# AMPA Receptor Inhibition by Medium-Chain Fatty Acids as a Mechanism for Seizure Control in the MCT Ketogenic Diet

Katrin Augustin

School of Biological Sciences

Royal Holloway, University of London


Research thesis submitted for the degree of Doctor of Philosophy

Declaration of Authorship

I, Katrin Augustin, hereby declare that this thesis and the work presented in it, are entirely my own. Where I have consulted the help of others, this is always clearly stated.

Signed:_____

Date:_____

# Abstract

Ketogenic diets are carbohydrate-restrictive diets developed in the early 20th century to control seizures in severe epilepsies. In these diets, calories obtained from carbohydrates are replaced with fats, thereby elevating fat and ketone levels in the blood. Seizure control by ketogenic diets is a well-established treatment which is most commonly used in children with hard to control epilepsy. The Medium-Chain Triglyceride (MCT) ketogenic diet, which this thesis focuses on, was developed as an alternative ketogenic diet and consists of medium-chain fatty acid that rapidly generate ketones. Ketogenic diets are a validated means of seizure control but despite their long-standing use, little is known about how the diets affect seizures. Whilst ketone levels have been used traditionally as a marker for transition into ketosis, conflicting evidence exists as to whether high ketone levels are associated with effective seizure control.

This thesis investigates the role of the medium-chain fatty acid decanoic acid supplied in the MCT ketogenic diet in seizure control. Using recombinant AMPA receptor expressed in *Xenopus laevis* oocytes, direct inhibition of AMPA receptors by decanoic acid is demonstrated and characterised for mode of inhibition. In addition, a range of branched fatty acid analogues are screened for their inhibition of AMPA receptors to assess improved AMPA receptor inhibition. The mechanism of inhibition is being assessed through competition assays with other AMPA receptor antagonists and mutated receptor subunits. These experiments show that decanoic acid inhibits AMPA receptors in a non-competitive, voltage-dependent and use-independent manner. In addition, competition assays that assess the potency of decanoic acid in the presence of the established AMPA receptor antagonists GYKI and spermine show that there is no decreased potency upon co-application of the antagonists and the medium chain fatty acids. Together with the maintained inhibition of GYKI-resistant AMPA receptor mutants by decanoic acids, these data suggest that medium chain fatty acids bind the receptor through a novel, not previously described mechanism. Altogether, these results strongly indicate that AMPA receptor inhibition by decanoic acid may contribute to seizure control in patients that are treated with the MCT ketogenic diet. In addition, experiments comparing the efficacy of the natural straight-chain fatty acids decanoic acid and octanoic acid to derivatives that contain a branching point at carbon 4, show that introduction of a methyl, ethyl or cyclohexyl group increases inhibition of AMPA receptors by the medium chain fatty acids. This suggests that AMPA receptor inhibition by decanoic acid could be increased and exploited for drug development.

In addition, AMPA receptor inhibition by the anti-epileptic drug perampanel is analysed and possible drug-fatty acid interferences are investigated in competition assays in oocytes. These data indicate a strong synergistic effect between perampanel and decanoic acid that increases the inhibition

by either antagonist tenfold. Lastly, effects of decanoic acid and perampanel on AMPA receptor subunit GluA1 localisation during seizures are investigated in primary hippocampal neurons. These experiments show that GluA1 localisation to the neuronal membrane is increased upon seizure initiation and that treatment with perampanel and decanoic acid reverses the augmentation of membrane co-localisation of this receptor subunit. This is the first evidence of a ketone-independent, seizure-protective mechanism at a molecular level for fatty acids provided in ketogenic diets. Future research and clinical trials could focus on investigating a possible role for compounds based on decanoic acid in drug treatment of epilepsy and analyse the implications of a combination-therapy for perampanel and decanoic acid for patients receiving both treatments

# Acknowledgments

*The trouble with having an open mind, of course, is that people will insist on coming along and trying to put things in it (Terry Pratchett, 1990)*. Over the last four years, people have relentlessly attempted to do so, and I can finally officially thank them for it. I am first and foremost grateful to my supervisor Robin Williams for the hours of work and support he has invested in consulting and guiding me, helping me to become the scientist I am today. I am also deeply grateful to my second supervisor, Philip Chen, for his invaluable advice, and stoic patience no matter how much glassware I broke. Special thanks go to my third and fourth supervisors, Matthew Walker and Simon Heales, and to my funding body Vitaflo, particularly Maura O'Donnell and Tricia Rutherford for all their enthusiasm for our work.

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# Abbreviations list

| | |
|---|---|
| 4-BCCA | Trans-4-butylcyclocarboxylic acid |
| 4-EOA | 4-ethyloctanoic aicd |
| 4-PCEA | 4-(pentylcyclohexyl)-ethanoic acid |
| AED | Anti-epileptic drug |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic |
| ANOVA | Analysis of variance |
| ATD | Amino – terminal domain |
| ATP | Adenosine trisphosphate |
| BSA | Bovine serum albumin |
| CA1 | *Cornu Ammonis* region 1 |
| CamKII | Ca2+/calmodulin-dependent protein kinase II |
| cDNA | Complementary DNA |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| CNS | Central nervous system |
| cRNA | Complementary RNA |
| CTZ | Cyclothiazide |
| DEPC | Diethylpyrocarbonate |
| *DIV* | Days *in vitro* |
| DMEM | Dulbecco's modified Eagle's minimal essential medium |
| DMSO | Dimethyl sulfoxide |
| DNQX | 6,7-dinitroquinoxaline-2,3-dione |
| EPSC | Excitatory post-synaptic current |
| EPSP | Excitatory post-synaptic potential |
| FBS | Fetal bovine serum |
| FDA | United States Food and Drug Administration |
| GABA | Gamma-aminobutyric acid |
| Glut1 | Glucose transporter 1 |
| GYKI-52466 | 4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine dihydrochloride |
| GYKI-53665 | 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride |
| HEPES | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| ILAE | International League Against Epilepsy |

| | |
|---|---|
| IntDen | Integrated Density |
| JaCoP | Just another Colocalisation Plugin |
| KLD | Kinase, ligase, Dpn I |
| LBD | Ligand-binding domain |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| MCT | Medium-chain triglyceride |
| MES | Maximal electroshock |
| MK-801 | Dizocilpine |
| MOPS | (3-(N-morpholino)propanesulfonic acid) |
| mRNA | Messenger RNA |
| NBQX | Nitroquinoxaline-2,3-dione |
| NINDS | National Institute of Neurological Disorders and Stroke |
| NMDA | N-Methyl-D-aspartate |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLD | Poly-D-Lysine |
| PPAR γ | Peroxisome proliferator-activated receptor gamma |
| PTZ | Pentylenetetrazol |
| PUFA | Polyunsaturated fatty acids |
| PVDF | Polyvinylidene fluoride |
| RIPA | Radio immunoprecipitation assay |
| rNTP | Ribonucleoside tri-phosphate |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SUDEP | Sudden unexpected death in epilepsy |
| TAE | Tris base, acetic acid and EDTA buffer |
| TARP | Transmembrane AMPA receptor regulatory protein |
| TEVC | Two-electron voltage clamp |
| TMD | Trans-membrane domain |

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# CHAPTER 1 – INTRODUCTION

# 1.1 Epilepsy

## 1.1.1 Symptoms, causes and classification

The general term epilepsy comprises a range of different conditions that result in repeated and transient seizures that are associated with a loss of control over movements, and sometimes awareness (Fisher *et al.*, 2005). It is one of the most common neurological disorders, affecting about 1% of the population worldwide and with developing countries having a slightly higher incidence than developed countries (Duncan *et al.*, 2006; Newton and Garcia, 2012). Seizures originate in the brain, where they can be localised to one region or spread throughout the CNS (Berg *et al.*, 2010).

Epilepsy as a diagnosis covers a wide range of cerebral syndromes, but in many cases, it is not well understood how these syndromes cause seizures. Some notable exceptions include conditions in which single gene mutations result in seizures, including some metabolic syndromes like Glut1 deficiency, where mutations in the glucose transporter result in neuronal death and seizures (Seidner *et al.*, 1998), or Dravet syndrome, where lack of functional voltage gated sodium channels causes abnormal neuronal signalling (Selmer *et al.*, 2009). This complexity and wide range of causes considerably complicates diagnosis and treatment. The International League Against Epilepsy (ILAE) has published guidelines for seizure classification to make the process of diagnosis more efficient. These recommendations are being revised annually by the ILAE to accommodate the most recent advances in research (Berg *et al.*, 2010; Trinka *et al.*, 2015; Scheffer *et al.*, 2016). It is currently recommended to base the primary classification on seizure types. These can be distinguishing between focal (limited to a specific region of the brain), generalised, generalised and focal, and unknown types (Scheffer *et al.*, 2016, figure 1.1). The seizure type, symptoms and EEG patterns are subsequently matched to potential syndromes, which may help provide indications for treatment and management of the particular range of symptoms (Scheffer *et al.*, 2016). Common syndromes include childhood absence epilepsy, Rett syndrome, autosomal dominant nocturnal frontal lobe epilepsy, and Dravet syndrome, all of which are distinct types of epilepsy (Berg *et al.*, 2010; Dravet and Oguni, 2013). The final classification, aetiology, refers to the underlying condition, and may provide further indications regarding treatment, or prognosis and outcome (Thomas and Berkovic, 2014; Scheffer *et al.*, 2016). Finally, the underlying aetiology is determined and classified as either genetic, structural, metabolic, immune, infectious, or unknown (Scheffer *et al.*, 2016). Identification of genetic causes has increased in recent years as genetic sequencing has become more time and cost efficient, and has helped identify the cause for many cases that would have previously been labelled as unknown (Thomas and Berkovic, 2014).

Today, studies estimate that 30% of patients with epilepsy have a monogenic mutation, with ca. 10% of patients presenting with de novo mutations without prior family history (Appenzeller *et al.*, 2014; Mercimek-Mahmutoglu *et al.*, 2015). Most frequently identified mutations affect proteins that are directly involved in synaptic transmission, such as potassium and sodium channels, or in cerebral development (Appenzeller *et al.*, 2014; Muona *et al.*, 2015; McTague *et al.*, 2016). A subset of these mutations results in metabolic syndromes, such as glucose-transport deficiencies, glycogen storage diseases and other deficiencies of metabolism (Engel, 2001; Berg *et al.*, 2010; Mercimek-Mahmutoglu *et al.*, 2015). Whilst structural causes for seizures can also have an underlying genetic origin, for instance in dysregulated cerebral development and Rett syndrome (Shorvon, 2011), more frequently, they are acquired or secondary, such as through cerebral trauma, tumours or cerebrovascular disorders (Berg *et al.*, 2010; Shorvon, 2011; Scheffer *et al.*, 2016). In addition, inflammation and infection can result in seizures, as in febrile seizures, or in meningitis and encephalitis (Millichap and Millichap, 2006; Shorvon, 2011; Vezzani *et al.*, 2013).

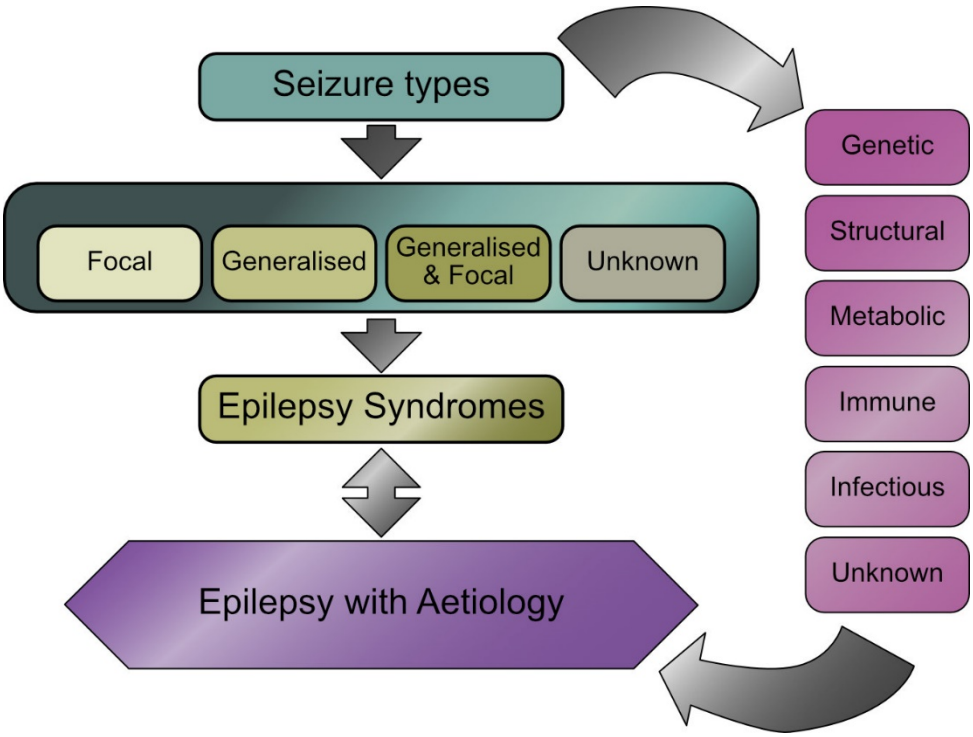



*Figure 1.1 Stepwise classification of epilepsy syndromes and aetiology as recommended by the ILAE.* Diagnosis should consider symptoms, seizure type, underlying aetiology and syndromes to accurately reflect underlying conditions and preferential treatment. Schematic based on Scheffer *et al.*, 2016.

The multitude of causes for seizures reflects the complexity of epileptic syndromes and encephalopathies. It is not uncommon for one aetiology to be associated with a range of syndromes, or for aetiologies to overlap, which is why an accurate description should reflect all three levels as defined by the ILAE, including seizure type, syndrome and aetiology (Scheffer *et al.*, 2016) to facilitate a tailored and suitable treatment for each patient.

### 1.1.2 Comorbidities, complications and quality of life

Epilepsy is associated with a range of comorbidities and complications that are thought to have a greater impact on the perceived quality of life of patients than seizures alone (Helmstaedter *et al.*, 2014; Selassie *et al.*, 2014). The incidence of comorbidities is much higher in patients with epilepsy than in people living with other chronic conditions, with studies suggesting that somatic comorbidities occur in 69% of patients, and neurodevelopmental comorbidities in up to 95% of patients (Selassie *et al.*, 2014; Srinivas and Shah, 2017). Due to the wide range of conditions associated with epilepsies, these comorbidities are correspondingly highly diverse including neuropsychological, neurobehavioral (autism spectrum disorders, mood disorders, attention deficit disorders, hyperactivity disorders), and neurological (cognitive impairment, stroke, migraine) conditions (Helmstaedter *et al.*, 2014; Selassie *et al.*, 2014; Srinivas and Shah, 2017).

Seizure strength and frequency are thought to be directly related to the occurrence and severity of comorbidities. Prolonged hyperexcitation likely causes neurobiological changes that affect a variety of neurological functions (Helmstaedter *et al.*, 2014). Lack of efficient seizure control, for instance due to failure to respond to anti-epileptic drug (AED) treatment, can lead to severely prolonged seizures (Trinka *et al.*, 2015). If seizures fail to terminate on their own or patients do not respond to AED intervention, epileptic activity may last for more than 10-30 minutes, a complication referred to as status epilepticus (Lewis *et al.*, 2014; Trinka *et al.*, 2015). Progression of seizures into status epilepticus is associated with long-lasting structural brain changes in children, increased health care costs, and sudden death in epilepsy (SUDEP, Lewis *et al.*, 2014; Betjemann and Lowenstein, 2015; Novak *et al.*, 2015; Trinka *et al.*, 2015). Thereby, epilepsy treatment is not only necessary to reduce the occurrence of seizures but also to reduce the long-term damaging effects that seizures can have on neurological function and neuronal health.

Importantly, comorbidities and complications, in addition to social aspects, are associated with a high incidence of perceived stigma in patients with epilepsy (Austin, Perkins and Dunn, 2014). The impact of social exclusion, bullying and activity restriction on the quality of life of patients with epilepsy

is severe, with many patients reporting fear and worries about living with epilepsy that frequently leads to concealment of their condition (Austin, Perkins and Dunn, 2014; England *et al.*, 2014). The impact of stigma on the quality of life is most severe in rural areas in the developing world where, due to lack of information and education, social exclusion and superstitious beliefs are commonly experienced, with some preconceptions persisting in parents and medical students (Maiga *et al.*, 2014; Bigelow *et al.*, 2015; Kakooza-Mwesige *et al.*, 2017). This is important as the impact of stigma on availability of treatment and disease progression cannot be underestimated, and can affect people irrespective of whether they live in high and low-to-medium-income countries (England *et al.*, 2014; Kakooza-Mwesige *et al.*, 2017).

# 1.2 Neuronal signalling

## 1.2.1 Healthy neuronal signalling

Neuronal function is optimised for the efficient and speedy transmission of signals from the central to the peripheral nervous system and back. A large proportion of signal transmission depends on specific neurotransmitters, the effects of which are mostly mediated by their respective receptor ion channels. Signal integration and response to neurotransmitters are facilitated by the organisation of neurons into cell bodies (soma), dendritic extensions, axons and synapses, which provides an optimised structure for the purpose of signal transmission (figure 1.2). Dendritic extensions "sense" changes in neurotransmitter levels through selective expression of ion channel receptor proteins that rectify ion concentrations inside the cell. Depending on the type of neuron, up to 15,000 dendritic spines, small protrusions that extend from the dendrites, can connect each cell with the synaptic terminals of other neurons (Koch and Zador, 1993). As will be discussed in detail below, axons, both presynaptic and postdendritic, are specialised structures that facilitate signal mediation from the dendritic spines to the synapses, which in turn efficiently transmit the signal to connecting neurons through the release of neurotransmitter. It is thought that on average, one neuron contains 7,000 synaptic terminals (Pakkenberg *et al.*, 2003). Through these high numbers of dendritic and synaptic connections, the neuronal structure allows a single neuron to form up to thousands of connections with other cells, and to specifically and precisely integrate and transmit a multitude of signals. However, the complexity of neuronal connectivity and its vulnerability to imbalances also directly contribute to seizure initiation and maintenance.

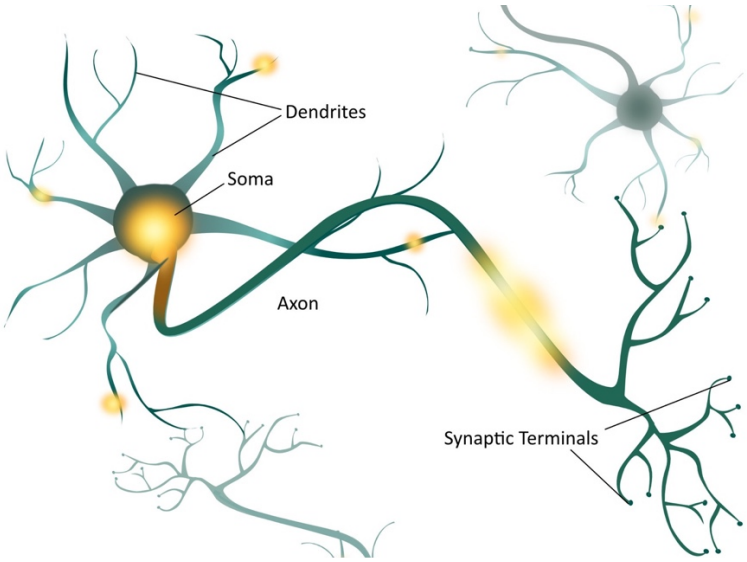



*Figure 1.2 Representation of a neuron showing the cell body (soma), dendritic outgrowths, axon and synaptic terminals.*

Rapid signal transmission is regulated through fast changes in intracellular and extracellular ion concentrations, which are mediated by specialised ion channels. Under resting conditions, positively charged ions are transported out of the neuronal cytosol into the extracellular space by ion transporters such as ATP-dependent cation pumps. As ions cannot diffuse passively back across the neuronal membrane, these transport proteins establish a strong polarisation of the cytosol relative to the extracellular space. This results in a negatively charged neuron with respect to the extracellular fluid and is defined as the resting state of the cell (figure 1.3). Upon excitation, channels that are expressed on the axons, open, and allow the flow of positively charged ions into the neuron. This effectively depolarises the membrane by establishing more equally charged intra- and extracellular fluids. If activation of the neuron is sufficiently strong, this depolarisation spreads across the axons to the synaptic terminals, where it triggers neurotransmitter release.

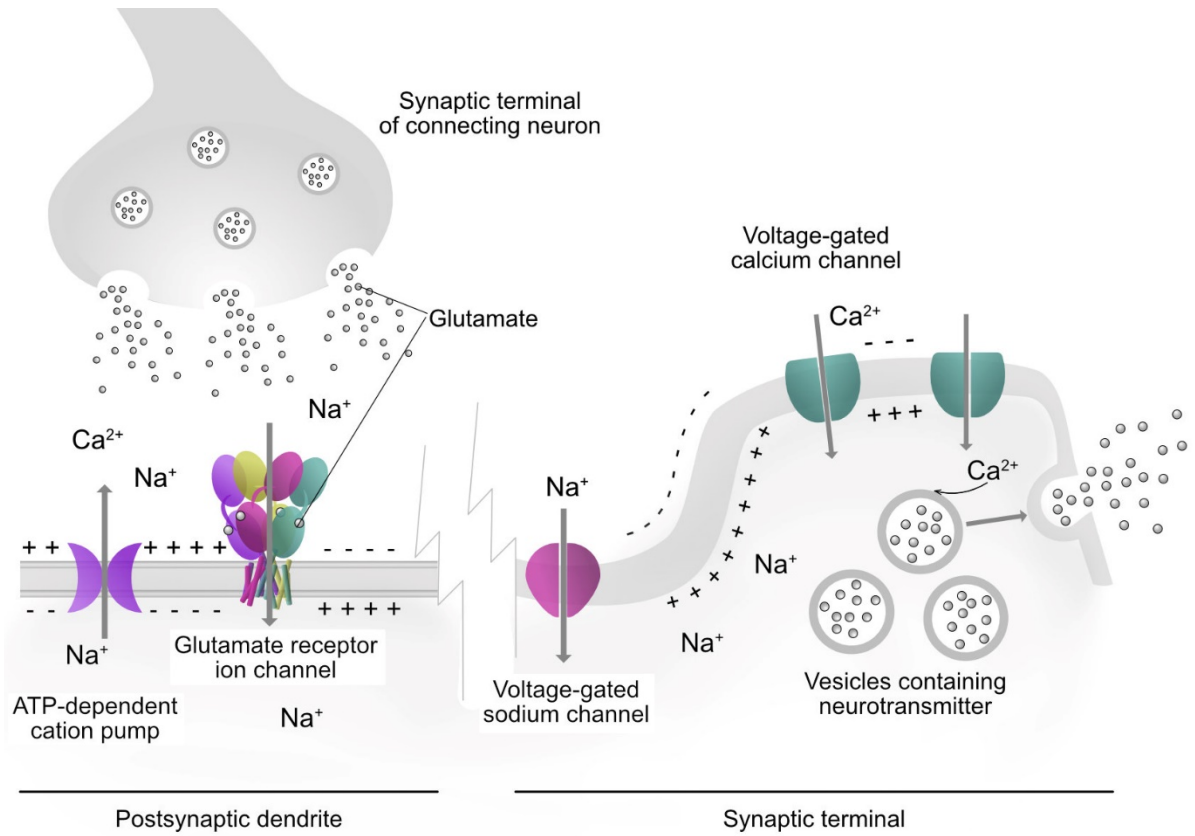



*Figure 1.3 Neuronal signal transmission upon activation of ionotropic glutamate receptors*. Glutamate binding at the receptor channel protein opens the channel pore and causes the influx of positively charged ions into the cell. Accumulation of positively charged ions in the cytosol depolarizes the membrane potential and gets propagated by voltage-gated sodium and calcium channels. Subsequent influx of calcium ions ultimately results in neurotransmitter release at the synapse.

For the excitation to spread along the axon, depolarisation of the membrane needs to be maintained by progressive (adjacent) influx of positively charged ions. Voltage-gated channel proteins that are expressed along the axon and respond to changes in membrane voltage contribute to maintaining the reversed membrane potential. Upon depolarisation, these channels open and selectively allow inward flow of sodium and calcium ions (reviewed by Wood and Baker, 2001). Rapid desensitisation of voltage-gated sodium channels, which is a period of inactivation following channel opening that is independent of membrane voltage, prevents bidirectional spread of the signal (Mantegazza *et al.*, 2010). Mutations and changes in voltage-dependent sodium channel expression lead to seizures in rats and have been identified in patients with epilepsy (Meisler *et al.*, 2000; Claes *et al.*, 2001; Ketelaars *et al.*, 2001; Ceulemans, Claes and Lagae, 2004; Klein *et al.*, 2004). Therefore, voltage gated sodium channels are key targets in epilepsy drug development (Errington *et al.*, 2007). In contrast to the axons, synapses contain mainly high and low-voltage activated calcium channels, the activation of which initiates neurotransmitter release through calcium ion influx. Increased intracellular calcium initiates steps resulting in the fusion of neurotransmitter-containing vesicles with the synaptic membrane, resulting in the release of transmitter into the synaptic cleft (Catterall and Few, 2008; Catterall, 2011).

Signal transduction is regulated by both excitatory and inhibitory neurotransmitters. The most abundant excitatory neurotransmitter in the brain is glutamate, which acts on four different known types of receptors, three of which – the NMDA, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) and kainate receptors – are ion conducting proteins that, upon neurotransmitter binding, allow excitatory ions such as sodium and sometimes calcium to enter the cell. NMDA, AMPA and kainate receptors therefore are critical proteins in neuron activation by increasing cation concentrations in the cell.

Inhibitory neurotransmitters inactivate signalling through opening channels that selectively conduct negatively charged ions into the cell, or increase the active transport efficiency of cations out of the cell, effectively increasing the polarity of the neuronal membrane (hyperpolarising it). GABA is the brain's major inhibitory neurotransmitter. It acts on two known types of receptors, accordingly referred to as $GABA_A$ and $GABA_B$ receptors. The $GABA_A$ receptor is a chloride channel protein that, when activated, facilitates hyperpolarisation by passive transport of chloride ions into the cell. The $GABA_B$ receptor on the other hand, is a metabotropic receptor that increases polarisation through activating the active transport of cations via intracellular signalling mechanisms. This way, both types contribute to a stronger concentration gradient of positively versus negatively charged ions across the neuronal membrane.

## 1.2.2 AMPA receptors

The glutamate receptor ion channels, including NMDA and AMPA receptors, are a ubiquitously expressed group of proteins that are made up of multiple highly-conserved subunits that are found throughout the brain. In the case of the AMPA receptor, named after the highly selective agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), four subunits (GluA1 to GluA4) are distinguished. The structure of all four subunits is highly similar, with three transmembrane domains (M1, M3 and M4), and one re-entrant loop (M2), that make up the channel pore. These membrane domains rearrange according to shifts and movement of the extracellular domains and thereby either close or open the pore, restricting or allowing ion conductance accordingly. The large extracellular part of the protein is formed by the two extracellular sequences S1 and S2. S1 makes up the N-terminal tail and S2 joins M3 and M4. Together, the two sequences form the two extracellular globular domains D1 and D2, also referred to as ligand-binding domain (LBD) and amino-terminal domain (ATD), respectively (Sobolevsky, Rosconi and Gouaux, 2009; figure 1.4 A). Binding at the LBD causes a conformational shift of the ATD that opens the channel via displacement of the M1-S1 and M3-S2 linker regions (Balannik *et al.*, 2005; reviewed by Traynelis *et al.*, 2010; figure 1.4 B).

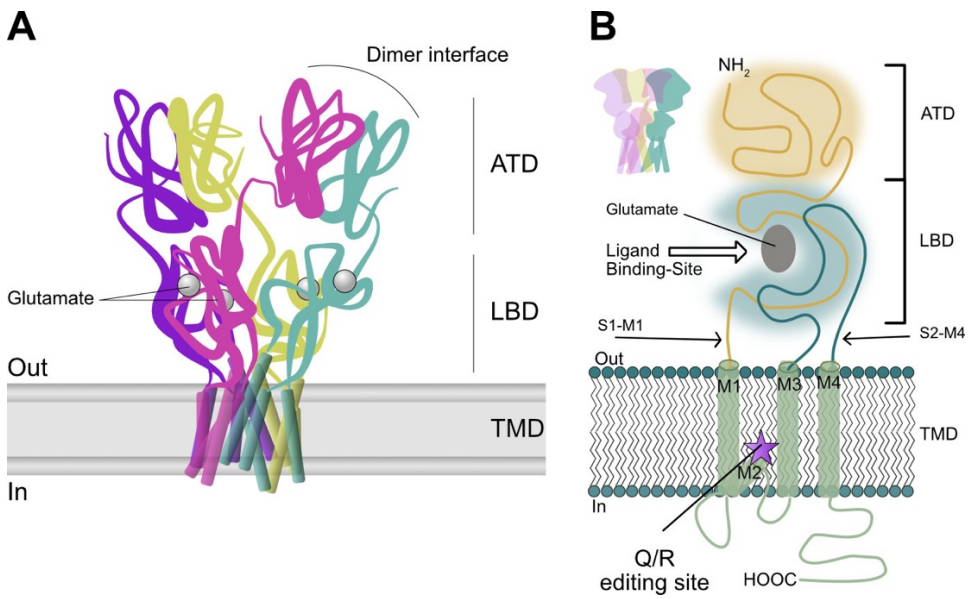



*Figure 1.4 Schematic representations of AMPA receptor assembly and subunit organisation.* (**A**) Fully functional AMPA receptor consisting of four subunits (purple, yellow, pink and green). The receptor assembles as a dimer of dimers (purple-yellow and pink-green), with each dimer binding two glutamate molecules at the ligand-binding domain (LBD). Changes in the transmembrane domain (TMD) are thought to be imitated by movement of the amino-terminal domain (ATD) following glutamate binding or modulation of the receptor structure by antagonists and agonists. The structural representation is based on Sobolevsky, Rosconi and Gouaux, 2009. (**B**) Schematic representation of a single subunit showing the four transmembrane domains (M1-M4), as well as arrangement of the extracellular sequences (S1 and S2) into ATD and LBD and ligand-binding site. The schematic is based on Balannik *et al.*, 2005; Rogawski *et al.*, 2016.

AMPA and kainate receptors are characterised by fast gating kinetics that result in activation and desensitisation rates in the millisecond time scale (Traynelis *et al.*, 2010). This mechanism critically contributes to open probability and the degree of membrane depolarisation. AMPA receptors assemble as a dimer of dimers, meaning that two subunits form a pair, which then combines with another pair to make up the fully functional tetramer (Traynelis *et al.*, 2010). This mechanism of AMPA receptor assembly is crucial for desensitisation because it allows the ATDs to form an interaction interface upon dimerisation (Sun *et al.*, 2002). Desensitisation is initiated by a rearrangement of this interface that effectively separates the ATDs from each other, resulting in a subsequent shift in the LBD which then displaces the linker regions. As a result, the membrane helices get pushed closer together, preventing channel opening (Sun *et al.*, 2002; Kovács *et al.*, 2004).

In addition to desensitisation as a means of regulating the flow of cations, AMPA receptors control ion influx in a number of ways. Most notably, the expression of a calcium-impermeable subunit (GluA2) is thought to be a regulatory mechanism that is critical in the control of excitation, and neuroprotection. The Q/R site in the M2 region (figure1.4 B) is a developmentally regulated site for posttranslational modification that determines calcium permeability. Editing of a glutamine (CAG; Q) to arginine (CIG; R) at this site renders GluA2 subunit containing AMPA receptors impermeable to calcium ions and reduces single channel conductance (Burnashev *et al.*, 1992; Traynelis *et al.*, 2010). Editing at this site is nearly complete for most species and mice express GluA2 subunits that cannot be edited develop epilepsy and die pre-maturely at the age of three weeks (Brusa *et al.*, 1995). All four AMPA receptor subunits are differentially expressed in the brain and throughout development (Ben-Ari *et al.*, 1997; Pickard *et al.*, 2000; Kumar *et al.*, 2002). In the mature brain, between 80 and 95% of AMPA receptors are made up of a combination of GluA1 and GluA2 (GluA1/2) with the majority of the remaining receptors being of the GluA2/3 type and a small percentage of homomeric GluA1 (Wenthold *et al.*, 1996; Lu *et al.*, 2009). Therefore, the vast majority of cerebral AMPA receptors in adults is calcium-impermeable, suggesting that GluA2 expression is critical for normal brain function. In animal studies, periods of low GluA2-expression correlate with an increased risk for ischemic injury. This links calcium-permeable AMPA receptors to a potential role in epileptogenesis and excitotoxicity as ischemic injury is often associated with the development of chronic seizures, and vice versa (Hossain, 2005; Talos *et al.*, 2006).

Localisation and the ion conducting properties of GluA2 are particularly important for neuronal depression or depotentiation. Knock-out mice that do not express GluA2 do not display long-term synaptic depression (LTD), a form of synaptic plasticity that results in lower synaptic strength and that is thought to be critically involved in memory formation. In GluA2 knock-out mice, all AMPA receptors are calcium permeable and therefore depolarise the membrane potential more strongly (Malinov and

Malenka, 2002; Chung *et al.*, 2003). This has long-lasting consequences for seizure susceptibility with overall stronger synaptic transmission that results in a lower seizure threshold (Kullmann, Asztely and Walker, 2000; Malinov and Malenka, 2002).

Whilst GluA2 expression is required for synaptic depotentiation, localisation of the calcium-permeable GluA1 subunit to synapses strengthens excitatory neurons by allowing stronger ion flow. In addition, calcium ions moving through GluA1 mediate synaptic transmission by inducing exocytosis and activating signalling cascades that can result in stronger depolarisation (Barria *et al.*, 1997; Lu *et al.*, 2009). For instance, ion flow through GluA1 is tightly regulated by calcium-dependent intracellular signalling cascades. The GluA1 subunit contains several phosphorylation sites on the C-terminal intracellular tail that have been shown to control synaptic localisation and receptor activity. Phosphorylation of serine 831 and serine 845, by calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC), is necessary for GluA1 to be localised at the synapse in the CA1 region of the hippocampus (Mammen *et al.*, 1997; Boehm *et al.*, 2006; Serulle *et al.*, 2007; Kristensen *et al.*, 2011). Serine 845 phosphorylation by protein kinase A (PKA) also increases the opening probability of the receptor resulting in stronger currents (Roche *et al.*, 1996; Banke *et al.*, 2000). Additional phosphorylation sites exist on the intracellular loops linking M2 and M3, and on the C-terminal tail. Modifications of these sites by PKC and CamKII are also thought to primarily affect GluA1 localisation to specific regions of the synaptic and extrasynaptic membranes (Hayashi *et al.*, 2000; Lee *et al.*, 2003; Lu *et al.*, 2009; figure 1.5).

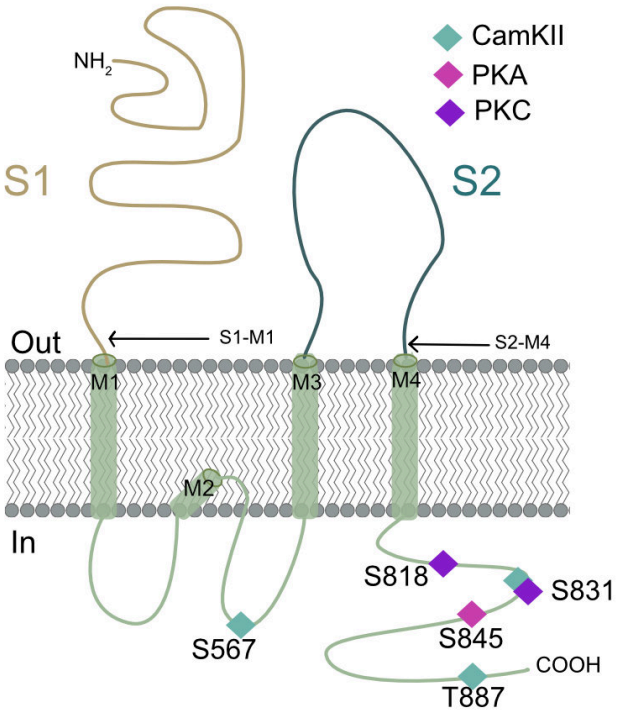



*Figure 1.5 Phosphorylation sites on the AMPA receptor GluA1 subunit.* Schematic representation of the GluA1 subunit showing S1 and S2 extracellular sequences, transmembrane sequences M1-M4 and intracellular phosphorylation sites that are recognised by CamKII (green), PKA (pink) and PKC (purple).

In addition to GluA1, localisation of subunit GluA2 is also tightly regulated by phosphorylation. Whilst fewer phosphorylation sites have been identified so far, the GluA2 Serine 880 site appears to be critically involved in regulating AMPA receptor activity, not through altering the channel and conductance properties, but by facilitating the interaction with scaffolding proteins at the membrane (Chung *et al.*, 2003). Phosphorylation of GluA2 at Ser880 has been shown to increase internalisation and localisation at the extra-synaptic membrane, therefore reducing the number of GluA2 subunits at the post synapse (Chung *et al.*, 2003). From these studies, it is therefore clear that both, the expression of distinct AMPA receptor subunits and the post-translational modification of these proteins, play a critical role in neuronal function. Changes in these two regulatory mechanisms are thought to be involved in seizure susceptibility and generation (Donevan and Rogawski, 1998; Rogawski, 2011).

### 1.2.3 AMPA receptor antagonists

Studying AMPA receptor function through the effects of specific antagonists has been used in the identification of complex structural and gating properties of AMPA receptors (Yamada and Tang, 1993; Iino *et al.*, 1996; Balannik *et al.*, 2005; Chen, Durr and Gouaux, 2014). The first studied antagonists of AMPA receptors were the quinoxalinediones CNQX, DNQX, and NBQX, which are selective antagonists that specifically block AMPA receptors (Honoré *et al.*, 1988; Wilding and Huettner, 1997; Traynelis *et al.*, 2010). These inhibitors bind to the glutamate binding pocket of the protein, which makes them competitive with respect to glutamate (Traynelis *et al.*, 2010), and means that they may be displaced by high glutamate concentrations. The suitability of competitive inhibitors in the treatment of conditions that are marked by excessive postsynaptic glutamate concentrations, such as epilepsy, may therefore be limited.

The study of AMPA receptor inhibitors for treatment of seizures has focused largely on non-competitive inhibitors, such as the 2,3-benzodiazepine which include the GYKI-related compounds, GYKI 53665 and GYKI 52466. These types of antagonists are well characterised for potency against AMPA receptors and binding mechanism (Bleakman *et al.*, 1996; Balannik *et al.*, 2005; Menuz *et al.*, 2007; Perrais *et al.*, 2009). Another well-defined non-competitive inhibitor, CP-465,022, is 100-times more potent than the GYKI-related compounds (Bleakman *et al.*, 1996; Lazzaro *et al.*, 2002; Menniti *et al.*, 2003; Balannik *et al.*, 2005). A site-directed mutagenesis study has found that replacing the M1-S1 and S2-M4 linker regions of AMPA receptors severely decreased inhibition by both GYKI and CP-465,022 (Balannik *et al.*, 2005), suggesting that these regions are involved in inhibition by both compounds.

Interestingly, a large number of natural AMPA receptor blockers are uncompetitive inhibitors. These include the spider and wasp toxins argiotoxin 636, Joro-spider toxin and philanthotoxin (Jones, Anis and Lodge, 1990; Blaschke *et al.*, 1993; Herlitze *et al.*, 1993). All of these compounds are structurally similar in that they contain a polyamine core, and their mechanism is thought to be identical (Traynelis *et al.*, 2010). They all bind only to GluA2-lacking AMPA receptors, and act as channel blockers that enter the receptor pore upon activation and get locked in the channel when it closes (Traynelis *et al.*, 2010). In order to overcome the block, the receptor has to be reactivated, upon which the toxin is released from the pore (Brackley *et al.*, 1993; Parsons *et al.*, 1995). This makes polyamines functionally distinct from non-competitive inhibitors which bind independently of receptor activation state and are therefore thought to be constitutively active and a more reliable choice for drug development (Rogawski, 1993; Fletcher and Lodge, 1996).

### 1.2.4 Two-electrode voltage clamp electrophysiology in *Xenopus laevis* oocytes

AMPA receptor antagonist studies have frequently relied on the two-electrode voltage clamp (TEVC) method in *Xenopus* oocytes. The African clawed frog, *Xenopus laevis*, has been used as a laboratory animal throughout the 20$^{th}$ century, as a tool for pregnancy testing (Shapiro and Zwarenstein, 1934; Elkan, 1938), or to study muscle cells and development (Hughes, 1957; Gurdon, 1960; Lewis and Hughes, 1960; Malick, Wilson and Stetson, 1975). It was only through pioneering work in the study of the early stages of *Xenopus* development, that Gurdon et al. (1971) observed the capacitance for oocytes to express foreign mRNA. Based on these findings, Miledi et al. (1982) were the first to report the successful expression and detection of heterologous protein in *Xenopus* oocytes in 1982.

Today, expression of recombinant or reconstituted channel proteins in *Xenopus laevis* oocytes is a useful and inexpensive approach to reliably study a variety of receptor and channel proteins in a near-isolated system outside their normal environment (Wagner *et al.*, 2000). Glutamate receptor ion channels, and especially AMPA receptors have been studied and characterised using this approach revealing important characteristics and functions such as calcium-impermeability of GluA2 subunits and desensitisation properties (Pellegrini-Giampietro, Bennett and Zukin, 1991; Partin *et al.*, 1993; Yamazaki *et al.*, 2004). TEVC in oocytes applies Ohm's law ($V = I/R$) where the voltage across the membrane is $V$, the current readout $I$, and the number of closed receptor channels corresponds to resistance *(R).* To get a cause effect relationship where $I = C$, the cell membrane is clamped at a fixed voltage, thereby allowing the current readout to be interpreted as a function solely of channel opening and closing. In addition, the oocyte system is efficient due to the fact that *Xenopus* frogs are easily kept and bred in captivity, whilst the eggs are sufficiently large and resistant to be easily prepared and kept *ex vivo* (Wagner *et al.*,

2000). *Xenopus* oocytes readily express endogenous mRNA, can tolerate repeated impaling by glass electrodes and, due to the independence of the single cell system, are usually also low in endogenous protein which facilitates the interpretation of the current readout (Baumgartner, Islas and Sigworth, 1999; Wagner *et al.*, 2000; Cohen and Zilberberg, 2006).

Use of TEVC must be assessed with a range of caveats. Oocytes are viable cells expressing foreign protein, therefore no readout will be entirely free of background modifications by the cell. For instance, protein phosphorylation has been shown to be altered in *Xenopus* oocytes (Cohen and Zilberberg, 2006), and interpretation of very large, and very small currents alike can be unreliable (Baumgartner, Islas and Sigworth, 1999). In addition, some endogenous protein may change the properties of the heterologous protein, or influence currents in other ways (Wagner *et al.*, 2000). Nevertheless, in terms of reliable pharmacological studies, the *Xenopus* TEVC systems constitutes a simple, robust system that provides a more isolated environment for channel protein function studies than can be assumed in cell or tissue culture. This makes the TEVC system a useful tool for the study of ion channel activity and direct modulation.

# 1.3 Seizure mechanisms

## 1.3.1 GABA/glutamate imbalance

A range of mechanisms that lead to seizures have been suggested, with long-term changes in synaptic plasticity and therefore strength, neuronal loss, mossy fibre sprouting and a general imbalance of signalling protein expression being among the most discussed and researched topics (Buckmaster, 2014; Schmeiser *et al.*, 2017). However, as diverse as the causes for epilepsy syndromes may be, they often result in dysregulation of the balance of the inhibitory/excitatory signal transduction mechanisms in the brain (Kaila *et al.*, 2014; Guerriero, Giza and Rotenberg, 2015).

Epilepsy affects adults and children alike. However, the mechanisms leading to seizures are thought to differ between mature and immature brains, making children more susceptible to seizures (Holmes and Ben-Ari, 2001; Haut, Veliškova and Moshé, 2004). This is thought to be due to the differential and development-dependent expression of inhibitory and excitatory neurotransmitter receptors (Pellegrini-Giampietro, Bennett and Zukin, 1991; Traynelis *et al.*, 2010). AMPA receptor calcium impermeable subunits are differentially expressed throughout development. In early development, GluA2 expression is very low, and AMPA receptors are predominantly assembled as homomeric GluA1 and GluA4 (Pellegrini-Giampietro, Bennett and Zukin, 1991). In postnatal development, GluA2 expression steadily increases, a process which is thought to protect neurons from neurotoxicity due to GluA2 subunit inclusion rendering AMPA receptors impermeable to calcium (Traynelis *et al.*, 2010). As a consequence of the developmentally regulated expression of GluA2, immature brains express predominantly calcium-permeable AMPA receptors, resulting in stronger ion influx into neurons. It is therefore thought that immature brains are more easily excitable than mature brains and more likely to be affected by neurotoxicity (Ben-Ari *et al.*, 1997).

Another theory regarding the development of epilepsy in children refers to GABAergic signalling. It has been shown that in infant brains, synaptic GABAergic signalling often does not result in hyperpolarisation but in depolarisation of the membrane (Leinekugel *et al.*, 1997). This is because the chloride ion transport via the $GABA_A$ receptor channel is not directional but dependent on the relative chloride ion concentrations in the cytoplasm and extracellular space. In infants, intracellular chloride is more concentrated than extracellular chloride because of a differential expression of chloride co-transporters that favours chloride transport into the cell in early development (Plotkin *et al.*, 1997). $GABA_A$ receptor activation therefore results in chloride ions leaving the cell, effectively depolarising the postsynaptic membrane (Ben-Ari *et al.*, 1989; Perrot-Sinal, Auger and McCarthy, 2003). In addition,

mutations in the $GABA_A$ receptor subunit γ2 have been shown to lead to seizures in primary hippocampal neurons (Harkin *et al.*, 2002; Kang, Shen and Macdonald, 2006).

A combination of developmentally regulated chloride-concentrations and AMPA receptor subunit expression provides an attractive theory for explaining some types of childhood epilepsies, such as febrile seizures. In addition, it is assumed that children who have had febrile seizures are more likely to develop epilepsy later in life (Kang, Shen and Macdonald, 2006; Patterson, Baram and Shinnar, 2014). The cause-effect relationship of increased likelihood for seizures following febrile status epilepticus is, however, controversial. For instance, genetically regulated changes in ion channel properties or expression levels, or neurotransmitter receptor expression could account for increased seizure susceptibility. On the other hand, as will be discussed in detail below, postictal (post-seizure) changes in neuronal signalling might make the brain more susceptible to subsequent seizures. Overall, however, there is evidence for the increased likelihood of childhood seizures in non-acquired epilepsies, and this makes the treatment of seizures in children an important and complex topic for drug development.

### 1.3.2 AMPA receptors in seizures

Given the central role of glutamate receptors in the mediation of fast excitatory signalling (Traynelis *et al.*, 2010), it is not surprising that they have repeatedly been suggested as key components in seizure generation. Both NMDA and AMPA receptors are equally widely expressed (Traynelis *et al.*, 2010), and have been shown to be involved in seizure generation. Initially, investigations of glutamate receptor modulation has focussed on NMDA receptor inhibition, due to the observation that activation of these receptors induces bursts that are similar to the depolarising shifts seen in ictal brain activity in patients (reviewed by M A Rogawski, 2013) and that some syndromes of epilepsy such as XYZ are caused by point mutations in the NMDA receptor (Carvill *et al.*, 2013; Lemke *et al.*, 2013; Lesca *et al.*, 2013). However, NMDA receptor antagonists, whilst showing some promise in seizure control, failed to fully block seizures and never reached clinical trial phases for epilepsy drug development (Mody, Lambert and Heinemann, 1987; Neuman, Cherubini and Ben-Ari, 1988). By comparison, AMPA receptor antagonists were more reliably potent anticonvulsants in animal models of epilepsy (Sveinbjornsdottir *et al.*, 1993; Yamaguchi, Donevan and Rogawski, 1993; Löscher and Hönack, 1994; De Sarro *et al.*, 2005). In fact, the two AMPA receptor antagonists talampanel and perampanel were the first glutamate receptor inhibitors to be assessed in clinical trials, and perampanel was subsequently released in 2012 for adjuvant treatment of seizures (Rogawski and Hanada, 2013).

As was discussed above, the differential developmental expression of calcium-impermeable AMPA receptor subunits may contribute to increased seizure susceptibility. This makes them a valid target in epilepsy drug development. However, regulation of AMPA receptor activity and expression exceeds developmental differences and can vary as a direct result of seizure activity. For instance, following an ictal event, GluA2 mRNA levels and protein expression are downregulated in kainate-kindled rats (Pollard *et al.*, 1993; Friedman and Koudinov, 1999). In addition, mice that are deficient in GluA2 Q/R editing, and therefore do not contain $Ca^{2+}$-impermeable AMPA receptors, develop severe seizures early on in life and die prematurely from neuronal injury and necrosis (Brusa *et al.*, 1995). A central role of AMPA receptors in epileptogenesis is further highlighted by the observation that treatment of less than two day-old rats with the AMPA receptor antagonist topiramate decreased long-term susceptibility to seizures (Koh and Jensen, 2001). Interestingly, in adults with epilepsy, GluA1 subunit expression is up-regulated, which suggests that calcium conductance is increased and excitation can spread more rapidly (Ying *et al.*, 1998; Valotta da Silva *et al.*, 2005). This suggests a role for AMPA receptors in seizure generation that goes beyond their short term, immediate effects on signal transduction and supports the development of AMPA receptor antagonists in the prevention of epileptogenesis.

### 1.3.3 AMPA receptors and neuronal plasticity

One explanation for the involvement of AMPA receptors in seizure susceptibility and possibly epileptogenesis is their function in synaptic plasticity. Due to a highly dynamic regulation of the gene expression and membrane localisation of the protein, AMPA receptors are important mediators of changes in plasticity. Effects on synaptic strength can either be mediated by generic synaptic activity or by AMPA receptor activation itself (Liu and Zukin, 2007), and AMPA-receptors are involved both in regulating long-term synaptic potentiation (LTP) of excitatory synapses (Gu *et al.*, 1996) and long-term depression (LTD) of inhibitory interneurons, depending on the strength and duration of receptor activation (Laezza, Doherty and Dingledine, 1999). Both mechanisms link AMPA receptors to a function in learning and memory (Lippman-Bell *et al.*, 2016; O'Leary *et al.*, 2016), but also to epileptogenesis (Nabavi *et al.*, 2014; Hell, 2016).

Activity-dependent self-regulation, on the other hand, requires short-term phosphorylation events at the AMPA receptor subunits. Repetitive synaptic activity results in increased GluA2 localisation to the synapse, a mechanism that is PKC-dependent and thought to be neuroprotective by reducing the net influx of $Ca^{2+}$ into the cell and thereby decreasing stimulatory capacity (Cull-Candy and Liu, 2000; Gardner *et al.*, 2005; Plant *et al.*, 2006). Interestingly, induction of status epilepticus in rats has been

shown to result in phosphorylation of the Serine 831 and 845 residues of the GluA1 subunit, which effectively strengthens the response to glutamate (Rakhade *et al.*, 2012). This suggests that seizures may potentiate glutamatergic synapses and make the brain more prone to subsequent seizures.

