the pro-oxidant effects of AEDs, or at least VPA, might be an increase in ROS via the activation of peroxisome proliferator-activated receptors (PPARs) that perform specific functions in fatty acid homeostasis and are known to upregulate CYP proteins (Chawla et al., 2001). Another important finding was that LEV, with or without additional H_2O_2, did not show any activation or inhibition of the expression Nrf2 and its related genes at any concentration, including those within and above the recognised therapeutic range of 45-180μM (Martinc et al., 2012). This finding contrasts those reported in chapter 2 in which higher concentrations of LEV had a significant effect on biochemical markers of oxidative stress in SH-SY5Y cells. To the best of our knowledge, very little is known about the pro-oxidant and/or antioxidant effects of LEV in *in vitro* studies (Johannessen and Landmark, 2010). It is possible that pre-incubation with LEV might somehow result in preservation of the activity of catalase, an antioxidant enzyme that catalyses the conversion of H_2O_2 to water and oxygen, which would explain the antioxidant behaviour of LEV against H_2O_2-induced oxidative stress in SH-SY5Y cells (Oliveira et al., 2016). Thus, LEV might help to counteract the overproduction of reactive oxygen species and the resulting oxidative damage.

The methods have been used in this experiment are highly reliable as they have been used by many researchers previously. However, the outcomes of this study are limited because of low n-numbers, lack of independent validation of findings, and potentially

by the use of different passage number of the used cell line, particularly with respect to comparison between these results and those in previous chapters. As described in the previous chapter, passage number has an impact on growth rates and on physiological and chemical characteristics of immortalised cell lines. In addition, and as mentioned before in chapter 3, a weakness of *in vitro* cell cultures is that they are studied in isolation and in the absence of their normal environment, which includes interactions with other cell types that might be critical to the process being tested. In such cases, *in vivo* testing is often preferred because it is better suited for observing the overall effects of experiment conditions or an intervention on a living subject.

4.4.1 Conclusion

This study has shown that several experimental inducers of oxidative stress, including H_2O_2, pilocarpine and LPS, caused an increase in the expression of Nrf2, HO-1 and NQO-1 gene in SH-SY5Y cells in a time and concentration-dependent manner. In addition, several widely used AEDs, including CBZ, VPA and LTG, also increased the expression of Nrf2, HO-1 and NQO-1 in SH-SY5Y cells following 24 hour treatment, particularly at high concentrations and irrespective of subsequent exposure to H_2O_2. Interestingly, another AED, namely LEV, was without effect on expression of the selected genes. These results indicate that H_2O_2, pilocarpine, and LPS and several AEDs most likely have pro-oxidant effects, especially at their highest concentrations, which in turn lead to activation of the Nrf2 pathway and the antioxidant response as a cellular defence mechanism against expected cytotoxicity. The exception is LEV which has pro-oxidant potential – see chapter 2 – but perhaps via a mechanism that does not recruit the Nrf2 pathway in response. These findings have gone some way towards enhancing our understanding of the effects of AEDs on Nrf2 as a master regulator of oxidative stress and which plays an important role in epileptogenesis.

Chapter 5

Effects of selected AEDs on Nrf2, HO-1, and NQO-1 protein expression in SH-SY5Y cells.

5.1 Introduction

Nrf2 is a basic leucine zipper transcription factor with a cap 'n' collar domain (Kasper et al., 2009). It is considered as a redox sensitive transcription factor that induces the expression of a variety of genes which serve to protect against the deleterious effects of oxidative and chemical stress, thus ensuring normal cellular functions are maintained or restored (Tonelli et al., 2017).

The activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1, a cytosolic repressor protein that facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (Itoh et al., 2003). Upon the exposure of cells to toxic insults and/or oxidative stress by electrophiles and oxidants, which cause an imbalance in the redox state of the cell, the Keap1-directed ubiquitination and degradation of Nrf2 is disrupted, leading to Nrf2 accumulation in the cytosol followed by its translocation to the nucleus. Here, Nrf2 binds to its transcriptional partner small masculoaponeurotic fibrosarcoma (Maf) proteins to form heterodimers (Motohashi et al., 2004). These Nrf2-Maf combinations then bind to the ARE in the promoter regions of cytoprotective target genes (Motohashi et al., 2004), which encode a host of antioxidants, xenobiotic detoxification and DNA repair enzymes, molecular chaperones, anti-inflammatory response proteins and other cytoprotective enzymes (Zhang et al., 2013). The coordinated expression of these genes converts toxic compounds to less harmful intermediates whilst repairing any damage caused within the cell.

The high frequency of cysteine residues in the Keap1 sequence confers a relatively high reactivity to oxidative inducers (Miseta et al., 2000). Several studies have shown that the covalent binding of cysteine residues by Nrf2 activators such as sulforaphane, nitric oxide and H_2O_2 alters the conformation of Keap1, disrupting its association with Nrf2

and allowing Nrf2 to translocate to the nucleus (Zhang et al., 2003; Wakabayashi et al., 2004). Nrf2 protein has a very rapid turnover, with a half-life of less than 20 minutes and thus, Nrf2 protein is minimally detectable in unstressed conditions (Kobayashi et al., 2004).

As for many proteins, the cellular mechanisms of Nrf2 and its downstream target genes have been largely characterized through western blot analysis. The field has been lacking a well-characterized, commercially available, and specific antibody with which to detect Nrf2. In western blotting, the proteins may instead be detected using colorimetric, chemiluminescence or fluorescence techniques (Kurien and Scofield, 2009).

The commonly used detection methods have involved the use of protein-specific primary antibody followed by detection using secondary antibodies conjugated with either horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzyme. In contrast, chemiluminescence is a commonly used detection method by which a chemical reaction results in the release of energy in the form of light. The emitted light can be captured on x-ray film (Kurien and Scofield, 2009).

There is a diverse body of literature on Nrf2 involvement in fields ranging from antioxidant defence, (Ma, 2004), neurological disorders (Gao et al., 2014) and cancer (Namani et al., 2014). Experimental evidence suggests that Nrf2 could be a promising therapeutic target for chronic neurological conditions like epilepsy (Patel, 2015). Epilepsy remains a major medical problem for which there is no curative treatment. Oxidative stress resulting from excessive free-radical release has been reported as an underlying mechanism in the initiation and progression of epilepsy and excessive oxidative stress contributes to neuronal degeneration in the epileptic focus (Shin et al., 2011). In addition, an increase in reactive oxygen species occurs in

response to sustained neuronal electrical activity and seizures (Mariani et al., 2005). Therefore, antioxidants have been suggested to have therapeutic potential for the treatment of epilepsy. The Nrf2-ARE signalling pathway regulates the expression of a group of cytoprotective enzymes, such as HO-1 and NQO1. Previous studies have shown that up-regulation of HO-1 (Ahmad et al., 2006) or NQO-1 (Lim et al., 2008; Chen et al., 2011) in neurons can protect them against oxidative and excitotoxic insults. Recent studies have demonstrated that the Nrf2-ARE signalling pathway could represent an important target in protecting the brain from the damage induced by ischemic stroke, and the resulting oxidative damage (Zhao et al., 2006), and also kainate toxicity (Kraft et al., 2006). However, to our knowledge, the neuroprotective effect of activating the Nrf2-ARE signalling pathway has not been studied in detail in epilepsy.

The role of mitochondria in acquired epilepsies, which account for about 60% of all seizure disorders, is important but less well understood. Oxidative stress is one of the possible contributory mechanisms to the pathogenesis of epilepsy, resulting from mitochondrial dysfunction making neurons more vulnerable to additional stress, and leading to energy failure and neuronal cell death (Khurana et al., 2013).

Until now, the major aim of epilepsy treatment has been to control seizures with the most appropriate AED without causing any significant side effects. Commonly used AEDs, such as CBZ, VPA, LTG and LEV, are employed to provide symptomatic relief by inhibiting seizure generation and/or reducing seizure frequency by targeting neuronal hyper-excitability. However, they are unable to modulate or alter the underlying epileptogenesis process or prevent it in high risk patients. Currently, there is no clinically-approved treatment to inhibit the process of epileptogenesis (Choi, 1993; Aparicio et al., 2015). Different studies have demonstrated the use of antioxidants in

epilepsy, indicating that pre-treatment with vitamin E, vitamin C, and various plant extracts or flavonoids can reduce lipid oxidation and restore the activities of SOD and CAT and the levels of GSH in the rat hippocampus, striatum and cortex (Aparicio et al., 2016).

5.1.1 Aims and objectives

The present study aimed to investigate the effects of CBZ, VPA, LTG and LEV on the expression of Nrf2, HO-1 and NQO-1 proteins as a marker of phase II detoxification antioxidant response in the SH-SY5Y cell line.

5.2 Materials and methods

5.2.1 Materials and chemicals

Lithium dodecyl sulphate (LDS) sample buffer, sample reducing agent, enhanced chemiluminescent substrate, NuPAGE 4-12% bis tris gel (1.5mm, 15-well), anti-NQO-1 antibody, monoclonal anti-β-actin antibody (raised in mouse), anti-HO-1 antibody, goat anti-rabbit IgG (H&L) HRP-conjugated, western blotting filter paper (8cm x 10.5cm), and NuPAGE MOPS SDS running buffer (20x) were purchased from Thermo Fisher Scientific, Loughborough, UK. Anti-Nrf2 antibody (rabbit polyclonal) was purchased from Proteintech, Manchester, UK. Nitrocellulose roll (type SCN, pore size 0.45µm, 30cm x 3m) was purchased from Gene Flow Ltd., Elmhurst, UK. Precision plus Protein Kaleidoscope was purchased from Bio-Rad, Hertfordshire, UK. Other chemicals were sourced as described in chapter 2, section 2.2.1 and chapter 3, section 3.2.1.

5.2.2 Cell culture

SH-SY5Y cells were cultured as described in chapter 2, section 2.2.2.

5.2.3 Cell treatment with AEDs

SH-SY5Y cells were seeded in 12-well plates at a density of 4 x 10^4 cells / well in 1ml culture medium and placed in an incubator containing humidified 95% air and 5% CO_2 at 37^oC for 2-3 days until they reached 80-90% confluence. Then, cells were treated with CBZ (0, 1, 3, 10, 30 and 100µM), VPA (0, 10, 30, 100, 300 and 1000µM), LTG (0, 1, 3, 10, 30 and 100µM) or LEV (0, 3, 10, 30, 100 and 300µM) for 24 hours. VPA and LTG were dissolved in serum-free medium (SFM; see chapter 2, section 2.2.4) whereas CBZ and LEV were dissolved in SFM containing 0.2% DMSO. A further batch of SH-SY5Y cells was treated with the same AEDs at the same range of concentrations but was subsequently exposed to 100µM H_2O_2 for an additional 4 hours. Cells were washed

twice with 1ml PBS, lysed by scraping in 500µl RIPA lysis buffer, transferred to 1.5ml Eppendorf tubes, and placed on ice for 30 minutes. Thereafter, the lysates were centrifuged at 12000 x g at 4^{o}C for 30 minutes and the resulting supernatant transferred to new 1.5ml Eppendorf tubes for protein quantitation.

5.2.4 Protein quantitation

Protein in cell lysates was quantified by BCA Protein Assay Kit as described in chapter2, section, 2.2.8.

5.2.5 Preparation of buffers

A 10x transfer buffer stock was prepared by dissolving 144.1g of glycine and 30.3g of Trizma base in 1L distilled water. A 1x working solution of transfer buffer was prepared by adding 200ml methanol to 100ml of 10x transfer buffer stock and diluting to 1L with distilled water. A 10x solution of Tris buffered saline (TBS) was prepared by dissolving 80g of NaCl, 2g of KCl and 30.3g of Trizma base in 800ml distilled water, adjusting the pH to 7.4 with 1M HCl, and adding distilled water to a final volume of 1L. Lastly, a 1x solution of TBS with Tween (TBST) was prepared by adding 1ml of Tween 20 to 100ml of 10x TBS and diluting to 1L with distilled water.

5.2.6 Western blotting

LDS sample buffer (70µl) and sample reducing agent (30µl) were pre-mixed and 5µl of the resulting mixture was added to individual samples, each containing 20µg of protein. Samples were then heated at 95°C for 5 minutes, centrifuged at 12000 x g at 4^{o}C for 30 minutes, and kept on ice for further 5 minutes.

Pre-cast gels (15-well) were used for loading of samples. The gel tank was loaded with 800ml 1X running buffer. A 5µl volume of protein ladder (Precision plus Protein Kaleidoscope) was loaded in the first and last well of each gel and 10µl of up to 13

individual samples were loaded in between. bufferThe gel was run at 80 volts in 1x NuPAGE MOPS running buffer for 15 minutes (or until samples had run past the stacking gel) and then at 200 volts for 1 hour (or until the samples reached the ridge at the bottom of the cassette).

Following electrophoresis, the gel was carefully removed from the tank and placed in 1x transfer buffer. A gel sandwich was prepared by opposing the gel to pre-cut nitrocellulose membrane and enclosing within layers of filter paper and fibre pad as illustrated in figure 5.1. The gel sandwich was then blotted in transfer buffer at 100 volts for 1 hour during which proteins were transferred to the nitrocellulose membrane. Thereafter, the gel sandwich was disassembled and the membrane blocked in 10% non-fat dried milk in 1x TBST for 1 hour (with gentle shaking) to prevent non-specific binding of the antibodies.

Primary antibodies were diluted (1:400 for Nrf2, 1:5000 for both HO-1 and NQO-1 and β-actin) in 10% non-fat dried milk in 1x TBST and the nitrocellulose membrane incubated with individual primary antibodies overnight at 4°C (with gentle shaking). After incubation, the membrane was washed 6 times with 1x TBST at 5 minute intervals. After washing, the membrane was incubated with a 1:5000 dilution of secondary antibody in 5% non-fat dried milk in 1x TBST at room temperature for 1 hour. The secondary antibodies were anti-rabbit HRP for Nrf2, HO-1, and β-actin, and anti-goat HRP for NQO-1. Thereafter, the membrane was again washed 6 times at 5 minute intervals in 1x TBST. Enhanced chemiluminescent substrate was then applied by mixing solution A and B in 1:1 ration and incubate for 1 minute and the western blot was visualised using the ChemiDoc™ Touch Imaging system (Bio-Rad, CA, USA) and quantitated by Image Lab™ software (Bio-Rad, CA, USA).

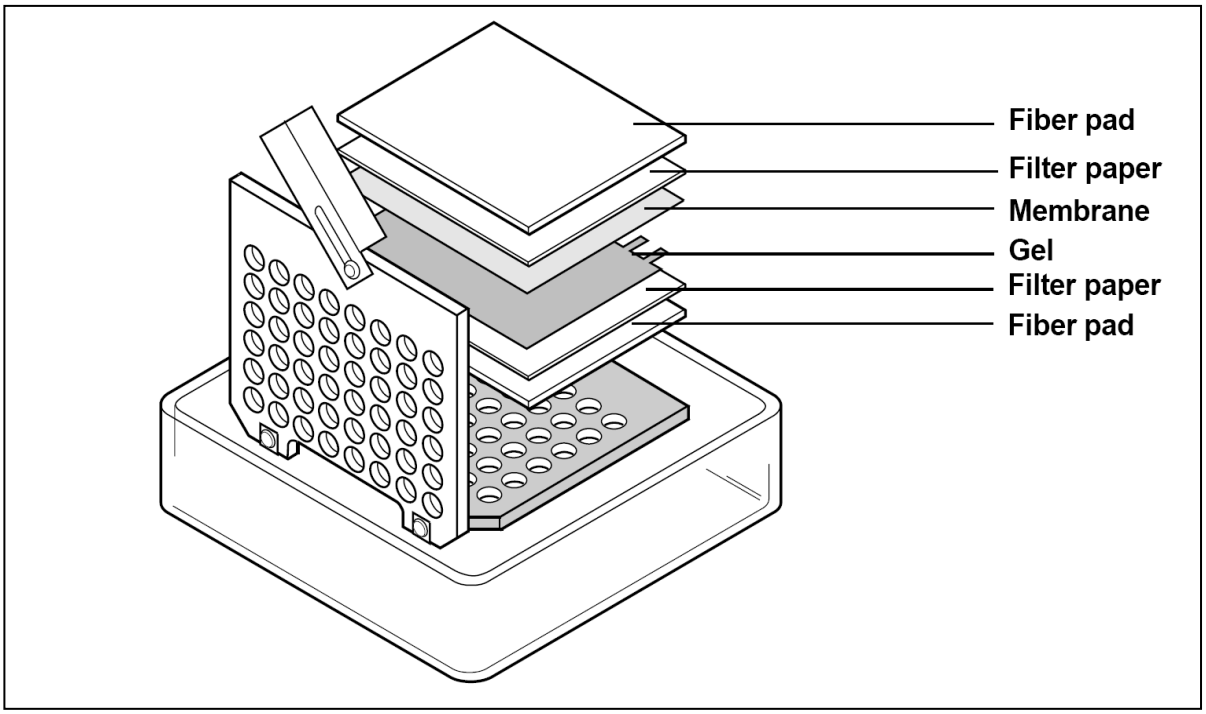


Figure 5.1 Gel sandwich cassette components. Image reproduced from Trans-Blot Turbo Instruction Manual (bulletin 10020688) for complete instructions.

