The role of auxiliary proteins in AMPA receptor function

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Declaration

This thesis is submitted to University College London in Faculty of Life Sciences for the degree of Doctor of Philosophy. I, Sarah Elizabeth Pearce, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:______________________________

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Abstract

Glutamate receptors of the AMPA-subtype mediate the majority of fast excitatory neurotransmission in the central nervous system (CNS). These receptors are associated with auxiliary proteins that have been shown to affect AMPA receptor (AMPAR) properties. Transmembrane AMPAR Regulatory Proteins (TARPs) were the first identified AMPAR auxiliary subunits, and since then, further auxiliary subunits continue to be identified. In this thesis, I investigate the effects of a novel auxiliary protein candidate – FRRS1L – on AMPAR function, as well as the involvement of TARPs in mediating AMPAR plasticity in a model of ischaemic stroke.

I show that when co-expressed with both homomeric GluA1 and heteromeric GluA1/GluA2 AMPAR subunits in tsA201 cells, FRRS1L slows recovery from receptor desensitization, without significant effect on other AMPAR properties. When the prototypical TARP stargazin is additionally co-expressed, the effect of FRRS1L is lost and the receptor properties appear as if only stargazin were associated. I examine the endogenous expression of FRRS1L in the CNS, and find that it is highly abundant in multiple brain regions including the hippocampus, cortex and cerebellum. In cultured hippocampal neurons, overexpressed FRRS1L does not influence mEPSC amplitude or frequency.

In a model of ischaemic stroke, direct ASIC1-a activation through lowering of the pH (acidosis) is sufficient to drive AMPAR plasticity in cultured hippocampal neurons. This manifests as a decrease in the GluA2 subunit, resulting in an increase of CP-AMPARs in the membrane. I show that this change in AMPAR...
subunit expression following acidosis is likely accompanied by an increase in TARP $\gamma$-8 expression. Using various techniques, I explore the change in CP-AMPAR expression and cell excitability. I show that following acidosis, there is an increase in Ca$^{2+}$ entry into the cells, which is likely mediated through CP-AMPARs.
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1. Introduction

AMPA Rs assemble with auxiliary subunits to form macromolecular complexes that when activated by the excitatory neurotransmitter glutamate are responsible for a majority of fast excitatory neurotransmission in the central nervous system. This thesis considers the way auxiliary proteins shape the post-synaptic response of AMPARs to this important excitatory transmitter in both health and disease. One of my main themes focuses on the newly AMPAR-interacting protein FRRS1L, how it influences receptor function and whether it can be defined as a novel auxiliary subunit. The other main theme of the thesis focuses on the TARPs that control the calcium permeability of AMPARs in the hippocampus following ischaemic stroke. In order to place this work into context, I will start my introduction with a general description of glutamatergic transmission, before summarising some of the existing knowledge on AMPARs and their auxiliary subunits relevant to this thesis. I will then briefly outline the circuitry of the hippocampus, and how AMPARs in this region of the brain are affected following ischaemic stroke.

1.1. Excitatory transmission in the central nervous system

Upon release from the presynaptic bouton, the excitatory neurotransmitter activates postsynaptic receptors that generate the excitatory postsynaptic currents required to propagate the neuronal signal (Curtis et al., 1959, Curtis et al., 1960). The properties of this postsynaptic signal vary greatly, which is attributed to a number of factors.
1.1.1. Neurotransmitter release

One of the factors influencing the properties of the postsynaptic signal is the release of neurotransmitter. Neurotransmitters are stored in vesicles at the synaptic terminal of the pre-synaptic neuron. When action potentials reach the nerve terminal, they induce the opening of voltage-gated Ca$^{2+}$ channels, leading to an accumulation of intracellular Ca$^{2+}$ near the synaptic terminal (Katz and Miledi, 1967, Sudhof, 2012). The increase of Ca$^{2+}$ is sensed by the membrane-trafficking protein synaptotagmin, which causes vesicles to fuse with the presynaptic membrane and release the neurotransmitter into the synaptic cleft (Geppert et al., 1994, Jahn and Sudhof, 1994). The number of vesicles releasing their contents into the cleft will influence the magnitude of the postsynaptic response.

1.1.2. The postsynaptic response

Once the neurotransmitter has been released, it diffuses across the synaptic cleft to the postsynaptic neuron, where it binds to specific ionotropic or metabotropic receptors embedded in the postsynaptic membrane. If the receptor is ionotropic, its probability of undergoing a change in conformational state (usually from closed to open) increases, allowing ions to flow and thereby directly affecting membrane properties. Depending on the number and the ionic selectivity of the postsynaptic channels, as well as the membrane voltage, this will lead to either an excitatory postsynaptic potential (EPSP) or an inhibitory postsynaptic potential (IPSP). Metabotropic receptors are linked to intracellular G-proteins that become activated upon ligand binding, causing an intracellular signalling cascade. In addition to the aforementioned factors of neurotransmitter release, diffusion across the synaptic cleft and the type of receptor present in
the postsynaptic membrane, the properties of the postsynaptic signal also rely on many other factors including transmitter removal and the sub-type of receptor present. Even among ionotropic glutamate receptors, there exist several distinct families that elicit different postsynaptic responses.

1.2. Ionotropic glutamate receptors

All ionotropic glutamate receptors are integral membrane proteins consisting of four subunits that assemble to form a central cation-permeable channel pore (Rosenmund et al., 1998, Traynelis et al., 2010). To date, at least three main ionotropic glutamate receptor families have been identified and named after their selective agonists: N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate receptors (AMPARs) and kainate receptors (KARs) (Figure 1.1) (Watkins, 1981). These can be grouped into NMDA receptors and non-NMDA receptors (AMPA- and KARs). As this thesis focuses on AMPA-type glutamate receptors, a more thorough introduction of these is given in the next section.
Figure 1.1. Classification of ionotropic glutamate receptors
Classification of ionotropic glutamate receptors and their receptor subunits.

1.2.1. NMDA receptors

Synaptic release of glutamate typically evokes a response with a fast rise and a
relatively slow decay. The decay consists of an early and a late component.
Application of the NMDAR antagonist DL-AP5 blocks the late component,
indicating the involvement of NMDARs in this phase of the response (Forsythe and
Westbrook, 1988, Lester et al., 1990). Activation of NMDARs requires the
binding of two agonists, glutamate and glycine (Johnson and Ascher, 1987), as
well as the voltage-dependent relief from extracellular magnesium block,
(Mayer et al., 1984, Nowak et al., 1984). Only then can the channels open to
allow the influx of positively charged ions, including Ca$^{2+}$, to which these
channels are highly permeable.

A variety of NMDAR subunits have been identified: GluN1, GluN2A-D and
GluN3A-B (Cull-Candy et al., 2001, Paoletti et al., 2013). Most native NMDARs
are thought to function as heterodimers consisting of two glycine-binding GluN1 and two glutamate-binding GluN2 subunits (Cull-Candy and Leszkiewicz, 2004, Monyer et al., 1992, Nakanishi, 1992), arranged in a 1-2-1-2 formation around the channel pore (Lee et al., 2014). The distinct NMDAR subtypes vary in their biophysical and pharmacological properties, their interacting partners and subcellular localisation (Cull-Candy et al., 2001, Paoletti et al., 2013).

1.2.2. Kainate receptors
Kainate receptors are tetrameric combinations from 5 subunits: GluK1-5 (Bettler et al., 1990). Of these, only GluK1-3 are able to form functional homomeric or heteromeric receptors, whereas GluK4-5 require the partnering of any of the GluK1-3 to form functional ion channels (Herb et al., Kumar et al., 2011, Werner et al., 1991). Like all other ionotropic glutamate receptors, KARs also form tetrameric assemblies, that display various properties depending on the subunit composition, and are differentially expressed throughout the CNS (Wisden and Seeburg, 1993). When activated in neurons, KARs generate excitatory postsynaptic currents (EPSCs) that are characterized by slow rise and deactivation times (Castillo et al., 1997). These, and almost every other KAR property, are largely shaped by the auxiliary subunits neuropilin and tolloid-like 1 (Neto1) and Neto2, which are expressed alongside KARs throughout the CNS (Copits and Swanson, 2012, Howe, 2015).

1.3. AMPA-type glutamate receptors
In the central nervous system, most of the fast excitatory neurotransmission is mediated by AMPA receptors. These are thought to underpin mechanisms involved in memory, learning, neuronal development, ageing and disease.
Rapidly activated upon glutamate release from the presynaptic bouton, AMPARs generate the initial excitatory postsynaptic currents required to propagate the neuronal signal. The properties of the signal vary within different CNS regions and at different synapses. This diversity in glutamatergic signalling arises partly from functional differences between the four AMPAR subunits (GluA1-4) (Figure 1.2) (Furuyama et al., 1993, Geiger et al., 1995, Tichelaar et al., 2004), the expression of which varies depending on the brain region, age and disease state (Cantanelli et al., 2014, Cull-Candy et al., 2006, Pagliusi et al., 1994).

1.3.1. AMPAR structure

AMPAR subunits assemble as homo- or hetero-tetramers, and the various compositional arrangements results in an array of different receptor properties (Dingledine et al., 1999, Traynelis et al., 2010). All subunits are approximately 100 kDa and share a 70% sequence homology (Rogers et al., 1991). They consist of a common structure with 3 transmembrane spanning domains (M1, M3 and M4) and a cytoplasmic re-entrant loop (M2) (Figure 1.2.A). Together with the M3 domain, this M2 re-entrant loop forms the lining of the channel pore (Hollmann et al., 1994, Sobolevsky et al., 2003, Wood et al., 1995). The extracellular N-terminal domain has been shown to be involved in the regulation of AMPAR assembly (Kuusinen et al., 1999). The ligand-binding domain resides within a conserved amino acid pocket (S1 and S2) and is linked the extracellular regions between the M1 and M3-M4 transmembrane domains. High-resolution analyses of crystal structures conclude that this region forms part of a clam shell-like structure, which contracts upon agonist binding, resulting in the opening of the channel (Armstrong and Gouaux, 2000).
The cytoplasmic C-terminal contains a PDZ binding domain, which is responsible for the binding of a number of AMPAR trafficking proteins (Barry and Ziff, 2002). While the N-terminal and transmembrane domains share large sequence homology, the C-tail differs among the various subunits (Malinow and Malenka, 2002). GluA1 and GluA4 usually possess a long C-terminus, while GluA2 and -3 have shorter C-tails. GluA2 also exists in a long form and GluA4 in a short form (Figure 1.2.B), resulting in altered trafficking properties (Gallo et al., 1992, Ziff, 2007).
Figure 1.2. AMPAR structure, RNA editing and post-translational modifications
A. Each AMPAR subunit contains 3 transmembrane spanning domains (M1, -3 and -4) and a cytoplasmic re-entrant loop (M2). S1 and S2 form the glutamate (green circle) binding domain. Amino-terminal domain (ATD). The flip/flop alternative splice region is highlighted in red. Figure adapted from Biggin (2002). B. Schematic representation of the flip/flop alternative splice regions (red), located between the M3 and M4 transmembrane domains. C-terminal length and splice variants (green) give rise to altered trafficking properties. The
blue rectangles depict the membrane spanning regions (M1-4). Depiction of the Q/R editing site on the GluA2 M2 region. Figure adapted from Dingledine et al. (1999).

1.3.2. RNA editing and post-translational modifications

RNA editing and post-translational modifications of subunits provide further diversity to AMPAR signalling properties. Each AMPAR subunit can exist as two splice variants “flip” or “flop” (Figure 1.2) (Monyer et al., 1991, Pei et al., 2009, Sommer et al., 1990). The affected short exon (38 amino acids) resides within the extracellular M3-M4 loop (Bennett and Dingledine, 1995, Stern-Bach et al., 1994), and has been shown to affect receptor kinetics. The main functional difference is the variation in receptor desensitization properties, with flop isoforms desensitizing more rapidly than flip (Sommer et al., 1990). The two isoforms also exhibit different sensitivity to drugs including cyclothiazide, aniracetam and thicyanate (Lomeli et al., 1994, Partin et al., 1996, Sommer et al., 1990). In rats, the flip isoform is expressed predominantly before birth and during postnatal brain development, until postnatal day 8, when flop mRNA expression begins to increase, reaching adult levels, which are similar to the flip levels, by postnatal day 14 (Monyer et al., 1991).

Probably the most striking AMPAR modification is mRNA editing at the Q/R site of GluA2. This single amino acid site resides within the channel pore lining (Figure 1.2.B), and its post-transcriptional modification changes the uncharged glutamine residue to a positively charged arginine (Burnashev et al., 1992, Seeburg and Hartner, 2003). Editing at this site is nearly 100% efficient in the adult brain, and consequently, any AMPAR that contains the edited GluA2
subunit has profound differences in receptor properties. Because the channel pore has a positively charged residue, edited GluA2-containing receptors are much less permeable to \( \text{Ca}^{2+} \) and exhibit relatively low single-channel conductance (Swanson et al., 1997). These receptors are also insensitive to intracellular polyamine block, giving rise to currents with a linear current-voltage (I-V) relationship. By contrast, AMPARs lacking the edited GluA2 subunit are \( \text{Ca}^{2+} \)-permeable, display high single-channel conductance (Swanson et al., 1997), and are blocked by intracellular polyamines in a voltage-dependent manner (Bowie and Mayer, 1995, Kamboj et al., 1995, Koh et al., 1995, Schmidt et al., 2014, Swanson et al., 1997), giving rise to an inwardly rectifying I-V relationship (Figure 1.3).

AMPARs undergo substantial post-translational N-glycosylation, palmotylation and phosphorylation. N-glycosylation has no intrinsic effects on AMPAR channel conductance, or ligand-receptor interactions, as recombinant non-glycosylated channels exhibit similar functions to glycosylated receptors (Everts et al., 1997, Pasternack et al., 2003). Glycosylation is, however, thought to play a role in receptor trafficking and stabilisation at the synaptic terminal, as site-specific mutations of N-glycosylation sites on GluA2 result in decreased cell surface expression (Takeuchi et al., 2015). Similarly, AMPAR palmotylation at two cysteine sites also regulates receptor trafficking. When the residue within the M2 transmembrane domain is palmotylated, there is an accumulation of the receptor in the Golgi and decreased receptor surface expression. Palmotylation of the C-terminal domain prevents receptor internalisation induced by NMDAR or AMPAR activation (Hayashi et al., 2005, Lussier et al., 2015). Several phosphorylation sites have been mapped onto the intracellular carboxy-terminal
of all four AMPAR subunits (Song and Huganir, 2002). In hippocampal neurons, phosphorylation by cyclic AMP-dependent protein kinase A (PKA), protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII) potentiates AMPAR currents (Greengard et al., 1991, Mao et al., 2014, McGlade-McCulloh et al., 1993, Roche et al., 1996).

Figure 1.3. Schematic representation of AMPAR subunit assembly to form functional tetrameric receptors
**Figure 3.1. Schematic representation of AMPAR subunit assembly to form functional tetrameric receptors**

Edited GluA2 expressing arginine (R) at the Q/R site is shown in pink, other unedited subunits expressing glutamine (Q) at the Q/R site are in blue. **A.** Individual AMPAR subunits, initially synthesized in the ER. **B.** Individual subunits undergo dimerization in the ER. Heteromeric dimers are favoured, but homomeric dimers also occur. **C.** Dimers undergo tetramerization. Pairs of identical dimers are favourably assembled, resulting in hetero-tetramers. A range of likely assemblies is depicted on the left. The resulting current-voltage plots are shown on the right, which depend on the inclusion of the GluA2 subunit. Numerals indicate individual subunit types. **D.** (Left panel) An uneven number of a single subunit, or the adjacent juxtaposition of two identical subunits results in an unstable receptor assembly. (Right panel) Homomeric assemblies are permitted. Figure adapted from Cull-Candy et al. (2006).

### 1.4. AMPAR auxiliary proteins

In the CNS, AMPARs are believed to exist as a multi-protein complex (Schwenk et al., 2012). The first discovery of a protein to associate within this complex occurred in the early 2000s (Chen et al., 2000, Tomita et al., 2005, Vandenberghe et al., 2005). Since then, further AMPAR-interacting proteins have been described, and are collectively termed *auxiliary proteins* or *auxiliary subunits*. Depending on the proteins present in this complex, AMPARs express an array of different properties, adding another layer of complexity to the function and regulation of these receptors.

#### 1.4.1. Definition of an AMPAR auxiliary protein

With the increasing discovery of AMPAR-interacting proteins, the question arises how to distinguish which of these become classified as auxiliary subunits. As described by Yan and Tomita (2012), an auxiliary subunit is defined according to the following four criteria: (1) Non-pore-forming subunit, (2) Direct and stable interaction with a pore-forming subunit, (3) Modulation of
channel properties and/or trafficking in heterologous cells, (4) Necessity *in vivo*. Based on these criteria, a number of AMPAR auxiliary proteins have been identified and characterised.

### 1.4.2. Transmembrane AMPAR regulatory proteins (TARPs)

The first auxiliary protein identified to interact with AMPARs was the transmembrane AMPAR regulatory protein (TARP) γ-2, or stargazin (Chen et al., 2000, Vandenberghe et al., 2005). Often referred to as the ‘prototypical TARP’, stargazin was first identified as a result of experiments in stargazer mice. This naturally occurring mutant lacks functional γ-2 (Letts et al., 1998). These mice display symptoms of absence epilepsy, unsteady gait and unusual head nodding (Letts et al., 1998), highlighting the importance of TARPs in normal neuronal function. Since then, 5 further TARPs have been characterized and are divided into the family of type I (typical) TARPs (γ-2, γ-3, γ-4 and γ-8) and type II (atypical) TARPs (γ-5 and γ-7) (Kato et al., 2007, Kato et al., 2008, Soto et al., 2009).

In-situ hybridisation reveals brain area-specific expression of TARPs. In adult brains, γ-2 and γ-7 are widely expressed, with the highest levels in cerebellar granule and Purkinje cells. The telencephalon shows high expression of γ-3 and γ-8, with the latter being at striking levels in the hippocampus. The γ-4 subunit predominates in the olfactory bulb, striatum, thalamus and hypothalamus, while γ-5 expresses highly in the olfactory bulb, hippocampal CA2, thalamus, inferior colliculus and Bergmann glia (Fukaya et al., 2005).
TARPs modify many AMPAR properties, including channel kinetics, open probability (Cho et al., 2007, Nicoll et al., 2006, Turetsky et al., 2005), single-channel conductance (Soto et al., 2007, Tomita et al., 2005), sensitivity to polyamine block (Soto et al., 2007), interaction with agonists and antagonists (Schober et al., 2011, Tomita et al., 2006) and receptor trafficking to the surface membrane (Bats et al., 2007, Chen et al., 2000, Coombs et al., 2017, Tomita et al., 2003, Turetsky et al., 2005).

1.4.2.1. The role of TARPs in AMPAR trafficking

Initial studies demonstrated that in stargazer mice, the fast AMPAR-mediated component of mEPSCs at glutamatergic synapses between mossy fibre and granule cells in the cerebellum was absent. Since the slow NMDA-mediated component remained largely unchanged, it suggested that the release of glutamate and formation of the synapse remained unimpaired (Hashimoto et al., 1999). Transfecting recombinant stargazin into cerebellar granule cells from stargazer mice fully restored synaptic AMPAR function, suggesting that stargazin plays a crucial role in trafficking and stabilising AMPARs at the synaptic terminal (Chen et al., 2000). Using chemiluminescence to detect HA-tagged GluA1 transfected into Xenopus oocytes, Tomita and colleagues showed that the co-injection of stargazin increases receptor surface expression by about 10-fold. By swapping the cytoplasmic C-tail of stargazin with that of γ-5 (which does not enhance glutamate-evoked currents of GluA1 in oocytes), they demonstrated that this region is crucial for AMPAR trafficking to the surface membrane (Tomita et al., 2005).
Another mechanism by which TARPs strengthen receptor surface expression is aiding receptor glycosylation, through increasing receptor trafficking to the Golgi apparatus. Cerebellar granule cells isolated from stargazer mice show an increased number of immature, un-glycosylated receptors that have do not reach the synapse (Tomita et al., 2003).