The importance of the GluA2 subunit in seizures is further demonstrated by studies that show that GluA2 expression is reduced in CA1 pyramidal cells in rats and children following status epilepticus (Rajasekaran, Todorovic and Kapur, 2012; Loddenkemper *et al.*, 2014). Also, GluA2 knock-down in mice results in spontaneous seizures (Friedman and Koudinov, 1999) and mice that have been treated with GluA2 antibodies will develop epilepsy (Ganor *et al.*, 2014). This suggests that expression of the GluA2 subunit is required to limit excitatory signalling and that insufficient expression and localisation at the synapse may be strongly involved in the development of epilepsy. Interestingly, decreased postictal GluA2 localisation to the synapse in rats is also associated with phosphorylation of serine 880 in GluA2, whilst increased GluA1 localisation coincides with increased phosphorylation of serine residues 831 and 845 (Rakhade *et al.*, 2008). Phosphorylation of these residues is PKC and CamKII dependent and further suggest the importance of AMPA receptor mediated calcium-influx and self-regulatory changes in synaptic localisation for plasticity events. Treatment with the AMPA receptor antagonists topiramate and GYKI reduced both the level of GluA1 phosphorylation as well as seizure susceptibility (Rakhade *et al.*, 2008) indicating a potentially central role for AMPA receptor antagonism in medium term seizure prevention and epileptogenesis.

### 1.3.4 Models of epilepsy

Animal models of epilepsy have been developed to resemble both acute seizures and chronic epilepsy. Acute seizures models are induced in rats and mice either by applying chemoconvulsants or through one-time electrical stimulation. Models of chronic epilepsy are often generated through kindling, which is the repeated application of chemoconvulsants or electroshock stimulation, or through trauma to mimic post-traumatic brain injury. In addition, specific kinds of epilepsy can be modelled with genetically modified mouse models.

To generate acute seizure models, chemoconvulsants can be applied in a single dose. This will cause secondarily generalised seizures with kainate application, and limbic seizures in pilocarpine application (Kandratavicius *et al.*, 2014). In addition, application of pentylenetetrazol (PTZ), which causes clonic seizures, has been in use for over 60 years and provides a reliable and quick seizure model for drug screening that can also be carried out in tissue culture to assess molecular effects of seizure-like activity (Löscher, 2002, 2011; Chang *et al.*, 2012). However, since chemoconvulsants are not

epileptogenic but induce seizures in healthy animals, they are not predictive of drugs that will treat epilepsy, but only of those that will stop seizures (Löscher, 2002). Application of chemoconvulsants in immature rodent brains can result in status epilepticus with subsequent spontaneous seizures; however, it needs to be noted that translation of acute seizure models to spontaneous human seizures may be limited as seizures in these animal models follow a chemical insult. Thereby, any changes in synaptic plasticity are likely to differ from those normally seen in epilepsy patients, with no neuronal loss and only mossy fibre sprouting (Löscher, 2002; Kandratavicius *et al.*, 2014; Huusko *et al.*, 2015). It can be assumed that this difference limits their use in identifying AEDs that have antiepileptogenic action in humans. This issue is even more pronounced in acute seizure models generated by PTZ, strychnine, aspartate or tetanus toxin, which are single-use assays that only reflect hyperexcitation and not underlying causes of epilepsy. However, application of these chemoconvulsants is easy, quick and inexpensive making them useful models for high-throughput screenings that narrow down drugs with general anti-seizure properties (Simonato *et al.*, 2014). Use of acute seizure models in combination with models of epileptogenesis and chronic epilepsy may therefore increase the likelihood of identifying an AED that can provide protection in a broad spectrum of seizures (Löscher, 2002; Kandratavicius *et al.*, 2014).

Kindled animal models can be used to mimic epileptogenesis by progressive increases in seizure susceptibility until spontaneous seizures occur. If the drug testing is carried out during kindling, these seizure models are much more likely to select medications that prevent epileptogenesis and progressive seizure aggravation than acute seizure models (Kandratavicius *et al.*, 2014; Simonato *et al.*, 2014). More commonly, though, kindling is carried out as a means to generate models of chronic epilepsy. The repeated application of an excitatory stimulus is thought to cause long-term synaptic changes and neuronal loss that results in spontaneous seizure occurrence that may be similar to the human epilepsies (Kandratavicius *et al.*, 2014). Closer resemblance to the human conditions and chronic epilepsies can be achieved through genetic rodent models, seizure induction by neonatal hypoxia and models that mimic posttraumatic brain injury (Simonato *et al.*, 2014). However, whilst these are chronic seizure models, they are also only approximations of human epilepsy as a primary insult is needed to cause seizures, which is not the cause for epilepsy in most human cases (Löscher, 2002).

A point that is often criticised is that a wide variety of these models, most notably the chemoconvulsant and electroshock models, were developed using first- and second-generation AEDs. These older models may therefore only select AEDs that act in a similar manner to early AEDs, which may contribute to the redundancy in epilepsy treatments focussing on a small range of targets (Golyala and Kwan, 2017). In addition, most animal models cannot account for short-lived, or non-convulsive seizures, which may lead to overestimation of drug efficacy as smaller seizures that persist during drug

treatment, are not being spotted. Despite these limitations, it is important to note the validity and need for fast, robust models to select drugs that can subsequently be assessed in clinical trials (Löscher, 2002), and for a combinatory approach that assesses drug efficacy in a range of models.

# 1.4 Treatment

## 1.4.1 Antiepileptic drugs

Epilepsy is most frequently and adequately treated with antiepileptic drugs (AEDs). To reduce the amount of excitatory signalling in the brain that leads to seizures, these drugs target multiple elements in the signal transduction pathway, and can be distinguished into modulators of voltage-gated channels, enhancers of inhibitory signalling, and inhibitors of synaptic excitation (Rogawski and Löscher, 2004). Modulators of voltage-gated ion channels act on either sodium or calcium ion channels. By inhibiting voltage-dependent sodium channels, drugs like phenytoin and carbamazepine reduce the degree of depolarisation of the neuron and thereby decrease the amount of neurotransmitter that is released at the synapse (Willow and Catterall, 1982; Segal and Douglas, 1997). As mentioned above, the main role of voltage-gated sodium channels is to sustain the membrane depolarisation along the axon. Crucial in regulating and limiting this propagation is their desensitisation state, in which the channel cannot open for a few milliseconds. AEDs targeting voltage-gated sodium channels enhance the refractory desensitisation resulting in a longer deactivation state of the ion channel (reviewed by Rogawski and Löscher, 2004). The other ion channel that is targeted by a number of anti-epileptic drugs is the voltage-dependent calcium channel. Opening of this channel specifically allows the entrance of calcium ions which then trigger the release of neurotransmitter from the synapse (reviewed by Rogawski and Löscher, 2004). Inhibiting voltage-gated calcium channels therefore indirectly reduces the amount of glutamate released from the synapse. Gabapentin, lamotrigine and topiramate are thought to act on high-voltage activated calcium channels (Greenberg, Cooper and Carpenter, 1984; Suzuki *et al.*, 1992; Gee *et al.*, 1996).

Another set of AEDs targets the GABA/glutamate imbalance more directly. The areas of the brain that are most prone to seizures are those which are also very rich in glutamatergic synapses, but contain few inhibitory neurons. Therefore, increasing the amount of inhibitory signalling from GABAergic neurons in the hippocampus and thalamus has been shown to reduce seizures. Drugs targeting GABAergic signalling either, like benzodiazepines or phenobarbital, activate the receptor (Mitchell and Martin, 1978; Pritchett *et al.*, 1989) or improve neurotransmitter release (reviewed by Rogawski and Löscher, 2004).

Due to their central role in seizure generation, NMDA and AMPA receptors have been a target in AED development for a long time. Some AEDs, such as topiramate and phenobarbital, which primarily target voltage gated ion channels, also have off-target effects on AMPA receptors (Angehagen *et al.*, 2005). It is, however, not clear how much such off-target effects contribute to seizure control in these

drugs. Unfortunately, clinical trials with novel, specific glutamate receptor inhibitors have long proven unsuccessful. NMDA receptor antagonists which were very promising in animal studies were discontinued due to insufficient seizure control. There is evidence that blocking of NMDA receptors only shortens epileptiform activity but cannot inhibit it (M. A. Rogawski, 2013). Further, NMDA antagonism causes severe side effects in patients including lack of concentration and ataxia (Sveinbjornsdottir *et al.*, 1993). In animal models, one very potent inhibitor, MK-801, has also been shown to lead to necrosis and symptoms of schizophrenia and interfere with memory and learning (Coan, Saywood and Collingridge, 1987; Fix *et al.*, 1993; Wiseman Harris *et al.*, 2003).

AMPA receptor inhibitors, on the other hand, proved more promising and drug development resulted in the release of perampanel, a selective, non-competitive AMPA receptor inhibitor that was found in placebo-controlled clinical trials to reduce seizures by 30-40% in refractory epilepsy (Bialer *et al.*, 2010; Ben-Menachem, 2014). However, since the mechanisms leading to epilepsy in the first place are still poorly understood, the currently available treatments are anti-convulsive rather than anti-epileptic drugs as they do not relieve the primary cause for epilepsy (Vezzani *et al.*, 2013).

### 1.4.2 Resistance, side effects, and other limitations of AEDs

Whilst in many cases AEDs can be used to successfully control epilepsy, roughly one third of all patients do not respond to treatment, or develop resistance to a drug they have been using (French, 2002; Schmidt and Löscher, 2005). This resistance may not be limited to one type of drug, leaving a considerable proportion of patients without satisfactory seizure control (Regesta and Tanganelli, 1999). These cases of pharmacoresistance occur with the same frequency in adult and children's brains (Regesta and Tanganelli, 1999). This is aggravated by the fact that, whilst children are just as vulnerable to resistance as adults, they have a much more restricted choice of alternative drugs due to the small number of approved AEDs for children and the lack of conclusive clinical guidelines (French *et al.*, 2004). Uncontrolled seizures in childhood are not only distressing but may also have far-reaching long-term complications. Severe and long-lasting seizures can result in delayed cognitive development and progress into status epilepticus. This may be particularly problematic if status epilepticus cannot be terminated with AEDs. Such refractory cases pose a higher risk of co-morbidity and mortality than non-refractory status epilepticus (Mayer *et al.*, 2002).

The exact mechanisms of drug resistance are not well understood and various possible models have been proposed including drug resistant or insensitive proteins and channels, and inability of the drug to reach the brain in the first place (Löscher and Potschka, 2002; Stables *et al.*, 2002). The so-called

target hypothesis, for instance, proposes that drugs over time and with repeated application lose efficacy due to reduced responsiveness in the target (Golyala and Kwan, 2017). This use-dependence has been demonstrated for valproic acid action against voltage-gated sodium channels (Vreugdenhil *et al.*, 1998; Vreugdenhil and Wadman, 1999). Another common theory suggests that overexpression of multidrug transporters in the blood-brain barrier results in rapid clearance of AEDs from the brain. Whilst co-administration of drug-transport protein inhibitors has been suggested to circumvent this effect, no clinical data exist so far to confirm that multidrug transporter overactivity actually contributes to drug resistance. Recent studies assessing expression levels of these proteins in resistant patient brains could not confirm overexpression (Bankstahl *et al.*, 2016; Golyala and Kwan, 2017). The third common theory of drug resistance mechanisms suggests that the changes in the brain that cause seizures are too general to be addressed by modulators of neurotransmission, and that current AEDs therefore cannot provide protection (Golyala and Kwan, 2017). Specifically, neurodegeneration due to chronic inflammation has been proposed to cause disruptions in healthy neuronal signalling which may not be prevented by targeting neurotransmission directly. Rather, the application of anti-inflammatory agents is now being investigated as an anti-epileptogenic therapy (Vezzani *et al.*, 2012, 2013; Dupuis *et al.*, 2015).

In addition to these gaps in treatment, side-effects of AEDs can be quite severe and often are cited as main reasons for poor compliance and discontinuation of treatments (French *et al.*, 2004). As all anti-convulsants target the neuronal signalling in the brain, they may affect basic cognitive functions and cause moderate to severe side effects which include nausea, cognitive defects, insomnia and psychomotor problems (Kwan and Brodie, 2001; Serratosa *et al.*, 2013; Villanueva *et al.*, 2016). Mood changing effects have been reported for perampanel, which, in addition to the common neurological side-effects mentioned, can cause aggression and homicidal behaviour in some patients (Serratosa *et al.*, 2013).

Another limitation of AEDs is the lack of indications in the use in paediatrics and pregnancy, which complicates treatment for a large group of patients. Few drugs have been tested in clinical trials in children and therefore few guidelines exist for their application and dosage (Stables *et al.*, 2002; French *et al.*, 2004). In addition, as was discussed above, it is well known that modulation of neuronal signalling by AEDs has side-effects on cognitive function in some patients, including memory loss and dizziness. With regard to developing brains, it can be expected that effects on cognitive function may be long-lived due to a potential disruption of normal network formation in the brain (Ijff and Aldenkamp, 2013). Some drugs that are regularly prescribed in paediatrics actually appear to affect the development of children, with long-term application of phenobarbital, phenytoin and carbamazepine in children with epilepsy being associated with reduced cognitive capabilities and psychomotor problems (Kwan and

Brodie, 2001). Long-term cognitive side-effects are also more likely to arise in polypharmacy, that is in administration of multiple types of AEDs (Ortinski and Meador, 2004), as is common practice in hard-to-control epilepsies. Since children make up a large group of patients with epilepsy, such treatment gaps and potentially long-lasting side effects are highly problematic.

In pregnancy, two factors strongly complicate AED treatment options. Firstly, due to the ethical issues of clinical trials to study the effects of AEDs in pregnancy, guidelines for the correct administration in pregnant people are limited (Tomson *et al.*, 2005). Hormonal changes in pregnancy are known to strongly affect absorption, metabolism and response to some AEDs, and since seizure control in pregnancy is a priority to protect foetus and parent, constant monitoring of patients is required (Tomson *et al.*, 2005). This is further complicated by the negative effect of some AEDs on normal cerebral development. Valproic acid is associated with decreased cognitive function and autism spectrum disorder in children that were exposed to it *in utero* (Bromley *et al.*, 2008; Meador *et al.*, 2009). In addition, valproic acid in particular, but also to a lesser extend carbamazepine, topiramate and phenobarbital, are all associated with an increased risk for congenital malformations in the foetus (Vajda and Eadie, 2005; Morrow *et al.*, 2006; Wide, Winbladh and Källén, 2007; Meador *et al.*, 2009; Hernández-Díaz *et al.*, 2012). Similar to the limitations in paediatric epilepsy care, these side effects cannot always be avoided by changing the AED, due to limited indications and a smaller selection of suitable medications in the two populations. Valproic acid is known to be a useful, broad spectrum AED that is often recommended as a suitable first-line treatment, and the decision to use it despite its contraindications in development may be due to the lack of other suitable AEDs (Davis, Peters and McTavish, 1994; Gerstner, Bell and König, 2008).

Finally, a large proportion of people with epilepsy do not have access to treatment at all. In areas with poor infrastructure or low-to-medium income countries, treatment for epilepsy is often not available or reflects only some treatment options. The example of Sub-Saharan Africa, where some estimated 65-95% of patients with epilepsy do not receive AEDs, is at the far end of the spectrum of treatment availability, but yet representative for a large proportion of people in low-income countries living with epilepsy (Scott, Lhatoo and Sander, 2001; Baskind and Birbeck, 2005; Ba-Diop *et al.*, 2014). This treatment gap due to lack of access to AEDs and insufficient health-care coverage and education forms a lethal duet with the aforementioned increased stigma faced by people with epilepsy (Maiga *et al.*, 2014; Bigelow *et al.*, 2015; Kakooza-Mwesige *et al.*, 2017). For this reason, low-income countries register a significantly greater incidence of premature and avoidable death due to epilepsy than high-income countries (Fazel *et al.*, 2013; Levira *et al.*, 2017). That being said, even across the high-income countries of Europe and the United States, treatment options vary due to local regulations and existing health care policies, impacting long-term outcomes of treatment, comorbidities of epilepsy and quality

of life (Fazel *et al.*, 2013; Baftiu *et al.*, 2015). All of these limitations in the use of AEDs highlight considerable treatment gaps that need to be addressed, since for a large proportion of people with epilepsy, despite the variety of drugs that are available, treatment is still unsatisfactory and seizure control is not always achieved.

### 1.4.3 Dietary treatments of epilepsy

In addition to AEDs, seizures can be treated with changes in diet. As described above in detail, the limitations of established and available AEDs affect children much more than adults, since recommendations for usage exist only in a proportion of AEDs. Therefore, alternative means of treating seizures in hard-to-control epilepsies do not use a solely medicated approach, but combine AEDs with modifications of the diet, by reducing carbohydrate intake and replacing the calories lost from sugar with fats. If blood glucose levels are low, ketones are generated from fats and released into the bloodstream (ketosis), and oxidised in mitochondria for energy. The ketogenic diets have been in use for almost 100 years, and have proven very effective in epilepsy treatment with ca. 25% of patients achieving full seizure control (Vining, 1999; Freeman, Kossoff and Hartman, 2007; Elizabeth G. Neal *et al.*, 2009; Kossoff, Zupec-Kania and Rho, 2009; Miranda, Turner and Magrath, 2012). The ketogenic diet was established as a treatment in epilepsy in the 1920s by Russell Wilder, long before anti-epileptic drugs became available (Wilder and Winter, 1922). Ketogenic diets are based on the observation that frequent epileptic seizures are controlled if a patient is fasting, an effect that has been known for centuries, and that has been misattributed to cleansing of evil spirits in the past (Hartman *et al.*, 2007). At the beginning of the 20$^{th}$ century, children were treated for severe seizures in hospital by withdrawing solid foods, and allowing only access to water for up to one week (Goldbloom, 1922). For obvious reasons, this approach had several limitations, including the recurrence of seizures upon dismissal of the children from the hospital and resumption of a normal diet. For this reason, Wilder proposed a novel approach to seizure control, whereby starvation is simulated in the body through glucose withdrawal, whilst ketones are supplied through fats (Wilder and Winter, 1922). As a result, ketone levels are elevated, similar to those that are observed in children during fasting.

To mimic the effects of glucose deprivation, the ketogenic diet is rich in fats but severely reduced in carbohydrates, with a daily allowance of only 10-15g of carbohydrates per kilogram of bodyweight and the remainder provided in fats (Wheless, 2008). By limiting glucose but allowing the rapid production of ketones from a rich supply of fatty acids, high blood ketone levels, or ketosis, are established rapidly and fasting is not necessary to persist for longer periods (Wheless, 2008; Kossoff, Zupec-Kania and Rho, 2009). For this reason, one of the strongest hypotheses regarding the mechanism

of action of the ketogenic diet has been a putative anti-convulsive effect of ketones. However, high fat diets are also difficult to maintain because of the high fat content which can lead to digestive problems, constipation, acidosis and kidney stones in long-term treatment (Wheless, 2001). These side effects together with the high fat content, that makes the diet unpalatable for some patients, can result in poor compliance (Vining *et al.*, 2002).

Due to the hypothesis that ketones are involved in seizure control, providing rapid and stable ketosis has been a principal focus in the development of alternate ketogenic diets. In the 1970s, the medium-chain triglyceride (MCT) ketogenic diet was established (P R Huttenlocher, 1976; Miranda, Turner and Magrath, 2012). By providing triglycerides made up of shorter fatty acids, such as octanoic, decanoic and lauric acid, the MCT ketogenic diet is considered more ketogenic and therefore more flexible than the classic KD allowing for a larger carbohydrate component (Huttenlocher, 1976; Miranda *et al.*, 2012; figure 1.6). This assumption is supported by studies in piglets and rats that have shown that medium-chain fatty acids are absorbed faster in the gut than long-chain fatty acids, with absorption peaking as soon as 15 minutes after feeding (Greenberger, Rodgers and Isselbacher, 1966; Guillot *et al.*, 1992). Therefore, the MCT ketogenic diet offers a much more flexible carbohydrate reduction with a smaller ratio of fats.

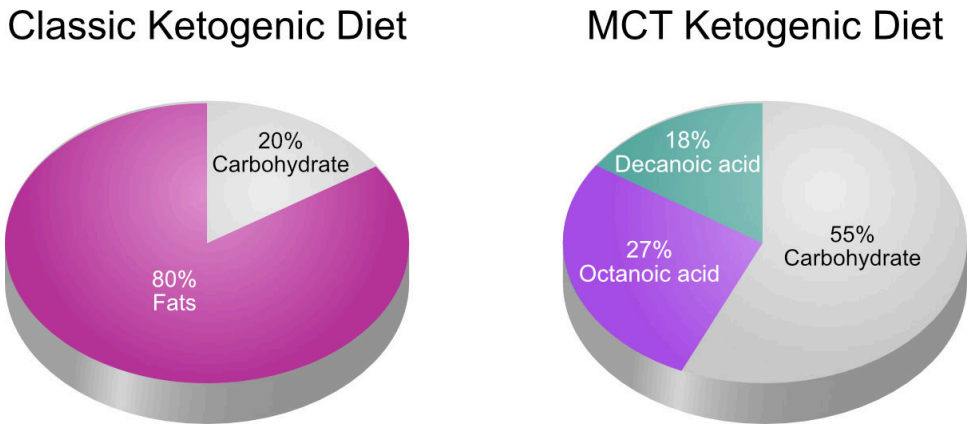



*Figure 1.6 Relative carbohydrate:fat ratios in the classic and MCT ketogenic diets.* Fat to carbohydrate ratios are relative to caloric uptake. The MCT ketogenic diet is more flexible, and carbohydrate intake may vary provided seizure control is achieved.

Dietary triglycerides are hydrolysed in the intestines by a variety of lipases that preferentially hydrolyse medium-chain over long-chain esters. Following hydrolysis, the medium-chain fatty acids are absorbed directly through the gut wall, and transferred to the liver via the hepatic portal vein. Therefore, first-pass metabolism is higher compared to long-chain fatty acids, making medium-chain fatty acids a better choice for rapid ketogenesis (Peter R Huttenlocher, 1976; Dean, Bonser and Gent, 1989; Eaton, Bartlett and Pourfarzam, 1996). In the liver, fatty acids are broken down into the ketone bodies, acetone, β-hydroxybutyrate and acetoacetate, with some long-chain fatty acids being re-esterified with mono-acylglycerol and entering the lymphatic system. Whilst medium-chain fatty acids do not undergo re-esterification, evidence from patient plasma samples shows that significant amounts of decanoic acid and octanoic acid enter the circulation in patients receiving the MCT ketogenic diet (Haidukewych, Forsythe and Sills, 1982; figure 1.7).

Just like the classic ketogenic diet, the MCT ketogenic diet has been demonstrated in clinical trials to be very effective at reducing seizures (Sills, Forsythe and Haidukewych, 1986; Elizabeth G. Neal *et al.*, 2009). However, because of the still high fat content and restriction in carbohydrate rich foods, side effects are similar to the classic ketogenic diet and compliance can be affected. In addition, there is a risk of reduced magnesium uptake and, contrary to the classic ketogenic diet, hypervitaminosis A (Christodoulides *et al.*, 2012). Children are also likely to be affected by reduced growth and slower weight gains (Elizabeth G. Neal *et al.*, 2009). Therefore, in terms of efficacy and reduction of side effects, there is still room for improvements of ketogenic diets. Understanding their mechanism may help to provide better seizure control, optimise for potential anti-epileptogenic effects and reduce side effects.

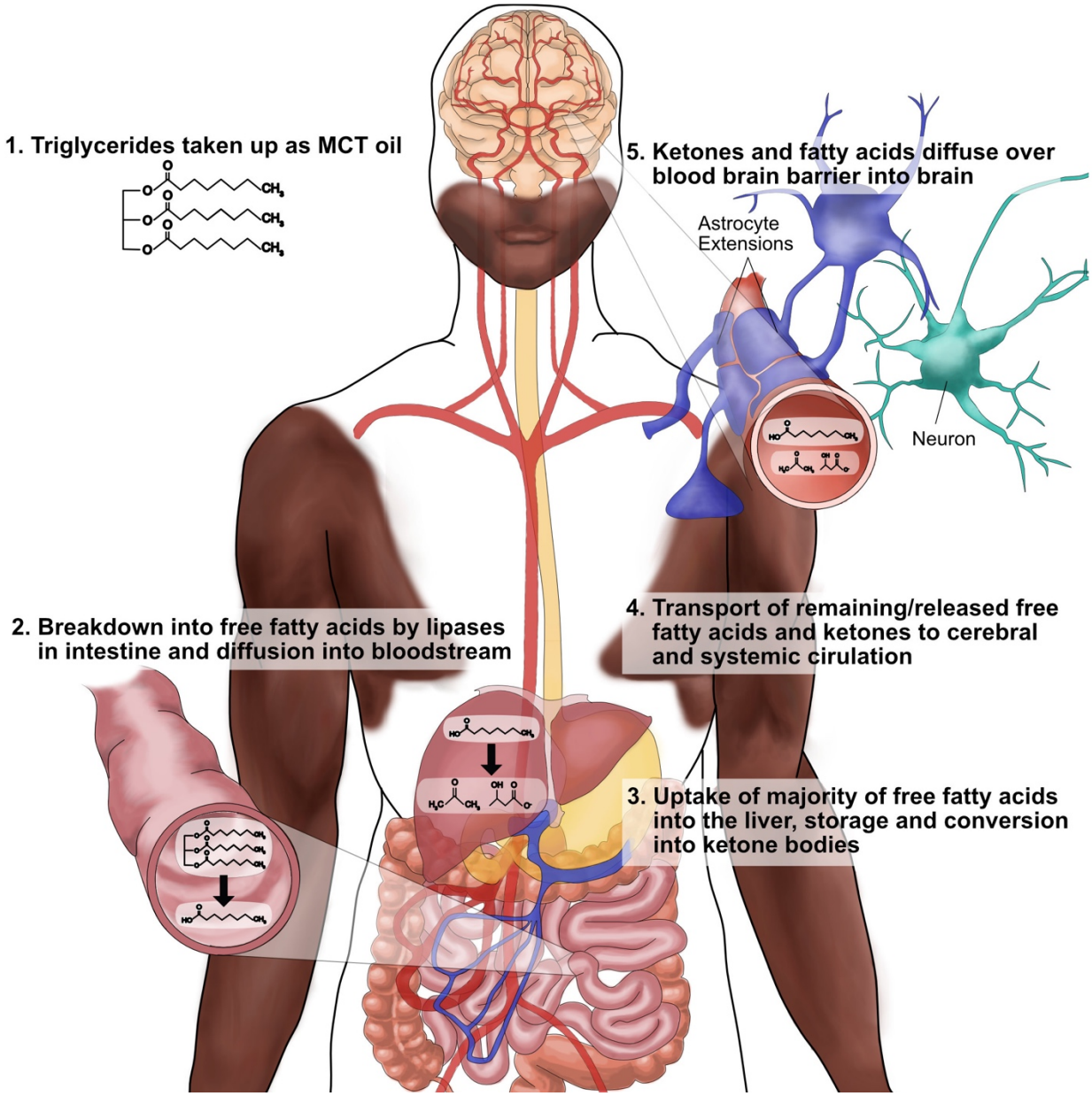



*Figure 1.7 Schematic summarising uptake, breakdown and circulation of fats upon glucose restriction.* Triglycerides are broken down into free fatty acids in the intestine, the majority of which is absorbed and transported to the liver. A proportion of free fatty acids and ketones generated from fatty acids in the liver, enter the circulation from where they can readily diffuse over the blood brain barrier to neurons and astrocytes.

# 1.5 Mechanism of action of ketogenic diets

## 1.5.1 Ketones

Today, almost a century after the development of the ketogenic diet for epilepsy, the diet is still being used successfully in children and adolescents that do not respond well to anti-epileptic drugs (Lefevre and Aronson, 2000; Freeman, Kossoff and Hartman, 2007), but how sugar reduction and ketone production help reduce seizures is not well understood. Ketones are rapidly generated from fats under carbohydrate restriction and reach plasma concentrations between 300 and 800 µM in patients (Urbain and Bertz, 2016). Since establishment of ketosis is the main measurable physiological change in ketogenic diets, determining a role for ketones in seizure control has been the primary focus of research investigating a mechanism of action for the diets.

The strongest evidence for ketones in seizure control currently exists for acetone and acetoacetate, both of which are elevated in patients receiving the ketogenic diet (Williamson *et al.*, 1962; Seymour *et al.*, 1999). Acetone has been shown to raise the threshold in acute seizure models where ictal activity was induced by PTZ and 4-aminopyridine (Gasior *et al.*, 2007), and in both acute (electroshock and PTZ) and chronic (amygdala kindling) animal models of epilepsy (Likhodii *et al.*, 2003). However, Gasior *et al.* also observed reduced motor function in their animals at anticonvulsive concentrations of acetone (Gasior *et al.*, 2007).

Nevertheless, it is possible that lower concentrations of ketones have long-lasting effects that cannot be detected in acute models of seizures. For instance, in a genetic mouse model of audiogenic seizures, which is thought to closely resemble the human condition (Kandratavicius *et al.*, 2014), beta-hydroxybutyrate and acetoacetate were both anticonvulsant (Rho *et al.*, 2002). In addition, ketones may have neuroprotective effects by enhancing mitochondrial respiration and reducing generation of reactive oxygen species, therefore making neurons more resilient to oxidative stress (Maalouf *et al.*, 2007).

Yet, evidence for ketones in seizure control is not conclusive, with experiments showing no effect of β-hydroxybutyrate or acetoacetate on PTZ-generated acute seizure models (Thio, Wong and Yamada, 2000), and thereby directly contradicting Likhodii *et al.* (2003). Other concerns relate directly to the translational, clinical aspects of seizure control by ketones. Whilst ketone generation can be demonstrated in patients that adhere to the MCT ketogenic diet (Courchesne-Loyer *et al.*, 2013), there is evidence that ketosis is only transient in the ketogenic diets. In rats that receive the ketogenic diet, blood ketone levels surge only at day 10 of the treatment and decrease permanently thereafter (Taha,

Ryan and Cunnane, 2005). Also, it has repeatedly been reported that the degree of ketosis does not correlate well with the actual seizure control, with patients that achieve high levels of ketosis not necessarily reporting effective seizure reduction (Seymour *et al.*, 1999; Sirven *et al.*, 1999; Likhodii *et al.*, 2000; Thavendiranathan *et al.*, 2000). However, recent reports suggest that monitoring of blood and breath ketone levels may correspond better to seizure control than the previous practice of assessing urine ketone levels (Gilbert, Pyzik and Freeman, 2000; Musa-Veloso *et al.*, 2006; van Delft *et al.*, 2010). Future reports on blood or breath ketone levels and seizure control will hopefully consolidate these reports.

Currently, the evidence for ketones in animal seizure models cannot be ignored and trials using ketone bodies rather than fatty acids will shed light on the realistic use of ketones for epilepsy treatment. Ketone-esters are being investigated as a means to reduce seizures without conforming to the ketogenic diets, but results are still outstanding (Hashim and VanItallie, 2014; Ciarlone et al., 2016). Nevertheless, the contradicting reports that have been published so far warrant investigating the extent to which other effects of the ketogenic diets may contribute to seizure control.

### 1.5.2 Glucose and carbohydrate restriction

Apart from increasing ketogenesis, glucose restriction is necessary for seizure control through ketogenic diets (Bough and Rho, 2007). Seizure control through glucose restriction may help explain the effect of fasting and starvation on seizures where ketosis is not maintained through fat intake. A proconvulsive effect of glucose could further explain the lack of correlation between ketone levels and seizure control. Studies assessing caloric restriction or intermittent fasting in mice report beneficial effects on susceptibility to neurotoxicity (Anson *et al.*, 2003). In addition, mice that were fed with either 15% or 30% calorie restricted diets were reported to have higher seizure thresholds than mice that were fed a classical ketogenic diet (Greene *et al.*, 2002). Other studies, however, that compared seizure control in animals fed ketogenic and standard diets, both ad libitum or restricted, showed that both ketogenic and standard diet could provide comparable levels of resistance to seizures if they were both restricted (Mantis *et al.*, 2004).

Interestingly, substitution of glucose with 2-deoxy-D-glucose, a glucose analogue that partially inhibits glycolysis and is not metabolised, reduces seizure susceptibility in the electroshock seizure model (Barton *et al.*, 2001), and has antiepileptogenic effects in progressively kindled rats (Garriga-Canut *et al.*, 2006). These data suggest that inhibition of glycolysis may provide some degree of seizure

protection, but due to the limited number of studies published so far, consensus is still outstanding (Bough and Rho, 2007).

### 1.5.3 Fatty acids

Interestingly, few studies have been carried out to investigate an effect of the fatty acids themselves on seizure control despite the fact that fatty acids readily cross the blood brain barrier (Kuge *et al.*, 1995). The fact that fatty acids do not rely on transporters which have been shown to be linked to drug-resistance may even explain their activity in pharmacoresistant epilepsy. Reports that describe modulation of glutamate receptor properties by free fatty acids exist, but their potential contribution to seizure prevention has not been studied (Voskuyl et al., 1998; Hamilton and Brunaldi Kellen, 2007). Wilding et al. (2010) have shown that *cis*-unsaturated fatty acids modulate kainate receptor gating, and arachidonic acid has been shown to reduce AMPA receptor generated currents in brain slices (Kovalchuk et al., 1994). Modulation of excitatory signalling by unsaturated fatty acids may therefore provide a mechanism in seizure control.

When assessing the suitability of unsaturated long-chain fatty acids in the context of the ketogenic diet, some promise was seen initially in polyunsaturated fatty acids (PUFAs) for seizure control after Fraser *et al.* (2003) found a correlation between elevated PUFA serum concentrations and improved seizure control in children on the ketogenic diet. This offered a novel angle to explain the poor correlation of ketones and seizure control by focusing on fatty acids themselves as mediators of reduced neuroexcitability. This observation was made after it had been suggested in animal studies that PUFAs had neuroprotective effects on $K^+$-channels, as well as sodium and calcium currents (Vreugdenhil and Wadman, 1999; Lauritzen *et al.*, 2000). However, more recently, studies investigating seizure protection in acute animal seizure models found no benefits of PUFAs (Taha *et al.*, 2006), and a pilot study that tested PUFA supplementation in adults with uncontrolled epilepsy reported that compared to a mineral oil placebo, PUFAs increased seizure occurrence, with no evidence for beneficial effects (Bromfield *et al.*, 2008). It is not clear what causes this discrepancy between animal studies and human effects, but current human data does not support a role for PUFAs in seizure control.

Data for medium-chain fatty acids supplied by the MCT ketogenic diet are more supportive of a function of fatty acids in seizure control. When comparing the effect that medium and long-chain fats have on cortical spreading depression, a mechanism that has been proposed to result in reduced synaptic input and therefore antiepileptogenic effects, medium-chain triglyceride feeding significantly reduced cortical spreading depressions. Long-chain triglycerides did not show this effect (de Almeida

Rabello Oliveira *et al.*, 2008). It is not clear if this effect is mediated directly by fatty acids or ketogenesis. However, blood plasma concentrations of decanoic and octanoic acid are elevated in patients taking an MCT oil preparation (Haidukewych, Forsythe and Sills, 1982). After taking 520 ml of the MCT oil, patients had average blood plasma concentrations of 157µM decanoic acid and 306µM octanoic acid (Haidukewych, Forsythe and Sills, 1982). No data is available for decanoic acid levels in patient brains, however animal experiments show that decanoic acid is being taken up into rodent brains rapidly and efficiently after feeding, reaching cerebral concentrations of more than 50% of plasma concentrations (Kuge *et al.*, 1995; Wlaź *et al.*, 2015). In addition, decanoic acid administration has recently been shown to decrease seizure-like activity in kindled animal models of epilepsy suggesting a beneficial role in chronic seizures (Wlaź *et al.*, 2015; Tan *et al.*, 2016).

In addition to direct effects on seizure control, decanoic acid may provide neuroprotection via indirect effects on mitochondrial respiration. Decanoic acid is known to activate PPARγ, which in turn is known to increase mitochondrial mass and function (Miglio *et al.*, 2009; Malapaka *et al.*, 2012). Experiments assessing citrate synthase activity as a marker of mitochondrial function in cultured neurons showed that following decanoic acid treatment, citrate synthase activity and mitochondrial mass were significantly increased as a result of PPARγ activation (Hughes *et al.*, 2014). Increased ATP availability through enhanced mitochondrial proliferation is neuroprotective and has been suggested to contribute to seizure resistance (Bough and Rho, 2007), whereas reduced mitochondrial mass and function have been shown in animal seizure models and post mortem patient brain examination (Kunz *et al.*, 2000; Cock, 2002).

Data for octanoic acid, the medium-chain fatty acid supplied in the greatest abundance in the MCT ketogenic diet, in seizure control is not as clear, with initial experiments suggesting a potential effect on adenosine receptors that could lead to significantly increased seizure thresholds (Socała *et al.*, 2015). It is possible that such beneficial effects of octanoic acid via adenosine receptor regulation are mediated by ketones rather than octanoic acid, as the study by Tan *et al.* (2016) reported no beneficial effects of octanoic acid on seizures if animals received the fatty acids without glucose restriction, implying that ketone generation would be limited in this case. Interestingly, though, experiments assessing branched derivatives of octanoic acid in PTZ-slice models of acute seizures report efficient reduction of ictal activity upon perfusion with compounds that contained side chains at the second or fourth C-atom of eight carbon-chain compounds (Chang *et al.*, 2013, 2015). These data suggest an immediate, direct effect of medium-chain fatty acids on neuroexcitatory signalling in addition to potential beneficial effects of ketones or glucose restriction.

This thesis will therefore test the hypothesis role that decanoic acid contributes to seizure control through inhibitory effects on AMPA receptors expressed in *Xenopus laevis* oocytes. In addition, major aims of the thesis include the investigation of a role for branched derivatives of medium-chain fatty acids to investigate a case for drug development based on AMPA receptor inhibition by decanoic acid. Since AMPA receptor inhibition is already used as a means of seizure control through the AED perampanel, this thesis will further investigate pharmacodynamic interactions between perampanel and decanoic acid to assess whether interactions between the AED and the MCT ketogenic diet ought to be reflected in recommendations and guidelines for practitioners and patients.

# CHAPTER 2 – MATERIALS AND METHODS

# 2.1 TEVC Electrophysiology

## 2.1.1 Solution recipes

10X Ringer's TEVC perfusion solution

| | |
|---|---|
| 960mM NaCl (Sigma #S7653) | 56.1g |
| 2mM KCl (Sigma #P9333) | 1.49g |
| 5mM HEPES (Sigma #H3375) | 11.9g |
| $ddH_2O$ | Up to 1L total volume |

1X Ringer's TEVC perfusion solution

100ml of 10X Ringer's solution was made up to 1L final volume with $ddH_2O$ and 1ml $MgCl_2$ (final concentration 1mM; Sigma #63069) and 1.8ml $CaCl_2$ (final concentration 1.8mM; Sigma #21115), and the pH was adjusted to 7.5 with 4M NaOH (Sigma #71687).

10X Barth's solution

| | |
|---|---|
| NaCl | 51.5g |
| KCl | 0.75g |
| $NaHCO_3$ (Sigma # S5761) | 2.02g |
| TRIZMA HCl (Sigma #T3253 ) | 23.7g |
| $ddH_2O$ | Up to 1L total volume |

1X Barth's solution

100ml of 10X Ringer's solution were made up to 1L final volume with $ddH_2O$ and 0.82ml $MgCl_2$ (final concentration 820µM; Sigma #63069) and 0.77ml $CaCl_2$ (final concentration 770µM; Sigma #21115), 5ml Penicillin/Streptomycin (10mg/ml; Thermo Fisher Scientific #15140122) and 5ml tetracyclin (10mg/ml; Sigma #T7660). The pH was adjusted to 7.4 with 4M NaOH.

DEPC water

1ml of diethylpyrocarbonate (Sigma # D5758) was dissolved in 1L of $ddH_2O$. The solution was sterilised by autoclaving.

5 X MOPS buffer

| | |
|---|---|
| MOPS acid (Sigma #M3183) | 20.6g |
| 50mM Na-Acetate (Sigma, #71183) in DEPC water | 800ml |
| 0.5M EDTA (#E5134) pH 8.0 in DEPC water | 10ml |

The pH of the solution was adjusted to 7.0 with 2M NaOH (Sigma #S8045) and made up to 1L with DEPC-treated water.

### 2.1.2 RNA synthesis from cDNA

The AMPA receptor cDNAs and mutant constructs were a generous gift from Prof Ralf Schoepfer (NPP, UCL) and Prof Yael Stern-Bach (Hebrew University of Jerusalem). All cDNAs and plasmids provided by collaborators are summarized in table 2.1. All centrifugation steps during RNA synthesis were done at 13,000rpm.

| cDNA | Plasmid | Promoter | Restriction enzyme used for plasmid linearisation | Source |
|---|---|---|---|---|
| **GluA1 (rat, flip isoform)** | pSP6T | SP6 | MluI | Prof Ralf Schoepfer (NPP, UCL) |
| **GluA2 (rat, flip isoform)** | | | | |
| **GluA3 (rat, flip isoform)** | | | | |
| **GluN1a (rat)** | | | | |
| **GluN2a (rat)** | | | | |
| **GluK2 (rat)** | pGEM-HE | T7 | NheI | Prof. Yael Stern-Bach (Hebrew University, Jerusalem) |
| **GluA3 (rat, flip isoform)** | | | | |
| **GluA3 (rat, flip isoform, R3(D543-E548, T810-I888))** | | | | |

*Table 2.1 Summary of plasmids used for in vitro synthesised RNA expression in Xenopus laevis oocytes.*

### *2.1.2.1 Plasmid linerisation and purification*

cDNA was linearised by adding 10µg cDNA, 10µl 10x enzyme buffer (Buffer H), and 30 units restriction enzyme (Mlu I, Sigma #MLUI-RO Roche) to a total of 100µl solution in DEPC-treated water, and incubating at 37˚C for 2 hours. Successful linearisation was assessed by running 1µl of sample on a 1% agarose gel in 1 x TAE buffer. The remainder was precipitated by adding 80µl of the organic layer of phenol:chloroform:isoamyl alcohol (25:24:1, VWR #101174-118) prior to vortexing and centrifuging for 2 minutes. Subsequently, 80µl chloroform (VWR #22706.326) was added to the supernatant, and the solution was centrifuged for 2 min. To 100µl of the supernatant, 300µl 100% ethanol (VWR # 20821.330), and 40µl 3M Na-Acetate (pH 4.0) was added prior to precipitation overnight at -20˚C. The next morning, cDNA was pelleted through centrifugation for 20 minutes at 13,000 rpm. The pellet was washed by centrifuging for 20 minutes with 50µl 70% ethanol, subsequently dried and resuspended in 8.5µl DEPC-treated water.

### *2.1.2.2 RNA synthesis*

RNA synthesis was carried out using the RiboMAX$^{TM}$ Large Scale RNA Production System SP6 or T7 (see table 2.1, Promega #P1280 and #P1300, respectively) according to the manufacturer's

instructions assuming that 5µg linearised DNA was obtained. For each reaction, to the 8.5µl DEPC-treated water with resuspended DNA, 4.0µl rNTPs, 1.5µl 10mM Ribo $m^7G$ Cap analogue (Promega #P1711), 4.0µl SP6 or T7 Transcription Buffer (5X) and 2.0µl SP6 or T7 polymerase were added and incubated at 37˚C for 20 min. Successful RNA synthesis was assessed by running 1µl of reaction sample on a 1% agarose DNA gel alongside a molecular weight marker (Bioline # BIO-33053).

#### *2.1.2.3 cRNA purification*

cRNA was precipitated by adding 480µl DEPC-treated water, 100µl 3M Na-Acetate (pH 4.0), 250µl water-saturated phenol (pH 4.0), and 50µl chloroform to the reaction mix and centrifuging for 2 minutes. After centrifugation, an equal volume of isopropanol (ca. 700-750µl depending on success of recovery, Fisher Scientific #P/7500/17) was added to the supernatant, and the mixture was vortexed and precipitated at -20˚C overnight. The next morning, RNA was pelleted by centrifuging for 20 minutes, and the pellet was subsequently resuspended in 200µl DEPC-treated water, 60µl 5M $NH_4$Acetate (Sigma # A1542) and 520µl 100% ethanol. RNA was precipitated at -20˚C for 20 minutes prior to pelleting by centrifugation for 20 minutes. Subsequently, RNA was washed by centrifuging for 20 minutes with 50µl 70% ethanol. Depending on pellet size, RNA was resuspended in 15-50 µl nuclease-free water (provided in kit) and kept in aliquots of 5µl at -80˚C.

### 2.1.3 RNA gels and quantification

#### *2.1.3.1 RNA denaturing agarose gel*

For 1% RNA agarose gels, 0.3g agarose (Bioline #BIO-41025) was heated in 18.5ml DEPC-treated water until fully dissolved, and 6ml 1X MOPS buffer and 5.5ml 37% formaldehyde solution (Sigma # F8775) were added (total volume 30ml) before the solution was poured into a sterile gel electrophoresis tank (BioRad) and allowed to cool.

#### *2.1.3.2 RNA gel electrophoresis*

1µl RNA sample added to 1µl 2X RNA gel loading dye (Fermentas #R0641) and 4µl RNA marker (Fermentas #SM0428) were heated to 65˚C for 3 min prior to loading into RNA gel wells. Samples were run at 90V for 30 min and visualised using the GeneFlash Bio Imaging Gel Documentation System (Syngene). To create heterologous AMPA receptor RNA samples, equal quantities of RNA for each subunit were mixed in a nominal ratio of 1:1 based on band fluorescent intensities from the RNA gels.

## 2.1.4 Injection and expression of AMPA receptor subunits in *Xenopus* oocytes

### *2.1.4.1 Oocyte preparation and injection*

*Xenopus laevis* oocytes were purchased from the European Xenopus Resource Centre, University of Portsmouth. Stage V to VI oocytes were manually separated into small ovary clumps and incubated with modified Barth's solution (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgCl_2$ 0.82, $CaCl_2$ 0.77, Tris-Cl 15, adjusted to pH 7.4 with NaOH (Sigma-Aldrich, # 71687), supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (Thermo Fisher Scientific, #15140122) and 50 µg/ml tetracycline (Sigma, #T7660) with 1% collagenase type 1A (Sigma #C9891) for 30 min at room temperature under gentle shaking. Oocytes were subsequently defollicated manually and injected with either homomeric GluA1 or GluA3 cRNA or GluA1/2 and GluA2/3 cRNA using an automated Drummond Nanoinject II injector (Broomall, PA). Approximately 5ng cRNA was injected per oocyte. Oocytes were incubated at 16˚C in modified Barth's solution for 72 hours.

### *2.1.4.2 Electrophysiological recordings from Xenopus laevis oocytes*

Two electrode voltage clamp (TEVC) recordings were performed at room temperature. One oocyte was placed in a recording chamber and perfused with Ringer's solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, with pH adjusted to 7.5). Current and voltage electrodes were made from borosilicate glass rods (GC150TF-7.5, Harvard Apparatus, Kent, UK) using a PC-10 electrode puller (Narashige Instruments, Japan) and filled with 300mM KCl. Electrode resistance was optimized to 0.5-2.0MΩ. Oocytes were voltage-clamped at a holding voltage of -50mV unless stated otherwise using a Turbo TEC-03 amplifier (npi electronics, Tamm, Germany). If different holding voltages were used within one experiment, control recordings to determine maximum response to glutamate only were carried out for each holding voltage and readings were normalized to control.

Stock concentrations of antagonists and fatty acids (table 2.2) were dissolved in DMSO, L-glutamate (Sigma #G5889), kainic acid (hello bio # HB0355) or cyclothiazide (CTZ, Insight Biotechnology # sc-202560A) in distilled water and diluted to the desired final concentration in perfusion solution (1X Ringer's). This amounted to a maximum DMSO concentration of 0.1%. For recordings, perfusion solution and chemicals were applied under gravity flow by using a multi-valve perfusion system (ALA Scientific Instruments, Farmingdale, NY). The bath solutions were perfused at a rate of ca. 10 ml/min (figure 2.1). Recordings were filtered at 10Hz and digitized at 100Hz (Digidata 1322A, Molecular Devices, Sunnyvale, CA) and data acquisition was performed using WinEDR v3.0.6 (John Dempster, University of Strathclyde, UK). Figure 2.1 illustrates TEVC and perfusion setup.

| Antagonist | Company/Distributor | Catalogue Number |
|---|---|---|
| Decanoic acid | Alfa Aesar | #A14788 |
| Octanoic acid | Alfa Aesar | #A11149 |
| Nonanoic acid | Alfa Aesar | #B21568 |
| Undecanoic acid | Alfa Aesar | #A11244 |
| Dodecanoic acid | Alfa Aesar | #A11672 |
| Heptanoic acid | Avocado Research | #17704 |
| 4-Ethyloctanoic acid | Sigma | #W380008 |
| 4-Butylcyclohexane carboxylic acid | TCI | #B1136 |
| 2-(4-pentylcyclohexyl)-carboxylic acid | Ukrorgsyntez, Ukraine | Custom-synthesis |
| 6-butylpiridine-3-carboxylic acid hydrochloride | | |
| 5-butylpiperidine-2-carboxylic acid hydrochloride | | |
| 4-[(ethylamino)methyl]cyclohexane-1-carboxylic acid hydrochloride | | |
| 4-(ethoxymethyl)cyclohexane-1-carboxylic acid | | |
| 4-propoxycyclohexane-1-carboxylic acid | | |
| 2-(4-propoxycyclohexyl)acetic acid | | |
| 2-[4-(propoxymethyl)cyclohexyl]acetic acid | | |
| 4-(3-methylbutyl)cyclohexane-1-carboxylic acid | | |
| 4-(2-methylbutyl)cyclohexane-1-carboxylic acid | | |
| 2[4-(4-methylpentyl)cyclohexyl]acetic acid | | |
| 2-[4-(3-methylpentyl)cyclohexyl]acetic acid | | |
| 2-[4-(2-methylpentyl)cyclohexyl]acetic acid | | |
| 4-(but-1-en-1-yl)cyclohexane-1-carboxylic acid | | |
| 2-(4-pentylidenecyclohexyl)acetic acid | | |
| Spermine | Sigma | #S3256 |
| GYKI 53655 | Abcam | #ab120490 |
| Perampanel | Apexmol Technology Co., Ltd | CasNo：380917-97-5 |

*Table 2.2 Summary of medium-chain fatty acids, analogues and AMPA receptor antagonists used.*

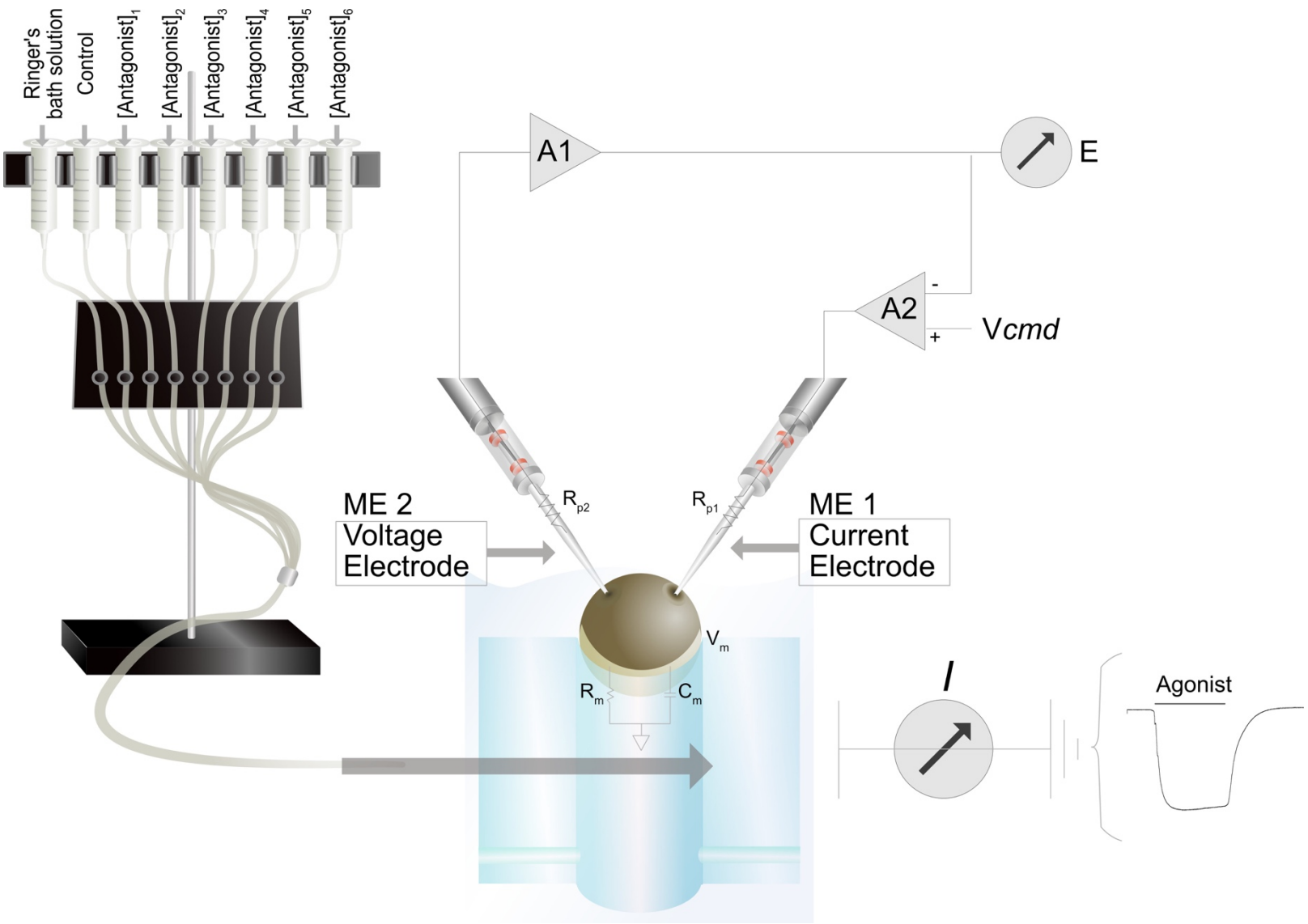



*Figure 2.1 TEVC setup showing the perfusion system and TEVC circuit*. ME = measuring electrode; A = amplifier; E = Voltage; Vcmd = Voltage input; Rp = pipette resistance; Vm = membrane voltage; Rm = membrane resistance; and Cm = membrane capacitance.