5.2.7 Statistical analysis

All experiments were performed using two technical replicates (i.e. analyses of all samples performed in duplicate) and three biological replicates (i.e. n=3 per group). Protein expression in individual samples was quantified by expressing optical density (OD) of the target protein band (Nrf2, HO-1, NQO-1) normalised to that of the corresponding control protein (β-actin) band using Image Lab™ software, version 4.1. Relative expression in technical replicates (n=2) was averaged and group results (n=3) reported as the mean (± SD) normalised OD for each target protein at each concentration of each AED. Statistical comparisons were undertaken using either one-way or two-way ANOVA, as appropriate, with a Dunnett correction for multiple comparisons, using Stats Direct software version 2.7.9. Differences were considered statistically significant at $p \leq 0.05$.

5.3 Results

5.3.1 Effect of carbamazepine on basal and H_2O_2-induced protein expression in SH-SY5Y cells

Exposure of SH-SY5Y cells to CBZ alone for 24 hours was without significant effect on the expression of Nrf2 at concentrations up to 30µM (figure 5.2). At 100µM CBZ there was a significant ($p<0.05$) increase in Nrf2 expression (1.7-fold) in comparison to the corresponding control (0µM) value (figure 5.2). When CBZ treatment was followed by exposure to 100µM H_2O_2 for 4 hours to induce oxidative stress, there was a statistically significant ($p<0.001$) elevation in Nrf2 expression at both 30µM CBZ (1.9-fold) and 100µM CBZ (2.1-fold) in comparison to control (figure 5.2).

Treatment with CBZ (1-10µM) for 24 hours was without significant effect on HO-1 expression in SH-SY5Y cells, with or without subsequent exposure to 100 µM H_2O_2 for 4 hours (figure 5.3). In contrast, 30µM CBZ and 100µM CBZ were associated with significant ($p<0.001$) increases in the expression of HO-1, compared to control, when administered alone (1.7-fold and 2.0-fold, respectively) and also following additional exposure to 100µM H_2O_2 for 4 hours (2-fold and 1.9-fold, respectively) (figure 5.3).

There was no significant change in NQO-1 expression in SH-SY5Y cells following 24 hour treatment with CBZ at concentrations up to 10µM, with or without subsequent exposure to 100µM H_2O_2 for further 4 hours (figure 5.4). However, 30µM and 100µM CBZ were associated with a statistically significant ($p<0.05$) increase in the expression of NQO-1, compared to control, when administered alone (1.6-fold and 2-fold, respectively). Significant increases in NQO-1 expression ($p<0.05$) were also observed when 30µM and 100µM CBZ was followed by exposure to 100µM H_2O_2 for 4 hours (2-fold and 2.3-fold, respectively) (figure 5.4).

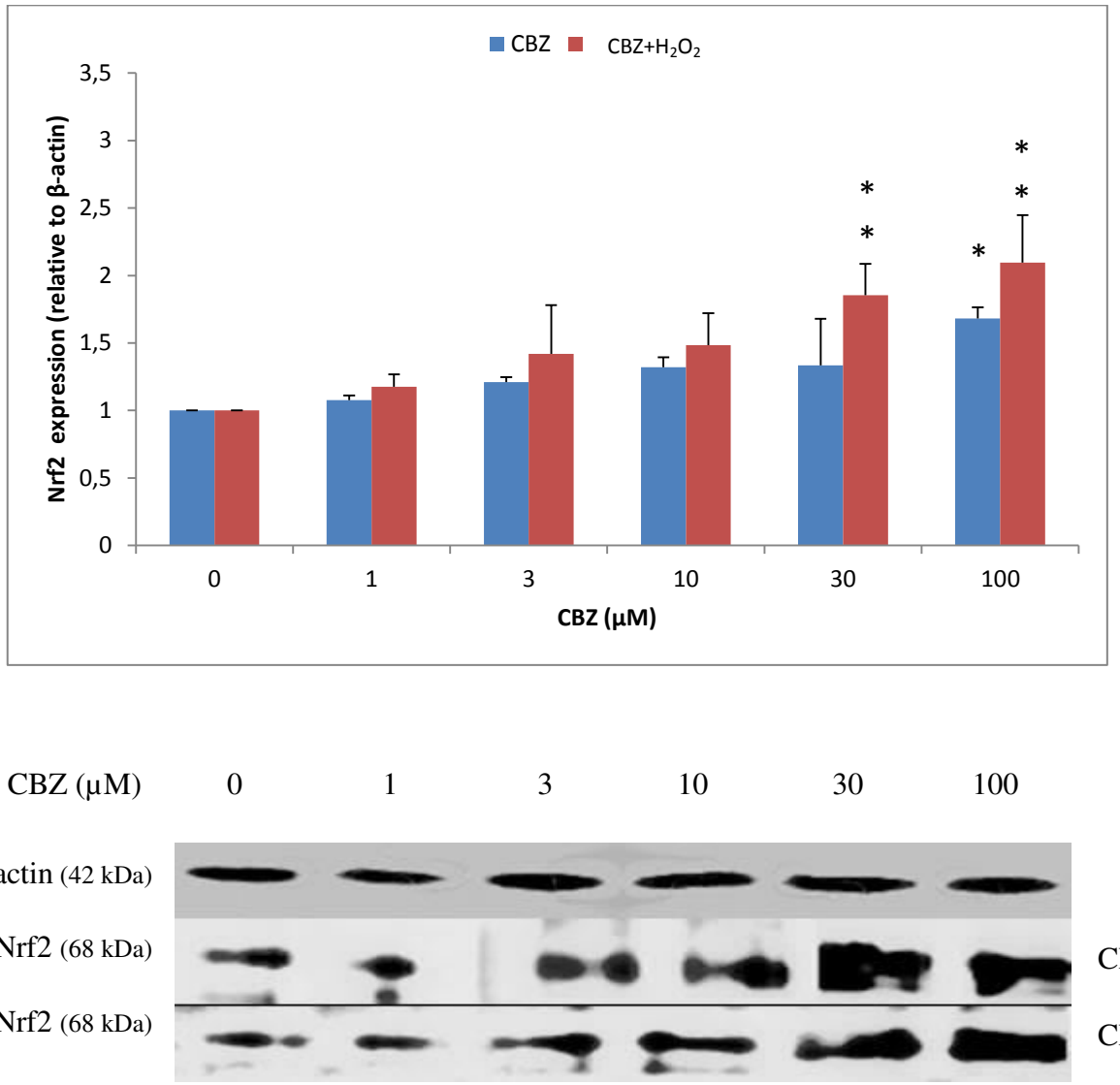
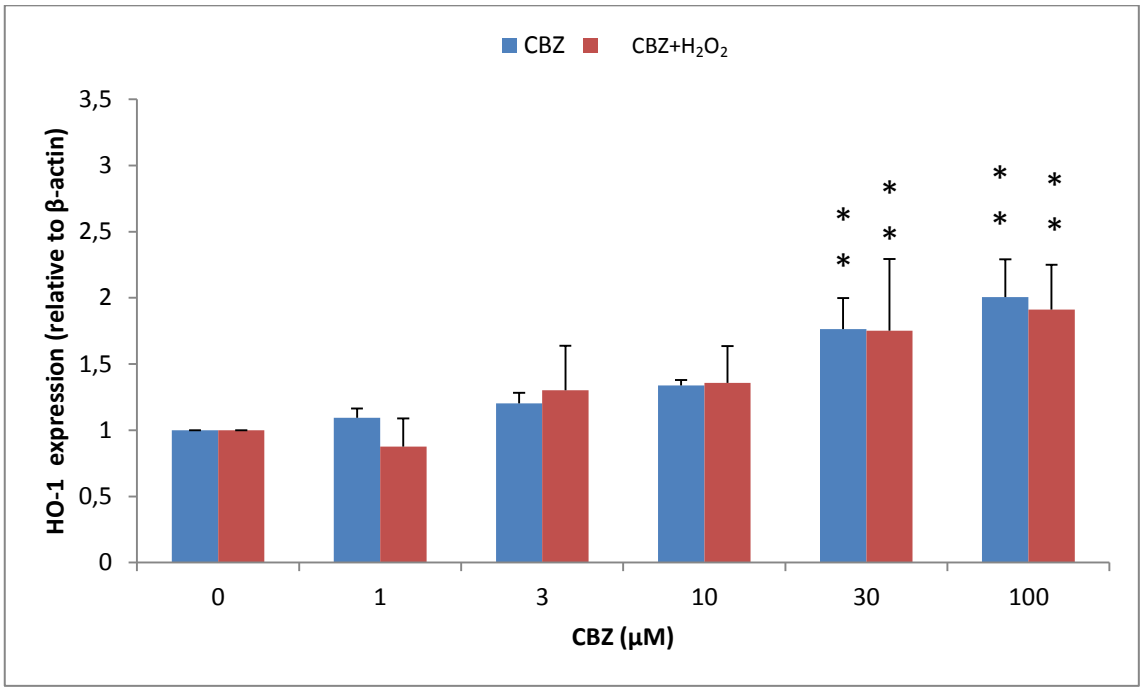


Figure 5.2: Effect of 24 hour treatment with CBZ (0-100μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on Nrf2 expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) expression of Nrf2, normalised to that of β-actin. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*p<0.05; **p<0.001).



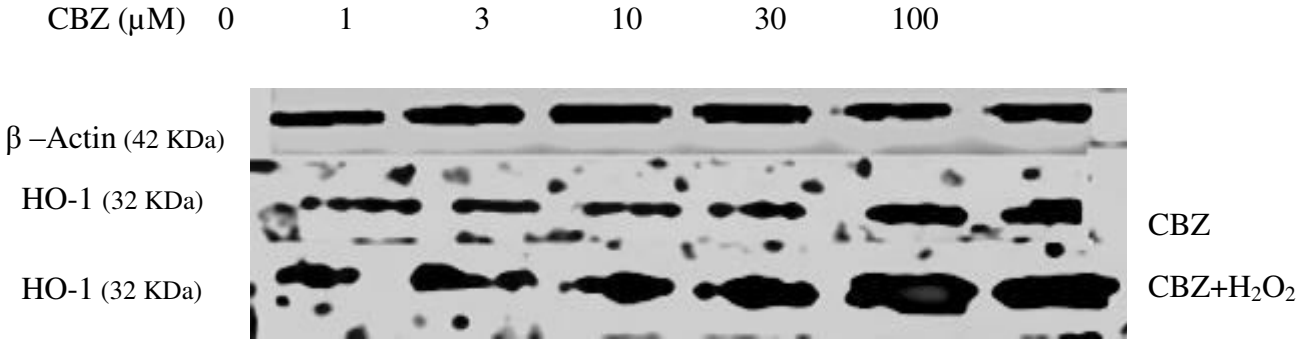


Figure 5.3 Effect of 24 hour treatment with CBZ (0-100µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on HO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in HO-1 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05; **p<0.001).

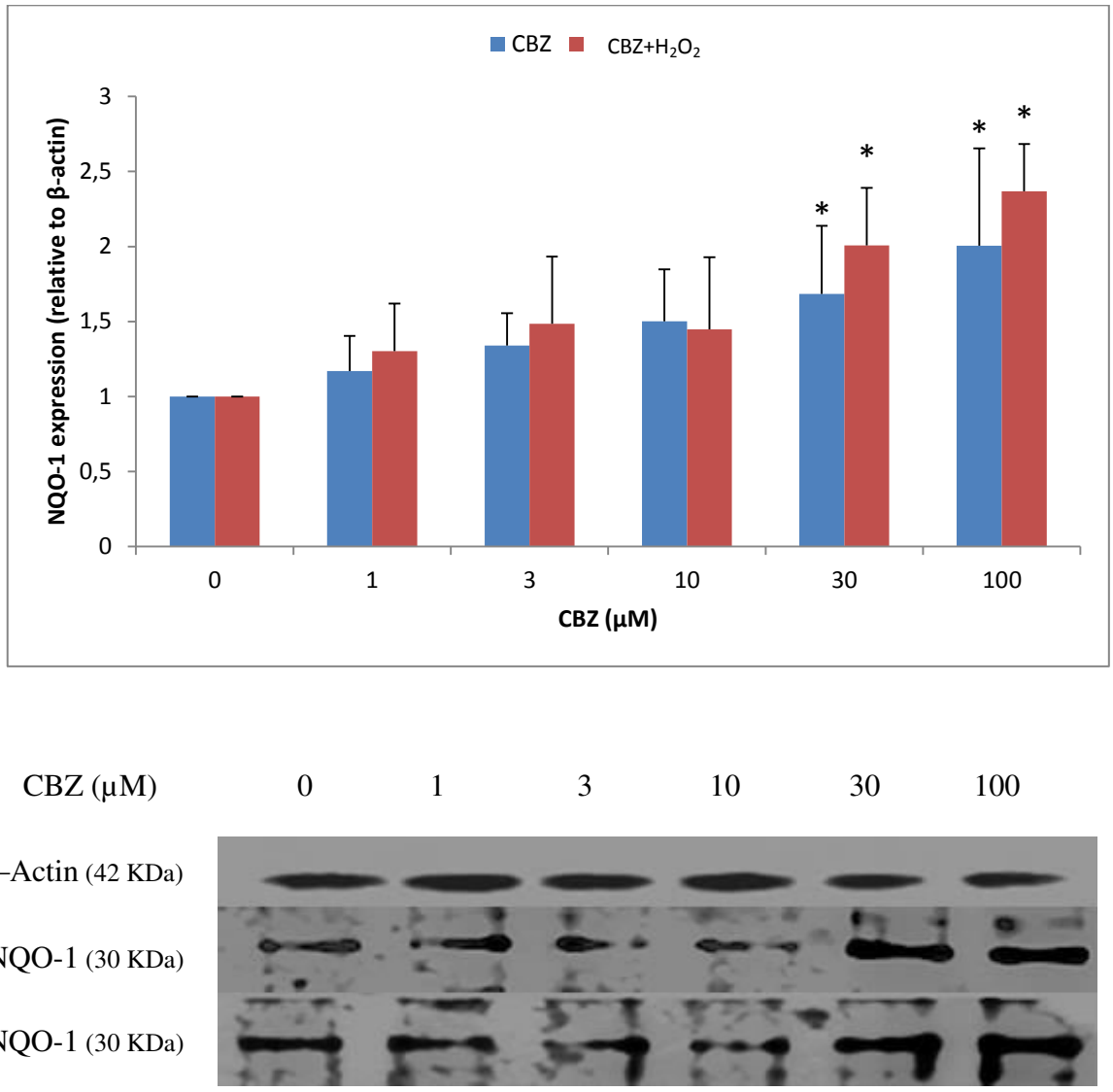


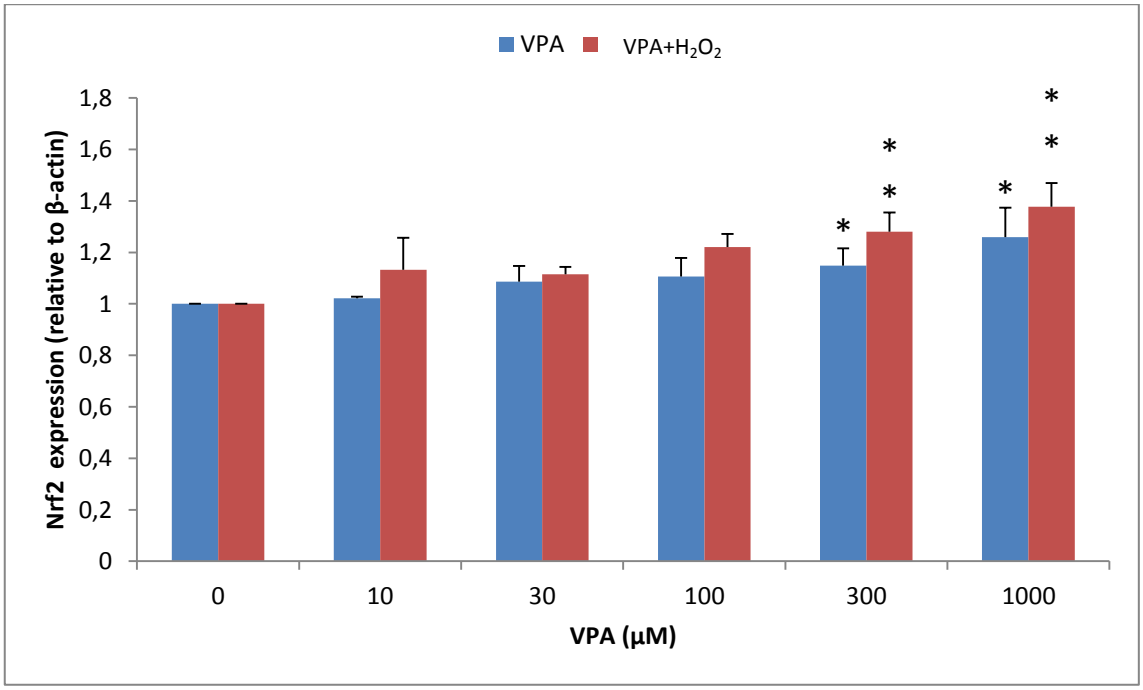
Figure 5.4 Effect of 24 hour treatment with CBZ (0-100μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on NQO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in NQO-1 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*$p<0.05$).

