Regulation of calcium-permeable AMPA receptors by auxiliary subunits in cerebellar neurons

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Declaration

I, Dorota Studniarczyk confirm that 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:
Abstract

AMPARs mediate the majority of fast excitatory synaptic transmission in the central nervous system. They exist as homo- or heterotetrameric assemblies of GluA1-4 subunits. Subunit composition is a crucial determinant of AMPAR biophysical and pharmacological properties. Notably, inclusion of the GluA2 subunit renders AMPARs impermeable to calcium ions as result of editing at the ‘Q/R site’. Despite the predominant expression of calcium-impermeable AMPARs (CI-AMPARs), calcium-permeable AMPARs (CP-AMPARs) play key roles in multiple physiological aspects of transmission and the influx of calcium ions through CP-AMPARs contributes to neuronal death in several neurological disorders. Given the critical roles played by CP-AMPARs in normal synaptic function and neurodegenerative processes, it is essential to understand their regulation. Neuronal AMPARs exist as complexes with accessory proteins such as transmembrane AMPAR regulatory proteins (TARPs), cornichons (CNIHs), cysteine-knot AMPA receptor-modulating proteins (CKAMPs/shisas) and GSG1L. These ancillary proteins not only govern receptor delivery to the cell membrane but also actively shape their biophysical and pharmacological properties. This thesis describes an investigation into subunit-specific AMPAR trafficking by TARPs in cerebellar granule cells (GCs). In the epileptic and ataxic stargazer mouse, a mutation in the stargazin (γ-2) gene and consequent loss of functional γ-2 protein results in a complete absence of AMPAR-mediated synaptic transmission at cerebellar mossy fibre (MF) to GC synapses. This led to the identification of γ-2 as the prototypical TARP. I used, siRNA constructs to manipulate the AMPAR subunit and TARP content in stargazer neurons to study the interplay between the type I TARP γ-2 and the less well understood type II TARP γ-7 in the synaptic targeting of CI- and CP-AMPARs. The last chapter of my thesis presents an investigation into the expression and function of AMPARs in a mouse model of juvenile Batten disease, a condition in which changes in cerebellar CP-AMPAR had been proposed by others. Overall, my results establish an important role of γ-7 in CP-AMPAR regulation in GCs and demonstrate a presynaptic rather than postsynaptic change, in the Batten disease model.
Impact Statement

AMPA-type glutamate receptors (AMPARs) mediate a majority of fast excitatory transmission between neurons in the brain. The dynamic regulation of AMPAR number and subunit composition is important for normal brain function. Any failure or defect in AMPARs can give rise to serious neurological, psychiatric and cognitive disorders. Furthermore, the calcium permeable AMPAR (CP-AMPAR) subtype underlies many forms of neurodegenerative disorder. Within the brain AMPARs are subject to regulation by auxiliary subunits such as TARPs (transmembrane AMPAR regulatory proteins TARPs). The importance of TARPs became apparent from a spontaneous mutant mouse designated the stargazer, in which a mutation of one member of the stargazin (γ-2) family of proteins abolished surface and synaptic AMPARs in cerebellar granule cells (GCs). Much of the work presented in this thesis investigates the regulatory role of two TARP proteins, γ-2 and γ-7 in the regulation of CP-AMPAR. The study has identified AMPAR subunit-specific trafficking mechanisms in cerebellar granule cells (GCs), where γ-7 supresses the expression of calcium impermeable (Cl-) AMPARs and enhances the expression of CP-AMPARs. Thus, I have established the importance of γ-7 in controlling AMPAR subunit composition, in shaping fast transmission, and potentially in influencing CP-AMPAR dependent forms of synaptic plasticity. Identifying the mechanisms and identity of auxiliary subunits that regulate CP-AMPARs in different brain regions is a basic neuroscience question of direct relevance to human health. The findings in this thesis are therefore of fundamental importance for Neuroscientists, Neurologists and Pharmaceutical Researchers interested in understanding synaptic signalling, and developing strategies for alleviating synaptic defects involved in disorders in the brain.
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Publications associated with this thesis


Chapter 1

Introduction

The main focus of this thesis is the function and regulation of AMPA-type glutamate receptors of the calcium-permeable subtype (CP-AMPARs). These receptors are an important class of ionotropic glutamate receptor with key roles in synaptic plasticity and neurological disease. The data presented comes primarily from experiments on cerebellar granule cells (GCs), a cell type with well-defined AMPAR subunit expression and limited repertoire of auxiliary AMPAR subunits. GCs are an appealing experimental model, and the discovery of the first class of AMPAR auxiliary subunits – the transmembrane AMPAR regulatory proteins (TARPs) – stemmed from work on these cells. In the initial part of this thesis (Chapters 3 and 4), I address a subunit-specific mechanism regulating cell surface AMPAR trafficking in the presence and absence of TARPs. In the later part of the thesis (Chapter 5), I examine whether mis-regulation of AMPARs is involved in juvenile Batten disease.

1.1 Synapses and synaptic transmission in the CNS

The central nervous system (CNS) consists of vast number of neuronal cells organized in interconnected networks. Efficient and precise signal transmission is crucial for proper communication between neurons and the maintenance of vital nervous system functions.

1.1.1 Historical overview

The term synapse was introduced in the 19th century by Sherrington and Foster to describe ‘the junction between nerve-cell to nerve-cell’ after it became clear that each neuron was a distinctive unit rather than, as was previously postulated, in continuity with other cells (Foster and Sherrington, 1897). The crucial evidence was provided by the Spanish neuroscientist Ramon y Cajal who visualised the complete structure of individual neurons using Golgi’s silver staining technique (Bennett, 1999). An image of a typical neuron emerged with its three main building blocks, all with unique roles in the
signal transmission. It was proposed that incoming information is gathered by a cell’s dendritic branches and transferred for processing into the cell soma. The signal then propagates along the axon towards the synaptic terminals. However, a question remained of how the assumed electrical signal was transmitted between two physically separated cells.

As the morphological studies were inconclusive, the need for a different approach became critical. In the middle of the 20th century, quickly advancing research techniques in the field of electrophysiology helped to provide substantial progress in understanding the nature of synaptic transmission.

The work of Sir Henry Hallett Dale and Otto Loewi established that the electrical signal in the peripheral nervous system is carried through the synaptic cleft indirectly, via a chemical agent – acetylcholine (ACh) (Fishman, 1972). However, an understanding of the way synaptic transmission takes place needs to be credited to Bernard Katz and his colleagues Paul Fatt, José del Castillo, Stephen Thesleff and Ricardo Miledi, founders of modern neuroscience. Equipped with an intracellular electrode recording technique, they demonstrated the presence of a specific type of activity – spontaneous on-going electrical ‘unrest’ in the form of endplate potentials (e.p.p.s) of reduced size. These tiny events, which are random, discrete and spontaneous in their nature, were termed miniature endplate potentials (miniature e.p.p.s) and ascribed to release of packets or quanta of ACh from the nerve terminal (Fatt and Katz, 1950; 1952). Further work by del Castillo and Katz (1954) examined the relationship between the e.p.p. and miniature e.p.p. and formed two important theories. First, the quantal theory, postulated that the e.p.p. is caused by synchronous release of multiple quanta of ACh (content of synaptic vesicles) from the presynaptic terminal. Second, the calcium theory, proposed that the release probability of individual quanta from the presynaptic terminal tightly depends on the extracellular calcium concentration. Nowadays, these findings form the foundation of our understanding of synaptic transmission at all chemical synapses.
1.1.2 Synaptic transmission at chemical synapses

Below, I provide a brief overview of general principles of synaptic transmission.

A simplified way to consider a synapse is as a site at which the information is transferred from one neuron to another. Morphologically, each synapse consists of: the presynaptic terminal packed with abundant vesicles containing neurotransmitter, the synaptic cleft (a narrow gap between two neurons), and the postsynaptic membrane containing pool of receptors activated by released neurotransmitter. As glia play an active role in synaptic function, they are often considered as part of the ‘tripartite synapse’ (Durkee and Araque, 2019). During synaptic transmission, an action potential invades the presynaptic terminal causing membrane depolarization, which triggers $\text{Ca}^{2+}$ influx via voltage-gated $\text{Ca}^{2+}$ channels. This rapid increase in $\text{Ca}^{2+}$ concentration initiates a cascade of cellular processes that lead to the fusion of synaptic vesicles with the presynaptic membrane. The neurotransmitter is released into the synaptic cleft where it rapidly diffuses. The typical neurotransmitter time course in CNS synapses is few hundreds of microseconds terminated by diffusion and uptake mechanisms (Barbour and Häusser, 1997; Clements, 1996; Scimemi and Beato, 2009). This brief but concentrated (1-5 mM) neurotransmitter pulse is sufficient to activate receptors situated in the postsynaptic membrane opposite the release sites. There are two broad types of neurotransmitter receptors – ionotropic receptors, also known as ligand-gated ion channels, and metabotropic receptors that depend on the action of receptor-associated G-proteins (Willard and Koochekpour, 2013). The binding of neurotransmitter – glutamate, GABA, glycine, acetylcholine and 5-hydroxytryptamine – to the relevant ligand-gated receptors causes their activation (Clements, 1996; Smart and Paoletti, 2012). Upon activation, postsynaptic currents, generated by the selective flow of ions down their concentration gradients. Excitatory postsynaptic currents (EPSCs) result in depolarizing excitatory postsynaptic potentials (EPSPs) that move the membrane voltage towards spike threshold, increasing the likelihood of an action potential. By contrast, inhibitory postsynaptic currents (IPSCs) typically result in hyperpolarizing inhibitory postsynaptic potentials (IPSPs) that move
the membrane voltage away from spike threshold. The efficacy of synaptic transmission depends strongly on the numbers and subunit composition of the postsynaptic receptors.

1.2 Glutamatergic ionotropic receptors

The neurotransmitter glutamate, upon release from presynaptic terminals, activates both ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors mediate excitatory synaptic transmission in the CNS. For these receptors, the binding of glutamate initiates channel pore opening that permits net cation influx causing membrane depolarisation and excitation of postsynaptic neuron. This important family of receptors can be subdivided into three major classes: N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate (KA) receptors – each named after their selective exogenous agonist (Collingridge et al., 2009; Dingledine et al., 1999; Ozawa et al., 1998; Traynelis et al., 2010) – and the fourth class, the delta receptors (Elegheert et al., 2016; Lomeli et al., 1993; Uemura et al., 2010). While much is known about AMPA, NMDA and KA receptors, less is understood about the ‘orphan’ delta receptors as their endogenous ligands (D-serine, cerebellin 1) were identified only recently (Matsuda et al., 2010; Yuzaki and Aricescu, 2017).

At excitatory central synapses the synaptic currents have a distinctive shape, with a typically fast rising and decaying phase followed by a much slower second component. The initial fast component is mediated by AMPARs (ms) whereas the slow component is mediated by NMDARs (hundreds ms) (Silver et al., 1992; Spruston et al., 1995). Although also present at some synapses, kainate receptors are considered to have more of a regulatory role (Smart and Paoletti, 2012). Molecular cloning techniques identified 18 iGluR subunits (see **Figure 1.1**) (Hollmann and Heinemann, 1994; Traynelis et al., 2010). Within each receptor class functional ion channels are formed as tetramers – four subunits surrounding ion channel pore. Native NMDARs are obligate
heteromers while native AMPARs exist as heteromers or homomers (with heteromeric assemblies being more common).

The iGluR subunits are synthesized in endoplasmic reticulum (ER), where they undergo sequential assembly – first dimers are formed followed by assembly of a pair of dimers (Ayalon and Stern-Bach, 2001). Generally, subunits from one class of receptors do not assemble with subunits from other (Traynelis et al., 2010). A typical iGluR subunit consists of the extracellular amino-terminal domain (ATD, or N-terminal domain, NTD), the extracellular ligand-binding domain (LBD – a clamshell structure defined by two extracellular amino acid segments S1 and S2), the transmembrane domain (TMD - three transmembrane domains and a re-entrant pore loop), and the intracellular carboxy-terminal domain (CTD) (Mayer, 2016; Wollmuth and Sobolevsky, 2004).

Figure 1.1 Ionotropic glutamate receptors. The diagram illustrates the 4 families of glutamate receptors and their corresponding subunits (each generated by a distinct gene). There are four AMPAR subunits GluA1-4; seven NMDAR GluN1, GluN2A-D, GluN3A-B; five KAR GluK1-5 and two deltaR GluD1-2.
The highly diverse family of iGluRs (Figure 1.1) is widely expressed throughout CNS, often with overlapping patterns. Although they share multiple homologies their unique pharmacological and electrophysiological properties allow their identification (Traynelis et al., 2010). The following section of the Introduction focuses on the main player of this thesis - namely the AMPAR.

1.3 What are AMPARs?

As already indicated, AMPARs are cation channels gated by glutamate that mediate fast excitatory synaptic transmission throughout the CNS (Traynelis et al., 2010). Changes in the AMPAR number and their functional properties play important roles not only in normal brain function but are also essential for the synaptic plasticity that is thought to underlie memory formation (reviewed in Cull-Candy et al., 2006; Park, 2018). Importantly, defective regulation and/or deficiency of AMPARs has been linked to multiple neurological and neurodegenerative disorders (Henley and Wilkinson, 2016; Kwak and Weiss, 2006).

1.3.1 Structure

AMPARs occur as tetrameric assemblies of combinations of GluA1, GluA2, GluA3 and GluA4 subunits. Each GluA subunit shares a conserved (among iGluRs) modular structure composed of amino terminal domain (ATD), ligand binding domain (LBD), channel pore-forming transmembrane domain (TMD) and cytosolic C-terminal domain (CTD) (Figure 1.2 A) (Greger et al., 2017; Sobolevsky et al., 2009). All AMPAR subunits occur in two alternative splice variants – flip and flop, with differences in the extracellular loop close to TM4 (Sommer et al., 1990) (Monyer et al., 1991) (Figure 1.2 B). Each splice variant confers distinct channel properties to the receptor. The flop isoforms (GluA2-4) desensitise faster and recover from desensitisation slower than do the flip isoforms (Koike et al., 2000; Mosbacher et al., 1994). The expression of flip and flop subunits varies among cell types (Geiger et al., 1995) and it is also controlled developmentally – cerebellar GCs show a developmental switch from GluA4 flip (before P9) to flop thereafter (Monyer et al., 1991; Mosbacher et al., 1994). The intracellular part of AMPAR subunits (the CTD) not only
varies in length between subunits but also exists in alternative splice variants (Figure 1.2 B).

Figure 1.2 AMPAR subunits. (A) AMPAR subunit structure. Each subunit consists of large extracellular amino terminal domain (ATD), ligand binding domain (LBD), four transmembrane domains (TM1-4) and intracellular C-terminal domain (CTD). (B) Alternative splicing and editing of AMPAR subunits. Schematic illustration of GluA flip/flop and CTD splice variants together with Q/R- and R/G-editing sites. (Modified from Dingledine et al., 1999).

The CTD splice variants are limited to GluA2 and GluA4 only and found in specific brain regions. GluA4c with its characteristic short CTD is predominantly expressed in cerebellar granule cells (Gallo et al., 1992)
whereas GluA2L, with the long CTD, in hippocampus (Kohler et al., 1994; Kolleker et al., 2003).

AMPAR subunits are also a subject to post-transcriptional editing which results in single amino acid changes. The vast majority of GluA2 mRNA undergoes adenosine-to-inosine editing at position 607 (pore forming segment TM2) where the codon for neutral glutamine (Q) is converted to one of the positively charged arginine (R) (Sommer et al., 1991) (Figure 1.2). This switch of charge at the Q/R site in the channel pore is particularly important as all AMPARs that incorporate edited GluA2(R) subunits in their complex are impermeable to calcium ions – a feature that will be discussed further in a later part of the Introduction (see 1.4). Another form of mRNA editing occurs at the R/G site, which precedes the flip/flop splice cassette. R/G editing replaces a codon for arginine with a codon for glycine and affects GluA2-4 subunits (Figure 1.2 B). Editing at the R/G site influences receptor kinetics – entry into and recovery from desensitisation: the edited (G) receptors recover faster from desensitisation and display slower desensitisation rates compare to unedited receptors (Lomeli et al., 1994). The extent of editing increases during development for all GluA2-4 subunits (Lomeli et al., 1994). The existence of different subunit splice variants combined with mRNA editing generates a heterogeneous population of AMPARs with unique biophysical and pharmacological properties.

1.3.2 Biogenesis and trafficking to synaptic locations

AMPAR subunits are synthesized in the endoplasmic reticulum (ER) where they first assemble as dimers and then subsequently form tetrameric ion channels (Greger and Esteban, 2007). The correct formation of dimers is initiated by NTD association, whilst the second step – tetramerization – involves contact points in all domains (Ayalon et al., 2005; Gan et al., 2016). Only properly folded and assembled receptors can leave the ER to be later trafficked to cell membrane (Sukumaran et al., 2012). The assembled GluA1-4 subunits form a channel pore with a diameter of approximately 0.8 nm (Burnashev et al., 1996). Although, the heterotetrameric assemblies of GluA1-
GluA4 dominate among native AMPAR channels, homotetrameric assemblies also yield functional channels (Buonarati et al., 2019; Nakagawa, 2010; Swanson et al., 1997). Of note, a recent study identified up to ten distinct native AMPAR complexes by single particle cryo-EM, including A1A2A1A2, A3A2A3A2, A1A2A3A2 and A2A2A2A2 (Zhao et al., 2019).

How do AMPARs travel to the cell surface and how are they targeted to synaptic sites? The trafficking of assembled AMPARs to the postsynaptic density (PSD) is thought to take place in the following steps. First, via exocytosis, AMPARs are delivered to extrasynaptic regions, where they operate as reserve pool of receptors. Second, depending on demand, the extrasynaptic AMPARs can be incorporated at a synapse via lateral diffusion to be finally stabilised at the PSD (Chater and Goda, 2014; Opazo and Choquet, 2011; Petrini et al., 2009). The number of synaptic AMPARs at a synapse varies from tens to hundreds (Cottrell et al., 2000; Matsuzaki et al., 2001). The process of receptor exocytosis is mediated by SNARE proteins (soluble NSF attachment protein receptors) (Luscher et al., 1999) and displays receptor subunit dependence – AMPARs with short CTDs (GluA2/3) are thought to cycle continuously in and out of the cell membrane in hippocampal pyramidal cells whereas those containing long CTDs (GluA1/2 and GluA2/4) are incorporated onto synapse in activity-dependent manner (Shi et al., 2001). Thus, the surface AMPARs remain in constant movement – moving laterally between extrasynaptic and synaptic regions together with constitutive trafficking to and from the cell surface (reviewed in Hastings and Man, 2018).

On the journey from ER to PSD, AMPARs interact with a wide range of proteins including AMPAR auxiliary subunits such as TARPs (described in more detail in 1.5) that not only regulate AMPAR trafficking but also actively shape the pharmacological and electrophysiological properties of the receptors (Greger et al., 2017; Haering et al., 2014; Jackson and Nicoll, 2011a).

1.3.3 GluA subunit expression in the cerebellum

AMPAR subunit variety is reflected not only in the heterogeneous composition of native receptors across various brain regions (Figure 1.3 A) but also in the
diversity between specific cell types or even individual synapses. In common with other brain regions, the cerebellum displays cell-specific AMPAR subunit expression (summarised in Figure 1.3 B).

**Figure 1.3 AMPAR subunit expression in distinct brain regions.** (A) Proportion of GluA1-4 subunits (see the legend) and the corresponding brain regions (adapted from Schwenk et al., 2014). (B) Cell type specific expression of GluA1-4 subunits in cerebellum (modified from Coombs and Cull-Candy, 2009).
Proteomics studies based on affinity purifications (of solubilized membrane fractions) identified GluA4 protein as the predominant AMPAR subunit of the cerebellum (Schwenk et al., 2014). The cerebellar cortex is arranged in three layers (the granule cell, Purkinje cell and molecular layers) (Eccles, 1973) and each layer is represented by a group of cells with a distinct set of AMPAR subunits. Thus, the granule cell layer is mostly comprised of granule cells expressing GluA2 and GluA4 (Bahn and Wisden, 1997; Hashimoto et al., 1999; Keinanen et al., 1990; Lambolez et al., 1992; Yamazaki et al., 2010) and a small proportion of Golgi cells containing GluA2 and GluA3 (Keinanen et al., 1990). The Purkinje cell layer shows predominant labelling for GluA1-3 subunits (Bahn and Wisden, 1997; Keinanen et al., 1990; Lambolez et al., 1992; Monyer et al., 1991; Yamazaki et al., 2010). The outer cerebellar layer – the molecular layer – contains stellate/basket cells, which are thought to express genes encoding all four AMPAR subunits (Rossi et al., 2008), although in some preparations the GluA2 subunit is either weakly stained or not detected (Keinanen et al., 1990; Petralia et al., 1997).

1.4 CP-AMPARs form a distinct group of AMPARs

1.4.1 Q/R editing dictates AMPAR permeability to Ca²⁺

In the CNS, expressed AMPARs can be functionally divided into two important groups: calcium permeable (CP-AMPARs) and calcium impermeable (CI-AMPARs). The calcium permeability is controlled by mRNA editing of the GluA2 subunit at the Q/R site in the pore-lining region of re-entrant loop TM2. The adenosine deaminase acting on RNA (ADAR2) enzyme converts a CAG codon for glutamine (Q) to a CIG codon for arginine (R) in the mRNA of GluA2 at position 607 (Melcher et al., 1996; Sommer et al., 1991). AMPAR tetramers, containing the edited GluA2(R) subunit, are calcium impermeable owning it to the positively charged arginine in the pore-lining region that prevents entry of divalent cations, including Ca²⁺ (Hollmann et al., 1991). By contrast, AMPARs containing the unedited GluA2(Q) subunit with a neutral glutamine are permeable to calcium ions. Interestingly, the overwhelming majority of GluA2 mRNA undergoes editing at the Q/R site (nearly 99%) leaving only a small
fraction of unedited GluA2 distributed across different brain regions (Seeburg et al., 2001; reviewed in Wright and Vissel, 2012).

1.4.2 Q/R editing controls receptor assembly and ER exit

AMPARs assemble within the ER to form heterotetrameric ion channels (see 1.3.2) mainly GluA2-containing. The preferential assembly mechanism of GluA2-containing receptors is strongly regulated by the Q/R editing (reviewed in Cull-Candy et al., 2006; Greger et al., 2017; Henley and Wilkinson, 2016). The presence of charged arginine at the Q/R editing site of GluA2 subunit affects subunit interaction during receptor tetramerization, hence the formation of homomeric GluA2 receptors is suggested to be highly unfavourable (Figure 1.5) (Greger et al., 2003). Studies comparing the trafficking mechanism of unedited GluA1 and edited GluA2 subunit showed that GluA1 receptors exit the ER rapidly and travel to the cell surface, while GluA2 receptors are retained in the ER. The GluA2 retention signal was lost for unedited GluA2 highlighting the importance of Q/R editing site in the assembly and trafficking process (Greger et al., 2003; Greger et al., 2002). The retained edited GluA2 provides a stable pool required for the formation of CI-AMPAR assemblies.

Figure 1.4 AMPAR assembly within ER. Schematic illustration of AMPAR assembly in the ER showing efficient tetramerization of GluA1/GluA2 and GluA1 receptors but with GluA2 retained in the ER. GluA2 subunits (red) and GluA1 (green) emerge from polyribosomes (grey). (Modified from Sukumaran et al., 2012)
1.4.3 GluA2 subunits regulate multiple properties of AMPAR channels

While edited GluA2 subunits are preferentially incorporated into many AMPAR assemblies in the CNS, a lack of GluA2(R) has profound consequences for AMPAR biophysical and pharmacological properties. Beyond calcium permeability, the absence of GluA2 endows AMPARs with a higher single-channel conductance and faster gating kinetics than their calcium impermeable counterparts (Feldmeyer et al., 1999; Geiger et al., 1995; Swanson et al., 1997) and a sensitivity to block by polyamines (Cull-Candy et al., 2006; Traynelis et al., 2010). Whilst, the expression of GluA2-containing Cl-AMPARs prevails, the GluA2-lacking CP-AMPARs play an important role not only in synaptic plasticity and learning but also in numerous neuronal disorders (reviewed in Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007; Wright and Vissel, 2012).

The susceptibility to block by polyamines allows a convenient means to identify CP-AMPARs during electrophysiological recordings. Polyamine block of CP-AMPARs occurs either from the intracellular or extracellular side (Blaschke et al., 1993; Kamboj et al., 1995; Koike et al., 1997; Twomey et al., 2018). At positive potentials the endogenous cytosolic polyamines (or those included in a patch pipette), such as spermine and spermidine are driven towards the CP-AMPAR channel pore, blocking it and producing an inwardly rectifying current-voltage (I-V) relationship (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh et al., 1995). At negative potentials, CP-AMPARs are blocked by the extracellular application of polyamine-containing toxins – e.g. Joro spider toxin or their synthetic analogues – e.g. 1-naphthyl acetyl spermine (NASPM) (Koike et al., 1997; Twomey et al., 2018; Washburn and Dingledine, 1996). The presence of edited GluA2 within the assembly gives rise to AMPARs that are insensitive to polyamine block, and exhibit a characteristic linear I-V relationship.

The GluA2 subunit presence strongly affects AMPAR channel gating and the single-channel conductance. Cells expressing predominantly CP-AMPARs display considerably faster kinetics and higher single-channel conductance.
than those expressing CI-AMPARs (Feldmeyer et al., 1999; Geiger et al., 1995; Swanson et al., 1997). For example, AMPARs expressed in Bergmann glial cells have a deactivation time (channel closure following agonist removal) of is ~1 ms and a desensitisation time (channel closure in the continued presence of agonist) of ~3 ms, while the corresponding values for CA3 pyramidal cells are ~3 and ~15 ms (Geiger et al., 1995).

1.4.4 CP-AMPAR expression is region- and cell-specific

Although the majority of AMPARs in the CNS are calcium impermeable, there are specific regions, cell types and even synapses enriched with CP-AMPARs (reviewed in Bowie, 2018). Interestingly, some neurons may express both CP- and CI-AMPARs, targeted to distinct synapses within the same neuron. In the hippocampus, individual stratum lucidum interneurons express CI-AMPARs at synapses with axon collaterals from CA3 pyramidal neurons and CP-AMPARs at synapses with mossy fibre axons (Toth and McBain, 1998).