### *2.1.4.3 Data analysis for electrophysiological recordings*

The number of repeats was defined as 2 biological with 6 experimental repeats each for standard dose-response assays, unless data sets were repeated in different experimental setups / controls and pooled. For mutant and screening data where only a rough understanding of response was required or expression was instable, repeats were limited to 3-6. Data points were measured as current in nA at t = 30sec/dose. Data generated from the electrophysiology experiments were analysed using GraphPad Prism software (GraphPad software, San Diego CA, USA). All data were normalized to control or control + synergist readings and were presented as mean ± standard error of the mean (SEM) and concentrations were transformed to a logarithmic scale. Dose-response curves were fitted using a sigmoidal standard curve for recordings with <5 readings (Y=100/(1+10^(X-LogIC50))), or a variable slope model for recordings with 5+ data points (Y=100/(1+10^((LogIC50-X)*HillSlope)))). Statistical analysis was performed using ANOVA with Dunnett's or Tukey post hoc tests for comparing 3 or more data sets, or paired or un-paired student's *t*-test for direct comparison of two data sets. Differences were considered as significant at $p < 0.05$.

# 2.2 Cell culture

## 2.2.1 Solutions and equipment

Dissociation media (5ml total volume)

- 4.5ml Dulbecco's Modified Eagle's Medium (Sigma #D5796)
- 50µl DNase I (100µg/ml final concentration, Roche # 10104159001)
- 0.5ml Trypsin (Thermo Fisher #12604021)
- 2ml Penicillin/streptomycin (Sigma #P4333)
- 2ml Glutamax (Thermo Fisher Scientific #35050061)

Plating media (50ml total volume)

- 50ml Dulbecco's Modified Eagle's Medium (Sigma #D5796)
- 2.5ml foetal bovine serum (10%; Gibco #10500-064)
- 0.5ml penicillin-streptomycin (100 units/ml penicillin final concentration; 100µg/ml streptomycin final concentration; Gibco #15140122)
- 0.5ml L-Glutamine (2mM final concentration; Gibco #A2916801)

Neurobasal media (50ml total volume)

- 48ml Neurobasal media (Thermo Fisher #21103049)
- 1ml B27 supplement (Thermo Fisher #17504044)
- 0.5ml penicillin-streptomycin (100 units/ml penicillin final concentration; 100µg/ml streptomycin final concentration; Gibco #15140122)
- 0.5ml Glutamax (2mM final concentration; Thermo Fisher #35050061)

SDS-loading dye

- 0.8ml 2M Tris, pH 6.8 (Sigma #TRIS-RO)
- 3ml 80% glycerol (Sigma #G5516)
- 5ml 10% Sodium dodecyl sulfate (SDS, Sigma #L3771)
- 1.25ml β mercaptoethanol (Sigma #M6250)

PBS and PBS-T

| | |
|---|---|
| $Na_2HPO_4$ | 14.4g |
| NaCl | 8.0g |
| KCl | 0.2g |
| $KH_2PO_4$ | 0.24g |
| $ddH_2O$ | Up to 1L total volume |

The pH of the solution was adjusted with HCl to 7.4. For PBS-T, 1ml of Tween20 (Sigma #8.22184) was dissolved in every litre of 1X PBS.

## 2.2.2 Primary hippocampal neurons

### *2.2.2.1 6-well and 24-well plate preparation*

Tissue plates were prepared by Dr Simona Ursu.

Coverslips (VWR #631-0149) were cleaned in acetone overnight and subsequently rinsed four times for 30 min with ethanol. Coverslips were then placed into the 24-well plates and left to dry at least for 2 hours in the hood, followed by UV-treatment for 20 min and coating with 0.5ml of Poly-D-Lysine (PLD, Sigma # P7886). PLD was prepared to 10mg/ml and stored at -20˚C. For coating, PLD was diluted to a final concentration of 100µg/ml in autoclaved sterile Borate buffer (1.24g Boric acid/1.9g Borax/400ml water pH 8.5) and applied either to coverslips or directly to the wells for more than 5 hours at 37˚C or overnight at 4˚C. After this, coverslips or wells were washed three times with autoclaved distilled water and left to dry in the incubator if used the same day, or in the fridge if used at a later stage.

#### *2.2.2.2 Primary neuron extraction*

Primary neurons were extracted by Dr Simona Ursu.

All animal experiments were in accordance with the Home Office regulations in appliance with the Animals Scientific Act 1986. Female Sprague-Drawley rats were housed individually and mated at the age of 4-5 months. At day 18 of gestation, rats were culled by concussion and embryos were removed immediately and placed into ice-cold Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, UK). The cerebrum was gently extracted from the cranium manually and the hippocampi were dissected from the meninges and stored in ice-cold HBSS until all embryos were dissected. Once all hippocampi were collected, HBSS was removed and replaced with pre-warmed (37˚C) filter-sterilised Dissociation media. Dissociation of the cells was aided by gentle trituration using a pipette until a homogenous cell suspension was attained. Trypsination was terminated by addition of Fetal Bovine Serum (FBS, Invitrogen Life Technologies #10082-147), and neurons were kept at 37˚C for another 5 min prior to centrifugation of the suspension at 1500 rpm for 3 min. The supernatant was discarded and cells were resuspended in pre-warmed (37˚C) DMEM. The cell number was estimated by counting in a haemocytometer and cells were plated at a density of 500.000 cells/well on 6-well plates and 80.000-100.000 cells/well on 24-well plates. Neurons were left in DMEM overnight at 37˚C and 5% $CO_2$.

#### *2.2.2.3 Primary neuron cell culture*

Primary neuronal cell culture was maintained by Dr Simona Ursu.

In the morning following the initial plating of the primary neurons, DMEM was removed by aspiration and replaced with 0.5ml/well and 2ml/well for 24-well plates and 6-well plates, respectively, of filter-sterilised and to 37˚C pre-warmed modified neurobasal medium. Neurons were left to mature for 14 days during which media were topped up once per week with the described modified neurobasal medium using 0.1ml/well for 24-well plates and 0.5ml/well for 6-well plates.

### 2.2.3 Seizure models and protein extraction

#### *2.2.3.1 Seizure termination model*

For the assessment of medium-term effects of seizures and treatment on AMPA receptor expression levels by western blot, 14 *DIV* primary hippocampal neurons from 6-well plates were treated with decanoic acid following PTZ insult. For this, and to allow for accurate dosage, neurobasal media were removed from up to 3 wells at a time and collected in two falcon tubes. For seizure groups, PTZ

(1M stock solution) was diluted in the collected media in one of these tubes to a final concentration of 5mM and 1ml of the modified media was re-applied to the wells and incubated at 37˚C and 5% $CO_2$. For control groups, media were removed as described and re-applied without added PTZ to mimic any potential stress due to media aspiration alone and optimize media volume for subsequent addition of treatments. After 20 min, seizures were terminated by aspiration of the PTZ-enriched media and replacement with the remaining pre-warmed media for PTZ controls or with added decanoic acid (1M stock concentration) to a final concentration of either 0.2mM or 0.3mM for treatment groups and incubated for 24 hours at 37˚C and 5% $CO_2$ (figure 2.2 A).

#### *2.2.3.2 Acute treatment model*

For the assessment of short-term effects of AED treatment on GluA1 localisation, an acute treatment model was used in which short-term application of AMPA receptor antagonists preceded seizure induction. For this, the neurobasal media from 14 *DIV* primary hippocampal neurons from 24-well plates were removed from up to 6 wells at a time and collected in a falcon tube. 500µl/well were re-applied to ensure accurate and even volume distribution throughout all wells. For intervention groups, either decanoic acid from 100mM stocks in DMSO or perampanel from 10mM stocks in DMSO was added to a final concentration of 0.2mM and 0.3mM or 0.2µM, 0.5µM and 1.0µM, respectively. This amounted to a maximum DMSO concentration of 0.1%. Untreated and intervention groups were incubated at 37˚C and 5% $CO_2$. After 30 min, PTZ was added to the media from 1M stock solution in DMSO to a final concentration of 5mM and neurons were returned to the incubator. Seizure-like activity was terminated after 20 min by aspirating the media and neurons were washed with ice-cold PBS (figure 2.2 B).

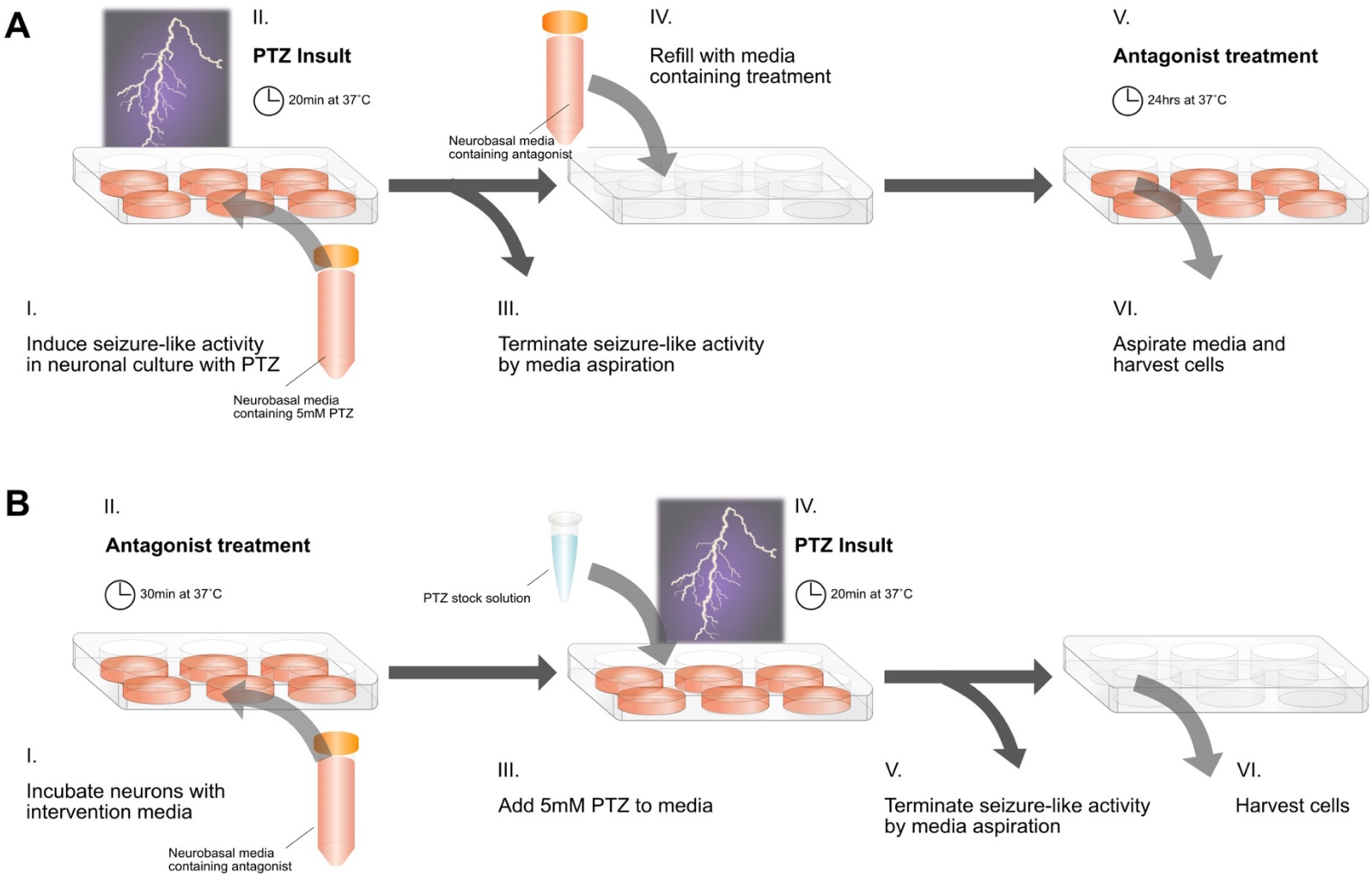



*Figure 2.2 Schematic showing treatment protocols of rat primary hippocampal neurons at 14 DIV.* (A) Seizure termination model used for quantitative analysis of AMPA receptor subunit expression by western blot. (B) Acute treatment model used for localisation analysis of GluA1 by immunofluorescent antibody staining.

### *2.2.3.3 Protein extraction and western blot*

Protein from primary neurons was extracted in ice-cold radio immunoprecipitation assay (RIPA) buffer (Sigma #R0278) with added 0.1% n-octyl-beta-D-glucopyranoside (Apollo Scientific #BIMB1075), phosphatase, and protease inhibitors (Roche #04906845001and #04693159001) and pelleted at 3000rpm for 5min. Supernatants were boiled at 95˚C for 10 min with SDS-loading dye and stored until subsequent use at -20˚C. Protein and pre-stained protein ladders (Fermentas #26619) were loaded into a 12.5% acrylamide/bisacrylamide (Sigma #A3699) gel and separated by SDS-PAGE under 100V for 1 hour. Protein was subsequently transferred to a PVDF membrane (Merck Millipore #IPFL00010) at 200mA for 1 hour. Membranes were blocked in 5% BSA V (Merck Millipore #112018) in PBS-T at room temperature for 1 hour and GluA1, GluA2 or GluA3 antibody as well as β-tubulin (table 2.3) were added directly to the blocking buffer at a final dilution of 1:1000 and incubated at 4˚C overnight. Membranes were washed 3 times for 5 minutes with PBS-T and incubated with secondary antibody (table 2.4) in PBS-T with 5% BSA at a final concentration of 1:10000 for 1 hour at room temperature followed by 3 more washes (5 minutes each). Protein bands were visualized using the Odyssey Sa system (LiCor). AMPA receptor subunit intensity was normalized to loading control and plotted using GraphPad Prism software (GraphPad software, San Diego CA, USA).

| Antibody | Species | Catalogue Number | Clone |
|---|---|---|---|
| **Anti-GluA1** | Rabbit mAb | NEB #13185 | D4N9V |
| **Anti-GluA2** | Rabbit, mAb | NEB #13607S | E1L8U |
| **Anti-GluA3** | Rabbit mAb | NEB # 4676S | D47E3 |
| **Anti-synapsin** | Mouse, mAb | Insight Biotech # sc-376623 | 4F6 |
| **Anti-ß tubulin** | Rabbit, mAb | NEB # 2128S | 9F3 |

*Table 2.3 Summary of primary antibodies.*

| Antibody | Species | Excitation wavelength | Catalogue number |
|---|---|---|---|
| **Anti-rabbit IgG (H+L)** | Goat | 555 | NEB #4413S |
| **Anti-rabbit IgG (H+L)** | Goat | 488 | NEB # 4412S |
| **Anti-mouse IgG** | Goat | 350 | Life Technologies #A-11045 |

*Table 2.4 Summary of secondary antibodies.*

### 2.2.4 Immunofluorescence

Immunofluorescent staining was carried out by Dr Simona Ursu and the antibody staining protocol was modified from the protocol published by Li *et al.* (2016).

Neurons in 24-well plates were blocked for 10 min at room temperature with 10% FBS in PBS. Rabbit anti-GluA1 recognising extracellular epitope was added directly in a 1:200 ratio and incubated for 30 min at 37˚C. Following one wash with 1X PBS, neurons were fixed with 4% PFA (Sigma #P6148) in PBS for 10 min at room temperature and subsequently washed 3 times with PBS for 5 min. The secondary antibody (Goat anti Rabbit 555) was added at a 1:500 ratio, incubated for 1 hour at room temperature and subsequently washed 3 times with PBS. To permeabilise the membranes, 1% Triton-X (Sigma #X100) in PBS was applied for 15 min at room temperature. For antibody-staining of internal GluA1 and synapsin, coverslips were blocked with 10% FBS in PBS for 30 min at room temperature and primary antibodies (Rabbit anti-GluA1 1:200 and Mouse anti-synapsin 1:100) were added to the blocking solution at a final concentration of 1:200 and incubated at 4˚C overnight. Coverslips were washed 3x with PBS for 5 min and secondary antibodies (Goat anti Rabbit 488, Goat anti Mouse 350) were added at a final concentration of 1:500 and incubated for 1 hour at room temperature. Coverslips

were washed 3x with PBS, mounted onto imaging slides using Fluorsafe (Merck Millipore, # 345789) and dried at room temperature and protected from light for 20 min.

### 2.2.5 Imaging

To ensure that all imaging and analysis were carried out blinded, slides were coded by Dr Simona Ursu prior to processing. Neurons were imaged using oil-immersion spinning-disk confocal microscopy (Olympus IX70; UPlanSApo 100x/1.40na Oil Objective; both from Olympus, Tokyo, Japan). Between two and four neurons were visualised per treatment and biological repeat. Secondary antibodies and excitation wavelengths are summarised in table 2.4. To minimize crosstalk between channels, z-stacks were taken sequentially by channel.

### 2.2.6 Image analysis

All image analyses were carried out using ImageJ software for Windows-based systems or Macintosh (Schneider, Rasband and Eliceiri, 2012).

#### *2.2.6.1 Surface/internal GluA1 ratio*

To estimate puncta intensity 2 to 3 identically sized dendritic portions were selected per neuron using a mask. The thresholds of individual channels and stacks were set to background using the ImageJ unbiased 'Moments' algorithm. The resulting number of pixels in the dendritic selections was measured as Integrated Density (IntDen) per stack. To calculate the surface vs. internal GluA1 IntDen ratios per dendritic selection, the total IntDen of puncta within one sequence in the red channel was divided by the total IntDen of puncta in the green channel.

#### *2.2.6.2 Total GluA1 IntDen in somas*

To assess the intensity of fluorescence in the soma, the somas of each neuron were selected and the threshold of the green channel (total GluA1) was set using the 'Moments' algorithm. The number of pixels was measured in each stack and the total was normalized to total area of each stack.

### *2.2.6.3 Colocalisation analysis*

Colocalisation of membrane bound GluA1 with synapsin was assessed using JaCoP plugin in ImageJ (Bolte and Cordelières, 2006). The dendritic sections analysed for surface/total GluA1 ratios were also used for colocalisation analysis. Red (surface GluA1) and blue channels (synapsin) were selected and the threshold was set to background. Pearson's coefficient analysis was selected to calculate the colocalisation correlation *r*. Three dendritic sections were analysed per neuron image.

### *2.2.6.4 Normalisation and data compilation*

Once all data acquisition and analysis was completed within each biological repeat, the code for blinding was provided by Dr. Simona Ursu and data were allocated to the treatment groups. Measurements within the control group were averaged and each treatment and control measurement within the respective biological repeat were normalized to this mean. Data were plotted as bar graphs in Prism, with each bar representing the mean normalised measurement and SEM. Statistical analysis was carried out as in 2.1.4.3.

# 2.3 Molecular biology

## 2.3.1 Molecular biology methods

### *2.3.1.1 Bacterial transformation*

*E. coli* competent cells were kept at -80˚C at all times, and for transformation, 50µl/reaction were defrosted on ice. Subsequently, 1µl of cDNA was added to bacteria and incubated on ice for 15 min. For heat-shock transformation, bacteria were transferred to a water bath at 42˚C for 1 min, and immediately returned to ice afterwards. For recovery, 400µl LB (Sigma #L3022 Sigma) was added per reaction and bacteria were kept at 37˚C for 30 min prior to spreading on selective LB agar plates + 200µg/ml Ampicillin (Sigma # A1593). Colonies were grown overnight at 37˚C.

### *2.3.1.2 Mini-prep of cDNA*

DNA mini-preps were carried out using the GeneJET Plasmid Miniprep Kit (Thermo Scientific #K0502) and following the provided manufacturer's protocol. Briefly, single colonies from LB Amp plates were grown in a shaker overnight at 37˚C in 5mL of LB broth and harvested the next morning by centrifugation at 8000rpm (6800 × g) for 2 min. All subsequent centrifugation steps were done at >12000 × g. Supernatant was discarded and the pellets were resuspended by vortexing in 250µL Resuspension Solution. The cell suspension was transferred to microcentrifuge tubes, 250µL Lysis Solution was added and mixed by inverting the tubes before addition of 350µL Neutralization Solution. Cell debris and chromosomal DNA were pelleted by centrifugation for 5 min and the supernatants were transferred to supplied GeneJET spin columns and centrifuged for 1 min. The flow-through was discarded and 500µL Wash Solution with added ethanol was added to the spin columns and centrifuged for 30-60 seconds after which the flow-through was discarded. After repeating this step, the columns were centrifuged for an additional minute to remove any residual ethanol. The cDNA was eluted by applying 20-50µl pre-warmed (70˚C) Elution Buffer to the columns and centrifuging for 2 min. cDNA was subsequently stored at -20˚C until further use.

## 2.3.2 Site-directed point mutagenesis

To introduce the point mutations, primers were designed using the NEBaseChanger® tool (v.1.2.6, http://nebasechanger.neb.com, primers summarised in table 2.5). Mutations were introduced using the Q5® High-Fidelity DNA Polymerase kit (NEB #E0554) following the provided protocol. Briefly,

10µM each of forward and reverse primer and 1-25ng of template DNA were added to 12.5µl of Q5 Hot Start High-Fidelity 2X Master Mix and 9µl of nuclease-free water. The reaction mix was transferred to a thermocycler and the reaction was carried out using the settings summarised in table 2.6.

| Purpose | Primer name | Sequence (5'-3') |
|---|---|---|
| **Site directed mutagenesis P610A** | P610A FW | TGACATTTCCGCCAGGTCCCTGT |
| | P610A RV | CATCCTTGCTGCATGAAGGC |
| **Site directed mutagenesis V644A** | V644A FW | GGAGAGGATGGCCTCTCCCATTGAGAGTGC |
| | V644A RV | ACAGTAGGAAGGCAGCC |
| **Site directed mutagenesis P646A** | P646A FW | GATGGTGTCTGCCATTGAGAGTG |
| | P646A RV | CTCTCCACAGTCAGGAAG |
| **Confirmation of point mutation** | SDM check | CTGTGGCTCCCTTGACCATA |
| **Sequencing** | GluA1 Seq 1 | TAACCTTATGTATCATACACAT |
| | GluA1 Seq 2 | AAATAAGTTCAAGGAGAGCGGA |
| | GluA1 Seq 3 | GGACCACAGAGGAAGGCATGAT |
| | GluA1 Seq RV | CACCACACCCGCCGCGCTTAAT |

*Table 2.5 Summary of primers used for generation of point mutations in the three GluA1 mutants and for sequencing.*

| Step | Temperature (˚C) | Time |
|---|---|---|
| **Initial Denaturation** | 98 | 30 seconds |
| **25 Cycles** | 98 | 10 seconds |
| | 62, 65, 68 | 30 seconds |
| | 72 | 2 minutes 40 seconds |
| **Final Extension** | 72 | 2 minutes |
| **Hold** | 4 | ∞ |

*Table 2.6 PCR settings for site-directed mutagenesis.*

To add 3'-phosphorylation, ligate and digest precursor cDNA, 1µl of the PCR product was added to 5µl of the 2X KLD Reaction Buffer provided in the kit, 1µl of the 10X KLD Enzyme Mix including kinase, ligase and Dpn I, and 3µl nuclease-free water and incubated for 5 min at room temperature. The reaction mix was transformed into NEB 4-alpha competent *E. coli* cells as described. Six single colonies were picked from the plate and grown in 5ml LB with 2X Ampicillin overnight at 37˚C and cDNA was extracted using the GeneJET Plasmid Miniprep Kit (see section 2.3.1.2).

Successful mutagenesis was confirmed by sequencing using the SDM check primer (table 2.5; APPENDIX I for sequencing results). Positive mutants were sequenced fully using the sequencing primers (see table 2.5, APPENDIX I for full sequences) to confirm absence of untargeted point mutations and transcribed into cRNA and expressed in *Xenopus laevis* oocytes as described in section 2.1.

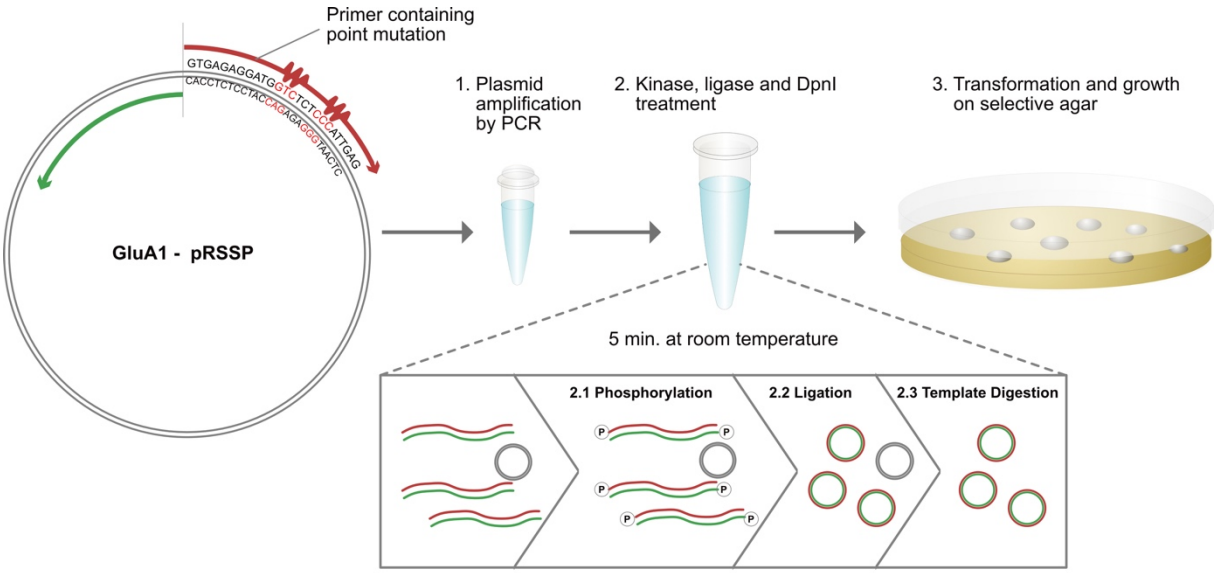



*Figure 2.3 Schematic showing primer design for mutagenesis.* Red and green arrows indicate the two mutations introduced in the M3-S2 linker regions of GluA1, and summarising the site-directed mutagenesis protocol based on NEB Q5® High-Fidelity DNA Polymerase kit protocol.

# CHAPTER 3 – INHIBITION OF AMPA RECEPTORS BY DECANOIC ACID AND OCTANOIC ACID

## 3.1 Introduction

To explain how the ketogenic diets exert their anti-seizure effects, research has focused largely on molecular targets of ketones in the brain. This approach was intuitive as ketones are rapidly generated from fats under carbohydrate restriction and have been demonstrated to be present at concentrations between 300µM and 800µM in patient plasma (Urbain and Bertz, 2016). However, clinicians have increasingly observed a poor correlation between ketone concentration and seizure protection (Haidukewych, Forsythe and Sills, 1982; Tan *et al.*, 2016). A wide range of possible neurological targets for ketones has been discussed as a mechanism for seizure control, and ketone-esters are being investigated as a means to reduce seizures without conforming to the ketogenic diets (Hashim and VanItallie, 2014; Ciarlone *et al.*, 2016). However, the discrepancy between ketone levels and seizure control raises the question whether the diet provides other molecules in addition to ketones that could be investigated for potential anti-seizure mechanisms.

Under healthy conditions, once absorbed into the bloodstream, the majority of free fatty acids in the body is directly transported into the liver where fats are either stored or converted into ketones. However, in high-fat diets, a significant amount of triglycerides and free fatty acids enter the systemic circulation and are transported to peripheral tissues and to the central nervous system (CNS). Since fatty acids diffuse across the blood brain barrier (Kuge *et al.*, 1995), they can readily enter the brain where they could influence neuronal signalling.

Despite these properties, research into modulation of neuronal signalling by fatty acids is scarce, and specifically medium-chain fatty acids have attracted little attention. This chapter therefore describes the modulation of excitatory postsynaptic signalling by medium-chain fatty acids and characterises AMPA receptor inhibition by decanoic acid and octanoic acid, the main constituents of the MCT ketogenic diet.

# 3.2 Results

## 3.2.1 AMPA receptor inhibition by decanoic acid and octanoic acid

Experiments carried out by Dr Pishan Chang in rat hippocampal slice models, in which seizure-like activity was induced with PTZ, show that decanoic acid potently inhibits excitatory post-synaptic potentials in the presence of NMDA, $GABA_A$ and $GABA_B$ receptor blockers (Chang *et al.*, 2016). This raises the question whether decanoic acid could reduce excitatory potentials through the interaction with AMPA receptors. To test this hypothesis, recombinant AMPA receptor subunits GluA1, GluA1/2 and GluA2/3 were expressed in *Xenopus laevis* oocytes. Currents were generated in the presence of glutamate and were measured using TEVC. Decanoic acid was prepared as 1M stock concentrations in DMSO and dissolved in TEVC solution (Ringer's). To control for an effect of DMSO on currents that were generated by glutamate, oocytes expressing GluA2/3 were perfused with Ringer's solution containing 1% DMSO which had only little effect on AMPA receptor generated currents (figure 3.1 A). Decanoic acid, applied at 1mM, reduced currents generated by GluA2/3 by 75.4 % (SEM = 3.8; n = 4; figure 1B and D). By comparison, octanoic acid reduced AMPA receptor activation by 13.7 %, which was significantly less compared to the reduction by decanoic acid (SEM = 5.19; n= 4; $p < 0.0001$ compared to 1mM decanoic acid; figure 1C and D). This suggests that AMPA receptor inhibition by medium-chain fatty acids depends on the carbon chain length and that efficacy is reduced in shorter chain fatty acid.

In order to more accurately compare the antagonism displayed by octanoic and decanoic acid and quantify inhibition by calculating an $IC_{50}$, full dose response assays were carried out using decanoic acid concentrations ranging from 0.001mM to 3mM, and data was plotted in a sigmoidal dose-response curve. In these experiments, decanoic acid inhibited AMPA receptors made up of subunits GluA2/3 (figure 1G) with an $IC_{50}$ of 0.52mM (SEM = 0.02; n=12). By comparison, decanoic acid inhibited GluA1/2 with an $IC_{50}$ of 1.16mM (SEM = 0.02; n=12), which was significantly weaker compared to the inhibition seen in GluA2/3 ($p = 0.0021$ for GluA1/2 compared to GluA2/3). This effect was even more pronounced in GluA1 expressing oocytes ($IC_{50}$ = 2.09mM; SEM 0.03; n=12; $p < 0.0001$). This suggests that decanoic acid inhibits AMPA receptors in a subunit-dependent manner with lower efficacy in GluA1 containing receptors.

The dose-response assay was also carried out for octanoic acid. Octanoic acid inhibited GluA2/3 with an extrapolated $IC_{50}$ of 3.82mM (SEM = 0.03; n = 10). This $IC_{50}$ is eight-fold higher than that calculated for decanoic acid, confirming significantly lower inhibitory potency of octanoic acid ($p < 0.0001$ compared to the $IC_{50}$ of decanoic acid against GluA2/3). In GluA1 homomeric AMPA

receptors, the $IC_{50}$ for octanoic acid was estimated to be similar to that shown in GluA2/3 ($IC_{50}$ = 4.82mM; SEM = 0.04, n = 5; $p$ = 0.4369 compared to GluA2/3). This confirms the initial findings obtained from single dose response assays that octanoic acid is the weaker antagonist of the two fatty acids. Since octanoic acid is found in the plasma of patients at mean concentrations of ca. 300µM (Haidukewych, Forsythe and Sills, 1982), these results indicate little significance of octanoic acid in seizure control at physiologically relevant concentrations. The experiments were therefore not repeated in GluA1/2, and subsequent assessment of AMPA receptor inhibition by medium-chain fatty acids was only carried out for decanoic acid unless stated otherwise.

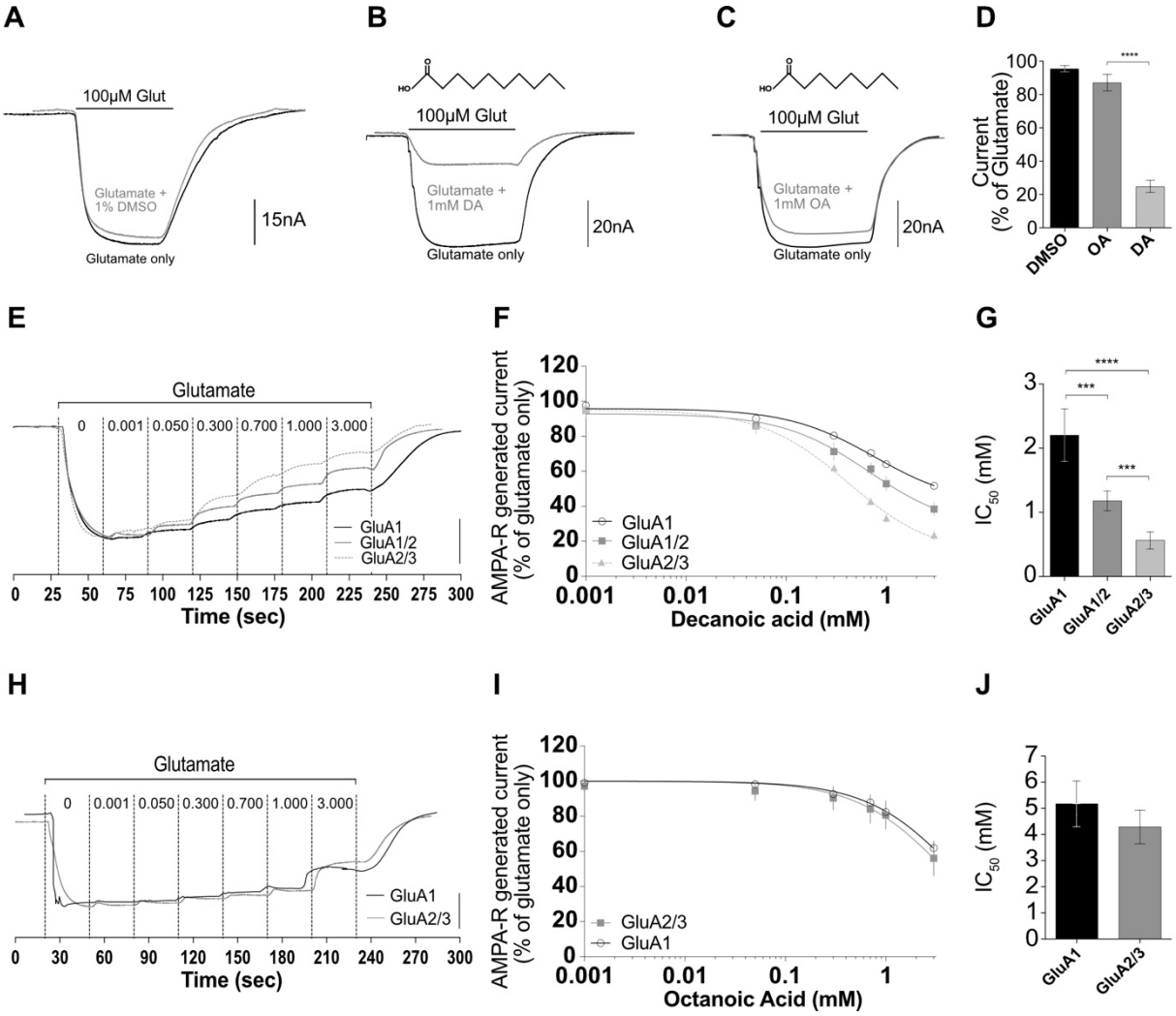



*Figure 3.1 Inhibition of AMPA receptors by the medium-chain fatty acids found in the MCT ketogenic diet.* (**A**) DMSO only control that shows small effect of DMSO on glutamate evoked AMPA receptor (GluA2/3) currents. (**B** and **C**) *Xenopus laevis* oocytes expressing recombinant AMPA receptor subunits GluA2/3 were perfused with either 100μM glutamate only (black line) or 100μM glutamate and 1mM decanoic acid (**B**) or octanoic acid (**C**, grey lines). (**D**) Bar graph shows the average current and SEM in the presence of the antagonists normalised to glutamate only of 4 recording. (**E**) Representative traces for dose-response curves of decanoic acid against GluA1 (black), GluA1/2 (grey) and GluA2/3 (grey, dotted). Scale bar corresponds to 200nA (GluA1), 250nA (GluA1/2) and 30nA (GluA2/3). (**F** and **G**) Sigmoidal dose-response curve and bar graph showing subunit specificity of decanoic acid. Each point represents the mean and SEM of 12 recording readings. (**H**) Representative trace recordings showing the dose-effect of octanoic acid against GluA1 (black) and GluA2/3 (grey). Scale bar corresponds to 200nA (GluA1) and 70nA (GluA2/3). (**I** and **J**) Sigmoidal dose-response curve and bar graph showing subunit specificity of octanoic acid. Each point represents the mean and SEM of 5 to 10 recording readings. Data were analysed using student's *t*-test in **D** and **J**, and ANOVA in **H**, where *** corresponds to $p < 0.001$, and **** corresponds to $p < 0.0001$.

### 3.2.2 Decanoic acid inhibits AMPA receptors in a non-competitive manner

Inhibition of AMPA receptors by decanoic acid may provide a mechanism through which the MCT ketogenic diet protects from seizures. To further characterise this antagonism and better evaluate a role and significance of medium-chain fatty acids in neuronal signalling, a non-competitive mode of inhibition was assessed by repeating the dose-response assay for currents evoked by a saturating concentration of glutamate (1mM). In competitive inhibition, increasing antagonist concentrations would be predicted to shift the dose-response curve to the right which would be reflected in a higher $IC_{50}$. However, higher glutamate concentrations did not significantly change the $IC_{50}$ of decanoic acid for GluA2/3 ($IC_{50}$ = 0.55mM; SEM = 0.31; n=10; $p$ = 0.6402 compared to 100µM glutamate; figure 3.2 A to C) nor shift the dose-response curve to the right. This suggests that inhibition by decanoic acid is not competitive to glutamate, and therefore would be maintained during seizures.

The experiments described in this paragraph were carried out by Dr Pishan Chang (Chang *et al.*, 2016). To confirm non-competitive inhibition, an agonist dose-response assay was carried out for glutamate in the absence and in the presence of 0.3mM and 1mM decanoic acid. In these experiments, competitive inhibition would be expected to shift the dose-effect curve to the right whilst not affecting the maximal response to glutamate irrespective of antagonist concentration. Addition of 0.3mM decanoic acid had a significant effect on the $EC_{50}$ of glutamate, significantly lowering it from 0.029mM (SEM = 0.002; n = 5) to 0.015mM (SEM = 0.001; n = 5; $p$ = 0.0006). The addition of 1mM decanoic acid had a similar effect on the activation of AMPA receptors ($EC_{50}$ = 0.017mM; SEM = 0.001; n = 5; $p$ = 0.0016). Moreover, the maximal response reduced noticeably in a dose-dependent manner showing that the block by decanoic acid was maintained throughout the tested glutamate concentration range (figure 3.2 D, E and F). The consistent reduction in maximum response to glutamate despite increasing antagonist concentrations shows that high glutamate concentrations did not overcome inhibition by decanoic acid and is therefore strongly indicative of non-competitive inhibition by decanoic acid.

The initial competition assay was repeated for octanoic acid to confirm the findings for decanoic acid. Increasing the concentration of glutamate to saturating levels (1mM) had no significant effect on inhibition of GluA2/3 by octanoic acid ($IC_{50}$ = 3.691; SEM = 0.272; n = 12; $p$ = 0.8117; figure 3.2 G, H, I), which is consistent with the findings reported for decanoic acid. Since octanoic acid only weakly inhibits AMPA receptors, the reversal of the competition assay was omitted for octanoic acid, and therefore these experiments do not conclusively demonstrate non-competitive inhibition. However, due to the high structural similarity of both fatty acids, it is very likely that the two compounds share a mode of inhibition.

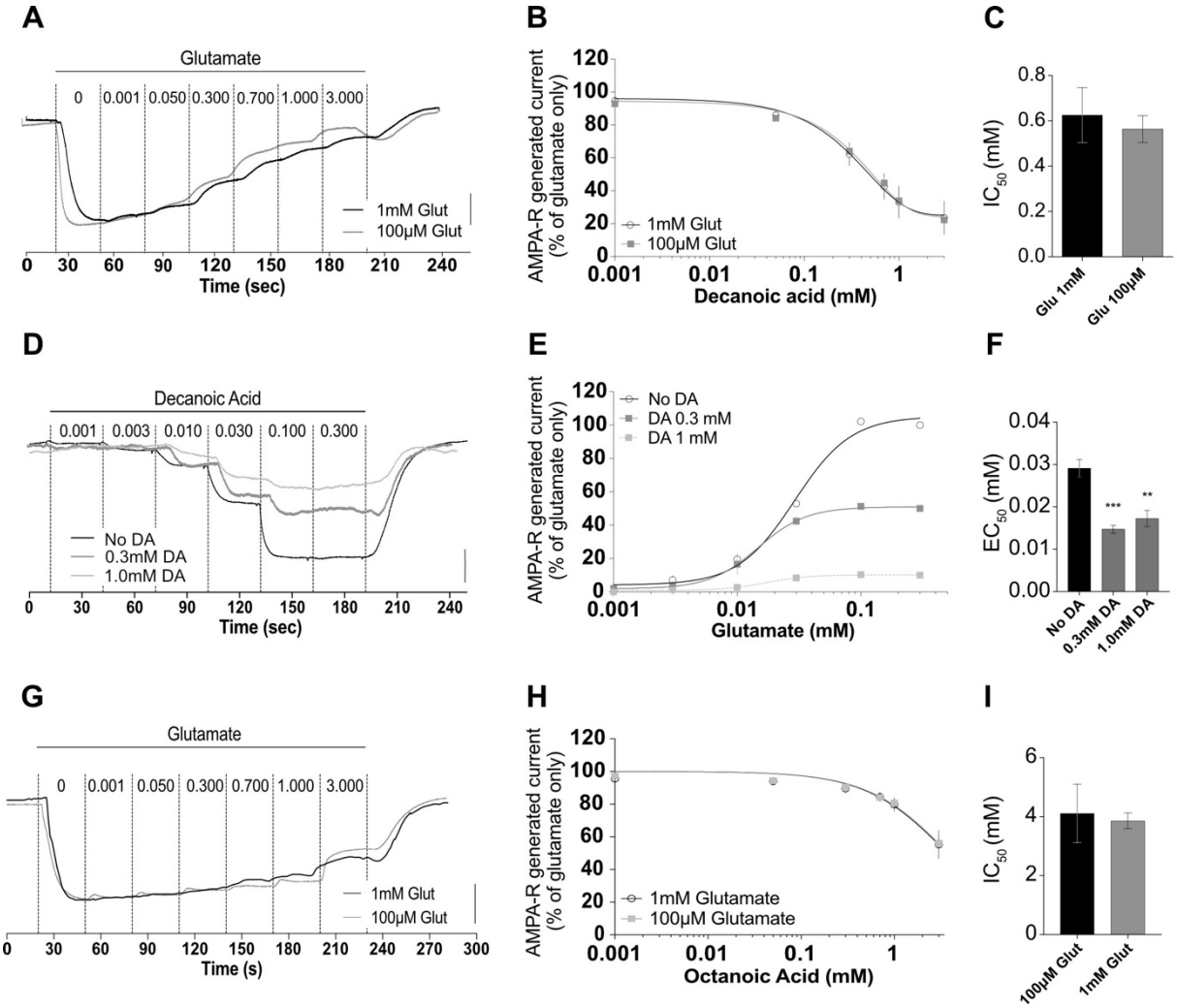



*Figure 3.2 Characterisation of type of AMPA receptor antagonism of decanoic acid in Xenopus laevis oocytes.* GluA2/3 was expressed in oocytes and currents were measured with TEVC. (**A**) Trace recordings representative of the dose-response assay carried out to investigate mode of inhibition by eliciting currents with either 1mM (black) or 100µM glutamate (grey line), and a range of decanoic acid concentrations. Scale bar corresponds to 25nA (1mM glutamate) and 30nA (100µM glutamate) (**B** and **C**) Dose response curve and bar graph showing no change in inhibition by decanoic acid under increasing glutamate concentrations where each point represents the mean and SEM of 10 to 12 recordings, and bar graph shows average IC50 and SEM generated from B. (**D**) Representative trace recordings showing the activation of GluA2/3 by a range of glutamate concentrations only (black) or in the presence of 0.3mM (grey) and 1.0mM (grey, dotted) decanoic acid. Scale bars correspond to 5nA (no decanoic acid), 2nA (0.3mM decanoic acid) and 4nA (1mM decanoic acid) (**E** and **F**). Dose response curve and bar graph showing no change in inhibition by decanoic acid under increasing glutamate concentrations where each point represents the mean and SEM of 5 recordings, and bar graph shows average EC50 and SEM generated from E. (**G**) Trace recordings representative of the dose-response assay carried out to investigate mode of inhibition by eliciting currents with either 1mM (black) or 100µM glutamate (grey line), and a range of octanoic acid concentrations. Scale bar corresponds to 70nA (100µM glutamate) and 40nA (1mM glutamate). (**H** and **I**) Dose response curve and bar graph showing no change in inhibition by octanoic acid under increasing glutamate concentrations where each point represents the mean and SEM of 12 recordings, and bar graph shows average IC50 and SEM. Data were analysed using student's *t*-test in **E** and **K**, and ANOVA in **H**, where ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$.

### 3.2.3 Inhibition of AMPA receptors by medium-chain fatty acids is carbon-chain length dependent

Compared to decanoic acid, a ten-carbon fatty acid, the eight-carbon octanoic acid, was significantly less potent against AMPA receptors. It is therefore likely that chain length is crucial in receptor antagonism and that seizure control by the MCT ketogenic diet may be improved through enrichment of the MCT oil in the most potent medium-chain fatty acid. Therefore, optimal aliphatic tail length in AMPA receptor inhibition of free fatty acids was assessed in single-dose response assays. The compounds that were screened included the seven-carbon fatty acid heptanoic acid, the nine-carbon fatty acid nonanoic acid as well as the eleven and twelve-carbon fatty acids undecanoic acid and dodecanoic acid (all compounds are summarised in table 3.1).

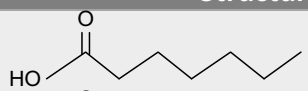
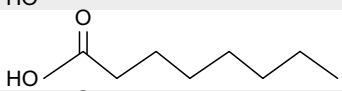
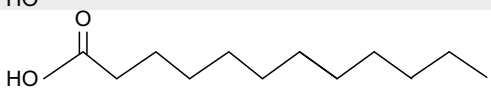
| Internal ID | Fatty acid | Length ($CH_3$) | Structure |
|---|---|---|---|
| RW41 | **Heptanoic acid** | 7 |  |
| RW26 | **Octanoic acid** | 8 |  |
| RW43 | **Nonanoic acid** | 9 |  |
| RW44 | **Decanoic acid** | 10 |  |
| RW76 | **Undecanoic acid** | 11 |  |
| RW39 | **Dodecanoic acid** | 12 |  |

*Table 3.1 Summary of the structures of the assessed medium-chain fatty acids.*

At 1mM, heptanoic acid reduced GluA2/3 generated currents by 6.82% (SEM = 2.761; n = 6; $p < 0.0001$ compared to inhibition by 1mM decanoic acid), whereas octanoic acid inhibited AMPA receptors by 13.05% (SEM = 5.19; n = 4; $p < 0.0001$ compared to inhibition by 1mM decanoic acid), and nonanoic acid inhibited currents by 39.99% (SEM = 1.97; n = 6; $p < 0.0001$ compared to inhibition by 1mM decanoic acid). By comparison, decanoic acid suppressed GluA2/3 generated currents by 75.08% (SEM = 3.43; n = 5). Increasing the length of the aliphatic tail further, however, reduced the efficacy in inhibition again significantly to 59.71% in undecanoic acid (SEM = 2.20; n = 6; $p = 0.0036$) and 50.41% in dodecanoic acid (SEM = 3.07; n = 6; $p = 0.0005$) (figure 3.4 A, B, C). Since all of the fatty acids tested here were statistically significantly less potent than decanoic acid, this supports the previous findings that suggested that AMPA receptor inhibition by medium-chain fatty acids is chain length dependent.