5.3.2 Effect of valproic acid on basal and H_2O_2-induced protein expression in SH-SY5Y cells

There was no significant change in Nrf2 expression in SH-SY5Y cells following 24 hour treatment with VPA at concentrations up to 100µM, with or without subsequent exposure to 100µM H_2O_2 for further 4 hours (figure 5.5). However, 300µM and 1000µM VPA were associated with a statistically significant ($p<0.05$) increase in the expression of Nrf2, compared to control, when administered alone (1.1-fold and 1.3-fold, respectively). Significant increases in Nrf2 expression ($p<0.001$) were also observed when 300µM and 1000µM VPA was followed by exposure to 100µM H_2O_2 for 4 hours (1.3-fold and 1.4-fold, respectively) (figure 5.5).

Treatment with VPA (10-100µM) for 24 hours was without significant effect on HO-1 expression in SH-SY5Y cells, with or without exposure to 100µM H_2O_2 for 4 hours (figure 5.6). In contrast, there was a significant ($p<0.05$) increase in the expression of HO-1, compared to control, following 300µM VPA alone (1.2-fold) and following further exposure to 100µM H_2O_2 (1.3-fold). Statistically significant ($p<0.001$) increases in HO-1 expression were also observed following treatment with 1000µM VPA alone (1.3-fold) and after additional exposure to 100µM H_2O_2 (1.4-fold) (figure 5.6).

Treatment with VPA (10-300µM) for 24 hours was without significant effect on NQO-1 expression in SH-SY5Y cells, with or without subsequent exposure to 100 µM H_2O_2 for 4 hours (figure 5.7). However, 1000µM VPA was associated with significant ($p<0.05$) increases in the expression of NQO-1, compared to control, when administered alone (1.3-fold) and also following additional exposure to 100µM H_2O_2 for 4 hours (1.32-fold) (figure 5.7).



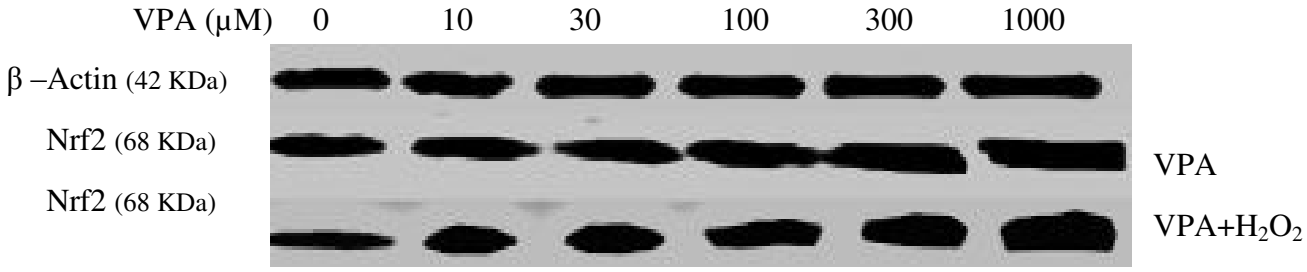


Figure 5.5 Effect of 24 hour treatment with VPA (0-1000μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on Nrf2 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in Nrf2 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*p<0.05; **p<0.001).

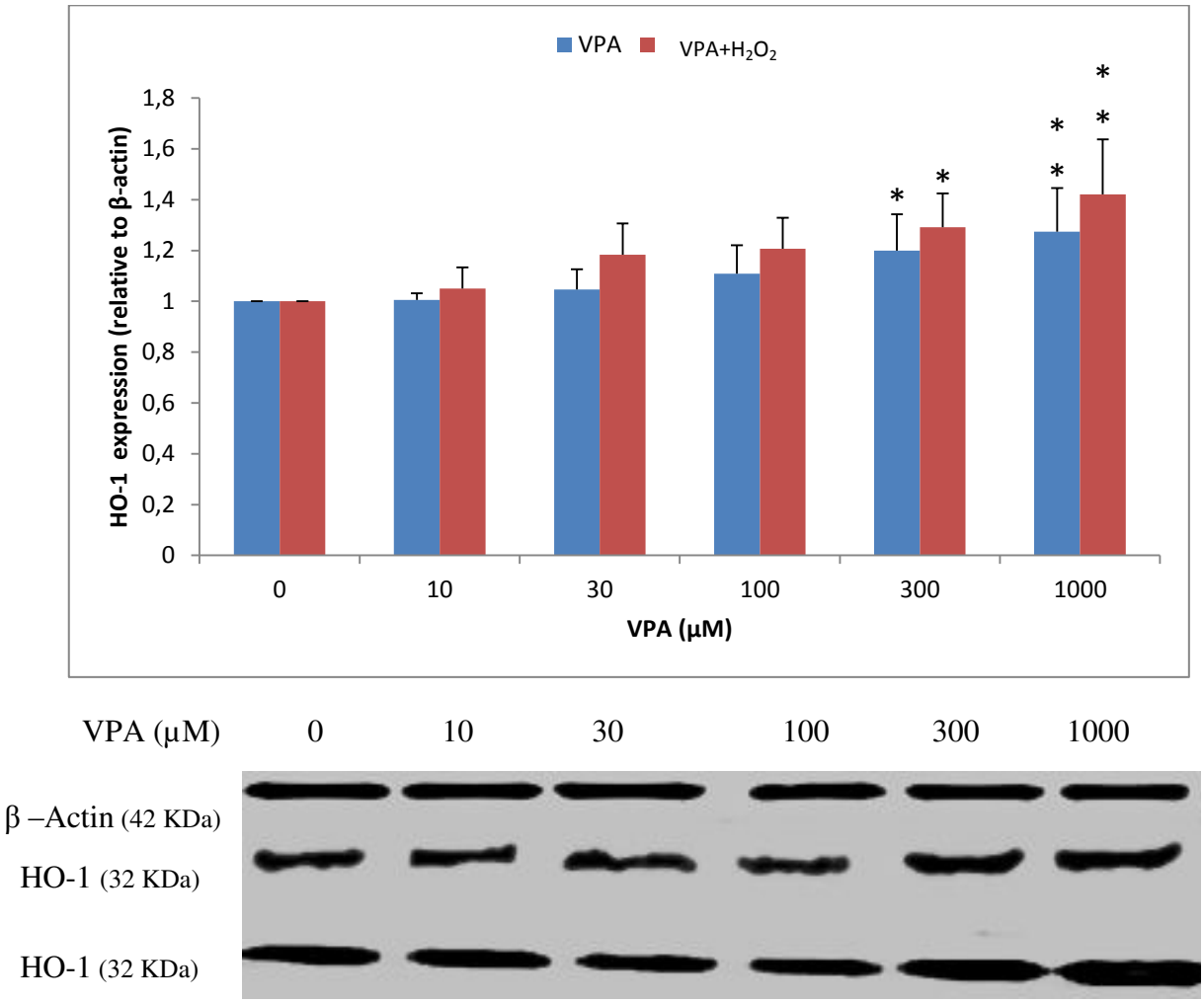


Figure 5.6 Effect of 24 hour treatment with VPA (0-1000μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on HO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in HO-1 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*p<0.05; **p<0.001).

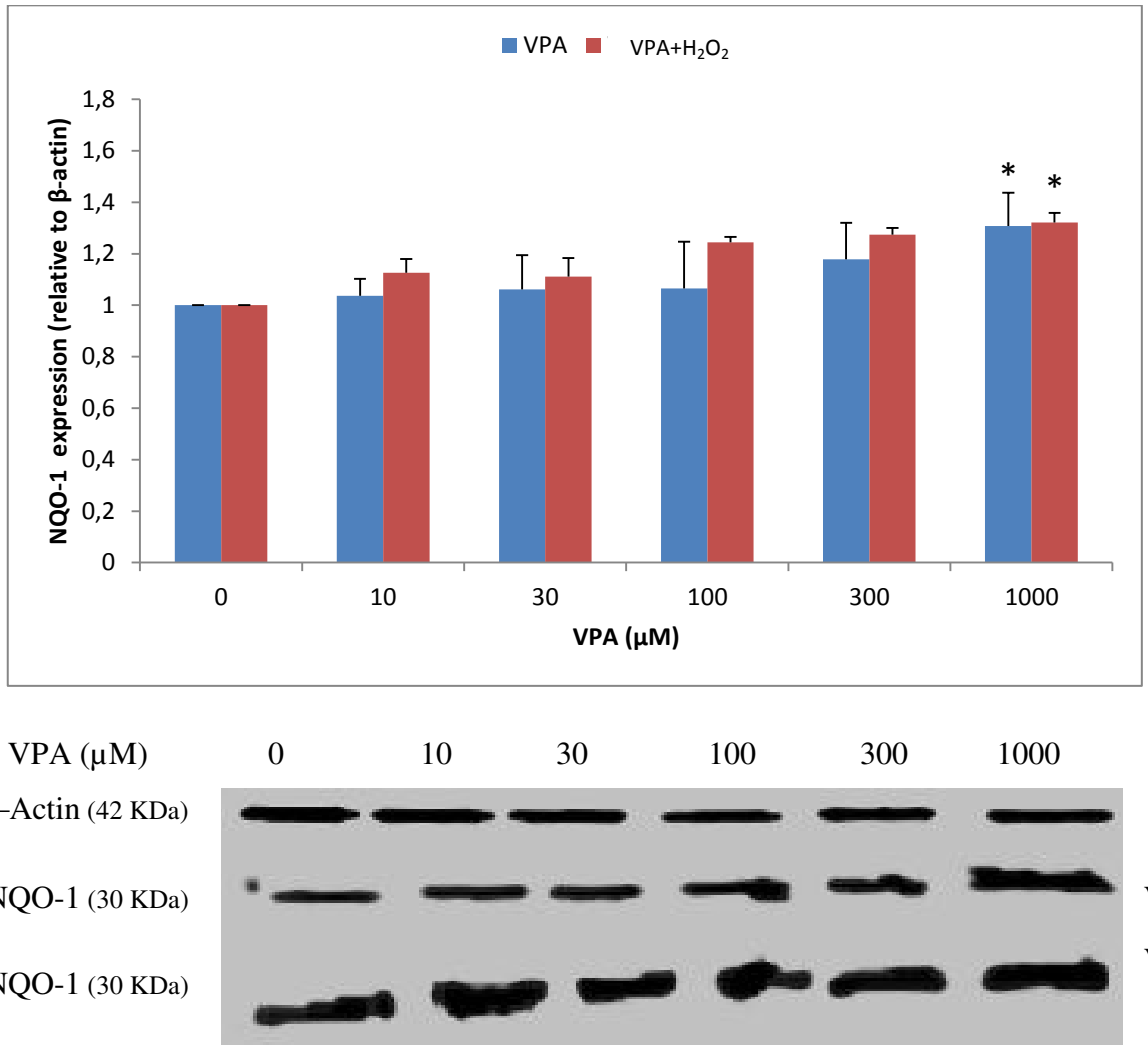


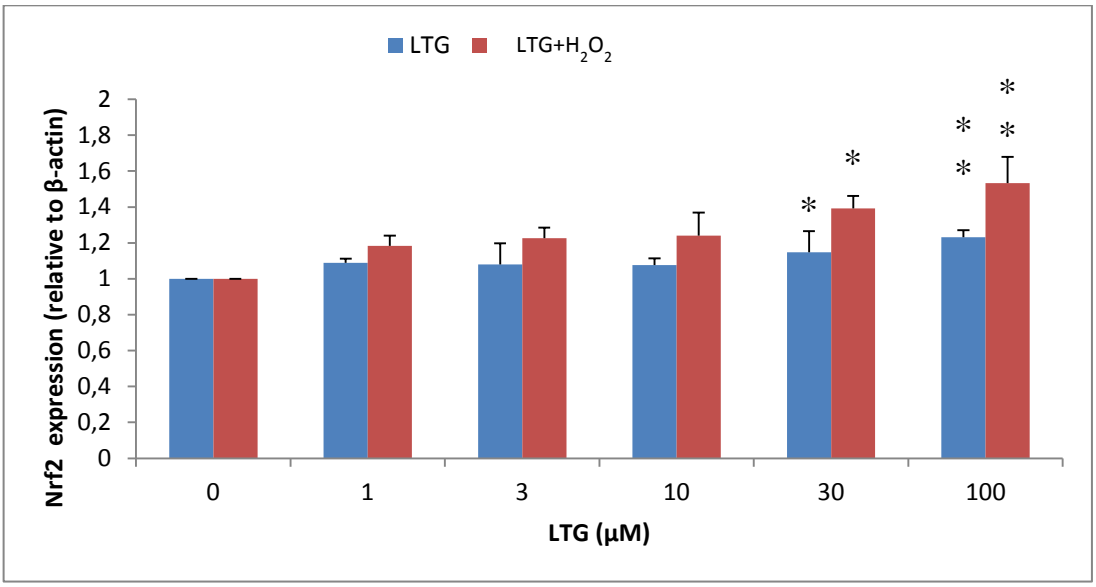
Figure 5.7 Effect of 24 hour treatment with VPA (0-1000µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on NQO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in NQO-1 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0µM) within each data series (*p<0.05; **p<0.001).

5.3.3 Effect of lamotrigine on basal and H_2O_2-induced protein expression in SH-SY5Y cells

Treatment with LTG (1-10μM) for 24 hours was without significant effect on Nrf2 expression in SH-SY5Y cells, with or without exposure to 100μM H_2O_2 for 4 hours (figure 5.8). In contrast, there was a significant ($p<0.05$) increase in the expression of Nrf2, compared to control, following 30μM LTG alone (1.1-fold) and following further exposure to 100μM H_2O_2 for 4 hours (1.4-fold). Statistically significant ($p<0.001$) increases in Nrf2 expression were also observed following treatment with 100μM LTG alone (1.2-fold) and after additional exposure to 100μM H_2O_2 (1.5-fold) (figure 5.8).

Treatment of SH-SY5Y cells with LTG (1-30μM) for 24 hours was without effect on the expression of HO-1, with or without subsequent exposure to 100 μM H_2O_2 for 4 hours (figure 5.9). However, treatment with 100μM LTG was associated with significant ($p<0.001$) increases in the expression of HO-1, compared to control, when administered alone (1.6-fold) and also following additional exposure to 100μM H_2O_2 for 4 hours (1.7-fold) (figure 5.9).

Expression of NQO-1 in SH-SY5Y cells was not significantly altered by treatment with LTG (1-10μM) for 24 hours, with or without subsequent exposure to 100μM H_2O_2 for 4 hours (figure 5.10). At 30μM LTG, there was a significant ($p<0.05$) increase in the expression of NQO-1, compared to control, following LTG treatment alone and also following further exposure to 100μM H_2O_2 (both 1.3-fold). Statistically significant ($p<0.001$) increases in NQO-1 expression were also observed following treatment with 100μM LTG alone (1.4-fold) and after 100μM H_2O_2 exposure (1.5-fold) (figure 5.10).