Various proteins aid TARPs in the synaptic targeting of AMPARs to the cell surface membrane. TARPs contain a PDZ binding domain, which binds to post-synaptic density protein 95 (PSD-95), a member of the MAGUK family of scaffolding proteins located in the post-synaptic density, thereby tethering AMPARs to the plasma membrane (Figure 1.4) (Bats et al., 2007, Chen et al., 2000, Dakoji et al., 2003, Ziff, 2007). The PDZ binding domain also interacts with membrane associated guanylate kinase 2 (MAGI-2) (Deng et al., 2006) and neuronal isoform of protein-interacting specifically with TC10 (nPIST) (Cuadra et al., 2004) – further proteins implicated in shuttling of transmembrane proteins, emphasizing the role of TARPs in AMPAR trafficking.
Figure 1.4. Trafficking of the AMPAR-TARP complex to the synaptic membrane

In the endoplasmic reticulum (ER), single AMPAR subunits undergo dimerisation, followed by tetramerisation (1). Upon the addition of TARPs to the AMPAR tetramers (2), the complexes are targeted to the Golgi apparatus (3). Here, the receptors undergo post-translational modifications including glycosylation. This is also where proteins such as nPIST, which associates with the C-tail of the TARP, are added to the complex. Transport vesicles subsequently shuttle the mature receptors to the extrasynaptic space (4), where they become inserted into the cell membrane (5). Phosphorylation facilitates mobilization to the synapse, where interactions with members of the MAGUK family such as PSD-95 anchor the receptors to the synaptic membrane (6). After internalization (7), potentially prompted by the dissociation of TARPs following agonist activation, AMPARs are either recycled back to the cell membrane (8), or sent to lysosomes for degradation (9). Figure adapted from Payne (2008) and Coombs and Cull-Candy (2009).
1.4.2.2. TARPs modulate AMPAR channel properties

The tight interaction between TARPs and AMPARs allows the auxiliary subunits to exert a wide range of effects on the biophysical properties of the receptors (Figure 1.5) by stabilising or altering the time the receptor remains in a certain state (Osten and Stern-Bach, 2006). In studies using dissociated neuronal cultures, Xenopus oocytes or human embryonic kidney (HEK) cells, the over-expression of stargazin reduces glutamate-evoked AMPAR channel desensitization and deactivation (Figure 1.5.A-B) and accelerates receptor recovery from desensitization (Nicoll et al., 2006, Priel et al., 2005, Turetsky et al., 2005). The response to the partial agonist kainate is also enhanced in the presence of stargazin (Turetsky et al., 2005). Pharmacological responses to various agonists and antagonists are also dependent on the presence of TARPs (Schober et al., 2011, Tomita et al., 2006). For example, the co-expression of stargazin renders both flip and flop isoforms of GluA1 sensitive to cyclothiazide and 4-(2-(phenylsulfonylamino)ethylthio)-2,6-difluorophenoxyacetamide (PEPA) – two AMPAR potentiators that in the absence of TARP show isoform-selectivity (Tomita et al., 2006). TARPs increase the rate of channel opening, as well as single-channel conductance and Ca\(^{2+}\)-permeability (Figure 1.5.A-C) (Kott et al., 2009, Soto et al., 2007, Tomita et al., 2005). One of the characteristics of Ca\(^{2+}\)-permeable AMPARs (CP-AMPARs) is that they are subject to a voltage-dependent block by intracellular polyamines (Bowie et al., 1998, Kamboj et al., 1995, Koh et al., 1995). When associated with TARPs, CP-AMPARs have reduced sensitivity to polyamines at both positive and negative potentials, resulting in more linear, non-rectifying current-voltage relationships (Figure 1.5.E-F) (Soto et al., 2007).
It was believed that while trafficking of AMPARs involves the C-tail of TARPs, the alterations in biophysical properties were attributed to the first extracellular loop (Tomita et al., 2005, Turetsky et al., 2005). However, since then the C-tail of TARPs has been shown to regulate the alleviation from polyamine block (Soto et al., 2014b), suggesting that multiple sites are important in regulating biophysical properties.

Overall, the current understanding is that AMPARs associated with TARPs are more receptive to synaptically released glutamate, and cause an overall larger, more prolonged current than receptors not associated with a TARP. Furthermore, recent work indicates TARPed AMPARs are also more sensitive to transmitter spillover (Coombs et al., 2017).
Figure 1.5. TARPs modulate AMPAR properties
A. Rapid application of 10 mM glutamate at -60 mV to outside-out patches from TsA201 cells recombinantly expressing GluA1/GluA2 heteromers in the absence (i) and presence (ii) of stargazin (Stg). Grey lines show representative
traces, black lines are averaged currents. Current-variance plots were obtained from non-stationary fluctuation analysis. Stargazin significantly increases single-channel conductance and peak open probability compared to control values. **B.** Superimposed traces from A (i) and (ii). Stargazin slows desensitization kinetics and increases the steady-state current. **C.** Single channel recordings obtained from homomeric GluA1 AMPARs expressed in the absence (upper panel) and presence (lower panel) of stargazin. Each panel shows three traces following the application of glutamate. After the initial desensitizing current, multiple single channel openings are evident in the presence of stargazin, but barely perceptible in its absence. This further illustrates how TARPs evoke increased single-channel conductance, slower desensitization kinetics and increased channel open probability. **D.** Dose-response curve of glutamate on GluA1 homomers illustrating the increased agonist affinity conveyed by the presence of stargazin. **E.** Representative currents at -60 and +60 mV evoked by fast application of 10 mM glutamate on outside-out patches of tsA201 cells recombinantly expressing homomeric GluA4 AMPARs in the absence and presence of stargazin. The intracellular solution contained 100 µM spermine, which in the absence of stargazin blocks the channel pore at +60 mV. **F.** Current-voltage plot of responses obtained in the same conditions as E at varying holding voltages, illustrating how stargazin relieves intracellular polyamine block. All currents are normalized to -80 mV values. Data obtained from (A-C) Coombs and Cull-Candy (2009), (D) Tomita et al. (2005) and (E-F) Soto et al. (2007).

**1.4.2.3. TARPs in neurological disorders**

Already from the phenotype of stargazer mice it is clear that TARPs are critical for the healthy functioning of the central nervous system. Indeed, stargazin has been linked to the genetic manifestation of absence epilepsy in rats (Kennard et al., 2011). Decreased stargazin expression is thought to underlie altered AMPAR membrane targeting, resulting in the pathophysiological phenotype. Studies on \( \gamma \)-8-knockout mice have demonstrated the importance of this TARP in epileptic seizures (Gleason et al., 2015). These mice showed reduced or absent responses to chemoconvulsants, suggesting a crucial role in enabling AMPAR-related seizures in epilepsy. Furthermore, this has led to the development of \( \gamma \)-8-specific antagonists LY3130481 and JNJ-55511118 that
decrease seizures without producing movement-related side effects such as ataxia, which are commonly observed in most other AMPAR-targeting anticonvulsants (Kato et al., 2016, Maher et al., 2016). These studies highlight how crucial TARPs are in regulating AMPAR function, and potentially could serve as future therapeutic targets for a more specific treatment of neurological disorders, with the prospective of causing fewer side effects.

1.4.3. Further AMPAR auxiliary proteins

The discovery of TARPs as AMPAR regulators prompted the search for further auxiliary subunits. This led to the identification of cornichons (CNIH) (Schwenk et al., 2009, Schwenk et al., 2012), CKAMP (von Engelhardt et al., 2010), SynDIG (Kalashnikova et al., 2010) and GSG1-L (Schwenk et al., 2012, Shanks et al., 2012) as AMPAR auxiliary proteins. A recent proteomics study (Schwenk et al., 2012) demonstrated an array of known proteins, as well as 21 novel interacting proteins that bind to AMPARs, giving rise to new targets for AMPAR regulation. A selection of these proteins is shown in figure 1.6.A.
Figure 1.6. AMPA receptor auxiliary proteins
Figure 1.6. AMPA receptor auxiliary proteins

A. Topology of AMPA receptor auxiliary proteins specifying transmembrane domains, as well as N- and C-termini. Figure adapted from Schwenk et al. (2012). B. Structure of the novel AMPAR binding protein FRRS1L. Numerals indicate amino acid positions. Shown in blue is the single transmembrane domain, and in orange is the DOMON domain, contained within the long N-terminal region.

1.4.3.1. Cornichons (CNIHs)

When co-assembled with AMPARs, CNIH homologues 2 and 3 increase receptor surface expression and prominently slow deactivation and desensitization kinetics (Schwenk et al., 2009). However, when CNIHs form part of the same receptor complex as TARPs, the subtype of TARP present dictates the resulting phenotype. AMPAR-CNIH2 complexes associated with a type Ia TARP (γ-2 or γ-3) show decreased sensitivity to cyclothiazide and a decreased \( \frac{I_{(KA)}}{I_{(Glu)}} \) ratio – the current elicited with the partial agonist kainate compared with the full agonist glutamate, while deactivation kinetics remain largely unaltered. By contrast, when associated with a type Ib TARP (γ-4 or γ-8), deactivation kinetics are slowed, cyclothiazide potency becomes increased, and TARP-mediated receptor resensitization is abolished (Gill et al., 2012, Kato et al., 2010a).

1.4.3.2. Cysteine-knot AMPAR modulating protein (CKAMP)

Proteomic searches for AMPAR-interacting proteins identified CKAMP-44 – a type I transmembrane protein, containing an extracellular N-terminal cysteine-rich motif, as a novel candidate (von Engelhardt et al., 2010). When expressed in Xenopus oocytes, this auxiliary subunit specifically reduces GluA1- and GluA2-mediated steady state currents, increases AMPAR desensitization time,
and slows recovery from receptor desensitization. Three further members of the CKAMP family (also known as Shisas) have now been identified and shown to interact with AMPARs: CKAMP-39, CKAMP-52 and CKAMP-59. Much like other families of auxiliary subunits, the subtypes follow a brain region-specific expression pattern, and exert different effects on AMPAR properties (Farrow et al., 2015). Indeed, when expressed in Xenopus oocytes, unlike CKAMP-44, the recovery from receptor desensitization of GluA1- and GluA2(Q)-mediated currents is increased by CKAMP-39, but only slightly increased and decreased respectively by CKAMP-52.

1.4.3.3. Synapse differentiation-induced gene (SynDIG)

SynDIG1 is a transmembrane protein that was found to interact with AMPARs to regulate clustering at the synapse (Kalashnikova et al., 2010). However, a recent study showed SynDIG1 does not alter AMPAR kinetics, pharmacology, or receptor trafficking (Lovero et al., 2013), suggesting it does not act as a bona fide AMPAR auxiliary subunit. Rather, SynDIG1 regulates the number of AMPAR-containing functional synapses, indicating it to be a protein critical for synaptogenesis. However, the related protein SynDIG4 (also known as proline-rich transmembrane protein 1 (PRRT1)), which shows high association with AMPARs in the hippocampal CA1 area, is de-enriched at the postsynaptic density, suggesting a role as an auxiliary factor for extrasynaptic AMPARs (Kirk et al., 2016).

1.4.3.4. Germ line specific gene 1-like protein (GSG1-L)

Another recently discovered AMPAR auxiliary protein is the membrane protein GSG1-L, a member of the claudin family and a distant homologue of TARPs
(Schwenk et al., 2012, Shanks et al., 2012). While GSG1-L shares structural similarities with TARPs, the functional effects it evokes on AMPAR properties are very different. Much like TARPs, GSG1-L increases AMPAR surface expression and decreases desensitization and deactivation kinetics. By contrast, GSG1-L profoundly slows recovery from receptor desensitization (Shanks et al., 2012). Furthermore, when transfected into cultured cerebellar stellate cells, GSG1-L enhances polyamine-dependent inward rectification of CP-AMPARs (McGee et al., 2015). Conversely, GSG1-L knockdown in cultured hippocampal neurons results in an increase in mEPSC amplitude, accompanied by a proportional increase in mean single channel conductance (Gu et al., 2016, McGee et al., 2015).

1.4.3.5. Ferric Chelate Reductase 1 Like (FRRS1L)

Among the novel AMPAR binding proteins, FRRS1L is predicted to bind with high stability and abundance to both the GluA1 and GluA2 subunits (Schwenk et al., 2012). Located on chromosome 9, the gene encodes a 344 amino acid protein (Figure 1.6.B) containing a single transmembrane domain close to the C-terminus (amino acids 322-342) (UniProt, 2014). The long N-terminal domain (NTD) includes a DOMON domain (UniProt, 2014) and four potential protein kinase C phosphorylation sites (Chadwick et al., 2000). FRRS1L is predicted to be highly expressed in many brain regions including the hippocampus, cerebellum, striatum and thalamus (Schwenk et al., 2014). However, its exact expression pattern and whether it is trafficked to the cell membrane, as well as its function still remain largely unknown.
1.5. AMPAR signalling in the hippocampus

The two main auxiliary subunits examined in this thesis are FRSS1L and TARP \( \gamma \)-8, both of which are highly prevalent in the hippocampus (Fukaya et al., 2006, Schwenk et al., 2012). It is therefore useful to next consider the function of AMPARs in this brain region, in order to understand how these proteins may influence hippocampal synaptic transmission.

1.5.1. Hippocampal structure and function

The hippocampus is a small neural structure buried deep in the medial temporal lobe and has a distinctive shape resembling a seahorse. A cross-section of its long axis reveals two C-shaped assemblies of neurons folded onto each other, as well as an area called the subiculum (Bliss and Collingridge, 1993). These C-shaped areas consist of the dentate gyrus (DG) and the cornu ammonis (CA), which is further subdivided into four regions, termed CA1, CA2 and CA3 (Figure 1.7) (Knierim, 2015, Bear et al., 2007).

One of the main cortical inputs to the hippocampus arises from the entorhinal cortex via the perforant path (Knierim, 2015). These axons synapse onto neurons in the DG, which in turn give rise to mossy fibres that project to the CA3 region. This area projects to the CA1 region via Shaffer collaterals, which provide input to the subiculum, as well as the DG, completing the trisynaptic loop (Figure 1.7) (Hjorth-Simonsen, 1973, Laurberg, 1979, Swanson et al., 1978). While it was originally believed that the flow of information was mainly contained within this uni-directional tri-synaptic loop, it has since been shown that longitudinal projections relay information throughout the hippocampal
structure, revealing a more widespread connectivity (Knierim, 2015, Li et al., 1994).

![Structure and microcircuits of the hippocampus](image)

**Figure 1.7. Structure and microcircuits of the hippocampus**
Coronal section through the transverse axis of the hippocampus. The black traces denote the ‘trisynaptic loop’. Information flows from the entorhinal cortex (EC) to the dentate gyrus (DG) via the perforant path. From the DG, mossy fibres project to pyramidal neurons in the CA3 area. Axons from the CA3 termed Shaffer collaterals synapse on pyramidal neurons in the CA1 area. The red lines denote other important pathways within the hippocampus, including projections from the EC to all three CA areas, the feedback from the subiculum to the EC, the longitudinal projections between CA3 areas, and the feedback from the CA3 area to the DG. Figure adapted from Knierim (2015).

1.5.2. Long-term potentiation (LTP)

The hippocampus is one of the most extensively studied areas of the brain. Since the case study in 1957, which famously reports patient H.M’s inability to
form new memories following bilateral hippocampal surgical removal (Scoville and Milner, 1957), much research has been devoted to unravelling the mechanisms involved in memory formation in the hippocampus. To date, the underlying process is believed to involve long-term potentiation (LTP), a mechanism by which the connection between synapses is strengthened, first described in 1973 by Bliss and Lomo (Bliss and Lomo, 1973, Lomo, 2003).

The underlying mechanisms are still not fully understood, but have been shown to involve both AMPARs and NMDARs. Upon high frequency stimulation, EPSCs through AMPARs lead to membrane depolarisation relieving synaptic NMDARs from Mg$^{2+}$ block. The resulting high levels of Ca$^{2+}$ influx lead to a local accumulation of intracellular Ca$^{2+}$, triggering a number of intracellular signalling cascades. This ultimately leads to an increase in synaptic strength, for example through the insertion of additional AMPARs into the postsynaptic membrane (Bliss and Collingridge, 1993, Gustafsson et al., 1987, Malenka et al., 1989). Additional signalling pathways activated by the NMDAR-mediated increase in intracellular Ca$^{2+}$ include calcium/calmodulin-dependent protein kinase II (CaMKII) (Blitzer et al., 1998), protein kinase C (PKC) (Hu et al., 1987, Linden and Routtenberg, 1989) and mitogen-activated protein kinase (MAPK) (English and Sweatt, 1996, English and Sweatt, 1997).

For LTP to be maintained, gene transcription and new protein synthesis must occur (Agranoff, 1967, Frey et al., 1988, Nguyen et al., 1994). Evidence suggests certain signalling molecules such as protein kinase A (PKA) and MAPK lead to the activation of the transcription factor CREB, as well as the

1.5.3. AMPARs in the hippocampus

In the hippocampus, most AMPARs are thought to be hetero-tetramers constituted of GluA1/GluA2 or GluA2/GluA3 subunits (Shi et al., 2001, Wenthold et al., 1996). There is also evidence for the presence of GluA1/GluA3 heteromers, as well as GluA1 homomers. However, their relative abundance appears to be low (Wenthold et al., 1996). The GluA4 and GluA2L subunits are expressed transiently in hippocampal pyramidal cells during early development (Huuupponen et al., 2016, Luchkina et al., 2014, Zhu et al., 2000).

AMPARs in the hippocampus play an important role in mediating LTP. A substantial number of hippocampal synapses that express NMDARs are lacking in AMPARs. These are referred to as 'silent synapses', and undergo rapid plasticity upon induction of LTP, which causes the all-or-none insertion of a population of functional AMPARs (Isaac et al., 1995, Kullmann, 1994, Kullmann, 2003, Liao et al., 1995, Nicoll, 2017). This rapid accumulation of AMPARs facilitates the neuronal depolarisation necessary for the activation of NMDARs and subsequent signalling cascade involved in LTP. Silent synapses may be particularly important during the early stages of life, as their abundance declines with age (Bliss and Collingridge, 2013, Durand et al., 1996, Nicoll, 2017), suggesting that pre-existing synapses containing AMPARs assume a more important role in the adult brain.
AMPARs inserted into silent synapses following the induction of LTP appear to have 2 sources. The first is from an intracellular reserve pool of AMPARs that undergo activity-dependent exocytosis and are subsequently inserted into the membrane. The second involves lateral diffusion of extrasynaptic AMPARs pre-existing on the neuronal surface to the PSD (Makino and Malinow, 2009, Nicoll, 2017, Patterson et al., 2010). The insertion mechanisms are believed to be mediated by trafficking and scaffolding proteins, such as stargazin and PSD-95 (Nicoll, 2003, Schnell et al., 2002).

Another mechanism by which AMPARs can contribute to LTP is by changes in subunit expression. In the hippocampal CA1 area, under physiological conditions, most AMPARs contain the GluA2 subunit resulting in CI-AMPARs. There is evidence to suggest that following LTP induction, these CI-AMPARs are rapidly replaced by GluA2-lacking, CP-AMPARs. These receptors are only expressed transiently, and subsequently are replaced again by GluA2-containing, CI-AMPARs (Plant et al., 2006). Additionally, GluA1 knockout mice can exhibit a striking deficit in LTP in the CA1 region (Zamanillo et al., 1999). However, LTP can also occur without any detectable changes in GluA2 expression, suggesting multiple mechanisms for AMPAR contribution to LTP in the hippocampus, of which not all may be necessary (Adesnik and Nicoll, 2007, Bliss and Collingridge, 2013, Granger et al., 2013).

1.5.4. AMPAR auxiliary subunits in the hippocampus
While there is evidence for the expression of multiple members of the TARP family in the hippocampus, the most enriched in this region is γ-8 (Fukaya et al., 2005, Tomita et al., 2003). In genetically generated γ-8 knockout mice, the CA1
region of the hippocampus shows a modest reduction in synaptic AMPARs, but a severe loss of extrasynaptic AMPARs, without a corresponding change in mRNA levels (Rouach et al., 2005, Jackson and Nicoll, 2011). Conversely, with the exception of γ-2, knocking down any of the other TARPs produces no obvious deleterious effects in this brain region (Jackson and Nicoll, 2011). In Schaffer collateral terminals in the CA1 region of stargazer mice, there is a significant loss of AMPARs in the postsynaptic density (Yamasaki et al., 2016). These data together suggest that γ-8 is required for basal expression of extrasynaptic AMPARs, and that γ-2 is essential for AMPAR trafficking and anchoring at the synapse.