To confirm the importance of chain length on AMPA receptor inhibition by medium-chain fatty acids, a full dose-response curve was calculated for nonanoic acid and compared to the above described data for octanoic and decanoic acid (figure 3.3 F, G, H). For undecanoic acid and dodecanoic, full dose-response curves could not be generated reliably due to their strong hydrophobicity and reduced solubility in aqueous solutions. With increasing carbon number the dose-effect curve was shifted further to the left reducing the $IC_{50}$ against GluA2/3 from octanoic acid ($IC_{50}$ = 3.818mM) to 1.477mM in nonanoic acid (SEM = 0.021; n = 12; $p$ <0.0001 to octanoic acid) and 0.52mM in decanoic acid. Combined with the evidence from single dose response assays, these data further support that, firstly, AMPA receptor inhibition by medium-chain fatty acids is dependent on the chain length and, secondly, that between the medium-chain fatty acids assessed here, decanoic acid provides the strongest inhibition of AMPA receptors.

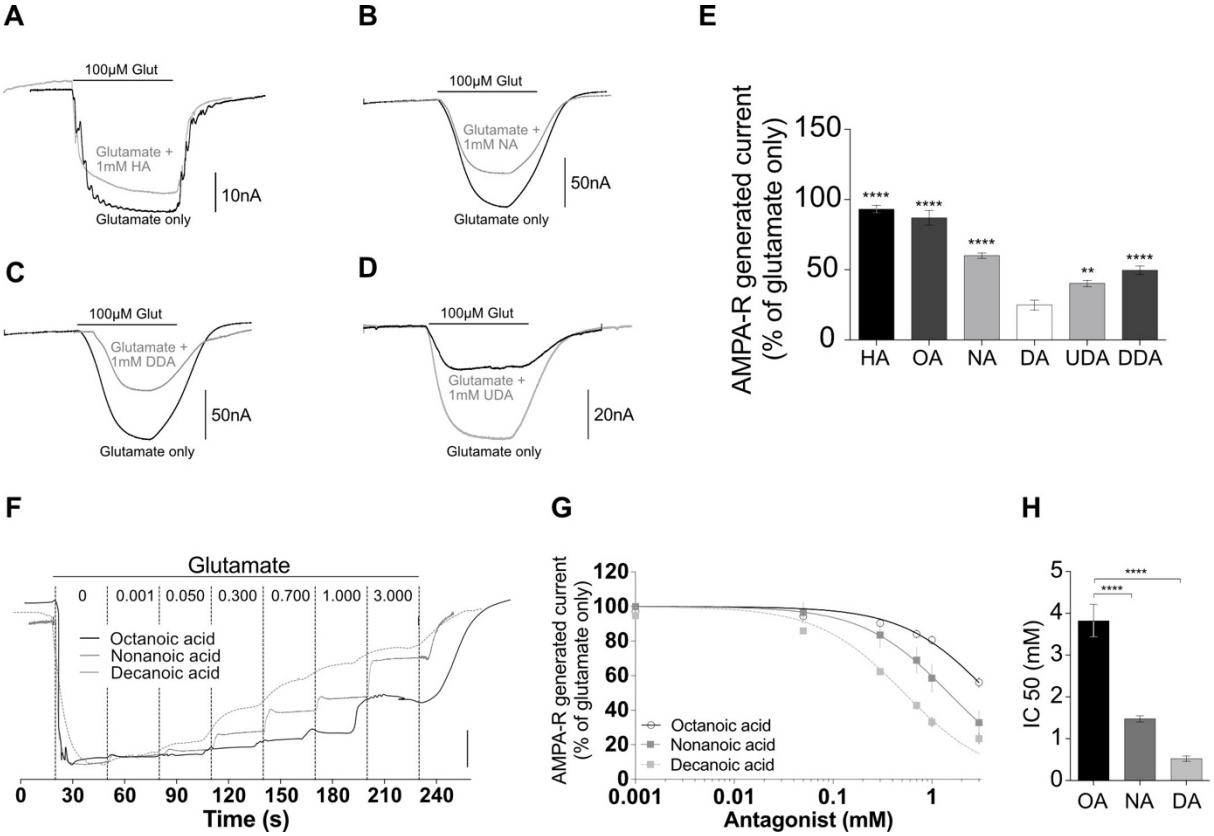



*Figure 3.3 Effect of varying chain length on inhibition of AMPA receptor generated currents.* Currents were evoked by 100μM measured in GluA2/3 expressing *Xenopus laevis* oocytes. (**A** to **D**) Representative trace recordings showing the reduction of currents generated by glutamate only (black line) by single dose applications of 1mM heptanoic acid (HA, **A**), nonanoic acid (NA, B), undecanoic acid (UDA, **C**) and dodecanoic acid (DDA, **D**). All fatty acids were dissolved at 1mM. (**E**) Bar graph summarising the mean receptor activity during application of heptanoic acid (HA), octanoic acid (OA), nonanoic acid (NA), decanoic acid (DA), undecanoic acid (UDA) and dodecanoic acid (DDA). Each bar represents the mean and SEM of 4 to 12 recordings, and statistical analysis was carried relative to DA. (**F**) Representative trace recordings showing the dose-effect relationship of a range of concentrations (mM) of octanoic acid, nonanoic acid and decanoic acid on AMPA receptors. Scale bar corresponds to 70nA for octanoic acid, 20nA for nonanoic acid and 30nA (decanoic acid). (**G** and **H**) Graphs showing effect of aliphatic chain length on inhibition of AMPA receptors for octanoic acid (OA), nonanoic acid (NA) and decanoic acid (DA). Each point and bar represents the mean reading and IC50, respectively, and SEM of 12 recordings. Statistical analyses were carried out using ANOVA, where ** corresponds to $p < 0.01$, *** corresponds to $p < 0.001$, and **** corresponds to $p < 0.0001$.

### 3.2.4 Identification of an optimal decanoic acid to octanoic acid ratio in *Xenopus* oocytes

More potent inhibition of AMPA receptors by decanoic acid compared to octanoic acid raises the question of whether the current composition of the MCT ketogenic diet can be optimised to account for this newly identified mechanism of seizure control. Currently, MCT oils that are administered in the MCT ketogenic diets consist of 60% octanoic acid and 40% decanoic acid (Chang *et al.*, 2016). Different ratios of decanoic acid to octanoic acid (DA:OA; figure 3.4) were tested against GluA2/3 expressed in oocytes, to determine a combination that most efficiently inhibits AMPA receptors. Decanoic and octanoic acid were mixed at 1mM concentrations to give 0:1 (octanoic acid only), 4:6 (mimicking the current composition of the MCT oil), 5:5, 7:3, 9:1 and 1:0 (decanoic acid only) ratios and currents were evoked with 100µM glutamate. To compare the efficacy of these ratios, single dose response recordings were normalised to glutamate control and compared to inhibition by 1mM decanoic acid (1:0). From these data, a ratio of 70% decanoic acid and 30% octanoic acid provides 66% inhibition (SEM = 2.7; n = 10) which is similar to the inhibition achieved by 1mM decanoic acid (70.8%; SEM = 4.3; n = 10; $p$ = 0.7919 compared to 70% decanoic acid) and 90% decanoic acid (67.7%; SEM = 4.1; n=10; $p$ = 0.9581). In contrast, equal amounts of decanoic and octanoic acid resulted in 54.9% inhibition (SEM = 1.8; n = 11; $p$ = 0.0020 compared to decanoic acid only) and at the ratio mirroring the current MCT oil composition (4:6) only 51.9% of AMPA receptor generated currents were inhibited (SEM = 3.8; n = 5; $p$ = 0.0038 compared to decanoic acid only). In addition, an 8:2 ratio was assessed in a single dose response assay. This combination inhibited AMPA receptors by 72.9% which is similar to the degree of inhibition seen for decanoic acid only (SEM = 1.1; n = 5, $p$ = 0.9248 compared to decanoic acid only; figure 3.4**,** D and E).

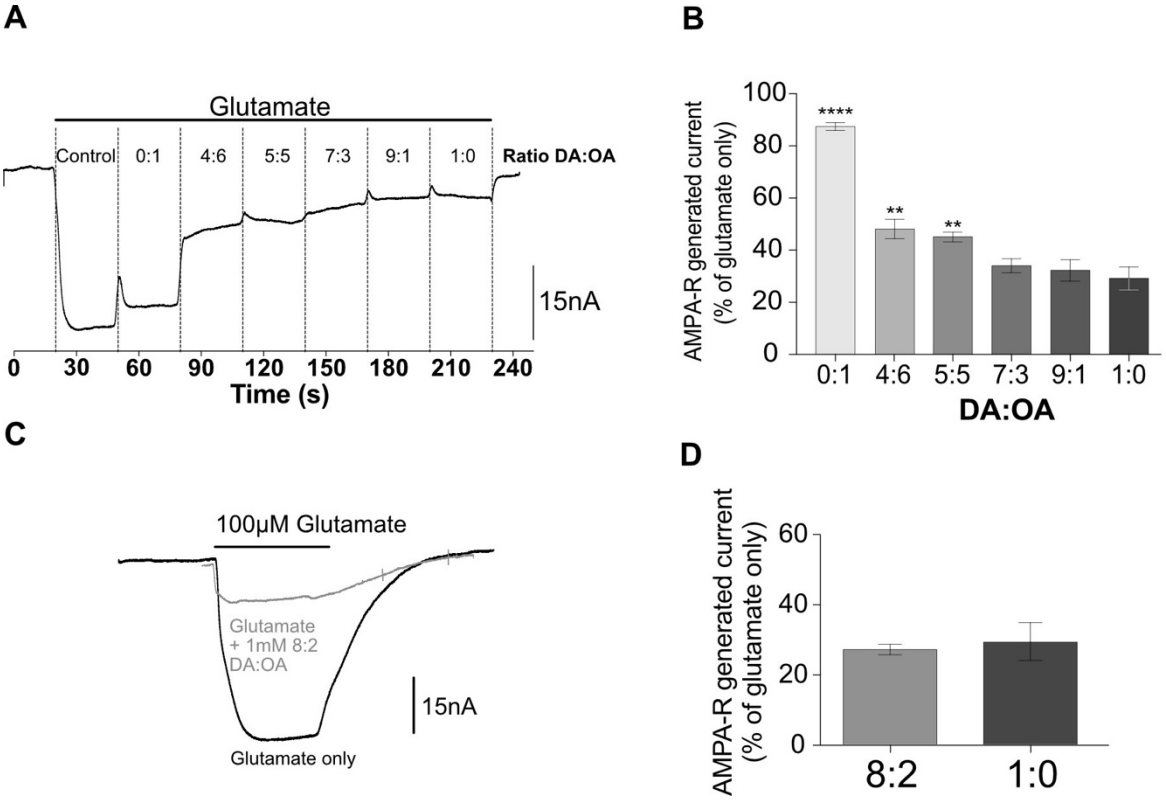



*Figure 3.4 Inhibition of GluA2/3 by different combinations of 1mM decanoic acid and 1mM octanoic acid.* Recombinant AMPA receptors were expressed in *Xenopus* oocytes and currents were evoked by 100μM glutamate. (**A**) Representative trace showing the effects of six different decanoic acid to octanoic acid ratios (Ratio DA:OA) on GluA2/3 generated currents. (**B**) Bar graph quantifying average AMPA receptor activity in the presence of the six ratios where each bar corresponds to the mean and SEM of 5 to 11 recordings. (**C**) Single dose response assay to assess inhibition of GluA2/3 by 1mM 8:2 (DA:OA). (**D**) Bar graph showing normalised mean inhibition and SEM of 5 to 10 recordings. Statistical analysis was carried out relative to inhibition by 1mM 1:0 (DA:OA) using ANOVA in B and student's *t*-test in D, where ** corresponds to $p < 0.01$ and **** corresponds to $p < 0.0001$.

### 3.2.5 AMPA receptor inhibition by decanoic acid is not use-dependent

A common issue in the therapeutic use of antagonists is use-dependence, which manifests in reduced responsiveness of the receptor to the antagonist following repeated target activation by the agonist (Kemp, Foster and Wong, 1987). Use-dependence cannot be conclusively determined using TEVC in *Xenopus* oocytes, but the TEVC setup can be applied to get a preliminary idea of whether repeated decanoic acid and glutamate application modulate receptor activation profiles.

The response of AMPA receptors comprised of GluA2/3 to glutamate was measured prior to and after repeated perfusion of *Xenopus* oocytes with 1mM decanoic acid and 100µM glutamate for 30 seconds. These were followed by 30 second washout phases with 1X Ringer's (figure 3.3 A). When decanoic acid was dissolved in 1X Ringer's solution, there was a significant decrease in response to the final application of 100µM glutamate (figure 3.3 B; n= 4; $p$ = 0.0068 compared to first time application), suggesting decreased responsiveness of the AMPA receptor to glutamate and therefore possibly use-dependence of inhibition by decanoic acid.

Since it is possible that decanoic acid due to its hydrophobicity was not efficiently cleared in washouts with Ringer's solution, the experiment was repeated with 0.5% BSA added to the perfusion for washouts and the final washout was prolonged to 90 seconds instead of 30 seconds. In these repeats, the loss in responsiveness to the final glutamate application was not observed (116.1% activity relative to control; SEM = 8.7; n = 3; $p$ = 0.2066 compared to first time application), suggesting that the initially observed decrease in activation was due to inefficient clearance of decanoic acid, and thus persisting AMPA receptor antagonism. These results therefore indicate no measurable reduction in responsiveness or maximal response of AMPA receptors due to decanoic acid application in the oocyte model.

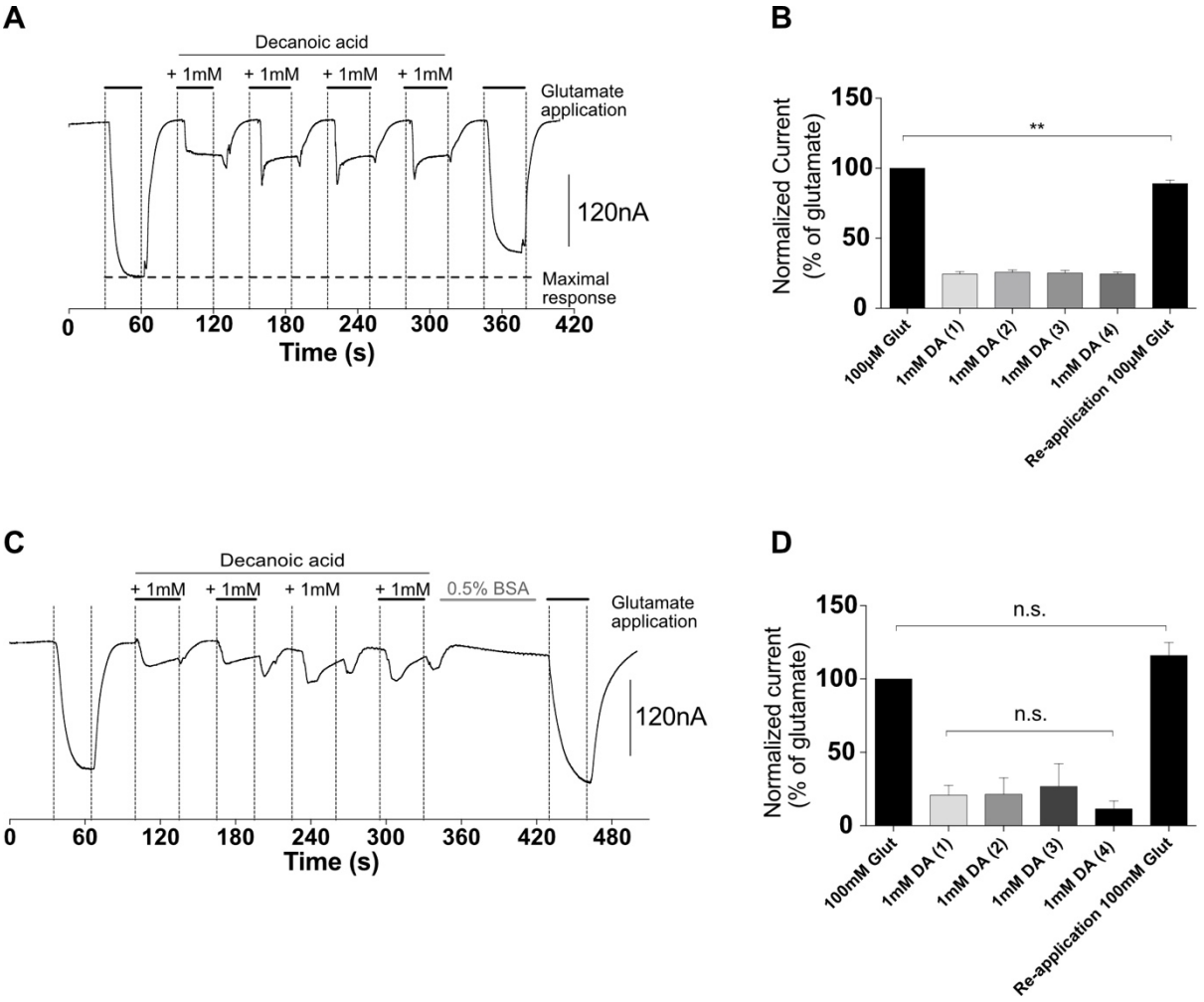



*Figure 3.5 Investigation of use-dependent inhibition of AMPA receptors by decanoic acid.* Use-dependence was assessed by repeated application of 100µM glutamate and 1mM decanoic acid in Xenopus oocytes expressing GluA2/3. (**A**) Representative trace recording showing the experimental setup and decreased maximal response of AMPA receptors to glutamate following repeated antagonist application. (**B**) Bar graph quantifying the AMPA receptor generated currents during use-dependence experiments where each bar represents the mean response and SEM of 4 recordings. (**C**) Representative trace recording showing maintained maximal response of AMPA receptors to glutamate following repeated antagonist application if final washout is prolonged and washout solution substituted with 0.5% BSA. (**D**) Bar graph quantifying the AMPA receptor generated currents second experimental setup where each bar represents the mean response and SEM of 3 recordings. Statistical analysis was carried using student's *t*-test for first and last application, where ** corresponds to $p < 0.01$.

### 3.2.6 AMPA receptor inhibition by decanoic acid is voltage-dependent

In the previous experiments described above, TEVC recordings in *Xenopus laevis* oocytes expressing recombinant AMPA receptor subunits were carried out at a holding voltage of -50mV. However, the voltage in neurons varies with the state of activation ranging from -70mV resting potential to +30mV during depolarisation. Whilst AMPA receptors are not known to be voltage-sensitive, the fact that they are embedded in a membrane that undergoes depolarisation warrants investigating the effects of voltage on inhibition by decanoic acid.

Therefore, single dose response assays were carried out at different holding voltages ranging from -20mV to -80mV, reflecting the membrane potentials of neurons during activation and resting state, respectively. A less negative holding voltage was not feasible as this would have reduced currents to non-detectable ranges. Oocytes were clamped at -20mV, -40mV, -60mV and -80mV and perfused for 30 seconds with 1mM decanoic acid and 100µM glutamate. At -20mV, currents were reduced to 35.3% (SEM = 6.8; n=4) compared to the maximal response observed in the glutamate only control. Changing the holding voltage to -40mV resulted in inhibition of currents averaging 41.3% of control (SEM = 7.3; n = 5; $p$ = 0.9918 compared to inhibition at -50mV). At holding voltages of -60mV and -80mV, currents were inhibited by 44.8% (SEM = 4.9; n = 5; $p$ = 0.8290), and 43.8%; respectively (SEM = 2.0; n = 4; $p$ = 0.6368 compared to -50mV). This suggests that inhibition is more efficient at less negative holding voltages compared to strongly depolarising voltages (figure 3.4 A to C).

To assess whether a change of AMPA receptor inhibition through changes in membrane voltage is more strongly reflected in a range of concentrations, full dose-effect assays were carried out at -40mV and -80mV. These voltages were selected for their compatibility with oocyte health over prolonged periods of clamping. The data from these dose response curves show that, at -80mV, decanoic acid inhibits AMPA receptors consisting of subunits GluA2/3 significantly less potently ($IC_{50}$ = 1.06mM, SEM = 0.03; n = 5) than at -40mV ($IC_{50}$ = 0.59mM; SEM = 0.03; n = 6; $p$ = 0.005 with respect to -80mV). This suggests that AMPA receptor inhibition by decanoic acid may be voltage-dependent and indicates that inhibition is more potent in receptors during depolarising conditions than during resting state.

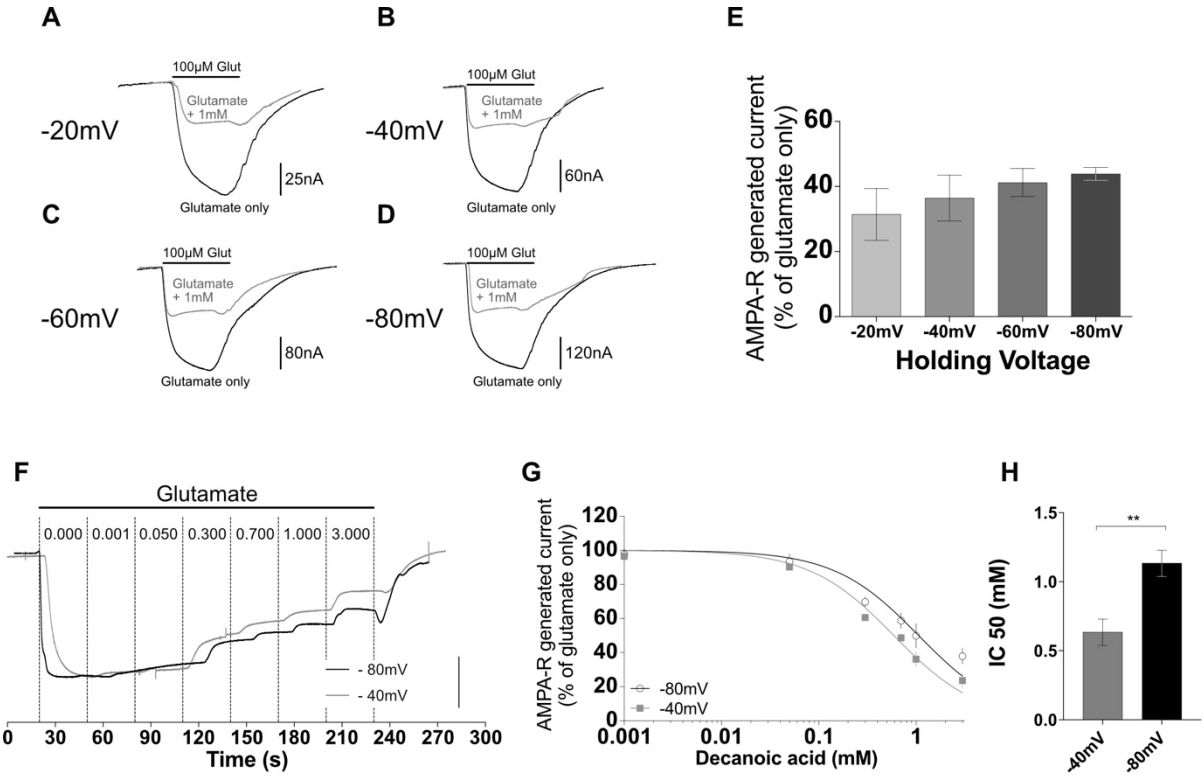



*Figure 3.6 Effect of variable holding voltage on GluA2/3 generated currents.* AMPA receptor subunits were expressed in *Xenopus laevis* oocytes and currents were evoked by 100µM glutamate. (A to D) Representative trace recordings from single-dose response assays comparing the currents of evoked by glutamate only (black line) and 100µM glutamate with 1mM decanoic acid (grey line) at -20mV (A), -40mV (B), -60mV (C) and -80mV (D) holding voltage. (E) Bar graph showing differences between GluA2/3 generated currents at four holding voltages. Each bar represents the mean and SEM of 4 to 5 recordings. (F) Representative trace recordings showing the effect of two different holding voltages (-80mV, black line; -40mV, grey line) on GluA2/3 inhibition by a range of decanoic acid concentrations. Scale bar corresponds to 40nA (-40mV) and 350nA (-80mV). (G and H) Dose-effect curve and bar graph showing the difference in IC50 of decanoic acid under two different holding voltages. Each point and bar represents the mean and SEM of 5 (-80mV) and 6 (-40mV) recordings. Statistical analyses were carried out using ANOVA in E with respect to -20mV, and using student's *t*-test in H, where ** corresponds to $p < 0.01$.

## 3.3 Discussion

These experiments show for the first time that medium-chain fatty acids inhibit AMPA receptors in a non-competitive, chain length specific and voltage-dependent manner. Of the medium-chain fatty acids tested here, decanoic acid showed the greatest potency. Decanoic acid makes up 40% of the fatty acids provided in the MCT ketogenic diet, and as a result substantial concentrations of free decanoic acid can be found in the blood of patients receiving this diet (Haidukewych, Forsythe and Sills, 1982). Octanoic acid, which provides the remainder of the fats in the MCT oil, provides reduced antagonistic action against AMPA receptors, with an eight-fold increased $IC_{50}$ compared to decanoic acid. Based on these data, it can be hypothesised that decanoic acid contributes to the reduction in seizure frequency and severity observed in patients who receive the MCT ketogenic diet.

The efficacy of the MCT ketogenic diet for epilepsy treatment has been confirmed repeatedly in clinical trials (Neal *et al.* 2009; Sills *et al.*, 1986), and it has further been shown that, despite having a lower fat to carbohydrate ratio compared to the classic ketogenic diet, both versions provide comparable seizure control (Elizabeth G Neal *et al.*, 2009). However, despite growing interest in determining a mechanism of action for the diets and numerous publications examining a seizure-preventive mechanism for ketones (Hasebe *et al.*, 2010; D'Agostino *et al.*, 2013; Kim *et al.*, 2015), the evidence for ketone bodies specifically and molecular targets of the ketogenic diets generally remains inconclusive. This is due to insufficient and conflicting data (Samoilova *et al.*, 2010) targeting highly heterologous mechanisms including mitochondrial respiration (Kim *et al.*, 2015), neurotransmitter release and synthesis (Suzuki *et al.*, 2009; Juge *et al.*, 2010), and neuronal excitability (Ma, Berg and Yellen, 2007; Lund *et al.*, 2015).

The results from this chapter propose an alternative mechanism for seizure control in ketogenic diets: Since medium-chain fatty acids have been shown to be present in the blood of patients receiving the MCT ketogenic diet, it is likely that this diet exerts its anti-seizure effects at least in part through the inhibition of AMPA receptors by decanoic acid. Fatty acids freely cross the blood brain barrier (Kuge *et al.*, 1995), and decanoic acid present in the blood could thereby reduce glutamatergic signalling in a concentration-dependent manner. Whilst for practical reasons quantification of decanoic acid in the human brain is not possible, experiments carried out in mice show that free decanoic acid is found in the brain mirroring plasma concentrations in a time-dependent manner and achieving more than 50% of the plasma concentration throughout (Wlaź *et al.*, 2012). From the data shown in this chapter, it can be extrapolated that at the mean plasma concentration of 160µM (Haidukewych, Forsythe and Sills, 1982), AMPA receptor generated currents are reduced by 10 to 20%, depending on the subunit

combination. Assuming that uptake into the brain is 50% efficient, the degree of AMPA receptor inhibition may therefore not exceed 10%. However, a small degree of inhibition may be sufficient to raise the threshold for seizures, especially in the early stages of seizure generation, by reducing the depolarisation of neuronal membranes sufficiently to prevent action potential initiation.

Data from animal experiments support a role for seizure protection by direct effects of decanoic acid. Wlaz *et al.* (2015) examined seizure control 30 minutes after oral administration of decanoic acid, which the authors argue minimised ketone generation and release into the bloodstream. In these experiments, decanoic acid increased seizure thresholds in the 6Hz and MES seizure tests. Both of these seizure models are animal models of acute seizures (Löscher, 2011; Kandratavicius *et al.*, 2014). These results therefore confirm a role for fatty acids in acute seizure control that is independent of ketone generation. Interestingly, the same group examined seizure control by octanoic acid under identical conditions, but found that octanoic acid was much less effective at reducing seizures (Wlaź *et al.*, 2012). These results therefore agree with the findings described in this chapter and confirm a mechanism for decanoic acid in seizure control.

Fatty acid antagonism of glutamate receptors has been described previously, but has so far not been linked to seizure prevention (Voskuyl *et al.*, 1998; Hamilton and Brunaldi Kellen, 2007). Wilding *et al.* (2010) have shown that *cis*-unsaturated fatty acids modulate kainate receptor gating, and arachidonic acid has been shown to reduce AMPA receptor generated currents in brain slices (Kovalchuk *et al.*, 1994). However, it is not clear how these long-chain fatty acids interact with the receptor proteins or how relevant these findings are for translation into human brain tissue, as the authors noted that during perfusion at concentrations that would generate a significant amount of inhibition, neurons that were imbedded deeper in the slice did not respond as well as those found on the surface (Kovalchuk *et al.*, 1994). This raises the question whether this discrepancy is due solely to experimental design or signifies that long-chain fatty acids do not efficiently reach deeper tissue in the brain. Also, these results are contradictory to the chain-length specific inhibition of AMPA receptors described in this chapter. However, it cannot be ruled out that contributing factors in the tissue culture that did not exist in the simple oocyte model resulted in inhibition of AMPA receptors by long-chain fatty acids. Therefore, it is possible that inhibition of glutamatergic signalling by long-chain fatty acids might indicate a mechanism for fatty acids found in the classic ketogenic diet. At this point, however, the scientific evidence for this hypothesis is only episodic and not sufficient to support a ketone-independent mode of action for long-chain fatty acids in seizure control.

### 3.3.1 AMPA receptor inhibition by medium-chain fatty acids is non-competitive

Inhibition of receptor proteins is a common target for drug development and the mode of inhibition is a key factor determining drug efficacy and side effects. Competitive inhibitors which bind at the ligand-binding site or at a site the access to which is affected by the ligand-binding state, may be overcome by high agonist concentrations when the ligand replaces the antagonist, rendering the inhibitor ineffective. Glutamate levels are highly variable depending on neuronal activity and high concentrations of glutamate over prolonged periods are a central aspect in seizure generation (reviewed by Donevan and Rogawski, 1993). Therefore, when designing a seizure treatment based on AMPA receptor inhibition, non-competitive inhibition is desirable and necessary to provide effective seizure protection independent of the amount of glutamate present in the post-synapse.

Due to the central role of AMPA receptors in seizure generation, the advance of non-competitive glutamate receptor antagonists has attracted a lot of interest in the past. So far, the only known non-competitive AMPA receptor inhibitor that has been confirmed for safety in clinical trials and approved by the United States Food and Drug Administration (FDA), is perampanel (Russo *et al.*, 2012; Rektor, 2013). The efficacy of perampanel in seizure protection is well documented (Krauss *et al.*, 2012; Kerling and Kasper, 2013; Steinhoff *et al.*, 2013), and this confirms that AMPA receptor antagonism provides a suitable approach for epilepsy seizure treatment. This suggests that inhibition of AMPA receptors by decanoic acid is a safe mechanism that is likely to contribute to the seizure control achieved in treatment with the MCT ketogenic diet.

### 3.3.2 AMPA receptor inhibition is most efficient in decanoic acid

This chapter further characterised the antagonism of medium-chain fatty acids with varying aliphatic chain lengths. These data strongly suggest that of the fatty acids tested here, decanoic acid most potently inhibits AMPA receptors. This finding has possible implications for the composition of the MCT oil which is the means for fat delivery in the MCT ketogenic diet. Therefore, a greater proportion of decanoic acid compared to octanoic acid may be desirable to raise the blood concentration of free decanoic acid.

The current composition of the MCT oil is optimised for rapid ketogenesis (P R Huttenlocher, 1976). Medium-chain fatty acids are considered more ketogenic as they, due to their shorter chain length, are more readily and quickly absorbed and broken down into ketone bodies. However, as the results of this chapter suggest, decanoic acid may provide seizure control through direct action against

AMPA receptors. Rapid metabolism of decanoic acid may therefore not be desirable in the treatment of seizures and it may be beneficial to provide a greater proportion of decanoic acid in the MCT oil. However, whether and how the ratio can be optimised can only be indicated from the data shown here and needs to be validated in animal experiments and clinical trials.

Whilst decanoic acid is the more active compound in AMPA receptor antagonism and this function may provide seizure reduction, a role for octanoic acid in seizure control may still be valid. Octanoic acid might contribute to seizure control through stabilising decanoic acid pools in the blood, for instance due to the fact that it preferably is metabolised over decanoic acid (Papamandjaris, MacDougall and Jones, 1998). However, insufficient data from patient blood samples exist to indicate whether this mechanism prevents rapid decanoic acid breakdown even at the current composition of the MCT oil.

In addition, the role of ketone bodies in seizure control needs to be considered. Ketones, which are readily generated from octanoic acid, are likely to contribute to seizure control despite the conflicting evidence for ketone levels predicting seizure reduction. The results shown in this chapter do not explain seizure control in the classic ketogenic diet, in which fats are provided predominantly as long-chain triglycerides, nor reduction of seizures during fasting (Rogawski 2013; Rogawski 1999). Further, there is increasing evidence that ketone ester administration without dietary changes can protect from seizures (D'Agostino *et al.*, 2013; Hashim and VanItallie, 2014; Viggiano *et al.*, 2015). Therefore, whilst decanoic acid is likely to contribute to seizure control, maintaining a steady supply of ketones generated from octanoic acid may very well add to this effect.

### 3.3.3 AMPA receptor inhibition by medium-chain fatty acids is influenced by membrane voltage but is use-independent

To evaluate how inhibition of AMPA receptors by medium-chain fatty acids may be influenced by neuronal activity *in vivo*, this chapter also investigates use- and voltage-dependence. From single-dose response assays, holding voltages of -40mV, -60mV and -80mV applied to *Xenopus* oocytes reduced inhibition by decanoic acid mildly but not significantly compared to a less negative holding voltage (-20mV). This difference was much more pronounced and significant when investigated in dose-response curves. These data suggest that decanoic acid may be more likely to potently inhibit AMPA receptors during action potentials than at resting state and could therefore more efficiently prevent receptor activation during high activation states such as would be expected during a seizure.

Further, these data allow some assumptions regarding the site of interaction of decanoic acid with AMPA receptors. Since AMPA receptor activity is not known to respond to changes in membrane voltage but the data presented here show effects of voltage on inhibition by decanoic acid, it follows that medium-chain fatty acids are likely to interact at a site that is known to respond to voltage changes in the membrane. One such site would be the transmembrane domain. Binding of decanoic acid to this part of the receptor would be in accordance with the findings for kainate inhibition by *cis*-unsaturated fatty acids which have been hypothesised to bind to the transmembrane domain where they are most likely to influence the receptor gating properties (Wilding, Chen and Huettner, 2010). Interestingly, one property of AMPA receptors that has been shown to be affected by voltage, is gating regulated through the transmembrane regions (Donevan and Rogawski, 1993; Prieto and Wollmuth, 2010). The data shown here therefore support that the transmembrane domain could be a likely site of interaction for decanoic acid.

In addition, this chapter shows data that suggest that decanoic acid inhibits AMPA receptors in a use-independent manner. Repeated activation of AMPA receptors in the presence of decanoic acid did not increase the inhibition nor did it reduce the maximal response of GluA2/3 to glutamate. Whilst it may seem beneficial to have augmented inhibition with repeated AMPA receptor activation in epilepsy treatment, where the synchronised and repetitive firing of neurons results in seizures, use-dependent inhibition of AMPA receptors may cause long-term changes in neuronal plasticity potentially increasing long-term depression in neurons that are not directly involved in seizure generation. This mechanism has been suggested to lead to deficits of memory formation and potentially memory loss (Lee *et al.*, 2003; Fleming and England, 2010). Whilst side-effects on memory are a concern in AMPA receptor antagonism, such effects might be augmented in use-dependent inhibition.

### 3.3.4 Indications and limitations of AMPA receptor inhibition by decanoic acid studied in *Xenopus laevis* oocytes

One limitation of pharmacological studies carried out in isolated systems such as the expression of recombinant proteins in *Xenopus* oocytes is that these data limit conclusions regarding the actual effect on seizure reduction in patients. Firstly, direct receptor inhibition studies disregard the complexity of fatty acid absorption, transport and metabolism in the body, as well as complex intra- and intercellular regulatory mechanisms. In addition, TARPs that are expressed *in vivo* and affect AMPA receptor gating, localisation and ion conducting properties were not co-expressed with the AMPA receptor subunits, which means that the overexpressed glutamate receptor may not have behaved in

the oocytes as they would *in vitro.* All of these factors may limit or enhance the efficacy of AMPA receptor inhibition and cannot be accounted for in the *Xenopus* system. Secondly, since every patient is different and the mechanisms leading to excessive excitatory signalling in epilepsy are numerous and dependent on the underlying cause for the seizures, it is not possible to estimate whether the shown reduction in AMPA receptor channel opening is sufficient to prevent seizures. However, in addition to the successful use of the AMPA receptor antagonist perampanel in seizure prevention (Kerling and Kasper, 2013), the findings presented here are supported by studies that have assessed seizure prevention in animals receiving decanoic acid. *In vivo* and *in vitro* animal studies have shown consistently that decanoic acid administration markedly reduces seizures within a short time frame and without the generation of ketones (Wlaź *et al.*, 2015; Chang *et al.*, 2016). The data presented here and the proposition that decanoic acid could contribute to seizure control in the MCT ketogenic diet through AMPA receptor inhibition are thereby also supported by data from animal studies.

# CHAPTER 4 – INHIBITION OF AMPA RECEPTORS BY BRANCHED ANALOGUES OF DECANOIC AND OCTANOIC ACID

## 4.1 Introduction

The previous chapter described AMPA receptor inhibition by medium-chain fatty acids, which constitutes a novel, heretofore insufficiently investigated mode of action for the ketogenic diets in seizure control. Ketogenic diets are reliable treatments for epilepsy with efficacies similar to most anti-epileptic drugs (Vining, 1999; Elizabeth G Neal *et al.*, 2009; Levy *et al.*, 2012). However, many patients struggle with the high fat content; and often the lack of palpability and the effort involved in food preparation as well as side effects such as constipation and nausea, are cited as main reasons for poor compliance and discontinuation (Elizabeth G Neal *et al.*, 2009; Levy *et al.*, 2012). It has been suggested that investigating the molecular mechanism of ketogenic diets might help to create a treatment option that optimises the diet and reduces side effects and thereby has less impact on the lifestyle of patients (Schwartzkroin, 1999; Chang *et al.*, 2016). The MCT ketogenic diet is an excellent example of dietary modifications that were developed to reduce the fat content, as the more ketogenic medium-chain fatty acids allow for a greater proportion of carbohydrates compared to the classic ketogenic diet (Huttenlocher, Wilbourn and Signore, 1971; Schwartz *et al.*, 2008; Elizabeth G Neal *et al.*, 2009).

The finding that decanoic acid, one of the main constituents of the MCT ketogenic diet, directly inhibits AMPA receptors, suggests a paradigm shift in ketogenic diet research and implies that the diet could be further improved through enrichment in fatty acids that provide stronger AMPA receptor inhibition. Research assessing how medium-chain fatty acids directly reduce seizure-like activity and influence secondary effects of seizures found that branched analogues of medium-chain fatty acids more potently exert these effects compared to their precursor straight-chain fatty acids (Chang *et al.*, 2013). Medium-chain fatty acids that contained a side chain at carbon 2 or carbon 4 more effectively reduced phosphoinositide levels, which are elevated after prolonged seizures, in the model organism *Dictyostelium discoidium* (Van Rooijen *et al.*, 1986; Chang *et al.*, 2012). They also more potently reduced seizure-like activity in low $Mg^{2+}$ and PTZ seizure models in hippocampal slices (Chang *et al.*, 2013; 2015).

Since introduction of a simple side chain to medium-chain fatty acids improves their effects on seizure-like activity and cell homeostasis, it is worth investigating whether this change could also affect AMPA receptor antagonism. Therefore, in this chapter, a range of analogues of medium-chain fatty acids containing ethyl branching, cyclic backbones, double bonds and substitutions that change the flexibility and binding porperties of the fatty acids, are screened in *Xenopus laevis* oocytes for their potency against recombinant AMPA receptors and compared to the straight chain fatty acids octanoic and decanoic acid.

# 4.2 Results

## 4.2.1 Methyl branching at carbon 4 of eight carbon chain fatty acids increases potency of AMPA receptor inhibition

Several recent papers suggest that reduction of phosphoinositide levels as well as inhibition of epileptiform activity by octanoic and nonanoic acid in hippocampal slice models of seizures is increased by introducing a methyl or ethyl side chain at carbon 4 (Chang *et al.*, 2012, 2015). To assess the effect of introducing an ethyl branching in medium-chain fatty acids on AMPA receptor inhibition and test the hypothesis that introduction of an ethyl side chain has a similar augmenting effect on potency in AMPA receptor inhibition as reported by Chang *et al* (2012; 2015) in hippocampal slice models, the branched analogue of octanoic acid, 4-ethyloctanoic acid was investigated. Dose response assays were carried out in *Xenopus laevis* oocytes expressing recombinant AMPA receptors consisting of either subunits GluA1/2 or GluA2/3. In these experiments, 4-ethyloctanoic acid inhibited AMPA receptors consisting of GluA1/2 with an $IC_{50}$ of 2.19mM (SEM = 0.04; n = 6, figure 4.1 A, B and C). Further, 4-ethyloctanoic acid inhibited AMPA receptors with comparable $IC_{50}$s for both GluA1/2 and GluA2/3 ($IC_{50}$ = 2.06mM; SEM = 0.03; n = 6; $p$ = 0.63 compared to GluA1/2; figure 4.1 A, B and C), which is similar to the straight chain fatty acid octanoic acid that inhibited AMPA receptors in a subunit independent manner (fig. 3.1 J, page 74). To compare the effect of branched medium-chain fatty acids on AMPA receptor inhibition to that of straight chain fatty acids, GluA2/3 inhibition by 4-ethyloctanoic acid was compared to GluA2/3 antagonism of octanoic acid (figure 4.2 G). From these data, ethyl branching at carbon 4 reduced the $IC_{50}$ of octanoic acid by 53.9%, making 4-ethyloctanoic acid significantly more potent than octanoic acid ($IC_{50}$ = 3.82mM; SEM = 0.03; n = 10; $p$ = 0.048 compared to $IC_{50}$ 4-ethyl octanoic acid against GluA2/3, figure 4.2.1 H and I).

To assess whether ethyl branching retains the non-competitive mode of inhibition observed in straight chain fatty acids, competition was evaluated by plotting normalised AMPA receptor generated currents elicited by 100µM glutamate and 1mM glutamate in the presence of 4-ethyloctanoic acid (figure 4.1 D, E and F). Increasing glutamate concentrations did not significantly change the $IC_{50}$ of 4-ethyloctanoic acid against GluA2/3 ($IC_{50}$ = 2.49mM; SEM = 0.04; n = 6; $p$ = 0.46 compared to 100µM glutamate). This suggests non-competitive inhibition of AMPA receptors by 4-ethyloctanoic acid.

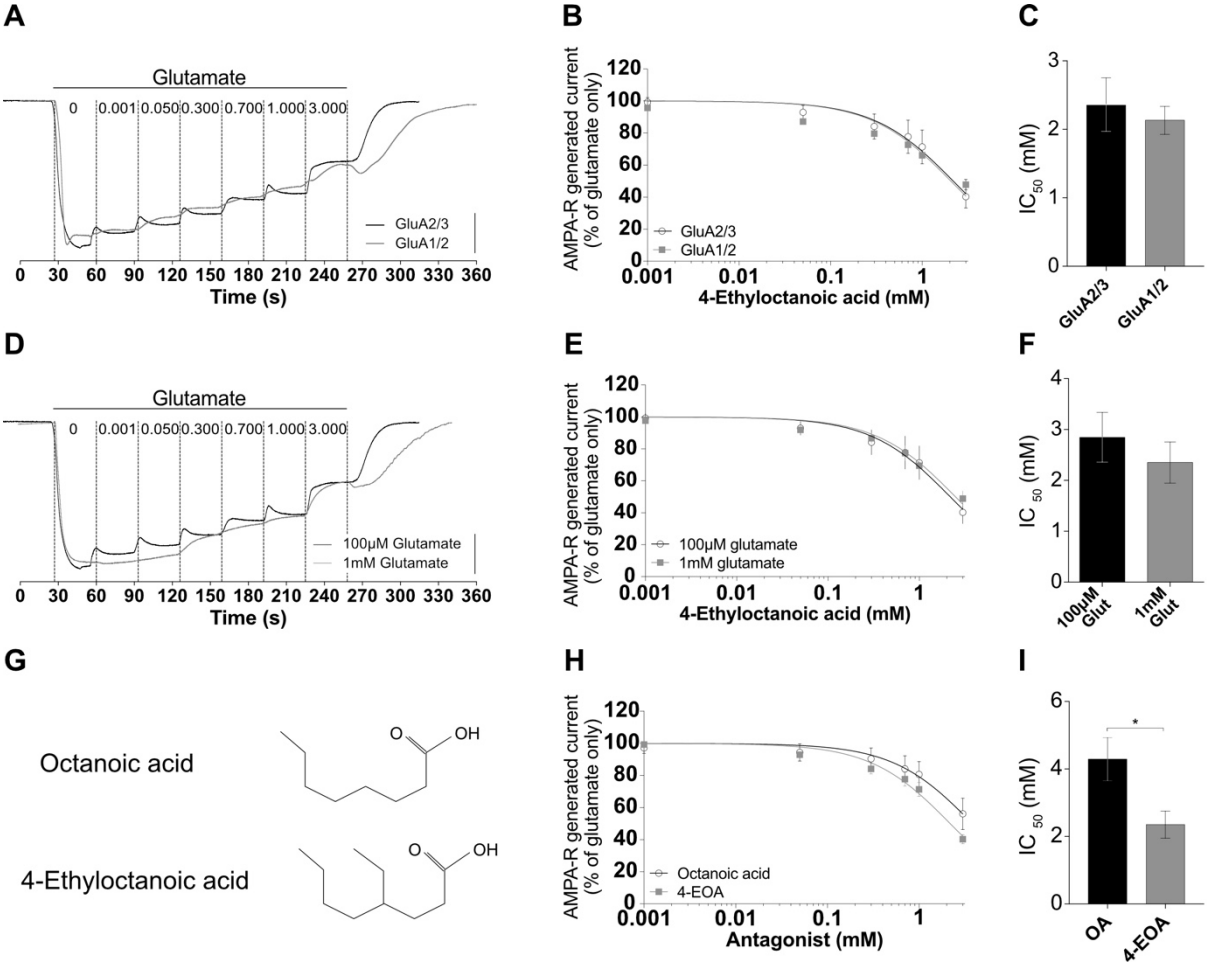



*Figure 4.1 Inhibition of AMPA receptors expressed in Xenopus laevis oocytes by the medium-chain fatty acid analogue 4-ethyloctanoic acid (4-EOA).* (**A**) Representative trace recordings for dose-response curves of 4-EOA (in mM) against GluA2/3 (black) and GluA1/2 (grey). Scale bar corresponds to 200nA (GluA1/2) and 25nA (GluA2/3). (**B** and **C**) Sigmoidal dose-response curve and bar graph showing subunit specificity of 4-EOA. Each point represents the mean and SEM of 6 recording readings. (**D**) Representative trace recordings for dose-response curves of 4-EOA (in mM) against GluA2/3 showing currents evoked by 100µM glutamate (black) and 1mM glutamate (grey). Scale bar corresponds to 25nA (100µM glutamate) and 30nA (1mM glutamate). (**E** and **F**) Sigmoidal dose-response curve and bar graph showing non-competitive inhibition by 4-EOA. Each point represents the mean and SEM of 6 recording readings. (**G**) Comparison of the molecular structures of octanoic acid (OA) and 4-EOA. (**H** and **I**) Comparison of the sigmoidal dose response curves and bar graph showing inhibition of GluA2/3 and IC50s for octanoic acid (estimated) and 4-EOA. Each point represents the mean and SEM of 6 (4-EOA) and 10 (OA) recording readings. Statistical analysis was carried out using student's *t*-test, where * corresponds to $p < 0.05$.

### 4.2.2 Cyclohexyl aliphatic chains more potently inhibit AMPA receptors than unbranched fatty acids

Introducing an ethyl side chain at carbon 4 is likely to reduce the number of possible secondary structures medium-chain fatty acids assume due to steric hindrance of the side chain with the aliphatic chain. To test this hypothesis further, the fatty acid 4-butylcyclohexyl carboxylic acid (4-BCCA) which contains a planar cyclohexyl group at carbon 2 was assessed for AMPA receptor antagonism. 4-BCCA inhibited GluA2/3 more potently than 4-ethyloctanoic acid with an $IC_{50}$ of 0.63mM against GluA2/3 (SEM = 0.04; n = 12) and an $IC_{50}$ of 1.13mM against GluA1/2 (SEM = 0.10; n = 6; figure 4.2.A, B and C). This difference is not statistically significant ($p$ = 0.1197 for GluA2/3 compared to GluA1/2).

Since the previous chapter showed that AMPA receptor inhibition is stronger in medium-chain fatty acids containing nine and ten carbon atoms compared to shorter chain fatty acids (chapter 3.2.3, page75), the analogue 4-(pentylcyclohexyl)-ethanoic acid (4-PCEA) which contains two additional $CH_2$ groups, was tested for AMPA receptor inhibition as described above. 4-PCEA inhibited AMPA receptors strongly with an $IC_{50}$ of 0.049mM against GluA2/3 (SEM = 0.003; n = 10). Consistent with the data described for 4-BCCA, potency against GluA1/2 was slightly lower with an $IC_{50}$ of 0.063mM (SEM = 0.004; n =5; figure 4.2. D, E and F), but this difference was not statistically significant ($p$ = 0.4165 for GluA1/2 compared to GluA2/3). Further, as described for 4-ethyloctanoic acid, non-competitive inhibition was assessed by repeating the dose-response assay with a higher glutamate concentration (1mM). As observed for 4-ethyloctanoic acid, cyclic branching did not affect non-competitive inhibition by 4-PCEA ($IC_{50}$ = 0.057mM; SEM = 0.008; n = 4; $p$ = 0.5992 compared to 100µM glutamate against GluA2/3; figure 4.2 G, H and I).

Compared to the unbranched decanoic acid and octanoic acid, 4-BCCA and 4-PCEA inhibit AMPA receptors significantly more potently ($IC_{50}$ of 0.63mM and $p$ = 0.0003 for 4-BCCA compared to octanoic acid; $IC_{50}$ of 0.049mM and $p < 0.0001$ for 4-PCEA compared to decanoic acid; figure 4.2 J and K). These data confirm that introducing a cyclohexyl group in the aliphatic chain increases AMPA receptor inhibition by medium-chain fatty acids.