As this thesis focuses on the regulation of the CP-AMPAR subtype in the cerebellum, I will now concentrate my description on this particular brain region. In the cerebellum, there are two types of cells predominantly expressing CP-AMPARs – Bergmann glial and stellate/basket cells (Burnashev et al., 1992; Geiger et al., 1995; Liu and Cull-Candy, 2000).

Bergmann glial cells play an important role in regulating the glutamate waveform seen by AMPARs at Purkinje cell synapses via both the physical barrier they create and by the high affinity glutamate transporters they express (Bergles et al., 1997). The CP-AMPARs present in these cells – comprised of GluA1 and GluA4 subunits – are activated by the glutamate spillover from parallel and climbing fibres and regulate both the Bergmann glial cell morphology and the expression of glutamate transporters (Iino et al., 2001; Lopez-Bayghen et al., 2003; Matsui et al., 2005; Sato et al., 1993). Thus, the expression of Bergmann glia CP-AMPARs is an essential element for normal synaptic transmission at these synapses.
Stellate and basket cells are inhibitory molecular layer interneurons that via feed-forward inhibition regulate output of Purkinje cells (Mittmann et al., 2005). CP-AMPARs found in these cells are most likely homomeric assemblies of GluA3 (Keinanen et al., 1990; Sato et al., 1993), however Gardner et al. (2005) showed the loss of GluA4 impacts the kinetics of EPSCs. In stellate cells, the expression of CP-AMPAR is activity-dependent as the high frequency stimulation of parallel fibre causes rapid switch of synaptic AMPARs from calcium permeable to impermeable (Kelly et al., 2009).

1.4.5 CP-AMPARs in disease

A finely tuned balance in the expression of CP-AMPARs and Cl-AMPARs maintains normal synaptic transmission in the CNS. CP-AMPARs fulfil important roles in synaptic plasticity (Cull-Candy et al., 2006; Henley and Wilkinson, 2016; Liu and Zukin, 2007) that is considered to underlie learning and memory formation (Bliss and Collingridge, 1993). Any dysregulation of GluA2 subunit editing or GluA2 trafficking itself has profound consequences for neuronal cells. As receptors lacking the edited GluA2 subunit are permeable to Ca\(^{2+}\), the elevated expression of CP-AMPARs might elicit unusually high levels of cytosolic Ca\(^{2+}\) and subsequent Ca\(^{2+}\)-mediated excitotoxic cell death. The excessive entry of Ca\(^{2+}\) via CP-AMPARs activates multiple processes that initiate cell apoptosis and eventually lead to neurodegeneration (reviewed in Cull-Candy et al., 2006; Liu and Zukin, 2007; Tanaka et al., 2000). In the following paragraph, I provide some examples of CP-AMPAR involvement in pathological disorders.

The entry of Ca\(^{2+}\) via CP-AMPARs is thought to contribute to the neurodegeneration observed in ischemia (Liu and Zukin, 2007) and following traumatic brain injury (Spaethling et al., 2008). CA1 pyramidal neurons are especially vulnerable to ischemic injury. Ischemia causes downregulation of the GluA2 subunit and increased numbers of surface CP-AMPARs. The involvement of CP-AMPARs in the ischemic neurodegeneration was confirmed by the reduced extent of neuronal death following application of the CP-AMPAR-selective blocker NASPM (Liu et al., 2004; Noh et al., 2005;
Quintana et al., 2015). Another study proposed that ischemic injury is triggered by defective editing of the GluA2 subunit. Specifically, a reduced efficiency of the ADAR2 enzyme was said to lead to predominant expression of GluA2(Q)-containing CP-AMPARs and subsequent neurodegeneration; the introduction of exogenous ADAR2 provided protection for vulnerable hippocampal neurons (Peng et al., 2006).

Inefficient Q/R site editing of GluA2 and the subsequent upregulation of CP-AMPARs has also been linked to motoneuron degeneration in amyotrophic lateral sclerosis (ALS) (reviewed in Cull-Candy et al., 2006; Yamashita and Kwak, 2018). ALS is a progressive neurodegenerative disorder affecting both spinal and cortical neurons. The rapid onset and motoneuron decline results in eventual respiratory problems and death within few years. Studies using RNA extracted from single motoneurons in ALS patients provided direct evidence for the defective GluA2 editing originating from downregulation of the ADAR2 enzyme (Kawahara et al., 2004). The subsequent expression of GluA2(Q)-containing receptors was suggested to initiate the excitotoxic motoneuron death (Kawahara and Kwak, 2005).

CP-APMARs have also been proposed to play a role in degeneration of synapses in Alzheimer's disease (AD) (Whitehead et al., 2017). This debilitating condition displays progressive degeneration of neuronal circuits leading to memory and cognition impairment (Spires-Jones and Hyman, 2014). The characteristic feature of the disorder is the accumulation of amyloid β–protein (Aβ) oligomers that impairs synaptic plasticity (inhibits LTP and facilitates LTD) in hippocampus, and interferes with the memory of a learned behaviour (Shankar et al., 2008). Although pathological concentrations of Aβ oligomers have been reported to induce overactivation of extrasynaptic NMDARs, leading to the abnormal elevation of intracellular Ca²⁺ and neuronal loss, the incorporation CP-AMPARs seems also to contribute to the neurodegenerative processes observed in AD (Tu et al., 2014). In hippocampal cell culture Aβ oligomers bind to GluA2-containing receptors causing their rapid internalization and surface loss (Zhao et al., 2010). Interestingly, the intracellular application of Aβ oligomers (1-5 nM) into CA1
hippocampal neurons increased the size of AMPAR-mediated EPSCs, primarily due to the insertion of GluA1-containing CP-AMPARs. The effect was absent in cells treated with IEM1460 (a CP-AMPAR blocker) or in cells transfected with shRNA against GluA1 (Whitcomb et al., 2015). These findings support the idea of Aβ oligomers incorporation of CP-AMPARs and consequent excitotoxicity in AD.

1.5 Auxiliary AMPAR subunits

A puzzling discrepancy between the biophysical and pharmacological properties of recombinant AMPARs in heterologous expression systems (such as HEK293 cells) and those of native AMPARs was noted early on (Jonas and Burnashev, 1995). The missing link was provided by the discovery of a small transmembrane protein stargazin (also known as γ-2), with a critical role in the expression of AMPARs in cerebellar GCs (Chen et al., 1999; Chen et al., 2000; Hashimoto et al., 1999). The discovery initiated a widespread search for other auxiliary proteins. To date, a large number of proteins have been identified and shown to associate with AMPARs. They include transmembrane AMPAR regulatory proteins (TARPs) (Chen et al., 2000; Tomita et al., 2003), cornichons (CNIH-2 and CNIH-3) (Schwenk et al., 2009), germ-cell-specific gene 1-like (GSG1L) (Schwenk et al., 2012; Shanks et al., 2012), cysteine-knot AMPAR modulating proteins (CKAMP39/shisa8, CKAMP44/shisa9, CKAMP52/shisa6 and CKAMP59/shisa7) (reviewed in von Engelhardt, 2019; von Engelhardt et al., 2010) and synapse differentiation-induced gene 1 (SynDIG1) (Kalashnikova et al., 2010) (Figure 1.5). The large family of auxiliary subunits contributes to remarkable AMPAR diversity in the brain (Bettler and Fakler, 2017).
1.6 Transmembrane AMPAR regulatory proteins (TARPs)

The discovery of key role of stargazin (γ-2) in the surface expression of AMPARs stemmed from experiments on the spontaneously occurring mutant mouse stargazer (Chen et al., 2000; Hashimoto et al., 1999; Letts et al., 1998) and began a new era in the field of AMPAR research.

1.6.1 Stargazin and identification of TARP family members

The stargazer mutant mouse is characterised by a distinctive head-tossing and ataxic gait implying a cerebellar defect. The mutation disrupts the Cacng2 gene encoding a small 36 kD protein γ-2 with a structure homology to the γ subunit of skeletal voltage-gated calcium channels (Letts et al., 1998). Later, it became clear that the cerebellar dysfunction in stargazer mice results from loss of AMPARs at MF-GC synapses (Hashimoto et al., 1999) revealing a critical function of γ-2 in AMPAR trafficking. Of note, simple transfection of γ-2 was able to fully rescue AMPAR-mediated transmission in GCs (Chen et al., 2000).
Subsequent studies identified an additional five members of the γ subunit family – γ-3, γ-4, γ-5, γ-7 and γ-8 (Kato et al., 2008; Kato et al., 2007; Soto et al., 2009; Tomita et al., 2003). Based on their functional differences and sequence homology, they were divided into type I TARPs comprising γ-2, γ-3, γ-4, and γ-8 and the type II TARPs comprising of γ-5 and γ-7 (Figure 1.6) (Kato et al., 2010).

### 1.6.2 Structure of TARPs and their role in AMPAR trafficking

TARPs share a conserved structure of four transmembrane domains with both N- and C-terminus facing the cytosolic site of the membrane and with two extracellular loops that are important for interaction with AMPARs (Herguedas et al., 2016; Twomey et al., 2016). The length of C-terminus varies between TARPs (Figure 1.7). Type I TARPs have longer C-tail with a characteristic PDZ-binding motif TTPV critical for targeting AMPARs to the synapse (Bats et al., 2007; Chen et al., 2000). On the other hand, type II TARPs have shorter C-tail ending with SSPC/TSPC and lack the classical PDZ-binding TTPV motif (Figure 1.7B) (Deng et al., 2006).
Introduction

Figure 1.7 Schematic illustration of TARP structure. (A) All TARPs consist of four transmembrane domains, two extracellular loops and the intracellularly orientated N and C termini. Although the size of γ subunits is similar, the length of their C terminus varies – the filled arrowhead denotes the beginning, and the open arrowheads denote the end, of each TARP C tail. (B) TARPs can be subdivided into type I and type II based on the length of their C tails. The upper box contains the amino acid sequences of type II TARP C termini, whereas the bottom box shows type I TARPs. In each sequence the amino acids crucial for interaction with PDZ proteins are highlighted in grey (modified from Deng et al., 2006).

Early work showed that transfecting γ-2 into cerebellar GCs from stargazer mice rescued AMPAR-mediated responses (Chen et al., 2000). Recovery of surface AMPARs in stargazer GCs was also seen with γ-3, γ-4, and γ-8 but not with γ-5 (Tomita et al., 2005; Tomita et al., 2003). Therefore, γ-5 and γ-7 were initially not considered to be functional TARPs (Tomita, 2004). Later, the work by Kato et al. (2008; 2007) and Soto et al. (2009) showed that γ-5 acts as a TARP but with different trafficking properties.

Type I TARPs are crucial for AMPAR trafficking. In heterologous expression systems, γ-2 enhances the surface expression of AMPARs of all subunit compositions (Chen et al., 2000; Priel et al., 2005; Tomita et al., 2005). Recombinant AMPARs are less well trafficked to the cell surface in the
absence of TARP and many are retained intracellularly (Hall et al., 1997; Vandenberghe et al., 2005b). The association of TARP and AMPARs starts within the ER where receptors assemble as tetramers (see 1.3.2) and where TARPs ensure misfolded AMPARs do not exit the ER (Tomita et al., 2003; Vandenberghe et al., 2005a). AMPARs can assemble with up to four TARPs (Kim et al., 2010; Schwenk et al., 2012; Shanks et al., 2012), although the preferred number may vary. For example, a recent cryo-EM structure study of a heteromeric GluA1/2 receptor in complex with TARP γ-8 subunits found that two γ-8 subunits associate with each receptor (Herguedas et al., 2019).

How do TARPs assist AMPARs in the ER exit? There are two possibilities: first by masking an ER retention signal of the receptor (Tomita et al., 2003; Vandenberghe et al., 2005a), second by their own ER export signals that activate transport of the AMPAR-TARP complex (Bedoukian et al., 2006; Bedoukian et al., 2008). In the cerebellum of the stargazer mouse but not in that of the heterozygous control mouse, a large fraction of GluA2 is sensitive to EndoH glycoside (which digests immature high mannose sugars only) signifying immature glycosylation of GluA2 subunits and a potential role of γ-2 in ER exit (Tomita et al., 2003). Indeed, Vandenberghe et al. (2005a) showed that absence of γ-2 results in upregulation of the ER chaperone Ig binding protein (BiP) indicative of misfolded or misassembled AMPARs. Studies of chimeric GluA1 with the C-terminal domain of γ-2 demonstrated the crucial importance of the TARP C-terminal domain in AMPAR exit from ER; the chimera trafficked as efficiently as GluA1 co-expressed with γ-2 (Bedoukian et al., 2008).

The TARP C-terminal domain is not only critical for AMPAR exit from ER and trafficking to cell surface (Bedoukian et al., 2008; Chen et al., 2000) but also for trafficking and accumulation at postsynaptic sites (Bats et al., 2007; Chen et al., 2000). Bats et al. (2007) used a truncated construct of γ-2 (γ-2ΔC) and established that PDZ domain of γ-2 acts as anchor necessary for AMPAR clustering. The interaction between γ-2 and PSD-95 enables trapping and stabilisation of otherwise laterally diffusing AMPAR at postsynaptic localisations.
1.6.3 TARPs modulate AMPAR biophysics and pharmacology

Native TARPs not only act as anchoring proteins, assisting in AMPAR ER exit and trafficking to cell surface but additionally also act as positive allosteric modulators of AMPAR channel function (outlined in Figure 1.8) (reviewed in Greger et al., 2017; Jackson and Nicoll, 2011a). In this section, I will summarize some of the modulatory features.

Studies from heterologous expression systems established that co-expression of TARPs with AMPARs strongly affects receptor gating kinetics and the single-channel conductance. TARP γ-2 and other type I TARPs slow deactivation and desensitisation of glutamate evoked responses (Bedoukian et al., 2006; Körber et al., 2007; Priel et al., 2005; Tomita et al., 2005; Turetsky, 2005). The extent of modulation strongly depends on the type of TARP, with γ-8 and γ-4 having more pronounced effects than γ-2 and γ-3 (Cho et al., 2007; Milstein et al., 2007). Type II TARPs, γ-5 and γ-7, do not affect the desensitisation and deactivation rates as type I TARPs do. γ-7 prolongs deactivation and desensitisation of GluA1 responses in HEK 293 cells but to a lesser degree than seen with γ-2, whereas γ-5 has no effect (Kato et al., 2007). Neither γ-5 nor γ-7 affect the deactivation and desensitisation rates of GluA4 currents (Soto et al., 2009).

AMPAR single-channel conductance is increased with co-expression of either type I or type II TARPs. Using non-stationary fluctuation analysis (NSFA) of macroscopic currents the weighted mean single-channel conductance of recombinant GluA4 receptors is enhanced by all TARP isoforms (Soto et al., 2007; Soto et al., 2009). TARPed AMPAR channels display long-lived high-conductance bursts in the tail of the macroscopic responses (Soto et al., 2009; Tomita et al., 2005). TARP γ-2 has been reported not to affect any of four of receptor conductance levels, but to increase the frequency of openings to the largest conductance state (Tomita et al., 2005). However, examination of single-channel recordings of homomeric GluA1 alone and in combination with γ-2, γ-4 or γ-5 revealed a doubling of each of the three conductance levels for each of the TARPs (Shelley et al., 2012).
In addition to affecting the receptors’ biophysical properties, TARPs modulate certain aspects of AMPAR pharmacology such as glutamate affinity, and kainate and CNQX efficacy (reviewed in Jackson and Nicoll, 2011a). The prototypical TARP γ-2 increases the potency of glutamate, visible as a shift in the dose-response curve to the left (Priel et al., 2005; Tomita et al., 2005; Turetsky, 2005; Yamazaki et al., 2004). Other type I TARPs share this feature, but in a GluA subunit-dependent manner. The extent of TARP-mediated potentiation depends on AMPAR subunit composition, splice variant, and associated TARP isoform (Kott et al., 2007). Unlike type I TARPs, type II TARPs do not increase the potency of glutamate (Kato et al., 2008; Kato et al., 2007).

Kainate is a partial AMPAR agonist that in the presence of type I TARPs evokes high amplitude non-desensitising currents (Tomita et al., 2005; Turetsky, 2005). The fact that TARP association changes this partial agonist into a full agonist has provided a useful research tool (Jackson and Nicoll, 2011b; Shi et al., 2010). The examination of the ratio of kainate and glutamate current amplitudes allows not only the identification of TARP presence in surface receptors but also allows estimation of AMPA/TARP stoichiometry (Shi et al., 2009). Similarly, to glutamate affinity, the kainate/glutamate ratio depends on the precise AMPAR and TARP subunit composition (Kott et al., 2009). Again, type II TARPs display a different behaviour since the kainate/glutamate ratio is not affected by γ-5 and only slightly increased by γ-7 (Kato et al., 2007; Tomita et al., 2005; Turetsky, 2005).
Figure 1.8 TAPR association modulates biophysical and pharmacological properties of AMPARs. Schematic summary of TARP modulatory actions on AMPAR properties. Traces in black represent GluA receptors expressed alone and those in red represent traces from TARP-associated receptors. As indicated in the text, the extent of modulation depends on the type TARP in the complex. (Modified from Jackson and Nicoll, 2011a).

Another interesting feature of γ-2 and other type I TARPs is that they convert the commonly used AMPAR antagonist – CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) – into a partial agonist (Kott et al., 2009; Menuz et al., 2007). The
CNQX-induced currents can be recorded from both recombinant and native AMPAR/TARP assemblies, and blocked by application of a potent non-competitive antagonist GYKI 53655 (Kott et al., 2009; Menuz et al., 2007). However, CNQX does not induce AMPAR mediated responses from GluA1, GluA3 and GluA4 receptors when they are co-transfected with type II TARP γ-7 (Bats et al., 2012).

TARPs modulate another important feature of AMPARs, namely they alter the polyamine block of CP-AMPARs (Jackson et al., 2011; Soto et al., 2007; Twomey et al., 2018). The intracellular polyamines such as spermine produce voltage-dependent block of TARPless CP-AMPARs generating inwardly rectifying I-Vs (for more details see 1.4.3). The co-expression of γ-2 markedly decreases the extent of inhibition of AMPAR mediated currents by polyamines – assessed as changes in rectification index (Soto et al., 2007). The attenuation of spermine block of CP-AMPARs is shared by all TARPs, however it differs between TARP subunits (Soto et al., 2009). TARPless AMPARs are also a subject to polyamine block by the extracellular application of polyamine toxins such as philanthotoxin 433 (PhTx-433) (1.4.3). The size of PhTx-433 block was markedly increased for TARPed CP-AMPARs compared to TARPless (Jackson et al., 2011). Of note the recent study by Twomey et al. (2018) reported three times higher IEM-1460 potency of current inhibition for GluA2(Q) alone than for GluA2(Q)-STZ. The situation differed for higher affinity blocker – AgTx-636, that produced increased current inhibition for GluA2(Q)-STZ.
2.1 Animals

Experiments were performed using wild-type C57BL/6 mice, mutant *stargazer* (*stg/stg*) mice on a C57BL/6 background and transgenic *Cln3* knockout mice (*Cln3^{Δex1-6}* ) also on a C57BL/6 background.

The mutation in *stargazer* mice arose spontaneously at the Jackson Laboratory on the A/J inbred mouse line (Noebels et al., 1990) and it was later linked to the *Cacng2* gene. The *Cacng2* mutation results in the loss of 38kD protein named stargazin and is associated with an insertion of an early transposon (ETn) retrotransposon into intron 2 ([Figure 2.1 A](#)) (Letts et al., 1998). *Stargazer* mice were bred by crossing heterozygous +/+stg males with heterozygous +/+stg females. To enable identification of mice, tail samples were collected and genotyping performed. The following set of primers was used:

ETn-OR 5' -GCCTTGATCAGGACTGTC-3'
109F 5' -TATTTCTGTCATCTCTTTG-3'
JS167 5' -GAGCAAGAGGTTCAGGC-3'
E/Ht7 5' -ACTGTCACCTCATCTCGGAC-3'

Forward 109F and reverse ETn-OR primers amplified 3' long term repeats LTR and generated a band around 300 bp whereas forward JS167 combined with reverse E/Ht7 amplified 5'LTR with a band of ~500 bp ([Figure 2.1 B](#)). The bands identify the *stg/stg* allele. For wild-type mice the intron was amplified using a forward primer 109F and reverse E/Ht7 with a band of approximately 600 bp being detected.
**Materials and Methods**

**Figure 2.1 Genotyping of stargazer mice.** (A) Schematic illustration of an ETn insertion into intron 2 and location of primers used for genotyping. (B) Image of a gel with electrophoresis of stg/stg (left hand side) and wild-type (right hand side) animals with corresponding bands highlighted.

The *Cln3*Δex1–6 mice were generated via targeted disruption of the *Cln3* gene involving the deletion of exons 2-6 and most of exon 1 via replacement with a neomycin resistance gene that was transcribed in the reverse orientation from a mouse PGK promoter (Mitchison et al., 1999). *Cln3*Δex1–6 mice were maintained in a colony of homozygotes and for experiments compared with age-matched wild-type animals.

In all experiments both male and female mice were used. All procedures for the care and treatment of mice were in accordance with the Animals (Scientific Procedures) Act 1986.
2.2 Cerebellar GCs in culture

2.2.1 Preparation of GC cultures

Cultures of dissociated cerebellar neurons were prepared from postnatal day (P)5–7 mice. The protocol of Levi et al. (1989) identified as producing a ‘98 % pure cerebellar granule cell culture’ was employed with some modifications. After decapitation of mice, their cerebella were rapidly removed and placed in ice-cold Krebs Ringer bicarbonate (KRB) solution (see Table 2.1) and then cleaned of meninges and blood vessels. The dissected tissue was cut into small pieces with sterile scalpel blade, transferred to Solution A (KRB supplemented with 3 mg/ml bovine serum albumin BSA and 1.2 mM MgSO₄) and centrifuged for 1 minute at 1000 rpm, room temperature (RT). The pellet was trypsinized at 37 °C for 15 minutes in Solution 1 (Solution A containing 0.25 mg/ml trypsin) with gentle agitation. To stop the trypsin digestion, Solution 2 (Solution A containing 12.8 µg/ml of DNase I, 83 µg/ml of soybean trypsin inhibitor and 2.7 mM MgSO₄) was added and the mixture centrifuged (1 min, 1000 rpm, RT). The formed supernatant was aspirated and Solution 3 (Solution A containing 80 µg/ml of DNase I, 0.52 mg/ml of trypsin inhibitor and 2.7 mM MgSO₄) added. The pellet of digested tissue was triturated (using fire polished glass pipettes with decreasing tip diameter) and left to stand for 15 min. The upper part of solution was collected and centrifuged (5 min, 1000 rpm, RT).

The resulting pellet was then carefully resuspended in Basal Medium Eagle (BME) supplemented with 10 % fetal bovine serum (FCS; vol/vol), 2 mM L-glutamine and 100 mg/ml gentamicin (all Gibco). Cells were plated onto poly-L-lysine-coated (Sigma) glass coverslips, at a density of 2.1 x 10⁵ cells per coverslip and maintained at 37 °C in 5 % CO₂ in a humidified incubator. Cells were kept in ‘high K⁺’ (25 mM KCl) to enhance cell survival and mimic the activity dependent granule cells maturation by mossy fibre (MF) inputs (Gallo et al., 1987). Cytosine arabinoside (10 µM; Sigma) was added 24 h after plating to inhibit glial proliferation, and recordings were made after 7–13 days (Figure 2.2).
Figure 2.2 Mouse cerebellar granule cells (GCs) in culture. Image of cells at 9 Days In Vitro (DIV).

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<tr>
<th>KRB solution</th>
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<td>Glucose</td>
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<td>NaHCO$_3$</td>
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<td>Phenol red</td>
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Table 2.1 Composition of Krebs Ringer bicarbonate solution.
2.2.2 Transfection

Cerebellar granule cells were transfected between 3-10 DIV using the calcium phosphate method (Hannan et al., 2013). Briefly, cells were moved to pre-warmed transfection medium (BME supplemented with 2 mM L-glutamine and 5 mM kynurenic acid) and incubated with calcium phosphate/DNA precipitates (2 µg cDNA per coverslip incubated for 30 min at 37 °C and 5 % CO₂). The DNA precipitates adhere to the cell membrane and are absorbed by endocytosis. After incubation, the transfection solution was gradually removed and replaced with warm serum-containing medium. The coverslips were transferred back to the original growth solution. After 48 hours, the first recordings were performed.

2.2.3 DNA and iRNA constructs

TARP γ-2 DNA (rat origin) was a gift from Roger Nicoll (University of California San Francisco). TARP γ-7 (human) was purchased from OriGene Technologies Inc. TARP γ-7 displays 100% sequence identity in human, mouse and rat (Chu et al., 2001). Cell cultures transfected with γ-2 or γ-7 had 50 µM NBQX (6-nitro-2,3-dioxo-1,4-dihydrobenzo[f]quinoxaline-7-sulfonamide) added to growth medium to prevent excitotoxic cell death. A Yellow Fluorescence Protein-γ-5 construct (YFP-γ-5) was created by Dr Ian Coombs, UCL. Full-length γ-5 was inserted into eYFP-tube vector (gift from Alastair Hoise, UCL). The YPF segment was linked to γ-5 N termini via 7-amino acid linker: SGLRLRG.

For knockdown experiments presented in this thesis, small interfering RNA against GluA2 (siGluA2) subunit and small hairpin RNA against γ-7 (shγ-7) were used. The siGluA2 construct was based on the protocol Passafaro et al. (2003) and generated by Dr Ian Coombs. Briefly, siRNA corresponding to nucleotides 400–418 of mouse GluA2 was incorporated into the U6 siStrike vector from Ambion according to the manufacturer’s instructions. The following primers from Sigma Genosys were used:
Materials and Methods

Fwd: ACCGAGCACTCTTAGCTTGATCTTCTGTAATCAAGCTAAGGAGTGCTCTTTTTC;
Rvs: TGCAGAAAAAGAGCACTCTTAGCTTGATTGACAGGAAGATCAAGCTAAGGAGTG.

