UNIVERSITAT DE BARCELONA

New insights into ionotropic glutamate receptors in physiological and pathological conditions

Federico Javier Miguez Cabello

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Tesi doctoral presentada per

Federico Javier Miguez Cabello

Programa de Doctorat en Biomedicina per la Universitat de Barcelona
Laboratori de Neurofisiologia, Departament de Biomedicina, Universitat de Barcelona

Desembre 2020

Treball realitzat sota la direcció del Dr. David Soto del Cerro i el Dr. Xavier Altafaj Tardío

Tutoritzat pel Dr. Xavier Gasull Casanova

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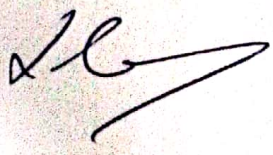

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CERTIFIQUEN:

Que el treball d'investigació corresponent a la tesi doctoral que porta per títol "New insights into ionotropic glutamate receptors in physiological and pathological conditions" del que és autor Federico Javier Miguez Cabello, ha estat realitzat sota la seva direcció al departament de Biomedicina de la Universitat de Barcelona.

I per a que així consti i als efectes oportuns, firmem el present certificat a Barcelona, el/de Desembre de 2020.

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INTRODUCTION

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1. Introduction

1. Introduction

Speaking metaphorically, the human nervous system is a complex ensemble of millions of parts operating together one with another. For this reason, each part is fundamental for the correct function of the whole system. In fact, it is not possible to understand the whole system without knowing about its components.

In this thesis project we have tried to give new insights into the ionotropic glutamate receptors field, which are a small but essential part in understanding how the nervous system works.

1. Glutamate: a non-essential amino acid but a key neurotransmitter

Glutamate is nowadays recognized as the most predominant excitatory neurotransmitter in central nervous system (CNS). Nevertheless, it was not until the middle of the XX century when this amino acid was recognized to be important in brain function: Hayashi and colleagues showed that exogenous glutamate application directly into the brain produced convulsions in treated animals (T. Hayashi 1954). The late discovery of glutamate as neurotransmitter contrasts with that of, for example, acetylcholine, which was described to be a chemical mediator of neurotransmission in the nervous system 40 years earlier. Indeed, this discovery by Henry Dale and Otto Lewi, permitted both researchers to win the Nobel Prize for their work about the chemical transmission of nerve pulses.

However, the role of glutamate in neurotransmission, disease and neurodevelopment have an extensive history. In the middle of the XX century early research showed that brain tissue possesses high concentrations of glutamate and glutamate uptake activity (Krebs 1935; Stern et al. 1949). Nevertheless, it was not enough to talk about glutamate as neurotransmitter and the idea of an essential amino acid acting as a chemical signal was not yet conceived.

As mentioned before, the first observation for a role of glutamate in neuron communication came from Hayashi‘s observations. With his work he realized that

glutamate injections into brain produced convulsions in treated animals. Due to that fact it was proposed that this amino acid was probably acting as a neurotransmitter (T. Hayashi 1954). Following this discovery, it was observed that L-glutamate on single cells *in vivo* produced excitatory effects. However, many years and various studies were needed to finally recognize this amino acid as a synaptic neurotransmitter.

During the following years, many experiments showed light into recognition of the glutamate receptors (GluRs) diversity. A key step was the synthesis of specific agonists related to glutamate. The first specific agonist was D-isomer of N-methyl-aspartate (NMDA), which showed 10 fold more potency than L-glutamate and was used to name a subclass of GluRs, the NMDA receptors (NMDARs) *vs.* the non-NMDARs, which showed less responses compared with the former group (Curtis, Phillis and Watkins 1960).In 1977 Dick Evans, made another key step in GluRs research. He observed that Mg^{2+} and other divalent cations limited channel conductance in a voltage dependent manner from these recent discovered receptors (for review see Watkins and Jane 2006). Furthermore, it was discovered later that glycine is required as a co-agonist for the NMDAR (J W Johnson and Ascher 1987).

In addition, due to the similarity of kainic acid with glutamate, Shinozaki and Konishi identified this compound as an excitatory molecule in CNS. They tested its effect and demonstrated that kainic acid can act as an excitatory molecule in the nervous system (Shinozaki and Konishi 1970). Later, similar to kainic acid, quisqualic acid was discovered to be a potent excitatory agent and a group of receptors were named as quisqualate receptors (Shinozaki and Shibuya 1974). By that time, as mentioned before, GluRs where classified as NMDARs and non-NMDARs, where the latter were subdivided into quisqualate and kainate receptors. Following up, quisqualate receptors were renamed as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (AMPARs) (Krogsgaard-Larsen et al. 1980) after some experiments that showed that the synthetic agonist AMPA could excite neurons through different receptors than kainate receptors. Then, after Krogsgaard work, finally three classes of glutamate receptors were established: NMDA, AMPA and kainate receptors, named after their prototypical agonists (Monaghan, Bridges, and Cotman 1989).

1. Introduction

Through the '90s, the development of molecular biology techniques permitted an important step forward in the glutamate receptor research field. Cloning techniques were key to reveal the amino acid sequence of GluRs and to start elucidating their function. In 1989 Steve Heinemann and Michael Hollmann cloned and sequenced for the first time a glutamate receptor subunit. This subunit was named GluR1-K1 because it was activated when kainic acid was used as agonist (Michael Hollmann et al. 1989). Later, it was confirmed that the cloned subunit by Steve Heinemann and Michael Hollmann belonged to an AMPA-selective family of glutamate receptors. Subsequently, more AMPAR/kainate subunits were cloned by distinct laboratories in a short period of time and named as GluR1-4 or GluRA-D. Later, GluR5-7 were sequenced and named. These gene products showed a kainate preference (it was later seen that they could form kainate receptors) and possessed near 70% homology between them. The cloned subunits with higher kainate affinities were further named as KA1 and KA2. In parallel, NMDARs subunits were cloned between 1991 and 2002 by different groups. In addition, several proteins were cloned and showed glutamate binding properties (δ1 and δ2) although being unable to form functional channels in combination with other subunits (Lodge 2009).

Finally, in 2008 the International Union of basic and clinical pharmacology (IUHPAR) agreed the new and final ionotropic glutamate receptors nomenclature which has been used to date (Collingridge et al. 2009; Table 1).

IUPHAR subunit nomenclature	Previous nomenclatures
AMPA receptors	
GluA1	GLUA1, GluR1, GluRA, GluR-A, GluR-K1, HBGR1
GluA2	GLUA2, GluR2,GluRB, GluR-B, GluR-K2, HBGR1
GluA3	GLUA3, GluR3,GluRC, GluR-C, GluR-K3
GluA4	GLUA4, GluR4,GluRD, GluR-D
Kainate receptors	
GluK1	GLUK5, GluR5, GluR-5, EAA3
GluK2	GLUK6, GluR6, GluR-6, EAA4
GluK3	GLUK7, GluR7, GluR-7, EAA5
GluK4	GLUK1, KA1, KA-1, EAA1
GluK5	GLUK2, KA2, KA-2, EAA2
NMDA receptors	
GluN1	GLUN1, NMDA-R1, NR1, GluRξ1
GluN2A	GLUN2A, NMDA-R2A, GluRε1
GluN2B	GLUN2B, NMDA-R2B, hNR3, GluRε2
GluN2C	GLUN2C, NMDA-R2C, GluRε3
GluN2D	GLUN2D, NMDA-R2D, GluRε4
GluN3A	GLUN3A, NMDA-R3A, NMDAR-L, chi-1
GluN3B	GLUN3B, NMDA-R3B,
Delta receptors	
GluD1	GluRδ1
GluD2	GluRδ2

Table 1. Nomenclature of ionotropic glutamate receptors family

When glutamate was identified as a neurotransmitter, it was first assumed that the neurophysiological effects of this amino acid were mediated exclusively by ionotropic receptors. However, Sladeczek and colleagues in 1985 changed this point of view and showed that glutamate could also mediate signalling cascades, throughout second messenger systems (Sladeczek et al. 1985). After this work, Nakanishi's laboratory cloned in 1992 the first metabotropic glutamate receptor. Subsequent studies, unravelled up to eight mammalian metabotropic glutamate receptors sharing a

common structure (Masu et al. 1991).

To date, many studies have revealed a vast range of GluRs functions beyond the transmission of excitatory postsynaptic currents (EPSCs) between neurons. Indeed, iGluRs are critical in early stages of brain development (Balázs, Jørgensen, and Hack 1988; Wilson and Keith 1998; Komuro and Rakic 1993) and their dysregulations has been also associated with several pathological conditions resulting from their persistent or overwhelming activation (Cull-Candy, Kelly, and Farrant 2006; Isaac, Ashby, and McBain 2007; S. J. Liu and Zukin 2007). Figure 1 shows in a chronological axis the fundamental findings in this research mentioned before.

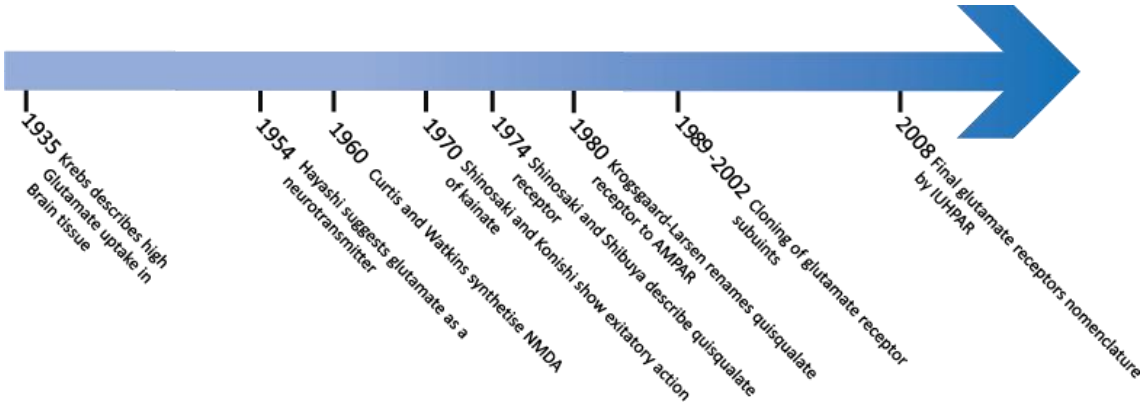


Figure 1. Milestones in iGluR research

2. Glutamatergic transmission

In the CNS, neuron-to-neuron communication occurs in highly specialized structures called synapses. This communication involves two main players: the neurotransmitter and the receptor. The chemical signal (neurotransmitter) is released by a first neuron (known as presynaptic neuron). The receptors for neurotransmitters are located post-synaptically and consist on transmembrane proteins that permit the transduction of an external chemical message in an electrical signal "understandable" for the postsynaptic neuron.

2.1 Neurotransmitter release

Neurotransmitter release occurs at the presynaptic terminal and it can be synchronous or asynchronous. The synchronous release occurs in a period of time of milliseconds after an action potential invades the presynaptic button. On the other hand, the asynchronous release persists for tens of milliseconds to tens of seconds after an action potential arrives at the presynaptic terminal. In addition, spontaneous neurotransmitter release can occur in absence of presynaptic depolarization (Kaeser and Regehr 2014). Each of these mechanisms are important in nervous system physiology. However, just to briefly introduce the basic ideas in neurotransmission release, the following lines will be focused in synchronous release, which is the mostly studied neurotransmitter release.