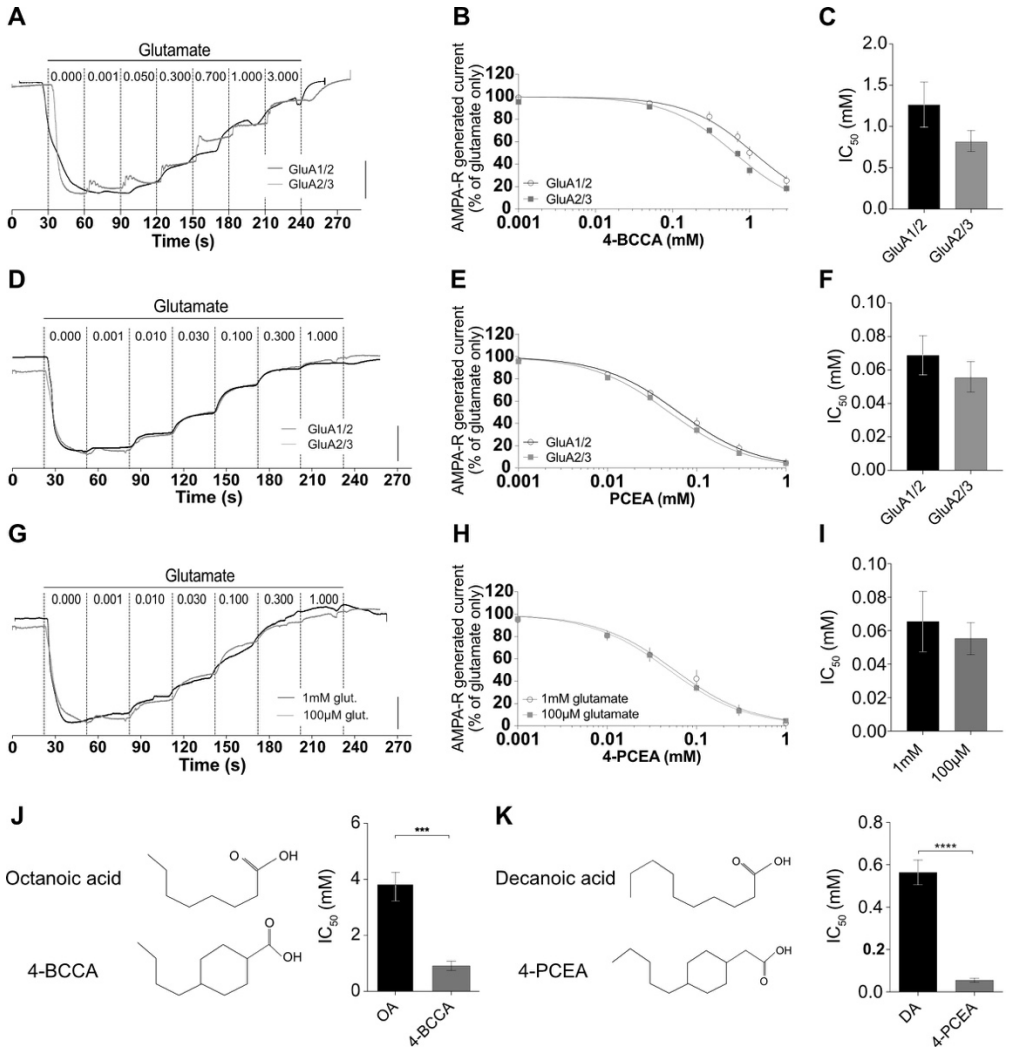



*Figure 4.2 AMPA receptor inhibition by the branched aliphatic compounds 4-BCCA and 4-PCEA.* (**A**) Representative trace recordings for 4-BCCA (in mM) showing dose-dependent inhibition of GluA1/2 (black) and GluA2/3 (grey). Scale bar corresponds to 150nA (GluA1/2) and 10nA (GluA2/3). (**B** and **C**) Dose response curve and bar graph showing lack of subunit specificity by 4-BCCA. Each point and bar represents the mean and SEM of 6 to 12 recordings. (**D**) Representative trace recordings showing inhibition of GluA1/2 (black) and GluA2/3 (grey) by 4-PCEA. Scale bar corresponds to 100nA (GluA1/2) and 20nA (GluA2/3). (**E** and **F**). Dose response curve and bar graph quantifying inhibition of GluA1/2 and GluA2/3 by 4-PCEA where each point and represents the mean and SEM of 5 and 10 recordings. (**G**) Trace recordings representative of the dose-response assay testing for mode of inhibition by 4-PCEA (in mM) with currents elicited with either 1mM (black) or 100µM glutamate (grey) in GluA2/3 expressing oocytes. Scale bar corresponds to 20nA (100µM glutamate) and 10nA (1mM glutamate). (**H** and **I**) Dose response curve and bar graph showing no change in inhibition by 4-PCEA under increasing glutamate concentrations where each point represents the mean and SEM of 4 and 10 recordings, and bar graph shows average IC50 and SEM. (**J**) Comparison of structures and IC50 against GluA2/3 of octanoic acid and 4-BCCA, where each bar represents the mean IC50 and SEM of 10 and 12 recordings. (**K**) Comparison of structures and IC50 against GluA2/3 of decanoic acid (DA) and 4-PCEA where each bar represents the mean IC50 and SEM of 12 and 10 recordings. Statistical analysis was carried out using student's *t*-test, where *** corresponds to $p < 0.001$ and **** corresponds to $p < 0.0001$.

### 4.2.3 Substitutions of cyclic medium-chain fatty acids

The results described above that medium-chain fatty acids which contain branching at carbon 4 in the form of an ethyl side chain or cyclohexyl group, more potently inhibit AMPA receptors, suggest that more rigid structures provide enhanced inhibition. Further, in addition to any effects the overall structure of the fatty acid may have on AMPA receptor antagonism, it is worth investigating the role the nonpolar methyl groups may have in binding to the receptor. Therefore, four sets of analogues that can be divided into two groups of changes affecting either the polarity of the aliphatic chain or the rigidity were screened in TEVC assays. In the first group, analogues of 4-BCCA and 4-PCEA containing amine and oxygen substitutions were assessed for their potency against AMPA receptors. The second group consists of compounds that in addition to the cyclohexyl group contain further methyl branching and double-bonds.

To provide a high throughput qualitative screen, single dose response assays were carried out with all analogues at 1mM concentrations against AMPA receptor currents evoked by 100µM glutamate. To select the most potent antagonists, the mean of these recordings was compared to the average effect of 1mM decanoic acid and 1mM 4-PCEA, which constitute the most potent so far identified straight chain and branched fatty acids, respectively.

### *4.2.3.1 Amine substitutions in cyclic fatty acids*

Three analogues of 4-BCCA containing secondary amine substitutions in both the ring structure and the butyl chain were assessed for AMPA receptor inhibition. All IDs, assigned for ease of identification, IUPAC names and structures are summarised in table 4.1.

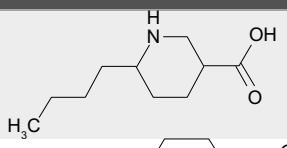
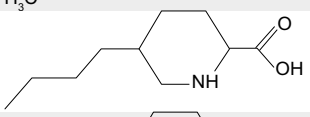
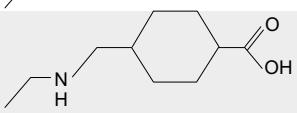
| Internal ID | ID | Name | Structure |
|---|---|---|---|
| RW173 | **A-1** | 6-butylpiperidine-3-carboxylic acid hydrochloride |  |
| RW178 | **A-2** | 5-butylpiperidine-2-carboxylic acid hydrochloride |  |
| RW179 | **A-3** | 4-[(ethylamino)methyl]cyclohexane-1-carboxylic acid hydrochloride |  |

*Table 4.1 Overview of allocated IDs for fatty acid analogues containing amine groups. Compounds are identified by their internal ID, allocated IDs (in bold), IUPAC names and chemical structures.*

In these experiments, addition of 1mM of all three compounds reduced GluA2/3 generated currents to a similar extend, with A-1 decreasing currents by 13.4% (SEM = 13.2; n = 6), A-2 by 25.7% (SEM = 2.9; n = 8), and A-3 by 33.0% (SEM = 1.6; n = 8; figure 4.3 A, B and C). All three compounds were significantly less efficient at inhibiting GluA2/3 than decanoic acid and 4-PCEA which reduced currents by 58.7% (SEM = 7.3; n = 5) and 95.6% (SEM = 1.9; n = 10), respectively. All *p*-values are summarised in table 4.2 and figure 4.3 F.

| ID | % normalized GluA2/3 inhibition | SEM | N | *p*-value compared to decanoic acid | *p*-value compared to 4-PCEA |
|---|---|---|---|---|---|
| **A-1** | 13.4 | 13.2 | 6 | 0.0008 | <0.0001 |
| **A-2** | 25.7 | 2.9 | 8 | 0.0085 | <0.0001 |
| **A-3** | 33.0 | 1.6 | 8 | 0.0471 | <0.0001 |

*Table 4.2 Summary of mean GluA2/3 inhibition by fatty acid analogues containing cyclohexyl-branching and amine-substitutions. Inhibition is normalised as % of control, SEM values, N-numbers and p-values for fatty acid analogues containing cyclohexyl branching and amine-substitutions.*

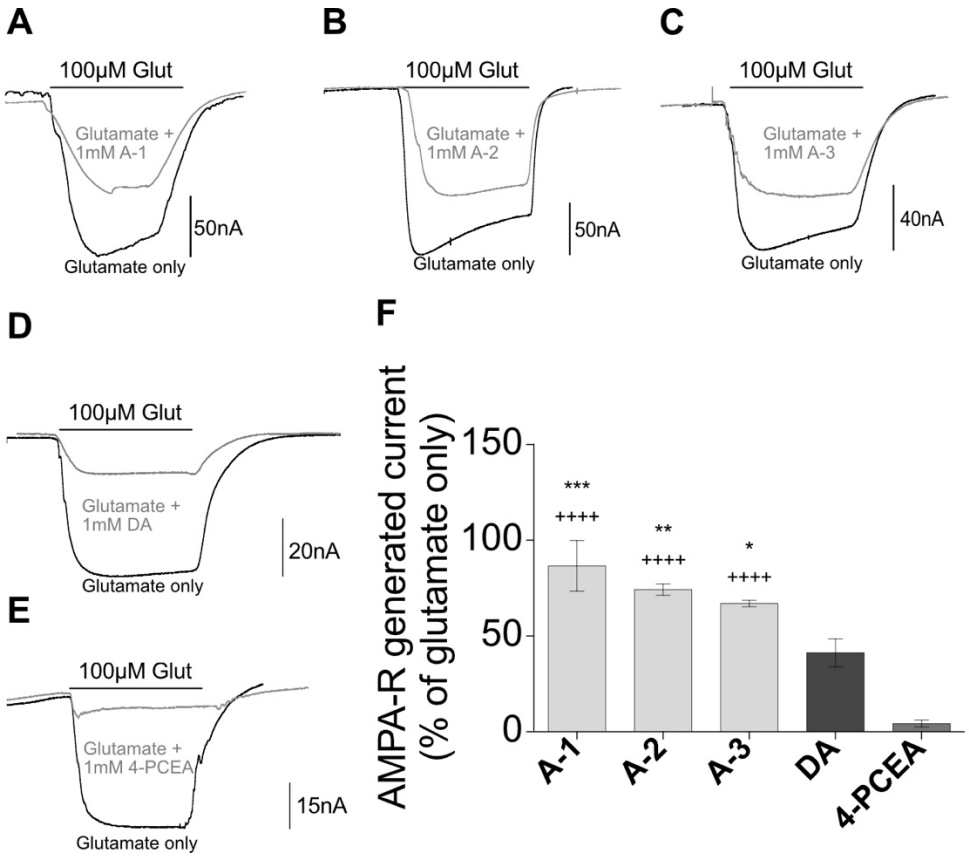



*Figure 4.3 Activity of fatty acid analogues containing amine substitutions against AMPA receptors.* Currents were evoked with 100μM in *Xenopus* oocytes expressing recombinant GluA2/3. (**A** to **C**) Representative trace recording showing the effects of 1mM A-1 (**A**), A-2 (**B**) and A-3 (**C**), as well as 1mM decanoic acid (DA, **D**) and 4-PCEA (**E**) (grey lines) on GluA2/3 evoked currents compared to glutamate only control (black lines). (**F**) Bar graph quantifying average AMPA receptor activity normalised to control in the presence of the three analogues and comparing it to the average inhibition by decanoic acid (DA) and 4-PCEA. Statistical analysis was carried relative to DA (stars) and 4-PCEA (crosses). Each bar corresponds to the mean and SEM of 5 to 10 recordings. Statistical analyses were carried out respective to DA (stars) or 4-PCEA (crosses) using ANOVA, where * corresponds to $p < 0.05$; ** corresponds to $p < 0.01$; *** corresponds to $p < 0.001$, and ++++ corresponds to $p < 0.0001$.

### *4.2.3.2 Oxygen substitutions cyclic aliphatic chains*

To further investigate the importance of nonpolar groups in the aliphatic chain of medium-chain fatty acids, analogues containing ether-groups in the butyl chain (summarised in table 4.3) were screened for inhibition of GluA2/3.

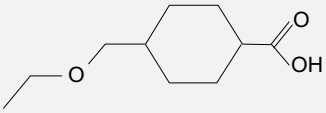
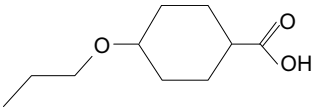
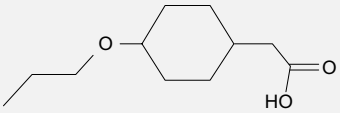
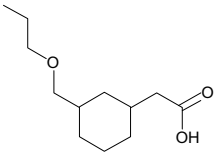
| Internal ID | ID | Name | Structure |
|---|---|---|---|
| RW177 | **O-1** | 4-(ethoxymethyl)cyclohexane-1-carboxylic acid |  |
| RW180 | **O-2** | 4-propoxycyclohexane-1-carboxylic acid |  |
| RW183 | **O-3** | 2-(4-propoxycyclohexyl)acetic acid |  |
| RW175 | **O-4** | 2-[4-(propoxymethyl)cyclohexyl]acetic acid |  |

*Table 4.3 Overview of allocated IDs for fatty acid analogues containing ether groups. Compounds are identified by their internal ID, allocated IDs (in bold), IUPAC names and chemical structures.*

For all four oxygen-substituted compounds, inhibition of GluA2/3 was less compared to decanoic acid, with O-1 and O-3 showing minimal reduction (13.3% and 2.8%, respectively) of GluA2/3 generated currents (SEM = 3.3 and n = 8 for O-1; and SEM = 1.0 and n = 8 for O-3). The longest chain analogue tested in this group, O-4, reduced currents by 22.6% (SEM = 8.8, n = 6). Surprisingly the strongest inhibition was seen in the compound with the shortest aliphatic chain, O-2, which inhibited GluA2/3 generated currents by 34.2% (SEM = 3.7, n = 8; figure 4.4 A to D). All of the compounds screened in this group were significantly less potent than both decanoic acid and 4-PCEA (figure 4.4 E, *p*-values summarised in table 4.4), supporting the hypothesis that nonpolar interactions are required for efficient AMPA receptor inhibition.

| ID | % normalized GluA2/3 inhibition | SEM | N | *p*-value compared to decanoic acid | *p*-value compared to 4-PCEA |
|---|---|---|---|---|---|
| **O-1** | 13.3 | 3.3 | 8 | <0.0001 | <0.0001 |
| **O-2** | 2.8 | 1.0 | 8 | 0.0078 | <0.0001 |
| **O-3** | 22.8 | 8.8 | 8 | <0.0001 | <0.0001 |
| **O-4** | 34.2 | 3.7 | 6 | 0.0002 | <0.0001 |

*Table 4.4 Summary of mean GluA2/3 inhibition by fatty acid analogues containing oxygen substitutions. Inhibition is normalised as % of control, SEM values, N-numbers and p-values.*

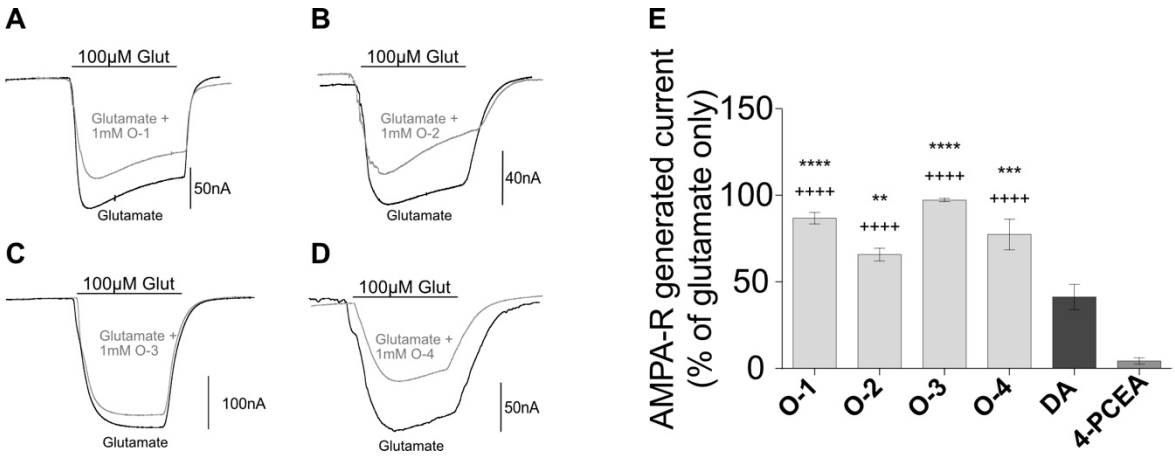



*Figure 4.4 AMPA receptor inhibition by of fatty acid analogues containing oxygen substitutions.* Recombinant GluA2/3 was expressed in *Xenopus* oocytes and currents were evoked using 100µM glutamate. (**A** to **D**) Representative trace recording showing the effects of 1mM O-1 (**A**), O-2 (**B**), O-3 (**C**) and O-4 (**D**) (grey lines) on GluA2/3 evoked currents compared to glutamate only control (black lines). (**E**) Bar graph quantifying average AMPA receptor activity normalised to control in the presence of the four analogues and comparing it to the average inhibition by decanoic acid (DA) and 4-PCEA. Statistical analysis was carried out relative to DA (stars) and 4-PCEA (crosses) using ANOVA, where ** corresponds to $p < 0.01$, *** corresponds to $p < 0.001$ and ****/++++ corresponds to $p < 0.0001$. Each bar corresponds to the mean and SEM of 5 to 10 recordings.

### *4.2.3.3 Additional methyl-branching in cyclic aliphatic chains*

Branching in the form of ethyl side chains and ring structures increased the inhibitory activity of medium-chain fatty acids. Therefore, cyclic aliphatic chains containing additional methyl branching in their butyl or pentyl chains (summarised in table 4.5) were assessed for GluA2/3 inhibition.

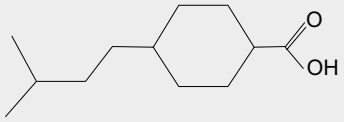
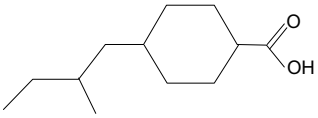
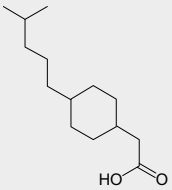
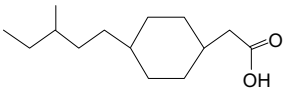
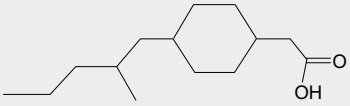
| Internal ID | ID | Name | Structure |
|---|---|---|---|
| RW181 | **M-1** | 4-(3-methylbutyl)cyclohexane-1-carboxylic acid |  |
| RW184 | **M-2** | 4-(2-methylbutyl)cyclohexane-1-carboxylic acid |  |
| RW172 | **M-3** | 2[4-(4-methylpentyl)cyclohexyl]acetic acid |  |
| RW176 | **M-4** | 2-[4-(3-methylpentyl)cyclohexyl]acetic acid |  |
| RW185 | **M-5** | 2-[4-(2-methylpentyl)cyclohexyl]acetic acid |  |

*Table 4.5 Overview of allocated IDs for fatty acid analogues containing methyl-branching groups. Compounds are identified by their internal ID, allocated IDs (in bold), IUPAC names and chemical structures.*

All compounds in this screening group showed significantly stronger AMPA receptor inhibition at 1mM than decanoic acid (all values summarised in table 4.6) with M-5 showing greatest potency and reducing currents to 91.1% (SEM = 1.1, n = 8; figure 4.5). This reduction is comparable to the inhibition measured with 1mM 4-PCEA ($p$ = 0.7505 compared to 4-PCEA; figure 4.5).

| ID | % normalized GluA2/3 inhibition | SEM | N | *p*-value compared to decanoic acid | *p*-value compared to 4-PCEA |
|---|---|---|---|---|---|
| **M-1** | 87.2 | 2.9 | 8 | 0.0012 | 0.0025 |
| **M-2** | 79.4 | 1.6 | 8 | 0.0031 | 0.0006 |
| **M-3** | 80.9 | 7.3 | 8 | 0.0029 | 0.0050 |
| **M-4** | 84.9 | 1.6 | 8 | 0.0002 | 0.0388 |
| **M-5** | 91.1 | 1.1 | 8 | <0.0001 | 0.7505 |

*Table 4.6 Summary of mean GluA2/3 inhibition by fatty acid analogues containing additional methy- branching. Inhibition is normalised as % of control, SEM values, N-numbers and p-values.*

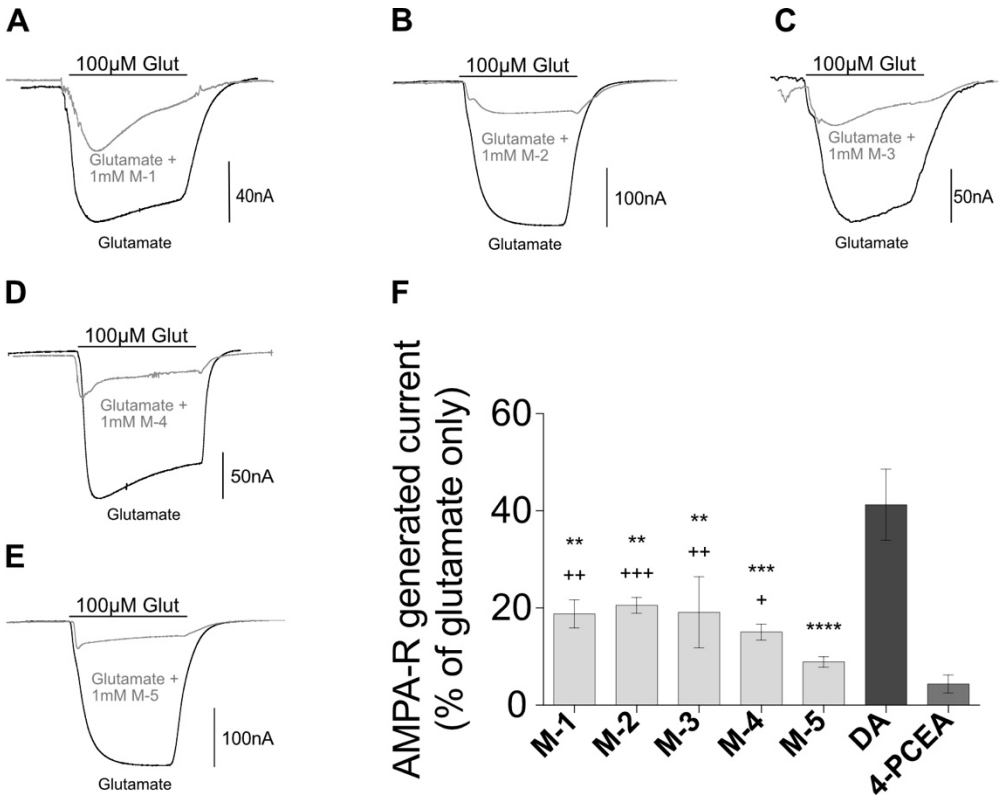



*Figure 4.5 AMPA receptor inhibition by a range of fatty acid analogues containing additional methyl-branching.* Single-dose response screening assays were carried out in GluA2/3 expressing *Xenopus* oocytes. Currents were evoked by 100µM glutamate (**A** to **E**) Representative trace recording showing the effects of 1mM M-1 (**A**), M-2 (**B**), M-3 (**C**), M-4 (**D**) and M-5 (**E**) (grey lines) on GluA2/3 evoked currents compared to glutamate only control (black lines). (**F**) Bar graph quantifying average AMPA receptor activity normalised to control in the presence of the five analogues and comparing it to the average inhibition by decanoic acid (DA) and 4-PCEA. Statistical analysis was carried out using ANOVA relative to DA (stars) and 4-PCEA (crosses), where + corresponds to $p < 0.05$, **/++ corresponds to $p < 0.01$, ***/+++ corresponds to $p < 0.001$ and **** corresponds to $p < 0.0001$. Each bar represents the mean and SEM of 5 to 10 recordings.

### *4.2.3.4 Introduction of double bonds into cyclic aliphatic chains*

As described above, fatty acid analogues that contain groups providing a greater degree of steric hindrance show stronger AMPA receptor inhibition than straight chain fatty acids. This may be due to a reduced flexibility of the aliphatic chain in these compounds. Therefore, two compounds that in addition to a cyclohexyl group contain a double bond in the butyl and pentyl aliphatic chain respectively (summarised in table 4.7), were screened for GluA2/3 inhibition.

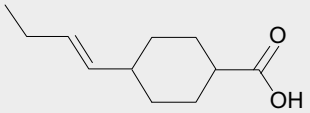
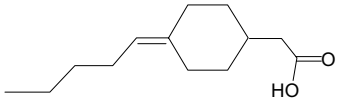
| Internal ID | ID | Name | Structure |
|---|---|---|---|
| RW174 | **D-1** | 4-(but-1-en-1-yl)cyclohexane-1-carboxylic acid |  |
| RW182 | **D-2** | 2-(4-pentylidenecyclohexyl)acetic acid |  |

*Table 4.7 Overview of allocated IDs for fatty acid analogues containing double bonds. Compounds are identified by their internal ID, allocated IDs (in bold), IUPAC names and chemical structures.*

Both compounds showed strong AMPA receptor inhibition with D-1 displaying similar potency to decanoic acid (all values summarised in table 4.8) and D-2 being significantly more potent ($p$ = 0.0005 compared to decanoic acid). However, even though structurally identical to 4-PCEA with the exception of the double bond, D-2 was significantly less potent than 4-PCEA, suggesting that the loss in flexibility in this analogue does not result in improved inhibition.

| ID | % normalized GluA2/3 inhibition | SEM | N | p-value compared to decanoic acid | p-value compared to 4-PCEA |
|---|---|---|---|---|---|
| **D-1** | 51.0 | 2.7 | 6 | 0.3841 | <0.0001 |
| **D-2** | 86.4 | 2.0 | 7 | 0.0005 | 0.0096 |

*Table 4.8 Summary of mean GluA2/3 inhibition by fatty acid analogues containing double bonds. Inhibition is normalised as % of control, SEM values, N-numbers and p-values.*

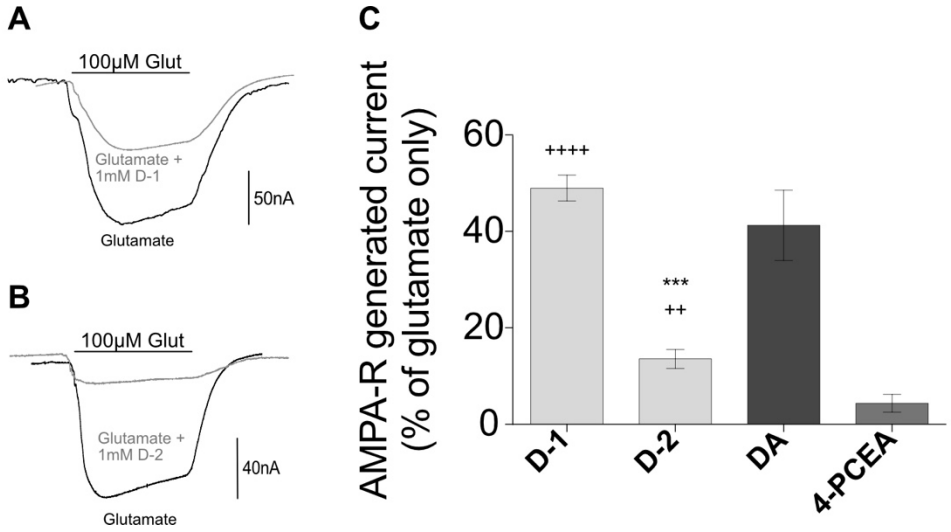



*Figure 4.6 AMPA receptor inhibition by a range of fatty acid analogues containing double bonds.* Summary of single-dose response screening assays assessing the inhibition of GluA2/3 generated currents in Xenopus oocytes. Currents were evoked by 100μM glutamate (**A** and **B**) Representative trace recording showing the effects of 1mM D-1 (**A**) and D-2 (**B**) (grey lines) on GluA2/3 evoked currents compared to glutamate only control (black lines). (**C**) Bar graph quantifying average AMPA receptor activity normalised to control in the presence of the two analogues and comparing it to the average inhibition by decanoic acid (DA) and 4-PCEA. Statistical analysis was carried out by ANOVA relative to DA (stars) and 4-PCEA (crosses), where ++ corresponds to $p < 0.01$, *** corresponds to $p < 0.001$ and ++++ corresponds to $p < 0.0001$. Each bar corresponds to the mean and SEM of 5 to 10 recordings.

## 4.3 Discussion

The results presented in this chapter show that modification of medium-chain fatty acids can improve AMPA receptor antagonism significantly, a finding that may provide a means to improve the existing MCT ketogenic diet or a new approach for drug development. Whilst the ketogenic diets are well-established treatment options in epilepsy, their side effects and time-consuming management make them a last resort option rather than a standard treatment for many patients (Rubenstein *et al.*, 2005; Kossoff, Zupec-Kania and Rho, 2009). Efforts to optimise the ketogenic diets for lower fat content and greater flexibility in food preparation have resulted in the use of alternative diets including the MCT ketogenic diet, low-glycaemic index diets and Modified Atkins diets, all of which have a lower fat to carbohydrate ratio than the classic ketogenic diet. However, side effects due to the high fat content are still one of the main reasons for patients to discontinue the treatment (Elizabeth G Neal *et al.*, 2009; Levy *et al.*, 2012). It may therefore be worthwhile to investigate whether using AMPA receptor inhibition by medium-chain fatty acids as a basis for future drug development could provide similar or better seizure control than the ketogenic diet, but without the need to adjust carbohydrate and fat intake.

### 4.3.1 Branching at carbon 4 increases AMPA receptor inhibition in a chain-length specific manner

The branched derivative of octanoic acid, 4-ethyloctanoic acid, was shown to be significantly more potent than octanoic acid. Inhibition of AMPA receptors by octanoic acid compared to the activity of the other main constituent of the MCT ketogenic diet, decanoic acid, is much weaker, as was shown in chapter 3 (page 72). In fact, the octanoic-dependent antagonism is negligible within the range of physiologically relevant concentrations (Haidukewych, Forsythe and Sills, 1982), with an extrapolated $IC_{50}$ estimated to be 3.82mM. Introduction of a single ethyl chain at carbon 4 in 4-ethyloctanoic acid, however, improved inhibition significantly and reduced the $IC_{50}$ by 48% to 2mM.

Initial studies that assessed the potential for medium-chain fatty acids in suppressing excitatory signalling in hippocampal slices have proposed that introducing a side chain to these fatty acids increases their ability to reduce seizure-like activity (Chang *et al.*, 2012, 2015). For instance, in slices where epileptiform activity was induced by PTZ, decanoic acid reduced seizures by 98% whereas octanoic acid provided no seizure protection. By comparison, in those experiments, the addition of the ethyl side-chain in 4-ethyloctanoic acid changed the profile for the eight-carbon chain fatty acid

dramatically, potently reducing seizures by 95% and therefore providing a degree of seizure prevention that was comparable to decanoic acid (Chang *et al.*, 2015). This effect is strikingly similar to the results shown here, and the fact that simple branching improves both inhibition of AMPA receptors and EPSCs to a similar degree relative to the unbranched compounds strongly supports a model of seizure control through AMPA receptor antagonism by medium-chain fatty acids.

Since it is not clear how medium-chain fatty acids interact with AMPA receptors, it can only be speculated at this point how the introduction of a side-chain results in improved inhibition. It is for instance possible that the ethyl-chain mimics a transient folding of the aliphatic chain that improves interaction with the receptor. Indeed, the significantly stronger inhibition of AMPA receptors by 4-BCCA and 4-PCEA, both of which contain a cyclohexyl-group at carbon 2 which constitutes a more rigid structure, suggests that improved binding of medium-chain fatty acids to the receptor by 4-ethyloctanoic acid may be due to a semi-circular structure formed by the side chain. Interestingly, 4-BCCA, which in chain-length is most similar to octanoic acid, inhibited AMPA receptors with an $IC_{50}$ of 0.6mM compared to 4mM by octanoic acid. This suggests that the branching alone results in an almost eight-fold increase in efficacy. For 4-PCEA, which in chain length is most similar to decanoic acid, the increase in efficacy is slightly more pronounced with a ten-fold stronger GluA2/3 inhibition compared to decanoic acid, giving an $IC_{50}$ of 0.05mM compared to 0.5mM, respectively. These results therefore support the hypothesis that targeted modifications to medium-chain fatty acids could be investigated to improve the existing ketogenic diets or even to provide a basis on which more effective treatments in epilepsy through AMPA receptor inhibition could be developed.

To confirm their potential role in seizure protection and epilepsy drug development, 4-ethyloctanoic acid, 4-BCCA and 4-PCEA were assessed in three different seizure models in the scope of the anti-epileptic drug screening programme carried out by the National Institute of Neurological Disorders and Stroke (NINDS). These experiments show promising efficacies for 4-ethyloctanoic acid in slices, kindled rats, and for 4-BCCA in the 6Hz seizure model when administered by intraperitoneal injection (Appendix II.i to II.iv). This confirms the potential for branched medium-chain fatty acids in seizure control and predicts a possible role in a range of seizure types since kindled animals are commonly used to mimic chronic, complex partial seizures, whereas the 6Hz model is thought to be useful to predict efficacy in treatment-resistant epilepsies (Barton *et al.*, 2001; Grone and Baraban, 2015). The efficacy of 4-PCEA was confirmed in the MES rat model of acute seizures. This suggests further applicability of branched medium-chain fatty acids in the treatment of generalised tonic-clonic seizures (Appendix II.v, Castel-Branco *et al.* 2009). However, when administered orally, seizure protection by 4-BCCA was lost (Appendix II.iv), a result that demonstrates that, despite promising

screening data, reduced bioavailability of branched medium-chain fatty acids compared to unbranched precursors could be a crucial limiting property in drug development.

### 4.3.2 Carbon substitutions reduce AMPA receptor antagonism by medium-chain fatty acids

Since of the medium-chain fatty acids initially screened in this chapter, 4-PCEA and 4-BCCA were the most potent AMPA receptor antagonists, further screening was based on these structures with additional substitutions of $CH_2$ with either oxygen atoms or amine groups. Surprisingly, introduction of a polar amine group either in the cyclic aliphatic chain or butyl chain significantly lowered AMPA receptor inhibition making the screened compounds less potent than decanoic acid. Similarly, introducing an ether group reduced inhibition dramatically with no noticeable effect on AMPA receptor activity at 1mM. These results suggest that increasing polarity reduces AMPA receptor inhibition, and that these residues are therefore important for binding to the protein.

### 4.3.3 Increasing sterical hindrance and chain fixation through additional branching and double bonds maintains inhibition of AMPA receptors

In contrast to substituting non-polar residues with more polar ones, decreasing the flexibility of the fatty acid carbon-chain through double bonds and additional methyl-branching maintained potent inhibition. However, compounds with chain-lengths comparable to 4-PCEA did not show increased inhibition compared to this analogue and, in fact, with the exception of M-5 (2-[4-(2-methylpentyl)cyclohexyl]acetic acid ) were significantly less potent than 4-PCEA.

To validate the efficacy of compounds containing additional methyl-branching and double bonds, M-1, M-2 and M-4, as well as D-2 were assessed in animal models. In these studies, which were carried out by the NINDS programme, compounds M-1, M-2 and M-4 showed limited efficacy against the 6Hz and MES seizure models, and significant toxicity as measured by behavioural toxicity screens was observed for M-2. Animals treated with D-2 showed no observable seizure control (Appendix II.v). These results clearly highlight the complexity of targeted drug design and the importance of validating screening data in animal models as simple screening models cannot predict loss of bioavailability due to absorption, first-pass metabolism or inefficient transport to the brain.

# CHAPTER 5 – INVESTIGATING THE MECHANISM OF AMPA RECEPTOR INHIBITION BY DECANOIC ACID

## 5.1 Introduction

Common treatments for epilepsy and seizures focus either on increasing inhibitory signalling in the brain or reducing excitation. Anti-seizure drugs that target excitatory signalling most commonly act by blocking voltage-gated sodium and calcium channels, with some having been reported to have off-target effects on the ion-channel glutamate receptors, AMPA, kainate and NMDA receptors (Rogawski and Löscher, 2004). The AED that was specifically developed to target glutamate receptors, the AMPA receptor antagonist perampanel, has only recently been approved for the treatment of drug-resistant epilepsy (M. A. Rogawski, 2013; Rogawski and Hanada, 2013).

Perampanel is recommended as an adjuvant treatment for drug-resistant epilepsy (Serratosa *et al.*, 2013), and may therefore be used in conjunction with other AEDs and ketogenic diets. The approval of perampanel and confirmation of its efficacy in clinical trials (French *et al.*, 2012, 2013) supports a role for medium-chain fatty acids in seizure control by direct AMPA receptor inhibition, but also raises the question of potential drug-diet interactions due to the shared target. In order to better understand AMPA receptor inhibition by decanoic acid and branched derivatives, and predict potential interactions with other AMPA receptor antagonists, it is therefore necessary to characterise the mechanism of inhibition by decanoic acid compared to other existing inhibitors.

Two types of inhibitors that do not bind to AMPA receptors in a competitive fashion are polyamines and the GYKI-related compounds. Polyamines non-selectively inhibit calcium-permeable ion channels by binding to the ion-conducting pore and thereby physically restricting ion flow (Donevan and Rogawski, 1998; Andersen *et al.*, 2006). The GYKI-related compounds belong to the group of benzodiazepines that have been shown to bind at the linker regions between transmembrane domains and extracellular sequences of AMPA receptors (Balannik *et al.*, 2005). In this chapter, a range of competition experiments were used to determine the effect of decanoic acid on the inhibitory activity of the polyamine spermine and GYKI-53665. In addition, specific residues in the AMPA receptor subunit GluA1 were mutated to assess potential binding sites of decanoic acid and. Finally, an effect on desensitisation was investigated through co-activation of AMPA receptor subunits 2/3 with the desensitisation inhibitor cyclothiazide (CTZ).

# 5.2 Results

## 5.2.1 Inhibition of AMPA receptors by decanoic acid and the AMPA receptor antagonist spermine is additive

The non-selective ion channel blocker spermine belongs to the group of polyamine channel blockers that obstruct calcium-permeable ion channels by binding to the channel-forming transmembrane regions, thereby physically blocking the flow of ions through the pore (figure 5.1 A). Structural similarities to straight-chain fatty acids (figure 5.1 B) suggests a possible shared mechanism of inhibition. To assess the likelihood of decanoic acid binding to the channel pore, a competition assay was carried out between spermine and decanoic acid. Initially, the potency of spermine against GluA1 homomeric AMPA receptor that was expressed in *Xenopus laevis* oocytes was assessed in dose-response assays. Spermine inhibited GluA1 with an $IC_{50}$ of 0.86mM (SEM = 0.14; n = 7). In order to determine any interference of decanoic acid with spermine, which could indicate competition for the same binding site, the dose-response assay was repeated with 1mM decanoic acid, which corresponds to half the $IC_{50}$ concentration, as determined previously for decanoic acid in competition assays with GluA1 ($IC_{50}$ = 2.1mM, page 71). All recordings were normalised to baseline inhibition by 100µM glutamate plus the respective decanoic acid concentrations to account for any increase in inhibition due to decanoic acid only. The addition of decanoic acid increased spermine potency against GluA1 slightly, but not significantly, lowering the $IC_{50}$ to 0.61mM (SEM = 0.07; n = 5; $p$ = 0.25 compared to spermine only). To determine if this additive effect was dose-dependent, the assay was repeated with 2mM decanoic acid added to the spermine dose-response assay. Again, spermine potency was increased, lowering the $IC_{50}$ significantly to 0.32mM (SEM = 0.09; n = 5; $p$ = 0.0099 for compared to spermine only; figure 5.1 C to E).

To determine if this additive effect was reversible, 250µM and 500µM spermine (concentrations that were optimised for solubility) were added to a decanoic acid dose-response assay against GluA1. The addition of spermine at either concentration increased overall decanoic acid potency and shifted the dose-response curves to the left in a dose-dependent manner, which is consistent with the data described above. Interestingly, the effect was more pronounced for decanoic acid than for spermine, significantly lowering the $IC_{50}$ of decanoic acid against GluA1 from 2.09mM (SEM = 0.05; n = 12) to 1.1mM when 250µM spermine was added (SEM = 0.1; n = 4; $p$ = 0.0021 when compared to decanoic acid only) and to 0.53mM when 500µM spermine was added (SEM = 0.03; n = 6; $p < 0.0001$ when compared to decanoic acid only; figure 5.1 F to H).

These results strongly indicate an additive effect of simultaneous administration of polyamines and medium-chain fatty acids in a dose-dependent manner and suggest that there is no competition between decanoic acid and spermine.

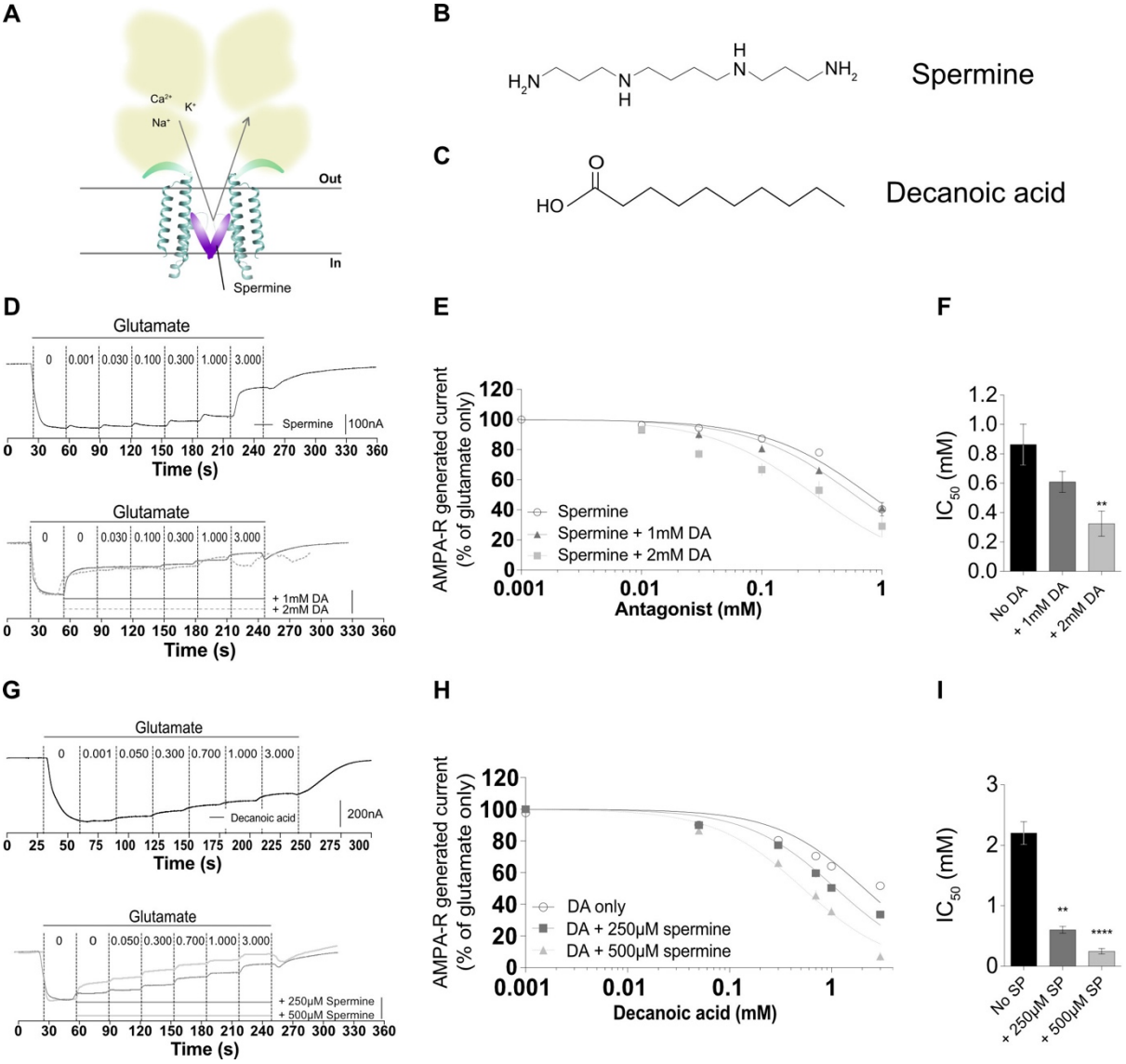



*Figure 5.1 Competition assay for the AMPA-receptor antagonists spermine (SP) and decanoic acid (DA).* Recombinant GluA1 was expressed in *Xenopus* oocytes and currents were evoked with 100µM glutamate. (**A**) Schematic showing proposed site of interaction of spermine at an AMPA receptor dimer. Chemical structures of spermine (**B**) and decanoic acid (**C**). (**D**) Representative trace recordings for dose-response curves of spermine only (in mM, black line, top) and spermine with added 1mM DA (in mM, solid line, bottom) and 2mM DA (dotted line, bottom) against GluA1. Currents were evoked with 100µM glutamate. Bottom scale bar corresponds to 110nA (+1mM DA) and 50nA (+2mM DA). (**E** and **F**) Sigmoidal dose-response curves and bar graph showing positive effect of DA co-application on GluA1 inhibition by spermine. Each point represents the mean and SEM of 5 to 7 recording readings. Statistical analysis was done by ANOVA where ** corresponds to $p < 0.01$. (**G**) Representative trace recordings for dose-response curves of DA only (in mM, black line, top) and DA with added 250µM spermine (in mM, dark grey line, bottom) and 500µM spermine (light grey line, bottom) against GluA1. Currents were evoked by 100µM glutamate. Bottom scale bar corresponds to 110nA (+250µM SP) and 70nA (+500µM SP). (**H** and **I**) Comparison of the sigmoidal dose response curves and bar graph showing a positive effect of added spermine on inhibition of GluA1 by decanoic acid and IC50s for the competition assay. Each point represents the mean and SEM of 4 to 12 recording readings. Statistical analysis was done by ANOVA, where ** corresponds to $p < 0.01$ and **** corresponds to $p < 0.0001$.

### 5.2.2 Co-application of decanoic acid reduces AMPA receptor sensitivity to GYKI-53665

The group of GYKI–related compounds comprises a number of analogues that contain multiple ring structures and have been shown to bind to residues in the linker regions between extracellular loop 1 and transmembrane domain 1 (S1-M1) as well as between extracellular loop 2 and transmembrane domain 4 (S2-M4; Balannik *et al.* 2005; fig 5.2 A and B). To assess whether decanoic acid and GYKI-related compounds share a binding mechanism, the AMPA receptor antagonist GYKI-53665 was assessed for potency against GluA2/3. In addition, the effect of ca. 50% and 100% $IC_{50}$ concentrations of decanoic acid against GluA2/3 as determined in chapter 3 ($IC_{50}$ = 0.52mM, page 70) was evaluated in a competition assay, where one concentration of decanoic acid is added to a dose-effect assay of GYKI-53665. As previously, data were normalised to baseline inhibition by 100µM glutamate plus the respective decanoic acid concentration to account for inhibition by decanoic acid only. In the absence of decanoic acid, GYKI-53665 potently inhibited AMPA receptors comprised of subunits GluA2/3 potently with an $IC_{50}$ of 4.9µM (SEM = 0.3; n = 6). The addition of 250µM decanoic acid reduced GYKI potency slightly shifting the dose-response curve to the right, but not significantly changing the $IC_{50}$ ($IC_{50}$ = 6.8µM; SEM = 0.8; n = 5; $p$ = 0.13 compared to GYKI only). Addition of 500µM decanoic acid lowered the $IC_{50}$ significantly to 8.2µM (SEM = 0.8; n = 5; $p$ = 0.0054 compared to GYKI-53665 only; figure 5.2 C to E), suggesting that, at concentrations higher than 250µM, decanoic acid negatively modulates the inhibition of the AMPA receptor by GYKI-53665.

To determine if this reduction in potency was independent of AMPA receptor subunit composition, GYKI-53665 potency against GluA1 was assessed and compared to its potency in combination with 1mM and 2mM decanoic acid, again corresponding to ca. 50% and 100% $IC_{50}$ concentrations against GluA1. In the absence of decanoic acid, GYKI-53665 inhibited AMPA receptors comprised of homomeric GluA1 less potently than AMPA receptors consisting of subunits GluA2/3 ($IC_{50}$ = 0.027mM; SEM = 0.0032; n = 6). Addition of decanoic acid reduced GYKI-53665 antagonism in a similar manner as described for GluA2/3 with addition of 1mM decanoic acid (ca. 50% $IC_{50}$ concentration), having no significant effect on GYKI-53665 potency ($IC_{50}$ = 0.024mM; SEM = 0.002; n = 6; $p$ = 0.84 compared to GYKI-53665 only), whereas addition of 2mM decanoic acid significantly lowered the $IC_{50}$ to 0.043mM (SEM = 0.005; n = 8; $p$ = 0.018 compared to GYKI-53665 only; figure 5.2 F to H).

These data indicate that decanoic acid reduces AMPA receptor inhibition by GYKI-53665 in a dose-dependent manner and suggest that the two antagonists potentially compete with each other.