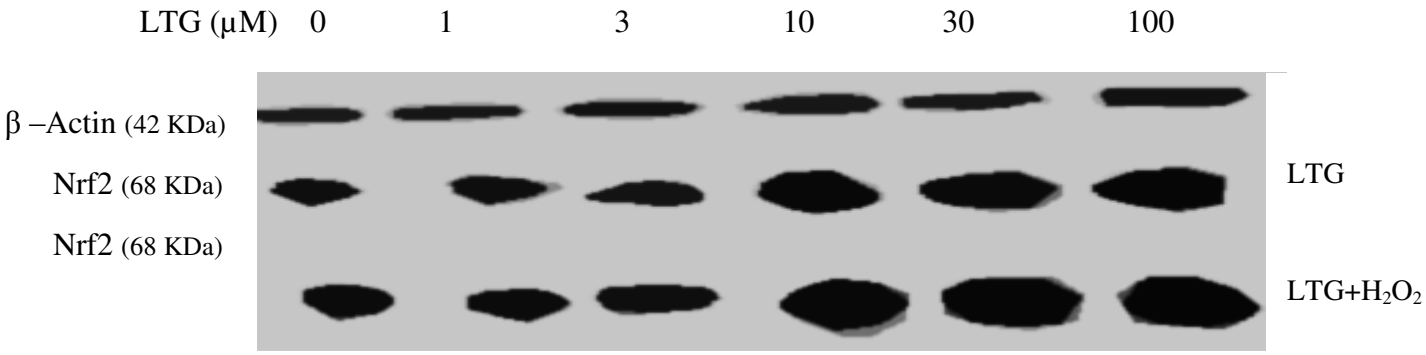


Figure 5.8 Effect of 24 hour treatment with LTG (0-100μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on Nrf2 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in Nrf2 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*$p<0.05$; **$p<0.001$).

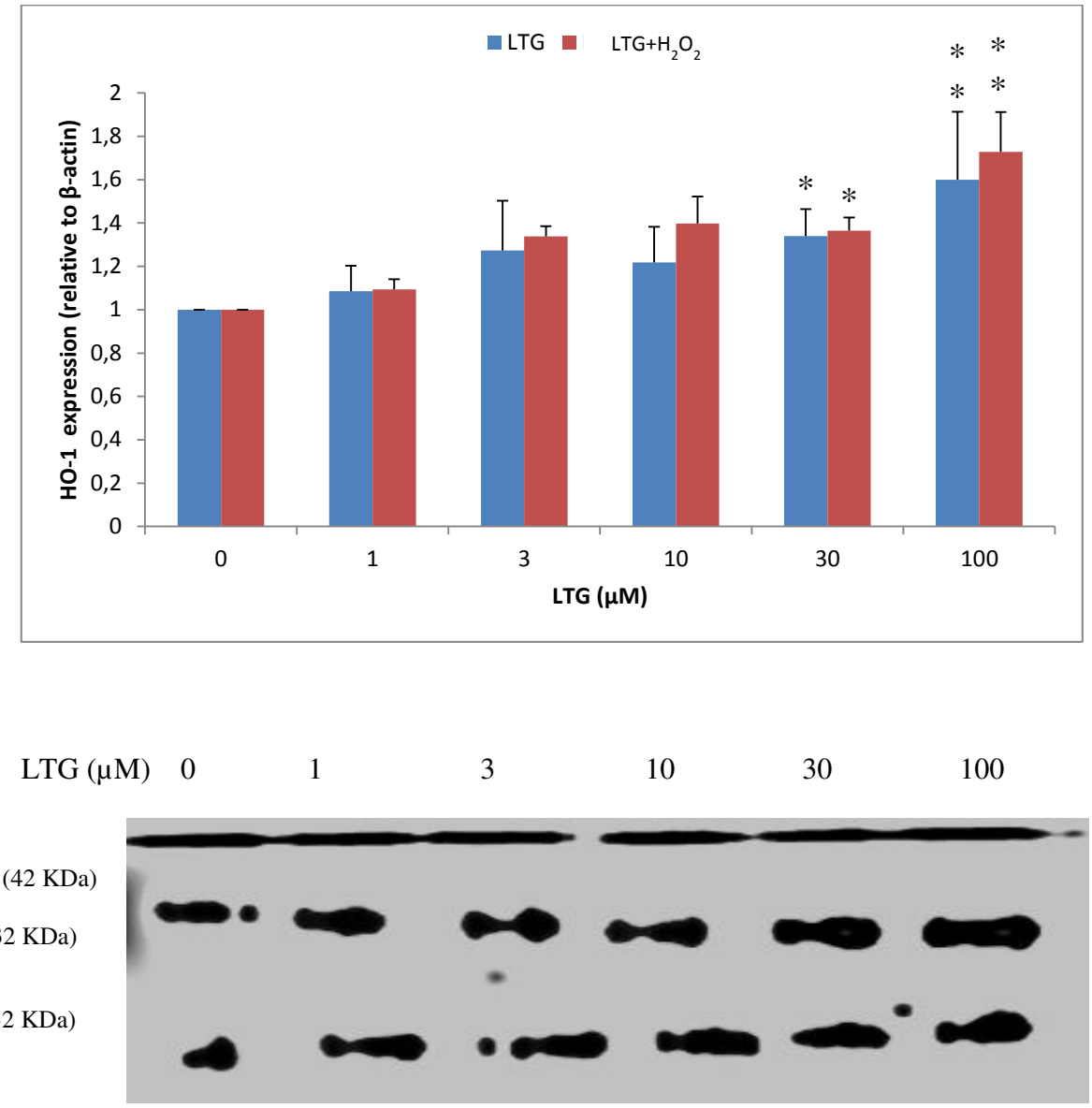


Figure 5.9 Effect of 24 hour treatment with LTG (0-100μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on HO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in HO-1 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*p<0.05; **p<0.001).

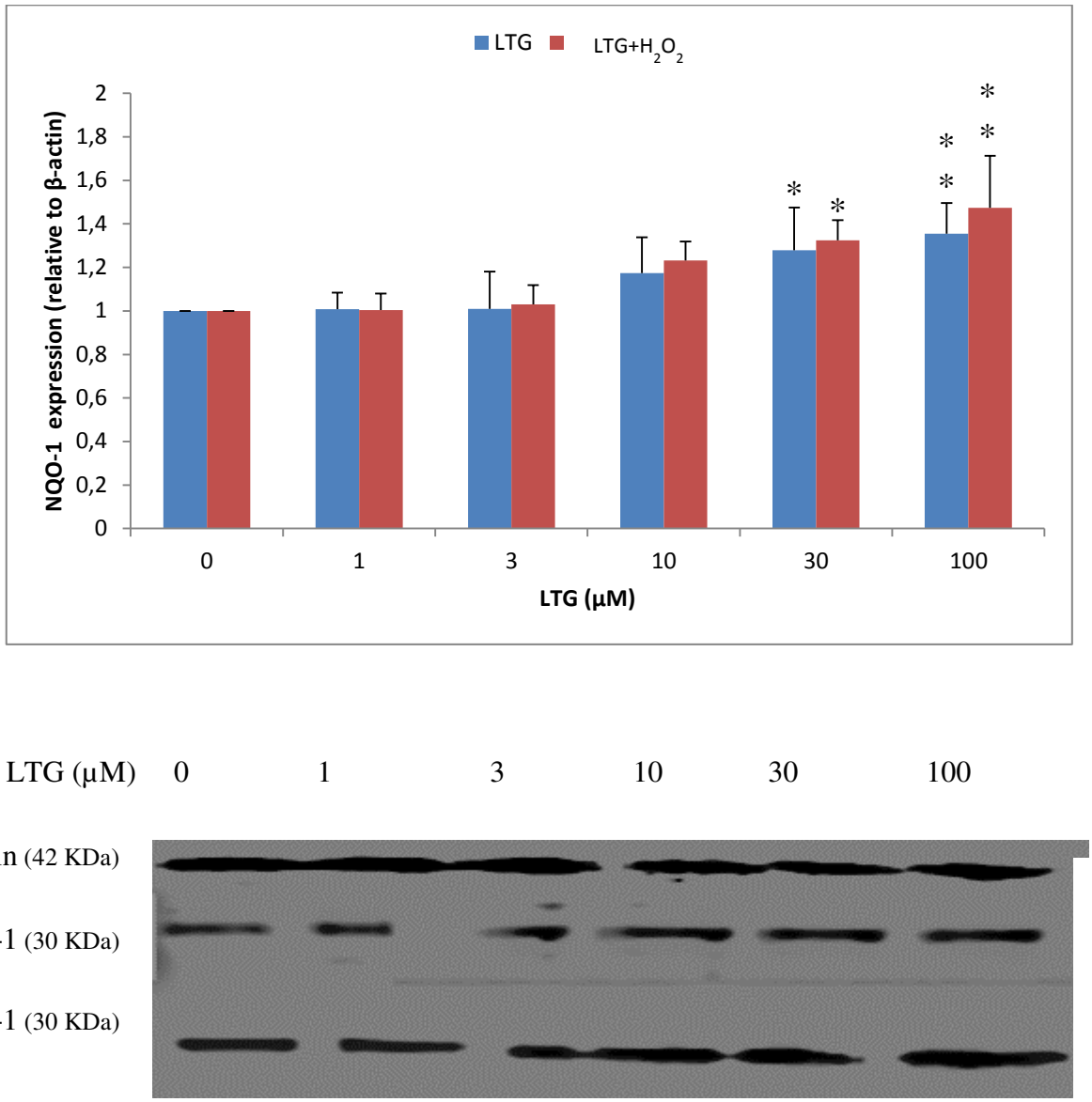


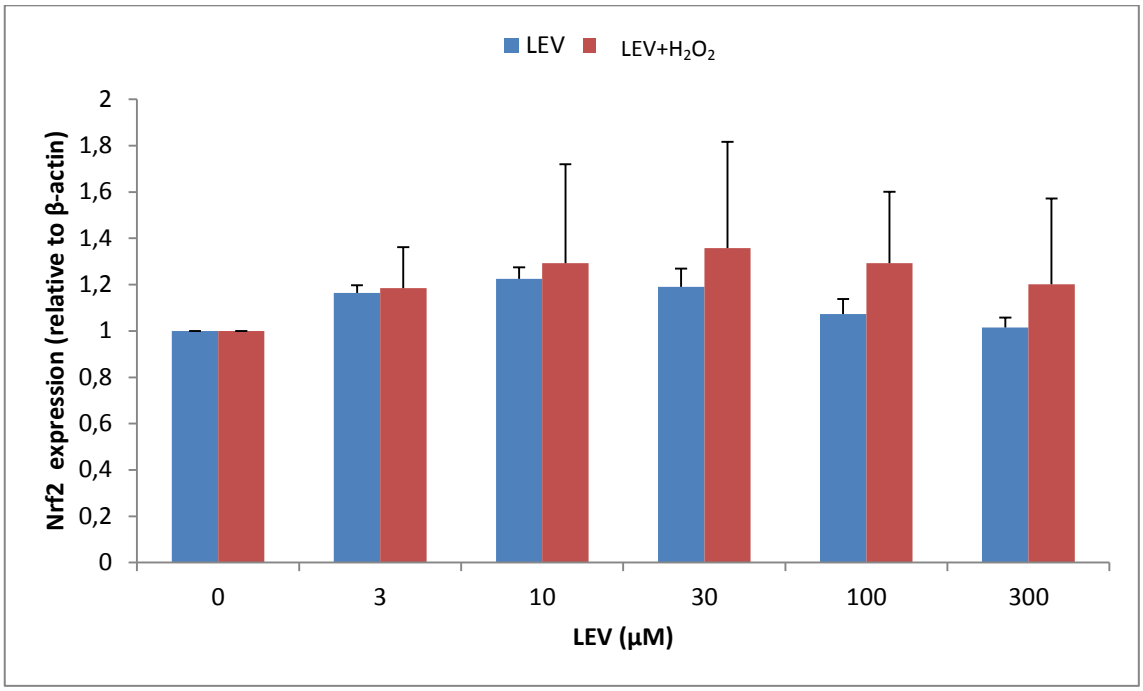
Figure 5.10 Effect of 24 hour treatment with LTG (0-100μM), with and without additional exposure to 100μM H_2O_2 for 4 hours, on NQO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in NQO-1 expression, relative to β-actin expression. Statistical significance was determined by one-way ANOVA with Dunnett correction for multiple comparisons with control (0μM) within each data series (*p<0.05; **p<0.001).

5.3.4 Effect of levetiracetam on basal and H_2O_2-induced protein expression in SH-SY5Y cells

There were no significant changes in Nrf2 expression, in comparison to control, following treatment of SH-SY5Y cells with LEV (3-300μM) for 24 hours, with or without subsequent exposure to 100μM H_2O_2 for a further 4 hours (figure 5.11).

Likewise, 24 hour exposure to LEV (3-300μM) was without significant effect on the expression of HO-1 protein in SH-SY5Y cells when compared to control, with or further exposure to 100μM H_2O_2 for 4 hours (figure 5.12).

Finally, the expression of NQO-1 in SH-SY5Y cells was not significantly altered, in comparison to control, following 24 hour treatment with LEV (3-300μM), with or without subsequent exposure to 100μM H_2O_2 (figure 5.13).



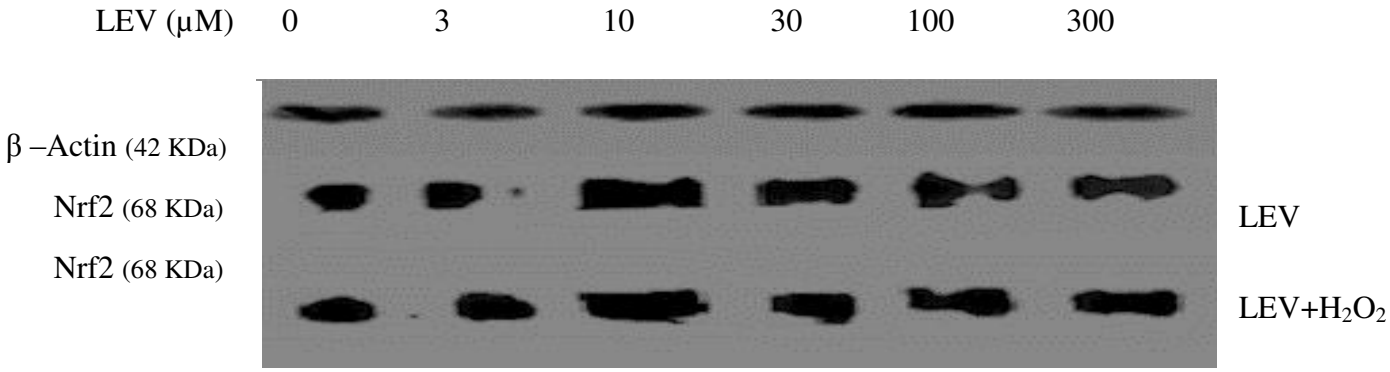


Figure 5.11 Effect of 24 hour treatment with LEV (0-300µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on Nrf2 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in Nrf2 expression, relative to β-actin expression.

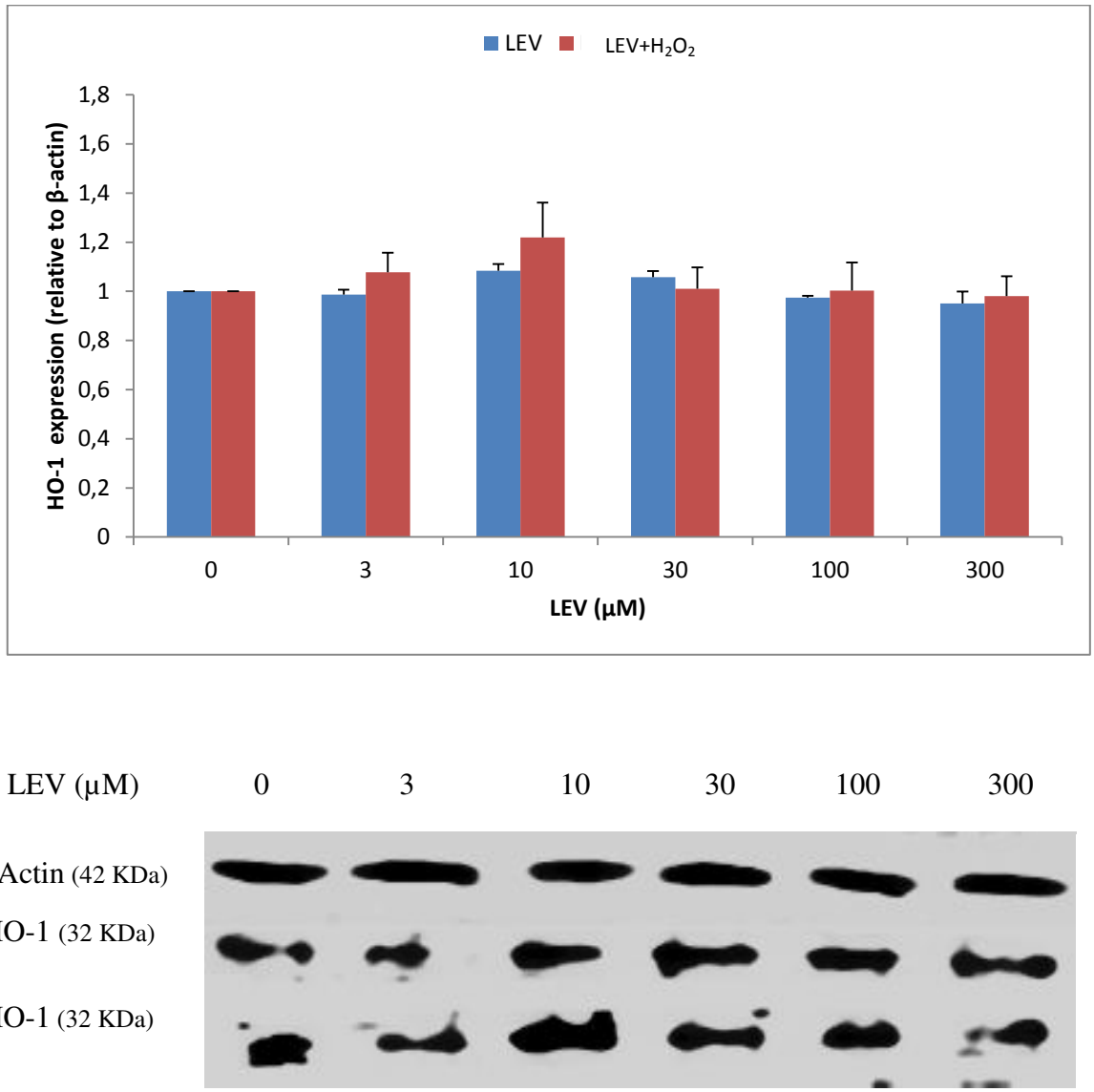


Figure 5.12 Effect of 24 hour treatment with LEV (0-300µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on HO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in HO-1 expression, relative to β-actin expression.