The vector contained the Green Fluorescent Protein (GFP) as a reporter gene. The shRNA against γ-7 was commercially available and purchased from GeneCopeia (MSH037278-mU6). It consisted from 4 separate shRNA expression constructs with the target sequences for mouse γ-7 (accession code NM_133189.3), 54 (5′-CTGCGGCCTGCTCCTTGTG-3′), 176 (5′-GGAGAGTCTGCTTTTGC-3′), 374 (5′-CTCAGAGGACCATTCTTG-3′) and 500 (5′-CTGAGCAGTACTTTCACTA-3′). Cerebellar granule cells were transfected with a mixture of all four, at 2 μg per coverslip. The shRNA construct encompassed the mCherry Fluorescent Protein (mCherryFP) as a reporter gene. Typically, cells were transfected with 2 μg of the cDNA of interest. For experiments involving double knockdown the following ratio was used 2 μg of siGluA2 to 1 μg of shγ-7 mix. Recordings were made from non-transfected and transfected (red or green fluorescent) cells after 2 to 4 days.

2.3 Electrophysiology of cerebellar GCs in culture

2.3.1 Cell visualisation and recording

The adapted cerebellar primary cell culture protocol allowed a highly pure culture of granule cells (GCs) to be obtained (98 %) (Aloisi et al., 1985; Levi, 1989). Cerebellar GCs (Figure 2.2) were visually identified according to previously described criteria, including the presence of a round cell body with large nucleus and lack of large neurites (Cull-Candy et al., 1988) when viewed under the upright microscope used on the recording set-up (Zeiss Axioskop FS1 or Olympus BX51WI).

Cells grown on glass coverslips were transferred from culturing medium to recording chamber filled with extracellular solution by means of fine tip forceps. Cells were continuously perfused by a gravity driven system with the flow of 1.5–2 ml/min (2 ml bath volume). The extracellular solution contained 145 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM 4-(2-
hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.3 with NaOH) (Table 2.3). Pipettes used for cell recordings were pulled with a two-step vertical puller (Narishige) from thick-walled borosilicate glass (1.5 mm o.d., 0.86 mm i.d., Harvard Apparatus). To reduce capacitance of the recording electrodes, a layer of Sylgard resin (Dow Corning 184) was applied to each pipette and hardened using a heating coil. The tips of the glass electrodes were fire-polished (MF-83 microforge; Narishige) to a final resistance of ~5–8 MΩ for whole-cell and ~8-10 MΩ for outside-out patch-clamp recordings. Pipettes were filled with a solution containing 145 mM CsCl, 2.5 mM NaCl, 1 mM cesium- ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Cs-EGTA), 4 mM adenosine 5′-triphosphate magnesium salt (MgATP) and 10 mM HEPES (pH 7.3 with CsOH) (Table 2.4). Spermine tetrahydrochloride (500 μM) was added to this intracellular solution immediately prior to each recording session. Currents were recorded at room temperature (22–26 °C) using an Axopatch 1D or Axopatch 200B amplifier and acquired using pClamp10 software and a Digidata 1200 interface (Molecular Devices). Series resistance and input capacitance were read directly from the amplifier settings used to minimize the current responses to 5 mV hyperpolarizing voltage steps.

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<tr>
<th>Compound</th>
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</tr>
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<td>MgCl₂</td>
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<td>VWR</td>
</tr>
<tr>
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<td>Sigma</td>
</tr>
<tr>
<td>HEPES</td>
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Table 2.2 External solution composition
Table 2.3 Internal solution composition

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<th>Concentration [mM]</th>
<th>Source</th>
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<tr>
<td>HEPES</td>
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<td>Sigma</td>
</tr>
</tbody>
</table>

2.3.2 Whole-cell current-voltage recordings

I used granule cells that were well isolated from others. A freshly pulled and polished pipette was mounted onto microelectrode holder (ISO-P-1.5G; G23 Instruments) and kept under continuous positive pressure to reduce tip contamination. This avoided small debris present in the recording chamber from attaching to the pipette tip and preventing tight gigaseal (GΩ) formation with the cell membrane. The patch-pipette was lowered into the solution until it reached cell level. The pipette offset was then set to zero. The positive pressure was maintained until a ‘dimple’ formed in the cell membrane. Subsequently, the positive pressure was released and negative pressure applied until a GΩ seal in ‘cell-attached’ configuration was achieved (Figure 2.3). Once the holding potential was set to –60 mV and pipette capacitance corrected, the cell membrane was ruptured by a rapid suction pulse and whole-cell configuration formed (Figure 2.3). As granule cells have a compact morphology (Cathala et al., 2003; Silver et al., 1996), the whole-cell configuration allowed accurate control of cell membrane voltage.

To examine current-voltage relationship the membrane potential was gradually ramped from –90 to +60 mV with the voltage maintained at –90 mV for 200 ms and corresponding current recorded. A series of ramps were delivered until currents had reached steady-state amplitude. Agonist-evoked currents were
Materials and Methods

recorded in the presence of 20 μM (S)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ((S)-AMPA) and 10 μM cyclothiazide (CTZ). Background currents were recorded in the absence of agonist. The background current included any endogenous voltage-gated channels were later subtracted from traces recorded in the presence of the agonist. All records were filtered at 2 kHz and sampled at 5 kHz.

![Diagram of pipette and cell configurations in patch-clamp recordings.](image)

**Figure 2.3 Pipette and cell configurations in patch-clamp recordings.** From top left, gigaseal formation in the cell-attached configuration. Right, gentle application of negative pressure removes small fragment of membrane and exposes cell inside. Patch-pipette solution rapidly replaces the cell internal medium and whole-cell configuration is achieved. Bottom right, gradually pulling the cell membrane attached to pipette tip forces separation of a small membrane patch. Bottom left, in the outside-out configuration a membrane patch containing a small number of channels is isolated.

The kainate/glutamate ratio experiments were recorded at holding potential of −60 mV. The currents were evoked by the application of 500 μM kainate or 500 μM glutamate, both in the presence of 100 μM cyclothiazide. The amplitudes of the agonist-evoked currents were measured and the ratio calculated for each cell.
2.3.3 Miniature EPSCs in cultured GCs

Miniature excitatory postsynaptic currents (mEPSCs) were recorded in whole-cell configuration at –60 mV. To block voltage-gated sodium channels, NMDA-, GABA<sub>A</sub>- and glycine receptors the following compounds were included in the external solution: 1 µM tetrodotoxin (TTX), 20 µM D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), 20 µM 2-(3-carboxypropyl)-3-amino-6-(4 methoxyphenyl) pyridazinium bromide (SR-95531) and 1 µM strychnine (Ascent Scientific). Prior to mEPSC recording, the cerebellar granule cells were briefly exposed (~2–3 min) to 200 µM LaCl<sub>3</sub> to enhance mEPSC frequency (Chung et al., 2008). The signal was filtered at 2 kHz and sampled at 20 kHz.

2.3.4 Fast agonist application to excised somatic patches

Outside-out patches were pulled from the soma of granule cells using thick-walled electrodes with resistance of 8-10 MΩ (Figure 2.3). Fast agonist application was achieved using a double-barrelled application tool made from theta glass (2 mm outer diameter; Hilgenberg GmbH) pulled to a tip opening of ~200 µm and mounted on a piezoelectric translator (Physik Instrumente) (Figure 2.4). The piezoelectric module was controlled by a precisely timed voltage step (pClamp; Axon). Adjusting the duration and magnitude of the voltage step allowed for a fast and reproducible solution exchange. To enable visualization of the solution interface and measurement of solution exchange (as changes in liquid junction potential), 2.5 mg/ml of sucrose was added to the agonist solution while the control solution was diluted by 5 %. The solution exchange rate for 10–90 % rise time was between 150 and 250 µs. Both, control and agonist solution were filtered with 0.2 µm cellulose syringe filters (Sartorius) and flowed continuously under gravity. The agonist solution contained 1 mM (S)-AMPA (Ascent). Recorded currents were filtered at 10 kHz and digitized at 50 kHz.
2.4 Electrophysiological data analysis – cerebellar GCs in culture

No analysis of electrophysiological data was blinded.

2.4.1 Whole-cell current-voltage ramps

To isolate the whole-cell AMPAR-mediated currents, the average background traces were subtracted from the agonist activated currents. Next, the steady-state parts of the curves (200 ms at –90 mV) were fitted with a linear function (y = a*x + b; a=0) and the average current amplitudes determined. The amplitude values were used for the current-voltage (I-V) plot normalisation. The rectification index (RIslope) was calculated as the ratio of slope conductance in positive (+20 to +40 mV) and negative (–40 to –20 mV) limbs of the I-V plot.

2.4.2 Miniature EPSC analysis

Event detection was performed using amplitude threshold crossing (Igor Pro 5, Wavemetrics Inc; NeuroMatic 2.02, www.neuromatic.thinkrandom.com), with the threshold set to 3× the baseline current variance (typically ~5 pA). The rectification index (RI_{+60/–60mV}) was calculated by dividing the mean mEPSC peak conductance calculated using all events detected at +60 mV and a matching number of the largest events at –60 mV. In some cases, to estimate the relative proportion of CP-AMPARs across synapses, the rectification index was also calculated as the ratio of summed mEPSC peak conductances from records of equal duration at +60 mV and –60 mV. This method takes into
account the full range of possible rectification at individual synapses, including those where currents might not be detected at +60 mV because of complete block by spermine (rectification index of 0) (Bats et al., 2012). When analysing charge transfer, any event with a distinct peak was included.

For mEPSC amplitude analysis, all events with a monotonic rise were included irrespective of overlapping decays. In the case of fluctuation analysis (see below) and kinetic analysis, only events that exhibited a monotonic rise and an uncontaminated decay were included. Such events were aligned on their rising phase prior to averaging. The decay of the averaged mEPSC was fitted with a double exponential, and the weighted time constant of decay \((\tau_{w, \text{decay}})\) calculated as the sum of the fast and slow time constants weighted by their fractional amplitudes. In some cases, mEPSCs were adequately fit with single exponentials.

Peak-scaled non-stationary fluctuation analysis (ps-NSFA) was used to estimate the weighted mean single-channel conductance of synaptic receptors (Hartveit and Veruki, 2007; Traynelis et al., 1993). Each mEPSC was divided into 30 bins of equal amplitude and, within each bin, the variance of the mEPSC about the scaled average was computed. The variance was plotted against the mean current value, and the weighted mean single-channel current was estimated by fitting the full parabolic relationship with the equation:

\[
\sigma_{\text{ps}}^2 = i\bar{I} - \bar{I}^2 / N_P + \sigma_B^2
\]

where \(\sigma_{\text{ps}}^2\) is the peak-scaled variance, \(\bar{I}\) is the mean current, \(i\) is the weighted mean single-channel current, \(N_P\) is the number of channels open at the peak of the EPSC, and \(\sigma_B^2\) is the background variance. The weighted mean chord conductance for each cell was calculated assuming a reversal of 0 mV.

2.4.3 Outside-out patches and single-channel recording

To estimate channel properties from macroscopic responses to 1 mM (S)-AMPA (100 ms duration, 0.83 Hz), stability analysis was performed in NeuroMatic to exclude any possible rundown of the response. Non-stationary
Materials and Methods

fluctuation analysis (NSFA) depends on the same number of channels being available at each agonist application. Once a stable selection of sweeps was identified, the ensemble variance of all successive pairs of current responses was calculated.

The single-channel current \( i \) and the total number of channels \( N \) present in the patch membrane were determined by plotting variance \( \sigma^2 \) against mean current \( \bar{I} \) and fitting with the equation

\[
\sigma^2 = i\bar{I} - \bar{I}^2 / N + \sigma^2_0
\]

where \( \sigma^2_0 \) is the background variance. The weighted-mean single-channel conductance was estimated from the single-channel current and the holding potential. No correction for liquid junction potential was made.

Some of the recordings displayed large single-channel openings in the tail of macroscopic patch currents. These records were first digitally filtered at 4 kHz and clear channel events (lasting longer than ~3 ms) were selected by eye. For each event, an all-point amplitude histogram was generated and fit with two Gaussians to determine the amplitude of the directly measured single-channel current.

2.5 Preparation of acute cerebellar slices

Cerebellar slices were prepared from both male and female mice aged P10–15. In accordance with UK Scientific Procedures Act 1986, mice were first deeply anaesthetized with isoflurane, then tested for their hind limb withdraw reflex and once deep anaesthesia confirmed, they were decapitated with sharp scissors. The brain dissection was performed on a block of ice submerged in ice-cold slicing solution (Table 2.4). Briefly, a cut was made along the middle of the scalp and skin was pulled away using small curved forceps. Once the skull was exposed, further cuts were made, one in the middle and other two to the sides of the head and the bone removed. The brain was transferred into a dish filled with ice-cold oxygenated slicing solution (bubbled with 95 % O\(_2\) and
5 % CO$_2$) using a small curved spatula. Meninges and blood vessels were removed and cerebellum isolated. Operating a pair of fine tip Dumont #5 forceps.

To obtain parasagittal cerebellar slices, the cerebellum was cut with scalpel along the midline and each hemisphere glued to the slicing platform of a vibratome (Campden 7000 smz). The platform was submerged in the ice-cold slicing solution in the slicing chamber. The cerebellum was sliced into 250 µm thick parasagittal sections. Slices were then transferred to incubation dish containing pre-warmed (35 °C) and oxygenated slicing solution supplemented with 40 µM D-AP5. After a 30 min incubation period, slicing solution was gradually replaced with oxygenated recording solution (23–26 °C) (Table 2.4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration [mM]</th>
<th>Source</th>
<th>Compound</th>
<th>Concentration [mM]</th>
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<tr>
<td>Sucrose</td>
<td>64</td>
<td>Fisher</td>
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</tr>
</tbody>
</table>

Table 2.4 Composition of slicing and recording solutions. Both solutions were continuously bubbled with 95 % O$_2$ and 5 % CO$_2$ to maintain pH at 7.4.

2.6 Slice electrophysiology

Slices were carefully placed in the recording chamber filled with oxygenated recording solution, continuously bubbled with 95 % O$_2$ and 5 % CO$_2$ to maintain pH at 7.4. A platinum ‘harp’ was placed over the slice, to prevent
tissue movements and subsequent cell loss during recording. Slices were viewed using a fixed stage upright microscope (Olympus BX 51WI) combined with Luigs Neumann photomultiplier tube and Hamamatsu CCD camera. Recordings were made from visually identified neurons in the internal granule cell layer (Kaneda et al., 1995).

To block NMDA and GABA receptors, 20 µM D-APV and 20 µM SR-95531 were added. The internal solution contained 128 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM Mg$_2$ATP, 0.5 mM CaCl$_2$, 2mM NaCl, 5 mM TEA, 1 mM N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314), and 0.1 mM spermine tetrahydrochloride (pH 7.3 with CsOH). The calculated free Ca$^{2+}$ of the internal solution is 5.3 nM (Bers et al., 2010). Currents were recorded using an Axopatch 200B amplifier, filtered at 2kHz and digitized at 20 kHz (pClamp 10.2 Molecular Devices). All currents were recorded at room temperature, with the exception of minimally-evoked EPSCs (below). Series resistance and input capacitance were read directly from the amplifier settings used to minimize the current responses to 5 mV hyperpolarizing voltage steps.

### 2.6.1 Quantal and evoked postsynaptic currents

To record quantal EPSCs (qEPSCs), the standard extracellular solution was replaced with a Ca$^{2+}$-free solution containing 5 mM SrCl$_2$ (Abdul-Ghani et al., 1996; Goda and Stevens, 1994). Mossy fibres (MFs) were stimulated (0.5 Hz) using a concentric bipolar tungsten electrode placed in the white matter tract (Digitimer DS/2A constant voltage stimulator). The cells were voltage clamped at −60 mV.

To record evoked EPSCs (eEPSCs), the standard extracellular recording solution containing 2 mM Ca$^{2+}$/1 mM Mg$^{2+}$ was used. The parameters used for MF stimulation were the same as for qEPSCs.

### 2.6.2 Minimally evoked postsynaptic currents

Minimally-evoked EPSCs (meEPSCs) were recorded at elevated temperature (30-34 °C). Mossy fibres were stimulated using constant voltage pulses
(80-100 μs; 20-48 V) delivered through a glass electrode filled with extracellular solution and positioned ~100-200 μm from the recorded granule cell. The criteria for minimal stimulation included an initial ~30% failure rate during repeated single stimuli at 0.25 Hz and invariant EPSC latency and amplitude with increased stimulus intensity. The mean voltage of the threshold stimulus was 32.7 V for wild-type cells and 34.2 V for Cln3Δex1–6 cells. For each cell, trains of 5 stimuli (100 Hz, ~2 V above threshold) were delivered at 3-second intervals and meEPSCs recorded at −70 mV in both ‘high’ and ‘low’ extracellular Ca\(^{2+}\) (2 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) and 1 mM Ca\(^{2+}\)/2 mM Mg\(^{2+}\)). In each case the amplitudes of evoked currents were normalized to the mean amplitude of the first response (meEPSC1) in 2 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\).

### 2.7 Electrophysiological data analysis – acute slices

Quantal events were detected using amplitude threshold crossing (Igor Pro 5, Wavemetrics Inc; NeuroMatic 2.02, www.neuromatic.thinkrandom.com), with the threshold (typically ~ 5 pA) set according to the baseline current variance. To avoid the inclusion of multiquantalt events, only qEPSCs occurring >10 ms after the mossy fibre stimulus were included. For event frequency analysis, any qEPSC with a distinct peak was included. For qEPSC amplitude analysis, all events with a monotonic rise were included, irrespective of overlapping decays. For kinetic analysis, only events with a monotonic rise and uncontaminated decay were included; they were aligned on their rising phase before averaging. The decay of the averaged qEPSC was fitted with a double exponential, and the weighted time constant of decay (\(t_w\), decay) calculated.

The meEPSC trains (5 stimuli) were averaged and the mean peak amplitude of individual evoked event determined. For each cell the amplitudes of evoked currents were normalized to the mean amplitude of the first response (meEPSC1) recorded in ‘high’ extracellular Ca\(^{2+}\). Cells where series resistance change was >33% were excluded from analysis.
2.8 Western blotting

Cerebellar tissue was homogenized in RIPA lysis buffer with proteinase inhibitors (Roche). Protein extracts were boiled for 5 min at 95 °C before loading onto 5-10 % gradient gels (50 μg of protein sample per lane). Gels were electrotransferred to 0.2 μm nitrocellulose membrane (Amersham). Blots were blocked in 4 % milk (wt/vol) in PBS-Tween 20 solution for 1 h, then incubated at 4 °C overnight with one of the primary antibodies listed in the Table 2.5.

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Table 2.5 Details of primary antibodies.

Transferred proteins were detected with appropriate horseradish peroxide-conjugated (HRP) secondary antibodies (Table 2.6) and reacted with chemiluminescent ECL substrate (Thermo Scientific Pierce), and visualized with a ChemiDoc MP System (Bio-Rad). In Chapter 5 band intensities of GluA2 and GluA4 were normalized to the respective cofilin bands or to the total protein determined by Ponceau S staining of the membranes (Image Lab 5.2, Bio-Rad Laboratories).
Table 2.6 Details of secondary antibodies.

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<td>Santa Cruz</td>
<td>sc-2438</td>
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2.9 Co-Immunoprecipitation

Cerebella of adult mice were homogenized in buffer I (0.32 M sucrose, 3 mM HEPES-Na and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7.4) and centrifuged at 16,000 g for 10 min at 4 °C. The pellets were solubilized with buffer II (50 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM PMSF and Complete Protease Inhibitor Cocktail (Roche)) for 1 h at 4 °C. After centrifugation (38,000 rpm; 40 min) the supernatant was collected, and the protein concentration determined using a Bradford assay. For each immunoprecipitation reaction 2 mg of protein was used. The lysate was incubated overnight at 4 °C with 2 µg of γ-7–specific antibody (Table 2.5). The next day, Protein G Sepharose (Sigma) was added and incubated for 1 h. The Protein G pellet was washed five times in buffer II. Adherent proteins were eluted with Laemmli sample buffer (Sigma) at 95 °C for 5 min. The proteins were transferred onto nitrocellulose and blotted with 2 µg of anti-GluA2, anti-GluA4 and anti γ-2 (Table 2.5).

2.10 Transmission electron microscopy

Cerebellar sagittal slices (200 µm) were prepared from P13 C57BL/6 mice (n = 3) and age-matched Cln3<sup>Δex1-6</sup> mice (n = 3), as described previously in 2.5. Thin slices were immediately transferred into freshly prepared 4 % paraformaldehyde and 0.5 % glutaraldehyde solution, and left overnight at 4 °C. Following primary fixation, the tissue was washed and osmicated for 1 h at 4 °C in 1 % OsO₄ in 0.1 M phosphate buffer, enblocked, stained in 2.0 %
uranyl acetate buffer for 30 min at 4 °C, dehydrated in ethanol, cleared in propylene oxide, and embedded in araldite. Cerebellar sections of 70–80 nm thickness were made. These were collected on copper mesh grids, counterstained with lead citrate, and examined using a JEOL 1010 electron microscope.

The identification of mossy fibre (MF) axons was based on previously published structural characteristics, namely a large number of mitochondria, the presence of multiple small vesicles and being encircled by granule cell dendrites (Xu-Friedman and Regehr, 2003). Release sites were identified by the presence of a presynaptic cluster of vesicles close to the membrane, active zone material and a postsynaptic density. The genotypes of electron micrographs were blinded and then analysed using ImageJ software (v1.46; https://imagej.nih.gov/ij/). To assess the density of vesicles in each MF terminal, a grid composed of multiple squares was overlaid on the image. Each square had an area of 0.1 µm². The number of vesicles (~ 30 nm radius) within each square was counted. Squares containing organelles, or those containing the border of the MF terminal were excluded from analysis. To determine vesicle diameter ‘plot profile’ (ImageJ) was used. It generated two peaks that correlated to the edge of analysed vesicle, thus the distance between the peaks corresponded to vesicle diameter. Active zones were identified visually by distinctive darkening of the postsynaptic site. The freehand line (ImageJ) was used to mark each active zone and measure the length. The straight line (ImageJ) was used to examine the shortest distance between vesicle and active zone. Vesicles were considered to be proximal to the release site if they were less than 100 nm from the presynaptic membrane of an active zone. The active zone vesicle density was then calculated as the number of vesicles per 50 nm of active zone length. Membrane adjacent vesicles were defined as those within 1 vesicle radius of active zone.

2.11 Statistical analysis

Summary data are presented in the thesis as the mean ± SEM from n cells or patches or mossy fibre terminals (Chapter 5). The data are displayed
graphically as box plots, showing the median (bar), mean (cross), interquartile range (box) and 10–90% range (whiskers) with superimposed data from individual cells or patches (open circles).

In Chapters 3 and 4, comparisons involving two data sets only were performed with a two-sided Welch two-sample t test. Although presented as selected pairwise comparisons in the figures, all analyses involving data from three or more groups were performed using one-way analysis of variance (Welch heteroscedastic $F$ test) followed by pairwise comparisons using two-sided Welch two-sample $t$ tests (with Holm’s sequential Bonferroni correction for multiple comparisons). Hierarchical cluster analysis was performed using the DIvisive ANAlysis Clustering algorithm (‘diana’) in the R package ‘cluster’, and grouping was identified using ‘cutree’.

In Chapter 5, comparisons involving two datasets only were performed using a Wilcoxon rank sum test. For the comparison of paired-pulse ratios at different frequencies and analysis of short-term plasticity, I used two- and three-way repeated measures ANOVA. For EM data, nested analysis was performed using a likelihood ratio test comparing two linear mixed-effect models (Bates et al., 2015).

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 R (the R Foundation for Statistical Computing; http://www.r-project.org/) and R Studio (RStudio). No statistical test was used to pre-determine sample sizes; these were based on standards of the field.
Chapter 3

Compartment-specific expression of CP-AMPARs in GCs

3.1 Introduction

The subunit composition of AMPARs tightly dictates their biophysical and pharmacological properties. The GluA2 subunit plays a particularly important role and its inclusion in AMPAR complexes renders them impermeable to calcium ions. Many neurons and glia predominantly express GluA2-containing AMPA receptors. Although CI-AMPARs prevail, their calcium permeable counterparts are also widespread in the central nervous system (CNS) and play an important role in multiple physiological and pathological processes. With the discovery of stargazin and other transmembrane AMPAR regulatory proteins (TARPs) and their strong modulatory effects on AMPARs, it is important to investigate the mechanisms involved in the specific regulation of not only CI-AMPAR but also CP-AMPAR subtypes. The experiments presented in this chapter, were designed to investigate the properties of AMPARs in cerebellar granule cells (GCs) from wild type and stargazer mice in which the level of GluA2 was modified by overexpression or knockdown techniques.

In the CNS, neuronal cells express a wide range of AMPAR subunits and auxiliary proteins, generating numerous and diverse assemblies of GluA-auxiliary subunits. In order to examine the regulation of CP-AMPARs by stargazin (TARP γ-2), we have selected a relatively ‘simple’ neuronal cell type - cerebellar granule cells (GCs). Their limited repertoire of GluAs and auxiliary subunits offers an attractive and well-defined research model (Bahn and Wisden, 1997; Coombs and Cull-Candy, 2009). The initial discovery of the prototypical TARP γ-2 and the subsequent studies were also completed in these cells. Importantly, the spontaneous mutant mouse stargazer, which is characterized by disrupted γ-2 expression, and lack of AMPAR mediated transmission at MF-GC synapses, permits the acute manipulation of TARP expression (Tomita et al., 2005). Thus, we took advantage of the relatively low
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complexity of AMPAR and auxiliary subunit expression in cerebellar GCs and the availability of the *stargazer* mouse, and combined both to investigate TARP specific regulation of CP-AMPARs.

The experiments described in this chapter were designed to achieve three main tasks. First, to alter the expression of AMPARs, from calcium impermeable to calcium permeable. Second, to examine importance of stargazin in the trafficking of CP-AMPARs in wild-type and *stargazer* GCs when GluA2 is absent. Third, to characterize the physiological and pharmacological properties of the AMPARs in these conditions.

3.2 Results

3.2.1 Expression of CP-AMPARs in ΔGluA2 GCs

Cerebellar GCs express predominantly Cl-AMPARs (Kamboj et al., 1995) and require TARPs for their successful synaptic expression (Chen et al., 2000). In order to investigate if similar regulatory mechanisms apply to CP-AMPARs we designed a small interfering RNA to disrupt production of endogenous GluA2 subunit in these cells (see Materials and Methods). To verify if AMPAR responses in granule cells transfected with siRNA against GluA2 were indeed mediated by CP-AMPARs, we compared properties of AMPAR currents in untreated (‘WT’) versus transfected (‘WT ΔGluA2’) cells.