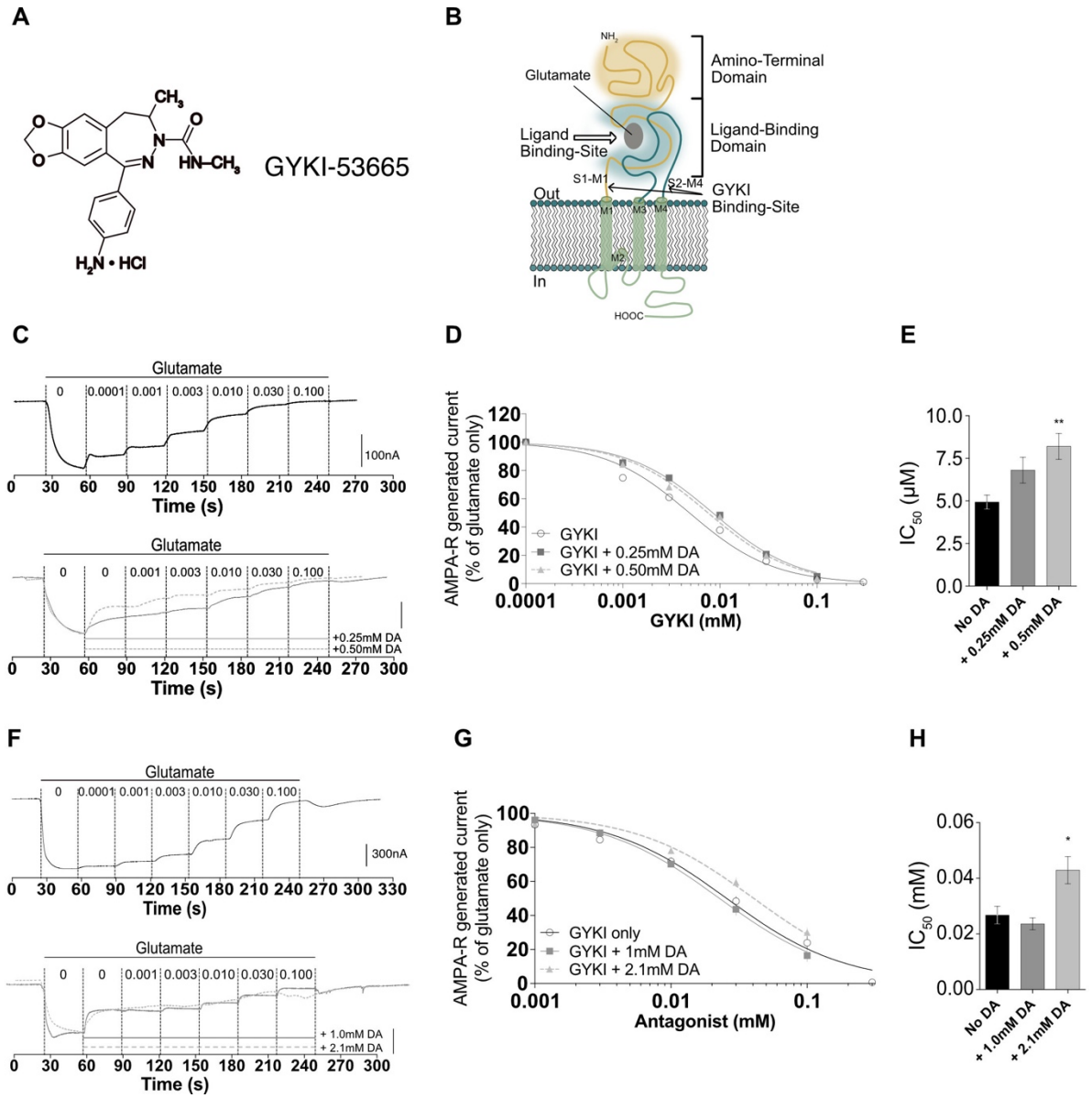



*Figure 5.2 Competition assays for the AMPA receptor antagonist GYKI-53665 (GYKI) and decanoic acid (DA*). GluA2/3 and GluA1 subunits were expressed in oocytes and currents evoked by 100µM glutamate. (**A**) Chemical structure of GYKI-53665. (**B**) Schematic of AMPA receptor subunit showing GYKI binding sites. (**C**) Representative trace recordings showing inhibition of GluA2/3 by GYKI only (in mM, black trace, top) and GYKI with added 0.25mM DA (solid trace, bottom) and 0.5mM DA (hashed trace, bottom). Bottom scale bars correspond to 35nA (+0.25mM DA) and 120nA (+0.5mM DA). (**D** and **E**). Sigmoidal plot and bar graph quantifying GYKI antagonism of GluA2/3 where each point and bar represents the mean and SEM of 5 to 6 recordings. Statistical analysis was carried out using ANOVA where ** corresponds to $p < 0.01$ (**F**) Representative trace recordings showing inhibition of GluA1 by GYKI only (in mM, black trace, top) and GYKI with added 1mM DA (solid trace, bottom) and 2.1mM DA (hashed trace, bottom). Bottom scale bars correspond to 300nA (+1mM DA) and 60nA (+2.1mM DA). (**G** and **H**) Sigmoidal plot and bar graph quantifying GYKI antagonism of GluA1 where each point and bar represents the mean and SEM of 6 to 8 recordings. Statistical analysis was carried out using ANOVA, where * corresponds to $p < 0.05$.

### 5.2.3 Co-application of the AMPA receptor antagonist GYKI reduces inhibition of GluA1 by decanoic acid

The competition assay between decanoic acid and GYKI-53663 was reversed to also assess whether GYKI-53663 modulates the potency of decanoic acid inhibition of glutamate receptors. In these experiments, two GYKI concentrations were selected on the basis of their similarity to the $IC_{50}$ calculated in section 5.2.2, whilst ensuring a sufficiently large difference between the two concentrations to minimise experimental error. Addition of either 4µM or 8µM GYKI-53663 to decanoic acid dose-response assays had no significant effect on GluA2/3 antagonism, with both assays returning similar $IC_{50}$s of 0.46mM (SEM = 0.06; n = 9; $p$ = 0.63 compared to decanoic acid only) and 0.62mM (SEM = 0.12; n = 9; $p$ = 0.88 compared to decanoic acid only), respectively. This is comparable to the $IC_{50}$ that was obtained for decanoic acid only in section 3.2.1 ($IC_{50}$ = 0.52mM, page 70).

The assay was repeated in GluA1-expressing oocytes with only one concentration of GYKI-53663. Here, addition of 25µM GYKI-53663 to decanoic acid significantly reduced inhibition, increasing the $IC_{50}$ from 2.1mM (see section 3.2.1, page 70) to 5.04mM (extrapolated; SEM = 1.20; n = 6; $p$ = 0.0058 compared to decanoic acid only). Since these results confirm the trend observed for GYKI-53663 that higher concentrations of co-applied antagonist reduce inhibition, repeating the assay with a second concentration of GYKI-53663 was omitted.

These results show that co-application of GYKI-53663 and decanoic acid reduces AMPA receptor inhibition.

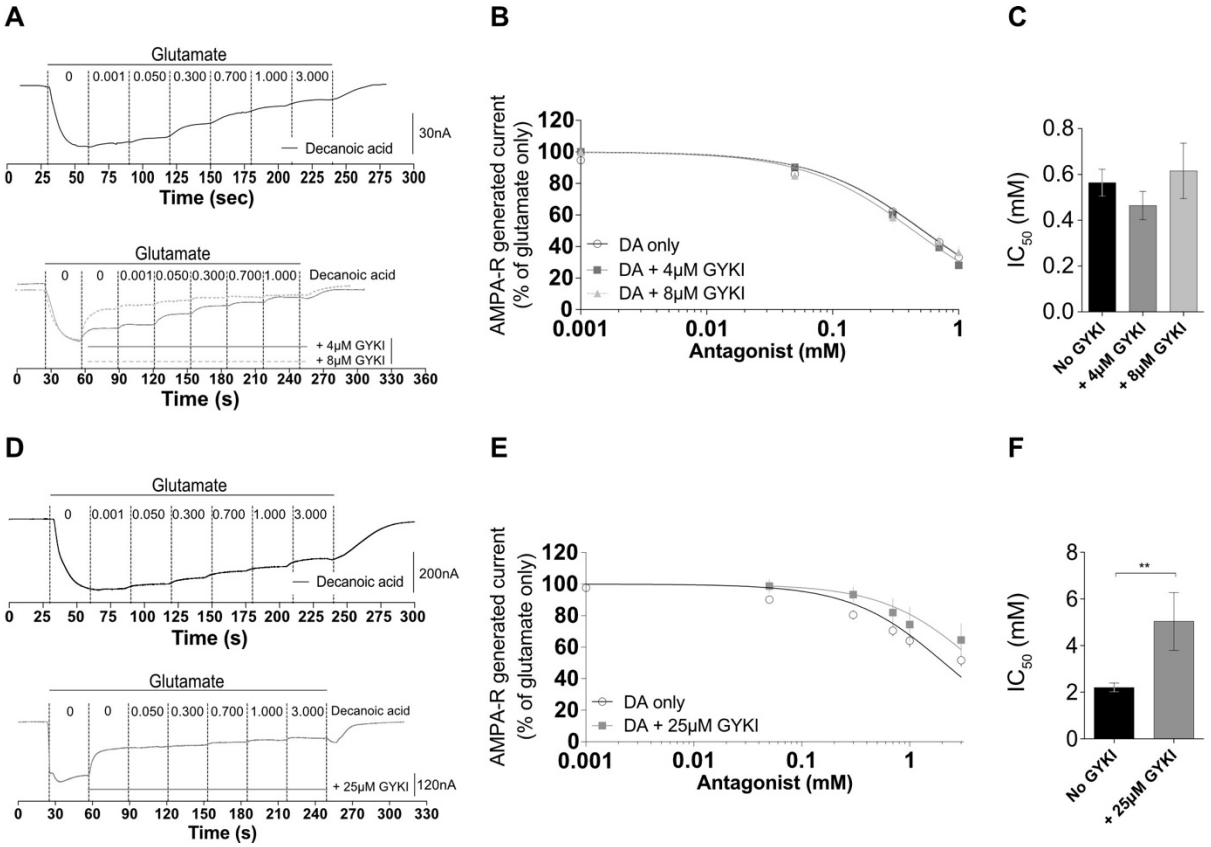



*Figure 5.3 Competition assays for the AMPA receptor antagonist decanoic acid (DA) with added GYKI-53665 (GYKI).* GluA2/3 and GluA1 subunits expressed in oocytes were activated by 100μM glutamate. (**A**) Representative trace recordings showing inhibition of GluA2/3 by DA only (in mM, black trace, top) and DA with added 4μM GYKI (solid trace, bottom) and 8μM DA (hashed trace, bottom). Bottom scale bars correspond to 35nA (+4μM GYKI) and 25nA (+8μM GYKI). (**B** and **C**) Sigmoidal plot and bar graph quantifying GluA2/3 antagonism by DA where each point and bar represents the mean and SEM of 9 to 12 recordings. Statistical analysis was carried out using ANOVA. (**D**) Representative trace recordings showing inhibition of GluA1 by DA only (in mM, top) and DA with added 25μM GYKI (bottom). (**E** and **F**) Sigmoidal plot and bar graph quantifying GluA1 antagonism by DA with and without GYKI where each point and bar represents the mean and SEM of 12 recordings. Statistical analysis was carried out using the *t*-test where ** corresponds to $p < 0.025$.

### 5.2.4 Decanoic acid inhibits AMPA receptors at a site distinct from the GYKI binding site

A shared binding site for decanoic acid and GYKI would explain the loss of potency against AMPA receptors that is observed upon co-application of the two compounds. A more direct way to determine a binding site for agonists or antagonists at a protein is the use of a mutated receptor protein. Therefore, a GYKI-resistant GluA3 mutant in which residues D543-E548 and T810-I888 were substituted for their homologues in kainate receptor GluK2 (figure 5.4 A), as well as AMPA receptors consisting of homomeric wild-type GluA3 (both cDNA constructs kindly provided by Prof. Stern-Bach, Hebrew University Jerusalem; Balannik *et al.* 2005) were assessed for sensitivity to both GYKI-53665 and decanoic acid in single-dose response assays. In these experiments, 100µM GYKI-53665 blocked wild-type GluA3-generated currents in *Xenopus* oocytes (99.6% inhibition; SEM = 0.4; n = 4), but showed significantly lower potency against the mutant GluA3 (35.4% inhibition; SEM = 13.7; n = 3; $p$ = 0.0025 compared to wild-type GluA3; figure 5.4 B and D). These data are consistent with the data published by Balannik *et al.* By comparison, 1mM decanoic acid reduced wild-type GluA3-generated currents by 74.5% (SEM = 6.4; n = 4), and showed no reduction in potency when assessed against the mutant GluA3 receptor (72.8% inhibition; SEM = 0.3; n = 3; $p$ = 0.839 compared to wild-type GluA3; figure 5.4 C and D). These data therefore show clearly that decanoic acid and GYKI-53665 do not share a binding site at the S1-M1 and S2-M4 linkers and that the negative regulation described in sections 5.2.2 and 5.2.3 is likely to be due to allosteric effects rather than a common binding site.

To conclusively rule out a shared binding site for medium-chain fatty acids and their analogues and GYKI, 4-ethyloctanoic acid, trans-4-butylcyclocarboxylic acid (4-BCCA) and 4-(pentylcyclohexyl)-ethanoic acid (4-PCEA) were assessed for potency against wild-type GluA3 and the GYKI-resistant mutant receptor. None of these analogues showed reduced activity against the mutant receptor (all data are summarised in table 5.1; figure 5.4 E to H), which is consistent with the data obtained with decanoic acid.

| Subunit | wtGluA3 | | | mutGluA3 | | | Statistical analysis |
|---|---|---|---|---|---|---|---|
| **Antagonist** | Inhibition (%) | SEM | N | Inhibition (%) | SEM | N | *p*-value |
| **4-EOA** | 38.0 | 4.9 | 9 | 40.5 | 9.5 | 8 | 0.900 |
| **4-BCCA** | 74.6 | 6.3 | 5 | 71.2 | 1.7 | 6 | 0.799 |
| **4-PCEA** | 78.5 | 6.1 | 7 | 72.2 | 5.9 | 7 | 0.436 |

*Table 5.1 Summary of experimental data for the branched analogues of medium-chain fatty acids. Data are summarised as average inhibition of wtGluA3 and the mutant (mutGluA3) by 1mM 4-ethyloctanoic acid (4-EOA), 1mM 4-BCCA and 100µM 4-PCEA. p-values refer to % inhibition of mutGluA3 compared to wtGluA3 of each antagonist.*

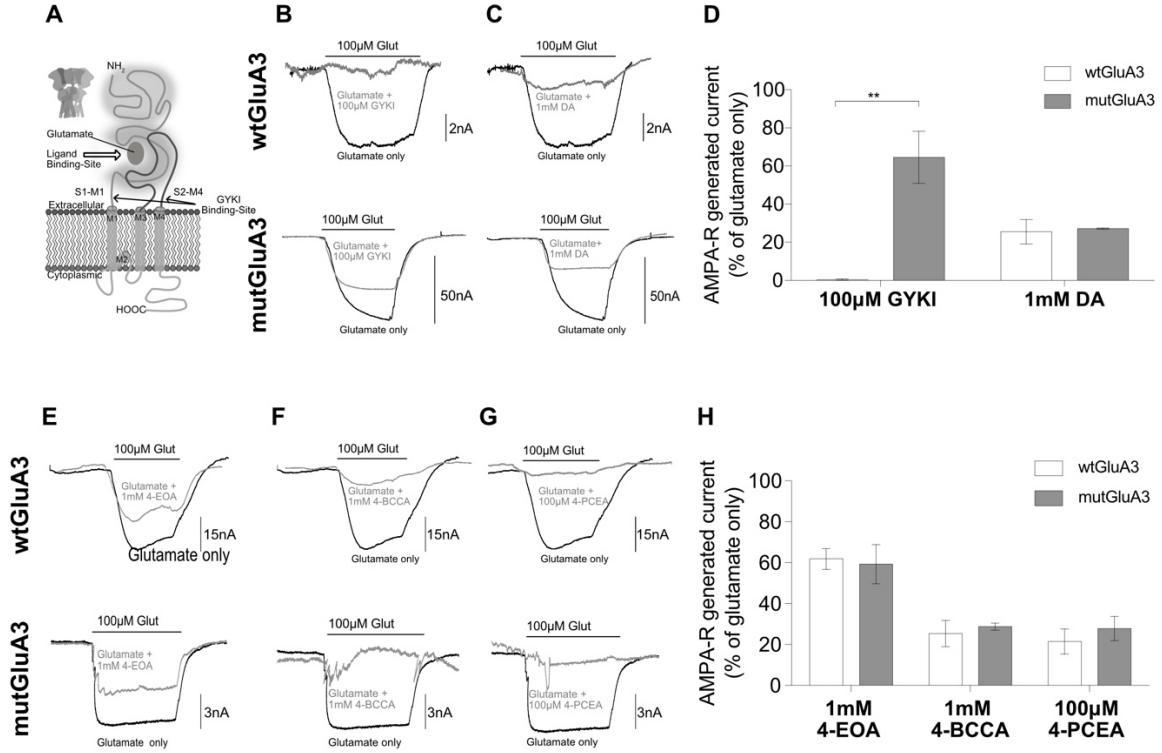



*Figure 5.4 Inhibition of the GYKI-resistant mutant GluA3 by decanoic acid.* Single-dose response assays were carried out in *Xenopus* oocytes expressing wild-type GluA3 (wtGluA3) and GYKI-resistant GluA3 (mutGluA3) subunits perfused with either 100µM glutamate (black lines) or 100µM glutamate + antagonist (grey lines). (**A**) Schematic showing single AMPA receptor subunit with mutated GYKI binding sites. (**B** and **C**) Traces representative of single dose response assays done in wtGluA3 (top row) and mutGluA3 (bottom row) for 100µM GYKI 53665 (GYKI, **B**) and 1mM decanoic acid (DA, **C**). (**D**) Bar graph quantifying average wtGluA3 (white bars) and mutGluA3 (grey bars) activity normalised to glutamate only control when perfused with either 100µM GYKI or 1mM DA. Each bar corresponds to the mean and SEM of 3 to 4 recordings. Statistical analysis was carried using student's *t*-test where ** corresponds to $p < 0.025$. (**E** to **G**) Traces representative of single dose response assays done in wtGluA3 (top row) and mutGluA3 (bottom row) for 1mM 4-ethyloctanoic acid (4-EOA, **E**), 1mM 4-BCCA (**F**) and 100µM 4-PCEA (**G**). (**H**) Bar graph quantifying average wtGluA3 (white bars) and mutGluA3 (grey bars) activity normalised to glutamate only control when perfused with either 1mM 4-EOA or 4-BCCA, or 100µM 4-PCEA. Each bar corresponds to the mean and SEM of 5 to 9 recordings. Statistical analysis was carried using student's *t*-test.

### 5.2.5 AMPA receptor inhibition by decanoic acid is reduced in the presence of the desensitisation inhibitor CTZ but unchanged if activated with kainate

One property that is characteristic of AMPA receptors is the fast rate of desensitisation, which is mediated through a shift of the extracellular domains that is triggered by glutamate binding (figure 5.6 A). The desensitisation inhibitor cyclothiazide (CTZ, figure 5.6 B) blocks this shift by binding to a serine residue in the S2 extracellular loop (Kovács *et al.*, 2004; Ptak, Ahmed and Oswald, 2009), resulting in prolonged AMPA receptor activation. To assess an effect of decanoic acid on receptor desensitisation, single dose-response assays were carried out for decanoic acid on GluA2/3 generated currents activated by 100µM glutamate, with and without 100µM CTZ, in *Xenopus laevis* oocytes (figure 5.5 C to H). In the absence of CTZ, the addition of 0.5mM decanoic acid reduced the generated currents by 55.4% (SEM = 6.6; n = 5). Addition of 100µM CTZ did not significantly affect decanoic acid potency (61.7% inhibition; SEM = 2.1; n = 5; $p$ = 0.0856 compared to 0.5mM decanoic acid only). However, addition of CTZ together with 1mM decanoic acid significantly reduced inhibition from a 78.4% reduction (SEM = 3.8; n = 5) to a 53.2% reduction when CTZ was added (SEM = 2.9; n = 5; $p$ = 0.0021 compared to 1mM decanoic acid only, figure 5.6 A to C).

To assess an effect of CTZ on branched derivatives of decanoic acid, GluA2/3 inhibition by the branched analogues 4-ethyloctanoic acid and 4-BCCA was determined during co-activation with 100µM glutamate and CTZ. Addition of 100µM CTZ reduced inhibition of GluA2/3 by 1mM 4-ethyloctanoic acid from 78.7% without CTZ (SEM = 6.1; n = 6) to a complete loss of inhibition with CTZ (-8.3% relative inhibition; SEM = 4.5; n= 5; $p$ = 0.0074 compared to 1mM 4-ethyloctanoic acid only; figure 5.6 D and F), which is consistent with the effect of CTZ on decanoic acid inhibitory activity of decanoic acid. Consistent with these results, addition of CTZ to 4-BCCA reduced inhibition from 64.1% without CTZ (SEM = 11.7; n = 6) to 37.9% with CTZ (SEM = 6.7; n= 5; $p$ = 0.0315 compared to 1mM 4-BCCA only; figure 5.6 E and F). Since the transition to the desensitised state is inhibited by CTZ, these data suggest that medium-chain fatty acids inhibit AMPA receptors by stabilising the desensitised conformation. Alternatively, the loss in activity could be explained by competition between CTZ and medium-chain fatty acids for a shared binding site at the receptor.

Reduced inhibition of AMPA receptors by decanoic acid upon co-application of CTZ may either be due to decanoic acid requiring the receptor to desensitise for efficient inhibition, or due to a shared binding site with CTZ. To confirm whether the loss of AMPA receptor inhibition by decanoic acid upon co-application of CTZ is due to the lack of desensitisation, a dose-response assay was carried out for decanoic acid. GluA2/3 generated currents were evoked by a saturating concentration of 1.7mM kainate (Traynelis *et al.*, 2010) instead of 100µM glutamate, and inhibition by decanoic acid was

compared to the inhibition provided against currents that were evoked by 100µM glutamate only or 100µM glutamate and 100µM CTZ. Consistent with the data from single-dose response assays, addition of 100µM CTZ significantly increased the $IC_{50}$ of decanoic acid from 0.52mM (see section 3.2.1 page 70) to 3.93mM (extrapolated $IC_{50}$; SEM = 0.56; n = 8; $p < 0.0001$ compared to decanoic acid only in glutamate-evoked currents). Decanoic acid-dependent AMPA receptor inhibition was independent of activation by glutamate or kainate, with similar potencies upon application of either agonist ($IC_{50}$ = 0.687mM; SEM = 0.047; n = 5; $p = 0.942$ compared to decanoic acid only in glutamate-evoked currents; figure 5.6 G to I). Kainate is thought to generate non-desensitising currents in AMPA receptors due to the involvement of distinct effects of kainate on protein interactions at the dimer interface compared to glutamate that result in weaker desensitisation (Hampson and Manalo, 1998; Levchenko-Lambert, Turetsky and Patneau, 2011). Therefore, these data which show no decrease in inhibition in kainate AMPA receptor activation imply that decanoic acid may not require desensitisation of the receptor to maintain potent inhibition.

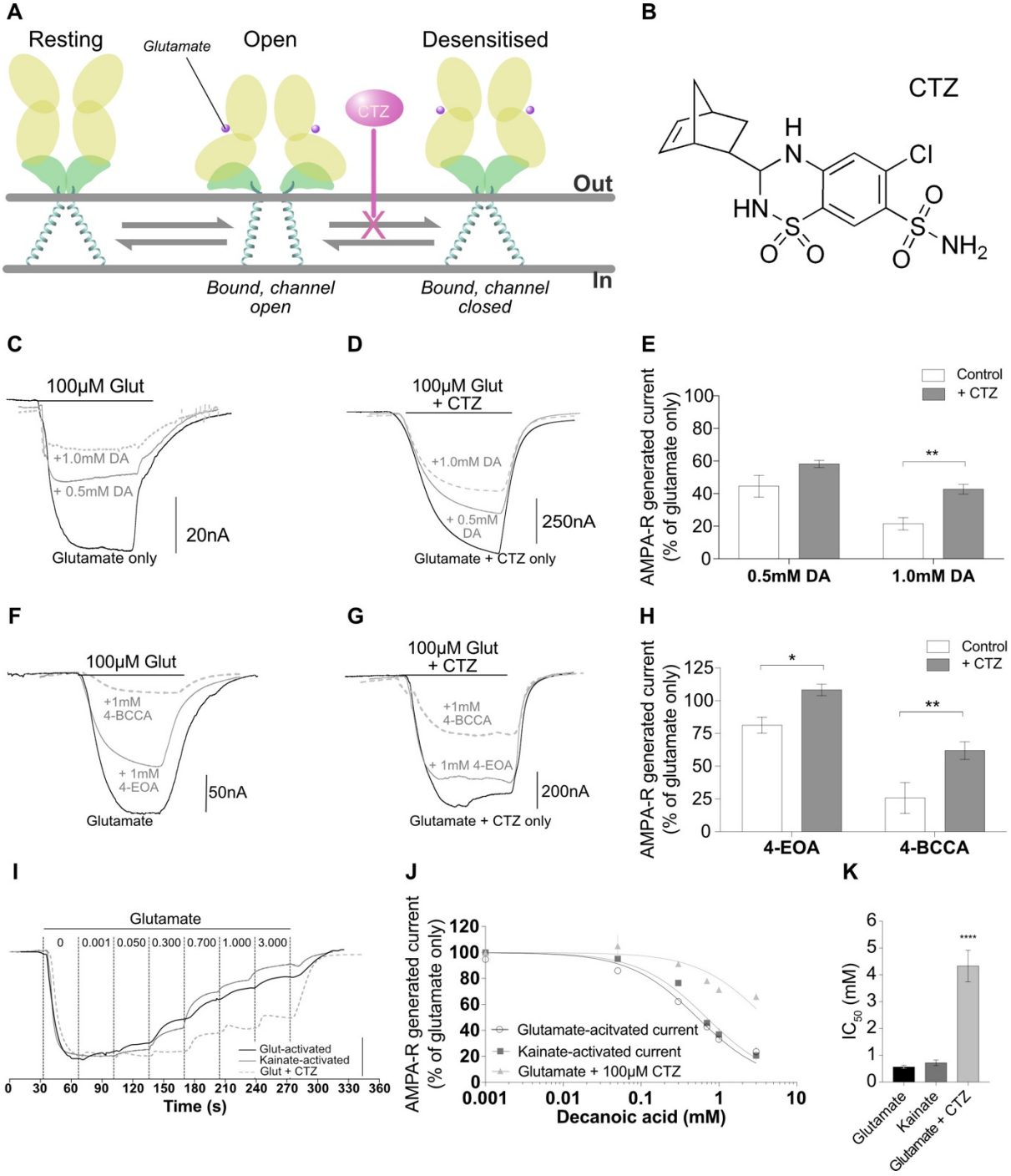

A
Resting
Glutamate
Open
Desensitised
CTZ
Out
In
Bound, channel open
Bound, channel closed
B
CTZ
H
N
Cl
HN
S
O
O
S
O
O
NH2
C
100μM Glut
+1.0mM DA
+ 0.5mM DA
20nA
Glutamate only
D
100μM Glut + CTZ
+1.0mM DA
+ 0.5mM DA
250nA
Glutamate + CTZ only
E
Control
+ CTZ
AMPA-R generated current (% of glutamate only)
0.5mM DA
1.0mM DA
F
100μM Glut
+1mM 4-BCCA
+ 1mM 4-EOA
50nA
Glutamate
G
100μM Glut + CTZ
+1mM 4-BCCA
+ 1mM 4-EOA
200nA
Glutamate + CTZ only
H
Control
+ CTZ
AMPA-R generated current (% of glutamate only)
4-EOA
4-BCCA
I
Glutamate
0
0.001
0.050
0.300
0.700
1.000
3.000
Glut-activated
Kainate-activated
Glut + CTZ
Time (s)
J
AMPA-R generated current (% of glutamate only)
Glutamate-acitvated current
Kainate-activated current
Glutamate + 100μM CTZ
Decanoic acid (mM)
K
IC50 (mM)
Glutamate
Kainate
Glutamate + CTZ

*Figure 5.5 The effect of the desensitisation inhibitor CTZ and non-desensitising receptor activation through kainate on GluA2/3 inhibition by decanoic acid (DA*). (**A**) Schematic showing AMPA receptor activation and desensitisation cycle. (**B**) Chemical structure of CTZ. (**C** and **D**) Traces representative of the single-dose response assays used to determine the effect of CTZ on DA potency, comparing to the effect of 0.5mM and 1mM DA (solid grey traces and dotted grey traces, respectively) on currents evoked by 100µM glutamate only (black traces, **C**) or by 100µM glutamate with added 100µM CTZ (black traces, **D**). (**E**) Bar graph summarising the average AMPA receptor activity in the presence of either DA concentration when activated by glutamate only (white bars) or glutamate + CTZ (grey bars). Each bar represents the mean and SEM of 5 experiments. Statistical analysis was carried out using the student's *t*-test where ** corresponds to $p < 0.01$. (**F** and **G**) Traces representative of the single-dose response assays used to determine the effect of CTZ on potency of 4-ethyloctanoic acid (4-EOA, solid grey traces) and 4-BCCA (dotted grey traces) on currents evoked by 100µM glutamate only (black traces, **F**) or by 100µM glutamate with added 100µM CTZ (black traces, **G**) (**H**) Bar graph summarising the average AMPA receptor activity in the presence of either 4-EOA or 4-BCCA when activated by glutamate only (white bars) or glutamate + CTZ (grey bars). Each bar represents the mean and SEM of 5 to 6 experiments. Statistical analysis was carried out using the student's *t*-test where * corresponds to $p < 0.05$ and ** corresponds to $p < 0.025$. (**I**) Trace recordings representative of the dose-response assay (in mM) carried out where currents were evoked by 100µM glutamate only (black trace), 1.7mM kainate (solid grey trace) and 100µM glutamate + 100µM CTZ (dotted grey trace). Scale bars correspond to 30nA (glutamate only), 200nA (kainate only) and 800nA (glutmate + CTZ). (**J** and **K**) Sigmoidal dose-response curve and bar graph quantifying GluA2/3-generated currents in the presence of the three agonist combinations and DA where each point and bar represents the mean and SEM of 5 to 12 recordings. Statistical analysis was carried out using the student's *t*-test where **** corresponds to $p < 0.0001$.

### 5.2.6 Investigation of a proposed novel binding site for decanoic acid at the AMPA receptor

Since the experiments described above suggest that a shared binding site for both decanoic acid and GYKI-related compounds at the linker regions is unlikely, alternative, new binding sites were investigated for decanoic acid. To determine potential target residues at the receptor, a computational analysis was carried out by Prof Jörg Hardege and Dr John Terschak (University of Hull, UK), which indicated an increased likelihood for medium-chain fatty acids to bind to residue P614 of GluA2 at the intracellular end of transmembrane domain 3 (TM3; Chang *et al.* 2016; figure 5.5 A). This residue corresponds to P610 in GluA1 (fig 5.5 B). To assess binding of decanoic acid to this residue, a point mutation was introduced by site-directed mutagenesis to replace the proline residue with an alanine (P610A, figure 5.5 C) in the GluA1 subunit. GluA1 was selected for consistently stable expression and reliable current stability.

In addition, the reduced activity of decanoic acid upon co-application of CTZ (as described above) suggests that decanoic acid may have a possible role in stabilising the desensitised conformation of the receptor. However, decanoic acid maintained potency towards AMPA receptors that were activated with kainate, arguing against this possibility. To assess a possible link between decanoic acid and receptor desensitisation more directly, the S2-M3 linker region was also mutated since desensitisation of AMPA receptors is transmitted through this region (Yelshansky *et al.*, 2004). Therefore, in addition to mutant P610A, two more mutants (V644A and P646A, figure 5.5 B and C) were generated through site-directed mutagenesis targeting the residues that have previously been suggested to be involved in receptor desensitisation.

To assess the inhibitory effect of decanoic acid on the three mutant GluA1 subunits, all three mutant cRNAs were expressed in *Xenopus laevis* oocytes. However, no loss of inhibition was observed for decanoic acid in any of the mutants when compared to the wtGluA1 subunit (summarised in table 5.2; figure 5.5 D to F). These data therefore do not support the computational analysis and, therefore, a potential binding site for decanoic acid remains to be identified. However, it is possible that single point mutations were not sufficient to disrupt interactions of decanoic acid with the receptor, if additional residues are involved in binding that were not identified or mutated. In addition, interestingly, the maximal responses measured in V644A were consistently lower than those measured in the wtGluA1 and the other two mutants (figure 5.5 D, collective data not shown), suggesting that V644 rather than P646 may be critically involved in transmitting channel gating properties.

| GluA1 type | $IC_{50}$ (mM) | SEM | N | *p*-value |
|---|---|---|---|---|
| **wtGluA1** | 1.45 | 0.03 | 8 | n/a |
| **P610A** | 1.47 | 0.04 | 4 | 0.720 |
| **V644A** | 1.23 | 0.03 | 4 | 0.958 |
| **P646A** | 1.18 | 0.02 | 4 | 0.989 |

*Table 5.2 Summary of $IC_{50}$s for decanoic acid against wtGluA1 and the three generated mutants. Data are listed as $IC_{50}$s, SEMs, number of repeats (n) and p-values for wtGluA1 and the three generated mutants. Statistical analysis was carried out relative to wtGluA1.*

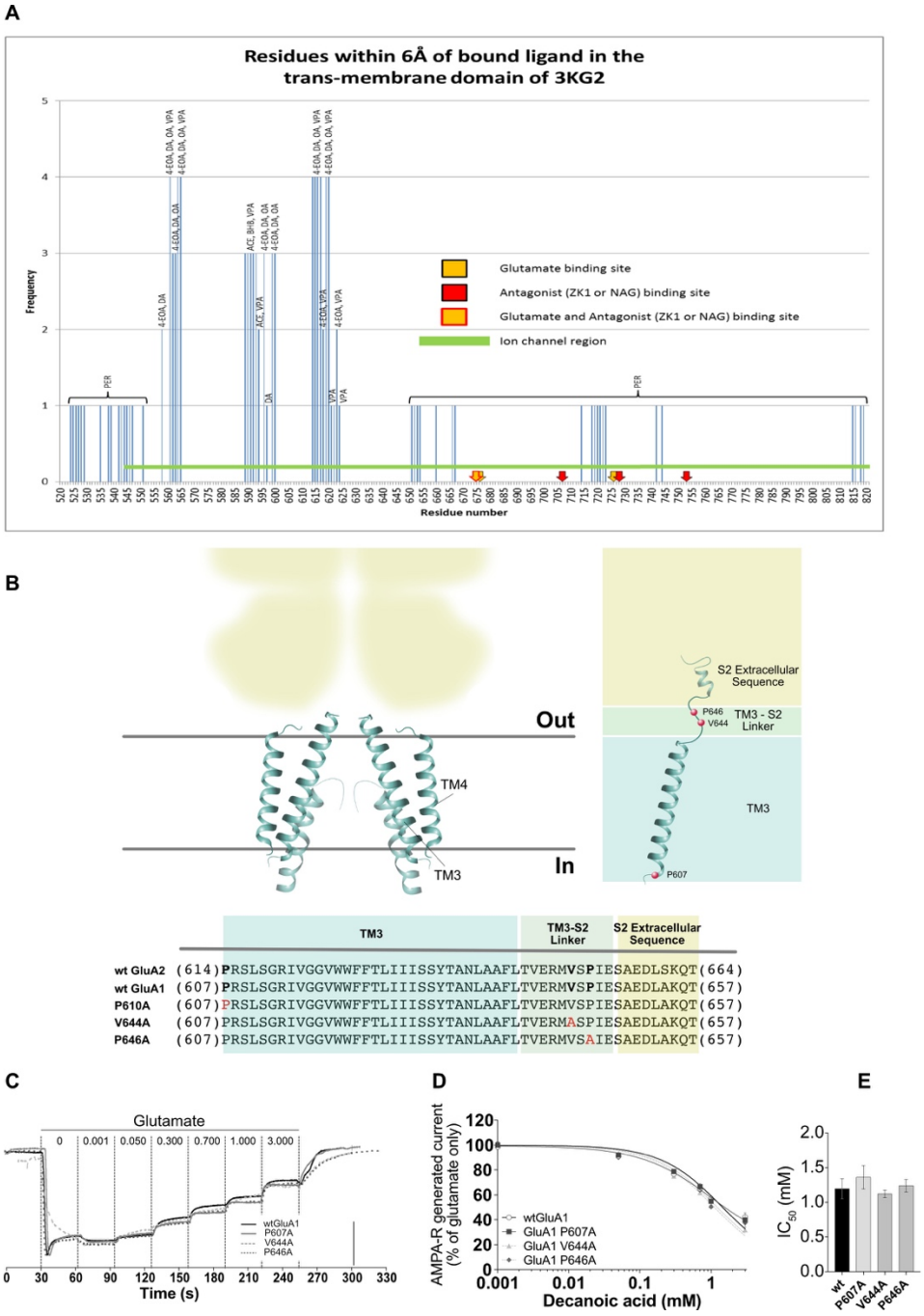



*Figure 5.6 Investigation of a potential binding site for decanoic acid against the AMPA receptor.* (**A** and **B**) Binding site as proposed by computational analysis estimating most likely sites of interaction based on frequency of residue approximation *in silico* (**A**) and schematic showing binding of decanoic (red) and octanic acid (green) at the intracellular part of transmembrane helix 3 (**B**). (**C**) Schematic showing the location of the mutated residues (pink circles) relative to transmembrane domain 3 (TM3) and their position in the amino acid sequence of rat GluA1. Schematic was based on structures developed by Zhao *et al.* (2016). (**D**) Representative trace recordings showing the inhibition of wtGluA (black trace), P610A (dark grey trace), V644A (dotted light grey trace) and P646A (dotted dark grey trace) by six doses of decanoic acid (in mM). Currents were evoked by 100µM glutamate and scale bars correspond to 120nA (wtGluA1), 150nA (P607A), 15nA (V644A) and 200nA (P646A). (**E** and **F**) Sigmoidal dose-reponse curve and bar graph quantifying the average inhibition and IC50s of the four different GluA1 variants by decanoic acid. Each point and bar represents the mean and SEM of 4 to 8 recordings. Statistical analysis was carried out using ANOVA relative to wtGluA1.

## 5.3 Discussion

AMPA receptor inhibition by decanoic acid is a novel finding that may have important implications for the future drug design for the treatment of epilepsy. In addition, other conditions could also benefit from AMPA receptor inhibition including amyotrophic lateral sclerosis (ALS), ischaemia and even Alzheimer's disease (reviewed by Chang *et al.* 2012). Whereas it is reported that AMPA receptors are the secondary targets of some anti-epileptic drugs, such as topiramate and benzodiazepines, the only the specific AMPA receptor antagonist that has been approved for medical use so far is perampanel (Rogawski and Löscher, 2004; M. A. Rogawski, 2013). However, the high potency of perampanel against AMPA receptors is counterbalanced by psychological contraindications, including irritability and violent behaviour (Rugg-Gunn, 2014). It is not clear whether these changes in behaviour are caused by AMPA receptor inhibition or whether they are a result of off-target effects of perampanel. No negative effects of ketogenic diets on mood and behaviour have been reported so far (Wheless, 2001; Elizabeth G. Neal *et al.*, 2009; Levy *et al.*, 2012) and preliminary data from clinical and animal studies suggest positive effects on behaviour by the ketogenic diets (Hori *et al.*, 1997; Evangeliou *et al.*, 2003). However, to warrant further investigations into drug design, it may be beneficial to confirm that the mechanism by which decanoic acid and branched analogues inhibit AMPA receptors is indeed novel, and that these compounds do not share a binding site with perampanel.

For this reason, this chapter summarises experiments that explored potential models of AMPA receptor binding sites for decanoic acid as well as modulation of AMPA receptor inhibition by decanoic acid through the use of competition assays with known AMPA receptor antagonists as well as drug-resistant mutants. Furthermore, an effect of decanoic acid on receptor desensitisation was also investigated using the desensitisation inhibitor CTZ (Kovács *et al.*, 2004; Ptak, Ahmed and Oswald, 2009).

It is noteworthy that the studies described here assume that medium-chain fatty acids directly affect the expressed receptors. Firstly, an effect of medium-chain fatty acids on the membrane in the immediate vicinity of the receptor can be excluded, as inhibition of AMPA receptors by medium-chain fatty acids is transient and disappears with application of the wash-out solution. Integration into the membrane would not be expected to be responsive to changes in perfusion and therefore is unlikely to contribute to AMPA receptor inhibition. Secondly, secondary effects through intracellular signalling were disregarded for the same reason, and a potential influence of decanoic acid on transmembrane AMPA receptor regulatory proteins (TARPs) was excluded since AMPA receptor subunits were

overexpressed in oocytes without co-expression of TARPs, which are therefore unlikely to have a strong effect on AMPA receptor currents.

### 5.3.1 Co-application of decanoic acid and spermine enhances the inhibition of calcium-permeable AMPA receptors

When applied together with a single dose of the polyamine spermine, AMPA receptor inhibition by decanoic acid was augmented, and this increase in inhibition was also observed for spermine with co-application of a dose of decanoic acid. This suggests that decanoic acid and the polyamine do not share a binding site, as competition for a binding site would result in less potent inhibition by either compound (Arai *et al.* 2000). However, the binding of medium-chain fatty acids to distinct residues inside the pore remains a possibility and the increased potency during co-application suggests some degree of positive modulation between the antagonists.

In fact, since medium-chain fatty acids are highly hydrophobic molecules, the most likely site of interaction with the receptor is the ion-conducting pore, which is formed by the lipophilic transmembrane domains (Sobolevsky, Rosconi and Gouaux, 2009). Moreover, glutamate receptor inhibition by polyamines is voltage-dependent (Lopatin, Makhina and Nichols, 1994; Cu, Bähring and Mayer, 1998), and increased potencies with less negative holding voltages were also observed for medium-chain fatty acids (section 3.2.6, page 82). Considering the increased potency in co-application of decanoic acid and polyamines and voltage-responsiveness of both compounds, it would be premature to exclude the channel pore as a binding site for medium-chain fatty acids.

The increased receptor antagonism in co-application of spermine and decanoic acid may have crucial physiological implications. Polyamines have been shown to be present in the human brain at varying concentrations (Morrison *et al.*, 2002) and it has been suggested that they contribute to neuroprotection by reducing ion conductance of excitatory ion channels (Washburn and Dingledine, 1996; Donevan and Rogawski, 1998). In accordance with this potential neuroprotective role, cellular spermine concentrations are up-regulated after seizures, presumably in order to provide further neuroprotection by limiting the entry of calcium into the cell (Hayashi *et al.*, 1993). It is therefore likely that AMPA receptor inhibition by decanoic acid is augmented *in vivo* due to intracellular spermine and that this synergy may result in efficient seizure control, even at low brain concentrations of decanoic acid. This is particularly relevant for homomeric GluA1, where spermine binds most efficiently but decanoic acid was shown to be the least potent.

### 5.3.2 Co-application of decanoic acid and GYKI53665 reduces AMPA receptor inhibition

When decanoic acid and GYKI-53665 were applied together, inhibition was reduced by both the highly potent GYKI-53665 and decanoic acid. GYKI-related compounds have been shown to bind to the AMPA receptor GluA3 subunit at the S1-M1 and S2-M4 linker regions where they cause significant changes in the receptor conformation (Balannik *et al.*, 2005). Upon glutamate binding, the extracellular domains shift into the open conformation, and this shift is transmitted via the linker region to the transmembrane domains, resulting in channel opening. However, if GYKI is bound to the linker regions, this shift is prevented and the receptor remains closed (Balannik *et al.*, 2005). A reduction in AMPA receptor inhibition upon co-application of decanoic acid and GYKI therefore could be due to direct competition of the two compounds for a binding site at the linker regions. Alternatively, binding of either compound to distinct sites could negatively affect accessibility of these sites through allosteric competitive inhibition.

### 5.3.3 Decanoic acid potency is decreased upon co-application with CTZ

Co-application of 100µM CTZ with decanoic acid significantly reduced inhibition of AMPA receptors by decanoic acid and increased the estimated $IC_{50}$ by 7.6-fold compared with that obtained for currents activated by glutamate only. This is a significant effect that very strongly suggests that inhibition of AMPA receptors by decanoic acid either requires accessibility of the CTZ-binding site or that decanoic acid affects the channel desensitising properties. To confirm a role for decanoic acid in desensitisation, currents were activated with 1.7mM kainate to generate only a small degree of desensitisation (Patneau, Vyklicky and Mayer, 1993; Hampson and Manalo, 1998). However, in these experiments, no decrease in the inhibition of AMPA receptors by decanoic acid was observed.

Retained antagonistic activity in response to kainate-induced currents does not, however, contradict an effect of decanoic acid on AMPA receptor desensitisation. The reduced desensitisation in kainate activation may in fact be a proportional effect of the partial receptor activation by kainate (Jin *et al.*, 2003). Agonism of AMPA receptors by kainate is distinct from glutamate activation and induces a unique receptor conformation that allows only restricted flow of ions through the channel (Swanson, Kamboj and Cull-Candy, 1997; Jin *et al.*, 2003; Levchenko-Lambert, Turetsky and Patneau, 2011). Incidentally, this partial activation was reflected in the much smaller currents that were measured in kainate-activated GluA2/3 compared to glutamate- and CTZ-activated GluA2/3. It has been proposed by Jin *et al.* (2003) that the reduced rate of desensitisation reflects the partial open state of the receptor, and retained responsiveness to CTZ shows that desensitisation is not fully blocked by kainate.

In this case, decanoic acid would not necessarily be expected to show a decrease in inhibition comparable to that seen in CTZ-co-activated currents.

In addition, since inhibition by GYKI has also been shown to be sensitive to desensitisation inhibitors (Balannik *et al.*, 2005), a role of decanoic acid in AMPA receptor desensitisation could further be supported by the decreased activity of decanoic acid upon GYKI co-application. Nevertheless, it needs to be noted that the interpretation of the results obtained here is complicated by the circumstance that TEVC measures macroscopic currents, which do not allow for a reliable prediction of modulation of gating properties (Jin *et al.*, 2003). To more conclusively study the effect of non-desensitising AMPA receptors on decanoic acid inhibition, the non-desensitising L483Y mutant GluA2 subunit (Sun *et al.*, 2002) could be assessed.

### 5.3.4 Existing mutants and site-directed mutagenesis did not confirm a binding site for decanoic acid

GYKI-53665 was shown previously to interact with residues in the linker regions S1-M1 and S2-M4 of the AMPA receptor (Balannik *et al.*, 2005). In the experiments described in this chapter, GYKI-53665 inhibition of this mutant was less potent than for the wild-type AMPA receptor. This confirms the results published by Balannik *et al.* (2005), which show full loss of inhibition of the mutant receptor when GYKI-53665 was applied together with CTZ, but residual activity when oocytes were perfused with only GYKI.

Interestingly, currents that were generated by the mutant homomeric GluA3 receptor, were up to 25-fold stronger than those seen in wild-type GluA3-expressing oocytes. This may be due to the fact that the mutations, which are located in areas that are involved in transducing the channel opening to the transmembrane domains, affect the receptor gating properties. This raises the possibility that these mutations, instead of reducing binding of GYKI-53665 to the receptor as was suggested by Balannik *et al.*, change the gating properties and reduce transmission of inhibition by GYKI-53665. The observation that the desensitisation inhibitor CTZ reduces GYKI-53665 activity (Balannik *et al.*, 2005) supports this idea, as it is possible that GYKI-53665 binding to the linker region stabilises the receptor in the desensitised state or facilitates transition into the desensitised conformation. In this case, blocking desensitisation with CTZ would reduce GYKI activity, as would a mutation that hinders this transition.

In contrast to GYKI-53665, decanoic acid did not lose inhibitory activity against the mutant GluA3 subunit compared with the wild-type GluA3, which strongly suggests that medium-chain fatty acids are unlikely to bind to the S1-M1 and S2-M4 linker regions. It is therefore unlikely that decanoic acid and GYKI-53665 inhibit AMPA receptors by the same mechanism, and the loss of activity that was observed upon co-application of the two antagonists is most likely not due to direct competition. That leaves the possibility that binding of decanoic acid to the receptor causes conformational changes that reduce inhibition by GYKI-53665, either directly by restricting access to the GYKI-binding site or by restricting the conformational changes to the linker regions normally induced by GYKI-53665.

Computational analysis of the GluA2 subunit suggested that decanoic acid and branched compounds are most likely to bind to the AMPA receptor at residues that are located at the intracellular end of transmembrane domain 3 and the proline-614 residue was calculated to be the most likely site of interaction. Since currents generated by calcium-impermeable homomeric GluA2 are not detectable in TEVC, GluA1 was used for site-directed mutagenesis. The P614 residue in GluA2 corresponds to the P610 in the GluA1 subunit, which was changed to an alanine by site-directed mutagenesis. However, this substitution did not affect inhibition by decanoic acid, either because decanoic acid does not require P610 for binding to GluA1, or because substitution of the nonpolar proline with a smaller, but similarly nonpolar alanine did not sufficiently interfere with binding affinity. Similarly, mutating the P646 and V644 residues at the extracellular end of transmembrane domain 3 did not reduce inhibition by decanoic acid. These residues were selected for their involvement in channel gating and desensitisation properties (Yelshansky *et al.*, 2004), which may affect binding of decanoic acid, as co-application of CTZ reduced decanoic acid inhibitory activity. Whereas a single site for direct binding of decanoic acid could therefore not be confirmed in these experiments, the use of well-characterised antagonists and mutants strongly suggests that decanoic acid inhibits AMPA receptors through a mechanism that is distinct from GYKI-related compounds. In addition, the reduced maximal response to glutamate seen in the V644A mutant compared to the wild-type or other mutant GluA1 constructs strongly suggests involvement of this residue in AMPA receptor gating.

In conclusion, the results presented in this chapter strongly suggest that the binding site of decanoic acid on AMPA receptors is distinct of that of GYKI-related antagonists. Importantly, the finding that co-application of GYKI-53665 and decanoic acid negatively affects AMPA receptor inhibition may have implications for patients receiving the MCT ketogenic diet and the anti-epileptic drug perampanel, since the development of perampanel was based on the GYKI-related compounds (Világi *et al.*, 2002; Hanada, 2014). The next chapter will therefore characterise AMPA receptor inhibition by perampanel and investigate possible interactions of decanoic acid and this AED in more detail.

# CHAPTER 6 – MOLECULAR EFFECTS OF DECANOIC ACID AND PERAMPANEL CO-APPLICATION

## 6.1 Introduction

When the anti-epileptic drug (AED) perampanel was approved by the FDA in 2012, its release was greatly anticipated. Drug resistant epilepsies have a major impact on the burden of disease and it was thought that through the new mechanism of seizure treatment provided by the AMPA receptor antagonist perampanel, an alternative approach to treating hard-to-control epilepsies was found (Hibi *et al.*, 2012; M A Rogawski, 2013; Hibi, 2015). Since perampanel is the first drug of its kind to be released, pharmacodynamic interactions with other AMPA receptor modulators were not of interest in the characterisation of the AED. However, the finding presented in this thesis that medium-chain fatty acids also inhibit AMPA receptors, is unprecedented, and warrants the question whether combined treatment of patients with perampanel and the MCT ketogenic diet could result in pharmacodynamic interactions against the target protein and thereby affect inhibition by perampanel. This could imply revising the guidelines for drug administration in this particular case.

Perampanel has repeatedly been shown to selectively block AMPA receptors at concentrations which fall well within the reported plasma concentrations of 1.06-3.26µM (Chen *et al.*, 2014; Hibi, 2015; Patsalos, 2015; Barygin, 2016). However, whilst its development was based on the non-competitive antagonist GYKI-52466, investigations into the mode of inhibition have been carried out in highly complex cell cultures, and demonstration of the perampanel binding site is still outstanding by site directed mutagenesis (Rogawski and Hanada, 2013; Chen *et al.*, 2014; Hibi, 2015).

Further, AMPA receptor localisation is highly dynamic and transient between the synaptic and extrasynaptic membranes, and intracellular vesicles. This makes AMPA receptors key effectors of synaptic plasticity and AMPA receptor mediated signalling is strongly implicated in excitatory synaptic strengthening (reviewed by Malinov and Malenka, 2002; Bredt and Nicoll, 2003; Shepherd and Huganir, 2007). Importantly, synaptic changes resulting in stronger glutamatergic and reduced $GABA_A$-mediated signalling have been linked to high-frequency AMPA receptor activation and are thought to be involved in the process of epileptogenesis (Brooks-Kayal *et al.*, 1998; Sanchez *et al.*, 2005; Ben-Ari, 2006).