At the presynaptic button, where neurotransmitters are stored in vesicles, synchronous neurotransmitter release is triggered by action potential arrival and occurs in a short period of time (in the milliseconds range). The upcoming of the amount of positive charges makes voltage-gated Ca^{+2} channels open briefly inducing a sharp local rise of Ca^{+2} in the presynaptic button. This transient Ca^{+2} influx in turn allows synaptic neurotransmitter vesicles fusion with the presynaptic membrane, with a delay of less than a millisecond. This rapid Ca^{+2} entry leads to fast release of neurotransmitters (reviewed by Kaeser and Regehr 2014). Synchronous release requires SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins) and Sec-1/Munc18 (S/M) proteins for fusion of synaptic vesicles with the presynaptic plasma membrane. More specifically, the increase in Ca^{+2} is sensed by the membrane-trafficking protein Synaptotagmin (Geppert et al. 1994) causing the vesicles to fuse with the presynaptic membrane and the release of neurotransmitter into the synaptic cleft.

Several substances are released into the synaptic cleft. Nevertheless, the term "neurotransmitter" would only refer to those compounds that fulfil the following criteria (Inoue 2009):

- A neurotransmitter must be synthesized in a neuron and released from a presynaptic terminal.

- Neurotransmitters should reproduce the specific responses evoked by a stimulation of the presynaptic neurons at the postsynaptic neuron or effector cells.
- Pharmacologically, their effect should be possible to block by antagonists in a dose-dependent manner.
- Neurotransmitters should be reabsorbed into by the presynaptic neuron or glia, or metabolized into an inactive form by enzymes to terminate the stimulation.

2.2. The postsynaptic response

Once the neurotransmitter is released by the presynaptic neuron, it exerts its signalling function by acting over the receptors at the postsynaptic neuron. Besides its effect on the receptors present at the postsynaptic site, they can also activate receptors present at the extrasynaptic region. Moreover, the same neurotransmitter can activate a wide range of different receptors that might be differentially distributed between synaptic and extrasynaptic sites.

An easy way to classify this large number of receptors is using their mechanism of action. Receptors that are ligand-opened channels (also called ionotropic receptors) allow ions to flow through membrane (inside or outside the cell) when a neurotransmitter binds in their ligand binding site. On the other hand, metabotropic receptors interact with other intracellular proteins and start a signalling cascade upon neurotransmitter binding. Thus, due to their intrinsic characteristics, ionotropic receptors have the capability to perform fast responses due to neurotransmitter release (millisecond range) while metabotropic receptors produce slower responses compared to ionotropic ones (seconds to minutes) but they amplify neurotransmitter-mediated response.

However, both groups of receptors act basically in the same manner if we look at their essential role. Basically, both are in charge to transmit a signal from outside the neuron to be integrated by this cell.

3. Glutamate receptors

The fast communication is carried out by the ionotropic receptors upon their binding to the neurotransmitter and their opening. One of the most important families of ionotropic receptors are the glutamate receptors due to the great importance of this neurotransmitter in brain function.

On the other hand, glutamate can elicit responses from a group of metabotropic receptors. They are classified as metabotropic glutamate receptors (mGluRs) and mediate slower but amplified signals as other metabotropic receptors.

3.1. Metabotropic receptors

Mentioned before, the role of metabotropic receptors differs from ionotropic receptors due to their mechanistic features. The last ones mediate fast responses and are ligand gated channels. By contrast, metabotropic receptors mediate amplified but slow responses by interacting with other proteins. In terms of glutamate signalling, metabotropic glutamate receptors (mGluRs) play also important roles in brain function but different from ionotropic glutamate receptors. They belong to the family of G protein-coupled receptors (GPCRs) and promote intracellular variations of second messenger levels upon activation (Pin, Galvez, and Prézeau 2003; Conn and Pin 1997). Moreover, it has been also revealed a multitude of additional intracellular components that assemble as scaffolding proteins around these type of glutamate receptors (reviewed at U. Gerber, Gee, and Benquet 2007).

Structurally, mGluRs are dimers composed by two cross-linked subunits by a disulphide bridge. Each dimer possesses 7 transmembrane domains and a long C-terminal domain. The dimer formation is crucial for mGluRs function (Kniazeff et al. 2011).

To date, eight mGluRs have been identified and classified into three subgroups depending on their sequence similarity and their capability to modulate phospholipase C or adenylyl cyclases. mGluR1 and 5 are classified into Group I mGluRs and couple to $G\alpha_{q/11}$ promoting

Ca^{+2} release from intracellular stores. They are located mostly at perisynaptic region in the postsynaptic terminal, surrounding ionotropic receptors and modulating neuronal excitability. mGluR2 and 3 belong to Group II mGluRs and mGluR4, 6, 7 and 8 are part of Group III mGluRs. Groups II and III negatively regulate adenylyl cyclase via $G\alpha_i$ and are localized, principally, at the presynaptic terminal. These two groups act as autoreceptors that inhibit glutamate or GABA release (see Ribeiro et al. 2017 for review).

Finally, it is worth to mention that, similarly to iGluRs, mGluRs are also involved in synaptic plasticity processes. At this aspect, in certain brain areas LTP and LTD induction has been reported to be dependent on mGluR activity (Oliet, Malenka, and Nicoll 1997).

3.2. Ionotropic glutamate receptors

Mentioned at the beginning of the introduction, in terms of fast synaptic transmission glutamate activates principally three types of receptors. These receptors are globally classified as ionotropic glutamate receptors (iGluRs) family with many commonalities and classified upon pharmacological and electrophysiological properties. The iGluRs family groups are as follows:

- α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (AMPARs)
- N-methyl-D-aspartate (NMDA) receptors (NMDARs)
- Kainate (KA) receptors (KARs)

The iGluRs share structural similarities, resulting from the conservation of topological domains. iGluR receptor subunits possess four transmembrane domains (TMDs) spanning the plasma membrane (TMD I-IV) with a TMDII domain that forms a re-entrant loop and do not fully crosses the lipidic bilayer. This fact gives to these receptor subunits an extracellular N-terminus (NTD) and an intracellular C-terminus (CTD). Moreover, they are all formed by tetrameric association of iGluR subunits. In addition, iGluRs share the property of being cationic channels, and are functionally involved in higher brain functions (Traynelis, Wollmuth, McBain, et al. 2010; Kumar and Mayer 2013). The figure 2

schematizes the topological structure of a prototypical subunit and a tetrameric iGluR through membrane.

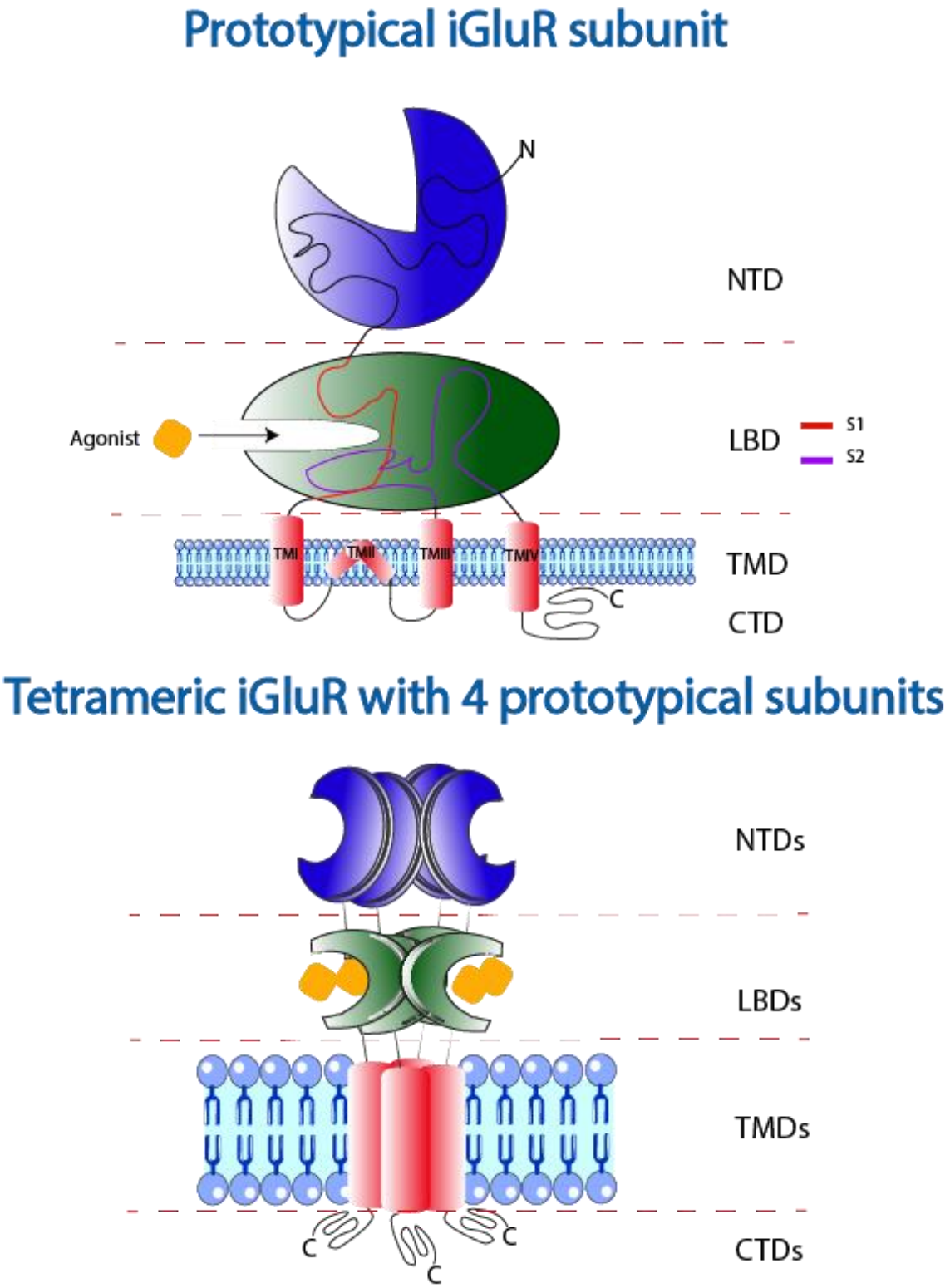


Figure 2. Scheme of the prototypical iGluR subunit and tetrameric assembly. Upper panel: the prototypical iGluR subunit with a large extracellular NTD, 4 TMD with a re-entrant TMDII and an intracellular CTD. NTD is

followed by the ligand binding domain (LBD) which is formed by S1 and S2 domains (in red and purple respectively). Lower panel: 4 prototypical subunits forming an iGluR.

3.3. Kainate receptors

As this thesis is focused in AMPA and NMDARs the following lines will make a brief introduction on kainate receptors.

Kainate receptors (KARs) form tetrameric combinations of GluK1-5 subunits. Like the other members of the iGluRs family, KAR subunits share the same topological structure. As AMPARs, KARs can form homotetramers or heterotetramers. Homotetramers can be composed by solely GluK1, GluK2 or GluK3 while GluK4 and GluK5 form obligatorily heterotetramers with GluK1-3 subunits. On the other hand, GluK1-3 and GluK4-5 can be differentiated by their affinity to agonist, which is lower for GluK1-3, compared to GluK4-5. In addition, mRNA editing at Q/R site in TMDII of GluK1 and 2 and alternative splicing of GluK1-3 sharply increases KARs possible combinations. On the other hand, GluK4-5 seems not to present variations due to alternative splicing (reviewed by Lerma and Marques 2013).