As well as TARPs, other auxiliary subunits show abundant expression in the hippocampus. Members of the cornichon family are highly enriched in the postsynaptic density of CA1 hippocampal neurons (Schwenk et al., 2009). In particular, CNIH-2 and γ-8 co-associate with AMPARs in this region to give AMPARs their characteristic ‘hippocampal’ properties. The deletion of γ-8 in these neurons markedly diminishes the expression of CNIH-2, while manipulating CNIH-2 levels alters the functional properties of AMPARs (Kato et al., 2010a). Similarly, in dentate gyrus granule cells CKAMP-44 and γ-8 can also be contained within the same AMPAR complex. The presence of both auxiliary proteins leads to increased receptor surface expression, as well as increased total dendritic length and spine number (Khodosevich et al., 2014). Hence, γ-8, γ-2 and CNIH-2 are particularly important in AMPAR expression and synaptic localisation in CA1 cells.
1.6. Ischaemia in the hippocampus

The hippocampal CA1 area is particularly vulnerable to ischaemic stroke, leading to neurotoxicity and cell death (Kirino, 1982, Petito et al., 1987). When the blood supply to the brain is interrupted during stroke, the reduced supply of oxygen and glucose renders cells unable to generate the energy necessary for healthy functioning. Neurons and glia are particularly vulnerable as they are unable to maintain the ionic gradients necessary for proper signalling. This leads to an excessive release of neurotransmitters, including glutamate, either from presynaptic terminals, or from glia as a result of ‘reversed uptake’. This in turn leads to neuronal excitotoxicity – associated with excessive Ca$^{2+}$ and Na$^+$ influx and cell death (Dirnagl et al., 1999, Orrenius et al., 2003, Szydlowska and Tymianski, 2010). Excessive Ca$^{2+}$ influx, in particular, is thought to contribute to cell excitotoxicity in ischaemia, as well as in many other neurological disorders (Bano and Ankarcrorna, 2017, Cull-Candy et al., 2006, Simon et al., 1984, Szydlowska and Tymianski, 2010).

Initial reports suggested that a main contributor to excess Ca$^{2+}$ influx following increased glutamate release during ischaemia was NMDAR ‘over-activation’ (Reynolds and Hastings, 1995). Subsequent studies have implicated a range of additional receptors, neurotransmitters and ion channels (Burke et al., 2004, Szydlowska and Tymianski, 2010), including AMPARs (Noh et al., 2005, Pellegrini-Giampietro et al., 1992, Quintana et al., 2006, Tanaka et al., 2002).

1.6.1. AMPARs in hippocampal ischaemia

The contribution of AMPARs to excitotoxicity following stroke is believed to arise from an increased expression of CP-AMPARs. Many of the AMPARs in
hippocampal CA1 neurons contain the GluA2 subunit, resulting in predominantly CI-AMPARs (Geiger et al., 1995, Wenthold et al., 1996), with a low level of CP-AMPARs (Mattison et al., 2014, Rozov et al., 2012). As evidenced by immunolabelling and Western blot analysis 72 h following induced global ischaemia, the CA1 region of the hippocampus displays a significant reduction in the expression of GluA2, while the level of GluA1 remains unchanged (Optiz et al., 2000). Furthermore, intra-hippocampal injection of the CP-AMPAR-selective blocker 1-naphthyl acetyl spermine (NASPM) 9-40 h after induced ischaemia markedly reduces the rise in intracellular cations, and affords partial protection against ischaemia-induced neuronal death (Noh et al., 2005). These and related findings have been taken to suggest that GluA2-lacking CP-AMPARs are expressed in vulnerable neurons following ischaemia, and may contribute to a large proportion of delayed excitotoxicity and cell injury.

1.6.2. Acid-sensing ion channels drive AMPAR plasticity following ischaemia

Following ischaemic insult to the hippocampus, the local pH descends to 6.0-6.5 under normo-glycaemic conditions, and can decline to below 6.0 following severe ischaemia, or during ischaemia accompanied by hyperglycaemia – abnormally high blood sugar levels (Nedergaard et al., 1991, Rehncrona, 1985, Siesjo, 1982). In the CA1 region of the hippocampus, there is an abundance of acid-sensing ion channel 1a (ASIC-1a) which becomes activated by low pH levels typically between 5.8-6.8 to allow the flow of Na\textsuperscript{+} and Ca\textsuperscript{2+} through the channel pore (Wemmie et al., 2013). Studies have shown that ASIC-1a activation plays a role in ischaemia-induced neurotoxicity, supposedly due to their Ca\textsuperscript{2+} permeability (Mari et al., 2010, Pignataro et al., 2007,
Sherwood et al., 2011, Xiong et al., 2004). Indeed, studies using ASIC-1a knockout animals or ASIC-1a antagonists demonstrate a neuroprotective effect after ischaemia (Pignataro et al., 2007, Quintana et al., 2015, Xiong et al., 2004), further consolidating their contribution to cell death following ischaemia. In a recent study, Quintana et al. (2015) revealed that direct activation of ASIC-1a drives AMPAR plasticity in the hippocampus. They demonstrated that acidosis – the brief treatment of cells with a solution of pH 6 – is sufficient to reduce the expression of GluA2-containing AMPARs (Figure 1.8), thereby highlighting a direct functional interaction between acidotoxicity and excitotoxicity (Quintana et al., 2015).

While it is established that AMPARs play a major role in eliciting excitotoxicity in the hippocampus following ischaemia, no studies have yet revealed whether any of the auxiliary proteins are necessary or sufficient to regulate the observed changes in AMPAR plasticity.
Figure 1.8. Direct activation of ASIC-1a drives AMPAR plasticity

A. Current-voltage plots of glutamate-evoked AMPAR currents in outside-out patches from hippocampal pyramidal neurons 12 h after being exposed for 15 min to pH 7.4 (control) or pH 6 (acidosis). More rectifying currents are observed in acidosis-treated cells. B. Representative current traces at -60 and +60 mV in control (top panels) and acidosis-treated (bottom panels) patches in response to fast application of 10 mM glutamate. In the left panels, acidosis-treated cells display inwardly rectifying currents compared to the control data. This effect is blocked by 100 nM psalmotoxin 1 (PcTx1), an ASIC-1a specific antagonist (right panels). For comparison, currents are scaled to the peak amplitude at -60 mV. C. AMPAR single channel conductance in control and acidosis samples. D. Representative Western blots showing total and surface expression of GluA2 in hippocampal cultures 12 h after control or acidosis treatment. Surface expression was measured by cell surface biotinylation followed by Western
The addition of PcTx1 to acidosis-treated cells reverses the decrease in both total and surface expression of GluA2. E-F. Pooled data from quantification of Western blot analysis as represented in D for cell surface (H) and total (I) GluA2 expression levels. Data from Quintana et al. (2015).

1.7. Aims of thesis

This thesis aims to enhance the understanding of how certain auxiliary proteins influence AMPAR properties. First, I have examined the influence of a novel auxiliary subunit on recombinant AMPARs. Second, I have addressed the influence of a form of neuropathological change on AMPARs and associated auxiliary subunits in hippocampal neurons.

The first results Chapter (Chapter 3) investigates the newly identified auxiliary subunit FRRS1L (also known as C9orf4). At the beginning of the project, no data had yet been published on this protein, except that it interacts with AMPARs (Schwenk et al., 2012). Using mainly electrophysiological, but also some biochemical approaches, this thesis aims to elucidate the interaction between FRRS1L and AMPAR subunits, as well as identify how it contributes to shaping AMPAR responses to glutamate.

The second results Chapter (Chapter 4) extends earlier studies using acidotoxicity as a model to provide insight into the role of ASIC1a and CP-AMPARs in neurodegeneration (Quintana et al., 2015). Using various techniques, this thesis aims to reveal the involvement of auxiliary subunits in AMPAR plasticity following the direct activation of ASIC1-a channels. Pinpointing which proteins drive the changes leading to increased excitotoxicity following ischaemic stroke will aid in understanding the possible underlying
mechanisms and potentially lead to more target-specific treatments in the future.

The thesis concludes with a general discussion (Chapter 5), highlighting the main findings and exploring ideas for future research.
2. Methods

2.1. Cell culture

2.1.1. TsA201 cell culture

TsA201 cells were cultured at 37 °C and 5% CO₂ in DMEM (Sigma-Aldrich Co. Ltd., Gillingham, UK) supplemented with 10% FBS and 1% penicillin/streptomycin (all from GibCO; Life Technologies Ltd., Paisley, UK).

2.1.2. Hippocampal dissection and dissociated culture

All animal procedures were carried out in accordance with the UK Home office Animals (Scientific procedures) Act (1968). P0 or P1 rodents were euthanised by decapitation in accordance with our project license. Hippocampi were dissected as described in the protocol by Kaech and Banker (2006). Once dissected, the tissue was immersed in cold Hank’s balanced salt solution (HBSS; Gibco) containing 1% sodium pyruvate, 0.1% glucose and 10 mM HEPES, bubbled with 95% O₂ and 5% CO₂. The hippocampi were chopped and transferred to warm papain solution (Earle’s balanced salt solution (EBSS, ThermoFisher) containing 20 units ml⁻¹ papain (Worthington), 0.5 mM EDTA, 1.65 mM L-cysteine, and 0.005% DNAse) and incubated for 1 h at 37 °C, with the tube gently swirled every 15 min. After incubation, the tissue was gently triturated and centrifuged at 300 g for 5 min at room temperature (RT). The cell pellet was re-suspended in ovomucoid medium (EBSS containing 10 µg ml⁻¹ ovomucoid (Worthington), 10 µg ml⁻¹ albumin and 0.005% DNAse) and incubated for 5 min at 37 °C. The cells were centrifuged at 300 g for 3 min, and the pellet re-suspended in pre-warmed plating medium (MEM Eagle with Earle’s...
BSS supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% GlutaMAX (all from GIBCO; Life Technologies Ltd., Paisley, UK) and 0.45% glucose). Cells were plated in 24 well plates at a density of 100’000 cells per well, unless indicated otherwise. After allowing at least 4 h for the cells to attach, the plating media was fully replaced with maintenance medium (Neurobasal-A supplemented with 1% penicillin/streptomycin, 1% B-27, 0.5% GlutaMAX (all from GIBCO) and 0.6% glucose). Cultures were maintained in maintenance medium at 37 °C with 5% CO₂.

2.1.3. Hippocampal slice culture

Hippocampal slice cultures were based on a protocol described by Opitz-Araya (2011). In accordance with our project license, P13 or P14 rats were fully anaesthetised using isofluorane and decapitated. The hippocampi were dissected as described previously (Kaech and Banker, 2006). Once dissected, the hippocampi were placed length-ways on a tissue slicer (Stoelting), cut into 300 µm slices and transferred to cold Hank’s balanced salt solution (HBSS; Gibco) containing 1% sodium pyruvate, 0.1% glucose and 10 mM HEPES, bubbled with 95% O₂ and 5% CO₂. After careful separation, the slices were transferred onto 0.4 µm cell culture inserts (Millipore) in 6 well plates, with 4-5 slices per insert, and maintained in 1 ml maintenance medium (see section 2.1.2.) in a 37 °C incubator with 5% CO₂.

2.2. Heterologous expression and hippocampal transfection

Transient transfection was done using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and the tsA201 cells were seeded in 4-well plates onto coverslips pre-coated with poly-L-lysine (100 µg ml⁻¹,
Sigma). For experiments using dissociated hippocampal cultures, cells were cultured as described above (2.1.2) and transfection was performed at 10-12 DIV using lipofectamine 2000 according to the manufacturer’s instructions. The ratio of AMPAR to FRSS1L DNA was 2:3, AMPAR to TARPs was 1:2, and AMPAR to TARP to FRSS1L was 1:2:3. AMPAR subunit cDNAs were gifts from S. Heinemann from (Salk Institute, La Jolla, CA, USA) and P. Seeburg (Max Planck Institute, Heidelberg, Germany), TARP cDNA were gifts from R. Nicoll (University of California, San Francisco, San Francisco, CA, USA) and FRSS1L cDNA was a gift from Bernd Fakler (University of Freiburg, Freiburg, Germany). A tandem construct of FRSS1L and GluA1 was made by directly linking the N terminus of FRSS1L to the C-terminal tail of the GluA1 subunit, incorporating a 9 aa linker (GluA1-GGGGGEFAT- FRSS1L) (Soto et al., 2014b). All cDNAs were rat.

2.3. Electrophysiological recordings

2.3.1. Fast application of glutamate to outside-out patches from tsA201 cells

Cells were placed in a bath of external solution containing 145 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES, brought to pH 7.3 with NaOH. The internal solution contained 145 mM CsCl, 2.5 mM NaCl, 1 mM Cs-EGTA, 4 mM MgATP, and 10 mM HEPES, adjusted to pH 7.2 with CsOH. Unless indicated, 100 µM spermine was added. Cells were viewed with a fixed stage upright microscope (BX50 WI, Olympus). Currents were recorded at -60 mV (unless otherwise indicated) at room temperature (22-25 °C) with an Axopatch 200 A amplifier (Axon Instruments), low-pass
filtered at 10 kHz, digitised at 50 kHz using a National Instruments interface with WinWCP software (University of Strathclyde, Glasgow, UK).

**Figure 2.1. Glutamate application to outside-out patches using a fast application tool**

The white arrows denote two fast flowing solutions of either a control or glutamate solution. 0.25% sucrose was added to the glutamate solution to help visualise the interface. The pipette with the outside-out patch was placed in the control solutions near the interface and the tool was piezoelectrically moved to briefly expose the patch to the glutamate solution. Figure generously supplied by Stuart Cull-Candy.

Rapid application of glutamate was achieved using a piezoelectric dual-channelled application tool (Colquhoun et al., 1992, Jonas and Sakmann, 1992) made from thick-walled theta glass (outer diameter: 2 mm, Hilgenberg GmbH, Malsfeld, Germany), with a tip opening pulled to 200 µm. On one side the solution contained external solution with 10 mM glutamate and 2.5% sucrose, the other side contained a control solution of external solution with 5% H₂O (Figure 2.1). Solution exchange occurred through a piezoelectric translator (Burleigh PZ-301) triggered by a voltage step (WinWCP). At the end of each experiment, the solution exchange was assessed by destroying the patch and
measuring the liquid-junction current. All recordings had a 10-90% rise time < 300 µs.

2.3.1.1. 2-pulse protocol to determine recovery from receptor desensitization

To determine recovery from receptor desensitization, 2 rapid pulses of glutamate were applied to out-side out patches with increasing time intervals from 10 to 5000 ms, or until the receptors showed complete recovery from desensitization. To estimate the weighted time constant for recovery ($\tau_{w\text{-rec}}$), the percentage recovery was plotted against time interval, and a single exponential function was fitted, from which the tau value was obtained.

2.3.2. Whole-cell recordings from dissociated hippocampal cultures

Cells were placed in a bath of external solution containing 145 mM NaCl, 2.5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM glucose, and 10 mM HEPES, brought to pH 7.3 with NaOH. To isolate AMPAR currents, the following drugs were added to the external solution: 0.5 µM TTX (Na$^+$ channel blocker), 20 µM AP5 (NMDAR blocker), 20 µM SR-95531 (GABA$_A$ receptor blocker), 1 µM Strychnine (glycine receptor blocker) and 10 µM D600 (voltage-gated Ca$^{2+}$ channel blocker). The internal solution contained 128 mM CsCl, 10 mM HEPES, 10 mM EGTA, 10 mM TEA-Cl (voltage-gated K$^+$ channel blocker), 2 mM MgATP, 2 mM NaCl, and 1 mM Qx-314 (voltage-gated Na$^+$ channel blocker), adjusted to pH 7.2 with CsOH. Unless indicated, 100 µM spermine was added. Cells were viewed with a fixed stage upright microscope (BX50 WI, Olympus). Currents were recorded at -60 mV (unless indicated otherwise) at room temperature (22-25 °C) with an Axopatch 200 A amplifier (Axon Instruments),
low-pass filtered at 2 kHz, digitised at 50 kHz using a National Instruments interface with WinEDR software (University of Strathclyde, Glasgow, UK). Whole-cell capacitance and series resistance were on average 23.2 ± 0.9 pF and 11.5 ± 0.7 MΩ. If the initial series resistance was > 20 MΩ the cell was not used. Series resistance compensation was 75%.

2.3.3. Rectification index (RI)

The rectification index was calculated by dividing the absolute value of the average peak response at +60 mV by the absolute value of the average peak response at -60 mV:

$$\text{RI}_{+60/-60 \text{ mV}} = \frac{|I_{+60 \text{ mV}}|}{|I_{-60 \text{ mV}}|}$$

2.3.4. Weighted time constant for desensitization kinetics

To determine the kinetics of desensitization of the AMPAR-mediated currents, a double-exponential function was fitted to the deactivation curve of the current. Using the amplitude and time constant of the fast component of desensitization ($A_f$ and $\tau_f$ respectively) and the amplitude and time constant of the slow component of desensitization ($A_s$ and $\tau_s$ respectively), a weighted time constant ($\tau_w$) was calculated according to the following equation (Cathala et al., 2000):

$$\tau_w = \tau_f \left( \frac{A_f}{A_f + A_s} \right) + \tau_s \left( \frac{A_s}{A_f + A_s} \right)$$

2.3.5. Non-stationary fluctuation analysis

Non-stationary fluctuation analysis allows the single-channel channel properties to be determined from macroscopic responses (Traynelis et al., 1993). Glutamate (10 mM) was applied to outside-out patches (100 ms pulse at
and the ensemble variance of all successive pairs of current responses was calculated. The single-channel current ($i$) and the total number of channels in the patch ($N$) were determined by plotting this ensemble variance ($\sigma^2$) against mean current ($I$) and fitting with a parabolic function:

$$\sigma^2 = il - \frac{I^2}{N} + \sigma^2_B$$

where $\sigma^2_B$ is the background variance. The mean single-channel conductance was determined from the single-channel current and the holding potential (-60 mV).

### 2.3.6. Data Analysis

Recordings were analysed using IGOR Pro (Wavemetrics) and Neuromatic (J. Rothman, University College London, London, UK). Data is presented as mean ± SEM. Statistical significance was determined using either a Mann-Whitney-U test or unpaired student t-test and differences were considered significant at $P < 0.05$.

### 2.3.7. Electrodes

Electrodes were pulled from thick-walled borosilicate glass (GC-150F; outer diameter: 1.5 mm, inner diameter: 0.86 mm, Harvard Apparatus) using a 2-stage vertical puller (PC-10, Narishige) and heat-polished to achieve a final resistance of 5-10 MΩ for outside-out patches, or 3-7 MΩ for whole-cell recordings.

### 2.4. Analysis of protein expression in mouse brain

Tissue from mouse hippocampus, cerebellum, cortex, spinal cord or retina was homogenised with RIPA lysis buffer (Pierce, Thermo Fisher Scientific Inc.,
Rockford, IL, USA) supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and solubilised for 1 h at 4 °C. Samples were centrifuged at 35,000 rpm for 35 min at 4 °C and the supernatant containing the protein was collected. Protein concentration was determined using a Bradford assay (Protein Assay Dye Reagent, Bio-Rad Laboratories GmbH, Munich, Germany). Laemmli sample buffer was added to the protein samples and boiled for 5 min at 95 °C. Proteins were then separated by SDS-PAGE followed by Western blotting with anti-FRRS1L (1:200; Novus Biologicals, Littleton, CO, USA), anti-GluA2 (1:500; Millipore), anti-γ-8 (1:2000; a gift from M. Watanabe (Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan)) and actin (1:6000; Abcam). Densitometry analysis was carried out using ImageLab (Biorad).