In this chapter, direct AMPA receptor inhibition by perampanel was characterised and the effect potential decanoic acid – perampanel interactions may have on inhibitory activity against AMPA receptors was assessed. In addition, a role for AMPA receptor mediated synaptic changes was examined by investigating GluA1 localisation following seizure-like activity and AMPA receptor antagonist treatment in rat primary hippocampal neurons.

# 6.2 Results

## 6.2.1 Perampanel is a non-competitive AMPA receptor antagonist without subunit specificity

Multiple studies have assessed AMPA receptor inhibition by perampanel in a variety of biological systems and with highly variable $IC_{50}$s. To characterise direct AMPA receptor inhibition by perampanel independent of potential secondary effects from differentiated cells or tissue, *Xenopus laevis* oocytes expressing recombinant GluA1/2 and GluA2/3 were perfused with 0.1µM to 100µM perampanel and activated with 100µM glutamate. In these experiments, perampanel inhibited GluA1/2 with an $IC_{50}$ of 6.27µM (SEM = 1.02; n = 13) and GluA2/3 with an $IC_{50}$ of 8.11µM (SEM = 2.05; n = 6; figure 6.1 A to C). The difference in $IC_{50}$ was not significant ($p$ =0.14 for $IC_{50}$ of GluA2/3 compared to GluA1/2). Therefore, there is no apparent subunit specificity in AMPA receptor inhibition by perampanel.

To confirm the mode of inhibition, the experimental setup was repeated in GluA1/2 expressing oocytes perfused with an increased, saturating concentration of glutamate (1mM). Perampanel maintained AMPA receptor inhibition with an $IC_{50}$ of 3.85µM (SEM = 0.98; n = 4; $p$ = 0.31 compared to 100µM glutamate; figure 6.1 D to F) despite the increased glutamate concentration, and there was no shift to either side of the dose-effect curve. This is strongly suggestive of absence of competitive AMPA receptor inhibition.

To conclusively demonstrate non-competitive inhibition, the effect of added perampanel on the $EC_{50}$ of glutamate against GluA1/2 was determined by adding two concentrations of perampanel to a glutamate dose-effect assay, as described in chapter 3.2.2 (page 73). In the absence of perampanel, glutamate activated GluA1/2 with an $EC_{50}$ of 15.3mM (SEM = 1.4; n = 6). Addition of 2.5µM perampanel raised the $EC_{50}$ to 22.8mM (SEM = 3.7; n = 5; $p$ = 0.34 compared to glutamate only), and addition of 5µM perampanel further increased the glutamate $EC_{50}$ significantly to 38.2mM (SEM = 6.0; n = 5; $p$ = 0.002 compared to glutamate only; figure 6.1 G to I). Addition of perampanel therefore had a dose-dependent negative effect on AMPA receptor activation by glutamate. Importantly, addition of perampanel also decreased the maximal response to glutamate to 80.1% when 2.5µM perampanel was added (SEM = 5.1, $p$ = 0.0004 compared to glutamate only) and 17.4% when 5µM perampanel was added (SEM = 1.0; $p < 0.0001$ compared to glutamate only). These data clearly show that AMPA receptor inhibition by perampanel is not overcome by increasing glutamate concentrations and therefore strongly suggests a non-competitive mode of antagonism.

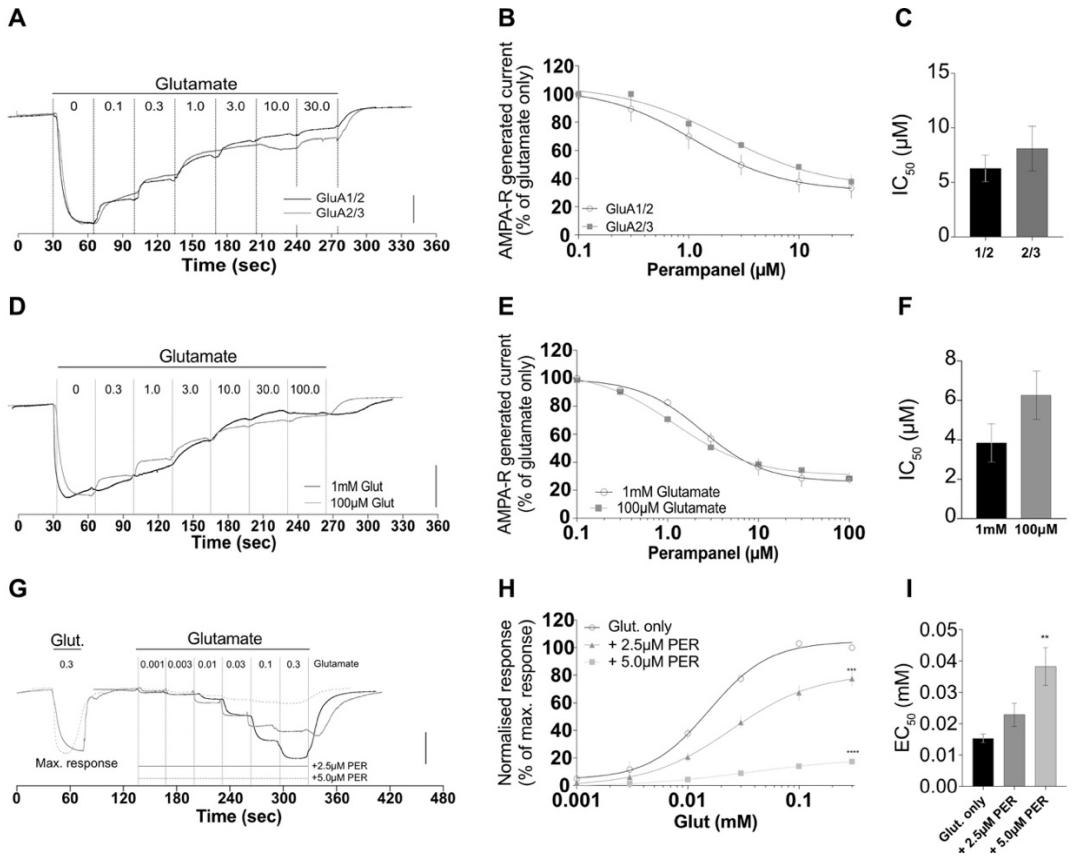



*Figure 6.1 Characterisation of AMPA receptor inhibition by the anti-epileptic drug perampanel (PER).* Recombinant GluA1/2 and GluA2/3 were expressed in *Xenopus* oocytes. (**A**) Representative trace recordings for dose-response curves of PER (in µM) against GluA1/2 (black line) and GluA2/3 (grey line). Currents were evoked with 100µM glutamate. Scale bar corresponds to 75nA (GluA1/2) and 20nA (GluA2/3). (**B** and **C**) Sigmoidal dose-response curves and bar graph showing similar inhibition of GluA1/2 and GluA2/3 by PER. Each point represents the mean and SEM of 13 and 6 recording readings, respectively. Statistical analysis was carried out using student's *t*-test. (**D**) Trace recordings representative of the competition experiments for PER (in µM) and glutamate in GluA1/2 expressing oocytes, where currents were either evoked by 1mM (black line) or 100µM glutamate (grey line). Scale bar corresponds to 30nA (1mM glutamate) and 120nA (100µM glutamate). (**E** and **F**) Sigmoidal dose-response curves and bar graph showing lack of competition between glutamate and PER. Each point represents the mean and SEM of 4 (1mM) and 13 (100µM) recording readings. Statistical analysis was carried out using student's *t*-test. (**G**) Representative trace recordings for dose-effect curves of glutamate only (in mM, black line) and glutamate with added 2.5µM PER (dark grey line) and 5µM PER (light grey line) against GluA1/2. Single dose response curve shows maximal response to 0.3mM glutamate without added PER. Scale bar corresponds to 250nA (glutamate only), 35nA (+2.5µM PER) and 160nA (+5µM PER). (**H** and **I**) Comparison of the sigmoidal dose response curves and bar graph showing decreased GluA1/2 activation by glutamate upon PER application and $EC_{50}$s for the competition assay. Each point represents the mean and SEM of 5 to 6 recording readings. Stars in (**H**) refer to statistical significance in maximal response relative to glutamate only. Statistical analysis was carried out using ANOVA where ** corresponds to $p < 0.01$, *** corresponds to $p < 0.001$ and **** corresponds to $p < 0.0001$.

### 6.2.2 Perampanel inhibits AMPA receptors at the S1-M1 and S2-M4 linker regions

Whilst perampanel development was based on the GYKI-related compounds, the only published studies assessing binding site were carried out using indirect GYKI displacement assays (Barygin, 2016). To conclusively demonstrate that perampanel shares a binding site with the well-characterised GYKI-related compounds, GYKI-resistant and wild-type GluA3 subunits were expressed in *Xenopus* oocytes, activated with 100µM glutamate, and assessed in single-dose response assays for sensitivity to 20µM perampanel compared to activation with glutamate only, as described in chapter 5 (5.2.4, page 119). Perfusion with 20µM perampanel blocked currents generated by the wild-type GluA3 (wtGluA3; 94.3% inhibition; SEM = 2.2; n = 5). However, inhibition of the drug-resistant subunit (mutGluA3) was significantly reduced to 54.2% (SEM = 1.6; n= 6; $p < 0.0001$ compared to wtGluA3; figure 6.2). This suggests loss of sensitivity to perampanel in the mutant. Therefore, it can be concluded that, like GYKI-53665, perampanel requires the residues D543-E548 and T810-I888 at the S1-M1 and S2-M4 linker regions to potently inhibit AMPA receptors.

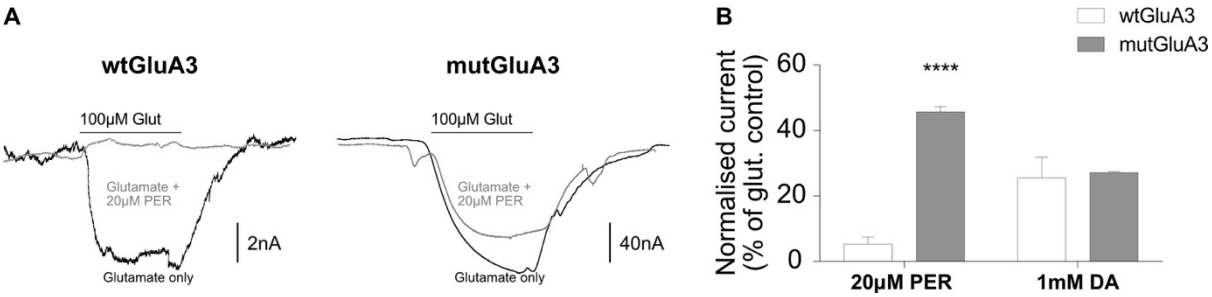



*Figure 6.2 Potency of perampanel against wild-type GluA3 and the GYKI-resistant mutant.* Single-dose response assays were carried out in *Xenopus* oocytes expressing wild-type GluA3 (wtGluA3) and GYKI-resistant GluA3 (mutGluA3) subunits for perampanel (PER) compared to the data presented in 5.2.4 for decanoic acid (DA). (**A**) Traces representative of the single-dose effect assay showing the response of either wtGluA3 or mutGluA3 to 100μM glutamate only (black) or 100μM glutamate and 20μM PER. (**B**) Bar graph comparing normalised wtGluA3 and mutGluA3 responses with added PER or DA. Each bar represents the mean and SEM of 5 (wtGluA3) and 6 (mutGluA3) recording readings. Statistical analysis was carried out using the student's *t*-test relative for mutGluA3 to wtGluA3 within each antagonist data set, where **** corresponds to $p < 0.0001$.

### 6.2.3 Co-application of decanoic acid and perampanel synergistically potentiates AMPA receptor inhibition by perampanel

To investigate a potential modulation of inhibition in simultaneous decanoic acid and perampanel application, a competition assay was carried out where two concentrations of decanoic acid were added to the perampanel dose-effect assay. Decanoic acid concentrations were selected based on sub-threshold doses that previously showed only minimal inhibition of AMPA receptors (3.2.1, page 70), as well as optimised solubility due to the high hydrophobicity of both antagonists. Taking these factors into consideration, 50µM and 200µM decanoic acid were added to dose-response assays against GluA1/2. All recording readings were normalised to the baseline inhibition by 50µM and 100µM decanoic acid, respectively, to account for inhibition by decanoic acid only. Interestingly, addition of decanoic acid significantly increased perampanel inhibition in both GluA1/2 and GluA2/3 expressing oocytes. In GluA1/2, addition of 50µM decanoic acid lowered the $IC_{50}$ from 6.27µM to 2.13µM (SEM = 0.25; n = 10; $p$ = 0.0032 compared to perampanel only), and addition of 100µM decanoic acid increased inhibition to a similar degree ($IC_{50}$ = 2.29µM; SEM = 0.06; n = 10; $p$ = 0.0045 compared to perampanel only; figure 5.3 A to C). This effect is contrary to the reduction in inhibition seen in the GYKI competition assay, where addition of GYKI to decanoic acid reduced AMPA receptors inhibition by decanoic acid (5.2.3, page 117).

The competition assay was repeated in GluA2/3 expressing oocytes, with the addition of 50µM and 100µM decanoic acid to the perampanel dose-response assay. Addition of 50µM decanoic acid to perampanel decreased the $IC_{50}$ significantly from 8.11µM to 1.65µM (SEM = 0.87; n = 10; $p$ = 0.011 compared to perampanel only). Addition of 200µM decanoic acid had a comparable synergistic effect to that observed in GluA1/2, decreasing the $IC_{50}$ to 1.63µM (SEM = 0.21; n = 5; $p$ = 0.028 compared to perampanel only). These findings are consistent with the pattern observed for perampanel inhibition of GluA1/2, indicating a strong synergistic effect of perampanel and decanoic acid against AMPA receptors that is independent of the subunit combination.

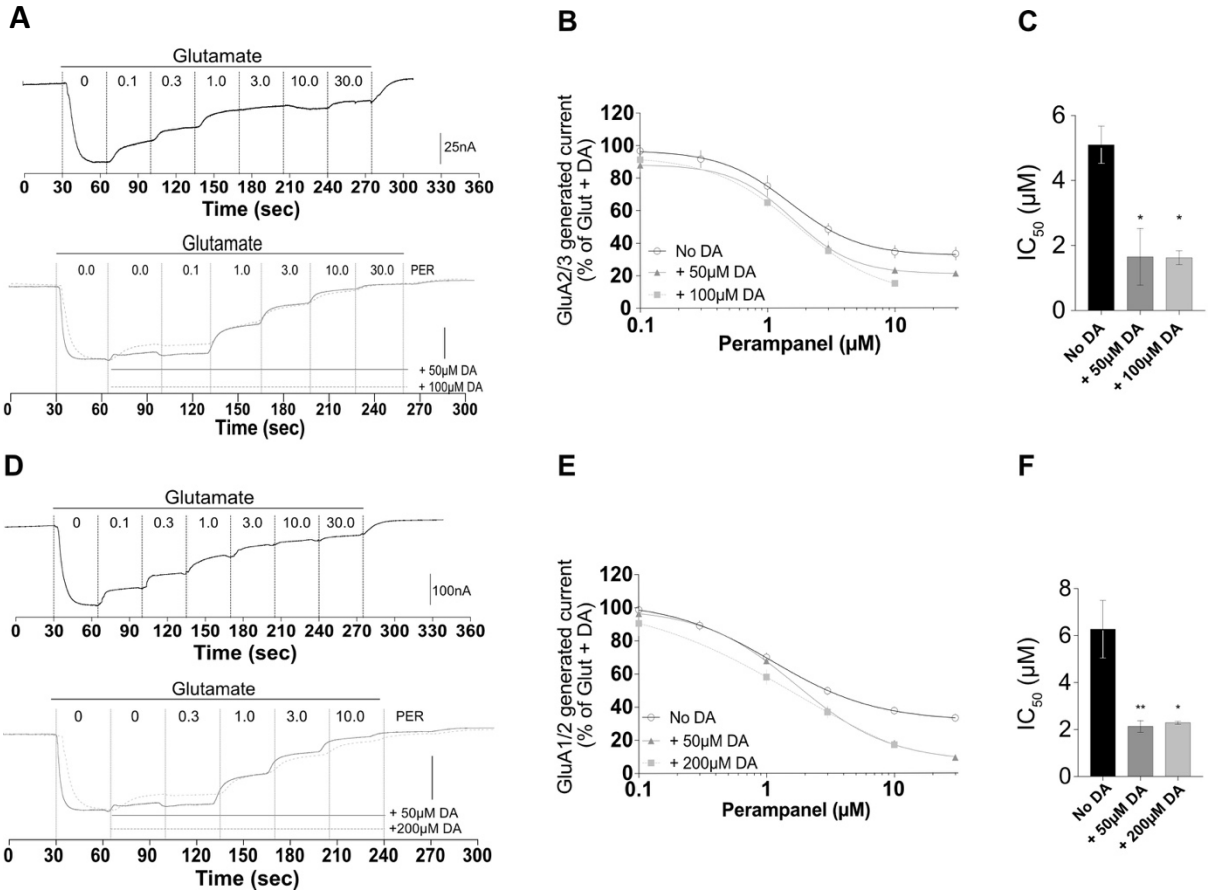



*Figure 6.3 Competition assays for the AMPA receptor antagonist perampanel (PER) with added decanoic acid (DA).* Assays were carried out in GluA2/3 and GluA1/2 expressing oocytes with currents evoked by 100µM glutamate. (**A**) Representative trace recordings showing inhibition of GluA2/3 by PER only (in µM, black trace, top) and PER with added 50µM DA (solid trace, bottom) and 100µM DA (hashed trace, bottom). Bottom scale bars correspond to 25nA (50µM DA) and 30nA (100µM). (**B** and **C**). Sigmoidal plot and bar graph quantifying PER antagonism of GluA2/3 only and PER antagonism with added DA, where each point and bar represents the mean and SEM of 10 to 13 recordings. Statistical analysis was carried out using ANOVA, where * corresponds to $p < 0.05$. (**D**) Representative trace recordings showing inhibition of GluA1/2 by PER only (in µM, black trace, top) and PER with added 50µM DA (solid trace, bottom) and 200µM DA (hashed trace, bottom). Bottom scale bars correspond to 120nA (50µM DA) and 125nA (200µM). (**E** and **F**) Sigmoidal plot and bar graph quantifying PER antagonism of GluA1/2 where each point and bar represents the mean and SEM of 5 to 10 recordings. Statistical analysis was carried out using ANOVA where * corresponds to $p < 0.05$ and ** corresponds to $p < 0.01$.

### 6.2.4 Addition of perampanel to decanoic acid dose-response assays synergistically potentiates AMPA receptor inhibition by decanoic acid

To determine if potentiation of inhibition between decanoic acid and perampanel is bidirectional, two doses of perampanel were added to the decanoic acid dose-effect assay. Currents were evoked by perfusion with 100µM glutamate and all recording readings were normalised to the baseline inhibition by 1µM and 4µM perampanel, respectively, to account for inhibition by perampanel only. Addition of 1µM perampanel to decanoic acid dose response assays had a potentiating effect on AMPA receptor inhibition by decanoic acid reducing the $IC_{50}$ against GluA2/3 significantly from 0.52mM to 0.15mM (SEM = 0.04; n = 8; $p < 0.0001$ compared to decanoic acid only). In addition, increasing the dose of perampanel to 4µM reduced the $IC_{50}$ to 0.12mM (SEM = 0.06; n = 10; $p < 0.0001$ compared to decanoic acid only; figure 6.4 A to C). These results suggest that the synergistic effect is not dose dependent. Therefore, these findings are consistent with the lack of a dose-dependent effect observed for co-application of decanoic acid to perampanel.

The synergy experiment for perampanel and decanoic acid was repeated in GluA1/2 expressing oocytes. Co-application of 1µM perampanel to decanoic acid dose-response assays lowered the $IC_{50}$ of decanoic acid significantly from 0.92mM (SEM = 0.06; n = 12) to 0.21mM (SEM = 0.02; n = 12; $p < 0.0001$ compared to decanoic acid only). Addition of 4µM perampanel reduced the $IC_{50}$ further to 0.10mM (SEM = 0.02; n = 10; $p < 0.0001$ compared to decanoic acid only; figure 6.4 D to F). Again, the potentiating effect was consistent with the synergistic effect of decanoic acid on perampanel, suggesting that the positive modulation of inhibition is bidirectional.

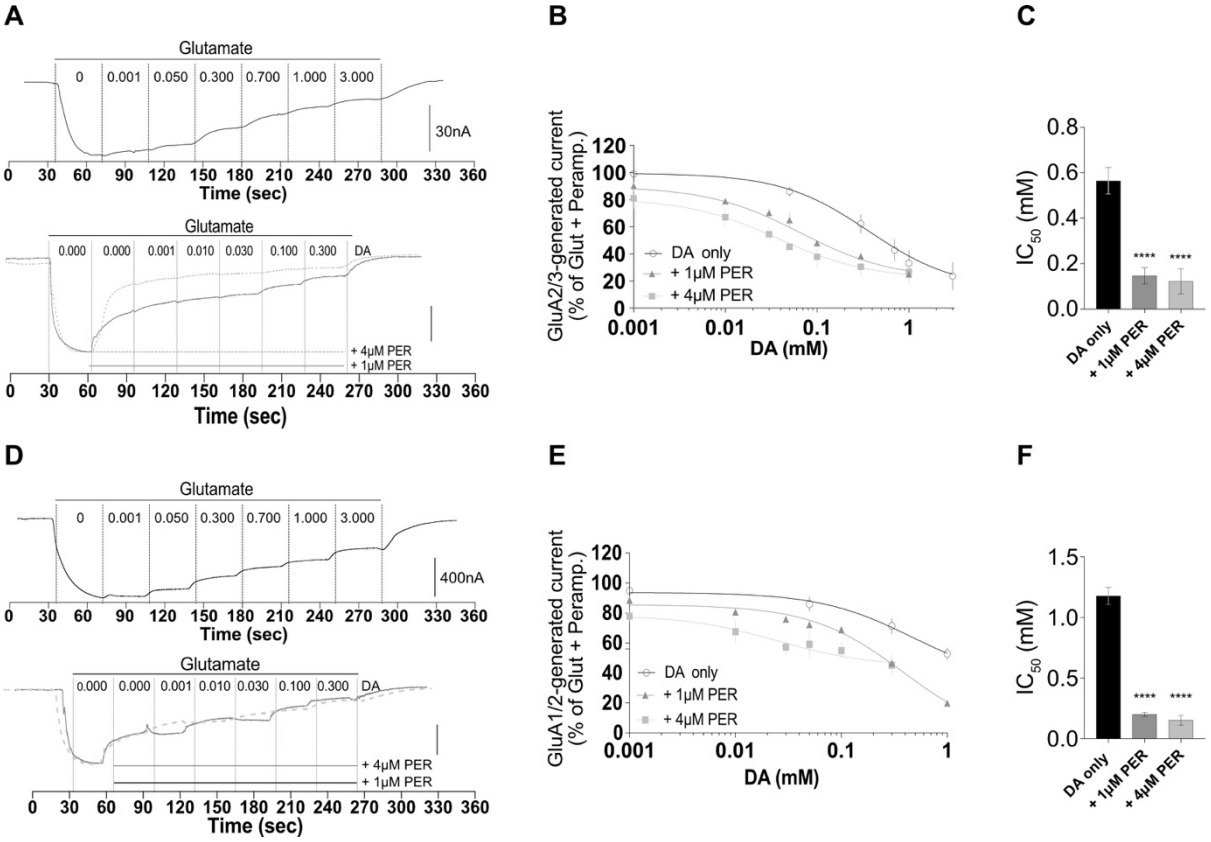



*Figure 6.4 Competition assays for the AMPA receptor antagonist decanoic acid (DA) with added perampanel (PER).* Dose-effect assays were carried out in GluA2/3 and GluA1/2 expressing oocytes with currents evoked by 100μM glutamate. (**A**) Representative trace recordings showing inhibition of GluA2/3 by DA only (in mM, black trace, top) and DA with added 1μM PER (solid trace, bottom) and 4μM PER (hashed trace, bottom). Scale bars correspond to 20nA (+1μM PER) and 30nA (+4μM PER). (**B** and **C**) Sigmoidal plot and bar graph quantifying GluA2/3 antagonism by DA where each point and bar represents the mean and SEM of 8 to 12 recordings. Statistical analysis was carried out using ANOVA, with **** corresponding to $p < 0.0001$. (**D**) Representative trace recordings showing inhibition of GluA1/2 by DA only (in mM, top) and DA with added 1μM PER (solid trace, bottom) and 4μM PER (hashed trace, bottom). Scale bars correspond to 100nA (+1μM PER) and 220nA (+4μM PER). (**E** and **F**) Sigmoidal plot and bar graph quantifying GluA1/2 antagonism by DA with and without PER where each point and bar represents the mean and SEM of 12 recordings. Statistical analysis was carried out using the *t*-test where **** corresponds to $p < 0.0001$.

### 6.2.5 Medium-term application of decanoic acid and seizure-induction do not affect AMPA receptor expression levels in primary neurons

Treatment with AEDs predominantly targets the symptoms of epilepsy by aiming to reduce seizure frequency and severity. Reports on whether early intervention with AEDs can help to reduce seizure recurrence and epileptogenesis give conflicting evidence (Hauser and Lee, 2002; Ben-Ari, 2006). However, due to the central role that the AMPA receptors play in synaptic strengthening (Barria *et al.*, 1997; Derkach, Barria and Soderling, 1999) and the fact that epileptiform activity has been reported to result in augmented AMPA receptor protein expression levels (Abegg *et al.*, 2004), it is worth investigating whether medium-term inhibition by decanoic acid affects overall AMPA receptor expression. For this purpose, primary hippocampal neurons (14 *DIV*) were treated with the seizure termination model in which ictal activity was invoked in neurons by addition of 5mM PTZ for 20 min to the media and subsequent incubation with either 200µM or 300µM decanoic acid for 24 hours. Protein levels were assessed by western blot and quantified by fluorescence intensity reading. The data were normalised to individual loading controls (ß-tubulin) and mean untreated control subunit/loading control ratios. All data are summarised in table 6.1. Briefly, treatment with either PTZ, decanoic acid, or a combination of both had no significant effect on GluA1 protein levels (figure 6.5 A and B). To control for other subunits, the protein levels of GluA2 and GluA3 were also assessed (all data are summarised in table 6.1). None of the treatments had a significant effect on subunit expression levels (figure 6.5 B to D).

| AMPA R subunit | Treatment | Normalised expression (fraction of control) | SEM | *p* (relative to control) |
|---|---|---|---|---|
| **GluA1** | PTZ only | 0.86 | 0.14 | 0.9768 |
| | PTZ + 0.2mM DA | 1.02 | 0.21 | >0.9999 |
| | PTZ + 0.3mM DA | 1.03 | 0.19 | >0.9999 |
| | 0.2mM DA only | 0.87 | 0.12 | 0.9838 |
| | 0.3mM DA only | 0.84 | 0.20 | 0.9648 |
| **GluA2** | PTZ only | 1.09 | 0.22 | 0.9987 |
| | PTZ + 0.2mM DA | 1.29 | 0.29 | 0.8131 |
| | PTZ + 0.3mM DA | 1.05 | 0.19 | >0.9999 |
| | 0.2mM DA only | 0.87 | 0.13 | 0.9923 |
| | 0.3mM DA only | 0.98 | 0.17 | >0.9999 |
| **GluA3** | PTZ only | 0.99 | 0.17 | >0.9999 |
| | PTZ + 0.2mM DA | 0.84 | 0.10 | 0.9421 |
| | PTZ + 0.3mM DA | 0.75 | 0.11 | 0.6936 |
| | 0.2mM DA only | 1.20 | 0.16 | 0.8679 |
| | 0.3mM DA only | 0.99 | 0.20 | >0.9999 |

*Table 6.1 Summary of normalised AMPA receptor subunit expression levels in primary hippocampal neurons 24 hours following seizure activity. Controls (not shown) were normalised to loading control (β-tubulin) within each experimental repeat, and data shown were normalised to normalised control. The table summarizes the average expression levels as fractions of control, SEM and p-values relative to control. Statistical analysis was carried out by ANOVA, and numbers are derived from 2 biological repeats with 3 experimental repeats each.*

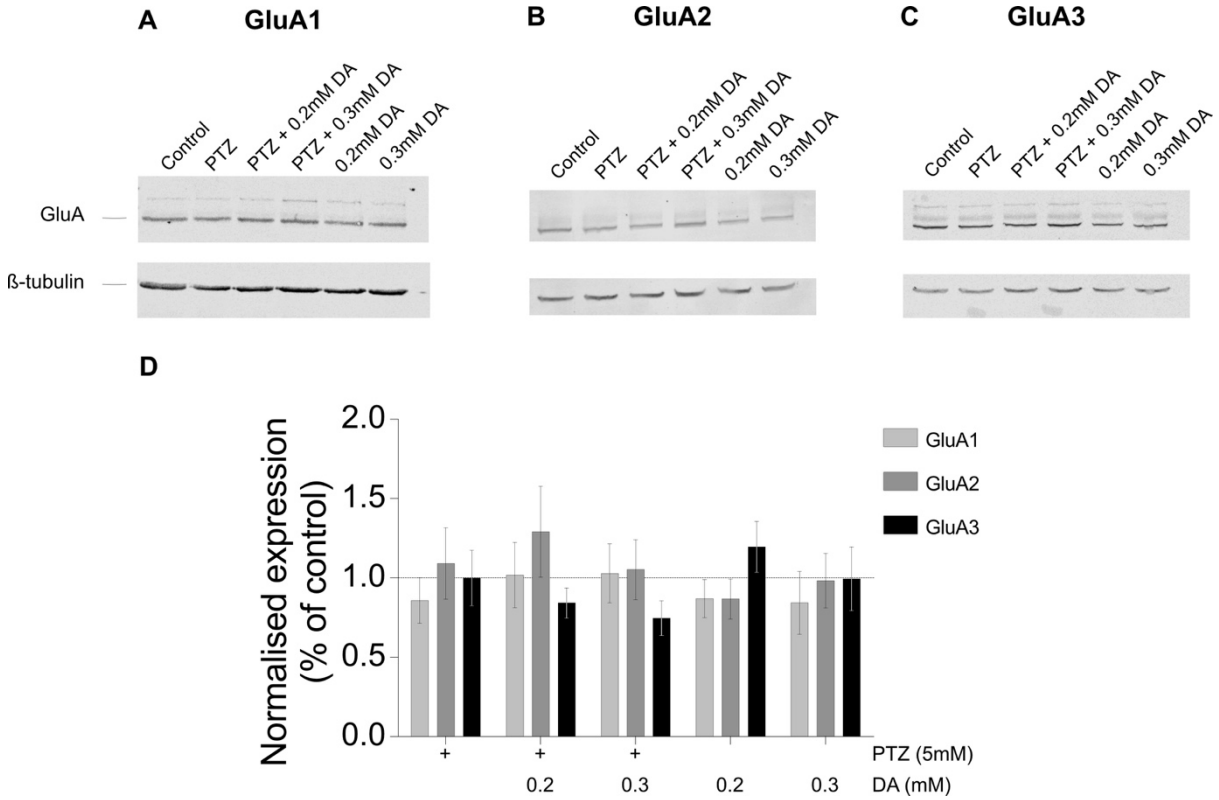



*Figure 6.5 Protein levels of the AMPA receptor subunits GluA1, GluA2 and GluA3 in rat primary hippocampal neurons.* Protein levels were assessed in hippocampal neurons (14 *DIV*) following seizure induction with 5mM PTZ and subsequent 24 hour incubation with decanoic acid (DA) determined by western blot. (**A** to **C**) Representative western blot images showing protein detection by GluA1 (**A**), GluA2 (**B**) and GluA3 (**C**) antibodies (top rows, 100kDa) and β-tubulin antibodies (bottom rows, 51kDa). (**D**) Bar graph summarising normalised (to control normalised to loading control) protein levels across the 5 treatment groups. Each bar corresponds to 2 biological repeats with each 3 experimental repeats.

### 6.2.6 GluA1 localisation to the synaptic membrane increases after seizures but this change is prevented by treatment with AMPA receptor antagonists

AMPA receptor localisation to the membrane is transient and tightly regulated by intracellular phosphorylation depending on the degree of receptor activation. To assess whether induction of seizure-like activity results in changes in GluA1 localisation, primary hippocampal neurons (14 *DIV*) were treated with the acute treatment model, where neurons were incubated for 30 min with decanoic acid, perampanel or a combinatory therapy prior to seizure induction with 5mM PTZ, and fixed after 20 min incubation (Chang *et al.*, 2013). Neurons were stained with anti-GluA1 antibody under both non-permeabilising and permeabilising conditions using a two-step protocol to selectively label membrane bound and internal GluA1 (Li, Xu and Pozzo-Miller, 2016).

To determine the effect of PTZ as well as perampanel and decanoic acid treatment on internalisation of GluA1, puncta intensity of membrane-bound vs. internal GluA1 was quantified using ImageJ. The data were compared to the control groups that were neither incubated with AMPA receptor antagonists nor PTZ (100% normalised Surface/Total GluA1; SEM = 6.9; n = 29). In neurons that were treated with PTZ in the absence of AMPA receptor antagonists, seizures increased membrane bound GluA1 in dendrites to 276 % of control (SEM = 30.3; n = 27). This effect was strongly significant ($p < 0.0001$ compared to control). The increase in membrane localisation in dendrites was prevented by treating the neurons with both decanoic acid and perampanel at physiologically relevant doses (0.2mM and 0.3mM decanoic acid; 0.5µM and 1.0µM perampanel; or combined treatment with 0.2mM decanoic acid + 0.2µM perampanel or 0.5µM perampanel; all data summarised in table 6.2) prior to seizure induction (figure 6.6 A and B). In addition, treatment with 0.3mM decanoic acid, combined treatment with 0.2mM decanoic acid + 0.2µM perampanel and treatment with 0.5µM perampanel in the absence of decanoic acid significantly reduced membrane localisation respective to control despite seizure induction ($p = 0.0168$, $p = 0.0097$, and $p = 0.0097$ respectively). All neuron images are shown in Appendix III.

Interestingly, whilst increasing membrane bound GluA1 in dendrites, PTZ treatment without antagonist application also reduced the total amount of GluA1 in the cell body to 48% of control (SEM = 18.3, n = 5), suggesting recruitment of AMPA receptor containing vehicles from the soma into the dendrites upon severe neuron stimulation. Treatment with decanoic acid or perampanel prevented this decrease in soma-localised internal GluA1 (data summarised in table 6.3). In addition, treatment with both perampanel and decanoic acid increased the relative amount of GluA1 in the cell body compared to control, with the combined application of 0.5µM perampanel and 0.2mM decanoic acid raising soma-localised GluA1 to a 174% intensity of control (SEM = 13.9; n = 9; $p < 0.0001$; figure 6.6 A

and C). This combination of perampanel and decanoic acid had the most pronounced effect on GluA1 localisation to the cell body of the treatments tested here. This suggests that AMPA receptor antagonism may down-regulate GluA1 localisation to dendrites and synapses in primary hippocampal neuronal cell culture.

AMPA receptor localisation has been suggested to be affected by seizure-like activity (Lippman-Bell *et al.*, 2016). To determine whether treatment with PTZ localises AMPA receptors predominantly to the synaptic or extrasynaptic membrane, colocalisation with synapsin was assessed in 3 dendrites per experimental repeat, by means of the unbiased ImageJ colocalisation analysis tool JaCoP. From these analyses, PTZ treatment, irrespective of antagonist application, appeared to increase synaptic colocalisation of GluA1 puncta (all data summarised in table 6.4), however, increased colocalistion was only significant in treatment of neurons with 0.2mM decanoic acid and PTZ (165% of control; SEM = 14.2; n = 7 experimental repeats; $p$ = 0.0084 compared to control; figure 6.6 A and D). This suggests that seizure induction by PTZ has no significant effect on synaptic localisation of GluA1 in primary hippocampal neurons.

| Intervention | Control | PTZ only | 0.2mM DA | 0.3mM DA | 0.2mM DA + PTZ | 0.3mM DA + PTZ | 0.5uM PER | 1uM PER | 0.5uM PER+ PTZ | 1uM PER + PTZ | 0.2uM PER+ 0.2mM DA + PTZ | 0.5uM PER + 0.2mM DA + PTZ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Surface/ Total GluA1** | 1.00 | 2.76 | 0.56 | 0.76 | 0.88 | 0.51 | 0.48 | 0.72 | 0.71 | 0.91 | 0.50 | 0.78 |
| **SEM** | 0.07 | 0.30 | 0.06 | 0.09 | 0.12 | 0.04 | 0.06 | 0.09 | 0.07 | 0.13 | 0.05 | 0.07 |
| **n** | 29 | 27 | 40 | 45 | 36 | 42 | 39 | 42 | 42 | 45 | 45 | 42 |
| ***p*(C)** | n/a | <0.0001**** | 0.0512 | 0.7005 | 0.9985 | 0.0168* | 0.0097** | 0.5103 | 0.4536 | 0.9999 | 0.0097** | 0.8158 |
| ***p*(PTZ)** | <0.0001 | n/a | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

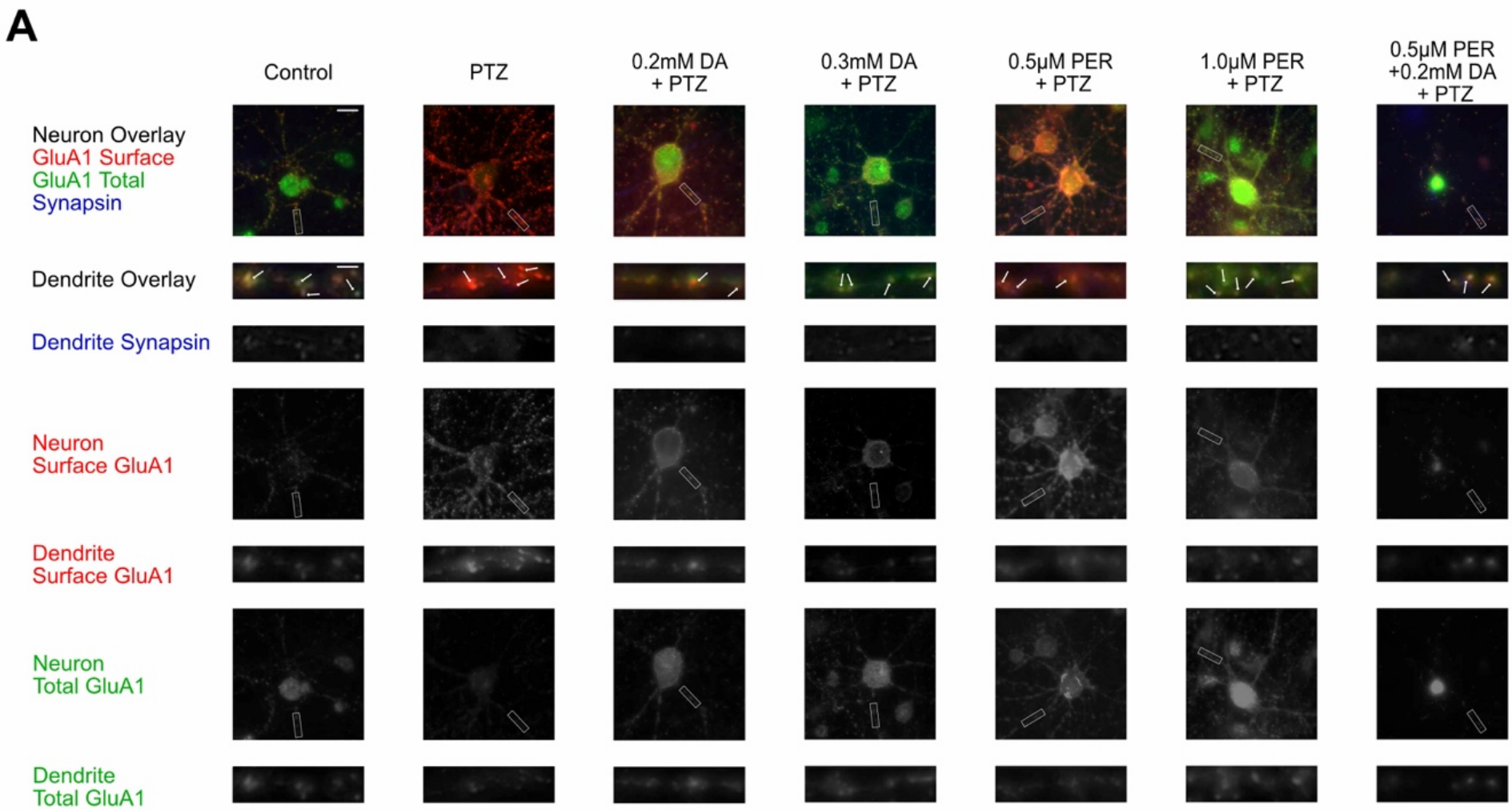
*Table 6.2 Summary of average surface/total GluA1 localisation after seizures and decanoic acid and perampanel treatment. Table lists surface/total GluA1 IntDen with SEM, n of repeats and p-values for the 12 treatment groups. All repeats were normalised to respective control. Statistical analysis was carried out by ANOVA with p-values relative to control (p(C)) and to PTZ treatment (p(PTZ)), where * corresponds to p < 0.05; ** corresponds to p < 0.01 and **** corresponds to p < 0.0001.*

| Intervention | Control | PTZ only | 0.2mM DA | 0.3mM DA | 0.2mM DA + PTZ | 0.3mM DA + PTZ | 0.5uM PER only | 1uM PER only | 0.5uM PER + PTZ | 1uM PER + PTZ | 0.2uM PER + 0.2mM DA + PTZ | 0.5uM PER + 0.2mM DA + PTZ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Soma IntDen** | 1.00 | 0.48 | 1.01 | 1.17 | 1.12 | 1.33 | 1.47 | 0.85 | 1.24 | 1.05 | 1.20 | 1.74 |
| **SEM** | 0.09 | 0.18 | 0.11 | 0.16 | 0.09 | 0.11 | 0.18 | 0.14 | 0.14 | 0.22 | 0.12 | 0.14 |
| **n** | 9 | 5 | 9 | 9 | 9 | 8 | 9 | 9 | 8 | 9 | 9 | 9 |
| ***p*(C)** | n/a | 0.275 | >0.999 | 0.995 | 0.999 | 0.716 | 0.190 | 0.999 | 0.949 | >0.999 | 0.986 | $0.004^{**}$ |
| ***p*(PTZ)** | 0.275 | n/a | 0.261 | $0.042^{+}$ | 0.081 | 0.007 | $0.001^{+++}$ | 0.748 | $0.022^{+}$ | 0.178 | $0.031^{+}$ | $<0.0001^{++++}$ |

*Table 6.3 Summary of average soma/neuron GluA1 localisation after seizures and decanoic acid and perampanel treatment. Table lists GluA1 IntDen with SEM, n of repeats and p-values for the 12 treatment groups. All repeats were normalised to respective control. Statistical analysis was carried out by ANOVA with p-values relative to control (p(C)) and to PTZ treatment (p(PTZ)), where * corresponds to p < 0.05; ** corresponds to p < 0.01; *** corresponds to p < 0.001 and **** corresponds to p < 0.0001.*

| Intervention | Control | PTZ only | 0.2mM DA | 0.3mM DA | 0.2mM DA + PTZ | 0.3mM DA + PTZ | 0.5uM PER only | 1uM PER only | 0.5uM PER + PTZ | 1uM PER + PTZ | 0.2uM PER + 0.2mM DA + PTZ | 0.5uM PER + 0.2mM DA + PTZ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Colocalisation correlation** | 1.00 | 1.17 | 1.37 | 1.09 | 1.65 | 1.04 | 1.39 | 1.07 | 1.42 | 1.26 | 1.28 | 1.26 |
| **SEM** | 0.109 | 0.186 | 0.111 | 0.084 | 0.142 | 0.126 | 0.149 | 0.101 | 0.124 | 0.119 | 0.068 | 0.148 |
| **n** | 18 | 15 | 27 | 27 | 21 | 27 | 24 | 24 | 24 | 24 | 27 | 27 |
| ***p*(C)** | | 0.9972 | 0.3685 | >0.9999 | 0.0084** | >0.9999 | 0.353 | >0.9999 | 0.2472 | 0.845 | 0.7527 | 0.8203 |
| ***p*(PTZ)** | 0.99 | | 0.97 | >0.99 | 0.17 | 0.99 | 0.97 | >0.99 | 0.92 | >0.99 | 0.99 | >0.99 |

*Table 6.4 Summary of co-localisation correlation for GluA1 with synapsin after seizures and decanoic acid and perampanel treatment. Table lists average Pearson correlation for GluA1 with synapsin normalised to control, including SEM, n of dendrites analysed and p-values for all 12 treatment groups. All repeats were normalised to respective control. Statistical analysis was carried out by ANOVA with p-values relative to control (p(C)) and to PTZ treatment (p(PTZ)), where ** corresponds to $p < 0.01$.*


A
Control
PTZ
0.2mM DA + PTZ
0.3mM DA + PTZ
0.5μM PER + PTZ
1.0μM PER + PTZ
0.5μM PER +0.2mM DA + PTZ
Neuron Overlay
GluA1 Surface
GluA1 Total
Synapsin
Dendrite Overlay
Dendrite Synapsin
Neuron Surface GluA1
Dendrite Surface GluA1
Neuron Total GluA1
Dendrite Total GluA1

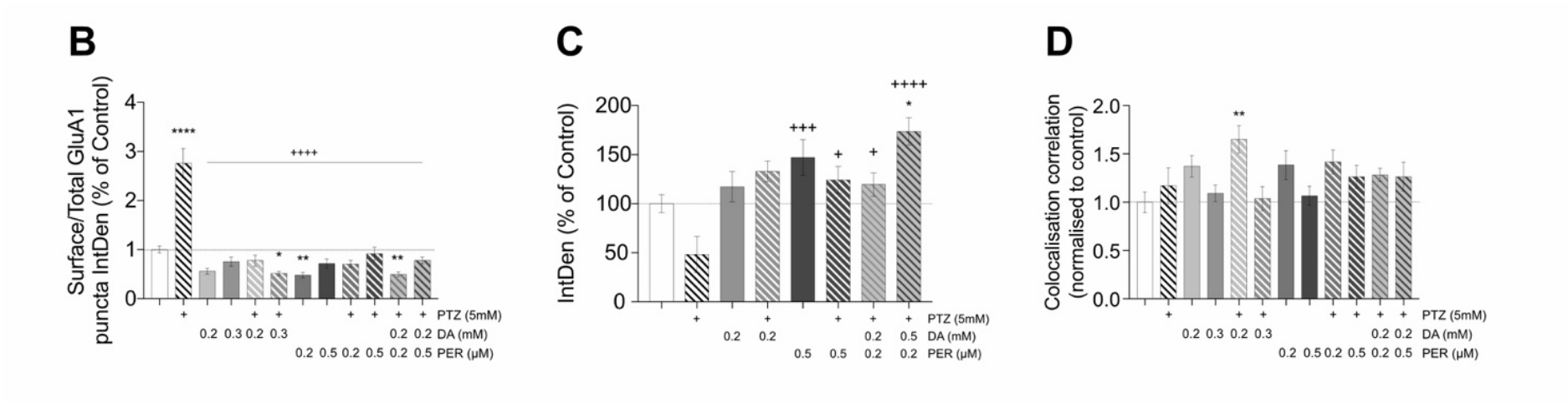



*Figure 6.6 Changes in AMPA receptor (GluA1) subunit localisation, under seizure-like conditions and in response to treatment with perampanel and decanoic acid*. Primary hippocampal neurons were treated with decanoic acid and/or perampanel for 30 min, and seizure-like activity was induced with addition of PTZ. Cells were fixed after 20 min, stained for surface GLuA1 (red); then lysed and stained for total GluA1 (green). (**A**) Representative images of double-labelling for surface GluA1 and total GluA1 in neurons showing whole neuron images as overlay (top row), surface GluA1 puncta (red) and total GluA1 (green). Enlargements show overlay with arrows indicating colocalisation of surface GluA1 to synapsin; synapsin staining (blue) surface (red) and total (green) dendrite puncta formation following treatment. Scale bars in control panel correspond to 12µm (low magnification) and 3µm (high magnification). (**B** and **C**) Quantification of average integrated density ratios of surface to total GluA1 expression in dendrites (**B**) and soma (**C**) normalised to untreated neurons. (**D**) Quantification of colocalistaion correlation as determined by Pearson's correlation coefficient. All data were normalised to control. Data are derived from 3-4 biological repeats, each with 2-3 experimental repeats with errors shown as SEM. Stars indicate *p*-values relative to control, and crosses show *p*-values relative to PTZ treatment only, where */+ corresponds to $p < 0.05$; **/++ corresponds to $p < 0.01$; ***/+++ corresponds to $p < 0.001$, and ****/++++ corresponds to $p < 0.0001$. All statistical analyses were carried out using ANOVA.

.

## 6.3 Discussion

This chapter for the first time fully characterises AMPA receptor inhibition by perampanel in *Xenopus laevis* oocytes and conclusively demonstrates that perampanel non-competitively inhibits the receptor. In addition, evidence is presented that supports the proposed binding site at the linker regions of the receptor. The AED perampanel is a potent and highly selective AMPA receptor antagonist (Ceolin *et al.*, 2012; Chen *et al.*, 2014), and its efficacy has been demonstrated repeatedly in clinical trials (French *et al.*, 2012, 2013; Krauss *et al.*, 2012). Multiple studies have investigated the mode of inhibition of AMPA receptors by perampanel, but due to a high heterogeneity of the models used and lack of isolated, direct systems, no consensus on the $IC_{50}$ could be established so far (Hanada *et al.*, 2011; Ceolin *et al.*, 2012; Chen *et al.*, 2014; Zwart *et al.*, 2014; Barygin, 2016).

In addition, data presented in this chapter demonstrates a significantly enhanced AMPA receptor antagonism upon the simultaneous application of decanoic acid and perampanel, which is most likely caused by pharmacodynamic interactions between the two antagonists. Pharmacodynamic drug interactions result from a shared mechanism of two or more drugs which can effect enhanced (synergistic) or reduced efficacy against the target (Rawlins, 1977; Jonker *et al.*, 2005). Prediction of pharmacodynamic drug interactions is therefore of considerable interest in clinical practice in order to advise on optimal drug administration. It is especially important to understand these dynamics as perampanel is used in adjuvant treatment in drug-resistant epilepsy (Steinhoff *et al.*, 2013; El Desoky, 2014; Villanueva *et al.*, 2016) and associated with a range of undesirable serious psychiatric and behavioural side effects including aggression, hostility, homicidal ideation and euphoria similar to ketamine (Fycompa FDA Approved Labeling Text, ID 3206660; Shih, Tatum and Rudzinski, 2013). Therefore, the combined use of perampanel treatment and the MCT ketogenic diet may very well occur in clinical practice and interactions between perampanel and decanoic acid may arise as a result of such a combinatory treatment. The purpose of this chapter was therefore to fully characterise perampanel inhibition of AMPA receptors and elucidate potential interactions with decanoic acid.