Interestingly, KARs have been shown to display special characteristics in terms of signalling. Compared to AMPARs, KARs are restricted to fewer synapses and their role in CNS is currently considered to be more modulatory, especially for those KARs localized at pre-synaptic terminals (Lerma et al. 2001; Huettner 2003; Pinheiro and Mulle 2006). At the postsynaptic level, contrary to AMPARs, KARs show small amplitude and slow kinetics. These kinetics are modulated by Neto proteins (KAR auxiliary proteins) and are important to modulate short-term plasticity, input integration and brain rhythms (Frerking and Ohliger-Frerking 2002; Goldin et al. 2007; Straub et al. 2011; Sylwestrak and Ghosh 2012). Moreover, in terms of KAR-mediated signalling, these receptors are capable to inhibit voltage-dependent K^+ channels that are responsible of K^+ outflow postsynaptically (Melyan, Wheal, and Lancaster 2002). That modulates firing frequency due to changes in membrane potential what finally is translated in changes of network excitability (Ruiz et al. 2005; Chamberlain et al. 2013).

On the other hand, KARs can also act presynaptically. KARs have shown capability to modulate both, excitatory and inhibitory synapses. For example, at CA1 and CA3 areas from hippocampus, KARs can inhibit GABA release, modulating inhibitory synapses (A Rodríguez-Moreno, Herreras, and Lerma 1997; Antonio Rodríguez-Moreno and Lerma 1998; Vignes et al. 1998). At excitatory synapses, KARs can modulate neurotransmission from mossy fibers to CA3 region (D. Schmitz et al. 2001; Contractor, Swanson, and Heinemann 2001).

An interesting and unique feature of KARs (among the iGluR family) is their ability to act both as ionotropic and/or metabotropic receptors. KARs are able to activate directly phospholipase C (PLC), protein kinase C (PKC) and G_o protein cascades, but not only due to ion influx like as AMPARs and NMDARS (Antonio Rodríguez-Moreno and Lerma 1998; A. Cunha, Malva, and Ribeiro 2000; Rozas, Paternain, and Lerma 2003).

Finally, this glutamate receptor in physiological conditions has shown to be implied in CNS disorders. On the one hand, GluK2 has been related in obsessive-compulsive disorder, schizophrenia and autism after comparing KAR amounts in diseased post-mortem tissue (Delorme et al. 2004; Bah et al. 2004; Strutz-Seebohm et al. 2006; S. A. Kim et al. 2007). On the other hand, GluK1 and GluK3 can be susceptible factor in depressive disorders (Schiffer and Heinemann 2007; Paddock et al. 2007). In addition, the alteration of KAR signalling has been linked to some types of mental retardation and episodic seizures (for extensive review see Derek Bowie 2008).

4. AMPARs

One of the most studied and the first cloned receptors of the iGluRs are the AMPARs. These receptors are responsible for the major fast excitatory neurotransmission in CNS (Palmer, Cotton, and Henley 2005). As for the other members of the inotropic glutamate receptors family, AMPARs consist on tetrameric structures formed by a combination of GluA subunits (named GluA1-4).

Physiologically, AMPARs are crucial for the correct development and normal function of the nervous system. Concomitantly, AMPAR dysfunction has been associated with multiple neurological conditions, such as epilepsy, addiction, amyotrophic lateral sclerosis, glioblastoma and others (Cull-Candy, Kelly, and Farrant 2006). Due to their importance, the understanding of the electrophysiological behaviour (biophysical and gating properties) of these receptors is needed to fully understand the synaptic transmission and neuronal physiology processes.

4.1. AMPAR Structure

Previously explained, AMPARs are tetrameric structures composed by 4 GluA subunits and they assemble as a dimer of dimers (Greger and Esteban 2007). The subunits that can form the receptors are named as GluA1-4 and they all have the same membrane topology although they have differences between them, especially at the intracellular C-terminal domain. Different from NMDARs but similar to KARs, AMPARs can be assemble by combination of different subunits forming a heterotetramer or by the same subunit forming a homotetramer (except by the GluA2 subunit).

In the first extracellular part it is found the **N-terminal domain (NTD)** which is composed by near 400 amino acid residues, with 14-33 residues that form a short signal peptide (Traynelis, Wollmuth, Mcbain, et al. 2010). Crystal structures have shown that separately expressed GluA1/A4 NTDs (Jin et al. 2009; Clayton et al. 2009) and tetrameric GluA2 (Sobolevsky, Rosconi, and Gouaux 2009) receptors form a “Venus flytrap” structure. In native receptors it mediates interactions with synaptic proteins as N-cadherin (Passafaro, Nakagawa, and Sala 2003) and neuronal pentraxins (Sia et al. 2007). Although less is known about the implications of the NTD in receptors biophysics it has been published that NTD deletion slows desensitization time and accelerates recovery from desensitization state, which results on increased steady state currents that can trigger glutamate-induced excitotoxicity in HEK293 cells (Möykkynen et al. 2014). Moreover, it has been

demonstrated recently that NTD plays a key role in synaptic transmission and plasticity by anchoring AMPAR at synapse (J. F. Watson, Ho, and Greger 2017).

Following the NTD starts the first part of the **ligand binding domain (LBD)** which is formed by the so-called S1 and S2 loops. These loops are located after the NTD and between TMDIII and IV respectively (Stern-Bach et al. 1994). At the end of S2 loop there is the flip/flop cassette which is determined by alternative splicing. These splice variants confer to the receptor different kinetics. The flop form generally produces fast desensitization kinetics and the opposite happens with the flip variation (Sommer et al. 1990).

All GluA subunits have four **transmembrane domains (TMDI-IV)**, as the other ionotropic glutamate receptors. It is also common for the four subunits that the TMDII forms a re-entrant loop lining the pore channel (Michael Hollmann and Heinemann 1994). Different from the other subunits, GluA2 has an arginine instead of a glutamine (as the other GluA subunits) in the cation selection filter at TMDII due to an mRNA editing process. This amino acid change confers special characteristics to GluA2 subunit (see below).

After TMDIV, AMPARs have an intracellular **carboxy terminal domain (CTD)** of about 50-100 amino acids length which determines specific subunits binding to other interacting proteins. CTD interactions determines functional receptors properties by interacting to other proteins and contains most of the characterized phosphorylation sites which are important in AMPARs regulation (Song and Huganir 2002). Differences in the CTD can also modulate GluA subunit trafficking (see 4.5 section).

However, despite their high structural similarities, the GluA subunits have many differences that confer to AMPARs a great variety of combinations. Most of their differences are a result of different processes of synthesis that are explained in more detail in the following section. The figure 3 illustrates the canonical GluA subunit topology and the different GluA regions that can be subject of variation.

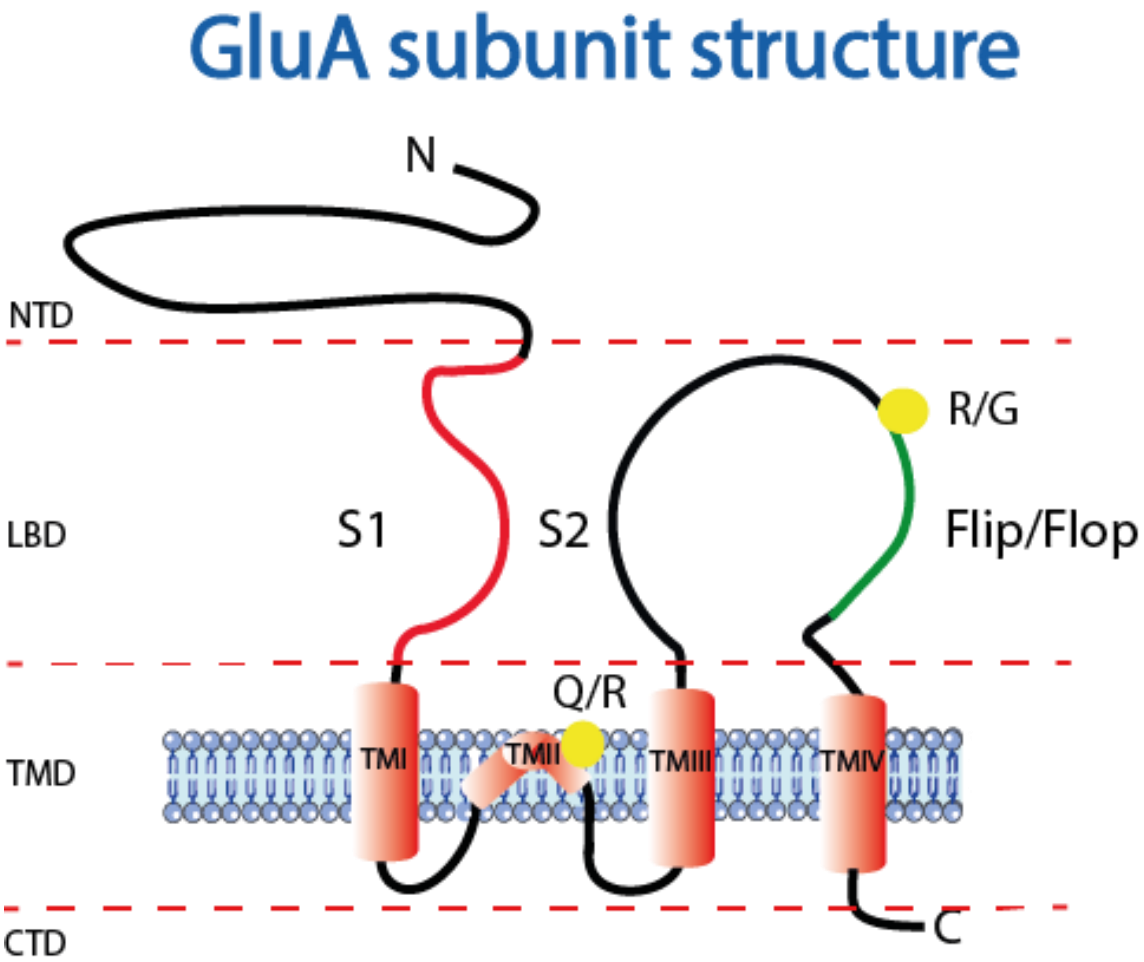


Figure 3. Structure of typical GluA subunits. At TMDII and S2 loop there are the Q/R and R/G sites that are positions subjected to an RNA-editing process. In addition, at the end of S2 loop and before TMDIV there a region called flip/flop cassette that gives name to the flip and flop isoforms of GluA subunits. These flip and flop isoforms are the product of an alternative splicing process.

Finally, as mentioned before, AMPAR subunits can be combined to form homo- or heterotetramers. However, despite the large variety of AMPARs combinations, the AMPARs predominant forms in CNS correspond to heteromers composed by GluA1/2 or GluA1/A3 (Boulter et al. 1990; Nakanishi, Shneider, and Axel 1990; Wenthold et al. 1996).

4.2. RNA processing modifications

In the process between gene expression to protein synthesis there are various steps until the final product is synthetized. First of all, this process starts with gene transcription to RNA which will become at the end, the messenger RNA (mRNA). The mRNA is what will be

translated into proteins. This molecule is the target of several proteins that modify it before is translated to protein.

Among other modifications of the RNA molecule like capping (addition of an altered nucleotide at 5' end of the mRNA) or polyadenylation (addition of an adenine nucleotide tail to the mRNA), alternative splicing and mRNA editing are processes of mRNA modifications that are common in AMPAR. In addition, both modifications in the mRNA that is going to be translated play an important role into GluA subunits synthesis and then in neurotransmission (Seeburg 1996). Moreover, these modifications add a wide range of possible combinations that can form an AMPAR.

4.2.1. RNA editing

The RNA editing process involves cellular mechanisms to modify single parts of the mRNA that is going to be translated. Different to RNA splicing, RNA editing do not operate over immature RNA extracting fragments. This process acts over the mRNA making punctual and acute modifications. In AMPAR there are 2 very well studied targets of RNA editing, the R/G site and the Q/R site. This punctual modification implies codon changes for later protein synthesis.