First, I recorded whole-cell currents evoked by bath-applied AMPA (20 μM) during voltage-ramps (–90 to +60 mV) with spermine (500 μM) included in pipette (intracellular) solution. The control group – untreated cells, generated linear *I-V* plots, typical of GluA2-containing AMPARs, with a rectification index (*RI*) of 1.00 ± 0.09 (*n* = 17) (*Figure 3.1A*). In contrast, the transfected cell group produced inwardly rectifying *I-V* plots, reflecting the surface expression of CP-AMPARs sensitive to a voltage-dependent block by intracellular spermine (*Figure 3.1 A*). The rectification index was significantly reduced compared with control cells (0.18 ± 0.06, *n* = 6; *P* < 0.0001) (*Figure 3.1 B*). To rule out the possibility of the transfection procedure influencing the shape of *I-V* relationship, cells transfected with GFP alone were also examined. These cells
displayed \( I-V \) relationships that were indistinguishable from control cells (RI of \( 0.81 \pm 0.10, n = 10; P = 1.00 \)) (Figure 3.1 B) indicating that the RI reduction seen in WT \( \Delta \text{GluA2} \) cells was due to siRNA successfully disrupting the production of GluA2.

Figure 3.1 Expression of CP-AMPARs in cerebellar GCs following GluA2 knock down. (A) A black and red trace represents respectively global average of current-voltage relationships recorded from control \( (n = 17) \) and \( \Delta \text{GluA2} \) cells \( (n = 6) \) in response to AMPA. Grey and pink shaded areas denote SEM. (B) Pooled data showing rectification index values for control, \( \Delta \text{GluA2} \), and GFP transfected cells \( (n = 10) \). *** \( P < 0.0001 \). (C) The application of 100 \( \mu \text{M} \) NASPM changes current-voltage relationship recorded from \( \Delta \text{GluA2} \) cells \( (n = 6) \). The plot shows the average current-voltage relationships before and after NASPM. (D) Pooled data showing percentage block by NASPM at negative \(-60 \text{ mV}\) and positive \(+60 \text{ mV}\) voltages in wild-type \( (n = 6) \) and \( \Delta \text{GluA2} \) cells \( (n = 6) \). *** \( P < 0.0001 \). In panels B and D, box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles represent individual values. (Modified from Studniarczyk et al., 2013).
The I-V curves obtained from WT ΔGluA2 granule cells displayed attenuated spermine block at positive potentials which might perhaps be interpreted as an indication of incomplete GluA2 knockdown resulting in a residual population of Cl-AMPARs. However, a reduced polyamine block is a well know feature specific to CP-AMPARs co-assembled with γ-2 (Soto et al., 2007). To determine that most of the AMPARs were indeed calcium permeable I used 1-naphthyl acetyl spermine (NASPM), a synthetic analogue of Joro Spider toxin known for its preferential voltage- and use-dependent block of CP-AMPARs (Koike et al., 1997; Washburn and Dingledine, 1996). Bath-applied 100 µM NASPM almost completely eliminated AMPA-evoked currents at –60 mV in ΔGluA2 cells (Figure 3.1 C), confirming that a majority of AMPARs were GluA2-lacking after siRNA transfection. The NASPM inhibiting effect was significantly greater in WT ΔGluA2 cells compared with WT control cells (n = 6 in each condition; P = 0.00017). Of note, a similar inhibition level was observed at a lower concentration of NASPM (3 µM) with 18.0% ± 5.6% (mean ± SEM) block in control cells (n = 5) compared with 51.2% ± 4.7% block after knockdown (n = 4; P = 0.016).

3.2.2 GluA2 knockdown increases single-channel conductance

The nature of the I-V plots in the presence of intracellular polyamines indicated that ΔGluA2 cerebellar GCs express predominantly CP-AMPARs and that these are co-assembled with TARPs. We next asked: Do CP-AMPARs in ΔGluA2 GCs display other properties indicative of TARP association?

One of the canonical properties of CP-AMPARs is that they display a considerably higher single-channel conductance than that of their Cl counterparts. Furthermore, their association with γ-2 typically enhances the conductance by a further 40% (Coombs and Cull-Candy, 2009; Soto et al., 2007; Tomita et al., 2005). To estimate the AMPAR conductance values in WT control and WT ΔGluA2 cells, we recorded responses evoked by rapid application of 1 mM AMPA onto outside-out membrane patches (Figure 3.2 A, B) and performed non-stationary fluctuation analysis (NSFA, see Materials and Methods). As anticipated, knockdown of the GluA2 subunit led to distinct
increase in the single-channel conductance from 9.2 ± 0.8 pS to 34.0 ± 3.1 pS (n = 7 and 4, respectively; \( P = 0.0060 \)) (Figure 3.2 B, C) characteristic of TARPed CP-AMPARs. Also, in patches from WT ΔGluA2 cells where the noise level was low, I was able to identify large bursts of single-channel openings in the tail of macroscopic currents (Figure 3.2 A). These long-lived bursts of large conductance openings are typical of CP-AMPARs co-assembled with TARPs (Coombs and Cull-Candy, 2009; Soto et al., 2007; Tomita et al., 2005). All-point amplitude histograms of these events gave a mean single-channel conductance of 43.0 ± 1.1 pS (n = 11) (Figure 3.2 D). The ~25% larger conductance estimate from resolved channel openings (when compared with NSFA) likely reflects a potential bias toward the selection of the largest and longest lived events, together with a possible small contribution of CI-AMPARs to the weighted-mean conductance from fluctuation analysis (Bats et al., 2012). The increased single-channel conductance in WT ΔGluA2 cells was consistent with the marked increase in the whole-cell current that we observed (at –90 mV) from 148 ± 37 pA to 773 ± 210 pA (n = 17 and 6, respectively). Of note, determining whether the brief flickers visible in the WT control recordings (Figure 3.2 A) are channel events would require further examination. The presented data comes from experiments designed to examine macroscopic currents, any more detailed analysis would require better resolution and improved signal to noise ratio. Of note, the nature of the observed events is uncertain as some are clearly seen prior to AMPA application (see Figure 3.2 A, c1).
Figure 3.2 CP-AMPARs in outside-out patches from ΔGluA2 cerebellar GCs. (A) Representative traces of macroscopic current responses to rapid application (100 ms) of 1 mM AMPA recorded at –60 mV. Three individual responses from each of three different patches (c1, c2 and c3) are shown for wild-type and ΔGluA2 cells (traces filtered at 2 kHz, for display). In the tail of the macroscopic currents from ΔGluA2 patches, long-lived directly resolved channel events were present (examples highlighted in red); in wild-type patches channel openings were short-lived and less apparent. (B) Representative current-variance relationships show estimated weighted mean single-channel conductance. Symbols denote mean variance and error bars SEM. The dashed lines indicate the baseline variance. (C) Pooled data showing a dramatic increase in single-channel conductance following GluA2 knockdown (n = 7 and 4 patches from wild-type and ΔGluA2 cells, respectively). ** P < 0.01. (D) Pooled data showing single-channel conductance extracted from directly resolved channel events. Directly resolved channel openings were present in 11 of 12 patches from ΔGluA2 cells but in 0 of 18 patches from wild-type cells. In panels C and D, box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. (Modified from Studniarczyk et al., 2013).
3.2.3 Synaptic CP-AMPARs in ΔGluA2 GCs

Having identified the presence of TARPed CP-AMPARs in extrasynaptic patches from WT ΔGluA2 cells, we next, asked if the synaptic AMPAR pool is also affected in the WT ΔGluA2 cells. If so, are these synaptic CP-AMPARs also associated with the prototypical TARP γ-2?

To address this, I examined miniature excitatory postsynaptic currents (mEPSCs) at negative (–60 mM) and positive (+60 mV) membrane potentials, in the presence of TTX, a specific blocker of voltage-gated sodium channels. In control cells, the average mEPSC amplitude was similar at both potentials and gave rectification index of 0.91 ± 0.08 (n = 9) (Figure 3.3 A). In WT ΔGluA2 cells mEPSCs displayed a nearly 2-fold increase in mean amplitude from 22.1 ± 3.4 pA to 46.4 ± 7.9 pA (–60 mV, P = 0.0056) (Figure 3.3 B) resulting in a pronounced inward rectification (RI 0.35 ± 0.06, n = 12) (Figure 3.3 C) signifying the presence of CP-AMPARs in the postsynaptic membrane.

To estimate the relative proportion of CP-AMPARs across synapses, I also calculated the RI as the ratio of summed mEPSC peak conductances from records of equal duration at +60 mV and –60 mV (Bats et al., 2012). This method considers the full range of possible behaviours at individual synapses, including those at which currents might not be detected at +60 mV because of complete block by spermine (RI of 0). This approach also showed a marked increase in rectification in ΔGluA2 cells (the RI shifted from 0.88 ± 0.11 to 0.19 ± 0.05). The similar outcomes from the two methods of analysis suggested that in granule cells, unlike in cerebellar stellate cells (Bats et al., 2012), synapses were relatively homogeneous in their AMPAR content, and after GluA2 knockdown there were no synapses where a substantial proportion of the current was carried by CI-AMPARs.
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Figure 3.3 The GluA2 knockdown alters mEPSC rectification and single-channel conductance. (A) Representative mEPSCs recorded at +60 and −60 mV from a WT and a ΔGluA2 cell. (B) Their corresponding mean events. (C) Pooled data showing a decrease in rectification index value after GluA2 knockdown (n = 9 and 12 WT and ΔGluA2 cells, respectively; *** P < 0.001). (D) Representative current-variance relationships (psNSFA) show estimated weighted mean single-channel conductance. Symbols denote mean variance and the dashed lines indicate the baseline variance. (E) Pooled data showing the increased single-channel conductance in ΔGluA2 compared to wild-type cells (n = 17 and 13 cells, respectively; ** P < 0.01). (F) Scatter plot illustrating the shift in single-channel conductance and rectification index following GluA2 knockdown. Open symbols denote individual cells and closed the corresponding mean values (error bars SEM). In panels C and E, box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. (Modified from Studniarczyk et al., 2013).

To decide whether the increase of mean mEPSCs amplitude (at −60mV) could be accounted for by the increase in postsynaptic single-channel conductance, we applied peak-scaled NSFA (psNSFA) to miniature currents (Figure 3.3 D) (Cathala et al., 2005; Traynelis and Jaramillo, 1998). Postsynaptic AMPARs in WT control cells gave relatively low single-channel conductance measurements of 13.9 ± 1.2 pS (n = 13) compared with 22.2 ± 2.2 pS (n = 17) in WT ΔGluA2 cells (P = 0.014) (Figure 3.3 E), a conductance value indicative of the expression of CP-AMPARs at postsynaptic sites. Also, the weighted
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decay ($\tau_{\text{w, decay}}$) time was reduced from 1.32 ± 0.10 ms (wild-type) to 0.99 ± 0.09 ms (WT ΔGluA2 cells) ($n = 13$ and 17, respectively; $P = 0.0052$). The difference in channel properties between CI- and CP-AMPARs is also evident from the scatter plot of rectification index against conductance (Figure 3.3 F) where low single-channel conductance values together with corresponding high RIs denote CI-AMPARs and high conductances with low RIs represent CP-AMPARs. The relatively large single-channel conductance values estimated for synaptic receptors in GluA2-lacking granule cells suggest that, similarly to the extrasynaptic CP-AMPARs, the synaptic CP-AMPARs were also associated with a TARP.

3.2.4 CP-AMPARs do not require γ-2 for surface expression in GCs

A number of studies have previously provided evidence for the critical importance of stargazin in the expression of CI-AMPARs in GCs. Here we asked if the same principle applies to CP-AMPARs. Can CP-AMPARs be expressed in cerebellar GCs if their canonical TARP – stargazin is absent? To address this, we made use of stargazer (stg/stg) mice, where the naturally occurring deficiency of γ-2 causes a selective loss of surface AMPARs in cerebellar GCs (Chen et al., 2000; Hashimoto et al., 1999). As anticipated, the bath application of 20 µM AMPA produced no detectable current ($n = 8$). However, the same application of AMPA in stg/stg GCs transfected with GluA2 siRNA generated clear AMPA responses (322.9 ± 40.4 pA at −90 mV, $n = 5$) (Figure 3.4 A). These AMPA-evoked currents displayed marked inward rectification, with a mean RI of 0.20 ± 0.04 ($n = 5$) (Figure 3.4 B) consistent with the expression of CP-AMPARs. This clearly demonstrated that stargazin was not required for the surface expression of CP-AMPARs in stg/stg GCs.

The selective loss of AMPARs from GCs in stargazer mice was generally taken to reflect the role of γ-2 in these cells, whereas many other neurons express additional TARP isoforms (γ-3, γ-4, or γ-8) that impart a measure of redundancy (Menuz et al., 2008). Although, this might seem to suggest that
Figure 3.4 Functional CP-AMPARs expressed in stg/stg cerebellar GCs following GluA2 knockdown. (A) Representative whole-cell responses (stg/stg cell in black and stg/stg ΔGluA2 in red) to 20 μM AMPA bath application during voltage ramp. Grey trace represents the voltage protocol used. (B) Inwardly rectifying mean current-voltage relationship (global) recorded from stg/stg ΔGluA2 cells (n = 5). Shaded area SEM. (C) Representative traces of macroscopic current responses to rapid application (100 ms) of 1 mM AMPA recorded from outside-out patches pulled from stg/stg ΔGluA2 cell. Directly resolved channel events were present in the tail (examples highlighted). (D) Representative current-variance relationships (NSFA) show estimated weighted mean single-channel conductance. Symbols denote mean variance and error bars SEM. The dashed lines indicate the baseline variance. (E) Pooled data showing single-channel conductance estimates from NSFA and directly resolved channel openings in patches from stg/stg ΔGluA2 cells (n = 7 and 9, respectively). ** P < 0.01. Box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. (Modified from Studniarczyk et al., 2013).
CP-AMPARs in \textit{stg/stg} ΔGluA2 GCs lack an associated TARP, it is now clear that the type II TARP γ-7 is also expressed in GCs (Fukaya et al., 2005; Kato et al., 2007; Yamazaki et al., 2010). Thus, to determine whether extrasynaptic CP-AMPARs in \textit{stg/stg} ΔGluA2 GCs were associated with γ-7, I recorded macroscopic currents from outside-out somatic membrane patches and determined the channel conductance, as described previously for WT GCs (see Figure 3.2).

Despite the lack of γ-2, AMPAR-mediated currents were reliably recorded in patches from \textit{stg/stg} ΔGluA2 GCs with the mean peak amplitude of $-17.9 \pm 5.6$ pA (at $-60$ mV, $n = 8$ patches) (Figure 3.4 C) and with a weighted mean single-channel conductance $22.3 \pm 2.4$ pS ($n = 7$) (Figure 3.4 D, E) suggesting that extrasynaptic CP-AMPARs are TARPed.

As seen in WT ΔGluA2 GCs, it was also possible to directly resolve large single-channel openings in the tail of the macroscopic currents; these gave a mean single-channel conductance of $39.7 \pm 2.4$ pS ($n = 9$) (Figure 3.4 C, E). The directly measured single-channel conductance was larger than that estimated from NSFA (as it was for WT ΔGluA2 GCs), likely reflecting the presence of a small population of lower conductance channels, possibly TARPless, that were too brief or small to be directly resolved. Importantly however, the conductance of the directly resolved channels was not significantly different from that obtained in WT ΔGluA2 cells (Figure 3.2 D) ($43.0 \pm 1.1$ pS, $P = 0.23$), supporting the view that a significant proportion of extrasynaptic AMPARs were associated with a TARP – in this case with γ-7 rather than γ-2. Additionally, the whole-cell I-V plots were similar in WT- and \textit{stg/stg} ΔGluA2 cells, as expected if the extrasynaptic CP-AMPARs were TARPed in both conditions (compare Figure 3.4 B with Figure 3.1 A). This situation resembles that previously described in \textit{stg/stg} cerebellar stellate cells (Bats et al., 2012), where extrasynaptic CP-AMPAR channels also exhibit properties indicative of co-assembly with γ-7.
3.2.5 Synaptic CP-AMPARs are TARPlless in stg/stg ΔGluA2 GCs

Stargazin is considered to be essential for AMPAR synaptic localisation but not for their surface delivery (Jackson and Nicoll, 2011b). Here, we tested whether CP-AMPARs can be trafficked to the postsynaptic membrane in stg/stg CG cells. Interestingly, the GluA2 knockdown rescued spontaneous miniature AMPAR-mediated excitatory transmission despite stargazin absence (Figure 3.5 A). The fact that the miniature events were readily detected at −60 mV but not at +60 mV suggests that not only were they mediated by CP-AMPARs but also that they had an unusually high sensitivity to block by intracellular polyamines, characteristic for TARPlless receptors (Soto et al., 2007). In line with this idea, the weighted mean single-channel conductance of the synaptic receptors, from psNSFA, was only 13.5 ± 0.9 pS (n = 7) (Figure 3.5 B, C), significantly less than that of synaptic CP-AMPARs in WT ΔGluA2 granule cells (P = 0.0093). Therefore, unlike CI-AMPARs, CP-AMPARs do not require γ-2 in order to form functional synaptic receptors in granule cells, and appear capable of doing so in the absence of a TARP.

Figure 3.5. Recovered mEPSCs in stg/stg cerebellar GCs are mediated by CP-AMPARs. (A) Representative mEPSCs recorded at positive and negative voltages from a stg/stg cerebellar GC following GluA2 knockdown and the corresponding average event (right-hand panel). At the positive potential (+60 mV) no miniature synaptic events were detected. (B) Representative current-variance relationships (psNSFA) show estimated weighted mean single-channel conductance. Symbols denote mean variance and the dashed line indicates the baseline variance. (C) Pooled data showing reduced single-channel conductance in stg/stg ΔGluA2 cells (n = 9) compared to wild-type ΔGluA2 cells (grey plot from Figure 3.3; ** P < 0.01). Box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. (Modified from Studniarczyk et al., 2013).
3.2.6 GluA2(Q) but not GluA2(R) rescues transmission in stg/stg GCs

The ability of GluA2 subunit to control calcium permeability depends strictly on RNA editing at position 607 where a codon encoding neutral glutamine (Q) is replaced by positively charged arginine (R) (Hume et al., 1991; Sommer et al., 1991). The inclusion of edited GluA2(R) subunits gives rise to receptors that are impermeable to calcium ions and hence insensitive to block by intracellular polyamines (Cull-Candy et al., 2006; Isaac et al., 2007). As GluA2 knockdown experiments suggested that stargazin is not a critical trafficking element for CP-AMPAR, I next tested this by examining the effect of a single GluA2 mutation at position 607 on synaptic AMPAR transmission in the absence of stargazin. Therefore stg/stg GCs, containing the endogenous levels of GluA2 (Chen et al., 1999; Hashimoto et al., 1999; Tomita et al., 2003), were transfected with either unedited GluA2(Q) or edited GluA2(R).

Cells expressing GluA2(Q) displayed clear mEPSCs at negative potentials (–60 mV, n = 5) (Figure 3.6 A) with no detectable events at positive potential (+60 mV, n = 3), indicating mEPSCs were rescued and exhibited strong polyamine block. By contrast overexpression of GluA2(R) failed to rescue synaptic transmission (n = 7) (Figure 3.6 A) (the mean frequency 0.04 ± 0.01 Hz). In stg/stg GluA2(Q) cells, the mean mEPSC amplitude was 11.9 ± 2.2 pA (n = 5) and the weighted mean single-channel conductance estimated from psNSFA was 21.2 ± 4.8 pS (n = 5) (Figure 3.6 B-D). The absence of mEPSCs at positive potentials together with elevated mean single-channel conductance suggests synaptic CP-AMPARs were most likely TARPless.
Figure 3.6 AMPAR-mediated mEPSCs present in cerebellar GCs following GluA2(Q) overexpression but not GluA2(R). (A) Representative mEPSCs recorded at −60 mV from a stg/stg cerebellar GC after GluA2(Q) (left-hand panel) and GluA(R) (right-hand panel) transfection. (B) Individual mEPSCs from the cell in panel A, aligned at their point of steepest rise. Middle, Color-coded image of all 243 events. Lower, Averaged mEPSC (black trace) with superimposed SEM (grey fill). (C) Representative current-variance relationships (psNSFA) show estimated weighted mean single-channel conductance. Symbols denote mean variance and the dashed line indicates the baseline variance. (D) Pooled data showing mean single-channel conductance (from psNSFA) in stg/stg GluA2(Q) cells (n = 5). Box-and-whisker plot indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values.

3.3 Discussion

CP-AMPARs play a central role in multiple physiological and pathological processes in the brain and spinal cord. Despite their importance, the regulation and trafficking of this group of AMPARs has previously received little attention. Stargazin (γ-2), the prototypical TARP, is widely recognised for its fundamental function in AMPAR trafficking. In the stargazer mouse, the lack of functional γ-2 manifests as a characteristic loss of surface AMPARs in cerebellar GCs. Here we showed that by switching the expressed AMPARs from predominantly calcium-impermeable to calcium-permeable rescued synaptic transmission in
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The recovery could be achieved not only by GluA2 subunit knockdown but also through overexpression of the unedited form of GluA2. The results presented in this chapter established that CP-AMPARs (unlike Cl-AMPARs) could be successfully trafficked and expressed at the cell surface without functional γ-2. Importantly, experiments in stg/stg ΔGluA2 cells revealed that while a relatively high proportion of expressed CP-AMPARs were γ-7 associated, TARPless receptors mediated synaptic transmission under these conditions. These findings resemble the situation in stg/stg stellate cells where TARPed CP-AMPARs are inserted in extrasynaptic locations and TARPless ones are present at synaptic sites (Bats et al., 2012). This compartmentalisation observed in stg/stg ΔGluA2 cells suggests that the atypical TARP γ-7, which is still present in stg/stg cells, may play a specific role in the CP-AMPAR trafficking.

3.3.1 ΔGluA2 GCs express CP-AMPARs

CP-AMPARs contribute to normal transmission at many central synapses (Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007) and are vital in many forms of synaptic plasticity (Bellone and Luscher, 2006; Gardner et al., 2005; Hanley, 2013; Liu and Cull-Candy, 2000; Plant et al., 2006; Wolf and Tseng, 2012). The voltage-dependent block by endogenous polyamines (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh et al., 1995), high single-channel conductance (Soto et al., 2009; Swanson et al., 1997), extracellular block by toxins and polyamine derivatives (Blaschke et al., 1993; Koike et al., 1997; Washburn and Dingledine, 1996) are all the characteristic features of GluA2-lacking AMPARs. The GluA2 knockdown in cerebellar GCs provided us with a relatively simple model system for studying mechanisms governing CP-AMPAR modulation and trafficking – specifically the TARP-dependent regulation of CP-AMPARs. The experiments on cerebellar GCs showed that by using siRNA against GluA2 subunit it is possible to successfully alter the predominance of expressed AMPARs from Cl- to CP-AMPARs. The responses recorded in ΔGluA2 GCs displayed a nearly 5-fold increase in AMPA-evoked current amplitude, elevated single-channel conductance at
extrasynaptic and synaptic sites, and voltage-dependent block by intracellular spermine and extracellular NASPM – confirming the presence of CP-AMPARs.

3.3.2 TARP γ-2 is not required for CP-AMPAR expression

Cerebellar GCs express high levels of γ-2, a tetraspanning transmembrane protein that plays a central role in AMPARs expression and function. Stargazin accompanies AMPARs on virtually every step of their ‘life cycle’ starting from biogenesis, cell trafficking to the cell surface and synaptic clustering (Bats et al., 2007; Chen et al., 2000; Shanks et al., 2010; Tomita et al., 2003; Vandenberghe et al., 2005b). The lack of functional γ-2 in the mutant mouse stargazer disrupts AMPARs delivery and causes complete loss of AMPAR-mediated transmission at MF-GC synapses even though cerebellar GCs maintain normal expression levels of GluA2 and GluA4 subunits (Chen et al., 1999; Hashimoto et al., 1999; Tomita et al., 2003). Considering the pivotal role γ-2 plays in cerebellar GCs, it was unexpected that increasing the prevalence of expressed CP-AMPARs rescued surface expression and synaptic AMPAR-mediated transmission.

The fact that loss of stargazin has such a marked effect on cerebellar GCs AMPARs was previously believed to reflect a very limited repertoire of TARPs expressed in cerebellar GCs whereas other neuronal types express stargazin homologues γ-3, γ-4 or γ-8 sufficient to preserve synaptic AMPARs (Menuz et al., 2008). Hippocampal pyramidal cells express multiple members of the TARP family (Fukaya et al., 2005; Tomita et al., 2003) including γ-8 as their primary AMPAR regulator (Rouach et al., 2005). In these cells γ-8 knock down leads to a marked decrease in AMPAR expression although ~ 65% of AMPAR-mediated synaptic transmission is maintained, presumably by the other type I TARPs (γ-2, γ-3 and γ-4) present (Rouach et al., 2005). This compensating mechanism is thought to operate in the majority of neuronal cells in the stargazer mouse, leaving cerebellar GCs, with their limited expression of type I TARPs, the only cell type to show a complete loss of postsynaptic AMPARs.
Our experiments on stg/stg ΔGluA2 cells provide an alternative explanation for the absence of AMPARs in stg/stg GCs. The rescue of synaptic AMPARs in stg/stg ΔGluA2 cells suggests that, unlike CI-AMPARs, CP-AMPARs do not require stargazin (or any type I TARP) to be efficiently inserted at postsynaptic sites. To support this view we can refer to previously published studies in Golgi cells in γ-2,3−/− mice (Menuz et al., 2008). Golgi cells express two members of type I TARP family, γ-2 and γ-3 (Fukaya et al., 2005), which co-assemble with GluA2-containing receptors. In wild-type animals the I-V relationships for evoked EPSCs were linear and remained so in γ-2−/− or γ-3−/− mice, indicating the presence of CI-AMPARs at the synapse. However, the deletion of both TARPs gave rise to inwardly rectifying CP-AMPARs (Menuz et al., 2008). This previous observation would also seem to support our proposal that, CP-AMPARs do not require type I TARPs for their synaptic localisations in either stg/stg ΔGluA2 cells or in γ-2,3−/− Golgi cells.