### 6.3.1 Characterisation of perampanel

The experiments described in this chapter confirm the highly potent, subunit independent and non-competitive inhibition of AMPA receptors by perampanel. Since antagonistic action against GluA1/2 and GluA2/3 was similarly potent, with half maximal inhibition ($IC_{50}$) achieved by 4-8µM, no apparent subunit preference was observed. This is consistent with data derived from patch-clamping experiments

in hippocampal neurons that showed no difference in inhibition of calcium-permeable and calcium-impermeable AMPA receptor generated currents (Barygin, 2016).

Multiple studies have shown the potency of perampanel against AMPA receptors in different systems, including hippocampal slices, single neuron electrophysiological studies and human neuronal membranes reconstituted in oocytes (Hanada *et al.*, 2011; Ceolin *et al.*, 2012; Chen *et al.*, 2014; Zwart *et al.*, 2014; Barygin, 2016). However, the $IC_{50}$s reported in these studies are highly variable, ranging from 60nM to 6.7µM. Interestingly, there is an apparent trend towards lower $IC_{50}$s reported in complex systems (neuronal cell cultures or hippocampal slices) that use secondary outcomes of receptor inhibition such as excitatory postsynaptic field potentials (field EPSPs) or $Ca^{2+}$-influx quantification. The only study that assessed perampanel activity in TEVC in *Xenopus* oocytes, carried out by Zwart *et al.* (2014)., used reconstituted patient-derived AMPA receptors, and reported the highest $IC_{50}$ (6.7µM), which is comparable to the results shown in this chapter. However, the tissue used in these experiments was derived from a single patient with epilepsy, and it is therefore questionable how representative these data are for a wide range of patients. In addition, in order to detect currents in this setup, co-application of 100µM CTZ was required, which has since been reported to reduce AMPA receptor antagonism by perampanel (Barygin, 2016). Therefore, it may well be that the $IC_{50}$ reported by Zwart *et al.* is an overestimation for inhibition in the system used in their study.

Whilst the $IC_{50}$ for perampanel presented in this chapter is higher than the previously reported $IC_{50}$s, the experiments summarised here are the first to determine perampanel potency against glutamate evoked currents in recombinant AMPA receptors. This is a crucial difference, as the previously published work relied on complex systems involving multiple ion channels, and in some cases used secondary, indirect effects of AMPA receptor activation (Hanada *et al.*, 2011; Ceolin *et al.*, 2012). In addition, all studies evoked currents with AMPA rather than glutamate to ensure specificity of AMPA receptor activation. AMPA is a highly selective, potent AMPA receptor agonist that binds at the same site as glutamate (reviewed by Traynelis *et al.*, 2010). Interestingly though, Chen *et al.* (2014) have found that perampanel inhibits AMPA–induced currents in a competitive manner with $IC_{50}$s ranging from 0.4µM to 1.2µM in systems activated with 10µM and 100µM AMPA, respectively. This finding has two theoretical consequences that clearly illustrate the limitations of the use of AMPA for perampanel characterisation: Firstly, the $IC_{50}$s determined in all AMPA-activated systems are subject to the AMPA concentration used in those experiments, and can therefore not be compared conclusively across the published reports. Secondly, since AMPA shares a binding site with glutamate, the competitive inhibition by perampanel shown by Chen *et al.* may well have indicated competitive inhibition in glutamate-activated currents as well. However, as shown in this chapter, perampanel inhibits AMPA receptors in a non-competitive manner.

The dose-effect assays that have been reported here, for perampanel with two doses of glutamate, and for glutamate with two doses of perampanel, clearly demonstrated non-competitive inhibition of perampanel. There was no displacement of the dose-response curve of perampanel upon increased glutamate application, showing that the mode of inhibition is most likely not competitive. The glutamate dose-effect assay confirmed this finding and conclusively demonstrates non-competitive inhibition of AMPA receptors by perampanel by showing a clear reduction in maximal response to glutamate with increasing antagonist concentrations. This demonstrates that high glutamate concentrations do not displace perampanel, and that therefore there is no competition, direct or allosteric, for the binding site. Perampanel is consistently being referred to as a non-competitive inhibitor (Rogawski and Hanada, 2013; Hanada, 2014; Patsalos, 2015), but since the experiments published so far were carried out in complex systems that may contain regulators of AMPA receptors and other membrane proteins, secondary effects on the chosen outcome measures cannot be ruled out. Activity of other proteins and ion channels in these systems thereby may obscure clear interpretation of mode of inhibition (Chen *et al.*, 2014). In contrast, the data shown here confirm non-competitive inhibition in a simple, nearly homogenous system and therefore support the published reports.

The non-competitive mode of inhibition by perampanel is substantiated by the finding that the AED is less active in the GYKI-resistant mutant of GluA3. This confirms the findings reported by Oleg Barygin (2016) that showed displacement of perampanel by increasing concentrations of GYKI, suggesting, but not proving, a shared binding site for the two antagonists. In addition, in the experiments using the GYKI-resistant mutant reported in this chapter, some residual sensitivity to perampanel was retained in the mutant, which is consistent with the findings described for GYKI in chapter 5. This suggests that the substituted S1-M1 and S2-M4 linker regions, while being involved in inhibition, may not solely constitute the binding site, and other amino acids that have yet to be identified may be involved in perampanel binding, as was discussed in 5.3.4 (page 132).

In summary, whilst the data shown here report a lower potency for perampanel compared to previously published reports, this is the first time that inhibition of glutamate-induced currents was assessed in a recombinant, largely homogenous system, and therefore comparability may be limited. Overall, these findings confirm the previously demonstrated effective concentration ranges, as well as the proposed mode of inhibition for perampanel. Further, data shown here support involvement of the AMPA receptor subunit residues that have previously been proposed to be involved in inhibition of the receptor by perampanel.

### 6.3.2 Co-application of decanoic acid and perampanel strongly potentiates AMPA receptor inhibition independent of dose and subunit composition.

To determine a potential effect of the co-application of decanoic acid and perampanel on the antagonism of AMPA receptors, inhibition in combined application of both antagonists was assessed. Surprisingly, co-application of perampanel and decanoic acid had a strong synergistic effect that increased inhibition significantly, reducing the $IC_{50}$ on average to 24.6% of that found in mono-application, across the concentrations and subunits tested. Within the doses used in the above experiments, there was no noticeable dose-dependent effect with the exception of the addition of perampanel to the decanoic acid dose-response assays in GluA1/2 expressing oocytes. This suggests that binding of one antagonist has an allosteric effect that, independent of the dose, enhances the antagonistic action of the other inhibitor, either by making the binding site more accessible or by changing the gating properties of the receptor and facilitating the shift into the closed conformation. A potential effect on gating is supported by the experiments presented in the previous chapter that demonstrated reduced inhibition of AMPA receptors by both GYKI and decanoic acid upon CTZ-co-application, and that therefore were indicative of an effect of both antagonists on gating. It is interesting that this effect is bidirectional, but due to the macroscopic scale of TEVC recordings, no clearer explanation can be given here. The exact nature of the synergistic effect between decanoic acid and perampanel could be further elucidated by using single channel recordings or crystal structure binding studies.

The synergistic enhancement of inhibition by perampanel and decanoic acid was all the more surprising for the co-application experiments between GYKI 53665 and decanoic acid that showed no augmentation of inhibition (chapter 5.2.2 and 5.2.3). It is not clear why GYKI, on which the development of perampanel was based (Hibi, 2015), reduces AMPA receptor inhibition by decanoic acid, whilst perampanel co-application enhances it. There are some structural differences between perampanel and GYKI-53665, but as the experiments using GYKI-resistant AMPA receptors show, both antagonists had very similar inhibition profiles. It is therefore very likely that perampanel and GYKI act through the same mechanism. The discrepancy between the two findings can therefore not be explained based on the TEVC experiments.

Importantly, the synergistic effect between perampanel and decanoic acid may have strong clinical implications. If co-application results in enhanced inhibition, it is possible that patients who receive both perampanel and decanoic acid may experience stronger AMPA receptor antagonism than expected. As will be discussed below (section 6.3.3), this could have serious side effects on memory and spatial awareness (Ferretti *et al.*, 2015; Olivito *et al.*, 2016). On the other hand, such enhanced activity

could also allow for a reduced dose of perampanel which might help to minimise unwanted side effects. Adverse events such as aggression, and suicidal and homicidal behaviour, have been described in conjunction with perampanel treatment, and may limit applicability of the AED in some patients (Steinhoff *et al.*, 2013; Coyle *et al.*, 2014; Villanueva *et al.*, 2016).

No behavioural side effects have been reported in clinical trials of the MCT ketogenic diet (Elizabeth G. Neal *et al.*, 2009). Although it cannot be ruled out that this may be due to lack of assessment of behavioural events in the trial design, it is noteworthy that ketogenic diets in general have been associated with improved behaviour in a variety of epilepsy syndromes (Hori *et al.*, 1997; Pulsifer *et al.*, 2001; Evangeliou *et al.*, 2003). In fact, beneficial effects of the MCT ketogenic diet on behaviour were described in a small study carried out in seven girls with Rett syndrome (Haas *et al.*, 1986). However, behavioural improvements also correlated with better seizure control and may therefore have been a secondary effect of improved quality of life rather than a direct effect of the diet. Yet, based on the findings presented in this thesis and the proof of significant levels of decanoic acid in the blood and in rodent brains (Haidukewych, Forsythe and Sills, 1982; Wlaź *et al.*, 2015), it can be assumed that use of the MCT ketogenic diet results in some degree of AMPA receptor inhibition without any reported adverse events on behaviour.

Interestingly, both GluA1 deficiency and AMPA receptor inhibition have been shown repeatedly to reduce aggression in hamsters and mice (Vekovischeva *et al.*, 2004, 2007; Fischer, Ricci and Melloni, 2007). In mice that were selected for severe aggressive behaviour, application of GYKI-52466, the antagonist on which perampanel development was based, completely eliminated all signs of aggression (Vekovischeva *et al.*, 2007). These findings make a compelling case for AMPA receptor antagonism improving behaviour and are in conflict with the clinical data reporting adverse behavioural events for perampanel. Since no animal studies on the effects of perampanel on aggression have been carried out so far, it cannot be predicted at this stage how co-application of decanoic acid and perampanel would affect behaviour. Future studies in animal models of aggression could help to better understand this relationship and provide advice on the clinical feasibility of combinatory treatment.

### 6.3.3 Seizure-like activity in hippocampal neurons increases AMPA receptor localisation to the membrane but is prevented by treatment with antagonists

The final experiments presented in this thesis that investigated changes in GluA1 localisation upon ictal activity in rat primary neuronal cell culture, show a 2.76-fold increase in GluA1 recruitment to the plasma membrane upon seizure induction with PTZ. This effect was prevented by preceding

treatment with the AMPA receptor antagonists decanoic acid and perampanel. The localisation of calcium-permeable GluA1 to the synapse critically regulates strength and frequency of signal transmission, and is highly dynamic and dependent on AMPA receptor activation (Mammen *et al.*, 1997; Ying *et al.*, 1998; Banke *et al.*, 2000). This dynamic regulation of GluA1 also provides a link to epileptogenesis, where seizure severity is thought to correlate with the likelihood for future events. The phrase "seizures beget seizures" coined by Gowers in 1881 illustrates this effect (Gowers, 1881; Hauser and Lee, 2002; Ben-Ari, 2006; Sills, 2007), and substantial evidence exists that demonstrates significant reorganisation of synapses, as well as strengthening of glutamatergic pathways following ictal activity (Cross and Cavazos, 2007; Zhou *et al.*, 2011; Bernard, 2012). One mechanism that has been suggested to contribute to synaptic strengthening is the localisation of GluA1 to the synaptic membrane, as a result of phosphorylation events. AMPA receptor hyperactivation increases calcium influx into the cell, which activates Ca2+/calmodulin-dependent protein kinase II (CamKII). Phosphorylation of serine residue 831 on the GluA1 subunit by CamKII has been shown to potentiate ion channel function and recruit GluA1 to the cell membrane (Barria *et al.*, 1997; Derkach, Barria and Soderling, 1999; Hayashi *et al.*, 2000). Increased GluA1 localisation to the membrane allows stronger cation conductance and effectively increases depolarisation. Importantly, in heteromeric receptors, trafficking of the calcium-permeable GluA1 subunit is dominant over calcium-impermeable GluA2 (Passafaro, Piëch and Sheng, 2001). For this reason, changes in GluA2 localisation due to ictal activity were not investigated in this thesis.

Treatment with decanoic acid and perampanel at physiologically relevant concentrations prevented increased GluA1 localisation to the membrane during seizure-like conditions. In primary cell culture, treatment with 0.2mM decanoic acid prior to seizure induction was sufficient to maintain GluA1 localisation levels at the membrane. In fact, treatment with decanoic acid reduced the membrane bound GluA1 population in dendrites independently of hyperexcitatory stimulation. Similarly, in perampanel treated neurons, application of 0.5µM and 1µM perampanel reduced GluA1 insertion into the membrane, with and without PTZ application. Decanoic acid has been found in the plasma of patients at average concentrations ranging from 87–552µM (mean = 157µM; Haidukewych, Forsythe and Sills, 1982). Since perampanel concentrations have been measured in the plasma of patients ranging between 1.06-3.26µM (Patsalos, 2015), the doses used in these experiments may represent physiologically relevant levels depending on the uptake efficiency into the brain.

To assess the effect of PTZ on synaptic strengthening, colocalisation of GluA1 with the synaptic marker synapsin was assessed. These experiments showed a trend towards increased synaptic localisation of GluA1 following PTZ treatment. However, in almost all cases, this was not significant. This finding suggests that increased GluA1 insertion into the membrane following seizures is a global event and not restricted to synaptic potentiation. AMPA receptor localisation is commonly differentiated into

the synaptic and the extrasynaptic membrane. Whilst insertion into the synaptic membrane is associated with synaptic potentiation, GluA1 subunits found in extrasynaptic regions are thought to serve as a pool for recruitment to the synaptic membrane, and are rapidly relocated to the synapse upon the induction of long-term potentiation (LTP, reviewed by Malinov and Malenka, 2002; Bredt and Nicoll, 2003). The consistently maintained distribution of GluA1 across the dendritic membrane therefore suggests that PTZ-triggered recruitment of AMPA receptors to the membrane is an event that is independent of LTP.

Interestingly, and contrary to the synergistic effect on AMPA receptor inhibition, the combined treatment with both perampanel and decanoic acid did not significantly reduce the amount of membrane localised GluA1 relative to total GluA1 in dendrites. Compared to mono-treatment, the ratio was in fact increased in this group. However, when assessing GluA1 localisation to the cell body, the intensity of GluA1 in the soma relative to the whole neuron was increased significantly in combinatory treatment. This suggests that GluA1 was trafficked away from the synapses and dendrites into the soma. This effect has not previously been described, and is difficult to explain. It does however imply significant depletion of glutamatergic AMPA-receptor induced synaptic transmission upon combinatory treatment.

In addition, the trend towards greater soma/total neuron GluA1 ratios was also seen in perampanel treatment without seizure induction, and could help explain some of the side effects described for perampanel. Memory loss and ataxia are associated with hippocampal long-term depression (LTD), and are common side effects of benzodiazepines and perampanel specifically (Cull-Candy, Kelly and Farrant, 2006; Krauss *et al.*, 2012; Baldwin *et al.*, 2013). Memory formation critically involves phosphorylation events on AMPA receptors, and particularly phosphorylation of serine residues on the GluA1 subunit has been shown to be required for long-term memory (Ferretti *et al.*, 2015). This is further supported by experiments in mice that contain alanine substitutions at both serine 831 and serine 845. This prevents phosphorylation of these residues by CaMKII and PKC and results in reduced spatial and object working memory, as well as decreased spatial long-term memory in these animals (Olivito *et al.*, 2016). Therefore, reduced membrane insertion of GluA1 following perampanel treatment and depletion of extrasynaptic pools of GluA1 may provide a link to some of the side effects that have been described for perampanel on memory through a near-silencing of synapses.

In conclusion, this chapter proposes a physiologically relevant synergistic effect upon the combined treatment with perampanel and decanoic acid, and shows evidence that application of either antagonist at physiologically relevant concentrations may contribute to reduced glutamatergic transmission through increased internalisation of GluA1.

# CHAPTER 7 – CONCLUSIONS

## 7.1 Summary

This thesis describes the characterisation of AMPA receptor inhibition by the medium-chain fatty acid decanoic acid and analogues thereof, and the study of pharmacodynamic interactions with the AMPA receptor antagonist perampanel. By using TEVC to measure currents that were generated in recombinant AMPA receptor subunits expressed in *Xenopus laevis* oocytes, direct and transient inhibition by decanoic acid was demonstrated, and the mode of inhibition characterised. TEVC in *Xenopus* oocytes is commonly used to study ion channel properties and receptor antagonism (Dascal, 1987; Wagner *et al.*, 2000). It is a direct system that allows real-time observation of changes in receptor activity as a readout of changes in current. At the same time, overexpression of the target protein minimises the contribution of endogenous channels to the current readout compared to studies carried out in neuronal cell culture. In this thesis, TEVC was used to define a range of parameters of AMPA receptor inhibition, including subunit specificity and voltage dependence. The expression of mutated recombinant cDNA also allowed confirmation of a GYKI- and perampanel-independent binding site for decanoic acid, suggesting a novel binding mechanism of AMPA receptor inhibition in seizure control.

In addition to the characterisation of direct AMPA receptor inhibition by decanoic acid and perampanel, TEVC was used to investigate possible pharmacodynamic effects of decanoic acid and other AMPA receptor antagonists. Using direct co-application of decanoic acid and perampanel, a synergistic effect on AMPA receptors was observed that suggests that the dosage of perampanel when applied together with the MCT ketogenic diet needs to be re-evaluated in order to avoid side effects due to the increased inhibitory activity of the drug in the presence of decanoic acid. To explore an effect of decanoic acid and perampanel treatment on seizure susceptibility, changes in GluA1 localisation following seizure-induction with PTZ and treatment with the two antagonists were examined using immunofluorescent staining. AMPA receptor antagonist treatment was found to significantly reduce GluA1 insertion into the neuronal membrane. Together with direct AMPA receptor inhibition, this may provide an explanation for the increased seizure threshold seen in mice receiving decanoic acid (Wlaź *et al.*, 2015). Overall, the results in this thesis suggest a new perspective on the mode of action of the ketogenic diet.

## 7.2 A novel mechanism for the ketogenic diets

The findings presented in this thesis strongly support a role for decanoic acid in seizure control through AMPA receptor inhibition. The experiments carried out in oocytes show that the $IC_{50}$ of decanoic acid varies between 0.5mM and 2.1mM depending on subunit composition and membrane holding voltage. In addition, increased inhibition was seen at voltages that mimicked depolarised membrane voltages. This means that at 200µM, decanoic acid can be extrapolated to reduce AMPA receptor activity by 5-20%. This degree of inhibition may be sufficient to reduce seizure susceptibility, since decanoic acid has recently been shown to significantly increase seizure thresholds in rats 30 minutes after feeding (Wlaź *et al.*, 2015). This timeframe minimised ketone generation, yet was sufficient for significant levels of decanoic acid to reach the brain, with concentrations peaking at 240µM after 60 minutes and reflecting 59-76% of plasma concentrations throughout the experiments (Wlaź *et al.*, 2015). Together with these *in vivo* data published by Wlaz *et al.*, the mechanism demonstrated in this thesis therefore makes a case for seizure control through AMPA receptor inhibition by medium-chain fatty acids and suggests a paradigm shift in the current theories on mechanisms of ketogenic diets in seizure control.

Aside from a direct effect on glutamatergic signalling, there is increasing evidence for modulation of cellular homeostasis by decanoic acid. Intracellular targets that have been suggested for decanoic acid specifically include anti-inflammatory activity (Huang *et al.*, 2014) and increased mitochondrial proliferation (Hughes *et al.*, 2014). Huang *et al.* (2014) found that treatment of human epithelial cells with decanoic acid decreased anti-inflammatory responses by reducing NF-$\kappa$B activation, which is a critical component of the cellular inflammatory response (Perkins, 2007). This finding provides a link to epileptogenesis since inflammation correlates with increased seizure-vulnerability (Gasior, Rogawski and Hartman, 2006; Vezzani *et al.*, 2013). Therefore, reduction of the inflammatory response by decanoic acid may provide another level of neuroprotection by the MCT ketogenic diet. In addition, Hughes *et al.* found that following treatment of SH-SY5Y cells with decanoic acid, activity of the mitochondrial enzyme citrate synthase was increased significantly, indicating increased mitochondrial proliferation and respiration rates, and thus potentially more efficient mitochondrial respiration (Hughes *et al.*, 2014). This adds another possible role for decanoic acid in seizure control as loss of mitochondrial activity has been shown following seizures (Kunz *et al.*, 2000; Cock, 2002), whilst increased mitochondrial proliferation was found following the treatment with ketogenic diets (Bough *et al.*, 2006). Therefore, decanoic acid may provide additional neuroprotection by enhancing mitochondrial function. Considering these findings, decanoic acid may have various roles in neuroprotection that may exceed its function in seizure control, which suggests a new, more direct angle on diet and brain health.

Research into the ketogenic diets and their effects on seizures has surged since their reestablishment into everyday clinical practice in the 1990s (Freeman *et al.*, 1998; Wheless, 2008). The strongest focus, however, has been on neuroprotective mechanisms of ketones, caloric and glucose restriction, and energy homeostasis in neuronal signalling (Bough and Rho, 2007; Freeman, Kossoff and Hartman, 2007; Hartman *et al.*, 2007). Whilst research into seizure control by fatty acids is currently novel and under-represented, the available data support a mechanism for fatty acids in seizure control. However, given the complex and varied ways in which fats and sugars are processed in the cells, it is highly unlikely that a single mechanism is responsible for the beneficial effect on seizure susceptibility and frequency (Bough and Rho, 2007). Therefore, these results add to the proposed mechanisms of ketogenic diets in seizure control, including a function for ketones in protection from seizures. Ketones have been shown to directly reduce seizures in both acute and chronic models of epilepsy (Rho *et al.*, 2002; Likhodii *et al.*, 2003; Gasior *et al.*, 2007), and to enhance mitochondrial function and thereby improving cell health (Maalouf *et al.*, 2007). Together with seizure reduction due to high ketone levels, decanoic acid may therefore contribute to seizure control in patients who receive the MCT ketogenic diet.

## 7.3 Limitations and opportunities

The findings described in this thesis clearly demonstrate a possible mode of action for the MCT ketogenic diet. However, decanoic acid is not supplemented in the other ketogenic diets, including the classical, Modified Atkins and low-glycaemic index diets. Yet all of these diets have been shown in clinical trials to be effective treatments in epilepsy, with comparable efficacies (Trauner, 1985; Pfeifer and Thiele, 2005; Kossoff *et al.*, 2006, 2008; Elizabeth G. Neal *et al.*, 2009; Muzykewicz *et al.*, 2009). Whilst evidence exists that shows modulation of glutamatergic receptors, including kainate and NMDA receptors by long-chain fatty acids (Kovalchuk *et al.*, 1994; Voskuyl *et al.*, 1998; Wilding, Chen and Huettner, 2010), a clinical trial assessing anti-convulsant action of long-chain fatty acids has found no superior action compared to placebo (Bromfield *et al.*, 2008). At this point, AMPA receptor inhibition by fatty acids can therefore only be suggested to contribute to seizure control in the specific case of the MCT ketogenic diet.

Another limiting factor is the fast breakdown of fats in the gut, and the rapid generation of ketones in the liver (Huttenlocher, Wilbourn and Signore, 1971). Whilst data exist that confirm significant levels of decanoic acid in the plasma and brain of rodents receiving decanoic acid orally by gastric gavage (Wlaź *et al.*, 2015), it is not clear how transient or steady these concentrations are in

humans. Efforts to study the mechanisms through which ketones and glucose restriction are involved in seizure control may therefore help to understand the complex effects the diets have on neuronal signalling.

A limitation of this thesis is the fact that the $IC_{50}$s that have been reported for perampanel are considerably higher than those presented by other studies (Hanada *et al.*, 2011; Ceolin *et al.*, 2012; Chen *et al.*, 2014; Barygin, 2016). One exception is the study published by Zwart *et al.* (2014) who studied AMPA receptor inhibition in reconstituted patient tissue in *Xenopus* oocytes and reported $IC_{50}$s in a similar range to those presented in this thesis. However, in the experiments presented by Zwart *et al.*, currents were co-activated with CTZ, which has since been shown to decrease perampanel activity (Barygin, 2016). On first glance, this contradicts the findings presented here and questions reproducibility. However, the experiments described here relied on overexpression of recombinant receptor protein. Therefore, data presented in this thesis can be assumed to predominantly reflect direct effects of the drug on AMPA receptors. This is not the case in heterogeneous tissues or reconstituted patient tissue, which contain a variety of natural intracellular modulators of AMPA receptors, including kinases and TARPs. In addition, in this thesis, currents were activated by glutamate, which reflects natural AMPA receptor activation (Traynelis *et al.*, 2010). The five published studies, on the other hand, used the highly selective agonist AMPA to activate receptor currents. AMPA was chosen to reduce cross-activation of other glutamate receptors in their models, so that readouts could be assumed to be largely representative of AMPA receptor activation only. However, since perampanel was shown to competitively inhibit AMPA-evoked currents (Ceolin *et al.*, 2012), it is likely that the $IC_{50}$s determined in these studies may not be representative of activity against glutamate-activated AMPA receptors.

Nonetheless, rather than seeing the heterogeneity of the available results as a limitation, the diversity of models used may help to better understand the bigger picture of how perampanel affects neuronal signalling *in vivo* and at the molecular level. Expression of recombinant AMPA receptors in this work cannot account for modulation of inhibitory activity *in vivo*. The positive modulation of decanoic acid inhibitory activity by spermine demonstrates this by showing augmentation of AMPA receptor inhibition by decanoic acid through a common intracellular molecule. This effect would have been undetected in the *Xenopus* oocyte model if these targeted experiments had not been carried out. Spermine occurs naturally at variable levels in cells (Hayashi *et al.*, 1993; Morrison *et al.*, 2002), and is therefore likely to contribute to AMPA receptor inhibition by decanoic acid *in vivo*. However, modulation by spermine is most likely not the only example of intracellular modulators, due to the wide regulatory network of AMPA receptors by various kinases and TARPs (Yamazaki *et al.*, 2004; Priel *et al.*, 2005; reviewed by Traynelis *et al.*, 2010). That being said, it can be expected that no single *in vivo*, *in vitro* or

*ex vivo* model accurately represents neuronal circuits or the activity in a patient's brain, and therefore some effects cannot always be modelled correctly in drug development.

The different effects of GYKI and perampanel addition to decanoic acid further illustrate the complexity of drug development. Whilst perfusion with GYKI reduced antagonism by decanoic acid, perfusion with perampanel significantly enhanced it. From the experiments presented here, this difference cannot be explained. Both antagonists had identical inhibition profiles regarding subunit independence, mode of inhibition and efficacy in the mutated GluA3 subunit. Interestingly, perampanel and GYKI also have different effects on aggression and irritability. Considering that GYKI-related compounds improve aggressive behaviour in a mouse model of aggression (Vekovischeva *et al.*, 2007), the fact that perampanel appears to increase irritability could not have been anticipated during drug development and is hard to explain. A better understanding of decanoic acid or perampanel interaction with AMPA receptors could be examined in binding studies that co-crystallise the compounds with the AMPA receptor. Specifically, inhibition of AMPA receptor generated currents by perampanel could be compared to GYKI *in vivo* animal models of aggression to determine if the opposing effect on behaviour is translational.

The complexity and unpredictability of translational drug discovery is further illustrated by the screening experiments carried out in this work. A wide range of medium-chain fatty acid derivatives identified in *Xenopus* oocyte screenings in this thesis were ineffective in animal models of seizures, either because they were not absorbed into the blood stream, could not cross the blood brain barrier, or were inactivated in firstpass metabolism. This illustrates that no single model can be used to accurately predict drug or antagonist profiles (Löscher, 2011).

Therefore, rather than contradicting the results published previously, the data presented here support them by demonstrating AMPA receptor inhibition by perampanel and conclusively proving non-competitive inhibition. It should therefore be considered that studies in diverse models and conditions may be combined to approximate an accurate representation of AMPA receptor antagonism by perampanel in the brain.

## 7.4 Future perspectives

AMPA receptor inhibition by decanoic acid is a fascinating, novel finding that raises the question of whether more efficient ketogenic diets could be developed in the future based on this mechanism, or if it could even contribute to the development of new AEDs. Whether enriching the currently used

MCT oil with decanoic acid or further reducing the fat:carbohydrate ratio can improve the efficacy of the MCT ketogenic diet, cannot be answered using *Xenopus* oocytes, or tissue culture. Multiple steps are involved in the process from ingestion of the MCT oil to molecular modulation of AMPA receptors by decanoic acid, which complicates predicting the factors that contribute to the breakdown of fats in the liver and transport to peripheral tissues. It is not clear how efficient the peripheral delivery of decanoic acid is, and it is very likely that this efficacy varies between patients depending on genetic variability, metabolic demand or diet composition. Various combinations of octanoic acid to decanoic acid ratios could be explored in animal models of epilepsies. However, due to interspecies variability in metabolism (Bernauer *et al.*, 2000), it is likely that not even an *in vivo* model of epilepsy can conclusively give estimates of effective ratios. Therefore, due to the established safety profiles of octanoic and decanoic acid, the most advisable mode of investigation may be through controlled clinical trials. The outcomes of the clinical trial currently assessing feasibility of different ratios of octanoic to decanoic acid are likely to provide first indications regarding the practicability of increased decanoic to octanoic acid ratios (*Identifier: NCT02825745 - Use of Betashot in Children and Adults With Epilepsy*, 2016).

Another interesting question is whether AMPA receptor inhibition by decanoic acid implies that carbohydrate restriction may be redundant in the future. However, at this point, this seems unlikely. There is evidence that carbohydrate restriction is necessary for seizure control. In a transgenic mouse model of epilepsy, seizure control though feeding of the ketogenic diet was reversed upon glucose administration (Masino *et al.*, 2011). In addition, research has been published that confirms a role for reduced glucose levels in seizure control (Williamson *et al.*, 1962; Greene *et al.*, 2002). Together with research showing the anticonvulsive action of ketone administration in seizure-susceptible transgenic animal models (Hasebe *et al.*, 2010; D'Agostino *et al.*, 2013; Kim *et al.*, 2015), these findings suggest that to bring about seizure protection, ketogenic diets most likely rely on more than one mechanism. In addition, it is possible that this spectrum of modes of action is necessary to achieve effective seizure reduction, or enable seizure protection in patients that may respond to one mechanism over another (French, 2002; St Louis, Rosenfeld and Bramley, 2009; Krauss and Sperling, 2011). Future research into the multiple aspects of ketogenic diets, and a possible role of long-chain fatty acids, could help elucidate the significance of glutamate receptor inhibition by fatty acids in epilepsy treatment.

Finally, these results show that while cost- and time-efficient screenings of drugs are optimal for rapid drug development (Heemskerk, 2004; Berghmans *et al.*, 2007), studying the molecular mechanism of drugs in detail should not be considered redundant. Whilst knowing the exact interactions of a drug or diet on the molecular mechanism may not change the existing treatment, it may improve further drug development, or our understanding of neuromodulation. From the co-localisation studies presented here, for instance, it may be concluded that, firstly, PTZ-induced seizures

do not affect LTP since there was no change in synaptic membrane co-localisation of GluA1 after PTZ treatment. However, this does not imply that seizures have no effect on LTP, and in fact, LTP-impairment in epilepsy has been reported previously (Moore, Barr and Wilson, 1993; Sørensen *et al.*, 2009). Therefore, a better understanding of the different seizure models on their effect may allow conclusions regarding their suitability and representation of actual *in vivo* seizures. In addition, following perampanel treatment, GluA1 localisation to synapses and dendrites was significantly reduced, suggesting a near-silencing effect of perampanel on hippocampal synapses in primary hippocampal neurons. This may provide a link to the memory loss commonly associated with benzodiazepine treatment (Cull-Candy, Kelly and Farrant, 2006; Krauss *et al.*, 2012; Baldwin *et al.*, 2013). Studying this effect using other benzodiazepines or in animal models of epilepsy could provide more clarity on synapse silencing in memory loss, and potentially provide suggestions on whether this mechanism is translatable to amnesia, or even dementia (Wang *et al.*, 2016). In addition, the synergistic effect of perampanel and decanoic acid should be investigated in animal models to confirm that augmentation of AMPA receptor inhibition is translatable *in vivo* and to outline a safety profile for the co-administration of the MCT ketogenic diet and perampanel.

Finally, it would be worth investigating if the synergistically increased inhibition is the result of differential modulation of both antagonists by AMPA receptor desensitisation. Results presented here show that decanoic acid and perampanel are both susceptible to the desensitisation inhibitor CTZ. This suggests that binding of the two antagonists is likely to be affected by gating. The involvement of the S1-M1 and S2-M4 linker regions in perampanel activity against GluA3 further supports this, since these regions are involved in transmitting desensitisation from the extracellular domains to the transmembrane domains (Balannik *et al.*, 2005). AMPA receptors are unique among the glutamate receptor ion channels in their ability to rapidly and strongly desensitise, a mechanism that is thought to be crucial in limiting ion conductance to millisecond periods, thereby regulating the spread of desensitisation along the neuron (Traynelis *et al.*, 2010). Interestingly, both perampanel and decanoic acid inhibit AMPA receptors selectively with no effect on NMDA receptors, and susceptibility to desensitisation could help explain this selectivity.

## 7.5 Concluding remarks

The findings presented in this thesis indicate a role for modulation of neurological signalling by medium-chain fatty acids. Whilst fatty acids have been shown previously to affect glutamatergic receptors (Voskuyl *et al.*, 1998; Wilding, Chen and Huettner, 2010), this line of research has not

attracted much interest since. Given the findings presented here, it may be time to re-evaluate the function of fats in the brain, and look beyond a role for energy provision and the detrimental effect triglyceride accumulation has on cerebrovascular perfusion. From the experiments described here, it cannot be said exactly how long-term exposure to fats affects neuronal survival and signalling. However, this thesis and the results presented here establish a role for the MCT ketogenic diet in direct inhibition of glutamatergic signalling, and thereby imply that diet may have more direct effects on brain health than has previously been assumed.

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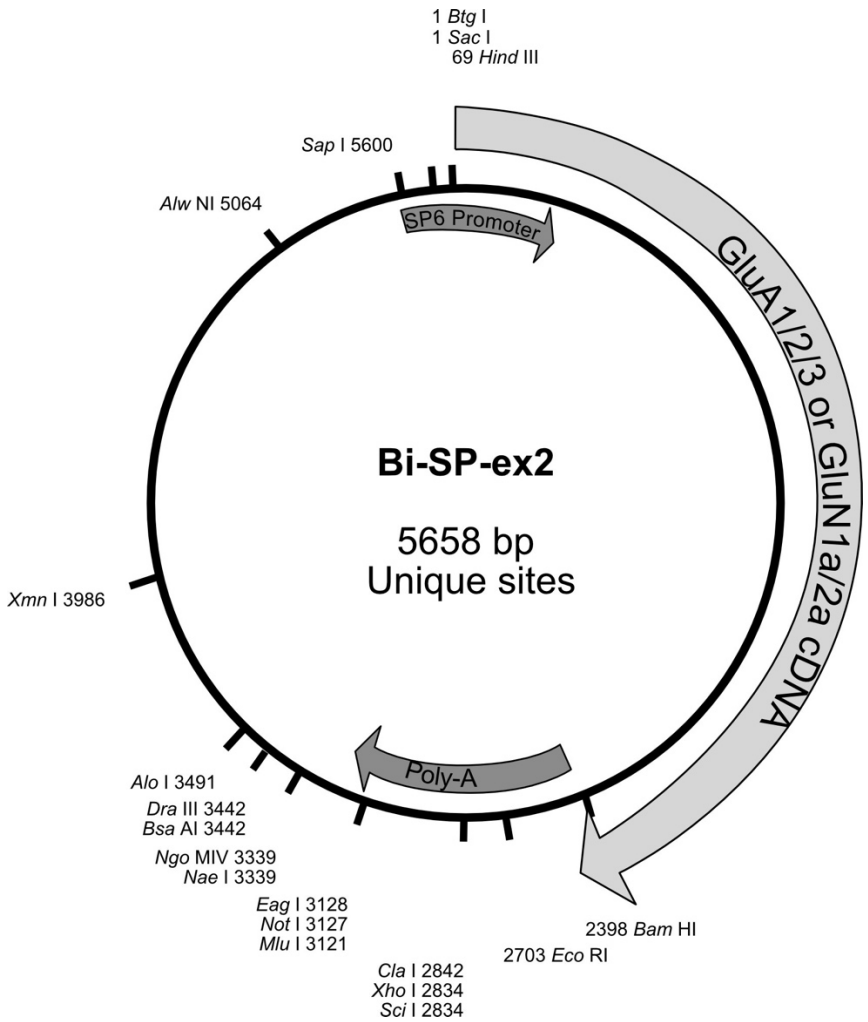
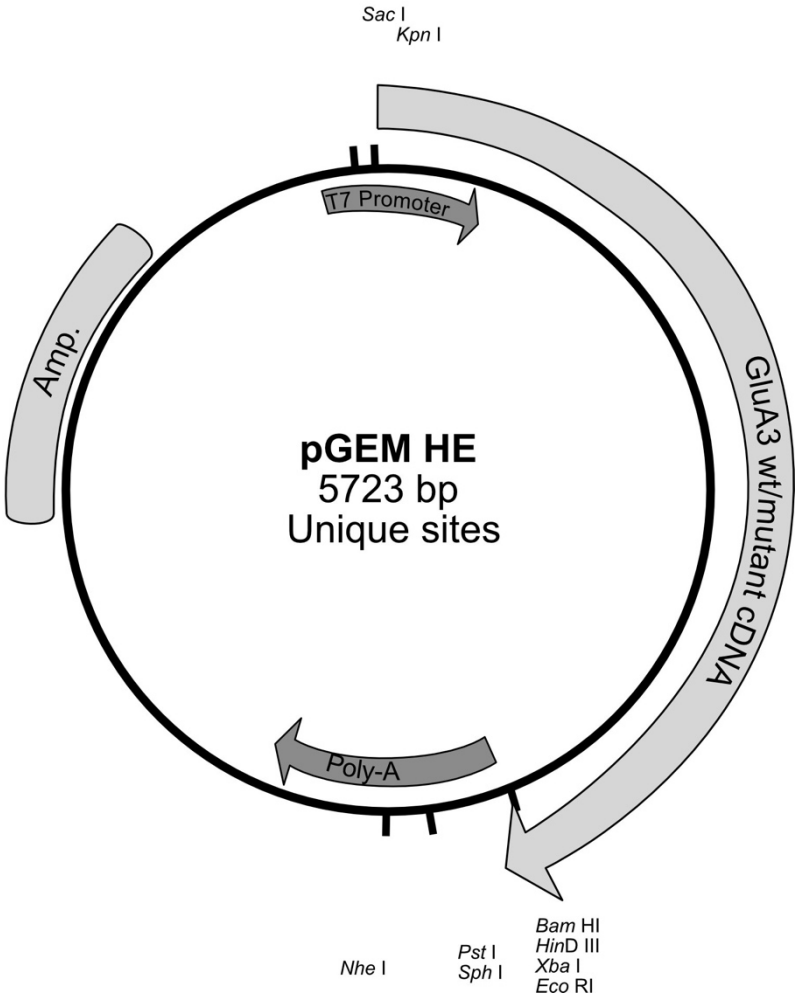
# APPENDICES

# Appendix I – Plasmids carrying cDNA used for RNA transcription for expression in *Xenopus laevis* oocytes and mutant sequences

I.i) sP6T plasmid map



I.ii) pGEM HE plasmid map (Liman, Tytgat and Hess, 1992)



## I.iii) Validation of point mutation in GluA1 mutants

### *I.iii.a) P610A*

```
cDNA sequence

GluA1  1741  AATGAGTTTGGCATATTCAACAGCCTGTGGTTCTCCCTGGGGGCCTTCATGCAGCAAGGA  1800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
P610A  1741  AATGAGTTTGGCATATTCAACAGCCTGTGGTTCTCCCTGGGGGCCTTCATGCAGCAAGGA  1800

GluA1  1801  TGTGACATTTCCCCCAGGTCCCTGTCCGGACGCATCGTCGGCGGCGTCTGGTGGTTCTTC  1860
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
P610A  1801  TGTGACATTTCCGCCAGGTCCCTGTCCGGACGCATCGTCGGCGGCGTCTGGTGGTTCTTC  1860

GluA1  1861  ACTTTGATCATCATCTCCTCGTACACAGCCAACCTGGCTGCCTTCCTGACTGTGGAGAGG  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
P610A  1861  ACTTTGATCATCATCTCCTCGTACACAGCCAACCTGGCTGCCTTCCTGACTGTGGAGAGG  1920

Protein sequence

GluA1   581  NEFGIFNSLWFSLGAFMQQGCDISPRSLSGRIVGGVWWFFTLIIISSYTANLAAFLTVER  640
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
P610A   581  NEFGIFNSLWFSLGAFMQQGCDISARSLSGRIVGGVWWFFTLIIISSYTANLAAFLTVER  640
```

### *I.iii.b) V644A*

```
cDNA sequence

GluA1  1861  ACTTTGATCATCATCTCCTCGTACACAGCCAACCTGGCTGCCTTCCTGACTGTGGAGAGG  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
V644A  1861  ACTTTGATCATCATCTCCTCGTACACAGCCAACCTGGCTGCCTTCCTGACTGTGGAGAGG  1920

GluA1  1921  ATGGTGTCTCCCATTGAGAGTGCTGAGGACCTGGCAAAGCAGACGGAAATTGCTTATGGG  1980
             ||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
V644A  1921  ATGGCCTCTCCCATTGAGAGTGCTGAGGACCTGGCAAAGCAGACGGAAATTGCTTATGGG  1980

GluA1  1981  ACATTGGAAGCAGGCTCCACTAAGGAGTTCTTCAGGAGATCTAAAATCGCTGTGTTTGAG  2040
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
V644A  1981  ACATTGGAAGCAGGCTCCACTAAGGAGTTCTTCAGGAGATCTAAAATCGCTGTGTTTGAG  2040

Protein sequence

GluA1   621  TLIIISSYTANLAAFLTVERMVSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFE  680
             |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
V644A   621  TLIIISSYTANLAAFLTVERMASPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFE  680
```

### *I.iii.c) P646A*

```
cDNA sequence

GluA1  1861  ACTTTGATCATCATCTCCTCGTACACAGCCAACCTGGCTGCCTTCCTGACTGTGGAGAGG  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
P646A  1861  ACTTTGATCATCATCTCCTCGTACACAGCCAACCTGGCTGCCTTCCTGACTGTGGAGAGG  1920


GluA1  1921  ATGGTGTCTCCCATTGAGAGTGCTGAGGACCTGGCAAAGCAGACGGAAATTGCTTATGGG  1980
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
P646A  1921  ATGGTGTCTGCCATTGAGAGTGCTGAGGACCTGGCAAAGCAGACGGAAATTGCTTATGGG  1980


GluA1  1981  ACATTGGAAGCAGGCTCCACTAAGGAGTTCTTCAGGAGATCTAAAATCGCTGTGTTTGAG  2040
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
P646A  1981  ACATTGGAAGCAGGCTCCACTAAGGAGTTCTTCAGGAGATCTAAAATCGCTGTGTTTGAG  2040


Protein sequence

GluA1   621  TLIIISSYTANLAAFLTVERMVSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFE  680

P646A   621  TLIIISSYTANLAAFLTVERMVSAIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFE  680
```

# Appendix II – Results from NIH NINDS Screening Program for medium-chain fatty acids in animal models of seizure control

II.i) *In vitro* slice electrophysiology studies

| Compound | Concentration tested (µM) | n | % Control Burst Rate | SEM | % Control Burst Duration | SEM |
|---|---|---|---|---|---|---|
| **4-EOA** | 100 | 5 | 87 | 4 | 90 | 3 |

II.ii) Hippocampal kindling studies

| Compound | Route | Time (Hrs) | $ED_{50}$ (µM) | 95% CI | Slope | STD Error |
|---|---|---|---|---|---|---|
| **4-EOA** | I.P. | 0 | 34.67 | 21.86-52.83 | 5.17 | 1.85 |

II.iii) Corneal kindling studies

| Compound | Route | Time (Hrs) | $ED_{50}$ (µM) | 95% CI | Slope | STD Error |
|---|---|---|---|---|---|---|
| **4-EOA** | I.P. | 0.25 | 71.43 | 46.75-101.18 | 3.77 | 1.14 |

II.iv) 6Hz Seizure test

| Compound | Route | Time (Hrs) | $ED_{50}$ (µM) | 95% CI | Slope | STD Error |
|---|---|---|---|---|---|---|
| **4-BCCA** | P.O. | n/a | >250 | - | - | - |
| | I.P. | 0.25 | 66.95 | 60.22-73.82 | 23.5 | 8.66 |

| Time (Hrs) | | | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 |
|---|---|---|---|---|---|---|---|
| Compound | Dose | Deaths | N/F | N/F | N/F | N/F | N/F |
| **M-1** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 0/4 | - |
| | 300 | | - | 3/4 | - | 1/4 | - |
| **M-2** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 1/4 | - |
| | 300 | | - | 4/4 | - | 3/4 | - |
| **M-4** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 0/4 | - |
| | 300 | | - | 1/4 | - | 0/4 | - |
| **D-2** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 0/4 | - |
| | 300 | | - | 0/4 | - | 0/4 | - |

II.v) MES seizures test

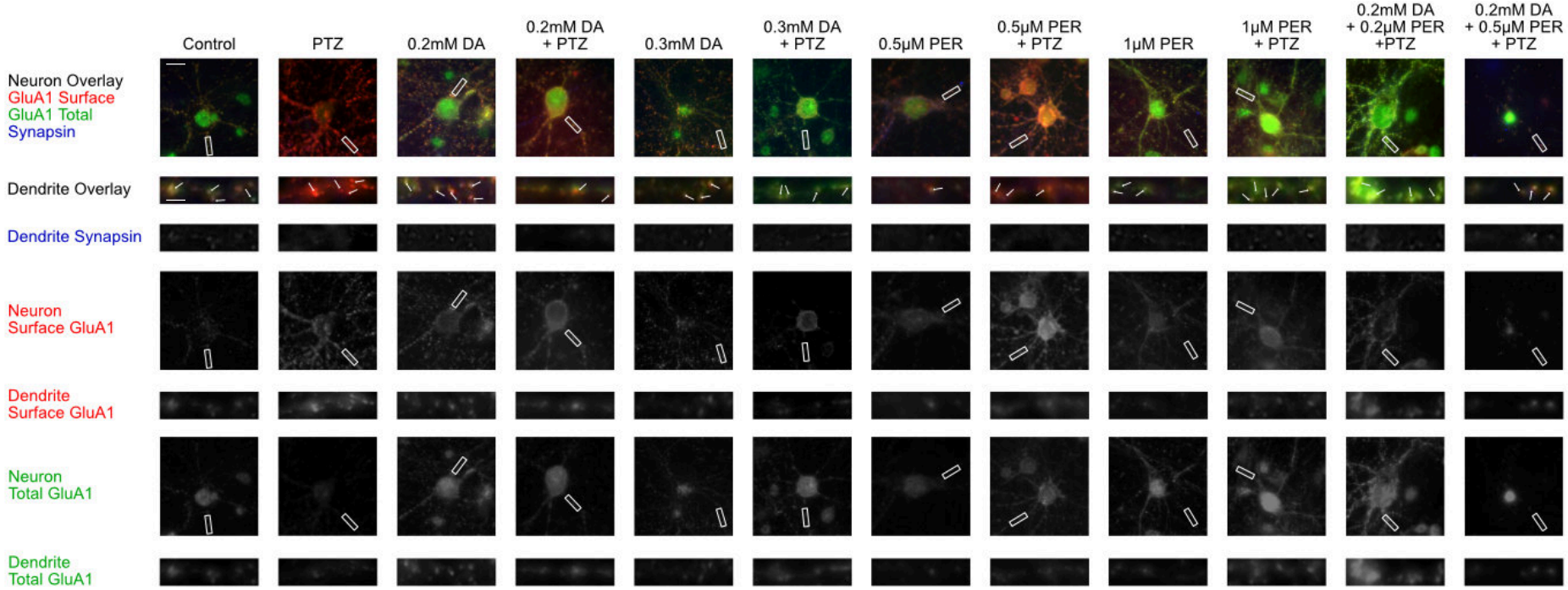
| Time (Hrs) | | | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 |
|---|---|---|---|---|---|---|---|
| **Compound** | **Dose** | **Deaths** | **N/F** | **N/F** | **N/F** | **N/F** | **N/F** |
| **4-PCEA** | 150 | | 0/4 | 0/4 | 3/4 | 0/4 | 0/4 |
| **M-1** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 0/4 | - |
| | 300 | | - | 3/4 | - | 2/4 | - |
| **M-2** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 1/4 | - | 1/4 | - |
| | 300 | | - | 4/4 | - | 3/4 | - |
| **M-4** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 0/4 | - |
| | 300 | | - | 0/4 | - | 1/4 | - |
| **D-2** | 30 | | - | 0/4 | - | 0/4 | - |
| | 100 | | - | 0/4 | - | 0/4 | - |
| | 300 | | - | 0/4 | - | 0/4 | - |

II.vi) Toxicity screen

Toxicity was assessed using rotorod assessment, minimal motor impairment assessment and automated locomotor activity assessment.

| Time (Hrs) | | | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 |
|---|---|---|---|---|---|---|---|
| **Compound** | Dose | Deaths | N/F | N/F | N/F | N/F | N/F |
| **4-PCEA** | 150 | | 3/4 | 4/4 | 0/4 | 0/4 | 0/4 |
| | 100 | | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| **M-1** | 30 | | - | 0/8 | - | 0/8 | - |
| | 100 | | - | 0/8 | - | 0/8 | - |
| | 300 | | - | 1/8 | - | 1/8 | - |
| **M-2** | 30 | | - | 0/8 | - | 0/8 | - |
| | 100 | | - | 0/8 | - | 0/8 | - |
| | 300 | | - | 6/8 | - | 3/8 | - |
| **M-4** | 30 | | - | 0/8 | - | 0/8 | - |
| | 100 | | - | 0/8 | - | 0/8 | - |
| | 300 | | - | 1/8 | - | 0/8 | - |
| **D-2** | 30 | | - | 0/8 | - | 0/8 | - |
| | 100 | | - | 0/8 | - | 0/8 | - |
| | 300 | | - | 0/8 | - | 0/8 | - |

# Appendix III – Immunofluorescent images for the acute treatment model of therapeutic AED administration




Representative images of double-labelling for surface GluA1 and total GluA1 in neurons showing changes in AMPA receptor (GluA1) subunit localisation, under seizure-like conditions and in response to treatment with perampanel and decanoic acid. Primary hippocampal neurons were treated with decanoic acid or/and perampanel for 30min, and seizure-like activity was induced with addition of PTZ. Cells were fixed after 20 minutes, stained for surface GLuA1 (red); then lysed and stained for total GluA1 (green). Images show whole neuron images as overlay (top row), surface GluA1 puncta (red) and total GluA1 (green). Enlargements show overlay with arrows indicating colocalisation of surface GluA1 to synapsin; synapsin staining (blue) surface (red) and total (green) dendrite puncta formation following treatment. Scale bars in control panel correspond to 12μm (low magnification) and 3μm (high magnification).