R/G site

The change of an arginine (R) for a glycine (G) residue (R/G site) at 743 position is targeted by RNA editing and alters assembly properties of AMPARs. Although the R/G has not the same decisive effect in AMPAR tetramerization as Q/R site, the unedited forms (with the arginine (R)) show an improvement of GluA2 folding and transport to cell membrane. Due to both RNA editing processes, Q/R and R/G sites, the GluA2 self-assembly results to be prevented. For this reason, GluA2 tends to be incorporated preferentially to heterotetramers at the endoplasmic reticulum (ER) (Greger and Esteban 2007).

Q/R site

The GluA2 subunit is the only GluA subunit subjected to this concrete mRNA editing process at the cation selection region in TMDII. This change implies a codon modification

after transcription. This codon modification exchanges a glutamine (Q) for an arginine (R) at 607 position, in a key place at the selectivity filter in the pore region of the TMDII (Seeburg, Higuchi, and Sprengel 1998). The process is catalyzed by adenosine deaminases (ADARs) 1 and 2 (Bass 2007). In neurons, the mRNA edition is nearly 100% efficient and configures decisively the AMPAR properties.

It has been extensively studied that the Q/R exchange alters dramatically AMPAR properties. For this reason, AMPARs are often classified in two subpopulations, upon the presence or absence of GluA2 into GluA2-containing or GluA2-lacking AMPARs. First of all, the Q/R site determines AMPARs permeability to Ca^{+2} (Verdoorn et al. 1991; Geiger et al. 1995; M Hollmann, Hartley, and Heinemann 1991; Kuner et al. 2001). In the edited GluA2 subunit, the positive charged arginine does not allow Ca^{+2} pass through the channel. For that reason, AMPARs that contain GluA2 subunit are also named as Ca^{+2} impermeable AMPARs (CI-AMPARs) and AMPARs that contain any other subunits combinations are Ca^{+2} permeable AMPARs (CP-AMPARs). Moreover, Q/R editing of GluA2 confers additional receptor properties. In CI-AMPARs, the current-voltage curve exhibits a linear relationship while CP-AMPARs have an inward rectifying current-voltage relationship (Koh et al. 1995; Derek Bowie and Mayer 1995; Geiger et al. 1995; Swanson, Kamboj, and Cull-candy 1997) (Kamboj, Swanson, and Cull-candy 1995). This difference on current-voltage (I-V) behavior is due to a differential channel block by intracellular endogenous polyamines (PAs). CP-AMPARs are blocked by endogenous PAs at depolarized membrane potentials because these compounds (present in every cell type) act as a voltage-dependent ion channel blockers (Derek Bowie and Mayer 1995; Koh et al. 1995; Kamboj, Swanson, and Cull-candy 1995). The presence of a positively charged arginine in GluA2-containing AMPARs makes these receptors insensitive (not blocked) by intracellular positively charged polyamines, thus showing the characteristic linear I-V relationship.

On the other hand, as Q/R site determines Ca^{+2} permeability it affects single channel conductance of AMPARs where CP-AMPARs (GluA2-lacking) have the larger pore unitary conductance (Geiger et al. 1995; Swanson, Kamboj, and Cull-candy 1997).

This editing process play a key role also in AMPAR tetramerization of the GluA2 subunit and retention (Greger, Khatri, and Ziff 2002) and implies specific characteristics in membrane traffic (Greger et al. 2003, 2006). Edited forms of GluA2 subunit homotetramerize inefficiently when exogenously expressed in neurons while non-edited forms assemble efficiently resulting in CP-AMPARs (Greger and Esteban 2007). For this reason, GluA2 subunit tends to form dimers with other GluA subunits and then form heterotetramers.

In mature CNS the GluA2 edited form is a dominant form expressed in synapses (Paschen and Djuricic 1995; Carlson et al. 2000; Kawahara et al. 2004; Barbon et al. 2010; Greger and Esteban 2007). In addition, different studies have demonstrated that unusual up-regulation of CP-AMPARs result in different neuronal diseases and synaptic plasticity (Cull-Candy, Kelly, and Farrant 2006; S. J. Liu and Zukin 2007). A summary of AMPAR biophysical properties conferred by GluA2 subunit are summarized in figure 4.

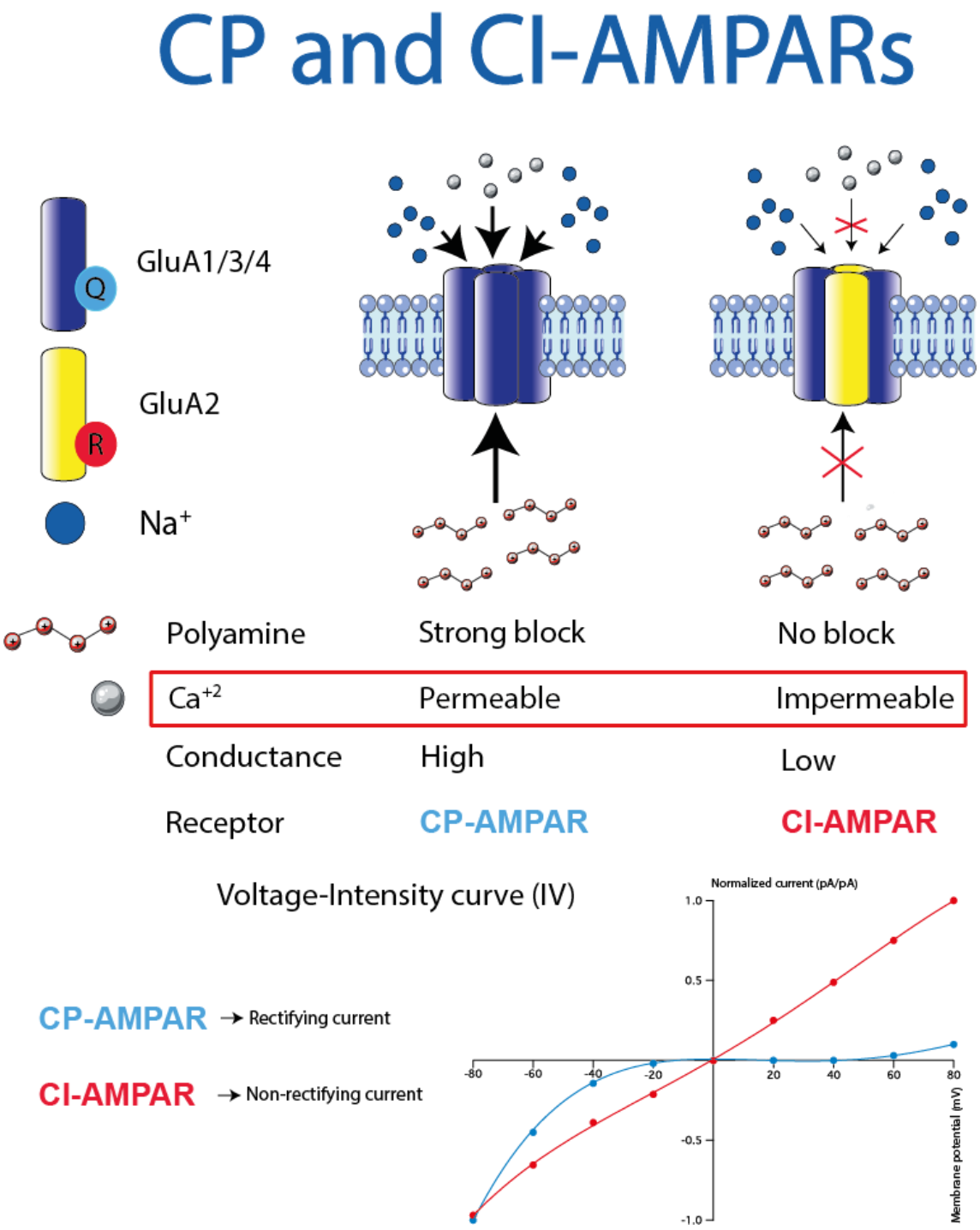


Figure 4. CP and CI-AMPARs and their biophysical properties

At this figure are summarized all biophysical properties that arise due to the presence of GluA2 subunit in an AMPAR. GluA2-containing AMPARs have low conductance and are not Ca^{+2} permeable. In addition, these receptors do not show inward rectifying currents because are not blocked by endogenous polyamines at depolarized membrane potentials. At the bottom of the figure there is an I-V curve where are shown normalized intensity current at different membrane potentials.

Altogether, AMPAR subunits composition as well as the mRNA modifications, endow AMPARs with substantial diversity and also determine the ion channel characteristics of the receptor.

4.2.2. Alternative splicing

One of the modifications in the mRNA that is going to be translated into a GluA subunit (increasing the molecular diversity of AMPARs) is the alternative splicing. It consists in the alternative selection of the exons that are going to form the mRNA that will be translated into proteins. In AMPAR the amino acidic chain of the S2 loop is a region which is subjected to be modified by an alternative splicing process (see figure 4.1).

As mentioned before, between the S2 loop (LBD) and the TMD-IV, there is a region called the flip/flop cassette that, by alternative splicing, results on either "flip" or "flop" isoforms of the GluA subunits (Sommer et al. 1990). These isoforms can modify the AMPAR kinetics, as for example in GluA2, which desensitizes and deactivates more rapidly when the flop variation is present. However, in GluA1 subunit the desensitization depending on the flip or flop isoform depends of the glutamate concentration (Mosbacher et al. 1994). Nevertheless, altogether can be concluded that the flip and flop isoforms produce distinct AMPAR kinetics.

The CTD is another region that can be modified by alternative splicing. GluA2 and GluA4 can have both, long and short C-tails depending on the splice form (called GluA4c the short isoform of GluA4 (Gallo et al. 1992). GluA1 and GluA3 have only one isoform in terms of the C-tail, which is long for GluA1 and short for GluA3 subunit. The C-tail in AMPAR has an important role in trafficking where long-tailed subunits traffic dominate over short-tailed subunits. Subunits containing receptors with long tails are rapidly mobilized from the ER pool and driven into the synapse during synaptic activity. By contrast, short-tailed GluA2 (short isoform) and GluA3 subunits are trafficked more slowly in absence of GluA1 (Coleman et al. 2006; Mah et al. 2005). At the following figure it is illustrated the GluA subunit diversity (figure 5).