2.5. Co-immunoprecipitation

Cells were washed with 1 x phosphate buffered saline (PBS) (Sigma), lysed in 1 ml of co-IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 100 mM PMSF, 0.5% Triton X-100 and 10 µl protease inhibitor cocktail (Roche)) and solubilised by rotating for 1 h at 4 °C. Solubilised membranes were then centrifuged for 10 min at 16’000 g (4 °C). The pellet was discarded and the lysate membranes were placed in a new centrifuge tube. 50 µl of the lysate was placed in an aliquot and denatured at 95 °C in 50 µl of Laemmli sample buffer (10% SDS, 0.1 M Tris HCl (pH 6.8), 20% glycerol, 294 mM β-mercaptoethanol and 0.004% bromophenol blue) to serve as the protein input sample. The remaining solubilised membranes were incubated with 1 µg of mouse anti-GluA1 (Millipore) at 4 °C. After 4 h, lysates were incubated with 15 µl of protein G bound to sepharose beads (Sigma) (2 h, 4 °C). The samples were centrifuged
for 3 mins at 500 g and the pellets were washed x 3 in co-IP buffer. The pellets containing the bound proteins were eluted with Laemmli sample buffer by boiling (5 min, 95 °C). Proteins were then separated by SDS-PAGE followed by Western blotting with anti-FRRS1L (1:200; Novus Biologicals, Littleton, CO, USA), anti-GluA1 (1:500; Millipore) and anti-γ-2 (1:500; a gift from M. Watanabe (Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan)) (Haukanes and Kvam, 1993, Karlsson and Platt, 1991).

2.6. Plasma membrane protein extraction

To isolate proteins expressed in the cell plasma membrane, we used a plasma membrane protein extraction kit (ab65400; Abcam) according to the manufacturer’s instructions, as follows: ‘Wash cells with ice cold PBS and lyse in 500 µl Homogenize Buffer Mix by mechanically homogenising on ice. Centrifuge the homogenate at 700 g for 10 minutes at 4 °C. Discard the pellet and centrifuge the supernatant at 10'000 g for 30 min at 4 °C. Collect the supernatant containing the cytosol fraction and elute the protein with Laemmli sample buffer by boiling at 95 °C for 5 min. Re-suspend the pellet containing the total cellular membrane protein in 200 µl of the Upper Phase Solution. Add 200 µl of the Lower Phase Solution and after thorough mixing, incubate on ice for 5 minutes. Centrifuge the samples at 1000 g for 5 min at 4 °C, transfer the upper phase to a new tube and keep on ice. To maximize the yield, extract plasma membrane proteins in the lower phase again by adding 100 µl of the Upper Phase Solution and centrifuging at 1000 g for 5 min at 4 °C. Combine the upper phase samples and keep on ice. To extract the combined upper phase, add 100 µl of the Lower Phase Solution, mix and centrifuge at 1000 g for 5 min at 4 °C. Carefully collect the upper phase, dilute in 5 volumes of water and keep
on ice for 5 min. Centrifuge the samples at top speed for 10 min at 4 °C. The pellet containing the plasma membrane protein was re-suspended in RIPA buffer (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and eluted with Laemmli sample buffer by boiling for 5 min at 95 °C. Proteins were then separated by SDS-PAGE followed by Western blotting with anti-GluA2 (1:500; Millipore), anti-γ-2 (1:500), anti-γ-8 (1:2000; both gifts from M. Watanabe (Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan)) and N-cadherin (1:2000; Millipore). Densitometry analysis was carried out using ImageLab (Biorad).

2.7. Acidosis treatment

Experiments on dissociated hippocampal cultures were carried out on cells 10-14 days in vitro (DIV), unless indicated otherwise. Experiments on organotypic hippocampal slice cultures were carried out after 24 h in vitro. Neurons were challenged for 15 min with maintenance medium (see section 2.1.2) of different pH (pH 7.4 for controls, and pH 6.0 for acidosis). Cells were then washed once and fully replaced with maintenance medium (pH 7.4) (Quintana et al., 2015).

2.8. Immunocytochemistry

Cells were washed x 2 with PBS and fixed with 4% PFA for 10 minutes at 25 °C. Cells were then incubated in blocking solution (1 x PBS containing 10% horse serum (Invitrogen UK), 0.5% BSA (Sigma UK) and 0.2% Triton (Sigma UK)) for 10 min. Coverslips were incubated with primary antibody (anti-FRRS1L (1:50; Novus Biologicals, Littleton, CO, USA), anti-GluA1 (1:200), anti-GluA2
(1:200; both from Millipore), anti-γ-2 (1:200), anti-γ-5 (1:200), anti-γ-7 (1:200), anti-γ-8 (1:1000; all gifts from M. Watanabe, Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan)) overnight at 4 °C. After three washes with PBS, cells were incubated with secondary antibody (donkey-anti-mouse Alexa-488, goat-anti-rabbit Alexa-555 and goat-anti-guinea pig Alexa-647 (all 1:1000; Invitrogen UK) for 1 hour at 25 °C. All coverslips were mounted on glass slides using gold anti-fade reagent (Invitrogen UK). Fluorescence images were acquired with a LEICA confocal microscope and a 40 x or 63 x oil immersion lens using LSF software. Image analysis was carried out with Image J software (public domain software developed at the US National Institutes of Health). For quantification, sets of cells were cultured, stained simultaneously and imaged using identical settings. The dendritic region of interest was randomly selected and measured with the Fiji image processing package. For graphical representation, individual values were normalised to the mean value obtained in control (pH 7.4-treated) cells.

2.9. Assessment of neuronal injury in hippocampal slice cultures

In experiments on organotypic hippocampal slice cultures, propidium iodide (PI) uptake was used to assess cell injury. PI (5 µg ml⁻¹) was added to the culture medium 12 h following acidosis treatment for 30 min to check slice viability. Image acquisition was performed using an inverted fluorescence microscope (BX50 WI, Olympus), with a 2.5 x magnification objective and a digital camera (C7500, Hamamatsu) (Quintana et al., 2015).
2.10. Live cell imaging

After plating, cells were placed in an IncuCyte® Zoom Live Cell Analysis System (Essen BioScience, Ann Arbor, MI, USA), and monitored for 12 days, during which a high definition phase contrast image was captured every 6 h. Cell body clusters, neurite growth and neurite branch points were analysed using IncuCyte® NeuroTrack Software (Essen BioScience) (Addis et al., 2017).

2.11. Single cell calcium imaging

Hippocampal neurons were cultured in 96 well plates at a density of 25'000 cells per well and subjected to acidosis treatment after 10-12 days in culture. The following day, the cells were loaded with 4 µM FLUO-4AM (Invitrogen) in HEPES-buffered tyrode solution (HBTS; Invitrogen, Paisley, UK) for 1 h in the dark at room temperature (RT). Cells were washed once and continuously perfused with HBTS throughout the recording a rate of 3 ml min⁻¹. Compounds were diluted in HBTS at the following concentrations: 0.5 µM TTX (Na⁺ channel blocker), 20 µM Gabazine (GABA_A channel blocker), 10 µM NVP (NMDAR blocker), 10 µM AMPA, 30 mM KCl, 30 µM Mibefradil (voltage-gated Ca²⁺ channel blocker) and 20 µM Philanthotoxin-74 (CP-AMPAR blocker). For experiments involving electrical field stimulation (EFS), a two-electrode stimulator was placed just above the cells. Each stimulus consisted of 5 stimuli of 50 V at 20 Hz. Cell fluorescence was excited by a 480 nm emitting light source (Polychrome II, TILL-Photonics, Gräfelfing, Germany) and viewed using an inverted epi-fluorescence microscope (Axiovert, 135TV, Zeiss, Cambridge, UK). Fluorescence emission at 506 nm was captured by an iXon 897 EMCCD camera (Andor Technologies, Belfast, UK), and the images were processed using Imaging Workbench 5.0 software (INDEC Biosystems, Santa Clara, CA,
USA). The change in intracellular Ca\(^{2+}\) following agonist application or electrical stimulation was measured by comparing the change in fluorescence compared to baseline intensity. Results were averaged from multiple cells from multiple wells (Addis et al., 2017).

2.12. Electrical field stimulation using FLIPR

For experiments in which cells were electrically stimulated in the fluorescence imaging plate reader (FLIPR; Molecular Devices Corporation, UK), hippocampal cells were plated in dark-walled, poly-D-lysine-coated 96 well plates at a density of 100'000 cells per well. After 11 days in culture, the cells were treated with acidosis. 16 h later, the cells were loaded with 4 µM FLUO-4AM (Invitrogen) for 1 h at RT in the dark, followed by a wash with HBTS. The cells were then analysed in the FLIPR device, set to a 384-well configuration, so that each well from the 96 well plate would provide 4 individual readouts. A set of 5 stimuli of 50 V at 20 Hz was applied to the bottom left quadrant, in which a ‘direct’ response was observed. The ‘synaptic’ response was defined as the response observed in the top right quadrant (furthest away from the stimulus site). Cells were excited by light at 488 nm from a 4W Argon-ion laser and the emitted fluorescence passed through a 510–570 nm band-pass interference filter before detection with a cooled charge coupled device (CCD) camera (Princeton Instruments). Drug dilutions in assay buffer were prepared in a separate 96-well, flat-bottom plate. NVP-AAM077 (NVP) and LY303070 were both used at 10 µM (both from Eli Lilly, UK). Parameters for drug addition to the cell plate were pre-programmed, and delivery was automated through a 96-well head pipettor. Drugs were added in 25 µl volumes by automated pipetting. Fluorescence data were exported and analysed in Microsoft Excel and
GraphPad Prism. Data are presented as normalised values, where the baseline fluorescence was subtracted from the peak fluorescence (Virdee et al., 2017).

2.13. Statistical analysis

All average data are expressed as mean ± the standard error of the mean (SEM). Data were not assumed to be normally distributed. Comparisons involving two datasets only were performed using a Mann-Whitney U test or an unpaired two-tailed student’s t-test. Analyses involving data from three or more groups were performed using one- or two-way ANOVA (Welch heteroscedastic F test), followed by pairwise comparisons using two-sided Welch two-sample t tests with Bonferroni correction where appropriate. Exact P values are presented to two significant figures, except when $P < 0.0001$. Differences were considered significant at $P < 0.05$. Statistical tests were performed using GraphPad PRISM 7.0b (GraphPad Software Inc, San Diego, CA) or R (3.3.2, the R Foundation for Statistical Computing; http://www.r-project.org/) and R Studio (1.0.143, RStudio).
3. Effects of auxiliary subunit FRRS1L on AMPA receptor function

3.1. Summary

AMPARs are associated with auxiliary proteins that have been shown to affect receptor properties. A novel AMPAR-interacting protein FRRS1L was shown in a proteomics study to interact with AMPAR subunits. In this chapter, we investigate the effects of FRRS1L on AMPAR function. We show that when co-expressed with both homomeric GluA1 and heteromeric GluA1/A2 AMPAR subunits in tsA201 cells, FRRS1L slows recovery from receptor desensitization, without significant effect on other AMPAR properties. When the prototypical TARP stargazin is additionally co-expressed, the effect of FRRS1L is lost and the receptor properties appear as if only stargazin were associated.

We examine the endogenous expression of FRRS1L in the adult mouse central nervous system, and find that it is highly abundant in multiple brain regions including the hippocampus, cortex and cerebellum. In whole-cell electrophysiological recordings of cultured hippocampal neurons, overexpression of FRRS1L does not influence mEPSC amplitude or frequency.

Elucidating the mechanisms by which FRRS1L slows AMPAR recovery from desensitization could provide a useful tool in studying channel desensitization kinetics.
3.2. Introduction

Throughout the CNS, AMPARs assemble with auxiliary subunits to form macromolecular complexes. Depending on the composition of subunits within these complexes, the receptors display an array of different properties. The first auxiliary subunits to be identified were the family of transmembrane AMPAR regulatory proteins (TARPs) (Chen et al., 2000, Tomita et al., 2005, Vandenberghhe et al., 2005). Upon co-assembly, TARPs alter AMPAR trafficking and channel properties (Nicoll et al., 2006), thereby influencing the synaptic response to glutamate.

The discovery of TARPs as AMPAR regulators prompted the search for further auxiliary subunits. This led to the discovery of an array of novel AMPAR-interacting proteins including cornichons (CNIHs) (Schwenk et al., 2009, Schwenk et al., 2012), cysteine-knot AMPAR modulating proteins (CKAMPs) (von Engelhardt et al., 2010), synapse differentiation-induced genes (SynDIGs) (Kalashnikova et al., 2010) and germ line specific gene 1-like protein (GSG1-L) (Schwenk et al., 2012, Shanks et al., 2012), which have all been shown to influence AMPAR function.

One of the more recent proteins revealed to bind to AMPARs is Ferric Chelate Reductase 1 Like (FRRS1L), also known as chromosome 9 open reading frame 4 (C9orf4). Identified by a proteomics study searching for proteins binding to GluA1 or GluA2, it was shown that FRRS1L binds with high affinity and stability to both subunits (Schwenk et al., 2012). Located on chromosome 9, the gene encodes a 344 amino acid protein (see introduction Figure 1.6.B) (UniProt, 2014). With one transmembrane domain near the C-terminus, FRRS1L
possesses a long extracellular N-terminal domain, which includes a DOMON domain – a domain involved in heme and sugar recognition (Iyer et al., 2007) – alongside four potential protein kinase C phosphorylation sites (UniProt, 2014).

At the beginning of this project, some studies had shown the potential expression pattern of FRRS1L (Schwenk et al., 2014). However, its function, whether it is expressed in postsynaptic densities, and what influence it has on AMPARs remained largely unknown. A recent publication has shown that FRRS1L associates with AMPARs and carnitine O-palmitoyltransferase 1c (CPT1c) in the ER, with a proposed role of a ‘catalyst’ in AMPAR biogenesis, priming the receptors for co-assembly with TARPs and CN1Hs for subsequent delivery to the synapse (Brechet et al., 2017).

This section of the thesis will consider whether FRRS1L can be defined as a novel AMPAR auxiliary protein. I will examine the interaction between FRRS1L and AMPARs, as well as explore the effects exerted on receptor properties when both proteins are co-assembled. As this study contains some overlap with the data recently published by Brechet et al. (2017), I will first present the experiments and results I obtained, and then compare the findings in the Discussion section.
3.3. Results

3.3.1. FRRS1L binds to GluA1 in tsA201 cells

To study the interaction between FRRS1L and AMPARs, we initially performed experiments in recombinantly transfected tsA201 cells. To ensure that FRRS1L successfully binds to AMPAR subunits in this heterologous system, tsA201 cells were transfected with GluA1 with or without FRRS1L, and GluA1 was immunoprecipitated. In cells expressing both GluA1 and FRRS1L, staining for FRRS1L produced a band around the 37 kDa mark (the expected size of FRRS1L), which was never present in the samples transfected with GluA1 alone (Figure 3.1). This indicates that FRRS1L interacts with GluA1 in tsA201 cells.

Figure 3.1. FRRS1L binds to GluA1 in tsA201 cells

GluA1 was immunoprecipitated from protein extracted from tsA201 cells transfected with either GluA1 or GluA1 + FRRS1L. The samples were run on a 10% polyacrylamide gel and stained for GluA1 and FRRS1L. This figure shows a representative outcome in which FRRS1L co-immunoprecipitates with GluA1 in tsA201 cells.
3.3.2. FRRS1L does not modify single-channel conductance, desensitization or deactivation kinetics of homomeric GluA1-containing AMPARs

To investigate the effect of FRRS1L on AMPAR properties, GluA1 was recombinantly expressed in tsA201 cells either alone, or together with FRRS1L. Using a fast application tool and a holding voltage of -60 mV, we applied 10 mM glutamate to outside-out patches excised from the membranes of the transfected cells. Initial observations of the general shape of the currents generated suggested no obvious difference between the two samples (Figure 3.2.A). Exploring various parameters, we found no significant correlation between the presence of FRRS1L and channel properties. Mean single-channel conductance estimated using non-stationary fluctuation analysis (NSFA) was $11.95 \pm 0.93$ pS versus $15.17 \pm 1.41$ pS in the absence and presence of FRRS1L, respectively ($P = 0.077$, Mann-Whitney U test; $n = 26$ and 16; Figure 3.2.B), desensitization time constant ($\tau_{w,des}$) was $1.972 \pm 0.072$ ms versus $2.123 \pm 0.117$ ms in the absence and presence of FRRS1L, respectively ($P = 0.34$, Mann-Whitney U test; $n = 27$ and 17; Figure 3.2.C), and deactivation time constant ($\tau_{deac}$) was $1.512 \pm 0.080$ ms versus $1.44 \pm 0.052$ ms in the absence and presence of FRRS1L, respectively ($P = 0.99$, Mann-Whitney U test; $n = 20$ and 18; Figure 3.2.D).
Figure 3.2. FRRS1L does not modify single-channel conductance, desensitization or deactivation kinetics of homomeric GluA1-containing AMPARs

A. Representative averaged current responses evoked by application of glutamate (10 mM, 100 ms, –60 mV) to outside-out patches from tsA201 cells transfected with GluA1 (left), or GluA1 + FRRS1L (centre) and an overlay of both traces (right). Each trace represents averaged currents from an individual patch. B-D. Pooled data for single-channel conductance estimated using non-stationary fluctuation analysis \((n = 26\) and 16) \((B)\), weighted desensitization time constant \((n = 27\) and 17) \((C)\), or deactivation time constant \((n = 20\) and 18) \((D)\). Individual data points are shown together with the mean (thick horizontal line) for tsA201 cells transfected with GluA1 or GluA1 + FRRS1L.
3.3.3. FRRS1L slows recovery from desensitization of GluA1 homomers

To examine whether FRRS1L affects the rate of receptor recovery from desensitization, outside-out patches from tsA201 cells were exposed to fast glutamate application using a 2-pulse protocol. This involved an initial application of 10 mM glutamate for 100 ms to fully desensitize all receptors in the patch, followed by a second application of 2 ms at increasing time intervals from 10 to 5000 ms, with a sweep interval of 17 s. Compared with cells expressing GluA1 alone, those expressing recombinant GluA1 homomers in the presence of FRRS1L were much slower to recover from desensitization (\(\tau_{w,rec}: 167.3 \pm 3.9 \text{ ms for GluA1 alone and } 919.8 \pm 33.4 \text{ ms for GluA1 + FRRS1L}; P < 0.0001, \text{ Mann-Whitney U test}; n = 13 \text{ and } 23; \text{ Figure 3.3}).
Figure 3.3. FRRS1L slows recovery from desensitization of GluA1 homomers
A. Representative averaged currents generated by applying a glutamate using a 2-pulse protocol on outside-out patches from tsA201 cells recombinantly expressing either GluA1 (black) or GluA1 + FRRS1L (red). The initial current was activated by a 100 ms application of 10 mM glutamate, to fully desensitize all receptors in the patch. The second pulse, applied at increasing time intervals from 10 to 5000 ms, consisted of 2 ms of 10 mM glutamate. A double exponential curve was fitted to the data to estimate weighted tau (time constant) values. B. Pooled data for weighted tau values (n = 13 and 23). Individual data points are shown together with the mean (thick horizontal line) for tsA201 cells transfected with GluA1 or GluA1 + FRRS1L. C. Mean percentage recovery from receptor desensitization at increasing time intervals fit with a double exponential curve.
3.3.4. FRRS1L does not alter the current-voltage relationship of GluA1 homomers

Ca$^{2+}$-permeable AMPARs are blocked in a voltage-dependent manner by endogenous polyamines, resulting in an inwardly rectifying current-voltage (I-V) relationship. Some auxiliary proteins, including stargazin, have the ability to relieve AMPARs from the polyamine block, giving rise to a less inwardly rectifying current-voltage (I-V) relationship (Soto et al., 2007). To explore whether FRRS1L also affected AMPAR rectification, outside-out patches from recombinant tsA201 cells were exposed to pulses of 10 mM glutamate while held at voltages from -80 to +60 mV, at 20 mV intervals. 3 traces were recorded per condition and averaged to obtain a final reading. Figure 3.4.A shows the traces generated at different voltages ($n = 5$ for GluA1 and $n = 4$ for GluA1 + FRRS1L). To generate an I-V plot, the peak current at each voltage was measured, normalised to the current generated at -80 mV, and plotted against the corresponding voltage (Figure 3.4.B). At none of the voltages recorded did the presence of FRRS1L evoke a significant change in amplitude compared to GluA1 alone.