The specific regulation of CP-AMPARs was apparent not only in stg/stg ΔGluA2 but also in stg/stg cells transfected with GluA2(Q). In addition to determining the channel calcium permeability, the Q/R editing site in the pore-lining region also plays a key function in AMPAR trafficking. Multiple studies have provided compelling evidence for Q/R editing site as a crucial regulator of AMPAR exit from the endoplasmic reticulum (ER). Greger et al. (2002) compared AMPAR trafficking events of two widely expressed AMPAR subunits – GluA1 and GluA2. They observed that GluA1 receptors travelled readily to the cell surface whereas GluA2 was mainly retained within the ER. Interestingly, the reversion to glutamine (Q) at position 607 caused rapid release of GluA2 subunits and elevated surface AMPAR expression in neuronal cells. Other studies from the Greger lab have provided further insight into a role of Q/R editing in AMPAR retention in that the Q/R site acts as a controlling element at the tetramerization step of AMPAR assembly. The edited GluA(R) subunits appear to be held as dimers whereas unedited subunits are tetramerized and trafficked to synapses (Greger et al., 2003).

Our experimental results suggest the presence of two main Q/R dependent pathways for AMPARs exit from ER. The first involves the GluA2-containing
receptors, which seem to require stargazin or type I TARPs to overcome their ER retention. This would ensure that only properly assembled receptors leave the ER. In the *stargazer* cerebellum, GluA2 is expressed in its immature (sensitive to EndoH) form signifying its ER retention. Stargazin protein is then necessary to enable expression of properly folded AMPARs on the neuronal plasma membrane (Tomita et al., 2003; Vandenberghhe et al., 2005a). The second pathway is the CP-AMPAR route (including GluA2(Q) containing receptors). This allows AMPARs to leave the ER and be expressed at synaptic and extrasynaptic sites. As most AMPARs in the CNS preferentially assemble as GluA2 heteromers they will normally require stargazin association to exit the ER.

The alteration of GluA2 subunit content (combined with its change to the unedited version) resulted in synaptic AMPAR rescue in *stargazer* cerebellar GCs. Further experiments are necessary to determine if all unedited GluA subunits can be expressed in neuronal cells in the absence of TARPs and if so, what are the intracellular signalling mechanisms involved.

### 3.3.3 Compartment-specific TARPed and TARPless CP-AMPARs

AMPARs found in cerebellar GCs are mostly expressed as GluA2/GluA4 complexes (Bahn and Wisden, 1997; Cathala et al., 2005; Gallo et al., 1992; Mosbacher et al., 1994). At MF-GC synapses, these CI-AMPARs associate with the ancillary subunit stargazin to mediate synaptic transmission. The TARP γ-2 not only critically determines the trafficking of AMPARs but also their pharmacological and functional properties (Coombs and Cull-Candy, 2009; Greger et al., 2017; Jackson and Nicoll, 2011a). In *stargazer* cerebellar GCs CI-AMPARs fail to reach synaptic locations owing it to the lack of functional stargazin. Our experiments on *stg/stg* ΔGluA2 cells revealed that increasing the prevalence of CP-AMPARs, by means of GluA2 siRNA transfection, reinstates AMPAR-mediated synaptic transmission. The close examination of I-V relationships, in the presence of intracellular spermine, and non-stationary fluctuation analysis of AMPAR-mediated currents revealed the existence of a heterogeneous population of CP-AMPARs in *stg/stg* ΔGluA2 cells. Based on
single-channel conductance values and their corresponding rectification indices, we were able to identify 2 groups of CP-AMPARs. Their properties were tightly linked to receptor localisation. The AMPAR channels from extrasynaptic compartments displayed properties typical for TARP assemblies: a high single-channel conductance (resolved single-channel openings as long-lived high-conductance bursts) and attenuated spermine block at positive potentials (Soto et al., 2007; Soto et al., 2009). Considering the fact that wild-type cerebellar GCs do not express AMPAR auxiliary subunits other than γ-2 and γ-7, it is highly likely that extrasynaptic CP-AMPARs in stg/stg ΔGluA2 cells are co-assembled with TARP γ-7. These CP-AMPAR/γ-7 complexes seem to fail to reach synaptic sites as the properties of synaptic CP-AMPARs – low single-channel conductance and pronounced inward rectification, suggest the presence of TARPIless receptors.

The compartment-specific expression of TARPed and TARPIless CP-AMPARs is not restricted to stg/stg ΔGluA2 cerebellar GCs. Also in stg/stg stellate cells, despite the presence of γ-7–associated CP-AMPARs extracellularly, these receptors also appear to be absent from synapses ((Bats et al., 2012) but see also (Jackson and Nicoll, 2011b)). We can assume, that either CP-AMPAR/γ-7 assemblies fail to cluster at synaptic sites, or that they are actively excluded from the subsynaptic membrane, or that TARPIless receptors are clustered preferentially.

3.3.4 TARP γ-7 as a regulator of CP-AMPAR trafficking

In stg/stg cerebellar GCs, although AMPAR subunits GluA2 and GluA4 are present, they fail to reach the cell surface (Chen et al., 1999; Hashimoto et al., 1999; Tomita et al., 2003). The fact that GluA2 knockdown rescued surface expression and synaptic targeting of AMPARs suggests that the observed retention is tightly linked to AMPAR subunit composition and that, in contrast with CI-AMPARs, CP-AMPARs can be efficiently trafficked to extracellular locations in the absence of γ-2. Furthermore, the analysis of currents mediated by CP-AMPARs revealed compartment-specific localisation of TARPed and TARPIless receptors. We found that currents mediated by extrasynaptic
AMPARs displayed characteristics typical of TARP associated CP-AMPARs and that these appeared to co-assemble with the atypical TARP γ-7. On the other hand, the AMPARs underlying synaptic activity behaved like TARPlless receptors in that they showed increased sensitivity to spermine block and reduced single-channel conductance. Despite earlier postembedding immunogold microscopy studies detecting both TARPs γ-2 and γ-7 on cerebellar postsynaptic membranes at MF-GC synapses (Yamazaki et al., 2010), our results suggest that TARP γ-7 is absent from synaptic CP-AMPARs in stg/stg ΔGluA2 cerebellar GCs. This observation might indicate a requirement for the presence of γ-2 in the AMPAR/TARP assembly to allow synaptic localisation of γ-7. The exclusion of γ-7 from postsynaptic places was also evident in stargazer stellate cells (Bats et al., 2012).

Thus, our results from stg/stg ΔGluA2 cells point to a potentially important function of γ-7 in the regulation of AMPARs. They also raise a number of important questions. Why does TARP γ-7 fail to traffic CI-AMPARs to the cell surface while clearly participating in the delivery of CP-AMPARs? Is γ-7 acting as AMPAR subunit selective TARP with specificity for CP-AMPAR? This interesting and so far, experimentally overlooked TARP is the main subject of the research presented in Chapter 4.
Chapter 4

AMPAR regulation by the type II TARP γ-7 in GCs

4.1 Introduction

The release of the neurotransmitter from MF terminals, activates numerous glutamate-gated ion channels situated at the postsynaptic membranes in cerebellar GCs. Fast transmission in these cells is mediated predominantly by AMPAR composed of GluA2 and GluA4 subunits (Bahn and Wisden, 1997; Keinanen et al., 1990; Lambolez et al., 1992) that appear be co-assembled with the auxiliary proteins γ-2 and γ-7 (Coombs and Cull-Candy, 2009; Fukaya et al., 2005; Schwenk et al., 2014; Yamazaki et al., 2010). Although, both γ-2 and γ-7 belong to the stargazin family of TARPs, they show differential regulation and control of AMPAR trafficking, gating and pharmacology. Based on their distinctive modulatory abilities, they have been classified, respectively, as type I (γ-2) and type II (γ-7) TARPs (Kato et al., 2010).

In the *stargazer* mouse, cerebellar GCs display a characteristic and selective loss of surface AMPAR caused by a deficiency in the functional γ-2 protein (Chen et al., 2000; Hashimoto et al., 1999). Thus, TARP γ-7, also expressed in cerebellar GCs (Fukaya et al., 2005; Yamazaki et al., 2010), appears incapable of compensating for the loss of γ-2 functions. Specifically, it is unable to escort AMPARs through the secretory pathways to the synaptic sites (Chen et al., 2000; Vandenbergh et al., 2005b). When expressed in heterologous cells, γ-7 strongly influences AMPARs in multiple ways. It increases AMPAR affinity for glutamate, it enhances glutamate-evoked currents, it modulates receptor gating by slowing both deactivation and desensitisation kinetics, and it alters receptor pharmacology (Bats et al., 2012; Kato et al., 2008; Kato et al., 2007). Importantly, γ-7 binds to neuronal AMPAR subunits GluA1-4 in the cerebellum (Kato et al., 2007) and is detected in the postsynaptic membranes at the MF-GC synapses (Yamazaki et al., 2010). Although γ-7 associates with
AMPARs, the previously attempted rescue of AMPAR-mediated EPSCs by γ-7 overexpression in stg/stg cerebellar GCs was unsuccessful (Kato et al., 2007).

In the previous chapter I described how the dramatic loss of extracellular AMPARs observed in stg/stg cerebellar GCs that reflects the γ-2 deficiency, is dependent on AMPAR subunit-specific trafficking mechanisms. The knockdown of GluA2, a subunit that dictates AMPAR permeability to calcium ions, rescued both surface and synaptic AMPAR expression in stg/stg GCs. We found that CP-AMPARs, unlike CI-AMPARs, were efficiently trafficked to the cell surface and mediated EPSCs despite the lack of γ-2. Additionally, receptors at extrasynaptic sites exhibited characteristics of TARPed assemblies whereas synaptic receptors appeared TARPless. By increasing the predominance of CP-AMPARs in stg/stg GCs, TARP γ-7 was able to traffic CP-AMPARs to the cell surface and influence receptor properties. It is unclear why the receptors co-assembled with γ-7 did not localise at postsynaptic regions. However, it is apparent that TARP γ-7 plays an important role in the trafficking of CP-AMPARs.

In this chapter, we investigated further the role of γ-7 in cerebellar GCs. I began by examining whether both γ-7 and γ-2 co-assemble with AMPA receptors or assemble individually in a competitive manner. Specifically, we asked whether γ-7 assembles with AMPARs only when γ-2 is absent (as seen in stg/stg ΔGluA2 cells)? Then, I examined whether γ-7 is essential for surface delivery of CI- and CP-AMPARs in these cells by recording whole-cell currents from cells transfected with shRNA against γ-7. To establish whether γ-7 has a function in regulating synaptic AMPARs, I studied the AMPARs that mediated the mEPSCs in Δγ-7 cells. I also looked at the effect of γ-7 overexpression on AMPARs in wild-type cerebellar GCs (that contained γ-2).
4.2 Results

4.2.1 Co-Immunoprecipitation of TARP γ-7 in the cerebellum

Type II TARPs are widely distributed across multiple brain regions signifying their potential importance in AMPAR handling (Fukaya et al., 2005). It has been suggested that type II TARPs cannot sustain efficient AMPAR trafficking in cells lacking type I TARPs (Menuz et al., 2008). However, our data from \( stg/stg \) ΔGluA2 cells showed that TARP γ-7 plays an important role in the delivery and expression of extrasynaptic CP-AMPARs (see Chapter 3).

It remained unclear whether γ-7 is able to co-assemble with AMPARs together with γ-2, or only when γ-2 is absent. Previous studies have been inconclusive regarding a possible interaction between γ-7 and γ-2 (Kato et al., 2008; Kato et al., 2007). In order to clarify this important point, I used co-immunoprecipitation assays and examined the γ-7 interaction with both GluA2 and GluA4 in cerebellar lysates from adult WT and \( stg/stg \) mice. I found evidence that γ-7 interacted with GluA2 and GluA4 AMPAR subunits in WT and \( stg/stg \) samples (Figure 4.1). Additionally, in WT samples, γ-7 also interacted with γ-2. And, as would be expected, I did not detect this interaction in \( stg/stg \) samples.

These results suggest that individual AMPA receptors can ‘bind’ both γ-2 and γ-7 within the same assembly. Furthermore, γ-7 co-assembled with GluA2 and GluA4 AMPAR subunits in the stargazer cerebellar samples but these assemblies failed to be delivered to the postsynaptic localisations (Bats et al., 2012) being ‘trapped’ intracellularly (Chen et al., 2000; Hashimoto et al., 1999; Tomita et al., 2003). Thus γ-7 clearly associates with AMPARs but what role does it play?
AMPAR regulation by the type II TARP γ-7 in GCs

Figure 4.1 TARP γ-7 binds to GluA2 and GluA4 AMPAR subunits in cerebellar lysates from both wild-type and stargazer mice. Western blotting analysis of the immunoprecipitates shows that γ-7 co-immunoprecipitates with γ-2 in wild-type cerebellum. The γ-7 protein complexes were immunoprecipitated (IP) with anti-γ-7 antibody then western blotted alongside input samples and IgG controls, using anti-GluA4, anti-GluA2 or anti-γ-2 antibodies (IB; immunoblot). Input represents 1.5% of the total, except for wild-type GluA2 and γ-2 where they were 0.5%. In each case the nearest molecular weight markers are indicated. Each immunoblot illustrated is representative of 2-5 replicates. No protein level was quantified. (Modified from Studniarczyk et al., 2013).
4.2.2 AMPAR surface expression following γ-7 knockdown

To establish whether γ-7 has a function in the AMPAR expression (both CI- and CP-AMPARs) in GCs, I used shRNA to knock down endogenous levels of γ-7. First, I transfected the shRNA construct into wild-type cerebellar GCs and recorded AMPA-evoked responses during voltage ramp. The Δγ-7 cells displayed a dramatic increase in the mean whole-cell current amplitude (Figure 4.2 A, B) together with essentially linear I-V relationship denoting the presence of CI-AMPARs (mean RI of 0.75 ± 0.06, n = 8) (Figure 4.2 C, D). This result suggests that, the γ-7 knockdown leads either to increase in AMPAR number, single-channel conductance or open probability, while the endogenous levels of γ-2 are retained. Clearly, γ-7 is not required for the surface expression of CI-AMPARs in cerebellar GCs; rather, it appears to suppress the expression of CI-AMPARs.

Figure 4.1 Altered surface expression of AMPARs in wild-type cerebellar GCs following γ-7 knockdown. (A) A black and blue trace represents whole-cell AMPA-evoked current during voltage ramp from a wild-type and Δγ-7 cell, respectively. Grey trace denotes the voltage protocol. (B) Pooled data showing increase in whole-cell current amplitude (–90 mV) following γ-7 knockdown (n = 17 wild-type and 8 Δγ-7 cells; # P = 0.055 Welch t-test and P < 0.05 robust methods. (C) Non rectifying global average of current-voltage relationship from Δγ-7 cells (n = 8). Shaded area SEM. (D) Pooled data showing rectification index (calculated as slope conductance ratio) wasn’t affected by knockdown of γ-7 (n = 8). The grey box-and-whisker plot represents data from wild-type cells (from Figure 3.1 B). Box-and-whisker plots denote the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles represent individual cells. (Modified from Studniarczyk et al., 2013).
Indeed, in \textit{stg/stg} granule cells, which lack surface AMPARs (see Figure 3.4 A), knockdown of \(\gamma-7\) was able to rescue AMPAR expression. AMPA application evoked large whole-cell currents, which, unlike those rescued by knockdown of GluA2 (see Figure 3.4 B), had \(I-V\) relationships displaying very little rectification (mean rectification index of \(0.67 \pm 0.07, n = 6\)), characteristic of CI-AMPAR predominance (Figure 4.3 A-D). The fact that CI-AMPARs are expressed at the \textit{stg/stg} cell surface after \(\gamma-7\) knockdown reinforces the concept that \(\gamma-7\) may normally be suppressing surface expression or delivery of CI-AMPARs.

In \textit{stg/stg} \(\Delta\text{GluA2}\) cells, the extrasynaptic CP-AMPARs appeared to be co-assembled with \(\gamma-7\) (see Chapter 3, Figure 3.4). But is TARP \(\gamma-7\) essential for the surface delivery of CP-AMPARs? We addressed this by knocking down both the GluA2 subunit and \(\gamma-7\) in \textit{stg/stg} cerebellar GCs. In \textit{stg/stg} \(\Delta\text{GluA2}\Delta\gamma-7\) cells, AMPA application produced responses of \(270.5 \pm 95.5\) pA (at \(-90\) mV; \(n = 7\)), which displayed strong inward rectification (\(0.28 \pm 0.06\)). These experiments, which involved both a single \(\gamma-7\) knockdown, or a double \(\gamma-7\) and GluA2 knockdown in wild-type and \textit{stg/stg} cells, suggested that TARP \(\gamma-7\) is not vital for the expression of either CP- or CI-AMPARs in these cells. Importantly, the experiments indicate that under appropriate conditions, both types of AMPARs (CP- and CI-AMPARs), can be expressed at the cell surface in a TARPlless form.
Figure 4.2 The γ-7 knockdown recovers whole-cell AMPAR-mediated currents in stg/stg cerebellar GCs. (A) A black and blue trace represents whole-cell AMPA-evoked current during voltage ramp from a stg/stg and stg/stg Δγ-7 cell, respectively. Grey trace denotes the voltage protocol. (B) Pooled data showing AMPA-evoked whole-cell current (−90 mV) following knockdown of γ-7 (n = 8 and 6 for stg/stg and stg/stg Δγ-7, respectively). Note the effective absence of whole-cell current in stg/stg GCs and its restoration following knockdown of γ-7. The recovery of whole-cell AMPA-evoked current was also seen in stg/stg ΔGluA2 cells as discussed in the Chapter 3. (C) Global average current-voltage relationship recorded from stg/stg Δγ-7 cells (n = 6) indicating a lack of rectification. Shaded area denotes SEM. (D) Pooled data comparing RI values following knockdown of γ-7 (n = 6) or GluA2 (n = 5) in stg/stg GCs. In panel B and D, pooled data showing the whole-cell current and RI in stg/stg ΔGluA2 cells are shown for comparison (n = 5; red). ** P < 0.01. Box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. (Modified from Studniarczyk et al., 2013).
4.2.3 Does γ-7 play a role in the delivery of synaptic AMPARs?

The data from \textit{stg/stg ΔGluA2} cerebellar GCs (Chapter 3), together with previously published findings from cerebellar stellate cells (Bats et al., 2012), back the idea that the extrasynaptic CP-AMPARs are co-assembled with TARP γ-7, while the synaptic CP-AMPARs are TARPlless in the \textit{stg/stg} cells. Although γ-7 is normally expressed in \textit{stargazer} cerebellum (see Figure 4.1), the synaptic AMPAR-mediated transmission is lost in cerebellar GCs. Interestingly, the γ-7 knockdown in both wild-type and \textit{stargazer} GCs resulted in pronounced AMPAR responses. These various findings raise a number of questions. Specifically, is γ-7 a redundant subunit in GCs, or a crucial component for a proper regulation of subunit-specific AMPAR expression? Is γ-7 vital for normal AMPAR synaptic targeting? Can we rescue synaptic AMPARs in \textit{stg/stg} cells by deleting γ-7?

To clarify whether γ-7 normally plays any role in the regulation or synaptic localization of CI-AMPARs I knocked down this subunit in wild-type GCs. Synaptic activity in the form of mEPSCs was still readily detected. The mEPSCs had a mean current amplitude of 11.2 ± 1.3 pA \((n = 9)\) (Figure 4.4 A) and RI 0.73 ± 0.09 \((n = 5)\) suggesting that synaptic targeting of CI-AMPARs is not affected by loss of γ-7. However, there was a pronounced reduction in synaptic single-channel conductance from 13.9 ± 1.2 pS \((n = 13)\) to 8.9 ± 0.6 pS \((n = 9; P = 0.0093)\) (Figure 4.4 A). While TARP γ-7 does not affect synaptic trafficking of CI-AMPARs it seems to influence their functional properties. It is unclear whether the observed drop in single-channel conductance reflects the presence of TARPlless CI-AMPAR assemblies (despite the presence of stargazin) or the presence of a population of low conductance receptors, for example homomeric GluA2 (Swanson et al., 1997).
AMPAR regulation by the type II TARP γ-7 in GCs

Figure 4.3 Synaptic Cl- and CP-AMPARs are affected by knockdown of γ-7. (A) Pooled data illustrating the effect of γ-7 knockdown in wild-type cells on mEPSC amplitude and single-channel conductance ($n = 13$ and $9$, wild-type and Δγ-7 respectively). Synaptic currents mediated by Cl-AMPARs. (B) Pooled data illustrating the effect of γ-7 knockdown in wild-type ΔGluA2 cells on mEPSC amplitude and single-channel conductance ($n = 17$ and $8$, ΔGluA2 and ΔGluA2Δγ-7 respectively). Synaptic currents mediated by CP-AMPARs. * $P < 0.05$, ** $P < 0.01$. Box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. (Modified from Studniarczyk et al., 2013).
To establish if γ-7 is important for the regulation and synaptic trafficking of the CP-AMPARs, I recorded mEPSCs in wild-type GCs where both GluA2 and γ-7 had been knocked down. ΔGluA2Δγ-7 cells exhibited clear mEPSCs. However, when compared with wild-type cells following GluA2 knockdown alone, both mEPSC amplitude (14.5 ± 3.4 pA, n = 10) and the underlying synaptic channel conductance (12.5 ± 0.8 pS, n = 8) were roughly halved (P = 0.049 and 0.0036, respectively) (Figure 4.4B). This reduction in amplitude and single-channel conductance was also accompanied by strong inward rectification of the mEPSCs (0.38 ± 0.07, n = 9) suggesting that synaptic CP-AMPARs are most likely expressed as TARPless channels after γ-7 knockdown. Again, as was the case for CI-AMPARs, the CP-AMPAR properties were susceptible to the loss of γ-7. This likely reflects the presence of a close interaction between both γ-2 and γ-7 in the AMPAR association. TARP γ-7 seems to represent a vital assembly component in the γ-2 association with AMPARs at synaptic sites. In other words, γ-2 does not appear to form part of synaptic AMPAR assembly in the absence of γ-7.

The present data reinforces the earlier postulated view that under certain conditions AMPARs can be targeted and expressed in the postsynaptic membrane without an associated TARP (see Chapter 3, (Bats et al., 2012)). To further test this hypothesis, I knocked down γ-7, the only remaining TARP in stg/stg GCs. Surprisingly, in these conditions AMPAR-mediated synaptic transmission was rescued (Figure 4.5A). mEPSCs could be detected at both positive and negative potentials, displaying relatively little inward rectification (RI 0.77 ± 0.09, n = 6), and a low single-channel conductance (9.6 ± 1.0 pS, n = 9) (Figure 4.5A, B). These results support the idea that CI-AMPARs can also reach the synapse in the absence of associated TARP.
AMPAR regulation by the type II TARP γ-7 in GCs

Figure 4.4 Rescued mEPSCs after γ-7 knockdown in stg/stg cells are mediated by TARPlless CI-AMPARs. (A) Representative mEPSCs recorded at positive (+60 mV) and negative (–60 mV) potentials from a stg/stg Δγ-7 cell and the corresponding mean events. (B) Pooled data showing mean rectification index value \((n = 6)\) and mean single-channel conductance \((\text{psNSFA, } n = 9)\) from stg/stg Δγ-7 cells. (C) Pooled data comparing mean mEPSC amplitude from stg/stg ΔGluA2 \((n = 9)\) and stg/stg ΔGluA2Δγ-7 \((n = 14)\) cells. (D) Pooled data comparing mean single-channel conductance \((\text{psNSFA})\) from stg/stg ΔGluA2 \((n = 7)\) and stg/stg ΔGluA2Δγ-7 \((n = 11)\) cells. Box-and-whisker plot indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values. ((Modified from Studniarczyk et al., 2013).

The data presented in Chapter 3 showed that by disrupting the production of GluA2 we were able to rescue synaptic AMPAR-mediated activity in stg/stg GCs (see Figure 3.5) and the resultant receptors underling mEPSCs were permeable to calcium ions. Furthermore, another manipulation in stg/stg GCs, namely γ-7 knockdown, can also rescue mEPSCs. However, unlike the situation in stg/stgΔGluA2 cells, the mEPSCs were mediated by receptors impermeable to Ca\(^{2+}\) (see Figure 4.5 A, B). Based on these results, TARP γ-7 appears to specifically suppress the expression of CI-AMPARs. Is co-assembly with γ-7 necessary for synaptic trafficking of CP-AMPARs? To test this, I made recordings from stg/stg GCs in which both GluA2 and γ-7 were knocked down. In these conditions, mEPSCs were still readily detected, and were strongly rectifying (rectification index 0.26 ± 0.06, \(n = 15\)), confirming that, in the absence of both γ-2 and γ-7, TARPlless CP-AMPARs can cluster at synaptic membranes.
The mean amplitude of mEPSCs (9.4 ± 1.0 pA, n = 14) (Figure 4.5 C) and the single-channel conductance from psNSFA (13.1 ± 1.6 pS, n = 11) (Figure 4.5 D) were not different from the values obtained from stg/stg ΔGluA2 cells (P = 0.06 and 1.00, respectively), supporting the previous view that synaptic CP-AMPARs are TARPless in the absence of γ-2, despite the presence of γ-7.

4.2.4 Kainate/glutamate ratio in wild-type Δγ-7 GCs

Knockdown of the γ-7 subunit had a profound effect on AMPARs expression in wild-type and stg/stg GCs, leading to an increase in the AMPA-evoked whole-cell currents and the synaptic expression of TARPEless AMPARs. The synaptic clustering of TARPEless CI- and CP-AMPARs in the presence of the prototypical TARP γ-2 is particularly unexpected and interesting. The properties of mEPSCs recorded from Δγ-7 GCs suggest that knockdown of γ-7 ‘negatively’ influenced the association of γ-2 with synaptic AMPARs. This behaviour was observed for both CI- and as CP-AMPARs. We next asked whether the loss of γ-7 influences the overall γ-2 association with AMPARs. Do all surface receptors display properties of TARPEless receptors in Δγ-7 cells?