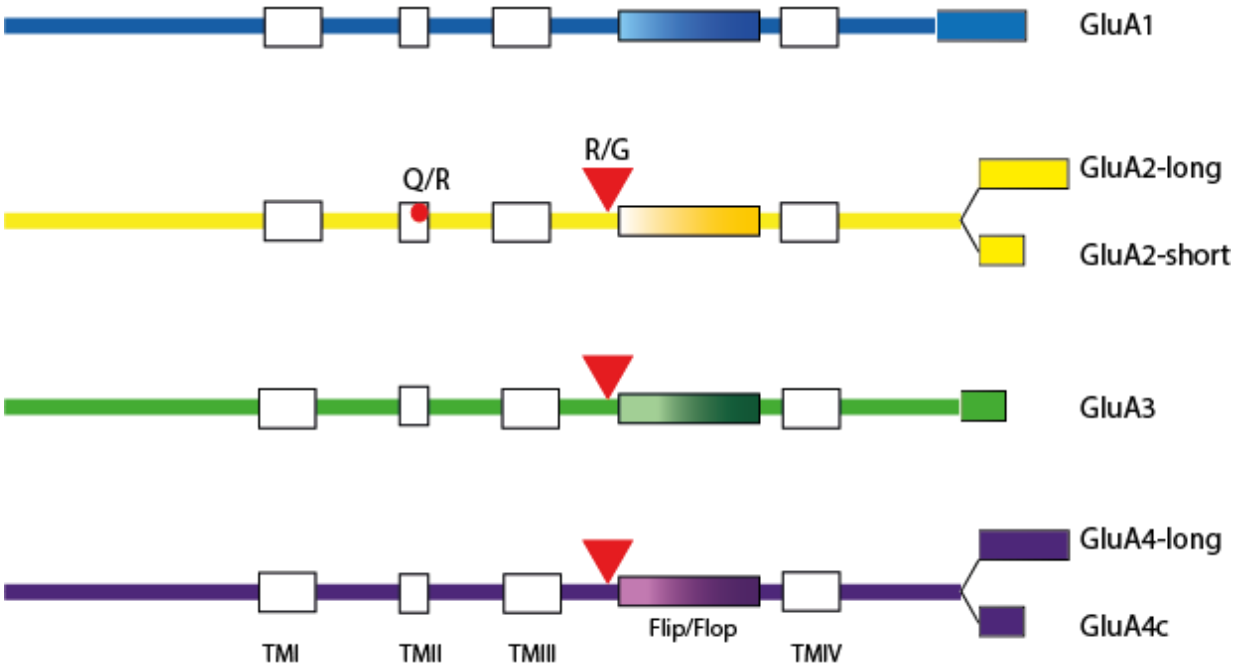


Figure 5. GluA subunits diversity

Different GluA subunits with their characteristic elements. Schematically illustrated the 4 TMD, the RNA editing sites Q/R and R/G sites, the flip/flop cassette and the short and long isoforms. In the figure it is shown how GluA1 and GluA3 have only long and short isoforms respectively; on the other hand, GluA2 and GluA4 have both, long and short isoforms (in GluA4 the short isoform is called GluA4c).

4.3. Posttranslational modifications

Following RNA modifications, mRNA is translated into proteins, which are also subjected to a series of modifications. Posttranslational modifications (PTMs) are a set of chemical modifications of proteins. These modifications imply the covalent union of different molecules to the amino acids that conform the protein at their side chains. The most common chemical groups posttranslationally modifying proteins, are: phosphate, acetyl or N-linked glucid (Khoury, Baliban, and Floudas 2011).

Not different from other proteins, AMPARs are subject also to posttranslational modifications. In AMPAR the most important modifications that take place are phosphorylation, palmitoylation and glycosylation.

4.3.1. Phosphorylation

This type of posttranslational modification implies the addition of a phosphate group (PO_4^{3-}) to –OH free amino acid residues (the most common amino acids that can be phosphorylated are tyrosine, serine or threonine. All GluA subunits can be phosphorylated and the reactions are catalysed by different protein kinases such as protein kinase A (PKA), protein kinase C (PKC) or calmodulin kinase II (CaMKII) at the CTD (H.-K. Lee 2006; J. Q. Wang et al. 2005; Lussier, Sanz-Clemente, and Roche 2015).

In the GluA1 subunit, it has been reported that phosphorylation can occur at two serine residues (831 and 845 amino acid positions, S831 and S845) within the CTD, resulting on a modification of the AMPAR properties. The S831 residue can be phosphorylated by PKC and CaMKII. This posttranslational modification in the GluA1 subunit increases single channel conductance of the receptor and it has been reported to be related with plasticity processes. In long term potentiation it was seen an increase of phosphorylation of the S831 residue following the induction of long-term potentiation (Barria et al. 1997; H. K. Lee et al. 2000).

On the other hand, when the GluA1 subunit is phosphorylated at S845 by PKA, this phosphorylation induces changes in receptor-mediated currents, the number of active channels, the open probability and the receptor kinetics of desensitization (Banke et al. 2000). In addition, this phosphorylation also affects plasticity processes where GluA1 subunit trafficking to cell membrane is activity-dependent (Derkach et al. 2007; Oh et al. 2006; Seol et al. 2007).

Also near CTD, the highly present AMPAR subunit GluA2 can be phosphorylated at serine residue 880 (S880). Protein kinase C catalyses this modification in the PDZ motif of the CTD. This residue has been shown to be important in AMPAR trafficking since it modifies the interaction of GluA2 with PICK1 and GRIP/ABP (see **5. AMPAR interacting proteins**; here ABP refers to *AMPAR binding protein* and should not confuse with actin binding protein) (Matsuda, Mikawa, and Hirai 1999; Chung et al. 2000; Dong et al. 1997). On the one hand, GRIP/ABP interactions stabilize GluA2 subunit at cell membrane while PICK1 seems to promote GluA2 internalization (however, see **5.**

AMPAR interacting proteins) (Matsuda, Mikawa, and Hirai 1999; Chung et al. 2000). For these reasons it has been purposed that S880 phosphorylation is related with GluA2 removal during process of long-term depression (reviwed Palmer, Cotton, and Henley 2005).

4.3.2. Palmitoylation

Palmitoylation is a posttranslational modification that consists in the covalent union of a palmitic acid (a carbon chain ended in a carboxy group highly apolar, a fatty acid) to cysteine residues (for this reason is also called S-palmitoylation). This is a dynamic and reversible modification. The palmitoylation of AMPAR subunits is catalysed by some palmitoyl acyl transferases (PATs) and the result is a cysteine linked to a palmitic acid via thiol linkage. The opposite process, the depalmitoylation is regulated by palmitoyl thiesterases (PTE) that break the thioester link (Shipston 2011; Fukata and Fukata 2010).

The AMPAR subunit GluA1 is target of this posttranslational modification at two cysteine residues, the 585 cysteine (C585) near pore domain and the 811 cysteine (C811) in the C-tail juxtamembrane region (T. Hayashi, Rumbaugh, and Huganir 2005). The C585 residue is plamitoylated by the PAT DHHC-3 (named by the Asp-His-His-Cys motive of the protein) and occurs in the early secretory pathway at ER. This modification regulates AMPAR stability and protects the receptor from degradation (Yang et al. 2009). On the other hand, depalmitoylation of this same residue contributes in AMPAR traffic (T. Hayashi, Rumbaugh, and Huganir 2005). By contrast, palmitoylation at C811 residue inhibits GluA1 interactions that are needed to stabilize receptor at cell surface (Shen et al. 2000). Therefore, this palmitoylation increases GluA1 endocytosis in an activity dependent manner. However, contrary to C585 palmitoylation, this reaction is not catalysed by DHHC protein (Lin et al. 2009).

4.3.3. Glycosylation

Glycosylation is a posttranslational modification that consists in the addition of a glucidic residue to the side chain of an amino acid from a protein. There are three types of glycosylation depending on the chemical group that links to the glucidic residue:

- **N-glycosylation**: the glucid is attached to an amino group from an asparagine.
- **O-glycosylation**: the glucid is attached to a hydroxy group from a serine or threonine.
- **C-glycosylation**: the glucid is attached to a carboxy group from a tryptophan.

In AMPARs, this posttranslational modification has shown to modify synaptic plasticity as the ones before mentioned. For example, the GluA1 subunit can be glycosylated at serine 831 residue (S831) by O-glycosylation by an N-acetylglucosamine (GlcNAc). This reaction is catalyzed by an O-GlcNAc transferase and its inhibition facilitates synaptic transmission in Schaffer collateral-CA1. This facilitation increases a 120% the synaptic transmission from basal level (Kanno et al. 2010).

On the other hand, the N-glycosylation take place at extracellular domains of GluA1 and play an important role in AMPAR trafficking. Mutations at asparagine residues 63 and 363 (N53 and N363) abolished AMPAR trafficking to cell surface in HEK293T expression system. However, in primary culture of cortical neurons of GluA1 KO mice the coexpression of GluA1 mutated at N363 (for a serine N63S) traffic to cell membrane but not GluA1 mutated at N63 (N63S). This implies a very sophisticated regulation in AMPAR traffic linked to the glycosylation pattern (Kandel et al. 2018).

In addition, just to mention two other posttranslational modifications that are not explained, AMPARs can also be modified by ubiquitination (addition of a small protein of 76 amino acid in a lysine residue) or subjected to S-nytrosilation (addition of a nitrogen monoxide (NO) group into a cysteine).

Altogether, the different posttranslational modifications that take place in GluA subunits are all important in AMPAR biogenesis; however, it is important to see that not always this chemical reaction are possible in expression systems due to the lack of

the enzymes that catalyzes the reactions.

4.4. AMPAR assembly and trafficking

The learning about AMPAR lifecycle has grown over the past years, elucidating more about this receptor from its synthesis and dendritic transportation to membrane insertion and removal.

All four AMPAR subunits folding and assembly take place in the ER. The assembly take place in a two-step process that implies dimerization of two GluA subunits that then, in dimeric structure, form a dimer of dimers formation that results in a tetramerization (Ayalon and Stern-Bach 2001). This first dimerization step at the ER is initiated by the NTD LIVBP-like domain which is adjacent to the LBD (Kuusinen et al. 1999). This interaction constitutes a step to ensure association between subunits from the same iGluR family (Ayalon and Stern-Bach 2001; Kohda et al. 2003). At this phase it is shown that the protein ABHD6 (α/β-hydrolase domain-containing 6) or porcupine (PORCN) intervene in AMPAR dimerization (Schwenk et al. 2019). Both proteins seems to interact without preference for any GluA subunit (Erlenhardt et al. 2016).
When this dimerization process has occurred the next step is the tetramerization. This process involves the S2 loop and the TMD both of which intimately participate in intersubunit contacts (Ayalon and Stern-Bach 2001).

Tetramerization itself does not seem to be enough to export AMPARs from the ER. An interesting 'quality control' occurs when conformational alterations that reflect gating motions are sensed by the ER. This quality control is needed to export AMPARs from the ER (Greger et al. 2006; Priel et al. 2006; Fleck 2006). One of this checks that is well established implies the ligand binding to AMPAR in the ER and is required for ER exit.

On the other hand, AMPARs require co-assembly with auxiliary subunits to be exported from the ER (Ziff 2007). The auxiliary subunits assembly with the receptor is catalysed by a priming complex formed by ferric chelate reductase 1 Like Protein

(FRRS1L) and carnitine palmitoyltransferase 1C (CPT1C) proteins. Once the receptor is associated with the auxiliary proteins its exit from ER is promoted (Brechet et al. 2017b; Schwenk et al. 2019). All of these proteins will be covered in the section **5. AMPAR interacting proteins**.

Next, before AMPAR arrives to synapse, the trafficking is expected to occur after receptors have completed their biosynthetic transport through the ER, Golgi apparatus and trans-Golgi network. Following exit from ER, AMPARs traffic to dendritic compartments via Golgi system by vesicular transport. Close to the cell membrane, vesicles containing AMPARs are conducted to fusion by members of the SNARE protein family.

This vesicle fusion delivers receptors to the dendritic plasma membrane or directly to the dendritic spine. Endo- and exocytosis as well as lateral diffusion are functional mechanisms involved in delivery and removal of AMPARs in cell membrane. That means AMPAR is not anchored permanently but acts dynamically at cell membrane.

The traffic to synapse will depend on the subunit composition of the receptor to target the membrane. At this point the specific signals in CTD play a fundamental role. As explained before, subunits with long tails traffic preferentially to synapses. AMPARs containing subunits with long tails are rapidly mobilized from the ER and conducted into the synapse in activity periods. On the other hand GluA2 (short isoform) and GluA3 subunits (the ones with short tails) traffic more slowly without of GluA1 in the receptor complex (Coleman et al. 2006; Mah et al. 2005). However, despite this important role in AMPAR targeting to synapse, the synaptic anchoring strongly depends in subunit specific NTD despite CTD exerts some modulation (J. F. Watson, Ho, and Greger 2017).