To calculate the rectification index (RI), the maximum current at +60 mV was divided by the maximum current at -60 mV. In some cells co-expressing FRRS1L, the RI reached values approaching 1, causing a more dispersed range of RI values. The mean value, however, did not differ between the two samples (RI$_{+60/-60}$: 0.114 ± 0.027 for GluA1 and 0.294 ± 0.091 for GluA1 + FRRS1L; $P = 0.16$, Mann-Whitney-U test; $n = 19$ and 14; Figure 3.4.C). We thus found no evidence that FRRS1L altered AMPAR rectification.
Figure 3.4. FRRS1L does not alter the current-voltage relationship of GluA1 homomers
Currents were evoked by application of glutamate (10 mM, 100 ms) to outside-out patches from tsA201 cells transfected with GluA1 or GluA1 + FRRS1L. The holding voltage ranged between -80 and +60 mV at 20 mV intervals.
A. Representative averaged current responses at 20 mV intervals between -80 and +60 mV for GluA1 (black) or GluA1 + FRRS1L (red), normalised to -80 mV.

B. Current-voltage plot of averaged data from outside-out patches from tsA201 cells expressing either GluA1 or GluA1 + FRRS1L. All values are normalised to the peak current obtained at -80 mV.

C. Pooled RI$_{+60/-60}$ values of tsA201 cells transfected with GluA1 or GluA1 + FRRS1L ($n = 16$ and 11). Individual data points are shown together with the mean (thick horizontal line).

### 3.3.5. A GluA1-FRRS1L tandem construct causes a decelerated recovery from receptor desensitization

Thus far, all experiments were performed by co-transfecting AMPAR subunits with auxiliary proteins. A disadvantage of this approach is that the number of FRRS1L molecules associated with receptors could be highly variable, depending on relative expression levels. Indeed, some receptors may be lacking in associated FRRS1L. While our co-immunoprecipitation experiments suggest that GluA1 and FRRS1L do co-assemble in tsA201 cells, to allow us to examine a homogenous AMPAR population, we created a GluA1-FRRS1L tandem, in which the two proteins were joined by a linker sequence. Consequently, any AMPAR expressed at the cell surface would be associated with four FRRS1L proteins.

In outside-out patches from tsA201 cells transfected with this tandem construct, AMPAR recovery from desensitization was prominently slowed compared with cells transfected with GluA1 alone ($\tau_{w,\text{rec}} = 167.3 \pm 3.9$ ms for GluA1 alone versus $2158.8 \pm 234.6$ ms for GluA1-FRRS1L tandem; $P = 0.016$, Mann-Whitney-U test; $n = 13$ and 5; Figures 3.5.A and B). Interestingly, the mean weighted tau for recovery ($\tau_{w,\text{rec}}$) from receptor desensitization indicated that these were slower than cells in which GluA1 and FRRS1L were co-transfected ($\tau_{w,\text{rec}}$:...
919.8 ± 33.4 ms and 2158.8 ± 234.6 ms for co-expressed and tandem GluA1-FRRS1L respectively; \(P = 0.023\), Mann-Whitney-U test; \(n = 23 \text{ and } 5\); Figure 3.5.B), suggesting fewer FRRS1L proteins are associated in co-transfected cells and that the time required for the receptor to recover may be proportional to the number of associated FRRS1L proteins.

In accordance with our findings for the co-expression system, there was no difference in single-channel conductance (11.95 ± 0.93 pS versus 12.65 ± 1.44 pS; \(P = 0.47\), Mann-Whitney-U test; \(n = 26 \text{ and } 10\); Figure 3.5.C), desensitization time constant (\(\tau_{\text{w,des}}\): 1.972 ± 0.072 ms versus 1.943 ± 0.162 ms; \(P = 0.98\), Mann-Whitney-U test; \(n = 27 \text{ and } 10\); Figure 3.5.D), or rectification (RI_{+60/-60}: 0.114 ± 0.027 versus 0.073 ± 0.053; \(P = 0.14\), Mann-Whitney-U test; \(n = 19 \text{ and } 5\); Figure 3.5.E) between cells transfected with GluA1 alone compared with the GluA1-FRRS1L tandem construct, respectively.
Figure 3.5. A GluA1-FRRS1L tandem construct causes a decelerated recovery from receptor desensitization.
Figure 3.5. A GluA1-FRRS1L tandem construct causes a decelerated recovery from receptor desensitization

A. Representative averaged currents generated by applying glutamate using a 2-pulse protocol on outside-out patches from tsA201 cells recombinantly expressing either GluA1 or a GluA1-FRRS1L tandem. The initial current was activated by a 100 ms application of 10 mM glutamate, to fully desensitize all receptors in the patch. The second pulse, applied at increasing time intervals, consisted of 2 ms of 10 mM glutamate. B. Pooled data for recovery from desensitization time constant for tsA 201 cells transfected with GluA1, GluA1-FRRS1L tandem or GluA1 and FRRS1L as separate constructs (n = 13, 5 and 23). C-E. Pooled data for single-channel conductance calculated by non-stationary fluctuation analysis (n = 26 and 10) (C), weighted desensitization time constant (n = 27 and 10) (D), or RI_{60/+60} (n = 19 and 5) (E). Individual data points are shown together with the mean (thick horizontal line) for tsA 201 cells transfected with GluA1 or GluA1-FRRS1L tandem.
3.3.6. FRRS1L slows recovery from desensitization of GluA1/GluA2 heteromers

In the mammalian CNS, many AMPARs are expressed as Ca\(^{2+}\)-impermeable heteromers containing the GluA2 subunit (Dingledine et al., 1999, Wenthold et al., 1996, Traynelis et al., 2010). We therefore decided to investigate whether FRRS1L also slows recovery from desensitization of heteromeric GluA1/GluA2 AMPARs in a recombinant system. TsA201 cells were transfected with GluA1 and GluA2, with or without FRRS1L. As with GluA1 homomers, FRRS1L slowed recovery from desensitization of GluA1/GluA2 heteromers (\(\tau_{w,\text{rec}}\): 56.2 ± 6.7 ms without FRRS1L and 754.7 ± 235.8 ms with FRRS1L; \(P = 0.025\), Mann-Whitney-U test; \(n = 12\) and 9; Figures 3.6.A and B), without any significant effect on single-channel conductance (3.45 ± 0.44 pS without FRRS1L and 3.15 ± 0.47 pS with FRRS1L; \(P = 0.60\), Mann-Whitney-U test; \(n = 16\) and 14; Figure 3.6.C), desensitization (\(\tau_{w,\text{des}}\): 5.60 ± 0.40 ms without FRRS1L and 4.95 ± 0.23 ms with FRRS1L; \(P = 0.50\), Mann-Whitney-U test; \(n = 17\) and 14; Figure 3.6.D) or deactivation kinetics (\(\tau_{\text{deac}}\): 2.40 ± 0.38 ms without FRRS1L and 2.13 ± 0.19 ms with FRRS1L; \(P = 0.79\), Mann-Whitney-U test; \(n = 7\) and 7; Figure 3.6.E).
Figure 3.6. FRRS1L slows recovery from desensitization of GluA1/GluA2 heteromers
Figure 3.6. FRRS1L slows recovery from desensitization of GluA1/GluA2 heteromers

A. Representative averaged currents generated by applying glutamate using a 2-pulse protocol on outside-out patches from tsA201 cells recombinantly expressing GluA1/GluA2 heteromers with or without FRRS1L. A heteromeric population was defined by an RI greater than 0.8. B-E. Pooled data for recovery from desensitization time constant ($n = 12$ and 9) (B), single-channel conductance calculated by non-stationary fluctuation analysis ($n = 16$ and 14) (C), weighted desensitization time constant ($n = 17$ and 14) (D), or deactivation time constant ($n = 7$ and 7) (E). Individual data points are shown together with the mean (thick horizontal line) for tsA 201 cells transfected with GluA1 + GluA2 or GluA1 + GluA2 + FRRS1L.

3.3.7. FRRS1L expression in the CNS

Having established that FRRS1L alters AMPAR recovery from desensitization of homomeric and heteromeric assemblies in a recombinant system, we next wanted to explore what effect FRRS1L has on AMPAR function in neurons. First, we considered which brain regions endogenously express FRRS1L. Mass spectrometry analysis indicates that FRRS1L is expressed in many regions of the mammalian brain including the hippocampus, cerebellum, striatum and thalamus (Schwenk et al., 2014).

To further establish the expression pattern of FRRS1L, we used Western blot analysis of FRRS1L protein extracted from mouse hippocampus, cerebellum, cortex and spinal cord. We observed clear bands indicating FRRS1L expression in the hippocampus, cerebellum and cortex (Figure 3.7.A). In the spinal cord sample, there was a very faint band, suggesting only low FRRS1L abundance. The band intensities for FRRS1L expression were measured and normalised to actin band intensities. The results were then normalised to the relative FRRS1L expression levels in the hippocampus. Our results suggested
that relative to the hippocampus, FRRS1L expression is 3-fold higher in the cerebellum, whereas in the cortex it was similar to the hippocampus, and in the spinal cord it was lower (only about 15% of hippocampal levels) (Figure 3.7.B).

Since both mass spec analysis and our Western blot data indicated that FRRS1L shows high abundance in the hippocampus, we decided to focus on this area specifically. Further Western blot analysis of protein extracted from mouse hippocampus confirm that FRRS1L is expressed here alongside γ-8 – the most abundant TARP in this region (Figure 3.7.C).

Another highly abundant TARP in the hippocampus is γ-2 (stargazin) (Fukaya et al., 2005). We therefore asked whether FRRS1L expression levels might vary when γ-2 is absent. We examined tissue from stargazer mice (lacking γ-2) and found that normalised FRRS1L expression levels did not vary compared with those in wild-type hippocampus (Figure 3.7.D), implying that stargazin is not necessary for FRRS1L expression.
Figure 3.7. FRRS1L expression in the CNS
A. Representative Western blot data depicting expression levels of total FRRS1L and GluA2 in the hippocampus, cerebellum, cortex and spinal cord of P30 mice. B. Quantification of FRRS1L in different brain regions. FRRS1L band intensities from Western blots were measured and normalised to actin band intensities. Relative expression levels were then normalised to expression levels in the hippocampus for comparison ($n = 2$). C. Representative Western blot showing that FRRS1L is expressed along with TARP γ-8 in the hippocampus of P30 mice. D. Western blot data showing FRRS1L is expressed in the hippocampus of both wt and stargazer P30 mice.
3.3.8. Immunofluorescent labelling of FRRS1L in dissociated hippocampal cultures

Knowing that FRRS1L is expressed in the hippocampus, our aim was to compare AMPAR currents in hippocampal neurons overexpressing FRRS1L. Before undertaking recordings, we considered whether we could label the cell types that endogenously expressed FRRS1L in dissociated cultures using immunocytochemistry. We also considered whether over-expressing FRRS1L in dissociated hippocampal cultures altered its expression levels as detected by immunocytochemistry.

To assess whether our antibody would specifically recognise FRRS1L immunocytochemically, we transfected tsA201 cells with either GluA1 or GluA1 + FRRS1L, and stained them with antibodies against FRRS1L and GluA1. As illustrated in Figure 3.8.A, the FRRS1L antibody stained only those cells expressing GluA1 + FRRS1L, with no expression in the cells transfected with GluA1 alone. This indicated that the antibody could be used to identify FRRS1L specifically.

To label endogenously expressed FRRS1L, dissociated hippocampal cultures were fixed and stained with FRRS1L and GluA2 antibodies and visualised with confocal microscopy. A strong signal was obtained for the GluA2 staining. However, the FRRS1L signal showed little or no staining (Figure 3.8.B). This suggested that either the antibody does not label endogenous FRRS1L, or dissociated hippocampal cultures do not express sufficient FRRS1L to be successfully visualised using the antibody.
Figure 3.8. Immunofluorescent labelling of FRRS1L in dissociated hippocampal cultures
Figure 3.8. Immunofluorescent labelling of FRRS1L in dissociated hippocampal cultures
A. TsA201 cells were transfected with GluA1 (upper panel) or GluA1 + FRRS1L (lower panel) and stained for GluA1, FRRS1L and the nuclear marker DAPI. FRRS1L staining (left) was only detected in cells transfected with FRRS1L, indicating good antibody specificity. GluA1 was successfully labelled in both conditions (centre). The overlay with DAPI (right) shows some overlap between GluA1 and FRRS1L expression. B. Dissociated hippocampal cultures were stained for endogenous FRRS1L and GluA2. The FRRS1L antibody (left) showed no staining in these cultures, while GluA2 (centre) and an overlay + DAPI (right) were labelled in both cultures. C. Dissociated hippocampal cultures were transfected with GFP (upper panel) or GFP + FRRS1L (lower panel) and stained for FRRS1L. Staining for FRRS1L (left) was observed in cells transfected with FRRS1L, indicating that the antibody could detect overexpressed FRRS1L in dissociated hippocampal neuronal cultures. Scale bars are 20 µm.

We then considered whether the antibody would label overexpressed FRRS1L in dissociated hippocampal cultures. After transfecting with either GFP or GFP + FRRS1L, cells were fixed and immunostained with a FRRS1L antibody. In the cells co-transfected with GFP + FRRS1L, there was a strong signal from the FRRS1L staining, compared with an absence of FRRS1L staining in cells transfected with GFP alone (Figure 3.8.C). Thus the antibody could successfully label overexpressed FRRS1L in dissociated hippocampal cultures.

3.3.9. Overexpression of FRRS1L in dissociated hippocampal cultures does not alter AMPAR mEPSC amplitude, deactivation time or frequency

We have shown that in tsA201 cells, FRRS1L slows recovery from desensitization of GluA1 homomers and GluA1/GluA2 heteromers. However, it is unknown whether FRRS1L has any effect on AMPAR mEPSCs in pyramidal cells in dissociated hippocampal cultures. To investigate this, we transfected hippocampal neurons with either GFP or GFP + FRRS1L and recorded
miniature excitatory post-synaptic currents (mEPSCs) in whole-cell patch-clamp configuration. Figure 3.9.A shows representative traces of both conditions, illustrating their similarity. We found no significant difference between the GFP control cells and those transfected with GFP + FRSS1L in terms of mEPSC amplitude (64.3 ± 5.3 pA for GFP versus 41.6 ± 8.4 pA for GFP + FRSS1L; $P = 0.086$, unpaired two-tailed student’s t-test; $n = 6$ and 3; Figure 3.9.B), deactivation kinetics ($\tau_{\text{deac}}$: 1.83 ± 0.23 ms for GFP versus 3.18 ± 0.91 ms for GFP + FRSS1L; $P = 0.28$, unpaired two-tailed student’s t-test; $n = 6$ and 3; Figure 3.9.C) and event frequency (0.81 ± 0.30 Hz for GFP versus 1.27 ± 0.58 Hz for GFP + FRSS1L; $P = 0.53$, unpaired two-tailed student’s t-test; $n = 6$ and 3; Figure 3.9.D).
Figure 3.9. Overexpression of FRRS1L in dissociated hippocampal cultures does not alter AMPAR mEPSC amplitude, deactivation time or frequency
A. Representative whole cell recordings of mEPSCs at -80 mV from pyramidal cells in dissociated hippocampal cultures after transfection with either GFP or GFP + FRRS1L. To isolate AMPAR currents, 0.5 µM TTX, 20 µM APV, 20 µM
SR-95531, 1 µM strychnine and 10 µM D600 were included in the external solution. Inserts show a larger magnification of individual currents for comparison. **B-D.** Averaged mEPSC amplitude \((n = 6 \text{ and } 3)\) \((B)\), deactivation time constant \((n = 6 \text{ and } 3)\) \((C)\) and event frequency \((n = 6 \text{ and } 3)\) \((D)\) for individual whole cell recordings from hippocampal cultures transfected with GFP or GFP + FRRS1L. Individual data points are shown together with the mean (thick horizontal line).

### 3.3.10. In the presence of stargazin, FRRS1L has no effect on GluA1 homomers

Most AMPARs in native neurons are associated with auxiliary subunits. Since overexpressing FRRS1L in dissociated hippocampal cultures had no effect on AMPAR mEPSC properties, we considered whether the presence of another auxiliary protein might be masking the effects of FRRS1L. In the hippocampus, there is an abundance of TARP \(\gamma-2\), among other auxiliary proteins, so we decided to investigate how the combination of \(\gamma-2\) and FRRS1L affected homomeric GluA1 AMPARs in tsA201 cells.

TsA201 cells were transfected with either GluA1 alone, GluA1 and \(\gamma-2\), or GluA1, \(\gamma-2\) and FRRS1L. Outside-out patches from these cells were exposed to 2 or 100 ms pulses of 10 mM glutamate to determine single-channel conductance, desensitization and deactivation time of GluA1 homomers. In keeping with previous findings, stargazin increased single-channel conductance \((11.95 \pm 0.93 \text{ pS for GluA1 alone and } 21.10 \pm 1.34 \text{ pS for GluA1 + } \gamma-2; P < 0.0001, \text{Mann-Whitney } U \text{ test}; n = 26 \text{ and } 13; \text{Figure 3.10.A})\), slowed receptor desensitization \((\tau_{w,\text{des}}: 1.972 \pm 0.072 \text{ ms for GluA1 alone and } 4.525 \pm 0.272 \text{ ms for GluA1 + } \gamma-2; P < 0.0001, \text{Mann-Whitney } U \text{ test}; n = 27 \text{ and } 13; \text{Figure 3.10.B})\) and slowed receptor deactivation \((\tau_{\text{deac}}: 1.512 \pm 0.080 \text{ ms for } \text{GluA1 alone and } 3.802 \pm 0.243 \text{ ms for GluA1 + } \gamma-2; P < 0.0001, \text{Mann-Whitney } U \text{ test}; n = 23 \text{ and } 13; \text{Figure 3.10.C})\).
GluA1 alone and 3.267 ± 0.256 ms for GluA1 + γ-2; \( P = 0.0012 \), Mann-Whitney U test; \( n = 20 \) and 5; Figure 3.10.C) of GluA1 homomers (Figure 3.9.A). The presence of FRRS1L in γ-2-associated GluA1 homomers had no significant effect on the channel conductance (21.1 ± 1.3 pS without FRRS1L versus 23.6 ± 1.8 pS with FRRS1L; \( P = 0.38 \), Mann-Whitney U test; \( n = 13 \) and 17; Figure 3.10.A), desensitization kinetics (\( \tau_{\text{w,des}} \): 4.53 ± 0.27 ms without FRRS1L versus 3.89 ± 0.38 ms with FRRS1L; \( P = 0.18 \), Mann-Whitney U test; \( n = 13 \) and 15; Figure 3.10.B), or deactivation kinetics (\( \tau_{\text{deac}} \): 3.27 ± 0.26 ms without FRRS1L versus 2.98 ± 0.33 ms with FRRS1L; \( P = 0.94 \), Mann-Whitney U test; \( n = 5 \) and 8; Figure 3.10.C).

Using a 2-pulse protocol to assess recovery from receptor desensitization, we found that recovery was accelerated by γ-2 (\( \tau_{\text{w,rec}} \): 167.3 ± 3.9 ms for GluA1 alone versus 125.8 ± 3.9 ms for GluA1 + γ-2; \( P = 0.034 \), Mann-Whitney U test; \( n = 13 \) and 9), whereas when both γ-2 and FRRS1L were co-expressed with GluA1, there was no difference compared with GluA1 + γ-2 (\( \tau_{\text{w,rec}} \): 125.8 ± 3.9 ms without FRRS1L versus 177.5 ± 6.5 ms with FRRS1L; \( P = 0.59 \), Mann-Whitney U test; \( n = 9 \) and 19; Figures 3.10.D and E). This data suggests that either the phenotype of stargazin masks the influence of FRRS1L when both auxiliary subunits exist in the same AMPAR complex, or that stargazin prevents FRRS1L from binding to the AMPAR subunit.
Figure 3.10. In the presence of stargazin, FRRS1L has no effect on electrophysiological properties of GluA1 homomers.
Figure 3.10. In the presence of stargazin, FRRS1L has no effect on electrophysiological properties of GluA1 homomers

Outside-out patches from tsA201 cells transfected with GluA1, GluA1 + FRRS1L, GluA1 + γ-2 or GluA1 + γ-2 + FRRS1L were exposed to short pulses of 10 mM glutamate at -60 mV A-C. Averaged single-channel conductance (n = 26, 16, 13 and 17) (A), weighted desensitization time constant (n = 27, 18, 13 and 15) (B) and deactivation time constant (n = 20, 18, 5 and 8) (C) * P < 0.05 D. Percentage recovery from receptor desensitization after increasing time intervals using a 2-pulse protocol. E. Averaged recovery from desensitization time constant (n = 13, 23, 9 and 19) * P < 0.05, *** P < 0.001. F. GluA1 was immunoprecipitated from protein extracted from tsA201 cells transfected with either GluA1, GluA1 + FRRS1L or GluA1 + FRRS1L + γ-2. The samples were run on a 10% polyacrylamide gel and stained for GluA1, FRRS1L and γ-2. This figure shows a representative outcome in which FRRS1L does not co-immunoprecipitate with GluA1 when γ-2 is co-transfected (n = 3).