To address TARP association with AMPARs, in a separate set of experiments, I used a well-defined pharmacological approach – measurement of the kainate/glutamate ratio. The presence of TARP changes the pharmacology of the AMPAR partial agonist kainate. In cells expressing TARPEless receptors, kainate elicits weak non-desensitising responses, whereas in cells expressing AMPARs co-assembled with a type I TARP, kainate acts as a full agonist giving dramatically larger currents (Tomita et al., 2005; Turetsky, 2005). To determine whether the loss of γ-7 influences the association between γ-2 and all surface AMPARs I sequentially bath applied 500 µM kainate and 500 µM glutamate (both in the presence of 100 µM cyclothiazide) and recorded AMPAR-mediated responses in wild-type and Δγ-7 cells (Figure 4.6 A, B). For each individual cell I measured the kainate and glutamate-evoked currents and calculated the ratio.
As expected from AMPARs co-assembled with TARPs, both kainate and glutamate applications evoked pronounced responses in wild-type cells (174.61 ± 33.3 and 151.37 ± 27.8 pA at –60mV, n = 7) with mean kainate/glutamate ratio of 1.18 ± 0.11. On the other hand, the mean kainate/glutamate ratio was reduced in Δγ-7 cells (to 0.71 ± 0.10, n = 7, \( P = 0.009 \)) (Figure 4.6 C).

**Figure 4.5** Kainate and glutamate evoked currents in wild-type and Δγ-7 cerebellar GCs. (A) Representative traces of whole-cell currents evoked by 500 μM kainate (grey bar) and 500 μM glutamate (red bar) from wild-type and Δγ-7 cells recorded at –60mV. (B) Pooled data showing whole-cell responses to kainate (black circle) and glutamate (red circle) recorded from wild-type (grey box) and Δγ-7 (blue box) cells. (C) Pooled data showing the reduction in the mean kainate/glutamate ratio following γ-7 knockdown. ** \( P < 0.01 \). Box-and-whisker plots indicate the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles show individual values.
The extent of reduction in the kainate/glutamate ratio did not match typically reported values for TARPIless AMPARs (HEK cells, (Shi et al., 2009)) but rather resembled previously published findings in heterozygous +/stg GCs (Jackson and Nicoll, 2011b; Shi et al., 2010). We can assume that either the TARP/AMPAR stoichiometry of expressed channels is affected by γ-7 knockdown or that there is a mixed population of TARPIless and γ-2 TARPed AMPARs. Determination of a kainate/glutamate ratio in stg/stg cells following γ-7 knockdown would provide more insight into the interactions between γ-7 and γ-2 in GCs. Interestingly in support of this idea, a recent paper reported values similar to ours for the kainate/glutamate ratio for γ-2/γ-7 double knockout stellate cells which are known normally to express both of these TARPs (Yamazaki et al., 2015).

4.2.5 Prevalence of CP-AMPARs following γ-7 overexpression

The experiments on stg/stg cells, where either GluA2 or γ-7 was knocked down, suggested that the atypical TARP γ-7 promotes expression of CP-AMPARs in the absence of γ-2. In wild-type cerebellar GCs, the tight interplay that occurs between endogenous γ-2 and γ-7 subunits presumably determines the fact that surface AMPAR are impermeable to calcium ions. We therefore next asked if shifting the TARP balance towards γ-7 (by making γ-7 the dominant TARP in the GCs) would alter the AMPAR subtype expressed. To test this, I transfected wild-type GCs with cDNA for γ-7. These cells gave whole-cell currents that were increased in amplitude at negative potentials (from 147.8 ± 37.2 pA to 1387.5 ± 277.1 pA at –90 mV, n = 17 and 5), and exhibited strong inward rectification characteristic of CP-AMPARs (RI = 0.24 ± 0.06, n = 5) (Figure 4.7 A, C and D).

To exclude the possibility that merely increasing TARP expression per se caused a non-specific increase in CP-AMPARs, whole-cells currents were also examined in WT cells in which γ-2 was overexpressed. Although, these currents were increased in amplitude (737.4 ± 190.2 pA, n = 9) they retained their linear I-V relationship (Figure 4.7 B and C), with a mean rectification index similar to that of untreated cells (1.12 ± 0.08, n = 8) (Figure 4.7 D). These
results confirmed that the overexpression of γ-7 selectively enhanced the surface expression of CP-AMPARs.

Figure 4.6 Predominance of CP-AMPARs in cerebellar GCs following overexpression of γ-7. (A) Representative whole-cell responses evoked by bath application of 20 μM AMPA during voltage ramp. The black trace represents a wild-type cell and the purple a cell transfected with γ-7 (+γ-7). Grey trace denotes the voltage protocol used. (B) As in panel A but showing the effect of γ-2 overexpression (+γ-2) (teal trace). (C) A teal and purple trace represents respectively global average of current-voltage relationships recorded from +γ-2 (n = 8) and +γ-7 cells (n = 5) in response to AMPA. The shaded areas denote SEM. (D) Pooled data showing rectification index values following overexpression of TARP γ-7 (purple, n = 5) and γ-2 (teal, n = 8) in granule cells. *** P < 0.0001. The grey box-and-whisker plot represents data from wild-type cells (from Figure 3.1 B). Box-and-whisker plots denote the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles represent individual cells. (Modified from Studniarczyk et al., 2013).
To test if AMPARs clustered at postsynaptic sites were also permeable to calcium, I recorded mEPSCs at negative (−60 mV) and positive (+60 mV) potentials. The overexpression of γ-7 markedly increased the amplitude of mEPSCs at −60 mV (Figure 4.8 A, B) resulting in their pronounced inward rectification (Figure 4.8 C). Consistent with the targeting of CP-AMPARs to the synapse, the weighted mean single-channel conductance obtained from psNSFA of mEPSCs was increased to 20.3 ± 1.4 pS (n = 6; P = 0.025 versus WT granule cells) (Figure 4.8 D). Overall, the mEPSCs recorded from WT cells overexpressing γ-7 displayed properties similar to those mediated by CP-AMPARs following knockdown of GluA2 (compare with Figure 3.3).

Our data support the view that increased expression of γ-7 results in a selective increase in the expression of CP-AMPARs, and that these receptors are targeted to the synapse. It is particularly striking that this can occur in cells that do not normally express synaptic CP-AMPARs. It is also of note that with γ-7 overexpression the synaptic CP-AMPARs exhibited properties indicative of TARPed receptors. This differs from the situation in stg/stg ΔGluA2 GCs, where only the extrasynaptic AMPARs appeared to be TARPed (Chapter 3, see also (Bats et al., 2012)). This difference presumably reflects the fact, that in GCs from wild-type mice, γ-2 was also present, and supports our finding that the presence of both TAPRs is required for synaptic CP-AMPARs to be TARPed.

4.2.6 AMPAR and TARP association at synaptic sites in GCs

This brief section aims to bring together and illustrate our main findings for synaptic AMPARs in GCs. AMPAR-mediated mEPSCs were examined in wild-type and stg/stg cells following TARP overexpression, and following single- and double knockdown (of TARPs and GluA2). For each experimental condition, RI and corresponding mean single-channel conductance (psNSFA) was calculated and graphed as a scatter plot (Figure 4.9 A). The plot of rectification versus conductance relationship allowed us to visualise the existence of four AMPAR groups: calcium impermeable (CI), calcium permeable (CP), TARPed and TARPless formations. The groups were
Figure 4.7 Overexpression of γ-7 results in a switch from Cl-AMPARs to CP-AMPARs at synaptic localisations. (A) Representative mEPSCs recorded at positive (+60 mV) and negative (−60 mV) potentials from a +γ-7 cell and the corresponding mean events. (B) Pooled data showing the increase in mean mEPSC amplitude following γ-7 overexpression (n = 7 cells), * P < 0.05. (C) The overexpression of γ-7 decreases RI value (n = 4 cells), ** P < 0.01. (D) Pooled data illustrating increased single-channel conductance (psNSFA) in +γ-7 cells (n = 6). The grey box-and-whisker plot represents data from wild-type cells (from Chapter 3). Box-and-whisker plots denote the median value (black line), the mean (red cross), the 25-75th percentiles (box) and the 10-90th percentiles (whiskers); open circles represent individual cells. (Modified from Studniarczyk et al., 2013).
verified by using divisive hierarchical cluster analysis and illustrated as a dendrogram (Figure 4.9 B).

The groups, which emerged from the clustering, suggest that synaptic AMPARs require both TARPs γ-2 and γ-7, in order to exist as TARPed receptors at postsynaptic membrane. The ablation of one TARP has a profound effect on whether the remaining one associates with AMPAR. Our data suggest that γ-2 and γ-7 are normally in a close relationship that shapes and fine tunes the expression and properties of synaptic AMPARs.

4.2.7 γ-5 overexpression reduces AMPAR surface expression

Type II TARPs (comprising γ-7 and γ-5 only) display distinctively different modulatory properties to type I TARPs (Kato et al., 2010). Since the overexpression of γ-7 in wild-type cerebellar GCs had such a profound effect on the surface expression and subunit composition of AMPARs, we decided, in a separate set of experiments, to examine briefly the effect of TARP γ-5 on AMPAR mediated current. To test this, wild-type GCs were transfected with cDNA for either γ-7 or γ-5. Again, the overexpression of γ-7 resulted in a dramatic increase in whole-cell current amplitude (from 109.8 ± 26.0 pA to 1005.0 ± 224.1 pA at –90 mV, n = 6 and 5; P = 0.03), and produced inwardly rectifying I-V plots, indicative of the surface expression of CP-AMPARs (mean RI: 1.10 ± 0.06 for wild-type and 0.55 ± 0.0.6 for γ-7 cells; n = 6 and 5). Intriguingly, cells transfected with γ-5 did not exhibit the same behaviour. The AMPA-evoked whole-cell current amplitude was considerably reduced to 18.1 ± 7.8 pA (n = 7; P = 0.03), possibly signifying a decrease in the number of surface AMPARs and/or their reduced single-channel conductance. The low amplitude of the responses observed in 5 out of 7 recorded GCs (+γ-5) prevented the calculation of their rectification index. The remaining cells displayed an RI of 1.21 ± 0.30, which suggests the presence of CI-AMPARs. It would clearly be of interest to follow this up to obtain further clues about the mechanism and mode of action of the type II TARPs in controlling surface AMPARs.
AMPAR regulation by the type II TARP γ-7 in GCs

Figure 4.8 Knockdown and overexpression experiments reveal the presence of TARPed and TARPless synaptic AMPARs in GCs. (A) Scatter plot of RI and single-channel conductance enables identification of four groups of synaptic AMPARs depending on the type of alteration introduced to the cell content. The following groups have emerged: TARPed CP-AMPARs, TARPless CP-AMPARs, TARPed CI-AMPARs and TARPless CI-AMPARs. (B) Dendrogram illustrating the result of divisive hierarchical clustering. (Modified from Studniarczyk et al., 2013).
4.3 Discussion

Transmembrane AMPAR regulatory proteins (TARPs) not only influence the channel properties and pharmacology of AMPARs but most importantly they are the crucial partners for their normal cell surface and synaptic targeting (Coombs and Cull-Candy, 2009; Kato et al., 2010). Here we examined the role of atypical TARP γ-7, which is the only other TARP found together with γ-2 in cerebellar GCs (Fukaya et al., 2005; Yamazaki et al., 2010). First, we have established that γ-7 co-assembles with GC AMPAR subunits GluA2 and GluA4 independently of γ-2’s presence. Second, the tight co-operation between γ-2 and γ-7 appears pivotal for the proper maintenance of AMPAR surface expression and synaptic trafficking in cerebellar GCs – suggesting a synergistic interaction of γ-2 and γ-7 in the regulation of AMPARs. Third, we identified a dual and subunit specific function of γ-7 in that it promotes the surface delivery of CP-AMPARs and suppresses CI-AMPARs. We will now consider these three findings in more detail.

4.3.1 Atypical TARP γ-7 co-assembles with AMPARs in GCs

Numerous studies have investigated TARP distribution across multiple brain regions, and both γ-2 and γ-7 have been shown to be highly expressed in the cerebellum in general, and in cerebellar granule cells in particular (Fukaya et al., 2005; Kato et al., 2007; Sullivan et al., 2017; Tomita et al., 2003; Yamazaki et al., 2010). Of these two proteins, only γ-2 was identified to play a central role in the trafficking of AMPARs after the discovery of a complete loss of AMPAR mediated EPSCs at MF-GC synapses in the mutant mouse stargazer (Chen et al., 2000; Hashimoto et al., 1999). The fact that neither endogenous γ-7, nor elevated levels of γ-7 (in transfected cells) were able to sustain normal AMPAR-mediated transmission in stg/stg cells (Kato et al., 2007) was taken to suggest that γ-7 plays no role in regulating AMPAR trafficking in cerebellar GCs.

The findings presented in Chapter 3 suggest this earlier view was incorrect. We found that modification of AMPAR subunit content (by GluA2 knockdown) in stg/stg cerebellar GCs rescued both surface and synaptic AMPAR
expression, even though the prototypical TARP γ-2 was absent. Interestingly, the expressed AMPARs were not only permeable to calcium ions but also displayed distinctive properties that depended on their location. Synaptic receptors behaved as TARPlless channels whereas CP-AMPARs situated at extrasynaptic sites exhibited characteristics of γ-7 association. This ‘compartment specific’ localisation of TARPed versus TARPlless CP-AMPARs receptors implies an apparently important and specific role of TARP γ-7 in the trafficking of CP-AMPARs. A similar compartment specific distribution of TARPed receptors was reported in stellate cells from *stargazer* mice, where extrasynaptic CP-AMPARs were also found to be associated with γ-7 (Bats et al., 2012).

It was previously unclear whether γ-7 co-assembled with AMPARs exclusively when γ-2 was missing (as in *stg/stg* cells), in other words binding to the receptor in a competitive manner. Earlier reports were inconclusive regarding the relationship between γ-7 and γ-2, showing a very weak level of interaction and postulating a model where AMPARs associate with one TARP isoform only – either γ-2 or γ-7 (Kato et al., 2008; Kato et al., 2007). Our co-immunoprecipitation experiments support the view that AMPARs can bind both TARP subunits γ-7 and γ-2. Moreover, TARP γ-7 co-assembles with AMPARs when cells lack γ-2 (as in *stargazer*), but also when they contain γ-2. The fact that γ-7 was detected (from Co-IP) in AMPAR complexes with and without γ-2, points towards an important function for γ-7 in the regulation of AMPARs in cerebellum.

### 4.3.2 Synergistic interaction of γ-2 and γ-7 in the regulation of AMPARs

Earlier studies have suggested that the two main TARP isoforms γ-2 and γ-7 actively shape AMPAR synaptic content at various asymmetrical synapses in the cerebellum, and that knockdown of either γ-2 or γ-7 results in pronounced AMPAR subunit reduction (Yamazaki et al., 2010). The prototypical and widely expressed TARP γ-2 (found in virtually all cerebellar neurons (Fukaya et al., 2005)) has been shown to tightly control the number of synaptic AMPA
channels at multiple cerebellar synapses such as mossy fibre-granule cell (Chen et al., 2000; Hashimoto et al., 1999), parallel fibre-Purkinje cell (Yamazaki et al., 2015), climbing fibre-Purkinje cell (Yamazaki et al., 2015) and parallel fibre-stellate cell (Bats et al., 2012; Jackson and Nicoll, 2011b), parallel fibre-Golgi cell (Menuz et al., 2008). Despite the fact that the atypical TARP γ-7 is expressed at equally high levels as γ-2 across cerebellar neurons (Fukaya et al., 2005), its role has remained elusive.

In this chapter we investigated the effect of γ-7 knockdown on the population of synaptic AMPARs in cerebellar GCs from wild-type and stargazer animals. Contrary to the effect of γ-2 loss (as occurs in stargazer mice), the knockdown of γ-7 alone did not abolish AMPAR-mediated mEPSCs. Indeed, it initially appeared that γ-7 is less important for AMPAR synaptic targeting than TARP γ-2. However, when we repeated knockdown of γ-7 in stg/stg GCs, the synaptic events were rescued and mediated by TARPlless AMPARs (the transfected cells lacked both γ-2 and γ-7). Intriguingly, the properties of these synaptic currents (their amplitude, RI and single-channel conductance) were not different from events detected in wild-type Δγ-7 cells (expressing γ-2) implying that TARPlless CI-AMPARs mediate synaptic transmission not only in stg/stg GCs but also in wild-type cells following γ-7 knockdown. Like the CI-AMPARs, synaptic CP-AMPARs were also sensitive to the loss of γ-7 subunit. Their increased sensitivity to block by intracellular polyamine and reduced single-channel conductance are typical features of CP-AMPARs when both TARPs are absent (see Chapter 3, (Bats et al., 2012)). Together these results suggest that the presence of both γ-2 and γ-7 is essential for synaptic AMPARs to exhibit their normal functional properties and to cluster at postsynaptic sites as TARPed assemblies. Of note, subsequent experiments carried out in the laboratory of Roger Nicoll (Yamazaki et al., 2015), where viral transfection was used, failed to observe any effect of γ-7 knockdown on AMPAR mediated currents in cerebellar GCs. The reasons for this discrepancy are unresolved, however the study used different culturing and transfection protocols. At 3 DIV culturing media containing high potassium was replaced with low 5 mM. The concentration of potassium ions affects the depolarisation of cell membrane of culturing neurons and their gene expression (Mellor et al.,...
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Importantly, the viral transfection yields a higher number of transfected cells compared with calcium phosphate. Accordingly, it seems possible that not only postsynaptic cells but also presynaptic cells will have been affected by knockdown of γ-7, hence the release of neurotransmitter might have been different in this condition. Additionally, the criteria used for the analysis differed – in the later study all cells with less than 20 events during the recording period were considered as lacking EPSCs.

It is unclear at what stage the interaction between AMPAR subunits and TARPs can become disrupted. The proper folding of AMPAR subunits and their exit from ER has been shown to be supported by γ-2 (Vandenberghhe et al., 2005a). However, the data from cells transfected with γ-7 shRNA indicate that the association of synaptic AMPARs with γ-2 is disrupted. It thus seems plausible that γ-2 TARPed AMPARs may travel to the cell surface where TARP γ-2 then dissociates/separates from the complex, allowing TARPless receptors to diffuse laterally towards synaptic sites. Our experiments involving kainate and glutamate application in wild-type Δγ-7 cells might seem to favour this view.

The kainate/glutamate current ratio appears to capture the phase where some of the cell surface AMPARs are either TARPless or represent a mixed population of γ-2 TARPed and TARPless receptors. This raises the question: what prevents receptors that are TARPed just with γ-2 from entering postsynaptic domain? The apparent ‘exclusion’ of γ-2 TARPed assembles from synaptic locations is particularly puzzling considering the important role known to be played by γ-2 in trapping AMPAR within the postsynaptic domain (Bats et al., 2007). Nevertheless, the findings presented in Chapter 3 and 4 strongly support the view that both γ-2 and γ-7 are essential for proper synaptic clustering of TARPed receptors in normal synaptic transmission in cerebellar GCs.

4.3.3 Dual action of γ-7 in subunit-specific AMPAR regulation

The results presented in this chapter revealed the vital and complex nature of γ-7 in the regulation of AMPARs in cerebellar neurons. The series of
knockdown and overexpression experiments identify γ-7 as a CP-AMPAR-specific TARP that supports the expression of CP-AMPARs while supressing the expression of CI-AMPARs.

Stargazer cerebellar GCs display a nearly complete loss of surface AMPARs, despite the fact that their expression of GluA2 and GluA4 subunits is preserved and receptors are retained intracellularly (Chen et al., 2000; Hashimoto et al., 1999; Tomita et al., 2003). The recovery of cell surface and synaptic AMPARs observed in stg/stg cells following γ-7 knockdown (mediated by CI-AMPARs) or following GluA2 knockdown (mediated by CP-AMPARs) strongly suggests that the intracellular AMPAR retention is due to the suppressive action of γ-7 towards GluA2-containing assemblies rather than the absence of γ-2 alone. Correspondingly, the knockdown of γ-7 in wild-type cells substantially increased the amplitude of the whole-cell current, which was mediated by CI-AMPARs – signifying that GluA2-containing receptors could then be trafficked to the cell surface. While TARP γ-7 clearly acts as a suppressor of CI-AMPAR expression, it concomitantly supports expression of CP-AMPARs. In wild-type cells ‘tilting’ the balance of expressed TARPs in favour of γ-7 (by γ-7 overexpression) resulted in a dramatic increase in AMPAR mediated whole-cell currents but in contrast to the γ-7 knockdown, the currents were mediated by inwardly rectifying CP-AMPARs. The shift in AMPAR calcium permeability was also evident with synaptic receptors – the mEPSC amplitude, rectification and weighted mean synaptic channel conductance were all significantly enhanced by the overexpression of γ-7.

Based on the findings presented in Chapter 3 and 4, TARP γ-7 emerges not only as a fine-tuning element in AMPAR regulation in cerebellar GCs, but also as an important TARP capable of selectively delivering CP-AMPARs. This subunit-specific TARP appears to reduce the prevalence of CI-AMPARs at both extrasynaptic and synaptic sites while promoting the expression of CP-AMPARs. Although γ-7 was found to bind to PSD-95 in the cerebellar PSD fraction (Kato et al., 2007) and was detected at multiple asymmetrical synapses in the cerebellum (Yamazaki et al., 2010), localization of TARPed AMPAR at synapse seems to require both γ-7 and γ-2 to be present within the
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AMPAR-TARP complex. Both CI- and CP-AMPARs associated with γ-7 alone appear to be ‘excluded’ from synaptic sites unless they are accompanied by the prototypical TARP γ-2. The data gathered from cells following knockdown and overexpression of γ-7 suggest that both TARPs (γ-2 and γ-7) are equally important for normal synaptic transmission in cerebellar GCs and both tightly control number and subunit specific receptor delivery to synaptic places.

4.3.4 Working hypothesis

To determine whether the ‘interaction’ between γ-2 and γ-7 occurs at the level of receptor biogenesis or receptor trafficking, and to reveal details of this process, will clearly require further experiments. Nevertheless, one speculative working hypothesis would be that γ-2 and γ-7 associate with all tetrameric assemblies, and that the GluA2/γ-7 interaction prevents GluA2-containing assemblies from leaving the ER unless this effect is relieved by the additional association of γ-2 (Figure 4.10).

Figure 4.9 Working hypothesis. The simplified model illustrating interactions between AMPARs and TARPs γ-2 and γ-7 in cerebellar granule cells. The GluA2 subunit and γ-7 interaction retains GluA2-containing AMPARs within ER unless γ-2 co-assembles. (A) Heteromeric CI-AMPARs. (B) Homomeric CP-AMPARs.

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Could such an interaction account for all of our observations? In this scenario, the preferentially assembled heteromeric CI-AMPARs in wild-type granule cells would exit the ER, and both extrasynaptic and synaptic receptors would display properties characteristic of TARPed receptors. In stg/stg GCs, the absence of γ-2 would result in retention of GluA2-containing CI-AMPARs mediated by γ-7. This is consistent with the near complete loss of surface expression of AMPARs and a complete absence of synaptic transmission (Figure 4.10 A; Figure 3.4 A; see also (Chen et al., 2000)). In stg/stg ΔGluA2 GCs, GluA2-lacking CP-AMPARs (both TARPed and TARPless) would be free to leave the ER; thus, surface expression would be increased and synaptic transmission rescued, as observed (Figure 4.10 B; Figure 3.4 A; Figure 3.5 A). Similarly, in stg/stg Δγ-7 cells, there would be no retention of GluA2-containing assemblies; this is consistent with the observed increase in CI-AMPAR-mediated whole-cell current (Figure 4.10 A; Figure 4.3 A) and the rescue of synaptic transmission (Figure 4.5 A). In wild-type Δγ-7 granule cells, the γ-7 mediated retention of GluA2-containing receptors would be abolished. In this condition, as the requirement for γ-2 is lifted, homo- and heteromeric CI-AMPARs (homomeric GluA2 and heteromeric GluA2/4 respectively), including TARPless receptors, would more effectively leave the ER and be trafficked to the cell surface, generating the observed increase in whole-cell current (Figure 4.2 A, B). The presence of TARPless AMPARs at the synapse is suggested by the reduced mEPSC amplitude and single-channel conductance (Figure 4.4 A). The fact that the channel conductance is very low in this condition may also reflect presence of some homomeric GluA2 receptors (Swanson et al., 1997).

What about TARP overexpression? In GCs where γ-7 is overexpressed there may be insufficient γ-2 to mask the increased γ-7-mediated retention of CI-AMPARs, thus predominantly GluA2-lacking CP-AMPARs receptors would leave the ER. This would account for the greatly enhanced inwardly rectifying whole-cell current (Figure 4.7 A). The current recorded at positive potentials in these cells (Figure 4.7 C) may reflect a small contribution of γ-2/γ-7 associated CI-AMPARs or partial relief of spermine block by TARP (Soto et al., 2007). In GCs where γ-2 is overexpressed, there would be greater relief of
γ-7-mediated AMPAR retention and thus greater ER exit of heteromeric receptors, leading to the observed increase in the whole-cell current, which remained non-rectifying (Figure 4.7 B, C).
Cerebellar AMPARs in a model of Juvenile Batten disease

AMPA receptors have been implicated in numerous neurological and neurodegenerative disorders mostly ascribed to the abnormal regulation or aberrant over-expression of their calcium permeable subtypes. The Ca²⁺ influx through CP-AMPARs is thought to activate multiple processes that lead to synaptic dysfunction and subsequently to neuronal cell death. Elevated expression of CP-AMPARs has been implicated in various pathological states and disorders, including ischaemic cell death (Liu and Zukan, 2007), epileptic seizures (Grooms et al., 2000), Alzheimer’s disease (AD) (Whitcomb et al., 2015), traumatic brain injury (Spaethling et al., 2008) and amyotrophic lateral sclerosis (ALS) (Yamashita and Kwak, 2018).

The dysregulation of AMPARs and an associated increased sensitivity to AMPAR-mediated excitotoxic damage has been proposed to contribute to the cerebellar dysfunction and degeneration of cerebellar GCs in juvenile Batten disease (Finn et al., 2011; Kovács et al., 2006). In this chapter I will examine the expression, synaptic transmission and properties of AMPARs in cerebellar GCs from CLN3 knockout mice (denoted as Cln3Δex1–6 or Cln3−/−) – an animal model of juvenile Batten disease. The experiments presented here were designed to compare not only the properties of individual AMPAR channels but also to assess the synaptic transmission mediated at MF-GC synapses under near-physiological conditions.