Once AMPARs are in the proximity of the synapse, the exocytosis of the receptors is a previous step before anchoring. This is the final step before the receptor arrives at cell membrane; however, when receptors arrive to membrane it is still dynamic and can be anchored or not. Two modulation pathways regulate AMPAR exocytosis. One of these

pathways is constitutive and it is determined by the AMPAR composition as explained before. The other and most studied pathway is the activity-dependent traffic. This is an exocytosis form that has been identified to relay in several forms of long-term synaptic plasticity (Carroll et al. 2001; Malinow and Malenka 2002). GluA2/A3 heteromers exhibit an in and out cycle maintaining the pool of the receptors (Passafaro, Piãch, and Sheng 2001; S.-H. Shi et al. 2001b). By contrast, long-tailed GluA1/A2 and GluA1/A4 require synaptic activity to membrane insertion

The final step in this trafficking involves their functional insertion and stabilization at the postsynaptic membrane. Members of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins are crucial in this step of synaptic targeting of AMPARs. Once at cell surface AMPARs are highly mobile and tend to diffuse laterally. There, AMPAR retention is regulated by PSD95-like MAGUK (PSD-MAGUK) protein family, which comprises PSD94, PSD95, SAP97 and SAP 102 proteins. The first proteins which bind directly to AMPARs is SAP97 On the other hand, PSD-95 has been shown crucial in AMPAR accumulation at synapse along with regulatory proteins Transmembrane AMPAR Regulatory Proteins (TARPs). Moreover, PSD-95 plays a key role trapping and anchoring wandering AMPARs at postsynaptic membrane between PDZ-dependent interactions with TARPs (Bats, Groc, and Choquet 2007; S.-H. Shi et al. 2001a) .

4.5. Gating

As ligand-gated ion channels, AMPAR gating due to agonist binding is one of the primary characteristic of these ionotropic glutamate receptors. The glutamate binding to the LBD is the first step in AMPAR gating. A minimum of two molecules of glutamate are required for AMPAR activation and the receptors display three conductance states, the amplitude peak of each depends on the number of glutamate molecules bound to the receptor (Rosenmund, Stern-Bach, and Stevens 1998). In the figure 6 (modified from Swanson, Kamboj, and Cull-Candy 1997) are shown different events evoked by agonists application where the three subconductance states can be seen..

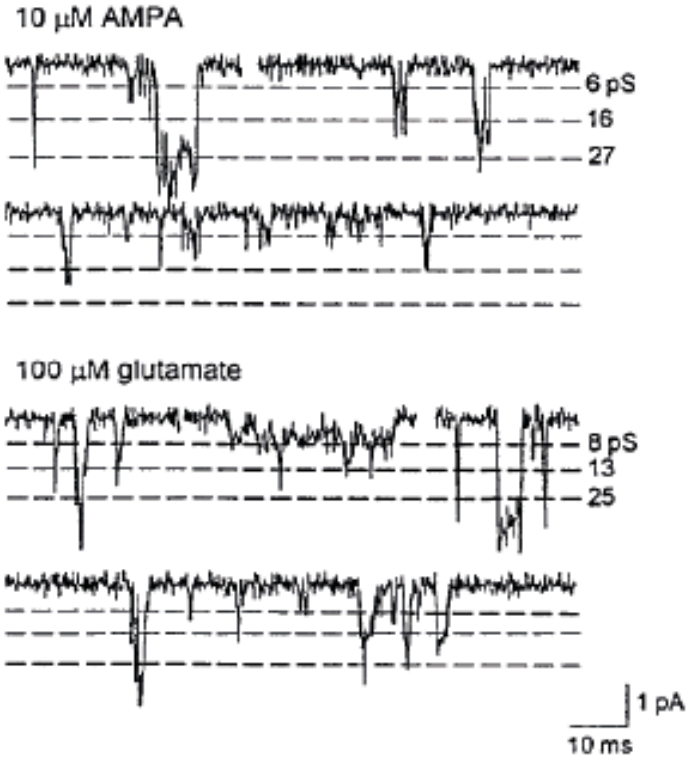


Figure 6. Different conductance levels in response to agonist. Modified from (Swanson, Kamboj, and Cull-candy 1997) It is shown in this figure different events evoked by 10μM AMPA or 100μM glutamate application in homomeric GluA4 AMPARs from outside-out patches. In dashed lines are pointed the subconductance levels resolved by single channel analysis. The different responses along the time have different channel conductance what suggests different opening states from AMPAR.

The LBD controls the activation, deactivation and desensitization of the receptor. It forms a self-contained clamshell-like structure in each GluA subunit (Armstrong and Gouaux 2000; Sobolevsky, Rosconi, and Gouaux 2009). Glutamate binds between S1 and S2 domains and conforms a two polypeptide domains association. When the receptor is at a resting state, LBDs of adjacent subunits form dimers linked back-to-back between their S1 domains (Sun et al. 2002; Armstrong and Gouaux 2000). LBD closure in a clamshell manner around the agonist causes separation of the S2 domains applying tension to linkers between the LBDs and the ion channel which opens the gate (Jin et al. 2003; Twomey, Yelshanskaya, and Sobolevsky 2019; Armstrong and Gouaux 2000), (Twomey et al. 2017c). It may be followed by desensitization, initiated

by the rupturing of the S1-S2 interfaces between subunits that relieves tension on the pore linkers imposed by glutamate binding which leads to the channel closing (Sun et al. 2002).

The activated conformation of the channel is unstable and tends to return to a more stable state. The re-opening of the LBD with the unbinding of glutamate (deactivation) or the re-arrangement of the dimer interfaces (desensitization) are two ways that permit receptor recovers stability. In deactivation, the receptor conformation changes leading to glutamate unbinding and channel closure. On the other hand, a prolonged exposure to glutamate drives to desensitization which is a process with a different time course where transmembrane helices are repositioned into a more relaxed conformation. Next it is shown in figure 7 the different states of AMPAR upon glutamate binding.

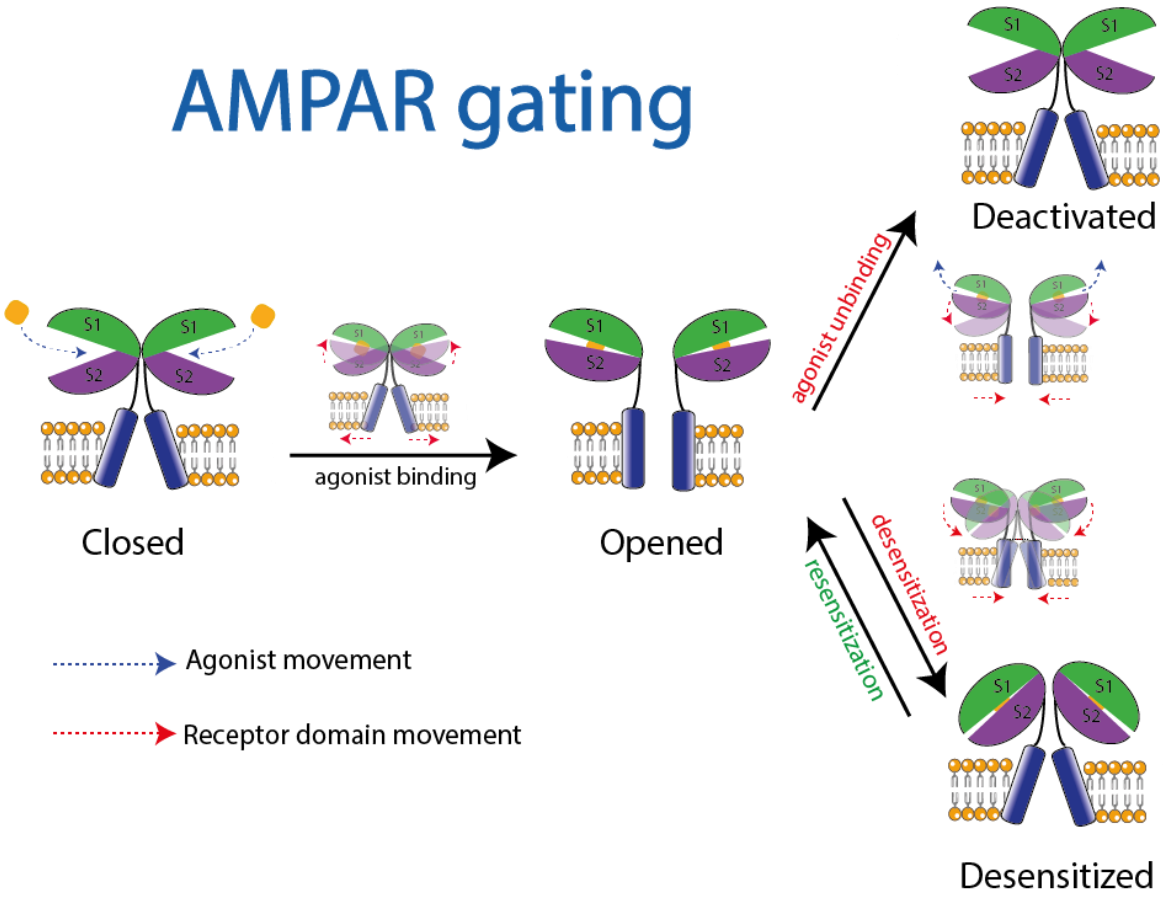


Figure 7. **AMPAR different conformations upon agonist binding**. The blue arrows indicate agonist movement while the red arrows show the movement of structural parts of the receptor. After glutamate binding to LBD, S2 loop pushes up closing with S1 loop the clamshell structure. It creates tension that opens channel and ions can flow through the AMPAR. At this point, receptor can display

two different configurations. First, if the agonist is unbounded, AMPAR returns to a closed state due to a relaxation in the structure that allows to close channel. Second, the rupture of the S1-S2 interfaces leads into a relaxation of tension caused by agonist bind. This relaxation while agonist is still bound produces a channel closure. However, this is a dynamic state and AMPAR channel can be opened again what is called resensitization.

Moreover, the AMPAR subunits show differences in terms of deactivation, desensitization and recovery due to alternative flip/flop isoforms.

In addition, AMPARs auxiliary subunits control receptors biophysical properties. These auxiliary subunits modulate amplitude and subconductance states of the receptor and will be explained later in more detail (Shelley, Farrant, and Cull-Candy 2012; Tomita et al. 2005). For example, auxiliary subunits like transmembrane AMPAR regulatory proteins are also responsible of AMPAR kinetics control.

4.6. Pharmacology

Many compounds have been studied as activators and modulators of AMPAR due to their effects on AMPAR gating. They have been classified as agonists, antagonists and positive or negative allosteric modulators.

4.6.1. Agonists

Glutamate is the natural agonist of AMPARs and acts as a full agonist. **Glutamate** binding results in peaks with small steady-state current (compared to peak current) and quick desensitization. **AMPA** is a specific compound for AMPARs that mimics effects of glutamate and gives the name of these receptors (AMPARs) due to their specific response to this agonist. **Kainate** can also evoke responses in AMPAR acting as a partial agonist that can bind to AMPAR but generates smaller and non-desensitizing currents (Patneau et al. 1992; Jin et al. 2003). It can also be considered as a competitive antagonist. **Quisqualic acid** can act also as agonist of AMPAR, KAR and mGluR group I (Jin et al. 2002; Kuang and Hampson 2006; Zhang et al. 2006). This

agonist is one of the most potent AMPAR agonist and it is used for its neurotoxicity to selectively remove neurons in nervous system (Muir et al. 1993; Giovannelli, Casamenti, and Pepeu 1998).

The full agonists cited before (Glutamate, AMPA and quisqualic acid) differ substantially in potency but produce similar binding domain closure (Armstrong and Gouaux 2000; Jin et al. 2003; Hogner et al. 2002).