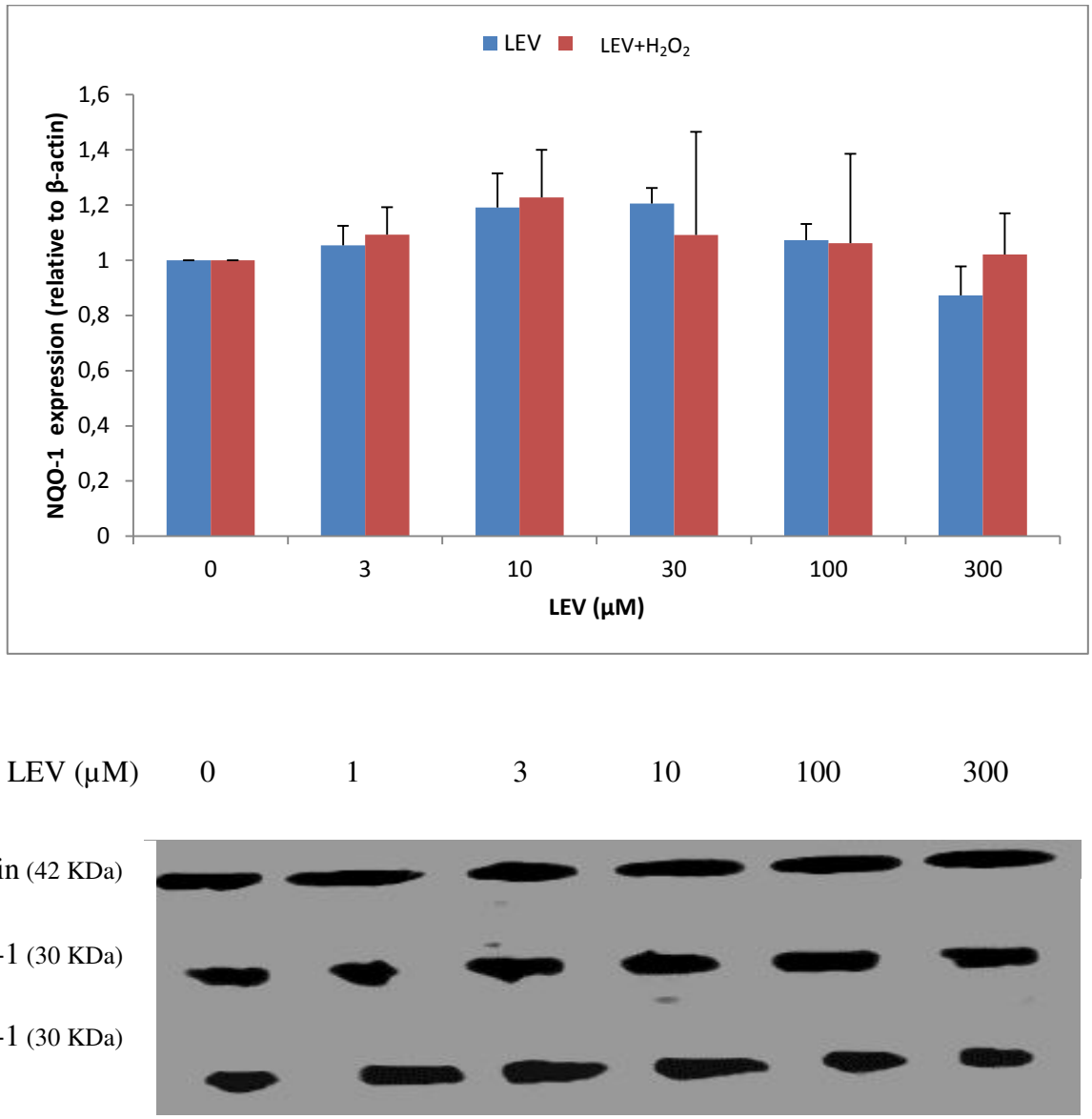


Figure 5.13 Effect of 24 hour treatment with LEV (0-300µM), with and without additional exposure to 100µM H_2O_2 for 4 hours, on NQO-1 protein expression in SH-SY5Y cells. Results (n=3) are expressed as the mean (± SD) protein in NQO-1 expression, relative to β-actin expression.

5.4 Discussion

The primary purpose of AEDs is to control the neuronal hyperexcitability that underlies the generation of epileptic seizures (Holsti et al., 2007). They are not designed nor intended to address the causes of epilepsy *per se*, or to prevent its development following epileptogenic brain insults. One mechanism that has implicated in epileptogenesis and in the perpetuation of seizures is oxidative stress (Pearson et al., 2015). Oxidative stress arises as a result of an imbalance between oxidation and anti-oxidation, which leads to excessive ROS and reduced anti-oxidative capacity (Aparicio et al., 2015). Excessive oxygen free-radicals generated during seizures have been shown to dramatically impair the structure and function of neurons (Freitas et al., 2005). Nrf2, as a transcription factor, controls the basal and inducible expression of an array of antioxidant and detoxification enzymes that act to degrade oxygen free-radicals and limit the associated cellular damage (Miller et al., 2015). Disruption of the Nrf2-ARE signalling pathway results in an increased susceptibility to oxidative insults and the effects of other toxicants (Kwak et al., 2002). Activation of the Nrf2-ARE pathway protects neurons against oxidative and excitotoxic damage (Ahmed et al., 2006). Thus, susceptibility of neurons to oxidative injury critically depends on effective Nrf2/ARE signalling but little is known about physiological signals that regulate this neuroprotective pathway in the CNS.

A growing body of evidence has been focused on the link between oxidative stress and neurological disorders (Santos et al., 2008; Khurana et al., 2013), and therefore it is essential to clarify if drugs used in treating those disorders can affect cellular responses to oxidative insults. If a drug is found to significantly up-regulate Nrf2 and its downstream antioxidant target genes, this could be suggestive of protective effects against oxidative stress. Nevertheless, this effect might also be observed with a drug

that possesses pro-oxidant activity, on the basis that the up-regulation of Nrf2 is part of the defence mechanisms of the cell to a pro-oxidant challenge. In this case, additional investigations at multiple clearly-defined time-points would be needed to assess oxidative stress and antioxidant response in drug treated cells to be able to distinguish these outcomes. Conversely, if a drug is found to down-regulate the expression of Nrf2, and that of its related target genes, this could point to that drug causing or predisposing cells to oxidative stress and the associated macro- and micro-cellular damage.

The aim of this study was to determine whether four commonly used AEDs (CBZ, VPA, LTG and LEV) had any significant effect on the expression of the transcription factor Nrf2, which is a master regulator of oxidative stress in living tissues, and on the expression of HO-1 and NQO-1 which are nuclear Nrf2-regulated genes. Experiments were conducted using SH-SY5Y cells, which were chosen as an *in vitro* model of neuronal-like cells. Drug effects against basal protein expression and also on expression induced by H_2O_2 were explored, the latter in anticipation of protective effects of prolonged AED exposure against oxidative stress.

The results obtained from this study revealed that exposure to a range of concentrations of CBZ, VPA and LTG for 24 hours, with and without further exposure to H_2O_2 for 4 hours, led to an upregulation in the expression of Nrf2, HO-1 and NQO-1 proteins in a largely concentration-dependent manner but which was typically only significant at the highest concentrations. Interestingly, and in contrast to other AEDs, there were no significant effects of LEV, at any concentration, on basal or H_2O_2-induced expression of any of these proteins. Interestingly, these results are highly cinsistent with the chapter4 results in which widely used AEDs, including CBZ, VPA and LTG, increased the gene expression of Nrf2, HO-1 and NQO-1 in SH-SY5Y cells following 24 hour treatment, particularly at high concentrations and irrespective of subsequent exposure to H_2O_2.

Interestingly, another AED, namely LEV, was without effect on expression of the selected genes.

These results reflect those of Pavone and Cardile (2003), who found that there was a time and concentration-dependent decline in cell viability and an increase in oxidative stress parameters, including total ROS, lactate dehydrogenase, TBARS, and nitric oxide (NO) concentration, following AED exposure in glial cells.

There are several possible explanations for the link between oxidative stress and AEDs. Recently, much attention has been paid to the influence of AEDs, and subsequent oxidative stress, on mitochondrial function (Khurana et al., 2013).

The involvement of oxidative stress in the pathogenesis of epilepsy likely results from mitochondrial dysfunction gradually disrupting the intracellular homeostasis, which in turn modulates neuronal excitability and synaptic transmission, making neurons more vulnerable to additional stresses, and leading to energy failure and neuronal loss. Both mitochondrial dysfunction and oxidative stress are considered critical determinants of the neuronal death associated with seizures (Cock, 2002) and may also play a role in subsequent epileptogenesis and chronic epilepsy. AEDs can also affect mitochondrial function in several ways (Khurana et al., 2013). A study by Santos and colleagues (2008) explored the *in vitro* effects of CBZ, phenytoin and phenobarbital on mitochondrial function and showed that aromatic AEDs can negatively affect mitochondrial function, via the generation of excessive ROS. This resulted in a decrease in mitochondrial respiration, respiratory control rate, ATP synthesis and membrane potential, impairment of Ca^{2+} uptake and release, and inhibition of Ca^{2+}-induced swelling. They suggested that the rare cases of hepatotoxicity seen with these aromatic AEDs might be linked to its mitochondrial toxicity and oxidative stress (Santos et al., 2008).

The effects of VPA in the current study are in accordance with a recent publication (Jafarian et al., 2013) that reported a detailed investigation of the metabolic effect of VPA in isolated rat liver mitochondria. The authors found that VPA treatment caused an increase in mitochondrial ROS production via impairment of respiratory chain complex II, which was followed by elevation of lipid peroxidation without GSH oxidation. VPA also induced mitochondrial permeability transition pore opening, mitochondrial membrane potential collapse, mitochondrial swelling, and cytochrome C release, which activates the intrinsic death signalling pathway (Jafarian et al., 2013). Another possible explanation for the pro-oxidant effects of VPA may be an increase in the generation of ROS via the activation of peroxisome proliferator activated receptors (PPARs) that perform specific functions in fatty acid homeostasis and are known to up-regulate CYP proteins (Chawla et al., 2001). This is additionally supported by previous observations from an *in vitro* study (Olson et al., 1986) which showed that administration of 500μM VPA to rat liver tissue cultures increased the rate of formation of H_2O_2, with the consequent oxidative stress expected to cause nuclear translocation of Nrf2 and an increase in the expression of its target genes and their protein products.

The results obtained with LTG in the current study are in agreement with a recent *in vivo* experiment by Abelaira et al (2011) who reported that acute administration of LTG to rats decreased mitochondrial respiratory chain complex I activity in the brain and increased the activity of complex II in the amygdala, complex II and III in the prefrontal cortex, and complex II and IV in the hippocampus. In contrast, chronic treatment with LTG increased activity of complex II and III in the amygdala alone. The authors suggested that the observed increases in the activity of complexes II, III and IV might arise, at least in part, as compensation for the decrease in complex I activity. Such effects of LTG on the mitochondrial respiratory chain might be considered positive,

taking into account the impairment in energy metabolism related to oxidative stress and cellular damage (Abelaira et al., 2011).

Thus, it is clear that several of the AEDs employed in the current study have the ability to negatively affect mitochondrial function (figure 5.14) and to cause excessive oxidative damage through liberation of ROS, which in turn can decrease sequestration of Nrf2 by Keap-1, preventing its degradation and increasing its nuclear translocation and activation of antioxidant target genes.

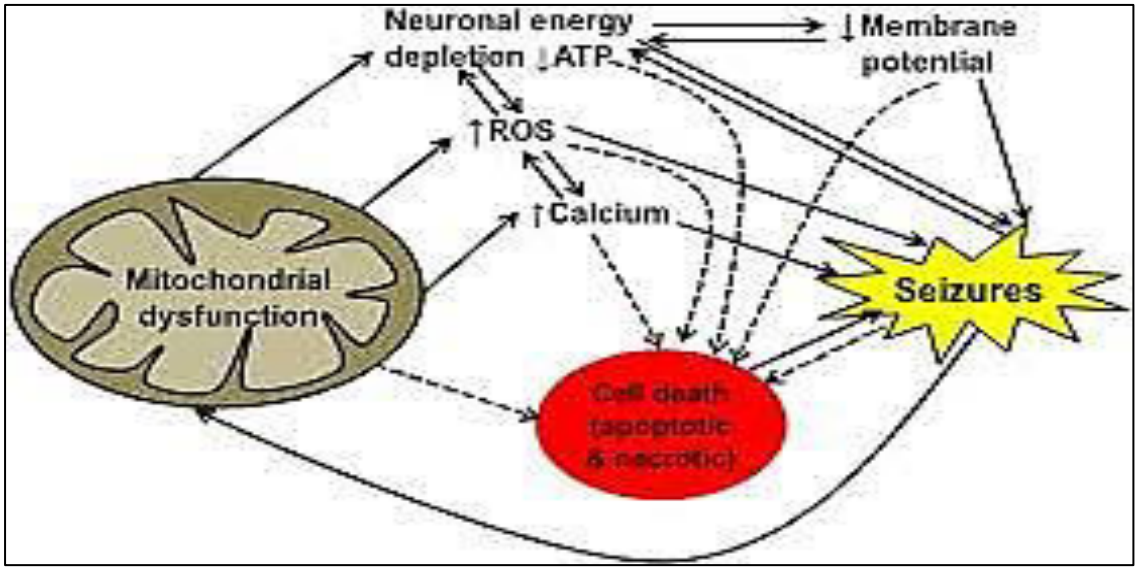


Figure 5.14: Disease mechanisms in mitochondrial epilepsy. There is a complex inter-relationship between mitochondrial dysfunction, seizures, and cell death. Key molecular mechanisms mediating epileptogenesis include neuronal ATP depletion, generation of reactive oxygen species (ROS), and abnormal calcium signalling. Complex molecular cascades are established, each feeding back on other components of the cascades, leading to a vicious spiral of mitochondrial dysfunction, seizure generation, and cell death (adapted from Rahman, 2015).

In contrast to other AEDs, LEV was without effect on protein expression in this study. The reason for this is unclear but there are some similarities between the behaviour of LEV reported here and that described by Oliveira and colleagues (2016). These authors found that LEV could help to protect against the oxidative stress induced by several ROS by reduction of lipid peroxidation and nitrite-nitrate contents, preservation of

catalase activity at control parameters and increase of GSH levels. These results allow us to infer the participation of this effect in the neuroprotective mechanism of LEV, and reinforce, even indirectly, the hypothesis of oxidative damage in the pathophysiology of epilepsy. These findings are also in agreement with other published work (Khurana et al., 2013) which showed that therapeutic concentrations of LEV have antioxidant and neuroprotective effects through increasing TAO, catalase, and Gpx activity. One possible explanation for these results is that pre-incubation of cells with LEV might result in preservation of the enzyme catalase, which is an anti-oxidant enzyme responsible for the breakdown of H_2O_2 into water and oxygen.