5.1 Introduction

The neuronal ceroid-lipofuscinoses (NCLs), also known as Batten disease, represent a group of rare genetically inherited diseases. To date, there have been fourteen CLN (ceroid-lipofuscinosis, neuronal type) genes identified (Cotman et al., 2013; Mole and Cotman, 2015; Nita et al., 2016) that when mutated cause an abnormal accumulation of storage material (ceroid and lipofuscin) in various cell types. Although the build-up occurs in many cell
types, the most devastating consequences are seen with neurons, as the mutations lead to neurodegeneration. The most common form of NCL is juvenile CLN3 disease or juvenile Batten disease (Williams and Mole, 2012). Children with this condition first exhibit symptoms at 4–7 years of age, suffer loss of vision, seizures, progressive motor and cognitive decline, and die prematurely in late adolescence (Haltia, 2003; Munroe et al., 1997).

Juvenile Batten disease is caused by mutations in the CLN3 gene – currently 67 have been characterised (Mole and Cotman, 2015). The most frequent mutation is a 1 kb deletion, which removes exons 7 and 8, that result in the loss of full-length CLN3 protein (1995; Kitzmüller et al., 2008; Munroe et al., 1997). The CLN3 gene encodes a transmembrane protein consisting of 6 transmembrane domains and N- and C-termini facing the cytosol (Ratajczak et al., 2014). The exact expression and localisation of CLN3 protein remains challenging to determine due to the overall low level of protein and lack of specific antibodies (Kollmann et al., 2013; Nelson et al., 2017). Similar to other NCLs, juvenile Batten disease is considered a lysosomal storage disorder and is characterized by lysosomal autofluorescent lipopigment inclusions (subunit c of mitochondrial ATP synthase complex) (Seehafer and Pearce, 2006). The accumulated material when observed under electron microscopy forms distinctive ‘fingerprint’ patterns. While the precise function of CLN3 continues to be unresolved, the protein has been implicated in various cellular processes, including endocytosis and endocytic trafficking, lysosomal pH regulation, autophagy, proliferation, cell-cycle control and apoptosis (Cárcel-Trullols et al., 2015).

Neurodegeneration observed in juvenile Batten disease patients occurs in multiple brain areas including the cerebellum. Cerebellar atrophy is a feature of juvenile Batten disease (Autti et al., 1996; Nardocci et al., 1995) that likely contributes to the eventual motor deficits (Raininko et al., 1990). Likewise, in mouse models of the disease, there are degenerative changes and neuronal loss in the cerebellum, seen most clearly in CLN3 knockout animals (termed Cln3Δex1–6 or Cln3−/−) (Kovács et al., 2006; Weimer et al., 2009) but also evident
Cerebellar AMPARs in a model of Juvenile Batten disease

in mice with knock-in of the most common human 1 kb deletion mutation \((Cln3^{\Delta ex7/8})\) (Cotman, 2002).

Several studies have provided evidence to support the idea of AMPA receptor’s involvement in juvenile Batten disease. In \(Cln3^{\Delta ex1–6}\) and \(Cln3^{\Delta ex7/8}\) mice, cerebellar GCs – neurons in the cerebellum that relay multisensory and motor-related information from mossy fibres (MFs) to Purkinje cells (PCs) (Eccles et al., 1967) (Huang et al., 2013) (Chabrol et al., 2015) – are reported to exhibit increased susceptibility to excitotoxic damage following activation of AMPARs (Finn et al., 2011; Kovács et al., 2006). Although majority of AMPARs expressed in the CNS are calcium impermeable, CP-AMPARs constitute a widely distributed subtype with key function in several forms of plasticity (Burnashev et al., 1992; Cull-Candy et al., 2006; Geiger et al., 1995) as well as in multiple neurodegenerative disorders (Liu and Zukin, 2007; Noh et al., 2005; Whitcomb et al., 2015; Yamashita and Kwak, 2018).

Increased AMPAR-mediated excitotoxicity in \(Cln3^{\Delta ex1–6}\) mice has been suggested to reflect altered AMPAR trafficking, an increase in CP-AMPAR number and enhanced AMPAR function (Kovács et al., 2006). However, recent experiments have described an increase in the GluA2 protein in the cerebellum of \(Cln3^{\Delta ex1–6}\) mice (Kovács et al., 2015), a change which is more usually associated with increased prevalence of CI-AMPAR subtypes.

Despite the suggestions of AMPAR involvement in various juvenile Batten disease mice (Finn et al., 2011; Kovács et al., 2006), and recent evidence of increased hippocampal field excitatory post-synaptic potentials in \(Cln3^{\Delta ex7/8}\) mice (Burkovetskaya et al., 2017), to date there have been no investigations of excitatory synaptic currents in mouse models of this condition. The experiments presented in this chapter were designed to characterise the expression and properties of AMPARs in cerebellar GCs. Multiple approaches were employed to compare AMPAR subunit levels in wild-type and \(Cln3^{\Delta ex1–6}\) cerebellum, to characterise AMPAR-mediated currents in GCs and to assess synaptic transmission at MF-GC synapses in cerebellar slices.
5.2 Results

5.2.1 GluA2 and GluA4 proteins in cerebellar lysate from \(Cln3^{\Delta\text{ex1-6}}\) mice

The previously reported increased AMPAR-mediated excitotoxicity in dissociated and slice cultures of cerebellum from 8–10-day old \(Cln3^{\Delta\text{ex1-6}}\) mice was originally ascribed to altered AMPAR trafficking, and a possible increase in the number of GluA2-lacking CP-AMPARs (Kovács et al., 2006). However, the same authors more recently described an increase in GluA2 protein in the cerebellum of 1 month-old \(Cln3^{\Delta\text{ex1-6}}\) mice (Kovács et al., 2015). To investigate possible AMPAR subunit changes, we initially measured protein levels for GluA2 and GluA4 in the cerebellum from wild-type and \(Cln3^{\Delta\text{ex1-6}}\) mice in the second postnatal week – around the age when the first structural and functional defects are observed in \(Cln3^{\Delta\text{ex1-6}}\) mice (Weimer et al., 2009).

Cerebellar tissue lysates were prepared from 12 wild-type and 12 \(Cln3^{\Delta\text{ex1-6}}\) mice (P14-16). For each group, 4 samples were generated by pooling tissue from 3 littermate mice. All 8 samples were run together and the membrane probed with the relevant antibodies (mouse anti-GluA2, mouse anti-GluA4, rabbit anti-cofilin; see Methods) (Figure 5.1 A, B). The analysis of blots showed no difference in total protein for either GluA2 (0.29 ± 0.04 for wild-type versus 0.29 ± 0.06 for \(Cln3^{\Delta\text{ex1-6}}\), normalized to the intensity of the cofilin band; \(P = 0.69\)) or GluA4 (0.33 ± 0.05 versus 0.30 ± 0.07 normalized to the intensity of cofilin; \(P = 0.89\)) (Figure 5.1 C, D). Similar results were obtained when values were normalized to total protein for GluA2 (0.0054 ± 0.0009 for wild-type versus 0.0036 ± 0.0004 for \(Cln3^{\Delta\text{ex1-6}}\); \(P = 0.11\)) or GluA4 (0.0040 ± 0.0006 versus 0.0027 ± 0.0004; \(P = 0.20\)).
Figure 5.1 GluA2 and GluA4 expression levels in the cerebellum of wild-type and Cln3Δex1-6 mice are similar. (A) The representative Western blot showing the expression of GluA2 subunit in the cerebellum from wild-type (WT) and Cln3Δex1-6 mice. Each lane illustrates pooled cerebellar homogenate obtained from 3 littermate mice. The upper bands located around 100 kD denote labelling for GluA2, whereas the lower at 20 kD denote the corresponding labelling for cofilin. (B) The representative Western blot showing the expression of GluA4 subunit in the cerebellum from wild-type and Cln3Δex1-6 mice. (C) Box-plot represents pooled data for GluA2 expression normalized to mean wild-type expression. Box-and-whisker plots indicate the median value (black line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); filled black circles are data from individual cells and open circles indicate means. (D) Same as in panel C but for GluA4. n.s., non-significant; Wilcoxon rank sum test. (Modified from Studniarczyk et al., 2018).
5.2.2 AMPA-evoked currents in cerebellar GCs from Cln3Δex1–6 mice

To establish whether the functional AMPARs expressed by cerebellar GCs were affected by the loss of CLN3 protein, I recorded whole-cell currents evoked by bath-applied 20 µM AMPA during voltage ramps (−90 to +60 mV) (Figure 5.2 A). The experiment was designed to compare the magnitude of AMPAR-mediated responses and their current-voltage relationship in the presence of intracellular polyamine (500 µM spermine). The inclusion of spermine in the pipette solution allowed the surface expression of CP-AMPARs to be determined as the polyamine blocks GluA2-lacking AMPARs pore at depolarising potentials (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh et al., 1995). The mean AMPAR current amplitude at −90 mV was not different in wild-type cells compare with Cln3Δex1–6 (wild-type 131.9 ± 41.4 and Cln3Δex1–6 126.4 ± 34.7 pA, n = 10 and 13, respectively; P = 0.95) (Figure 5.2 A, B). During voltage ramps (−90 to +60 mV) both groups of cells maintained linear I-V relationship with the mean RI value of 0.91 ± 0.08 for wild-type and 0.85 ± 0.07 for Cln3Δex1–6, (n = 9 and 10, respectively; P = 0.60) (Figure 5.2 C-E). The similar amplitude and RI values suggest that CLN3 loss does not affect the number of surface AMPARs in cerebellar GCs in culture and the AMPAR currents are mediated by CI-AMPARs.
Figure 5.2 Unaltered surfaced AMPARs in Cln3Δext-6 GCs in culture. (A) A blue and red trace represents global average of whole-cell AMPA-evoked current during voltage ramp from wild-type and Cln3Δext-6 cells, respectively. Grey shaded areas denote SEM. (B) Pooled data showing unchanged whole-cell current amplitude recorded at −90 mV in Cln3Δext-6 cells. (C) Representative I-V relationship from a wild-type cell. The blue lines fitted between −40 to −20 mV and +20 to +40 mV denote the slope conductances (G_slope) for the negative and positive limbs of the I-V relationship. The slopes were used to calculate R_I_slope. (D) Same as in panel C but a representative I-V relationship recorded from a Cln3Δext-6 GC. (E) Pooled data showing similar rectification values between wild-type and Cln3Δext-6 GCs. Box-and-whisker plots indicate the median value (black line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); filled black circles are data from individual cells and open circles indicate means; n.s., non-significant; Wilcoxon rank sum test. (Modified from Studniarczyk et al., 2018).
5.2.3 Properties of synaptic AMPARs are unaltered in \( \text{Cln}^{\Delta \text{ex}1-6} \) GCs

To determine whether lack of functional CLN3 protein impacts the properties of synaptic AMPARs, I recorded miniature excitatory postsynaptic currents (mEPSCs) in cerebellar GCs in culture (Figure 5.3). Both wild-type and \( \text{Cln}^{\Delta \text{ex}1-6} \) cells displayed similar levels of synaptic activity.

![Figure 5.3 AMPAR-mediated mEPSCs in wild-type and \( \text{Cln}^{\Delta \text{ex}1-6} \) cerebellar GCs.](image)

The amplitude and frequency of mEPSCs, recorded at the negative membrane potential (–60 mV) were similar for cells cultured from wild-type and \( \text{Cln}^{\Delta \text{ex}1-6} \) mice. The mean amplitude was 10.7 ± 0.8 pA for wild-type and 9.8 ± 0.5 pA for \( \text{Cln}^{\Delta \text{ex}1-6} \) cells (\( P = 0.49 \)), whereas frequency was 3.1 ± 1.1 versus 2.5 ± 0.9 Hz (\( n = 24 \) and 28 respectively; \( P = 0.22 \)) (Figure 5.4).
Cerebellar AMPARs in a model of Juvenile Batten disease

Figure 5.4 Amplitude and frequency of miniature synaptic events remain unchanged in GCs from Cln3\(\Delta e1\)–6 mice. (A) Pooled data showing cumulative probability distribution for mEPSC amplitudes from wild-type cells. The blue trace denotes the averaged distribution. (B) Same as in panel A but for mEPSC amplitudes from Cln3\(\Delta e1\)–6 cells. (C) Box plot represents pooled data for mEPSC amplitudes with similar synaptic event amplitude values for wild-type and Cln3\(\Delta e1\)–6 cells. (D) Box plot showing unaltered mEPSC frequency in Cln3\(\Delta e1\)–6 cells (log\(_{10}\) scale). Box-and-whisker plots indicate the median value (black line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); filled black circles are data from individual cells and open circles indicate means; n.s., non-significant; Wilcoxon rank sum test. (Modified from Studniarczyk et al., 2018).

Next, to establish if the basic properties of synaptic AMPARs expressed in Cln3\(\Delta e1\)–6 cells were affected by the loss of CLN3, we closely analysed kinetics, voltage-dependence and underlying single-channel conductance of
mEPSCs. The AMPAR kinetics – rise time (10-90%) and weighted decay ($\tau_{w, \text{decay}}$) – did not differ between wild-type and $Cln3^{\Delta\text{ex}1-6}$ cells ($0.33 \pm 0.02$ versus $0.34 \pm 0.02$ ms; $P = 0.76$ and $1.27 \pm 0.10$ versus $1.42 \pm 0.12$ ms, $P = 0.57$; $n = 10$ and 8). Also, peak scaled non-stationary fluctuation analysis did not show difference in the weighted mean single-channel conductance with $11.5 \pm 1.5$ pS for wild-type cells versus $11.2 \pm 0.9$ pS for $Cln3^{\Delta\text{ex}1-6}$ ($n = 10$ and 8 respectively; $P = 1.00$; Figure 5.5). To assess the voltage dependence of synaptic AMPARs in $Cln3^{\Delta\text{ex}1-6}$ granule neurons, the rectification index ($\text{RI}_{\text{CM}}$) was calculated (see Materials and Methods) and compared with that from wild-type cells. The $\text{RI}_{\text{CM}}$ remained unchanged with $0.99 \pm 0.05$ (wild-type) and $1.05 \pm 0.08$, ($n = 12$ and 7, respectively; $P = 0.65$) (Figure 5.6). The fact that the mEPSCs remained non-rectifying and their underlying single-channel conductance remained low in $Cln3^{\Delta\text{ex}1-6}$ mice suggests that, in keeping with the data from whole-cell AMPA-evoked currents, CI-AMPARs are the predominant subtype present at GC synapses despite the absence of CLN3.

Figure 5.5 Comparable single-channel conductance values of synaptic AMPARs recorded from $Cln3^{\Delta\text{ex}1-6}$ cells. (A) Representative current-variance relationship (psNSFA) showing estimated weighted mean single-channel conductance for a wild-type cell. Symbols denote mean variance and the dashed line indicates the baseline variance. (B) Same as in panel A but for a $Cln3^{\Delta\text{ex}1-6}$ cell. (C) Pooled data illustrating similar values for single-channel conductance in wild-type and $Cln3^{\Delta\text{ex}1-6}$ cells. Box-and-whisker plots indicate the median value (black line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); filled black circles are data from individual cells and open circles indicate means; n.s., non-significant; Wilcoxon rank sum test. (Modified from Studniarczyk et al., 2018).
5.2.4 Quantal EPSCs at MF-GC synapses in \( Cln^3\Delta ex1\text{-}6 \) mice

While cerebellar GCs in culture provide an attractive model to investigate AMPAR-mediated currents, the synaptic connections formed between neurons differ from those formed in vivo. In the cerebellum, GCs receive an excitatory input from mossy fibres (MF) whereas in vitro the synapses found on GCs are from other GCs (Losi et al., 2002). To address further the effect of CLN3 loss onto AMPAR-mediated synaptic transmission, the next set of experiments were carried out in acute slices, where the original neuronal network is largely preserved and thus more relevant to the in vivo conditions.

In GCs in acute cerebellar slices spontaneous AMPAR-mediated EPSCs occur at a very low frequency. Therefore, to increase glutamate neurotransmitter release, MFs were stimulated in external solution in which \( \text{Ca}^{2+} \) was replaced with \( \text{Sr}^{2+} \) (5 mM \( \text{SrCl}_2 \)). As strontium ions trigger evoked
release less efficiently than calcium ions (Babai et al., 2014; Goda and Stevens, 1994; Miledi, 1966; Xu-Friedman and Regehr, 2000), release becomes asynchronous, allowing individual quantal events (qEPSCs) to be identified and the size and the number of quanta released per stimulus determined (Figure 5.7). The raw EPSC recordings used in the next two sections were kindly provided by Dr Elizabeth Needham.

The preliminary assessment of responses evoked by MF stimulation showed striking changes in both the initial evoked EPSC amplitude and number of subsequent quantal events in cells from mice deficient in CLN3 (Figure 5.7). In slices from Cln3Δex1–6 mice the initial EPSC amplitude was reduced from 52.3 ± 6.9 pA (wild-type) to 21.2 ± 6.3 pA (Cln3Δex1–6) (n = 6 and 7, respectively; P = 0.014) and accompanied by a reduction in number of quantal events per stimulus from 10.0 ± 2.6 to 2.5 ± 0.7 (n = 6 and 7; P = 0.0023) (Figure 5.8 A). The observed decrease in the amplitude and number of qEPSCs might reflect a change in postsynaptic AMPARs and/or presynaptic changes in neurotransmitter release.

While, the amplitude of the initial eEPSC was dramatically reduced in cells from Cln3Δex1–6 mice, the amplitude of qEPSC remained unchanged (wild-type: 14.0 ± 1.6 pA versus Cln3Δex1–6: 12.4 ± 1.5 pA, n = 6 and 7; P = 0.73) (Figure 5.8 B, C). Importantly, when the mean quantal events were normalized to the peak amplitude, they showed overlapping patterns (Figure 5.8 D), suggesting that AMPARs expressed at postsynaptic locations were not affected by the loss of CLN3. Indeed, the analysis of qEPSC kinetics showed that both 10-90% rise-time (RT10-90%; 0.34 ± 0.01 versus 0.33 ± 0.03 ms; P = 0.20) and weighted decay time (τw, decay; 2.11 ± 0.23 versus 2.79 ± 0.44 ms; P = 0.29) to be unaltered (Figure 5.8 E, F).

The apparent reduction in the number of quantal events, together with their unaltered amplitude and kinetics strongly suggest that the observed change may be associated with presynaptic impairment of transmitter release rather than postsynaptic AMPARs at MF-GC synapses in Cln3Δex1–6 mice.
Figure 5.7 Evoked quantal EPSCs at MF-GC synapses from wild-type and Cln3Δex1–6 mice. (A) Representative qEPSCs recorded from a GC from wild-type animal that were evoked by stimulation of local MFS. Symbols I-III denote three consecutive records for the cell. The highlighted in red section is enlarged and displayed below. The red dots indicate position of individual quantal events. (B) Same as in panel A but for a Cln3Δex1–6 cell. (Modified from Studniarczyk et al., 2018).
Figure 5.8 Number of qEPSC is reduced but their kinetics remained unaltered. 
(A) Pooled data showing reduced number of quantal events evoked by MF stimulation in slices from Cln3Δex1–6 mice. (B) Pooled data showing cumulative probability distribution for qEPSC amplitudes. The blue trace in bold denotes the averaged distribution for wild-type and the red trace for Cln3Δex1–6. Shaded areas indicate SEM. (C) Pooled data showing unchanged qEPSCs in Cln3Δex1–6 cells. (D) Normalized global mean qEPSCs from wild-type (blue) and Cln3Δex1–6 (red) cells showing overlapping patterns. (E) Pooled data comparing qEPSC 10–90 % rise time and (E) decay time (τ_{w, decay}). Box-and-whisker plots indicate the median value (black line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); filled black circles are data from individual cells and open circles indicate mean; ** P <0.01; n.s., non-significant; Wilcoxon rank sum test. (Modified from Studniarczyk et al., 2018).

5.2.5 Release probability – paired-pulse ratio in Cln3Δ^{ex1–6} GCs

The kinetic properties and amplitude of qEPSCs evoked by MF stimulation, suggest that the postsynaptic AMPARs remained unaffected in Cln3Δex1–6 GCs. Therefore, to determine if the observed decrease in the frequency of quantal
events indeed reflected presynaptic impairment, responses to paired stimuli were examined next.

Paired-pulse stimulation is widely used to assess changes in neurotransmitter release (Fioravante and Regehr, 2011). Assuming the postsynaptic receptor population stays unaltered in GCs from Cln3Δex1–6 mice, the paired-pulse ratio should expose any change in the probability of release. Paired-pulse eEPSC recordings were performed with MF stimulation at 5, 10, 20 and 100 Hz and the paired-pulse ratio (PPR) calculated for each condition. Both wild-type and Cln3Δex1–6 cells displayed a similar level of paired-pulse depression (PPD) (PPR < 1) across the frequency range examined (Figure 5.9). For example, at 100 Hz the PPR was 0.41 ± 0.13 for wild-type cells and 0.30 ± 0.05 for Cln3Δex1–6 cells (n = 5 and 4, respectively; P = 1.00). The comparison of PPR across all the stimulation frequencies used, showed an effect of inter stimulus interval (F3,21 = 16.88, P < 0.0001), no effect of genotype (F1,7 = 0.24, P = 0.64) and no interaction (F3,21 = 0.80, P = 0.51) (two-way repeated-measures ANOVA) (Figure 5.9).

![Figure 5.9 Paired-pulse depression at MF-GC synapses.](image)

(A) In blue, relationship between mean (PPR) and inter stimuli interval (ISI) (10-200 ms) recorded in slices from wild-type mice (n = 5). (B) Same as in panel B but recorded in slices from Cln3Δex1–6 mice (n = 4). Error bars denote SEM.
5.2.6 Short-term plasticity in ‘near-physiological’ recording conditions

While the qEPSC data clearly pointed towards a presynaptic defect at MF-GC synapses in \( Cln3^{\Delta ex1-6} \) mice, the paired-pulse stimulation did not show any differences. These seemingly inconsistent results might have been caused by the dissimilar recording conditions of the two experiments. qEPSCs were obtained in an external solution containing 0 mM Ca\(^{2+}\)/5 mM Sr\(^{2+}\) (low probability release), whereas eEPSCs were recorded in 2 mM Ca\(^{2+}\) (higher probability release). Considering that calcium ions play a fundamental role in synaptic transmission in CNS, we next chose to examine eEPSCs in two different extracellular Ca\(^{2+}\) concentrations under near-physiological conditions, specifically with minimal stimulation at elevated temperature MF-GC synapses are known to sustain high bandwidth transmission; however, the majority show an initial short-term depression during high frequency stimulation. The depression appears usually over the first few stimuli (Chabrol et al., 2015; Nieus et al., 2006; Saviane and Silver, 2006), hence a protocol of high frequency trains – 5 stimuli at 100Hz, was employed. To capture the impact of calcium ions on short-term plasticity, the cells were exposed to both standard recording solution (2 mM Ca\(^{2+}\)/ 1 mM Mg\(^{2+}\)) and ‘physiological’ (1 mM Ca\(^{2+}\)/ 2 mM Mg\(^{2+}\)) (Borst, 2010) (Figure 5.10).

As expected, wild-type cerebellar GCs exhibited short-term depression (Figure 5.10 A) with meEPSC\(_2\)/ meEPSC\(_1\) of 0.46 ± 0.07 (\( n = 6, P = 0.0028 \) versus 1). However, when the same cells were exposed to solution containing 1 mM extracellular Ca\(^{2+}\), the depression was lost (meEPSC\(_2\)/meEPSC\(_1\) was 0.81 ± 0.12; \( n = 6, P = 0.18 \) versus 1) (Figure 5.11 A). Interestingly, cells recorded in slices from \( Cln3^{\Delta ex1-6} \) mice (Figure 5.10 B) maintained paired-pulse depression in both 2 and 1 mM extracellular Ca\(^{2+}\) (2 mM Ca\(^{2+}\): meEPSC\(_2\)/ meEPSC\(_1\) 0.30 ± 0.05; \( n = 6, P = 0.0022 \) versus 1; 1 mM Ca\(^{2+}\): meEPSC\(_2\)/ meEPSC\(_1\) 0.31 ± 0.06; \( n = 6, P = 0.0028 \) versus 1) (Figure 5.11 B). Three-way repeated measures ANOVA was run to examine the effect of stimulus number, extracellular Ca\(^{2+}\) concentration and genotype on meEPSC amplitude (normalized to meEPSC1 in 2 mM Ca\(^{2+}\)).
Figure 5.10 High frequency stimulation at MF-GC connections in wild-type and Cln3Δex1-6 mice. (A) Representative averaged minimally evoked EPSCs (meEPSCs) from a wild-type GC in response to 5 stimuli at 100 Hz train recorded in two external solutions. The 2 mM Ca\(^{2+}\) trace (left) is an average of 428 sweep and 1 mM Ca\(^{2+}\) trace (right) average of 110 sweeps. The cell displayed typical short-term depression in 2 mM Ca\(^{2+}\) that was lost when cell exposed to 1 mM Ca\(^{2+}\) solution. The red arrows indicate the position of stimuli applied. (B) Same as in panel A but recorded from a Cln3Δex1-6 GC (197 and 111 sweeps). (Modified from Studniarczyk et al., 2018).

There was a significant three-way interaction, $F_{4,80} = 3.67, P = 0.0085$. The high frequency stimulation data strongly suggest that short-term plasticity at MF-GC connections is affected by loss of CLN3 when examined in the ‘physiological’ calcium concentration (1 mM). Moreover, the mean amplitude of meEPSC\(_1\) in 2 mM Ca\(^{2+}\) did not differ between wild-type and Cln3Δex1-6 cells ($77.3 \pm 26.0 \text{ pA}$ and $69.7 \pm 12.0 \text{ pA}$, respectively; $P = 0.59$), but amplitudes of meEPSC\(_1\) in 1 mM Ca\(^{2+}\) (normalized to those of meEPSC\(_1\) in 2 mM Ca\(^{2+}\)) were different ($0.42 \pm 0.06$ in wild-type and $0.82 \pm 0.07$ in Cln3Δex1-6; $P = 0.0087$).