In heterologous expressed AMPARs, in the absence of auxiliary subunits, it has been shown that agonist potency and kinetics are strongly affected by the agonist applied. EC_{50} has been used to determine agonist potency. Looking at AMPA and quisqualic acid EC_{50} curves the affinity of these agonists are higher than glutamate. It shows that can produce the same response in AMPARs than glutamate but with less concentrated solution. When the potency of the agonist is increased it is translated into slower deactivation kinetics. In addition, in the double exponential used to calculate receptors kinetics, the weight of the slow component in percentage is increased as more potency has the agonist. By contrast, the desensitization kinetics in AMPARs without auxiliary subunits shown not to be affected by agonist potency (Zhang et al. 2006).

4.6.2. Positive allosteric modulators

These molecules allow extending the open state of the receptor only in presence of glutamate and as they are AMPAR positive allosteric modulators are called AMPAR PAMs (positive allosteric modulators). These molecules have potential medical application in the treatment of cognitive impairment, dementia or depression. They have shown memory and cognition-enhancing effects and also antidepressant-like effects also at preclinical models (K. Lee et al. 2016; Ranganathan et al. 2017).

Cyclothiazide (CTZ), one of the best described (a member of benzothiazides family), is a modulator of AMPAR/KAR that avoids receptor desensitization which permits a sustained current flow throughout channel. This allosteric modulator binds to the flip/flop cassette and prevents conformational change required for desensitization

(Sun et al. 2002). It is used to facilitate detection of AMPARs in cell surface by means of electrophysiology (Partin, Bowie, and Mayer 1995).

On the other hand, there are a set of compounds called **ampakines** which are also AMPAR PAMs. Ampakines are also called 'CX compounds' and have a benzamide or a structuraly related chemical structure (Staubli, Rogers, and Lynch 1994). Many members of this family of AMPAR PAMs have a clear subunit preference which is a plus if it is taken into account that AMPAR subunit combinations varies along the nervous system (Yan-Fang Xia, Kessler, and Arai 2005; Y-F Xia and Arai 2005). Ampakines act slowing deactivation and desensitization kinetics of AMPARs but their effect can vary depending on the structure of the ampakines (Lynch 2002). In addition, they can also modify AMPARs peak current but also depending on the member of the ampakines family who exerts the modulation (A. C. Arai et al. 2002). Less is known about where ampakines bind but using X-ray crystallography it has been showed that CX614 can bind between AMPAR subunits (Jin et al. 2005). While agonists bind at the LBD of the clamshell, the ampakines binding site is near the hinge of the clamshell of each subunit. This binding stabilizes the agonist union and prolongs the attachment of the agonist to the receptor.

Another family of compounds that can act as AMPAR PAMs are a group of molecules that share a pyrrolidine nucleus called **racetams** (Löscher and Richter 2000). There is not a universally accepted mechanism of action but these PAMs have been shown to act over glutamate and cholinergic receptors. As AMPARs allosteric modulators are considered to have a weak effect (Copani et al. 1992).

4.6.3. Antagonists

An antagonist is a molecule or compound that can block or diminish the response of a cellular receptor. In terms of their action, antagonists can be classified as **competitive**, **non-competitive**, **uncompetitive** (**open-channel blockers**), **partial agonists** (in presence of full-agonists) and **inverse agonist** (activates different downstream response).

In AMPAR pharmacology exists many antagonists. At this thesis the attention will be focused at **competitive**, **non-competitive** and **open-channel blockers**.

Competitive antagonist. Competitive antagonists are a set of compounds that act over the same binding site of a receptor than an agonist or an endogenous ligand, nevertheless, an antagonist does not produce a receptor response. To simplify, this kind of antagonist 'compete' for the same binding site.

A widely used and studied group of compounds that act over AMPARs are derivate from quinoxiline. In this subset of competitive antagonists are found DNQX (6,7-dinitroquinoxaline-2,3-dione), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and NBQX (2,3-dioxo-6-nitro-7-sulfamoilbenzo(f)quinoxaline) which have been widely used to elucidate biology and physiology of AMPARs (Armstrong and Gouaux 2000). These compounds bind to AMPAR with high affinity at the agonist binding site (ABS) preventing their activation by glutamate. However, each of these compounds show different properties. NBQX was the first reported in 1988 and shown to have useful therapeutic effects in animal models of neurological disease in 1990 (reviewed at Catarzi, Colotta, and Varano 2007). This AMPAR antagonist is highly selective for AMPARs (Donevan and Rogawski 1996). CNQX can act also over KARs and it has been reported that can act as a partial agonist of AMPAR channel when is associated with TARP auxiliary subunits (Menuz et al. 2007; Kott et al. 2009).

Non-competitive antagonists. The non-competitive antagonists are compounds that can be also called as allosteric antagonist (Neubig et al. 2003). They act at an allosteric site of the receptor so they no compete for the binding site with the ligand. Non-competitive antagonists reduce the maximum response evoked by any amount of agonist as these compounds do not bind at the pocket.

This family includes several compounds, being GYKI52466 and their derivate and Perampanel two of the most widely used to modulate AMPAR responses either *in vivo* or *in vitro*. GYKI52466 is a 2.3-benzodiazepine that shows selectivity for AMPARs over

Kainate receptors (KAR). It is used for chronic blockade of neuronal AMPARs and as other AMPAR antagonists as an anticonvulsant (Donevan and Rogawski 1993; Szabados et al. 2001).

On the other hand, Perampanel was the first AMPAR antagonist to receive regulatory approval and it has been used as an anti-epileptic drug sold under brand name Fycompa (Hanada et al. 2011). This compound has a bipyridine core that distinguish Perampanel from other AMPAR antagonists and blocks excitatory neurotransmission by targeting AMPARs. As it cannot be displaced by high agonist concentration it can block still at raised glutamate levels; what confers to Perampanel potent antiseizure activity (Hanada et al. 2011; Rogawski 2011). However, this drug does not show brain-region specificity as it blocks AMPARs independently from GluA subunits or auxiliary subunits. Recently, more specific non-competitive agonists are being synthetized. LY3130481 is an example of AMPAR non-competitive antagonist that can block selectively those subunits guided by auxiliary subunit γ8 TARP but not γ2 (see 5 AMPAR interacting proteins). That brings to this compound brain-region selectivity as their action is focalized to a specific subset of AMPARs. Concretely it can block AMPARs at forebrain region (Kato et al. 2016).

· **Open-channel blockers**. Another group of antagonists with a different action mechanism are the open-channel blockers that can also be included as uncompetitive agonists. The fundamental difference with non-competitive antagonists is the fact that uncompetitive antagonists need the receptor previous activation so they can act. In this case, open-channel blockers act once the receptors are opened due to agonist binding.

In this subgroup of antagonists there are natural or synthetic polyamines (PAs) like spermine and spermidine. These positive charged compounds have been shown to block AMPARs when the channel is opened. These compounds are group of small positively charged molecules that weight less than 1kD and are isolated from spiders and wasps venom (Shaw 1979; D Bowie and Mayer 1995). They are present in every

cell type and are commonly used to measure rectification currents in AMPAR, which allows to detect presence of CP-AMPARs in cells from the nervous system. PAs block CP-AMPARs in a voltage dependent manner generating a strong inward rectification of CP-AMPARs (see 4.2. RNA processing; (Donevan and Rogawski 1995; Isa et al. 1995; Kamboj, Swanson, and Cull-candy 1995; Koh et al. 1995; D Bowie and Mayer 1995). These molecules block channels when the PA head group is positioned in the ion channel vestibule external to the selectivity filter with the PA tail permeating the selectivity filter (Nelson et al. 2009) in a voltage-dependent manner. The blockage is stronger as the membrane potential depolarizes. This is translated into a characteristic inwardly rectifying current-voltage relationship curve (IV curve). Over positive 50mV membrane potential, outward currents tend to increase by pushing polyamines to pass through the pore and unblock the channel (Koh et al. 1995; D Bowie and Mayer 1995). It has been demonstrated that members of TARP (Transmembrane AMPAR Regulatory Protein) family associated with AMPARs can attenuate PAs blocking (Soto et al. 2007). These compounds are an important tool to study native AMPAR because of their selectively blockage of GluA2-lacking AMPARs.

Other open-channel blockers are toxins that contain acylpolyamines like argiopin (also known as argiotoxin-636), Joro spider toxin (JSTX-3) philanthotoxin-433 and their natural or synthetic analogs also (Twomey et al. 2018).

Although the promising effects for therapeutic uses of the open-channel blockers, there is still a limited comprehension about the structural bases of channel block by these molecules. A recent publication tries to address this gap of knowledge by means of cryo-EM microscopy and electrophysiological techniques in CP-AMPARs (Twomey et al. 2018) It is shown in this work that IEM-1460, NASPM (1-Naphthylacetyl spermine) and AgTx-636 toxins anchor with their tails at the upper and central region of the pore which is an electroneutral surface. This space is mostly formed by TMDII and TMDIII domains of AMPAR subunits. When the channel is opened due to agonist binding, blockers can anchor their tails at different points along the region before mentioned. However, this anchoring does not allow the blocker pass through the channel, plugging the ion flow. Interestingly, when the receptor desensitizes, these compounds remain

trapped at the pore. Then it is need a channel re-opening for the blocker to leave the channel (Twomey et al. 2018). The following figure (figure 8) illustrates how IEM-1460, NASPM or AgTx-636 block receptor and are trapped.

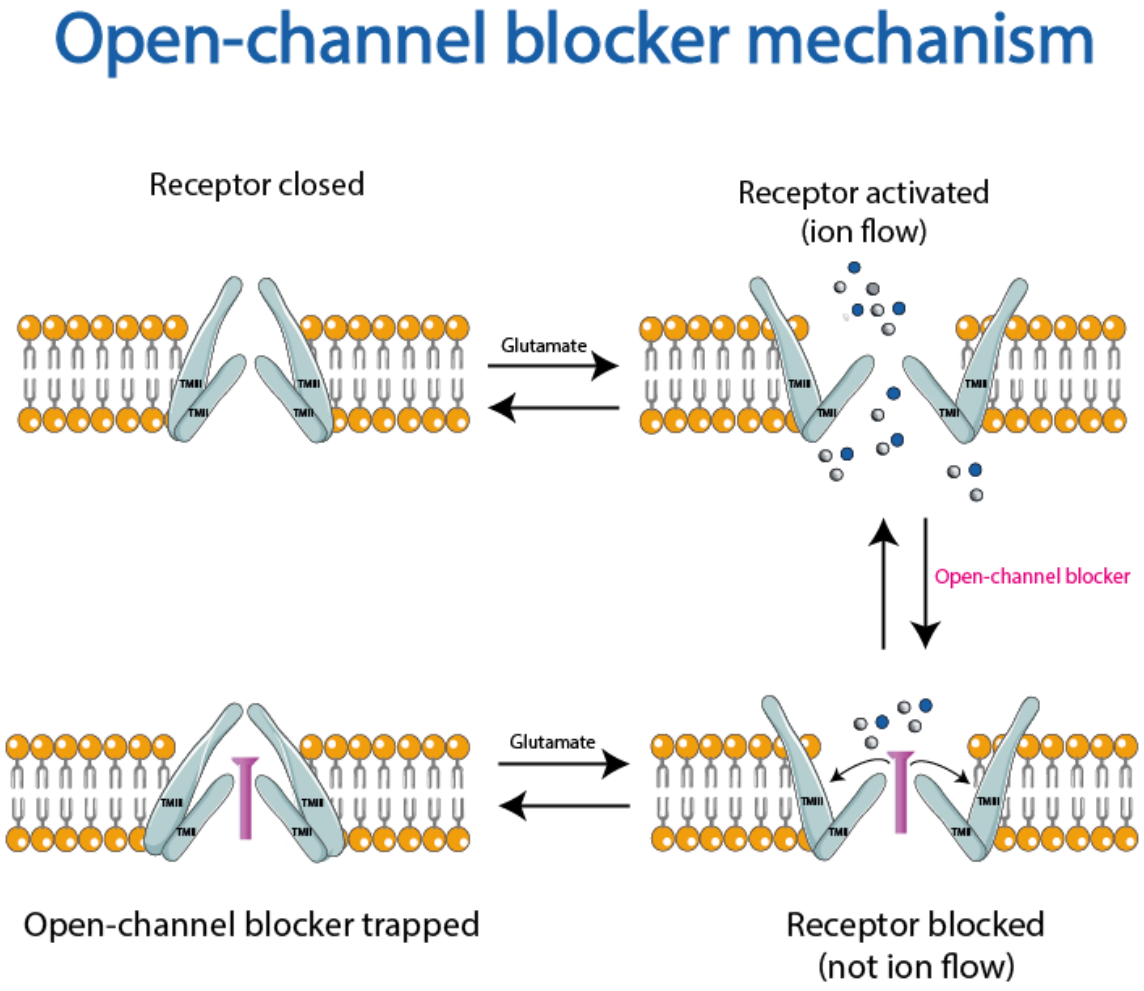


Figure 8. **Mechanism of action of an CP-AMPAR open-channel blocker**. Here it is shown how molecules like IEM-1460, NASPM or AgTx-636 act blocking channel once it is activated. These compounds interact with TMDII and TMDIII AMPAR domains and anchors to channel pore not allowing ion flux. After the block, these molecules are trapped across the receptor and need receptor activation to leave the pore (based on the figure 8 Twomey et al. 2018).