To address whether FRRS1L and stargazin can both bind to GluA1 simultaneously, we decided to transf ect all three proteins in tsA201 cells, and immunoprecipitate GluA1 to assess protein binding. Our results showed that when γ-2 is co-transfected, FRRS1L no longer binds to the GluA1 subunit (Figure 3.10.F). This suggests that both proteins likely compete for the same binding site of the AMPAR subunit, and γ-2 binds with higher affinity.
3.4. Discussion

These experiments, exploring the influence of FRRS1L on AMPAR signalling, confirm that this protein interacts with AMPARs and might be able to modify their activity. When co-expressed in tsA201 cells, FRRS1L markedly slows AMPAR recovery from desensitization, without affecting single-channel conductance, rectification, desensitization or deactivation kinetics. FRRS1L is abundant in many brain regions including the hippocampus, cerebellum and cortex. Overexpression in dissociated hippocampal cultures does not affect AMPAR mEPSC amplitude, deactivation kinetics or frequency. As our data suggests that stargazin and FRRS1L cannot simultaneously bind AMPAR subunits, the lack of effect of overexpressed FRRS1L may be due to the presence of TARPs (possibly stargazin) that might negate any effect of FRRS1L.

3.4.1. Overexpression of FRRS1L affects AMPAR function in tsA201 cells, but not in dissociated hippocampal cultures

The ubiquitous expression of FRRS1L in the brain (Figure 3.7) (Schwenk et al., 2014) suggests an important role for the auxiliary protein in normal brain function. When examined in tsA201 cells, most of the electrophysiological properties remained unaltered by the presence of FRRS1L, except for recovery from receptor desensitization, which was drastically slowed (Figure 3.3). Since AMPARs in neurons are part of a larger protein complex, it would be difficult to predict the exact effect of FRRS1L on mEPSCs in dissociated hippocampal cultures. Upon examination, mEPSC amplitude, deactivation kinetics and frequency were unaltered in FRRS1L over-expressing hippocampal neurons (Figure 3.9).
One possibility is that FRRS1L does not interact with AMPAR subunits expressed in the postsynaptic membrane. Evidence suggests that FRRS1L successfully binds to AMPAR subunits in both tsA201 cells (Figure 3.1) and various brain regions (Schwenk et al., 2014, Schwenk et al., 2012), but there is little evidence as to whether this interaction is present in the postsynaptic membrane.

It is also possible that we observed no significant difference in FRRS1L-overexpressing dissociated hippocampal cultures because there is already a saturating level of FRRS1L in these neurons. Thus, any additional expression of the protein would not be expected to cause further change when compared with controls. To test this, these experiments could be repeated using a knockdown of FRRS1L, allowing insight into how AMPAR mEPSCs behave in the absence of this auxiliary subunit.

Properties of mEPSCs in dissociated hippocampal cultures depend on numerous factors including number of synapses on to the postsynaptic neuron, and quantal release (Raghavachari and Lisman, 2004). To fully understand the function of FRRS1L in neurons, and specifically whether it may affect AMPAR recovery from desensitization, one approach would be to expose outside-out patches from dissociated cultures over-expressing FRRS1L to glutamate using a 2-pulse protocol, similar to the experiments we performed in tsA201 cells.
3.4.2. The phenotype elicited by FRRS1L overexpression may depend on cell type

In a recent study, recordings from mossy cells (MCs) in hippocampal slices over-expressing FRRS1L showed increased mEPSC amplitude, while leaving the current decay time unaffected (Brechet et al., 2017). Together with our findings, this data can provide further insight as to how FRRS1L may be influencing AMPAR function in hippocampal neurons. It is possible that FRRS1L exerts its effects only in certain cell types. For example, Brechet et al. recorded mEPSCs from MCs over-expressing FRRS1L in hippocampal slices and observed an increase in current amplitude, whereas we recorded from FRRS1L over-expressing pyramidal cells (PCs) in dissociated hippocampal cultures and observed no significant effect. Brechet et al. also examined extrasynaptic currents in PCs overexpressing FRRS1L by exposing outside-out patches to short pulses of glutamate, in which they observed an increase in current amplitude. It is therefore possible that FRRS1L overexpression in PCs increases the amplitude of extrasynaptic, with no effect on synaptic EPSCs.

3.4.3. Is FRRS1L incorporated in the postsynaptic AMPAR complex in hippocampal neurons?

Our experiments in tsA201 cells co-expressing GluA1 with γ-2 and FRRS1L suggest that the two auxiliary subunits do not assemble in the same AMPAR complex (Figure 3.10). This implies distinct populations of FRRS1L-containing and γ-2-containing AMPARs. Likewise, the findings of Brechet et al. are consistent with the existence of two mutually exclusive populations of AMPAR assemblies: one population comprising FRRS1L, CPT1c, Sac1 as well as ABHDs 6/12 and PORCN, the other containing the core auxiliary subunits
(CNIHs, TARPs, GSG1-L) and the remainders of the peripheral constituents. They propose a ‘priming’ role for FRRS1L in AMPAR biogenesis in the ER, in which it acts as a catalyst for receptor co-assembly with TARPs and other auxiliary proteins. This would be consistent with the view that FRRS1L may be absent from the post-synaptic membrane.

Particularly striking is the strong correlation between FRRS1L and CPT1c expression (Brech et al., 2017, Schwenk et al., 2014). When overexpressed in tsA201 cells or hippocampal neurons, CPT1c increases the current amplitude in response to glutamate application (Gratacòs-Batlle et al., 2015). This is thought to arise from an increase in AMPAR trafficking to the cell surface. The selective knockdown of either FRRS1L or CPT1c in hippocampal neurons using shRNA decreases AMPAR current amplitude by similar percentages (Brechet et al., 2017), suggesting that both proteins are important in controlling AMPAR function.

3.4.4. **GluA1-FRRS1L tandem orientation in the cell membrane**

Subsequent to performing the current experiments with the GluA1-FRRS1L tandem construct, we noticed a flaw in its design: the amino acid linker connected the C-terminal of the GluA1 subunit and the N-terminal of FRRS1L. As the C-terminal of GluA1 normally resides in the intracellular space, and the N-terminal of FRRS1L in the extracellular space, the linker would appear to prevent normal physiological receptor assembly. Surprisingly, outside-out patches from tsA201 cells transfected with this construct still displayed currents in response to glutamate application, and showed very slow recovery from desensitization. This suggests that there was assembly of functional receptors.
Previous studies have also reported tandem constructs yielding results that suggest unexpected stoichiometry (McCormack et al., 1992, Schorge and Colquhoun, 2003). These reports provide some explanations for the mechanisms behind the observed phenotypes, which could also apply to the results we observed with the GluA1-FRRS1L tandem. For example, the linking sequence could be cleaved by a peptidase, such as a signal peptidase (Robakis et al., 2008), permitting the linked proteins to exist as two separate, fully functional proteins that assemble correctly.

While FRRS1L is mostly described with a single membrane-spanning domain (Schwenk et al., 2012, UniProt, 2014), other protein prediction databases indicate a second putative transmembrane domain between amino acids 60-78, albeit with a lower degree of probability (Center for Biological Sequence Analysis, 2013, Swiss Institute for Bioinformatics, 2014). It is therefore plausible that when its NTD is linked to the intracellular part of GluA1, FRRS1L crosses the membrane twice, thereby allowing normal AMPAR assembly.

Although it seems unlikely due to the very short amino acid sequence in the C-tail, it is possible that FRRS1L exerts its effect of slowing the recovery from desensitization of AMPARs solely through an interaction with this intracellular domain. This would provide an explanation of how FRRS1L can exert its effects on the AMPAR, while the entire protein is trapped inside the cell. However, further experiments would be required to fully understand how this GluA1-FRRS1L tandem can produce functional receptors.
3.4.5. Is FRRS1L a novel AMPAR auxiliary subunit?

One of the main aims of this study is to investigate how FRRS1L influences AMPAR function, and whether it may be classified as a novel AMPAR auxiliary subunit. As defined by Yan and Tomita, an auxiliary subunit possesses the following four criteria: (1) Non-pore-forming subunit, (2) Direct and stable interaction with a pore-forming subunit, (3) Modulation of channel properties and/or trafficking in heterologous cells, (4) Necessity *in vivo*. From our current knowledge of this protein, does FRRS1L possess these four criteria, and can it thus be defined as a novel AMPAR auxiliary subunit?

(1) Non pore-forming subunit: FRRS1L is a 344 amino acid protein containing a single transmembrane domain close to the C-terminus (UniProt, 2014). Glutamate application to outside-out patches from tsA201 cells heterologously expressing FRRS1L do not evoke any currents (data not shown), suggesting that FRRS1L is not a pore-forming subunit.

(2) Direct and stable interaction with a pore-forming subunit: When transfected in tsA201 cells, C9orf4 co-immunoprecipitates with GluA1 (Figure 3.1), implicating a direct and stable interaction between these two proteins. Furthermore, proteomics analysis shows that FRRS1L binds with high stability and abundance to both the GluA1 and GluA2 subunits (Schwenk et al., 2012).

(3) Modulation of channel properties and/or trafficking in heterologous cells: When co-transfected with C9orf4, GluA1 homomers and GluA1/GluA2 heteromers show a marked recovery from receptor desensitization in tsA201 cells (Figures 3.3 and 3.6).
(4) Necessity *in vivo*: Overexpression of FRRS1L in pyramidal cells in dissociated hippocampal cultures does not alter amplitude, deactivation kinetics or frequency of AMPAR mEPSCs (Figure 3.9).

Our data supports that FRRS1L fulfils three of the four criteria of an auxiliary subunit: it is a non pore-forming subunit, forms a direct and stable interaction with GluA1 and GluA2 subunits and slows recovery from receptor desensitisation in heterologous cells. While our data is not able to confirm the necessity of FRRS1L *in vivo*, other studies have shown that knockdown of FRRS1L in hippocampal mossy cells significantly reduces the amplitude of AMPAR-mediated currents by around 40% (Brechet et al., 2017). Furthermore, genetic mutations in the FRRS1L gene can lead to severe cognitive disability (Brechet et al., 2017, Madeo et al., 2016, Shaheen et al., 2016), suggesting an important role for FRRS1L in normal brain function. Combining our findings with data from other research groups provides strong evidence to define FRRS1L as a novel AMPAR auxiliary protein. However, further experiments, in particular to establish the role of FRRS1L *in vivo*, are necessary to consolidate our findings.

### 3.4.6. FRRS1L: future directions

While it seems FRRS1L is unlikely to assemble with AMPARs in the postsynaptic membrane, there is still much to learn about the physiological role of this novel auxiliary subunit. The abundant expression throughout the brain indicates an important role for this protein.
Because of its robust effect on receptor recovery from desensitization in a recombinant system, this protein could be used to gain insight how AMPAR recovery from desensitization is controlled. Thus, knowing the region and sequence of FRRS1L that binds to the AMPAR subunit could shed light on the mechanisms underlying the recovery from receptor desensitization phenotype.

Previous work has shown that intellectual disability can arise from AMPAR mutations (Soto et al., 2014a). Furthermore, patients carrying mutations in auxiliary subunits γ-2 (Hamdan et al., 2011) or CNIH2 (Floor et al., 2012) also suffer from cognitive impairment. The recent discovery that mutations in the FRRS1L gene can result in severe cognitive disability (Brechet et al., 2017, Madeo et al., 2016, Shaheen et al., 2016) now further extends our understanding of the importance of auxiliary subunits in regulating and maintaining normal cognitive behaviour.
4. The involvement of TARPs in AMPAR plasticity in a model of ischaemic stroke

4.1. Summary

The hippocampal CA1 region is particularly vulnerable to injury following ischaemic stroke. This damage arises in part from ‘over-activation’ of ligand-gated ion channels, resulting in an excessive influx of Na\(^+\) and Ca\(^{2+}\) into the cell. AMPARs are among the channels shown to be involved in this process, as are acid-sensing ion channels 1-a (ASIC1-a), which are activated by the low pH that accompanies ischaemia.

Recently, it has been shown that direct ASIC1-a activation by reduced pH (acidosis) is sufficient to drive AMPAR plasticity in cultured CA1 neurons. 12 h after acidosis, there is a decrease in the GluA2 subunit, resulting in a relative increase in CP-AMPARs present in the membrane. The aberrant influx of Ca\(^{2+}\) into the cell through CP-AMPARs is thought to contribute to excitotoxicity.

In this chapter, we show that the acidosis-induced decrease in GluA2 expression is likely accompanied by an increase in TARP \(\gamma\)-8 expression. Using various techniques, we explore the change in CP-AMPAR expression and cell excitability. Lastly, we show that following acidosis, there is an increase in Ca\(^{2+}\) entry into the cells, which is likely mediated in part through CP-AMPARs.
4.2. Introduction

Ischaemia in the hippocampus leads to excessive release of excitatory neurotransmitters, including glutamate, invoking neuronal toxicity and death. This damage is partially due to excitotoxicity – the ‘over-activation’ of ligand-gated ion channels – resulting in excessive Na\(^+\) and Ca\(^{2+}\) influx (Dirnagl et al., 1999, Orrenius et al., 2003, Szydlowska and Tymianski, 2010). NMDARs were among the first receptors linked to ischaemia-induced excitotoxicity (Reynolds and Hastings, 1995). Subsequently, a range of further receptors were implicated, suggesting a more complex situation in which many ion channels and receptors contribute to this excitotoxicity. Both CP-AMPARs and acid-sensing ion channels (ASICs) have been shown to be involved (Gorter et al., 1997, Mari et al., 2010, Optiz et al., 2000, Pignataro et al., 2007, Sherwood et al., 2011, Xiong et al., 2004). However, how these channels contribute to excitotoxicity is still not well defined.

AMPARs are thought to contribute to excitotoxic neuronal death in the hippocampus by two distinct mechanisms. First, there is an alteration of the normal cycling of glutamate leading to a persistent increase in glutamatergic neurotransmission within the first few hours following ischaemia. This arises from an increase in the release of glutamate into the extracellular space, a decrease in glutamate uptake from the synaptic cleft and an increase of spillage of glutamate from injured neurons. As a result, both AMPAR and NMDAR expression is markedly increased, resembling the changes that accompany LTP, thereby enhancing excitatory transmission (Quintana et al., 2006). The second mechanism involves a down-regulation of GluA2 several hours later. This results in an increase in GluA2-lacking, Ca\(^{2+}\)-permeable AMPARs (CP-
AMPARs) (Optiz et al., 2000). The long-lasting increase in the relative proportion of CP-AMPARs is believed to contribute to the over-activation of calcium-dependent mechanisms that follow ischaemia and ultimately lead to cell death.

During ischaemic stroke, the reduced blood flow to the hippocampus leads to a build-up of CO₂, causing the pH to decline to 6.0-6.5. Following severe ischaemia, or during ischaemia accompanied by hyperglycaemia (abnormally high blood sugar levels), the pH can descend to levels of less than pH 6.0 (Nedergaard et al., 1991, Rehncrona, 1985, Siesjo, 1982). Na⁺ and Ca²⁺-permeable ASIC1-a channels expressed in hippocampal pyramidal neurons are activated at this low pH (Wemmie et al., 2013) and have also been implicated in neurotoxicity induced by ischaemic acidosis, possibly due to their Ca²⁺ permeability (Mari et al., 2010, Pignataro et al., 2007, Sherwood et al., 2011, Xiong et al., 2004). More recently, it has been shown that direct activation of ASIC-1a channels is sufficient to drive the changes in AMPAR plasticity in the hippocampus, demonstrating a direct functional interaction between acidotoxicity and excitotoxicity (Quintana et al., 2015). It is also of note that protons markedly suppress NMDAR activation, making their contribution less likely at low pH levels (Traynelis and Cull-Candy, 1990).

While the effect of acidosis on AMPAR plasticity and subsequent excitotoxicity have been reported, it still remains unknown whether any auxiliary proteins are necessary to regulate these changes. This section of the thesis will further examine AMPAR plasticity following acidosis, and explore whether TARPs are involved in this process.
4.3. Results

4.3.1. GluA2 expression levels decrease following acidosis

Direct activation of ASIC-1a leads to a decrease in the expression of GluA2, resulting in an increase in the proportion of CP-AMPARs (Quintana et al., 2015). To investigate whether any TARPs are involved in regulating the change in AMPAR subunit expression levels, we first examined whether GluA2 decreased following our method of ASIC-1a activation. Dissociated hippocampal cultures were treated for 15 min with culture medium at pH 7.4 (control), or culture medium titrated with HCl to pH 6 (acidosis). 16 h after treatment, the cells were fixed and stained for GluA2. Our results confirm previous findings, showing that a 15 min decrease in pH from 7.4 to 6 is sufficient to drive AMPAR plasticity (Figure 4.1.A). This decrease in the expression of GluA2 appears to occur not only in the cell bodies, but importantly also shows a change in dendritic spines, where the relative GluA2 abundance decreases to about 40% (Normalised fluorescence: 1.000 ± 0.109 and 0.381 ± 0.038 after treatment with pH 7.4 and pH 6 respectively; \( P = 0.012 \), Mann-Whitney U test; \( n = 5 \) and 5; Figure 4.1.B).
Figure 4.1. GluA2 expression levels decrease following acidosis
A. Representative confocal images of GluA2-labelled dissociated hippocampal cultures 16 h after treatment (DIV 15) with either pH 7.4 (left panel) or pH 6 (right panel) and subsequent staining for GluA2. Scale bars represent 10 µm. Inserts show an enlargement of dendritic spines with GluA2 staining at synapses. B. Quantification of dendritic GluA2 expression in pH 7.4- or pH 6-treated dissociated hippocampal cultures (n = 5 and 5) * P < 0.05.

4.3.2. Acidosis does not alter cell morphology
We next wanted to ensure that the underlying change in AMPAR plasticity did not result from acidosis treatment affecting cell morphology or health. After plating, cells were placed in an Incucyte plate reader, which allowed the cells to be visually monitored every 4 h, enabling us to track the growth and branching
of neurites and to measure cell body area. 3 days after plating – when the cells had attached to the plate and begun neurite outgrowth – acidosis treatment was performed. Cells were treated for either 15, 30, 60, 120 or 240 min. Figure 4.2.A shows representative examples of images taken at different time points of cells treated with pH 7.4 for 15 min. Initially, at DIV 0, there were very few neurites and the cell bodies appeared small. Around the time of the treatment (DIV 3), more neurites protruded from the cell body, which by DIV 12 had grown extensively to form a dense network.

Comparing cells from the different treatment conditions at 9 days after treatment (DIV 12), there appeared to be no obvious difference in the growth rate or morphology between control and acidosis-treated cells. A selection of representative images of either untreated cells, cells treated for 15 min or 4 h with pH 7.4 or pH 6 (DIV 12) are shown in Figure 4.2.B. For quantitative analysis, we applied tracking software that would detect any changes in neurite or cell body size. The data confirmed that there was no significant difference in neurite length, neurite branch points and total cell body area between any of the treatment groups (Figure 4.2.C and Table 4.1). This suggests that the observed differences in GluA2 expression were not due to a secondary artefact involving a change in cell morphology, and therefore more likely reflect molecular changes induced directly by the shift in pH.
Figure 4.2. Acidosis does not alter cell morphology
Figure 4.2. Acidosis does not alter cell morphology
Rat hippocampal neurons were plated in 96-well plates at a density of 10'000 cells per well, and placed in an Incucyte plate reader to image the cells every 4 h. After 3 days, cells were treated for 15 min with pH 7.4 or pH 6 solutions and continuously imaged. **A.** Representative images of cells after plating (DIV 0; left panel), < 6 h after treatment with pH 7.4 solution for 15 min (DIV 3; middle panel) and 9 days after treatment (DIV 12; right panel). **B.** Representative images of cells at DIV 12 either untreated (left panels), 9 days after 15 min (middle panels) or 4 h (right panels) treatment with either pH 7.4 (top panels) or pH 6 (bottom panels). **C.** Change in neurite length, neurite branch points and cell body area as measured by the Incucyte tracking software normalised to the time of treatment for either untreated, 15 min, or 4 h treatments with either pH 7.4 or pH 6 solutions. Data points show mean values from 3 sets of experiments.