As with all experimental investigations, there are a number of limitations that must be considered when interpreting the results of this study. The western blot method used to quantify protein expression is employed widely and is highly reliable but occasional inconsistencies in gel quality and in band resolution can influence results. This is evident when comparing differences between individual blots, but is compensated (at least in part) by individual normalisation to β-actin expression. Low n-numbers potentially undermine the reliability of the findings but this was necessary to accommodate the large number of experimental conditions to be tested and the limits on available time and resources. The majority of experiments were performed using cells in earlier passage numbers, below 10, at a time when SH-SY5Y cells are considered to be stable, functional and healthy (Shipley et al., 2016), but nevertheless differences in passage number may have introduced a degree of heterogeneity in relevant properties of the cells.

5.4.1 Conclusions

This study has shown that CBZ, VPA and LTG can increase the expression of Nrf2, HO-1 and NQO-1 proteins in SH-SY5Y cells, particularly at higher concentrations that are likely above the normal therapeutic range. Interestingly, LEV was without effect, suggesting that it possesses a different profile of cellular effects from other commonly used AEDs. These findings enhance our understanding of the effects of selected AEDs on the anti-oxidant / pro-oxidant balance and on the expression of Nrf2, which acts as a master regulator of oxidative stress and has been suggested to play an important role in epileptogenesis.

Chapter 6

Association between *NFE2L2* gene variants and treatment outcome in people with epilepsy

6.1 Introduction

Despite the availability of more than 20 AEDs, at least 30% of patients suffering from epilepsy show therapeutic failure, which highlights the need for new AEDs with novel targets (Kambli et al., 2017). A master transcriptional regulator, Nrf2, has emerged as a potential new therapeutic target in epilepsy (Calkins et al., 2009). In 1994, Nrf2 was discovered as a cap'n'collar (CNC) basic-region leucine zipper (bZIP) transcription factor which modulates the cellular redox status (Hirotsu et al., 2012). Nrf2 recognizes AREs in DNA to increase the transcription of phase II antioxidant enzymes which include GST, HO-1, NQO-1, glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modulatory subunit (GCLM) (Itoh et al., 1997; Thimmulappa et al., 2002).

The Nrf2-ARE signalling pathway is not fully understood. However, recent studies have shown that it offers important targets which might be potentially helpful for protecting the brain from ischemic stroke and kainate-induced toxicity (Shih et al., 2005; Kraft et al., 2006; Zhao et al., 2006). The role of Nrf2 in relation to the contribution of oxidative stress during epileptogenesis justifies its selection as a potential target along with the pathways that it mediates (Milder et al., 2010; Rowley and Patel, 2013).

NFE2L2 gene encodes Nrf2. This gene is located on chromosome 2 (Acosta-Herrera et al. 2015). Upon activation by an increase in cellular levels of ROS, Nrf2 translocates to the nucleus and binds to the AREs, inducing the transcription of *NFE2L2*-regulated

genes (Acosta-Herrera et al. 2015**).** There is good evidence to suggest that this process is activated by brain insults and seizures and that it might be relevant during epileptogenesis and as a consequence of recurrent epileptic seizures (Aparicio et al., 2015).

Recent epidemiological and association studies have revealed significant associations between *NFE2L2* sequence variations and the risk of a variety of diseases, which further supports *NFE2L2* as a susceptibility gene (Cho. 2013). Recent data from Otter et al (2014) suggested that there was association between *NFE2L2* SNPs and Parkinson's disease risk. Further studies have provided important information on the association between *NFE2L2* SNPs and chronic gastritis (Arisawa et al., 2007), chronic obstructive pulmonary disease (COPD; Hua et al., 2010), ulcerative colitis (Arisawa et al., 2008), vitiligo (Guan et al., 2008) and many other diseases. Although *NFE2L2* gene variants have not previously been reported in epilepsy studies, the current study has chosen to investigate several SNPs in this gene and whether they associate with clinical characteristics and treatment outcome in a large cohort of people with epilepsy. This association study is consistent with the focus on antioxidant mechanisms throughout this thesis and any positive association will give a strong signal that Nrf2-mediated antioxidant response is of relevance in epilepsy.

6.1.1 Aims of study

To assess the association between the genotype of selected SNPs in the *NFE2L2* gene and a variety of demographic variables and clinical characteristics in people with epilepsy, including AED treatment outcome (defined as remission on any AED).

6.2 Materials and methods

6.2.1 Reagents and chemicals

TaqMan® genotyping mastermix and TaqMan® SNP genotyping assays for selected SNPs (rs7557529, rs1806649, rs2886161, rs2001350 and rs10183914) in the *NFE2L2* gene were purchased from Thermo Fisher Scientific, Loughborough, UK. Other chemicals were sourced as described in chapter 2, section 2.2.1.

6.2.2 Patients and DNA samples

A total of 1523 DNA samples were used in this study. The study population comprised unselected individuals with epilepsy recruited via out-patient clinics at the Western Infirmary, Glasgow, UK (Glasgow cohort, 914 samples) and the UK-wide Standard and New Antiepileptic Drugs trial (SANAD I cohort, 609 samples). These cohorts include most of the demographic and clinical variables which were assessed in this association study (table 6.1). All patients provided written informed consent for analysis of their DNA. Clinical and demographic data on the patient population was derived from existing databases that had been compiled as part of the EpiPGX project (www.epipgx.eu).

Table 6.1: Demographic and clinical variables of SANAD I and Glasgow cohorts involved in this study.

Outcomes	SANAD cohort	Glasgow cohort
No. of samples	609	914
Sex		
Male %	53	52
Female %	47	48
Ethnicity		
European %	64	99
Unknown %	36	1
Newly diagnosed		
Yes %	83	53
No %	17	47
Epilepsy type		
Focal %	71	78
Generalised-Unclassified %	29	22
EEG outcome		
Normal %	45	28
Abnormal %	47	51
Unknown %	8	21
Imaging outcome		
Normal %	60	53
Abnormal focal %	5	21
Abnormal-unclassified %	14	19
Unknown %	20	6
Aetiology		
Cryptogenic %	48	51
Idiopathic %	30	22
Symptomatic %	22	27
MTLE-HS		
Yes %	1	3
No %	99	97
Family history		
Yes %	9	31
No %	91	69
Neurological exam.		
Normal %	92	92
Abnormal %	8	8
Learning disability		
Yes %	4	2
No %	96	98
Remission on any AED		
Yes %	61	66
No %	39	34
Age average	34	30

6.2.3 SNP selection and inclusion criteria

The rs7557529, rs1806649, rs2886161, rs2001350 and rs10183914 SNPs in *NFE2L2* (table 6.2) were selected for genotyping on the basis of a global minor allele frequency (MAF) exceeding 10% in the dbSNP database (https://www.ncbi.nlm.nih.gov/snp) and prior published evidence of association with a common brain disorder. At the time of SNP selection, there were no common SNPs (MAF>10%) in *NFE2L2* of known or predicted functional significance (i.e. in coding sequence, promoter, splice site or untranslated regions). The rs7557529 SNP lies upstream of the *NFE2L2* gene and has a MAF of 39.5%, while rs1806649, rs2886161, rs2001350 and rs10183914 are all intronic variants with MAFs of 10.5%, 37.6%, 12.8% and 23.2%, respectively. All five SNPs have been implicated, as part of an *NFE2L2* haplotype, in the pathogenesis of Parkinson's disease (Van Otter et al., 2010; Van Otter et al., 2014).

SNP ID	Chromosome	Gene	Position (bp)	SNP type	Allele	MAF
rs7557529	2	NFE2L2	177270369	Upstream	G/A	0.395
rs1806649	2	NFE2L2	177253424	Intron	G/A	0.105
rs2886161	2	NFE2L2	177263111	Intron	A/G	0.376
rs2001350	2	NFE2L2	177235697	Intron	G/A	0.128
rs10183914	2	NFE2L2	177232938	Intron	G/A	0.232

Table 6.2: Details of the SNPs genotyped in this study. Global minor allele frequency (MAF) is derived from the 1000 Genomes project, as reported by the dbSNP database (https://www.ncbi.nlm.nih.gov/snp).

6.2.4 Genotyping

Genomic DNA samples were genotyped for the five candidate SNPs at the Department of Molecular and Clinical Pharmacology, University of Liverpool. TaqMan® SNP Genotyping Assays (Thermo Fisher Scientific, Loughborough, UK) were used in accordance with the manufacturer's instructions, which can be found at:

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0009593_TaqManSNP_UG.pdf

The assay is based on the 5' to 3' exonuclease activity of DNA polymerase. TaqMan® assay contains two specific primers targeting the region flanking the SNP site and two TaqMan fluorescent probes with a minor groove binder (MGB). Each probe is labelled with a different fluorophore (VIC or FAM). These reporters are attached covalently to the 5' end of the two probes. Near the 3 'end, there is a non-fluorescent quencher (NFQ) that prevents liberation of the reporter fluorescence if the probe is not degraded. The MGB serves to stabilize the double-stranded structure formed between the target and the probe. During the PCR reaction, probes that hybridize specifically to DNA fragments are destroyed and the fluorescence of corresponding fluorophore is liberated (figure 6.1)

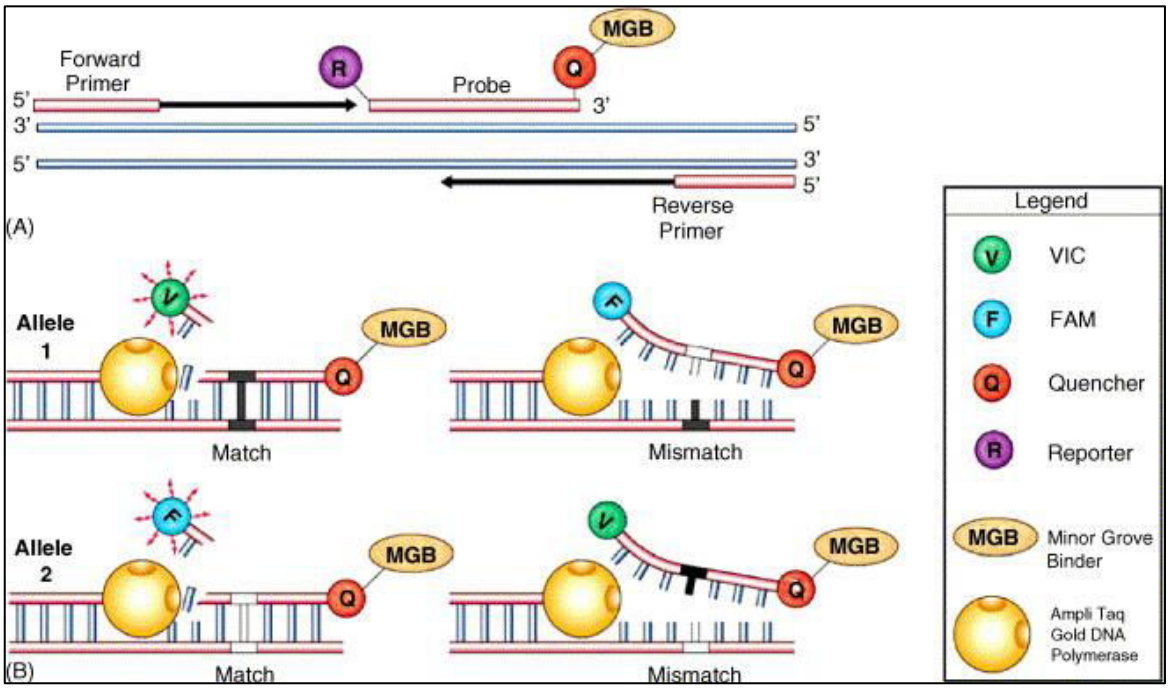


Figure 6.1 A) Probe binding and primer extension in a TaqMan® SNP Genotyping Assay. (B) Allelic discrimination is achieved by the selective annealing of matching probe and template sequences, which generates an allele-specific (fluorescent dye-specific) signal. V=VIC, F=FAM, R=Reporter, Q=Quencher, MGB=Minor grove binder (adapted from De La Vega et al., 2005)
.

6.2.5 Experimental details

The genotyping was performed on an Applied Biosystems ABI 7900HT Real Time PCR machine using Taqman® genotyping assays and Taqman® Genotyping mastermix in 384 well plates. Individual DNA samples were plated in a single well using 1 µl of a 20 ng/µl solution and allowed dry overnight. DNA was reconstituted by the addition of 5 µl of reagent mix, which comprised 2.5 µl of Taqman® mastermix (2X), 0.125 µl of Taqman® genotyping assay (20X), and 2.375 µl of MilliQ water.

PCR was performed as follows: activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 sec and extension at 60°C for 1 minute. Alleles were clustered using fluorescent signals (VIC™ and FAM™). Each PCR plate contained 10% duplicates, two negative controls and one known heterozygous control for each SNP. Samples with undetermined calls in the initial analysis were repeated under the same conditions. Any self-priming of negative control(s) was considered to be contamination, and genotyping was repeated.

6.2.6 Statistical analysis

All experiments were performed using two technical replicates (i.e. all samples were genotyped in duplicate). For univariate analyses, statistical comparisons were undertaken using one-way ANOVA or Chi-square test, as appropriate, using Stats Direct software version 2.7.9. P-values below 0.05 were considered to indicate associations of statistical significance, For multivariate analyses, statistical comparisons were undertaken by binary logistic regression analysis using SPSS software version 24, with predictors added to or removed from the predictive model using a stepwise approach depending on their degree of impact, as indicated by the p value. The significance threshold for identification of predictors was set at p=0.05. The MAF for each SNP was compared to the global MAF reported on the dbSNP website (https://www.ncbi.nlm.nih.gov/snp) and deviation from Hardy Weinberg equilibrium

(HWE) was explored using an online calculator (http://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl), with p<0.01 assumed to indicate deviation from expected values.

6.3 Results

6.3.1 Association between remission on any AED and *NFE2L2* genotypes

A series of univariate analyses were conducted to explore associations between individual *NFE2L2* SNP genotypes and any reported period of 12-month remission from seizures on any AED at any point during clinical follow-up in all patients for whom this information was available. All analyses were undertaken using the Chi-square test and unadjusted p-values below 0.05 were considered to indicate associations of statistical significance. Results are summarised below and reported in full in table 6.3.

rs7557529: There was no statistically significant association between rs7557529 genotype frequencies and remission on any AED (p=0.597) (table 6.3).

rs1806649: There was no statistically significant association between rs1806649 genotype frequencies and remission on any AED (p=0.372) (table 6.3).

rs2886161: There was a statistically significant association between rs2886161 genotype frequency and remission on AED treatment (p<0.0001). Genotype frequencies in patients who achieved 12-month remission from seizures during follow-up were

37.4% AA, 50.4% AG and 12.2% GG, compared to 55.5% AA, 35.5% AG and 8.9% GG in those who did not achieve any sustained period of remission (table 6.3).

rs2001350: There was no statistically significant association between rs2001350 genotype frequencies and remission on any AED (p=0.304) (table 6.3).

rs10183914: There was no statistically significant association between rs10183914 genotype frequencies and remission on any AED (p=0.674) (table 6.3).

6.3.2 Association between remission on AED treatment and clinical and demographic covariates

All clinical and demographic covariates were assessed, using multiple univariate analyses, against any reported period of 12-month remission from seizures on any AED at any point during clinical follow-up in all patients for whom this information was available. Analyses were undertaken using the Chi-square test for categorical variables and one-way ANOVA for continuous variables. Unadjusted p-values below 0.05 were considered to indicate associations of statistical significance. Results are summarised below and reported in full in tables 6.4.

There was a significant association between abnormal neurological exam (p=0.0006), presence of learning disability (p=0.0077), presence of MTLE-HS (p=0.048), symptomatic aetiology (p=0.0016), abnormal imaging (p=0.0071), focal epilepsy (p=0.0012), and a higher number of pre-treatment seizures (p=0.0001) and the

likelihood of not achieving a 12-month period of remission on any AED at any point during clinical follow-up. In contrast, there was no association between sex, family history, EEG outcome (table 6.4) or age at epilepsy onset (figure 6.2).

Table 6.3: Univariate analysis of rs7557529, rs1806649, rs2886161, rs2001350, and rs10183914 *NEFEL2* SNP genotypes and remission on AED treatment within the study population.

Remission on any AED	***NEFLE2* SNPs**			**χ2 - statistic**	***p value***
	rs7557529 genotypes				
	AA	**AG**	**GG**		
Yes	232	372	169		
No	147	268	121	1.0313	0.5971
	rs1806649 genotypes				
	AA	**AG**	**GG**		
Yes	43	276	455		
No	40	184	314	1.972	0.372
	rs2886161 genotypes				
	AA	**AG**	**GG**		
Yes	255	344	83		
No	360	230	58	44.127	**<0.0001**
	rs2001350 genotypes				
	AA	**AG**	**GG**		
Yes	630	152	5		
No	427	105	8	2.379	0.304
	rs10183914 genotypes				
	AA	**AG**	**GG**		
Yes	90	343	345		
No	71	238	232	0.788	0.674

Table 6.4: Univariate analysis of clinical and demographic variables with remission on any antiepileptic drug during follow-up.