From these results, it is apparent that the process of MF-GC synaptic transmission is affected in the mouse model of juvenile Batten disease in a manner that is strongly influenced by the extracellular calcium concentration.
Figure 5.11 Short-term plasticity at MF-GC synapses in standard and physiological calcium concentrations. (A) Global average showing normalised meEPSC amplitudes in wild-type cells in response to high frequency 5-pulse trains in 2 mM and 1 mM Ca\(^{2+}\) recording solutions. (B) Same as A but in Cln3\(^{Δex1-6}\) cells. ** \(P < 0.01\) and n.s., non-significant. (Modified from Studniarczyk et al., 2018).
5.2.7 Reduced vesicle number at MF-GC synapses of Cln3Δex1–6 mice

To determine if the presynaptic impairment observed at MF-GC connections during five-pulse trains was accompanied by structural changes we used 2D transmission electron microscopy. Mossy fibre rosettes were identified based on their specific appearance (abundant small vesicles and mitochondria), distinctive size and multiple contacts with a large number of granule cell dendrites (Rothman et al., 2016; Xu-Friedman and Regehr, 2003).

Parasagittal slices from 13-days old wild type (n = 3) and Cln3Δex1–6 mice (n = 3) were viewed under the electron microscope. The initial examination of the MF did not expose any obvious anatomical differences between two genotypes (Figure 5.12 A, B). However, the presynaptic vesicle quantification revealed reduced average density of vesicles per MF terminal in Cln3Δex1–6 mice, from 131.7 ± 8.9 to 92.6 ± 6.0 µm⁻² (n = 16 and 21 terminals; P = 0.0025). The decrease in the vesicle number was not accompanied by a change in vesicle size. The mean vesicle diameter per MF was unchanged, being 32.2 ± 0.5 nm in wild-type mice (n = 19 terminals) and 33.5 ± 0.5 nm for Cln3Δex1–6 mice (n = 20) (P = 0.094) (Figure 5.12 C). As the vesicle density in MF terminals from Cln3Δex1–6 mice was reduced, we also quantified the number of vesicles proximal to each active zone. We classified proximal vesicles as the vesicles situated within distance of 100 nm of the active zone and (due to various active zone lengths captured on electron micrographs) the average proximal vesicle number was calculated per 50 nm length of active zone. We found that the number of proximal vesicles in terminals from Cln3Δex1–6 mice was reduced by approximately 30% (from 2.70 ± 0.19 to 1.92 ± 0.16, n = 9 terminals each genotype; P = 0.013) (Figure 5.12 D). Additionally, the number of membrane adjacent vesicles – vesicles within 1 vesicle radius of the presynaptic membrane, was also reduced by approximately 40% (from 1.24 ± 0.16 to 0.71 ± 0.15 per active zone; P = 0.022 and from 0.37 ± 0.04 to 0.22 ± 0.04 per 50 nm of active zone; P = 0.034) (Figure 5.12 D).
Figure 5.12 Structural changes at MF-GC synapses in Cln3Δex1-6 mice. (A) Representative electron micrograph of MF terminal making a synaptic connection (darker area marked by arrows) with a GC dendrite (d) in a slice from a wild-type mouse. (B) Same as in panel A but from a Cln3Δex1-6 mouse. (C) Pooled data showing unchanged vesicle diameter and reduced vesicle density in Cln3Δex1-6 MF terminals. (D) Pooled data showing decreased number of vesicles proximal to active zones (AZ) and reduced number of membrane adjacent vesicles in Cln3Δex1-6 MF terminals. Box-and-whisker plots indicate the median value (black line), the 25–75th percentiles (box) and the 10–90th percentiles (whiskers); filled black circles are data from individual cells and open circles indicate means; ** P <0.01; * P <0.05; n.s., non-significant; Wilcoxon rank sum test. (Modified from Studniarczyk et al., 2018).
5.3 Discussion

An elevated expression of calcium-permeable AMPARs (CP-AMPARs) and a consequent excess calcium influx has been linked to many neuropathological conditions (Cull-Candy et al., 2006; Kwak and Weiss, 2006; Wright and Vissel, 2012). Here we sought to determine whether a change in expression of CP-AMPARs in cerebellar GCs contributes to neurodegenerative process occurring in juvenile Batten disease. Contrary to earlier suggestions (Kovács and Pearce, 2008; Kovács et al., 2006), we found that expression levels, rectification index and basic functional properties of AMPARs expressed in GCs of a mouse model of juvenile Batten disease were unaltered and synaptic transmission is mediated by CI-AMPARs, as in wild-type cells. However, the examination of eEPSCs at MF-GC synapses in standard and physiological condition revealed presynaptic changes in cells from Cln3Δex1–6 mice. Further presynaptic disruptions were detected with electron microscopy and showed a reduction in synaptic vesicle numbers. Thus, while the experiments did not detect any changes in postsynaptic AMPARs, they identified a potentially important presynaptic defect in cerebellar neurons from Cln3Δex1–6 mice.

5.3.1 Unaltered AMPARs in Cln3Δex1–6 GCs

Previous studies have reported selective AMPAR-mediates neurotoxicity in cerebellar GCs from Cln3Δex1–6 mice (Kovács et al., 2006), together with improved motor skills in these animals following administration of an AMPARs antagonist (Kovács and Pearce, 2008; Kovács et al., 2010). These authors proposed that in the absence of functional CLN3 protein, AMPAR function and surface expression is enhanced (reflecting an increase in number of CP-AMPARs) and that this contributes to cerebellar dysfunction and progressive neurodegeneration. Yet, the more recent study by the same group also reported increased levels of GluA2 subunit in slices from Cln3Δex1–6 mice and proposed a reduction in CP-AMPAR numbers (Kovács and Pearce, 2015). Neither the biochemical analysis nor the patch-clamp data presented in this chapter, support the idea of an increase or reduction in CP-AMPARs number. The quantification of GluA2 and GluA4 proteins in wild-type and Cln3Δex1–6 cerebellar lysates did not identify any difference. Likewise, the examination of
surface AMPARs, assessed by whole-cell current recordings in the presence of intracellular spermine, showed no change. Both wild-type and $Cln3^{\Delta ex1-6}$ neurons in culture produced responses of similar magnitude and with linear $I-V$ relationships typical of Cl-AMPARs. Similarly, the synaptic pool of glutamatergic AMPARs was not affected by CLN3 loss. The frequency and basic functional properties, including the current amplitude, rise time, decay time, single-channel conductance and rectification, also remained unaltered in $Cln3^{\Delta ex1-6}$ neurons. Based on these findings we conclude that the number and subunit composition, together with permeability to calcium ions, of AMPARs in cultured GCs are unaffected by the loss of CLN3 protein.

5.3.2 Quantal events reveal presynaptic changes at MF-GC synapses

To assess AMPAR properties at synapses formed in vivo, evoked EPSCs were recorded from GCs in acute slices. By replacing extracellular calcium ions with strontium, the stimulation of MFs lead to asynchronous neurotransmitter release and enabled the capture of quantal responses (Babai et al., 2014; Goda and Stevens, 1994; Miledi, 1966; Xu-Friedman and Regehr, 2000). However, in common with mEPSCs in cell culture, the mean amplitude and kinetics of the AMPARs underlying qEPSCs remained unaltered in $Cln3^{\Delta ex1-6}$ mice. Intriguingly, cells deficient in CLN3 protein displayed a profound decrease in the number of quanta released per action potential. The observed reduction might reflect a change in the probability of release and/or morphological/anatomical changes such as fewer MF terminals. Interestingly, the CLN3 protein has been detected in synaptosomal fractions (Luiro et al., 2001), axonal compartments and synaptic spines (Oetjen et al., 2016) and was proposed to play important role in endocytosis (Luiro, 2004; Uusi-Rauva et al., 2012) – suggesting a possible role in neurotransmitter release. The morphological aspect of our study, showing a reduction in quantal number also seems plausible as Weimer et al. (2009) previously reported several deficits in the cerebellum from one-week old $Cln3^{\Delta ex1-6}$ mice. These included atypical dendritic orientation of Purkinje cells, fewer large projection neurons of deep cerebellar nuclei or thinning of internal granule cell layer (Weimer et al., 2009). However, a recent study by Burkovetskaya et al. (2017) appears to argue
against such a morphological defect as the authors report increased axonal excitability in mice deficient in CLN3 (Burkovetskaya et al., 2017).

The number of quantal events was greatly reduced in cerebellar GCs from Cln3Δex1–6 mice indicating a change in release probability. Surprisingly, the reduction was not maintained when the paired pulse ratio of eEPSCs was recorded in standard recording condition of 2 mM Ca^{2+}. The discrepancy between qEPSC and eEPSC results, suggests that the change in release probability detected in Cln3Δex1–6 mice might depend on an important additional factor – namely, the extracellular calcium concentration.

5.3.3 Impaired short-term synaptic plasticity in physiological conditions

The clear relationship between extracellular calcium concentration and changes seen in MF-GC synaptic transmission in Cln3Δex1–6 mice was further examined by recording high frequency trains in standard (2 mM Ca^{2+} / 1 mM Mg^{2+}) and ‘physiological’ (1 mM Ca^{2+} / 2 mM Mg^{2+}) recording conditions. In the standard calcium concentration, the high frequency MF stimulation produced short-term depression in wild-type and Cln3Δex1–6 mice. However, when the cells were exposed to a ‘physiological’ calcium concentration (lower release probability than in 2 mM Ca^{2+}) the wild-type group showed loss of depression (Nieux et al., 2006; Saviane and Silver, 2006) while short-term depression was still maintained in Cln3Δex1–6 cells. The fact that the absence of CLN3 appeared to have a functional impact on transmission only when extracellular Ca^{2+} was reduced (‘physiological’ calcium) suggests the possibility of a deficit in Ca^{2+} handling or sensing.

Calcium ions act as a crucial messenger in neuronal cells and affects numerous cellular processes such as synaptic vesicle exocytosis, induction of activity dependent synaptic plasticity and gene transcription in the nucleus. The intracellular calcium concentration is carefully balanced by calcium influx and efflux, combined with exchange of calcium with internal stores. Calcium ions invade the cell via voltage gated calcium channels, glutamate receptors, nicotinic acetylcholine receptors and transient receptor potential type C. Its
removal occurs via plasma membrane calcium ATPase and the sodium-calcium exchanger. The main intracellular calcium source is from the endoplasmic reticulum (ER) and its high level is maintained via sarco-endoplasmic reticulum calcium ATPase (SERCA) (Grienberger and Konnerth, 2012).

Our data clearly suggest that CLN3 loss of function disrupts calcium sensing or handling. Indeed, elevated intracellular calcium levels following potassium chloride-induced depolarisation have been reported in CLN3 deficient cortical neurons. These abnormal calcium levels have been suggested to trigger cell death as application of the voltage gated calcium channel blocker – amlodipine, inhibit neuronal apoptosis (Warnock et al., 2013). Another study has suggested that calsenilin-binding CLN3 protein regulates the intracellular calcium concentration where the binding disruption resulted in increase of intracellular calcium concentration and calcium mediated cell death (Chang et al., 2007). More recently, a comprehensive study examined the effect of Cln3 gene mutation on intracellular Ca\textsuperscript{2+} handling in mouse cerebellar neuronal progenitor cells. In a series of experiments where the activity of thapsigargin (SERCA Ca\textsuperscript{2+} pump inhibitor), general ER stress response, mitochondrial store operated Ca\textsuperscript{2+} uptake and lysosomal Ca\textsuperscript{2+} stores were examined. The authors found that cells deficient in CLN3 protein are unable to maintain the cytosolic calcium homeostasis, and the frequency of spontaneous calcium spikes was increased (Chandrachud et al., 2015).

It is unclear whether Ca\textsuperscript{2+} handling in MF terminals could account for the differences in short-term plasticity between Cln3\textsuperscript{Δex1–6} and wild-type mice but our data would seem to strongly support a model of disturbed calcium management that has been previously proposed (Chandrachud et al., 2015; Chang et al., 2007; Warnock et al., 2013). Unlike wild-type, Cln3\textsuperscript{Δex1–6} cells failed to detect and respond to changes in the extracellular calcium concentration of the recording solution thus maintaining short-term depression in both standard and ‘physiological’ calcium.
If we assume that the loss of function of CLN3 disturbs the intracellular calcium homeostasis in neurons (Chang et al., 2007; Warnock et al., 2013) and that the affected cells experience spikes of high calcium concentration (Chandrachud et al., 2015) then the unbalanced calcium would influence multiple cellular processes potentially leading to neurodegeneration. Why are certain types of neurons particularly susceptible to cell damage caused by CLN3 loss? The fact that neurons require calcium entry to mediate synaptic transmission may make them particularly vulnerable. In which case, both inhibitory and excitatory neuronal cells would be expected to exhibit synaptic transmission deficits and experience high intracellular calcium concentrations leading to neurodegeneration. Indeed both excitatory and inhibitory synaptic transmission is affected by CLN3 loss (Grünewald et al., 2017)(DS, MF and SGC-C, unpublished observations).

5.3.4 Ultrastructural changes at MF terminals in Cln3Δex1–6 mice

Our EM studies of MF terminals revealed a number of structural changes in Cln3Δex1–6 mice – a reduction in the neurotransmitter vesicle density, a reduction in the number of vesicles proximal to active zones and a reduction in the membrane adjacent vesicles. The observed disturbances in vesicle numbers are not exclusive to juvenile Batten disease, as similar findings have been reported in a different NCL’s. In cortical neurons from palmitoyl-protein thioesterase-1 knockout mice (Ppt1⁻/⁻), a model of infantile CLN1 disease, a decrease in vesicle number has been described and linked with persistent membrane association of palmitoylated synaptic vesicle proteins preventing endocytosis (Kim et al., 2008; Virmani et al., 2005). Conversely, in cathepsin D knockout mice (Ctsd⁻/⁻), a model of congenital CLN10 disease, there is a reported increase at hippocampal CA1 synapses in the total vesicle number and in the number of docked vesicles (Koch et al., 2011). Thus, changes in the presynaptic vesicle pool may be a common feature of multiple NCLs.

We cannot exclude that the reduction in synaptic vesicle number observed in MF terminals of Cln3Δex1–6 mice is associated with changes in intracellular vesicular trafficking (Fossale et al., 2004; Metcalf et al., 2008; Tecedor et al.,
However, an alternative scenario could also account for the changes seen in CLN3 deficient cells. In 1 mM Ca\(^{2+}\) normalized amplitudes of meEPSC in \(Cln3^{\Delta ex1-6}\) mice were greater than those of wild-type mice. Thus the reduced vesicle numbers may constitute a compensatory mechanism to overcome the tendency towards elevated release under 'physiological' conditions, where the concentration of extracellular Ca\(^{2+}\) is thought to be closer to 1 rather than 2 mM (Borst, 2010).

### 5.3.5 Early presymptomatic changes detected in \(Cln3^{\Delta ex1-6}\) mice

The mouse model of juvenile Batten disease (\(Cln3^{\Delta ex1-6}\)) exhibits abundant accumulation of autofluorescent lysosomal storage material at ~3 months of age (Mitchison et al., 1999; Seigel et al., 2002). Although the original studies did not identify any clinical symptoms (even at 12 months) (Mitchison et al., 1999), the recent study of Grunewald et al. (2017) demonstrated that \(Cln3^{\Delta ex1-6}\) mice show increased anxiety-related behaviour, reduced learning abilities and motor deficits. These symptoms were initially detected at 7 months and became more pronounced with age. The first signs of impaired motor coordination assessed with rotarod testing were identified as early as P14 (Kovács et al., 2006), and were preceded by thinning of the cerebellar granule cell layer and Purkinje cell loss (Weimer et al., 2009). Our data obtained from P10-P15 mice, clearly suggest that there are indeed early changes in synaptic transmission in the cerebellum of \(Cln3^{\Delta ex1-6}\) mice. Since the presynaptic deficits were identified in the presymptomatic phase, they likely reflect the initial causative changes rather than degenerative processes.

It remains unclear whether the observed changes in synaptic transmission are a direct consequence of CLN3 loss or an effect of multiple cellular processes being disrupted. It is noteworthy, that the absence of CLN3 significantly impacts the expression of 756 genes in the cerebellum (Brooks et al., 2003) and affects the levels of various proteins in other brain regions (Llavero Hurtado et al., 2017). Considering how extensive these changes are, there is high probability of multiple cellular processes being disrupted. Nevertheless, our findings in a mouse model of juvenile CLN3 disease complement
molecular, structural, and functional studies in various animal models of infantile CLN1 disease (Kielar et al., 2009; Kim et al., 2008; Pezzini et al., 2017; Virmani et al., 2005), late infantile CLN6 disease (Kielar et al., 2009), congenital CLN10 disease (Koch et al., 2011), and late infantile CLN5 disease (Amorim et al., 2015), and suggest that early synaptic alteration is a characteristic feature of NCLs.

5.3.6 Summary

Although the precise mechanism underlying synaptic changes in Cln3Δex1–6 mice remains unclear (Cárcel-Trullols et al., 2015) our findings provide a better understanding of the early changes in juvenile Batten disease. We have identified a presynaptic release defect apparent in 1 mM extracellular Ca^{2+} that is expected to be relevant in vivo (Borst, 2010). The loss of CLN3 protein not only affects neurotransmitter release at MF-GC synapses but has also been shown to affect excitatory and inhibitory synapses in amygdala and hippocampus (Grünewald et al., 2017). It is noteworthy, that our findings were obtained in 2-week-old mice (that lack of clinical symptoms), whereas those of Grunewald et al. (2017) were from 14-month-old Cln3Δex1–6 mice – an age when the mice display clinical symptoms. Our functional and EM data capture changes in the presymptomatic phase of the disease, thus they are unlikely to reflect early degenerative processes. Importantly, contrary to previous reports (Finn et al., 2011; Kovács et al., 2015; Kovács et al., 2006) our biochemical and patch-clamp results did not reveal any changes in AMPAR function, expression or subunit composition. Instead of postsynaptic changes, the data provide compelling evidence for presynaptic defect in Cln3Δex1–6 mice. Rather, our data provide strong evidence for a presynaptic defect in a model of juvenile Batten disease.
Chapter 6 Appendix: The structures of TARPed AMPARs

Single-particle cryo-electron microscopy (cryo-EM) and X-ray crystallography have provided substantial insight into three-dimensional organization of AMPARs, in their various functional states and in association with auxiliary subunits. Here, I present a brief overview of AMPAR structures, including those obtained recently with TARPs.

As described in the Introduction, AMPARs exist as tetrameric assemblies where each subunit consists of four modules: amino terminal domain (ATD), ligand binding domain (LBD), channel pore-forming transmembrane domain (TMD) and cytosolic C-terminal domain (CTD). The structure of full-length receptors resembles a capital Y with its broad and narrow ‘faces’ (Figure 6.1A) (Chen et al., 2017; Sobolevsky et al., 2009; Zhao et al., 2016). The AMPAR tetramers are assembled with two pairs of diagonally opposed subunits denoted as A/C and B/D, that display differential symmetries at the respective receptor domains (Figure 6.1B). Thus, the ATD and LBD adapt 2-fold symmetry, whereas the TMD 4-fold (Figure 6.1B) (Sobolevsky et al., 2009). In heteromeric receptor assemblies, the GluA2 subunits were shown to occupy preferentially positions B/D (He et al., 2016; Herguedas et al., 2019; Zhao et al., 2019).

At the level of ATD, the receptor subunits are arranged as A/B and C/D dimers with additional cross-dimer interface between B and D subunits. The subunits in the position B/D are proximal to the pore axis whereas the A/C subunits are distal (Figure 6.1B) (Sobolevsky et al., 2009).

Immediately below the ATD is the LBD, also arranged as non-equivalent pairs of A/C and B/D dimers within an overall 2-fold symmetric LBD ‘gating ring’. However, as a consequence of domain swapping the subunit arrangement differs compared with the ATD. The local dimer pairs are switched to A/D and B/C, and the cross-dimer interface is positioned between subunits A and C. The A/C subunits are now proximal to the pore axis whereas the B/D subunits are distal (Figure 6.1B) (Sobolevsky et al., 2009). Each LBD adopts a
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‘clamshell’ structure, with upper D1 and lower D2 lobes, where the D1 lobes of adjacent subunits dimerize. Upon glutamate binding the clamshells close, causing separation of lower D2 lobes which mechanically force the channel gate – formed by four M3 helices – to open. The motion is mediated by the direct connection between D2 lobe and the transmembrane domain M3. With continued agonist exposure the receptor enters a desensitised state that involves the rupture of the interface between the D1 lobes.

The domain swapping continues further into the hydrophobic TMD, where the subunits are organised with 4-fold symmetry. The TMD of each subunit is formed out of three helical segments M1, M3, M4 and a cytoplasm facing re-entrant loop M2 with the Q/R editing site, that controls channel permeability to calcium ions. The TMD is linked with LBD via flexible polypeptide linkers S1-M1, M3-S2 and S2-M4, essential to accommodate domain swapping. The M1 and M4 segments are peripherally located from the channel pore, whereas the M2 loop faces the pore. The Q/R editing site is orientated in such a way that the side chains point into the channel pore beneath the channel gate formed by the crossover of M3 helices (Figure 6 E).

Native AMPAR complexes exists predominantly in combination with auxiliary subunits such as TARPs that actively shape receptor properties (Jackson and Nicoll, 2011a; Greger et al., 2017). The initial structures of AMPARs in association with TARPs were of homomeric GluA2/γ-2 receptors in the presence of the antagonist MPQX (Twomey et al., 2016; Zhao et al., 2016). AMPARs are thought to associate with between one and four TARP γ-2 subunits. Analogous to core AMPAR subunits, TARPs are also arranged in two non-equivalent pairs termed A’/C’ and B’/D’ (Figure 6.1C). At the TMD level, TARPs interact with AMPARs via transmembrane domains TM3 and TM4 that are situated against receptor helices M1 and M4. This interaction is thought to influence receptor conductance and rectification. At the LBD level, the interaction is mediated by the two extracellular loops Ex1 and Ex2, that form a five-stranded beta sheet that engage with LBD (Figure 6.1D) (Twomey et al., 2016; Zhao et al., 2016).
Appendix: The structures of TARPed AMPARs

Figure 6.1 AMPAR structure from X-ray crystallography and cryo-EM. (A) View of the broad (left) and the narrow (right) faces of the receptor with its distinctive layered arrangement of domains: ATDs, LBDs and TMDs. Each subunit is shown in a different colour. (B) A view of receptor domains from the top together with a schematic indicating the subunit arrangement. As a consequence of domain swapping, each domain section displays a different subunit arrangement and symmetry. The dimer-to-dimer interactions are labelled as large black ovals, the intradimer interactions as smaller ovals. Both the ATD and LBD layers have two-fold symmetry while the TMD layer has four-fold. (C) View of broad (left) and narrow (right) faces of the receptor associated with two γ-2 subunits (STZ). (D) The 3D structure of a γ-2 subunit: the extracellular loops form a five-stranded beta sheet. (E) A schematic of AMPAR/γ-2 illustrating the interaction between TARP and AMPAR subunit with agonist bound. TARPs are thought to physically limit the extent of LBD movement towards the membrane thus maintain the tension on the M3 linkers acting on the channel gate. Modified from (Chen and Gouaux, 2019; Sobolevsky et al., 2009; Twomey et al., 2016).
The cryo-EM structures of GluA2/γ-2 in a ligand bound state provided insight into how TARPs modulate AMPAR properties (Chen et al., 2017; Twomey et al., 2017a). TARP subunits, positioned underneath LBD clamshells, prevent the movement of LBD D2 domains towards the membrane, thus slowing the closing of the channel gate (Figure 6.1E).

Neuronal AMPAR assemble primarily as heteromers. The recent work by Greger’s group (Herguedas et al., 2019) determined the structure of the GluA1/2 receptor associated with two γ-8 subunits. They showed that GluA2 preferentially occupies position B/D and that the first extracellular loop of γ-8 interacts with the upper lobe of GluA2 LBD. In the transmembrane region, the M2 pore loops, with Q/R editing site at their apexes, constrain the channel pore. The R586 side chains of GluA2 subunits project into the cavity above the selectivity filter, hence control calcium ion permeation and polyamine block. The GluA1 Q582 chains line the entrance to the selectivity filter formed by pore loops.

The functional data presented in Chapter 3 and Chapter 4, not only confirm the important role of TARPs in the AMPAR trafficking but also highlight the AMPAR subtype-specific TARP regulation (of Cl- and CP-AMPARs). As the calcium permeability is controlled by mRNA editing of the GluA2 subunit, an important question arises: does the Q/R site affect the interaction with TARPs? The recent cryo-EM findings provide detailed 3D structures of the edited GluA2(R) homomers in association with γ-2 (Figure 6.2A) (Chen et al., 2017) and the unedited GluA2(Q)/γ-2 (Figure 6.2B) (Twomey et al., 2017a). Both structures capture the receptor in the agonist bound state in the presence of a desensitisation blocker.
Figure 6.2 Structures of homomeric GluA2 receptors co-assembled with γ-2 subunits. (A) A transmembrane region of edited GluA2 receptor in the complex with γ-2 subunits. The image shows only the GluA2 subunits that occupy the positions B and D (cyan and yellow), accompanied by γ-2 subunits at the corresponding positions B’/D’ (purple and pink). The Q/R editing sites with arginine face channel pore (red circle). PDB code: 5VOT (Chen et al., 2017) (B) Same as in panel A but for the unedited GluA2 receptor. PDB code: 5WEO (Twomey et al., 2017a).

Comparison of these structures using PYMOL 2.3.2 software does not reveal any striking differences (see Figure 6.2). The spatial arrangement of γ-2 TM1-4 helices towards the core receptor M1-M2 helices seems to be similar in both
GluA2(R) and GluA2(Q) assemblies. It is highly unlikely that a single amino acid change in the pore would be in the position to affect the AMPAR/TARP interface. Nevertheless, the recent findings suggest that a GluA2 subunit, the subunit controlling calcium permeability, preferentially occupies position B/D in the heteromeric receptor assemblies (He et al., 2016; Herguedas et al., 2019; Zhao et al., 2019). Interestingly, the site (B'/D') is also favoured by TARPs γ-2 (Twomey et al., 2017b) and γ-8 (Herguedas et al., 2019) as well as GSG1L (Twomey et al., 2017b) rising a possibility that the receptor-auxiliary subunit interactions at transmembrane region are particularly important. Additionally, to date, there is no clear evidence to suggest that the positioning of type I and type II TARPs in the AMPAR might differ, and further studies are necessary to address this.
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