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**Table 4.1. Acidosis does not affect neurite growth, neurite branch points or cell body area**
Averaged measurements for neurite length, neurite branch points and cell body area for different treatment conditions 9 days after treatment (DIV 12). Results were normalised to the first measure after treatment (DIV 3). One-way ANOVA ($F_{8, 18} = 0.28, P = 0.96$) suggests there was no significant difference between the various treatments. N represents the number of experiments, where data for each experiment was obtained from 4 wells per treatment.
4.3.3. Acidosis-induced decrease in GluA2 is associated with an increase in the type I TARP γ-8

TARPs regulate many properties of AMPARs, including trafficking and tethering to the cell membrane (Bats et al., 2007, Chen et al., 2000, Coombs et al., 2017, Tomita et al., 2003, Turetsky et al., 2005). We therefore considered whether TARPs were involved in mediating the decrease in GluA2 levels following acidosis. As the most abundant TARPs in the hippocampus are the type I TARPs γ-2 and γ-8, we examined these first. Dissociated hippocampal cultures were treated with either pH 7.4 or pH 6 medium for 15 min, and 16 h later fixed and stained for γ-2, γ-8 and GluA2. While there was no significant shift in γ-2 levels (Normalised fluorescence: 1.000 ± 0.045 and 1.106 ± 0.052 after treatment with pH 7.4 and pH 6 respectively; \( P = 0.30 \), Mann-Whitney U test; \( n = 5 \) and 5; Figure 4.3), γ-8 was consistently up-regulated by nearly 2-fold following acidosis (Normalised fluorescence: 1.000 ± 0.043 and 1.92 ± 0.138 after treatment with pH 7.4 and pH 6 respectively; \( P = 0.012 \), Mann-Whitney U test; \( n = 5 \) and 5; Figure 4.3). This was accompanied by an overall decrease in GluA2 levels, consistent with the idea that γ-8 might be involved in acidosis-induced AMPAR plasticity.
Figure 4.3. Acidosis-induced decrease in GluA2 is associated with an increase in the type I TARP γ-8.
**Figure 4.3. Acidosis-induced decrease in GluA2 is associated with an increase in the type I TARP γ-8**

A. Representative confocal images of dissociated hippocampal cultures 16 h after acidosis treatment (DIV 15), fixed and stained for γ-8 (top panels), γ-2 (middle panels) and GluA2 (bottom panels). Scale bar is 10 µm. B. Quantification of dendritic GluA2, γ-8 and γ-2 expression in pH 7.4- or pH 6-treated dissociated hippocampal cultures (n = 5 and 5) * P < 0.05.

**4.3.4. Acidosis does not change expression levels of type II TARPs**

Hippocampal neurons also express type II TARPs (γ-5 and γ-7) (Fukaya et al., 2005), which are often co-expressed with type I TARPs (Kato et al., 2010b). To explore whether any of the type II family of TARPs are involved in mediating GluA2 levels following acidosis, we repeated the experiments with antibodies against γ-5 and γ-7. We found no change in expression levels of either TARPs between the control and acidosis-treated samples (Normalised fluorescence of γ-5: 1.000 ± 0.073 and 1.074 ± 0.090 after treatment with pH 7.4 and pH 6 respectively; P = 0.053, Mann-Whitney U test; n = 5 and 5; Normalised fluorescence of γ-7: 1.000 ± 0.086 and 1.121 ± 0.0.086 after treatment with pH 7.4 and pH 6 respectively; P = 0.53, Mann-Whitney U test; n = 5 and 5; Figure 4.4), while the GluA2 expression levels were still diminished. This suggests that if the changes in GluA2 expression levels following acidosis involve a TARP, it is most likely mediated by γ-8.
Figure 4.4. Acidosis does not change expression levels of type II TARPs A+C. Representative confocal images of dissociated hippocampal cultures 16 h after acidosis treatment (DIV 15), fixed and stained for γ-5 and GluA2 (A) or γ-7 and GluA2 (C). Images provided by Rebecca Jones. Scale bar is 20 μm. B+D. Quantification of dendritic GluA2 and γ-5 (B) or GluA2 and γ-7 (D) expression in pH 7.4- or pH 6-treated dissociated hippocampal cultures (n = 5 and 5) * P < 0.05.
4.3.5. Membrane quantification of the change in γ-8, γ-2 and GluA2 expression levels in dissociated hippocampal cultures following acidosis

As the cells had been permeabilised prior to immunolabelling, the results thus far indicated a global decrease in GluA2, accompanied by a global increase in γ-8 expression levels. To establish whether the changes were occurring in the cell membrane, we decided to extract the membrane fraction of acidosis-treated hippocampal neurons and analyse protein expression levels using Western blotting.

When repeating this method multiple times, we obtained inconsistent results. In most experiments, expression levels of all three proteins increased when we directly compared pH 6-treated with pH 7.4-treated cells (Figure 4.5.A). However, the range of values obtained for percentage change among experiments was very scattered: percentage change in γ-8 expression ranged from 5.6 to 82.6 %, γ-2 from 9.6 to 810.3 % and GluA2 from -44.9 to 59.9 % (n = 4; Figure 4.5.B). On average, γ-8 membrane expression increased by 39.4 ± 18.4 % (P = 0.10, unpaired student’s t-test; n = 4), γ-2 by 258.1 ± 186.0 % (P = 0.45, unpaired student’s t-test; n = 4) and GluA2 by 30.4 ± 26.5 % (P = 0.40, unpaired student’s t-test; n = 4; Figure 4.5.B).
Figure 4.5. Membrane quantification of the change in γ-8, γ-2 and GluA2 expression levels in dissociated hippocampal cultures following acidosis

Hippocampal dissociated cultures were exposed to acidosis treatment after 14 DIV. 16 hours later, the cells were lysed and membrane proteins extracted. Membrane samples were run on a Western blot and stained for γ-8, γ-2 and GluA2. A. Representative Western blot. B. Change in protein expression levels in acidosis-treated (pH 6) cells represented as a percentage of protein expression levels in control (pH 7.4) cells. Circles represent values from individual experiments and thick horizontal lines represent the mean values. Overall, there was no significant change in γ-8 ($P = 0.10$, $n = 4$), γ-2 ($P = 0.45$, $n = 4$) or GluA2 ($P = 0.40$, $n = 4$) expression levels.

One concern we had with this method was the overall low levels of protein yielded by the hippocampal culture. We speculated that this could have been the cause of the scattered results, as low total protein levels would have increased the variability of this test, and thus any slight changes would have been magnified. In order to increase our protein yield, we decided to examine acidosis-induced changes in TARPs and AMPARs in organotypic slice cultures.

We reasoned that culturing organotypic slices involved less handling of the
tissue samples, allowing more of the cells to be conserved, thereby giving a greater protein yield.

4.3.6. Membrane quantification of the change in $\gamma$-8 and GluA2 expression levels in organotypic hippocampal slice cultures following acidosis

As in the previous experiments, organotypic hippocampal slice cultures were treated with either pH 7.4 or pH 6 media for 15 min, and 16 h later were subjected to protein extraction. A small sample was set aside to determine total protein expression. From the remaining protein, the membrane protein fraction was isolated, and together with the total protein sample this was run on a 10 % polyacrylamide gel and stained for $\gamma$-8 and GluA2.

Again, the results we obtained showed a lot of variability. Figure 4.6.A shows an example of a Western blot of membrane expression levels, in which both $\gamma$-8 and GluA2 expression decreased, along with the membrane protein N-cadherin. As with the dissociated cultures, there was a large spread in the values of % change following acidosis: the percentage change in membrane $\gamma$-8 expression ranged from -26.1 to 84.1 % ($n = 6$); membrane GluA2 ranged from -57.7 to 54.2 % ($n = 6$); total $\gamma$-8 ranged from -57.8 to 279.8 % ($n = 5$) and total GluA2 ranged from -26.6 to 90.6 % ($n = 4$; Figure 4.6.B). On average, $\gamma$-8 membrane expression increased by $46.3 \pm 39.3$ % ($P = 0.74$, unpaired student’s t-test; $n = 6$); membrane GluA2 increased by $13.2 \pm 15.8$ % ($P = 0.53$, unpaired student’s t-test; $n = 6$); total $\gamma$-8 increased by $123.5 \pm 67.3$ % ($P = 0.14$, unpaired student’s t-test; $n = 5$) and total GluA2 increased by $32.5 \pm 24.4$ % ($P = 0.27$, unpaired student’s t-test; $n = 4$; Figure 4.6.B).
Hippocampal organotypic slice cultures were subjected to acidosis treatment after 1 DIV. 16 hours later, the slices were homogenised and total protein extracted. After setting aside a small sample, membrane proteins were purified. Both total and membrane protein samples were run on a Western blot and stained for γ-8 and GluA2. As a control, N-cadherin was used instead of actin due to its high abundance in the cell membrane. A. Representative Western blot. B. Change in protein expression levels in acidosis-treated (pH 6) cells represented as a percentage of protein expression levels in control (pH 7.4) cells. Circles represent values from individual experiments and thick horizontal lines represent the mean values. Overall, there was no significant change in...
membrane γ-8 ($P = 0.74$, $n = 6$), membrane GluA2 ($P = 0.53$, $n = 6$), total γ-8 ($P = 0.14$, $n = 5$) or total GluA2 ($P = 0.27$, $n = 4$) expression levels. **C.** Hippocampal slice cultures (DIV 1) untreated or treated with pH 6 overnight and stained with 5 µg/ml propidium iodide for 30 min to assess cell death.

To assess the health of the organotypic slice cultures, and determine whether the acidosis treatment might be giving rise to cell death, we stained slices with propidium iodide (PI) – a non-permeant nuclear stain used as a marker for cell death. Slices were treated with pH 6 overnight – to assess maximal potential damage induced by the acidosis treatment, and compared with untreated slices using PI staining. As seen in Figure 4.6.C, both conditions showed very little PI staining, implying that even a long treatment of pH 6 did not cause cell death in organotypic hippocampal slice cultures. Thus the slice cultures appeared to be healthy, and cell death could not explain the wide variation in the expression level of γ-8 and GluA2 that we observed after acidosis. We therefore used an electrophysiological approach to determine changes in AMPAR composition. Using patch-clamp to measure changes in the rectification of AMPAR-mediated currents is a well-established method for measuring changes in the expression of CP-AMPARs.

### 4.3.7. Acidosis does not alter rectification of AMPAR mEPSCs

To determine whether acidosis treated cells evoked a change in expression of CP-AMPARs at synapses, we treated dissociated hippocampal cultures with either pH 7.4 or pH 6 solution. 12-24 h later, we recorded mEPSCs at -60 and +60 mV. Including the polyamine spermine in the intracellular solution allowed us to identify the expression of GluA2-containing Cl-AMPARs, as the edited
Q/R site on this subunit renders the receptor insensitive to polyamine block at positive potentials.

Figure 4.7.A shows averaged mEPSCs at -60 and +60 mV for cells treated with pH 7.4 or pH 6. To calculate rectification, the current amplitude was compared at -60 and +60 mV. For cells treated with pH 7.4, the RI_{+60/-60} value was 0.576 ± 0.050. For pH 6-treated cells, the RI_{+60/-60} value was 0.706 ± 0.050 (P = 0.12, unpaired student's t-test; n = 4 and 4; Figure 4.7.B). Furthermore, when we constructed a current-voltage (I-V) plot with maximum currents measured at -80, -60, +40 and +60 mV, there was no obvious difference between pH 6-treated cells and control cells (Figure 4.7.C).
Figure 4.7. Acidosis does not alter rectification of AMPAR mEPSCs
Electrophysiological mEPSC recordings with 100 µM intracellular spermine from dissociated hippocampal cultures 16 h after treatment with either pH 7.4 or pH 6 solutions. A. Averaged mEPSC traces recorded at -60 and +60 mV from pH 7.4- (black) or pH 6-treated (red) dissociated hippocampal pyramidal cells. Each trace represents averaged currents from multiple experiments (n = 4 for both conditions). B. RI values calculated by dividing the amplitude at +60 mV by the amplitude at -60 mV for pH 7.4- (black) or pH 6-treated (red) cells. Circles represent RI values from individual experiments and the thick horizontal lines represent the mean values. There is no significant change in RI_{+60/-60} between recordings from pH 7.4- and pH 6-treated cells (P = 0.12; n = 4 for both conditions). C. Current-voltage (I-V) plot of mEPSCs recorded at -80, -60, +40 and +60 mV. Values recorded from pH 7.4-treated cells are shown in black and pH 6 in red.
4.3.8. Acidosis does not affect AMPAR rectification in the absence of intracellular polyamines

As there was no difference in rectification between pH 7.4- and pH 6-treated cells in the presence of spermine, we wanted to ensure there was also no change in rectification when polyamine was absent. This control was to confirm that there was no change in rectification between the two groups of treated cells that may be masked by the presence of polyamine. We thus repeated the experiments described in section 4.3.7 without spermine in the intracellular solution.

When we compared cells treated with pH 7.4 and pH 6 solutions, we found no obvious difference in the average currents at -60 and +60 mV (Figure 4.8.A). When we compared rectification, cells treated with pH 7.4 solution had an $RI_{+60/-60}$ value of 0.605 ± 0.060, and cells treated with pH 6 had an $RI_{+60/-60}$ value of 0.634 ± 0.052 ($P = 0.73$, unpaired student’s t-test; $n = 5$ and 4; Figure 4.8.B). Furthermore, when we constructed a current-voltage (I-V) plot with maximum currents recorded at -80, -60, +40 and +60 mV, there appeared to be no difference between pH 6-treated and control cells (Figure 4.8.C).
Figure 4.8. Acidosis does not affect AMPAR rectification in the absence of intracellular polyamines

Electrophysiological mEPSC recordings without intracellular polyamines from dissociated hippocampal cultures 16 h after treatment with either pH 7.4 or pH 6 solutions. A. Averaged mEPSC traces recorded at -60 and +60 mV from pH 7.4- (black) or pH 6-treated (red) dissociated hippocampal pyramidal cells. Each trace represents averaged currents from multiple experiments (n = 4 for both conditions). B. RI values were calculated by dividing the amplitude at +60 mV by the amplitude at -60 mV for pH 7.4- (black) or pH 6-treated (red) cells. Circles represent RI values from individual experiments and the thick horizontal lines represent the mean values. There is no significant change in RI_{+60/-60} between recordings from pH 7.4- and pH 6-treated cells (P = 0.73; n = 5 and 4)
**C.** Current-voltage (I-V) plot of mEPSCs recorded at -80, -60, +40 and +60 mV. Values recorded from pH 7.4-treated cells are shown in black and pH 6 in red.

### 4.3.9. Assessing Ca\(^{2+}\) influx through CP-AMPARs after acidosis

If CP-AMPAR expression was increased by exposure to pH 6, it might be possible to detect this functionally as an increased Ca\(^{2+}\) influx when cells were exposed to AMPA. To address this, we next used fluorescence-based single-cell calcium-flux imaging. Dissociated hippocampal cultures were loaded with the Ca\(^{2+}\)-sensitive dye Fluo-4, 16 h after pH 7.4 or pH 6 treatment. We bath-applied both KCl and AMPA to eliciting a Ca\(^{2+}\) signal; KCl was used to depolarise the cell and evoke responses; AMPA was used to distinguish Ca\(^{2+}\) currents generated primarily through AMPARs. Additionally, we used Philanthotoxin-74 (PhTx-74), a use-dependent blocker of CP-AMPARs.

AMPA generated similar responses in pH 7.4- compared to pH 6-treated cells when PhTx-74 was absent (\(\Delta F/F_0\): 3.10 ± 0.22 versus 2.99 ± 0.12; \(P = 0.68\), unpaired student’s t-test; \(n = 4\) and 4), as did KCl (\(\Delta F/F_0\): 3.25 ± 0.33 versus 3.01 ± 0.23; \(P = 0.53\), unpaired student’s t-test; \(n = 4\) and 4; Figure 4.9.A).

When AMPA was applied in the presence of PhTx-74, the normalised fluorescence intensity of pH 7.4- and pH 6-treated cells was again similar (\(\Delta_{\text{norm}} F/F_0\): 1.22 ± 0.02 versus 1.18 ± 0.01; \(P = 0.22\), unpaired student’s t-test; \(n = 4\) and 4; Figures 4.9.A and B).
Figure 4.9. Assessment of Ca\(^{2+}\) influx through CP-AMPARs after acidosis
Figure 4.9. Assessment of Ca\textsuperscript{2+} influx through CP-AMPARs after acidosis A+C+E. Representative single-cell traces from calcium-flux imaging for hippocampal neurons treated with either pH 7.4 or pH 6 solutions in response to 10 µM AMPA (A) or 30 mM KCl, with the application of 20 µM Philanthotoxin-74 (A), 30 µM Mibefradil (C), or 20 µM Philanthotoxin-74 and 30 µM Mibefradil (E). Individual cell traces are in cyan and the mean responses in red. B+D+F. Peak fluorescence of cells treated with pH 7.4 (black) or pH 6 (red) in the presence of 20 µM Philanthotoxin-74 (B), 30 µM Mibefradil (D), or 20 µM Philanthotoxin-74 and 30 µM Mibefradil (F) in response to activation by AMPA or KCl. All values are normalised to the peak fluorescence evoked by the respective agonist before antagonist application. Error bars indicate SEM.

These results could suggest that only a small proportion of Ca\textsuperscript{2+} enters the cell through CP-AMPARs upon neurotransmitter binding and cell depolarization. Application of both AMPA and KCl evoked large fluorescent responses even in the presence of PhTx-74, suggesting there was considerable Ca\textsuperscript{2+} influx through other channels, potentially masking Ca\textsuperscript{2+} entry through CP-AMPARs and thus making it difficult to detect if there were any changes in CP-AMPAR expression levels following acidosis.

We next considered whether we could distinguish the fraction of Ca\textsuperscript{2+} entering the cell solely through CP-AMPARs by lowering the overall fluorescent response by blocking voltage-gated Ca\textsuperscript{2+} channels (VGCCs) using the use-dependent blocker Mibefradil. We therefore bath-applied Mibefradil to the treated hippocampal cultures. We found no difference in normalised peak fluorescence between pH 7.4- and pH 6-treated cells with the third activation by AMPA (\(\Delta_{\text{norm}} F/F_0\): 0.169 ± 0.005 versus 0.161 ± 0.005; \(P = 0.27\) Mann-Whitney U test; \(n = 8\) and 8; Figures 4.9.C and D). Application of KCl after three applications of AMPA in the presence of Mibefradil still generated a small fluorescent response (\(\Delta_{\text{norm}} F/F_0\): 0.110 ± 0.021 for pH 7.4 and 0.103 ± 0.017 for
pH 6; \( P = 0.43 \) Mann-Whitney U test; \( n = 8 \) and 8; Figures 4.9.C and D), suggesting that the addition of Mibefradil was not sufficient to block all \( \text{Ca}^{2+} \) entering the cell through voltage-gated channels. These residual \( \text{Ca}^{2+} \) currents could still be large enough to mask the changes in the contribution of CP-AMPARs we expected to see.

To deduce whether the small currents observed with the application of Mibefradil were occurring through CP-AMPARs, we co-applied PhTx-74, which would block any currents through these receptors. We found no difference in normalised peak fluorescence between pH 7.4- and pH 6-treated cells with the third activation by AMPA (\( \Delta_{\text{norm}} F/F_0: 0.179 \pm 0.011 \) versus 0.194 \( \pm 0.011; P = 0.39 \), unpaired student’s t-test; \( n = 4 \) and 4; Figures 4.9.E and F). These currents were not significantly different from currents treated with Mibefradil alone (\( P = 0.44 \) and \( P = 0.050 \) for pH 7.4- and pH 6-treated cultures respectively, unpaired student’s t-test). This suggested either that both pH 7.4- and pH 6-treated cultures both predominantly express Cl-AMPARs, or that the technique is not sensitive enough to detect \( \text{Ca}^{2+} \) influx through CP-AMPARs.