Categorical clinical variables	Remission on any AED		χ^2 - test	*p*-value
	No	Yes		
Sex				
Male	285	419	0.087	0.767
Female	265	377		
Family history of epilepsy				
No	424	640	2.153	0.142
Yes	126	156		
Neurological examination				
Normal	491	751	12	**0.0006**
Abnormal	59	45		
Learning disability				
No	529	784	7.261	**0.0077**
Yes	21	12		
Mesial temporal lobe epilepsy with hippocampal sclerosis				
No	537	788	3.909	**0.048**
Yes	13	8		
Aetiology				
Cryptogenic	288	391	12.905	**0.0016**
Idiopathic	110	226		
Symptomatic	150	179		
Imaging outcome				
Normal	299	478	9.886	**0.0071**
Abnormal focal	89	92		
Abnormal unspecified & non-specific changes	104	118		
EEG outcome				
Normal EEG	204	288	1.833	0.399
Abnormal - non-specific EEG	133	207		
Abnormal - epileptiform EEG	121	208		
Epilepsy type				
Focal epilepsy	438	572	10.502	**0.0012**
Generalised & unclassified epilepsy	112	224		
Pre-treatment seizure groups				
1-5 seizures	66	175	27.723	**0.0001**
6-20 seizures	86	104		
20 + seizures	131	134		

AED = antiepileptic drug; EEG = electroencephalogram.

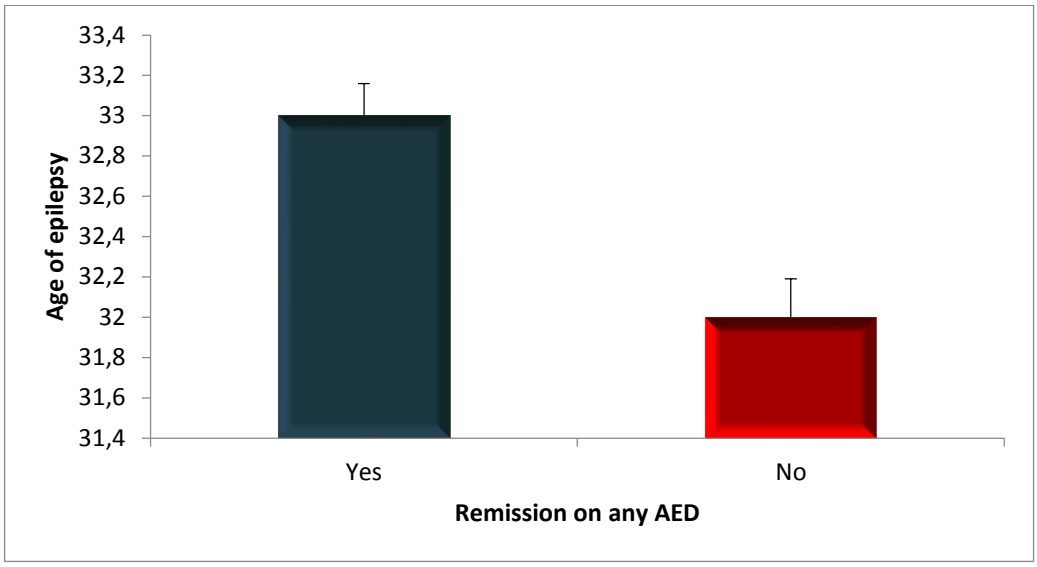


Figure 6.2 Univariate analysis of age of epilepsy variable with remission on any antiepileptic drug during follow-up of the study population.

6.3.3 Multivariate analysis of drug treatment outcome

A multivariate analysis using binary logistic regression was performed to further explore associations between *NFE2L2* SNP genotypes, demographic and clinical variables and the response to AED treatment. All demographic, clinical and genetic covariates were assessed, using a multivariate regression model, against any reported period of 12-month remission from seizures on any AED at any point during clinical follow-up in all patients for whom this information was available (table 6.5). Despite observing multiple statistically significant associations using univariate analysis, in which each covariate was assessed independently, the multivariate model failed to identify any association between demographic, clinical or NFE2L2 genetic variables and the likelihood of achieving a 12-month period of remission on any AED (table 6.5).

Table 6.5: Multivariate analysis of clinical, demographic and *NFE2L2* genetic covariates and remission on any antiepileptic drug during follow-up. The relative impact of each predictor is denoted by the Wald statistic. The p-value indicates the probability that the predictor influences remission.

Predictor	Wald statistic	*P value*
Constant	1.647	0.199
Sex	0.221	0.638
Family history of epilepsy	2.063	0.151
Neurological examination	3.549	0.160
MTLE-HS	0.717	0.397
Aetiology	1.165	0.558
Imaging outcome	0.089	0.957
EEG outcome	0.963	0.326
Epilepsy type	0.379	0.538
rs7557529 G>A	2.033	0.362
rs1806649 G>A	0.052	0.974
rs2886161 A>G	2.854	0.240
rs2001350 A>G	4.284	0.117
rs10183914 G>A	2.382	0.304

AED = antiepileptic drug; EEG = electroencephalogram; MTLE-HS= mesial temporal lobe epilepsy with hippocampal sclerosis

6.4 Discussion

The identification of biomarkers for the prediction of disease outcome and better drug response, as well as avoidance of adverse drug effects, is a relatively new field of interest in medicine in general and, in particular, in epilepsy research (Larson et al., 2013). The concept of personalized therapy is increasingly recognised in the treatment of people with epilepsy, but predictive markers for its prognosis are still very limited (Weber et al., 2014). The Nrf2-ARE pathway has been shown to play an important role in protection against several diseases, including epilepsy, through opposing oxidative imbalances associated with mitochondrial dysfunction (Rowley and Patel, 2013).

Differences in AED response can be seen between types of epilepsy, seizure types and particular seizure syndromes (Semah et al., 1998). Any genes causing epilepsy are thus potential candidates for genetic variation that may also influence differences in AED response (Spear, 2001; Depondt, 2006). In recent years at least a dozen genes have been identified in rare forms of monogenic epilepsies (Graves, 2006). AED target genes, as sources of genetic variation, have only recently been the focus of AED pharmacogenetics (Depondt and Shorvon, 2006). Mutations in the α-subunit of the voltage-gated sodium channel (NaV) were first associated with familial and sporadic epilepsies (Wallace et al., 2001b) and early observations indicated that these NaV channel mutations could also affect the clinical response to AEDs in genetic epilepsies (Guerrini et al., 1998). Further research has additionally implicated the astrocytic GABA transporter GAT-3 (encoded by *GAT3*) with drug responsiveness (Meldrum and

Rogawski, 2007, Kim et al., 2011a). *GAT3* variation was associated with the pharmacoresistance phenotype in a candidate gene association study (Kim et al., 2011a). Numerous pharmacogenetic studies have implicated ATP binding cassette subfamily B member 1 (*ABCB1)* polymorphisms in multi-drug resistant epilepsy, with *ABCB1* polymorphisms that affect expression or functionality, including the well-known 3435C>T polymorphism (Siddiqui et al., 2003), apparently more frequent in AED non-responders than in responders (Loscher and Schmidt, 2009).

Currently, more than 200 genes are known to be regulated by Nrf2, including those involved in mechanisms of cytoprotection, metabolism, and mitochondrial function (Aparicio et al., 2015). Moreover, a recent study has suggested that therapies targeting the Nrf2 pathway possess significant potential for the treatment of epilepsy, neurodegenerative and inflammatory diseases (Mazzuferi et al., 2013).

At present, the selection of a specific AED for any given patient is based predominately on seizure type, syndrome and likely adverse effects (Engel et al., 2013). However, the response to that AED at an individual patient level is almost unpredictable, hence a degree of trial and error is part of the standard care provided to patients undergoing treatment for epilepsy (Weber and Lerche, 2008). There is therefore an urgent need for predictive biomarkers, whether demographic, clinical, genetic or otherwise, which may guide personalized treatment strategies for each patient (Sueri et al., 2018).

With this in mind, the analysis reported in this chapter, focusing on genetic biomarkers of disease and treatment outcome, is entirely in line with many ongoing epilepsy research studies and with the requirements of epilepsy in general. The analysis took advantage of the availability of a large cohort of epilepsy patients who had recently undergone detailed clinical phenotyping of their basic demographic characteristics, key clinical aspects of their epilepsy, and examination of their response to treatment with AEDs. Many of the patients had been enrolled in prior studies of newly-diagnosed epilepsy, in which information had been collected prospectively from the point of diagnosis and initiation of AED treatment. In addition, a DNA sample was available for all patients, which was genotyped for five common variants in the *NFE2L2* gene, which encodes Nrf2. Demographic variables (i.e. age, sex), clinical variables (i.e. type of epilepsy, EEG outcome), and genetic variables (i.e. genotype of *NFE2L2* SNPs) were then combined in a multi-dimensional (univariate & multivariate) analysis that was designed to find biomarkers for treatment outcome in epilepsy. A similar strategy is being pursued by many other researchers (Lagarde et al., 2018; Rawat et al., 2018).

Genetic variants have been the focus of many epilepsy research studies, particularly in recent years, and have been studied in various populations (Lee, 2018). In the current study, it was shown that a specific SNP (i.e. rs2886161) in the *NFE2L2* gene was associated with remission on AED treatment (table 6.3). There was, however, no other notable association between *NFE2L2* genotypes (rs7557529, rs1806649, rs2001350, and 10183914) and remission in this study. To our knowledge, there is no previously

published work that has directly investigated the contribution of *NFE2L2* gene variants in epilepsy or in the context of response to treatment in epilepsy, so there is no opportunity to validate these observations. Moreover, the absence of association might be due to clinical heterogeneity (variability in the clinical characteristics of the two different cohorts) as two different disease cohorts have been analysed together. Analysis of clinical data in the current study suggested that normal neurological examination, absence of learning disability, idiopathic aetiology, absence of MTLE-HS, generalised or unclassified epilepsy, normal neuroimaging investigations, and a low number of seizures prior to initiation of drug treatment were all associated with a higher likelihood of remission on AED therapy, which is in line with already published data (French, 2002).

Seizure freedom (i.e. remission) is the primary goal of AED therapy and a number of prognostic clinical factors have been reported in previous studies. It is widely recognised that nearly 50% of newly diagnosed epilepsy patients become seizure-free on the first ever AED, with >90% doing so at moderate or even modest dosing (Kwan and Brodie, 2001). Bonnett and colleagues (2012) investigated several prognostic factors (i.e. age, sex, number of seizures, EEG outcomes, etc.) in a large randomised controlled trial of patients with newly-diagnosed epilepsy commencing their first ever AED. Treatment failure increased as age and total number of pre-treatment seizures increased and was significantly more likely in women than in men and in patients with EEG abnormality than in patients with a normal EEG (Bonnett et al., 2012).

Interestingly, the current analysis failed to show an association between treatment outcome and sex, age or EEG findings, even though many of the same patients (i.e. those from the SANAD study) were included in both cohorts. Similarly, the current analysis also showed an association between treatment outcome and the presence of MTLE-HS and/or learning disability, which was not reported by Bonnett et al (2012), although this has also been recognised in other studies, in which patients with MTLE-HS may never experience complete seizure freedom, even after surgery (Wiebe et al., 2001; Tellez-Zenteno et al., 2005; Wendling et al., 2013).

Despite observing multiple independent associations with AED treatment outcome following univariate analyses, a multivariate approach in which clinical, demographic and genetic variable were combined using stepwise regression within a single statistical model was without significant findings. This would suggest that none of the associations observed in the univariate analyses was particularly strong or that the inclusion of non-significant genetic covariates in the multivariate model may have reduced the sensitivity (or power) to detect true associations. A different approach, where only those covariates shown to be statistically significant at the univariate level were included in the multivariate model may have been more revealing.

Thus, the attempt to identify genetic predictors of treatment outcome within the *NFE2L2* gene was largely unsuccessful, with no evidence to suggest that common genetic variants in this gene had any influence on the response to AED therapy. This is maybe unsurprising as the functional effects of the selected SNPs on Nrf2 function are

unclear. Although the cohort size was significantly larger than many other published studies, the approach of using selected SNPs from candidate genes is no longer favoured. The majority of previous studies that have used this methodology to identify significant associations between genotype and treatment outcome have never been replicated. Epilepsy genetics is now moving towards much larger-scale, international collaboration style studies and epilepsy researchers are beginning to understand the challenges of small scale studies (Ottman et al., 2018), but still there is a lot to be done in this regard.

Most of the biomarker studies published to date have included heterogeneous populations of epilepsy patients which might make it difficult to a genetic biomarker (Engel et al., 2013). They are often small-scale and driven by specific hypotheses that do not apply to all patients. There is also some confusion in the epilepsy research community about what constitutes a biomarker and what we can learn from studies conducted in humans and in animals. Animal models of epilepsy are useful for testing drugs but they do not always offer a good insight into the clinical situation (Kandratavicius et al., 2014). There is a need for a much more standardized approach and for larger, well-defined cohort studies that maximise the potential, statistical likelihood, and speed at which potential biomarkers for epilepsy can be discovered.

6.4.1 Conclusions

This analysis has shown expected associations between a number of clinical and demographic variables (i.e. number of pre-treatment seizures, abnormal neurological examination, presence of a learning disability, aetiology of epilepsy, etc.) and remission on AED treatment. There was also a modest univariate association between *NFE2L2* genotype rs2886161 and treatment outcome. However, there were no statistically significant associations between the rs7557529, rs1806649, rs2001350 and rs10183914 SNPs and the remission on any AED. Similarly, a multivariate predictive model did not show any significant contribution of *NFE2L2* SNP genotypes, demographic and clinical predictors and the response to AED treatment.

Although derived from analysis of a large cohort of patients, these findings should be treated with caution because of concerns about false-positive findings and the failure of a detailed multivariate analysis to identify any reliable interaction between *NFE2L2* genotype and treatment outcome. Despite our hypothesis about the involvement of the Nrf2-ARE pathway in epilepsy, there was no evidence from this pharmacogenomics investigation that common variants in the *NFE2L2* gene, which encodes Nrf2, have any influence on the presentation of epilepsy or on its prognosis.

Chapter 7

General discussion

7.1 Background

Epilepsy continues to be a major burden on the global health system. The high rates of pharmaco-resistance in addition to a lack of specific measures to predict treatment response have increased the need for sensitive and specific biomarkers for this disease. Epilepsy is associated with increased mortality, significant comorbidities, unique stigmatization of affected individuals, and high societal cost. Moreover, common AEDs merely provide symptomatic treatment without having any influence on disease course (Brandt et al., 2006). Thus, there is a pressing need to develop alternative therapeutic approaches that prevent epileptogenesis after brain insults. New antiseizure treatments are unlikely to bridge this treatment gap. Therefore, it has been suggested that the next generation of drugs should potentially be able to delay or prevent the onset of epilepsy in susceptible individuals (anti-epileptogenesis) or to halt or reverse its progression and/or improve the neuropathology and the associated comorbidities (disease-modifying) (Varvel et al., 2015). Oxidative stress has been shown to play a key role in epileptogenesis after the first seizure. Through progressive neurobiological changes, the first seizure becomes a cause for recurrent seizures (Martinc et al., 2012). Much evidence supports the idea that oxidative stress is involved in the pathogenesis of epilepsy and that antioxidants or free radical scavengers can ameliorate seizure activity and neuronal damage in various epilepsy models (Shin et al., 2011). Hence, new agents with antioxidant properties can be expected to have therapeutic potential against epilepsy. Breaking this vicious cycle through the neutralization of ROS and/or RNS might help in termination of the progression of oxidative insults during epileptogenesis.

7.2 Overview of experiments

The work presented in this thesis was designed to explore various neurobiological aspects of oxidative stress and the effects of antiepileptic drugs on key molecules and proteins involved in this insidious process. The first experimental chapter (chapter 2) set out to determine the effect of H_2O_2, LPS, and pilocarpine on several markers of oxidative stress, i.e. MDA concentration, SOD activity, and GSH/GSSG ratio, in SH-SY5Y cells in order to establish the optimal experimental conditions (i.e. times, concentrations, preferred inducer, etc.) with which to later assess drug effects. The subsequent experimental chapter (chapter 3) focused on the biochemical actions of the four most widely prescribed AEDs, i.e. CBZ, VPA, LEV and LTG, on MDA, SOD and GSH/GSSG as markers of oxidative stress. Chapters 4 and 5 then explored AED effects on the expression of several phase II detoxification antioxidant genes (Nrf2, HOX-1, and NQO-1) at the mRNA and protein level, respectively. The final experimental chapter (chapter 6) had a somewhat different focus, looking instead at the potential contribution of known common variants in the *NFE2L2* gene (which encodes Nrf2) to multiple clinical characteristics, including treatment outcome, using DNA samples and clinical information from a large cohort of people with epilepsy. This work has accordingly provided an excellent training in experimental design, in the use of several established and contemporary laboratory techniques, and in translational research that relies on clinical data and human biological samples.