4.3.10. Synaptic activity is enhanced following acidosis

We next considered the possibility that enhanced CP-AMPAR expression may have a more marked effect on synaptic AMPAR expression. Therefore, we used a Fluorescence Imaging Plate Reader (FLIPR) to measure any changes in synaptic activity following acidosis treatment. pH 7.4- or pH 6- treated hippocampal neurons were plated in a 96-well plate and loaded with Fluo-4. The FLIPR was set to a 384-well configuration, so that each well from the 96-well plate would provide four individual read-outs (Figure 4.10.A). By stimulating
in the bottom left quadrant, we were able to distinguish between a direct response, and a response due to synaptic activity, observed in the other three quadrants. As the top right quadrant is furthest from the stimulus site, this quadrant was selected for 'synaptic' read-outs.

When a 50 V stimulus was applied to the cells, we found consistently higher peak fluorescence intensity in the 'direct' quadrant in pH 6- compared with pH 7.4-treated cells (F/F₀⁻¹: 0.289 ± 0.028 for pH 7.4 versus 0.536 ± 0.050 for pH 6, P < 0.0001, Mann-Whitney U test; n = 42 and 42; Figures 4.10.B and C). Furthermore, peak fluorescence intensity in the 'synaptic' quadrant was also markedly increased following acidosis (F/F₀⁻¹: 0.106 ± 0.013 for pH 7.4 versus 0.358 ± 0.057 for pH 6; P = 0.0040; n = 42 and 42, Figures 4.10.B and C).
Figure 4.10. Synaptic activity is enhanced following acidosis.
Figure 4.10. Synaptic activity is enhanced following acidosis

Dissociated hippocampal cultures were plated into 96-well plates and after 14 DIV, subjected to control (pH 7.4) or acidosis (pH 6) treatment. 16 h later, the cells were loaded with Fluo-4 and analysed using a FLIPR. A. Experimental setup. The 96-well plate analysed with a 384-well setting, so each well gave four individual read-outs. The stimulus was applied in the bottom left quadrant, where the ‘direct’ response was measured, and the ‘synaptic’ response was measured in the top right quadrant. B. Representative traces from individual wells for the different pH treatment conditions, in the absence of any drugs, with 10 µM of the NMDAR antagonist NVP, or 10 µM of the AMPAR antagonist LY303070. Three examples of direct responses (blue) and synaptic responses (red) are shown. C. Change in peak fluorescence for the different conditions of both direct (left) and synaptic (right) responses. For the direct response, two-way ANOVA indicated significant main effects for pH treatment ($F_{1, 138} = 22.57$, $P < 0.0001$) and for drug application ($F_{2, 138} = 4.37$, $P = 0.014$) but no interaction between pH treatment and drug application ($F_{2, 138} = 1.07$, $P = 0.34$). For the synaptic response, two-way ANOVA indicated a non-significant main effect for pH treatment ($F_{1, 138} = 3.57$, $P = 0.061$), but a significant main effect for drug application ($F_{2, 138} = 33.16$, $P < 0.0001$) and a significant interaction between pH treatment and drug application ($F_{2, 138} = 8.92$, $P = 0.00023$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Welch t-test).

Applying the NMDAR antagonist NVP or the AMPAR antagonist LY303070 showed that neither drug had any significant effect on the directly mediated response to the 50 V stimulus when compared with the control condition ($F/F_{0-1}$ for pH 7.4: 0.289 ± 0.028 for control; $n = 42$; 0.219 ± 0.032 for NVP; $n = 15$; $P = 0.11$ compared to control, Mann-Whitney U test; 0.333 ± 0.054 for LY303070; $n = 15$; $P = 0.98$ compared to control, Mann-Whitney U test; Figures 4.10.B and C). Both drugs however significantly decreased the synaptic response ($F/F_{0-1}$ for pH 7.4: 0.106 ± 0.013 for control; $n = 42$; 0.0145 ± 0.0013 for NVP $n = 15$, $P < 0.0001$ compared to control, Mann-Whitney U test; 0.017 ± 0.001 for LY303070; $n = 15$; $P < 0.0001$ compared to control, Mann-Whitney U test; $F/F_{0-1}$ for pH 6: 0.358 ± 0.057 for control, $n = 42$; 0.0170 ± 0.0016 for NVP $n = 15$, $P < 0.0001$ compared to control, Mann-Whitney U test; and 0.0215 ±
0.0030 for LY303070, n = 15, P < 0.0001 compared to control, Mann-Whitney U test; Figures 4.10.B and C), suggesting that both NMDARs and AMPARs are essential in generating the enhanced synaptic response.

4.3.11. Acidosis increases spontaneous activity and Ca^{2+} entry into hippocampal neurons

After we had analysed the cells in the FLIPR, we applied the same stimulus under the single-cell Ca^{2+} imager. We gave two 50 V stimuli 1 min apart from another, and compared the peak fluorescence between the control and acidosis-treated cells. Using this method, we observed no significant difference in peak fluorescence between the two samples (ΔF/F₀ for second stimulus: 0.395 ± 0.010 versus 0.415 ± 0.078; P = 0.58, Mann-Whitney U test; n = 6 and 6; Figure 4.11.A and B). However, cells treated with pH 6 displayed much higher overall activity during the time when the stimulus was not applied (Figure 4.11.A and C), suggesting there is an increased level of spontaneous synaptic activity following acidosis.
Figure 4.11. Acidosis increases spontaneous activity and Ca^{2+} entry into hippocampal neurons
A. Representative single-cell traces from calcium-flux imaging for hippocampal neurons treated with either pH 7.4 or pH 6 solutions in response to two 50 V stimuli. Individual cell traces are in cyan and the mean responses in red. B. Peak fluorescence of cells treated with pH 7.4 (black) or pH 6 (red) in response to a 50 V stimulus. Error bars indicate SEM. C. Averaged raw traces from individual wells.
4.4. Discussion
In this chapter, we investigated AMPAR plasticity following acidosis, with a focus on whether any TARPs are involved in mediating any changes in AMPAR subunit expression. Our immunocytochemistry data indicates that γ-8 levels increase following acidosis, with γ-2, γ-5 and γ-7 levels unaffected. While some of the experimental setups were either too sensitive or not sensitive enough to detect specific changes in CP-AMPAR expression levels and allow further investigation of specific TARP effects on CP-AMPAR expression, the results demonstrate that hippocampal neurons show greater synaptic activity following acidosis.

4.4.1. Western blotting to determine shifts in γ-8 and GluA2 expression levels
Since the first description of Western blotting (Towbin et al., 1979), this technique has become an extremely popular tool in determining protein expression levels. Recently however, quantification of this multi-step method has become increasingly criticised, with many factors leading to possible inaccuracies in protein measurement (Gassmann et al., 2009, Taylor et al., 2013). From sample preparation, sample loading, protein transfer, antibody selection, incubation and wash times, densitometry analysis to normalisation, Western blotting contains many stumbling points that could lead to inaccurate results.

To ensure the protein concentration used to compare between samples is within the linear range, Taylor et al. (2013) recommend performing serial dilutions of the protein and running these samples on the same gel. This should
ensure that the appropriate dilutions of samples are used for accurate and normalised quantitation of the target proteins. However, when we performed such experiments, it became apparent that this quantification method produced inconsistent results.

Figure 4.12. Densitometric analysis of Western blot bands
Protein extracted from mouse cerebellar lysates were ran in duplicate on a 10% polyacrylamide gel and stained for GluA2. Circles represent the normalised density of each band from 20, 50 or 180 µg samples, and fit with a linear function. The two different colours represent the two different samples.

We performed serial dilutions of protein samples extracted from mouse cerebellar lysates to obtain 180, 50 and 20 µg samples. We then ran these samples in duplicate on a 10% polyacrylamide gel and stained for GluA2 (data generously provided by Dorota Studniarczyk). Using the same analysis parameters as described previously, we found a difference in density between the two equally loaded samples (Figure 4.12). Especially at the higher concentrations of 180 and 50 µg protein per well, the normalised density values were 1.98 and $1.40 \times 10^8$, and 1.13 and $1.01 \times 10^8$ respectively. The
difference was less obvious at the lower concentration of 20 µg protein per well (8.05 and 7.31 x 10^7). This demonstrates that two lanes of the exact same sample produce different values, indicating a source of error beyond the sample preparation. Possible sources of error include sample loading, protein transfer or antibody binding. This data could explain the inconsistency of the results we obtained in our Western blot densitometry analysis.

4.4.2. Washout of intracellular polyamines in whole-cell electrophysiological recordings

In our electrophysiological recordings examining the rectification of pH 7.4- and pH 6-treated hippocampal neurons, the RI_{60/-60} values in the absence of intracellular polyamine were 0.605 ± 0.060 and 0.634 ± 0.052 respectively. This suggests a non-linear relationship, with the receptors partially blocked by intracellular polyamines.

Upon critical review of our methods, we realised that we may not have allowed sufficient time for the solution inside the cell to exchange with the solution inside the pipette. The exchange time constant can be calculated using the equation

\[ \tau_{\text{exchange}} = 0.6 \times \frac{C_m}{5.91} \times R_s \times (M)^{1/3} \]

(Bowie and Mayer, 1995, Pusch and Neher, 1988), where \( C_m \) is the cell capacitance (23.18 ± 0.89 pF), \( R_s \) the series resistance (11.46 ± 0.65 MΩ; \( n = 37 \)) and \( M \) the molecular weight (202 for spermine). Values for \( \tau_{\text{exchange}} \) ranged from 23.8 to 643.2 s (Mean ± SEM: 314.1 ± 23.4 s, \( n = 37 \)). Apart from time taken to adjust series resistance compensation and an initial few sweeps at -80 mV to briefly assess the quality of the patch, no additional time was allowed for solution exchange between the pipette and the inside of the cell. As a result, the actual intracellular solution
may not have been completely polyamine-free, resulting in inwardly rectifying I-V relationships. Ideally, we would repeat these experiments allowing more time for solution exchange.

4.4.3. Change in GluA2 expression levels following acidosis might be extrasynaptic

In the recordings examining the rectification of pH 7.4- and pH 6-treated hippocampal neurons, we observed no significant difference between the two groups (Figure 4.7). This contrasts the findings by Quintana et al. (2015) that show that rectification is reduced following acidosis, suggesting a change in AMPAR subunit composition. Comparing the two experimental setups, our experiments assessed mEPSCs from whole-cell recordings, whereas Quintana et al. generated their data by exposing outside-out patches to 10 mM glutamate. This means that the different data sets reflect post-synaptic and extrasynaptic currents respectively. Therefore, it is possible that the increase in GluA2-lacking, CP-AMPARs occurs extrasynaptically, while AMPARs at the postsynaptic membrane remain unaltered in terms of Ca\(^{2+}\)-permeability. Furthermore, previous studies have reported differential regulation of synaptic and extrasynaptic AMPARs in disease. For example, in AI1 amacrine cells, diabetic hyperglycaemia significantly reduces Ca\(^{2+}\) permeability of extrasynaptic, but not synaptic, AMPARs (Castilho et al., 2015).

4.4.4. \(\gamma\)-8 regulation of extrasynaptic AMPARs

Our immunocytochemistry data suggests that the down-regulation of GluA2 is accompanied by an increase in \(\gamma\)-8 levels (Figure 4.3). \(\gamma\)-8 is critical for AMPAR delivery and anchoring to the extrasynaptic membrane (Jackson and Nicoll,
2011, Rouach et al., 2005), further strengthening our hypothesis that the changes in AMPAR subunit expression levels following acidosis may only be occurring extrasynaptically. Here, increased levels of γ-8 may be involved in tethering CP-AMPARs to the membrane. In γ-8 knockout mice, extrasynaptic AMPARs are reduced by ~ 95% (Rouach et al., 2005). However, this only reduces synaptic transmission by ~ 35%, indicating that synaptic AMPARs may be partially immune to extrasynaptic loss of AMPARs. Therefore, it still remains unclear what direct effect acidosis has on synaptic transmission involving AMPARs.

One way to specifically test whether γ-8 is involved in regulating extrasynaptic AMPAR subunit expression in the hippocampus following acidosis would be to record evoked currents from outside-out patches from γ-8 knockout mice. If our predictions are correct and γ-8 is involved, the decrease in GluA2 expression levels may no longer occur, resulting in the insertion of non-rectifying, Cl-AMPARs into the membrane.

4.4.5. Assessing Ca^{2+} entry through AMPARs using single cell imaging

When we applied Mibefradil as well as Philanthotoxin-74 to assess Ca^{2+} influx following acidosis, we observed a small fluorescent response when activating with KCl (Figure 4.9), suggesting that there was still Ca^{2+} entry through channels other than CP-AMPARs. Mibefradil is a potent blocker of T-type calcium channels (IC_{50} 2.7 µM), but less effective at blocking L-type channels (IC_{50} 18.6 µM) (Mehrke et al., 1994). Therefore, it is plausible that Ca^{2+} was still entering the cell through L-type channels. To overcome this, we could use an additional antagonist of L-type calcium channels, such as Nifedipine. However,
since the levels of Ca$^{2+}$ entering a single cell through CP-AMPARs account for a small percentage of total Ca$^{2+}$ influx (around 1.2% in medial septal neurons (Schneggenburger et al., 1993)), this technique is probably not sensitive enough to use as a tool to detect any changes in CP-AMPAR expression.

4.4.6. Acidosis increases cell excitability

We show single-cell fluorescent responses to Ca$^{2+}$ influx in pH 7.4- and pH 6-treated hippocampal neurons in both Figures 4.9 and 4.11. In Figure 4.9, there is no difference between the two samples, whereas Figure 4.11 clearly shows greater Ca$^{2+}$ influx in pH 6-treated cells. While the cell types were the same, a few differences in the experimental design could account for these contrasting findings.

Firstly, the cultured cells were plated at different densities: the cells for Figure 4.9 were plated at 25,000 cells per well, whereas those for Figure 4.11 were plated at 100,000 cells per well. A higher cell density could lead to a greater number of synapses, and thus increased synaptic activity and higher levels of Ca$^{2+}$ influx.

Secondly, the external solution of the cells for Figure 4.9 contained a cocktail of channel inhibitors in order to extract the response produced solely through AMPARs. These drugs included TTX (a sodium channel blocker), NVP (an NMDA channel blocker), and Gabazine (a GABA$_A$ receptor antagonist), all of which would decrease synaptic transmission. Since we did not include these drugs in the experiments conducted for Figure 4.11, it is possible that some of the currents were mediated through sodium, NMDA or GABA$_A$ channels. While
this points to receptors other than AMPARs to be involved in the increased activity elicited through pH 6 treatment (Figure 4.11), we have shown that this response can also be blocked with the AMPAR antagonist LY303070 (Figure 4.10). Therefore, we can confirm that AMPARs are also important in mediating these differences.

Thirdly, the stimulation we applied varied between these two experiments. To generate the data for Figure 4.9, we perfused AMPA or KCl directly onto the cells, whereas for the data in Figure 4.11, we applied an electrical stimulation of 50 V. While these distinct activation methods would lead to the activation of different receptors and thus elicit different responses, the pH 6-treated cells at higher density in the absence of channel inhibitors (Figure 4.11) were more intrinsically active in the absence of a stimulus. It is therefore more plausible that the difference in fluorescent responses between the two experimental setups was due to either the higher cell density or the presence of channel blockers.

4.4.7. Future experiments

In this chapter, we used many different techniques in order to attempt to quantify changes in CP-AMPARs. The plan was to establish a method that allowed us to confirm and quantify a change in CP-AMPARs, which would subsequently allow us to explore whether any TARPs were involved in the controlling the insertion and removal of AMPARs in the membrane. Having identified γ-8 as a likely candidate (Figure 4.3), we were hoping to repeat the experiments in dissociated hippocampal cultures from γ-8 knockout mice, to see whether the change in GluA2 expression was still affected following acidosis. In
particular, it would be of interest to repeat the experiments showing enhanced synaptic activity following acidosis (Figure 4.10) in dissociated hippocampal cultures from γ-8 knockout animals.
5. General Discussion

In this thesis, we have explored how auxiliary subunits influence synaptic transmission through the regulation of AMPAR function.

In Chapter 3, we further established that the newly identified AMPAR-interacting protein FRRS1L might act as a novel AMPAR auxiliary subunit, that greatly slows recovery from receptor desensitization when recombinantly expressed in tsA201 cells. While we did not find any significant changes in AMPAR mEPSC properties in dissociated hippocampal cultures over-expressing FRRS1L, more research is required to fully understand the function of this ubiquitously expressed protein in the brain.

Reports of mutations in the FRRS1L gene describe a phenotype of severe intellectual disability (Brechet et al., 2017, Madeo et al., 2016, Shaheen et al., 2016), signifying a crucial role for this protein in brain function and development. Schwenk et al. (2014) show that in the rat brain, FRRS1L has a moderately high molecular abundance of around 38% compared to AMPAR levels during development, which increases to around 65% during adulthood. This spike could indicate a more crucial role for FRRS1L in regulating the pool of AMPARs designated for mature synapses.

The experiments we described in Chapter 4 explored the shift in expression levels of CP-AMPARs following acidosis, and whether any TARPs are involved in this process. While further experiments are required to consolidate our
findings, our immunocytochemistry data suggest \( \gamma \)-8 as a potential candidate involved in this process.

This is not the first report of \( \gamma \)-8 being implicated in a neurological disorder. For example, the consumption of naturally occurring glutamate analogs, such as kainate and domoate can lead to excitotoxic shellfish poisoning. This includes symptoms of seizures, coma and memory loss, and in severe cases can be fatal (Perl et al., 1990). This is due to excitotoxicity induced by non-desensitizing AMPARs currents. Recently, it has been shown that \( \gamma \)-8, along with \( \gamma \)-2, potentiates these responses, adding to the overall excitotoxicity (Tomita et al., 2007). The knockout of \( \gamma \)-8 in mice provides a neuroprotective effect by selectively abolishing these sustained depolarisations in the hippocampus.

Studies on \( \gamma \)-8 knockout mice have also revealed how this protein might influence synaptic activity in epilepsy and depression. The convulsant effects of kainate and the antidepressant-like effects of the AMPA receptor potentiator LY392098 are reduced or absent in these mice (Gleason et al., 2015). Indeed, using antagonists that selectively inhibit AMPARs associated with \( \gamma \)-8 significantly decreases seizures. Furthermore, unlike other AMPAR modulators used in the treatment of epilepsy, these compounds do not produce motor-related side effects such as ataxia (Kato et al., 2016, Maher et al., 2016).

If we were to consolidate our findings and demonstrate that \( \gamma \)-8 is involved in mediating CP-AMPAR plasticity following hippocampal ischaemia, these \( \gamma \)-8-selective AMPAR modulators could potentially be therapeutically beneficial.
Preventing these changes and reducing AMPAR-mediated excitotoxicity, could provide a neuroprotective effect specific to γ-8-associated AMPARs in the hippocampus.

Overall, our data further highlights the important role of auxiliary subunits in AMPAR function. Discovering and characterising these proteins is crucial for understanding the molecular mechanisms that control synaptic transmission. Despite the growing understanding of auxiliary proteins and their influence on AMPAR function, many questions still remain. Particularly challenging is the continuous emergence of novel auxiliary subunits, rendering the overall picture more complex than previously thought. Combined with other factors that influence AMPAR function, such as the expression of different subunits, splice variants and RNA editing, auxiliary proteins add another layer of complexity, resulting in a vast array of different AMPAR combinations with diverse properties. Only further experiments will explain the extensive array of receptor combinations, helping us understand the bigger picture of AMPAR function.

Finally, understanding auxiliary protein function could have implications for the future development of drugs that target specific AMPARs. As a result of the ubiquitous expression of AMPARs in the CNS, most modulators affect receptors in all areas, bringing about unwanted side effects. Tailoring drugs to target solely those AMPARs involved in the underlying pathology could hold great therapeutic potential in both neurological and psychological disorders.
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