7.3 Optimising induction of oxidative stress

The initial results showed that incubation of SH-SY5Y cells with H_2O_2, LPS, and pilocarpine decreased the cell viability and increased oxidative stress by increasing MDA concentration, and by decreasing SOD activity and GSH/GSSG ratio in a time- and concentration-dependent manner. Later studies also showed corresponding effects

on the expression of Nrf2 and its target genes HO-1 and NQO-1. Taken together, these experiments indicated that exposure of SH-SY5Y cells to 100μM H_2O_2 for 4 hours resulted in optimum induction of oxidative stress, i.e. maximal change in biochemical markers of oxidative stress at acceptable cell viability (>80%). This became the standard experimental approach in later experiments. These results reflect those of Kang et al (2014) who also showed a concentration-dependent decrease in SH-SY5Y cell viability after exposure to H_2O_2, with cell viability remaining above 80% even after 24 hours exposure to 100μM H_2O_2. The strong pro-oxidant activity of H_2O_2 has been suggested to be due to its direct damaging effect, as it is a highly diffusible ROS that can cross the plasma membrane (Forman, 2007). In addition, treatment with H_2O_2 has been shown to produce more O^- by activation of NADPH oxidase and subsequently the formation of $ONOO^-$ through superoxide scavenging of nitric oxide (Beckman et al., 1990; Pryor and Squadrito, 1995). These findings, and also those described in chapter 4, are similarly consistent with a previous study which reported an up-regulation in Nrf2 expression after H_2O_2 exposure (Fourquet et al., 2010), which may be explained by a direct effect of H_2O_2 on the Nrf2-Keap 1 dissociation and Nrf2 nuclear translocation.

In contrast to H_2O_2, cellular exposure to LPS may trigger the release of inflammatory mediators, such as TNF-α, which can, in turn, result in oxidative stress and inflammation leading to a reduction in cell viability (Choudhury et al., 2015; Ruiz-Miyazawa, 2015). In line with the current results, previous studies have demonstrated a similar increase in MDA concentration and decreases in GSH concentration and SOD activity in different experimental models and cell lines after exposure to LPS (Pourganji et al., 2014; Gou et al., 2015; Frimmel et al., 2016). The key event that leads to Nrf2 pathway activation appears to be TLR receptor activation by LPS, which results in ROS release, with the Keap-1 protein undergoing oxidation and dissociation of Nrf2 (Hu et

al., 2011). When using pilocarpine as an inducer, the current work again supported evidence from previous observations which showed concentration-dependent effects of pilocarpine on lipid peroxidation, decreases in GSH level and SOD activity, plus increased NO, TNF-α, and IL-6, all of which play an important role in inflammation and oxidative stress (Freitas et al., 2004; Santos et al., 2011; Kabel. 2014; Noor et al., 2015). In addition, it has been suggested that ROS are immediately increased after M1 receptor stimulation by pilocarpine, with ROS potentially participating as second messengers in the regulation of ARE-driven genes by disrupting the Nrf2/Keap1 interaction (Mangelus et al., 2001; Nguyen et al., 2003).

In addition to optimising the conditions for induction of oxidative stress in terms of preferred inducer, concentration and duration of exposure, early experiments also considered morphology of the SH-SY5Y cells themselves. There was no significant difference in the change in MDA concentration, SOD activity and GSH/GSSG ratio caused by 100μM H_2O_2 when comparing differentiated and undifferentiated SH-SY5Y cells. This confirmed that undifferentiated SH-SY5Y cells are sensitive to oxidative damage and are a suitable *in vitro* system for these experiments. Undifferentiated SH-SY5Y cells are often considered more appropriate for studying neurotoxicity and neuroprotection in experimental neurological disease research (Cheung et al., 2009) because differentiation can be associated with a number of issues that might impact on the interpretation of experimental results, including proteomic changes, cellular proliferation and apoptosis. It is also reported that in studies assessing cellular stresses, including oxidative and metabolic stress, differentiation may be undesirable due to the potential for artefacts from the differentiation process (Hermann, 2014).

7.4 Effects of antiepileptic drugs

Much of the experimental work in this thesis focused on the effects of four AEDs, CBZ, VPA, LEV and LTG, which are the four most commonly-prescribed drugs for the treatment of epilepsy in the UK and amongst the most widely used across the globe. All four AEDs decreased the viability of SH-SY5Y cells in a time-and concentration-dependent manner and negatively affected oxidative stress markers by increasing MDA concentrations and decreasing SOD activity and GSH/GSSG ratio, with and without additional exposure to 100μM H_2O_2 for 4 hours. Three of the four AEDs (CBZ, VPA, LTG) also increased the expression of Nrf2, HOX-1, and NQO-1 at both gene and protein level following 24 hour exposure, again with or without 100μM H_2O_2 for a further 4 hours. In contrast, LEV had no effect on gene or protein expression.

These findings are consistent with those of other researchers who also showed concentration-dependent cytotoxic effects of CBZ and VPA (Ambrósio et al., 2000 Dodurga et al., 2014). Pro-oxidant effects of CBZ and VPA have also been reported, which may be linked to their cytotoxic effects (Thomas et al., 2006; Santos et al., 2008). One possible explanation for these results may be the propensity for CBZ and VPA to generate toxic epoxy and peroxy metabolites which are believed to play an important role in cellular mitochondrial toxicity and ROS liberation (Tong et al., 2005; Tutanc et al., 2015). In respect of LTG, there is, to the best of our knowledge, just one report that has assessed its effect on oxidative damage. Using a rat liver tissue model, Poorrostami et al (2014) showed that LTG could increase MDA concentration 2-fold and decrease catalase activity 3-fold in comparison to control. The mechanism behind LTG-induced liver injury is not clearly explained, although some reports have suggested this may be caused by an immune reaction to the arene oxide metabolite of LTG, leading to an inflammatory process and oxidative stress (Maggs et al., 2000; Mecarelli et al., 2005).

These findings are in agreement with the up-regulation in the expression of Nrf2, HO-1 and NQO-1 genes and proteins after exposure to these AEDs reported in chapters 4 and 5 of this thesis. Accordingly, one can speculate that the pro-oxidant effects of CBZ, VPA and LTG leads to an increase in the expression of Nrf2 and its downstream target genes as an attempt to provide protection against cytotoxic damage.

No previous laboratory studies have assessed the pro-oxidant and/or anti-oxidant behaviour of LEV. There is a single report that investigated the effect of LEV on oxidative stress markers in epilepsy patients and which showed an increase in the urinary level of 15F-2t-isoprostane, which is a reliable marker of oxidative damage (Ozden et al., 2010). Contrary to our expectations, LEV did not have any effect on the expression of Nrf2 or its target genes in the current study. It is possible that pre-incubation with LEV may have resulted in the preservation of the activity of catalase, an antioxidant enzyme that catalyses the conversion of H_2O_2 to water and oxygen, which might then explain the antioxidant behaviour of LEV against H_2O_2-induced oxidative stress in SH-SY5Y cells (Oliveira et al., 2016). Thus, LEV might help to protect against the overproduction of ROS and the resulting oxidative damage.

7.5 Genetic variation in the antioxidant response

The work described in the final experimental chapter of this thesis (chapter 6) took advantage of the availability of DNA samples and related clinical information from a large cohort of people with epilepsy. It focused on the association between common variants in the *NFE2L2* gene, which encodes Nrf2, and characteristics of the study population, including several demographic and clinical variables related to epilepsy and its response to AED treatment. *NFE2L2* SNP genotypes were generated *de novo* using Taqman genotyping, while phenotypes were extracted from existing databases. A variety of modest but significant associations were identified using univariate analysis.

These included a higher prevalence of the rs2886161 in those experiencing remission on any AED treatment. In addition, associations were found between a number of clinical and demographic variables (i.e. number of pre-treatment seizures, abnormal neurological examination, presence of a learning disability, aetiology of epilepsy, abnormal EEG, etc.) and remission on any AED. It is found that fewer seizures prior to initiation of AED treatment, earlier age at onset of epilepsy, less frequent seizure (1-5 seizures) and normal neuroimaging, and EEG outcomes are all associated with a greater likelihood of achieving seizure freedom (i.e. remission), which is the primary goal of AED therapy. Moreover, this study indicated that patients with mesial temporal lobe epilepsy with hippocampal sclerosis and learning disability are less likely to respond to drug treatment, and therefore less likely to achieve remission.

The additional SNPs in *NFE2L2* (rs7557529, 1806649, rs2001350, rs10183914) failed to show any association with any feature of the epilepsy and none of the above associations survived re-analysis using a multivariate approach, suggesting that the initial observations may have been false positive findings. This conclusion was reinforced by the fact that several known associations between clinical characteristics and treatment outcome in epilepsy remained evident after multivariate regression analysis. These included abnormal neurological examination, learning disability, aetiology, epilepsy type, age at onset, and the number of pre-treatment seizures, all of which have been shown in previous studies to have a significant influence of the likelihood of achieving remission from seizures following AED treatment or the time to achieve remission (Kwan and Brodie, 2001; Bonnett, 2012). To the best of our knowledge, no previous studies have specifically explored the association between *NFE2L2* genotype and treatment outcome in epilepsy and the results reported here suggest that any such investigation is not warranted.

7.6 Summary of experimental findings

(1) H_2O_2, LPS and pilocarpine decreased cell viability and attenuated basal antioxidant mechanisms in a time-and concentration-dependent manner in human neuroblastoma (SH-SY5Y) cells. Exposure to 100μM H_2O_2 for 4 hours was considered optimal for further investigations because it showed efficient induction of oxidative stress with minimum cell death (less than 20%) within a manageable timeframe, when compared with other experimental conditions investigated.

(2) No significant differences were noted between differentiated and undifferentiated SH-SY5Y cells in terms of their response to exposure to oxidative challenge with 100μM H_2O_2. On that basis, and given previous reports highlighting concerns associated with the differentiation process and their potential impact on experimental results, undifferentiated SH-SY5Y cells were used in all subsequent investigations.

(3) CBZ, VPA, LEV, and LTG had negative effects on the pro-oxidant / anti-oxidant balance which were both time- and concentration-dependent, were especially pronounced at the highest concentrations, and were exacerbated by subsequent exposure of cells to H_2O_2. Oxidative stress produced by H_2O_2 was enhanced, rather than ameliorated, by exposure to AEDs. The pro-oxidant behaviour of AEDs that was observed throughout these experiments was unexpected but may be related, at least in part, to the generation of toxic metabolites, resulting in cell stress and apoptosis.

(4) H_2O_2, LPS and pilocarpine increased the expression of Nrf2, HO-1 and NQO-1 at both mRNA and protein level in a time-and concentration-dependent manner. CBZ, VPA and LTG did likewise and their effects were further exacerbated by subsequent exposure to H_2O_2. In contrast, LEV did not induce expression of Nrf2, HO-1 or NQO-1. It is reasonable to speculate the pro-oxidant effects seen with several AEDs leads, in turn, to activation of the Nrf2-ARE pathway as a cellular defence mechanism against

cytotoxicity. LEV also showed pro-oxidant potential in biochemical assays but no evident activation of the Nrf2-ARE pathway, suggesting fundamental differences in the cellular response to this drug which remain unexplained.

(5) There were modest statistical associations between known common variants in the *NFE2L2* gene, which encodes Nrf2, and isolated clinical characteristic in a large cohort of people with epilepsy. These included associations between one intronic variant in *NFE2L2* (i.e. rs2886161) and the outcome of AED treatment. However, no significant association was found between other intronic variants in NFE2L2 (i.e. rs7557529, 1806649, rs2001350, rs10183914) and the AED treatment outcome. This analysis has shown expected associations between a number of clinical and demographic variables (i.e. number of pre-treatment seizures, abnormal neurological examination, presence of a learning disability, aetiology of epilepsy, abnormal EEG, etc.) and remission on any AED. Similarly, a multivariate predictive model did not show any significant contribution of *NFE2L2* SNP genotypes, demographic and clinical predictors and the response to AED treatment.

Although there is good evidence to support a role of oxidative stress and the Nrf2-ARE pathway in epilepsy, it would appear that this is not influenced by common variation in the gene encoding the master regulator Nrf2.

7.7 Limitations and challenges of the work

As with any series of experiments, there are inevitable inconsistencies and limitations which mean that results should be interpreted with a degree of caution.

A major limitation of the work described in this thesis was the low number of biological replicates, with many studies employing an n-number of just 3. This was necessary to accommodate the complex experimental design used and the large number of concentrations, conditions and comparisons explored but it has an inevitable impact on the statistical validity of the findings. As such, more emphasis should be placed on the pattern of results (i.e. time- and concentration-dependence) than on the p-value associated with any isolated observation. Other related concerns include the lack of independent verification of results in another batch of SH-SY5Y cells (or in a different cell line), which would give important validity to the observations, and a limited range of time-points, which perhaps failed to capture the full extent of the cellular response to oxidative challenge or AED exposure. In the interests of time, it was not possible to explore either of these issues.

SH-SY5Y cells were always used at a passage number of between 6 and 12 in an effort to standardise experiments and enable comparison between results obtained in different phases of the work. Even within this relatively narrow window, it is recognised that passage number has an influence on growth rates and on physiological and chemical characteristics of immortalized cell lines. Although its influence is questionable, this is another potential confounder whose effect could have been minimised by increased n-numbers and independent verification. Another concern about the use of *in vitro* cell cultures is the fact that they are studied in isolation and in the absence of their normal environment, which includes interactions with other cell types that might be critical to the process being tested.

The laboratory methods used in the experiments described in this thesis are widely used in biomedical research and can be considered reliable. However, the western blot method used to investigate protein expression was occasionally associated with inconsistencies in gel quality and in-band resolution, which had the potential to influence quantification of expression. This was most evident when comparing data between blots, but is compensated (at least in part) by 'within gel' normalization to β-actin expression.

The biochemical assays used to quantify oxidative stress are long-established and robust but the use of just three markers (i.e. MDA concentration, SOD activity and GSH/GSSG ratio) might not be sufficient to reflect the actual cellular redox status. Measuring total antioxidant (TAO) activity and including assays for catalase activity (which is specific for H_2O_2 neutralization) and Gpx activity may have provided a more comprehensive picture of cellular response to oxidative challenge and AED exposure and may have helped to elucidate the distinct action of LEV.

Finally, it is recognised that the candidate gene approach and SNP selection strategy used in the association analysis reported in chapter 6 was not particularly modern or robust, with SNPs selected predominantly on the basis of minor allele frequency rather than on any known or predicted functional effects on Nrf2 expression or activity. Furthermore, clinical heterogeneity within the two selected study cohorts may be another reason for the lack of association observed with Nrf2 SNPs. This was however an exploratory analysis, seeking verification of Nrf2 involvement in epilepsy and therefore justifying a candidate gene analysis, and the number of samples/cases included greatly exceeded those typically reported in similar studies, improving the sensitivity to detect genetic signals.

7.8 Recommendations and future work

Exploration of the relationship between oxidative stress, epilepsy and commonly used AEDs is an important and potentially fruitful area for future research that aims to understand the underlying mechanisms of epileptogenesis and to identify novel drug targets. Future studies should seek to validate their findings using *in vivo* models of seizures and epilepsy. This would allow chronic exposure to AEDs, which is much more clinically relevant, concomitant investigation of drug pharmacokinetics and their pharmacodynamic effects, and a better understanding of how seizures themselves affect oxidative stress and whether this has any impact on the behaviour of AEDs. Likewise, more effort is required to explore the full range of oxidative stress markers, with the addition of measures of TAO activity, Gpx activity, catalase activity and LDH activity likely to give a more complete picture of the cellular response to oxidative insults and AED treatment. Finally, for any future genetic association analysis, it would be reasonable to target a panel of genes that more broadly represent mitochondrial function, cellular redox status and the anti-oxidant response, rather than just a single gen like Nrf2. More robust SNP selection, focussing more on functional significance rather than just allele frequency, would increase the likelihood of identifying associations between inter-individual genetic variation, epilepsy and response to AED therapy.

7.9 Conclusions

These findings imply that several existing AEDs have pro-oxidant rather than antioxidant effects, albeit at relatively high concentrations. This may limit their antiseizure activity and/or contribute to their adverse effect profile. There was no evidence from this pharmacogenomics investigation that common variants in the *NFE2L2* gene have any influence on the presentation of epilepsy or on its prognosis.

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