On the other hand, completely different from the compounds explained before, the divalent cation Zn^{+2} has the capability to block AMPAR current under some conditions. This ion has been shown to be endogenously released along with glutamate at synapses and modulate AMPAR currents (Qian and Noebels 2005; Kalappa et al. 2015). In a very recent publication it was found that Zn^{+2} can inhibit AMPARs depending on

the receptor activity, Q/R editing of GluA2 and also depending on auxiliary subunits (Carrillo et al. 2020). In addition, it was shown that Zn^{+2} block is higher at negative membrane potentials (Carrillo et al. 2020).

However, till date, the role of Zn^{+2} have been variable in terms of AMPAR inhibition. On the one hand, Zn^{+2} was found to not have effect at mossy-fibber-CA3 but a large effect on other hippocampal synapses in mice model (Pan et al. 2011; Fukaya et al. 2006). That fact corresponds to the variability in AMPAR complex (AMPAR plus auxiliary subunits) that can be found through nervous system (as it will be explained in '**5. AMPAR interacting proteins**').

Interestingly, Zn^{+2} cannot block AMPAR currents when GluA2 (Q) (non-edited GluA2 form at the Q/R site) AMPARs are expressed in expression systems. However, co-transfection with auxiliary subunits permits Zn^{+2} to reduce steady-state currents but not peak amplitude. In addition, Zn^{+2} dramatically diminish AMPAR currents when CTZ and glutamate are applied to the receptor. When co-agonist and glutamate are released AMPARs evoke a sustained response because receptors do not desensitize. There, in absence of auxiliary subunits, Zn^{+2} can act dramatically diminishing AMPAR currents. Altogether indicates that Zn^{+2} blocks these receptors when they are in an open state and in a more physiological level when the receptor is associated with auxiliary subunits (Carrillo et al. 2020).

4.7. Physiological roles of AMPARs

4.7.1. Role in synaptic transmission

As explained before, AMPARs are tetrameric ligand-gated glutamate receptors that are permeable to Na^+, K^+ and Ca^{+2} (depending on subunit composition of the receptor). Contrary to other members of the glutamate receptors family, AMPARs participate in fast excitatory transmission between neurons. Due to this, AMPARs function is fundamental in rapid synaptic communication and in signalling for action potential firing.

However, as explained below, these basic electrical signals are not only a key part in action potential firing but also in pathological and physiological condition (see below long term potentiation).

4.7.2. AMPAR mediated synaptic plasticity

As explained previously, synapses are specialized structures where it takes place the chemical neuron-to-neuron communication. These complex cell connections are in addition not static but dynamic. In the dynamics of synaptic modulation intervenes the synaptic plasticity. This is a biological process that involves several players acting in a coordinated way. The synaptic plasticity is explained by specific patterns of synaptic activity and result in changes in synaptic strength. These changes involve the rearrangement of membrane and intracellular components, for example variations in the population of membrane receptors (reviewed at (Huganir and Nicoll 2013).

The best understood of these modifications that cause synaptic reorganization of component, involves changes in AMPAR-mediated currents following NMDAR (T. V Bliss and Collingridge 1993) or CP-AMPARs activation (Mahanty and Sah 1998; J. G. Gu et al. 1996). These changes occur postsynaptically due to a specific pattern of stimulation.

In this section, however, I will focus in plasticity induced by AMPAR itself, independently of NMDAR activity since long term potentiation induced by NMDARs (where AMPARs are also an active part) will be explained later.

As mentioned before, maybe the most commonly used way to classify AMPARs is between CP and CI-AMPARs. The presence or not of GluA2 rules Ca^{+2} in these glutamate receptors and then the inflow through the cell. The importance of Ca^{+2} ions to trigger many proteins function is widely known. At synapses it is has also been studied that Ca^{+2} entry is needed to start many processes that finally rearrange synaptic structure. For this reason, it has been investigated the role of CP-AMPARs in

synaptic plasticity.

When CP-AMPARs are localized at synapses they can mediate postsynaptic Ca^{+2} influx and trigger for changes in synaptic strength. It has been shown that in specific neuronal populations Ca^{+2} mediated currents can form activity-dependent modulation of synaptic strength. For example, in dorsal horn neurons or cerebellar stellate cells there are data about this new type of synaptic reorganization (J. G. Gu et al. 1996; S. Q. J. Liu and Cull-Candy 2000). Indeed, in cerebellar stellate cells it has been shown that after activity, Ca^{+2} were diminished while amplitude of excitatory post-synaptic currents was modified and GluA2-containing AMPARs were replacing non-containing GluA2 AMPARs.

4.7.3. AMPAR subunit expression during development

During the development AMPAR subunit expression varies markedly in time and space in rodent models.

AMPAR expression seems to start at embryonic levels in brain as mRNAs for all subunits can be detected by *in situ* hybridization (Monyer, Seeburg, and Wisden 1991). In early stages, around P4, CP-AMPARs are the most predominant at neocortex, striatum and cerebellum. However, the expression of these receptors declines with age. At hippocampus their expression increases until more advanced stages compared to other regions, nevertheless, their expression also declines, at early stages of life. Then on, are exchanged for CI-AMPARs after few postnatal weeks (Pellegrini-Giampietro, Bennett, and Zukin 1992).

These findings are consistent with low expression levels of GluA2 compared to GluA1 at neonatal stages showed by Pickard and colleagues (Pickard et al. 2000). In addition, in rat model, at postnatal day 14 almost all AMPAR-positive synapses are GluA2-containing and the predominant GluA1 expression is highly developmentally restricted (Monyer, Seeburg, and Wisden 1991).
On the other hand, at early stages of development GluA4 homomers are inserted

preferentially at silent synapses depending in activity and in NMDAR function. Then on, these CP-AMPARs (GluA4 homomers) are exchanged by GluA2-containing AMPARs in a constitutive process that maintains synaptic strength (J. J. Zhu et al. 2000).

At more advanced stages occurs another variation in AMPAR composition, at approximately P21, when GluA3 levels increase while GluA1 decline. That clearly modifies AMPARs biophysical properties as GluA3-containing AMPARs show slower deactivation and desensitization kinetics compared to GluA1-containing AMPARs (in presence of the auxiliary subunits γ4 and γ8 as will be explained in more detail later on)(E. Suzuki, Kessler, and Arai 2008). This fact presents a longer duration in AMPAR response that probably leads into an increase of postsynaptic excitability and LTP threshold reduction (Blair et al. 2013).

In terms of subunits expression, GluA2 seems to be widely expressed throughout the grey matter, the other subunits appear to be expressed differentially present in different cells population. For example in rodents, pyramidal cells of V layer express higher levels of GluA2/3 (Geiger et al. 1995) than GluA1 which is highly expressed in basket and chandelier interneurons (Kondo et al. 1995); or GluA3 has been reported to be present high levels in fast and regular-spiking somatostatin neurons (Cauli et al. 2000).

As a result, in adult brain the vast majority of AMPARs contain the GluA2 subunit and that implies they are CI-AMPARs. However, it cannot be negligible the presence of CP-AMPARs in mature neurons (Wenthold et al. 1996) and it is important to mention that CP-AMPAR subunits are differentially expressed depending on cell type in contrast with GluA2 subunit that is widely expressed (for more extensive review about AMPAR expression during development and disease see (Henley and Wilkinson 2016; Hanse, Seth, and Riebe 2013; Hadzic, Jack, and Wahle 2017) .

Finally, to configure a more complex scenario, flip and flop isoforms vary in time expression taking into account mRNA levels. While flip variant remains almost invariant during postnatal brain development in rodents, flop versions mRNA levels are

low until P8. At this moment, flop forms mRNA increases throughout the brain reaching adult levels by P14.

4.8. AMPARs in pathological conditions

Pathological conditions in the nervous system are strong related with defects in neuronal communication. For this reason, it is not weird that receptors can be a fundamental piece of the puzzle in research about pathological conditions and then to try to treat theme.

As a fundamental player in glutamatergic signalling, AMPAR correct function is mandatory. This fact implies that deregulation of synaptic AMPARs and their abnormal function is involved in a variety of neurological disorders and neurodegenerative diseases. For example, glutamatergic signalling leads into seizure events when the glutamatergic and GABAergic transmission balance is altered (Bonansco and Fuenzalida 2016). In this aspect AMPAR have a significant role in ictogenesis (the process of generation of a seizure episode) (Kaminski, Banerjee, and Rogawski 2004; Turski et al. 1998) and for this reason are a an important pharmacological target to maintain normal brain activity.

On the other hand, AMPARs have been related in a variety of neurodegenerative diseases. In amyotrophic lateral sclerosis motor neuron death is caused due to toxic levels of Ca^{+2} entries in the cell. This Ca^{+2} is explained by a deregulation of GluA2 mRNA editing process that provokes a change in the cation selection pore and makes GluA2-containg AMPARs be CP-AMPARs (in physiological conditions, GluA2-containing AMPARs are CI-AMPARs) (Kawahara et al. 2004).

Moreover, AMPAR cytotoxic effect has been described in Parkinson's and Huntington's diseases as well as schizophrenia (Michael Hollmann and Heinemann 1994; Lees 2000; Goff, Freudenreich, and Evins 2001; Yamada 1998)(Danysz and Parsons 2002)(K. A. Johnson, Conn, and Niswender 2009). Alzheimer's disease (AD) is characterized by a

diminished AMPAR activation and synapse loss. It has been demonstrated that a deregulation in AMPAR endocytosis at synapse can contribute to a progressive loss of memory in AD (T. T.-T. Tang et al. 2009). Thus, β-amyloid, a key player in AD development, has been shown to disrupt AMPAR activity-dependent trafficking (Z. Gu, Liu, and Yan 2009).

Finally, as glutamatergic signalling is fundamental for neuron-to-neuron communication it is normal to assume that AMPARs are involved in more pathological conditions than the ones mentioned above.

5. AMPAR interacting proteins

In mammalian brain, native AMPARs associate with more than 30 different proteins forming macromolecular complex (Schwenk et al. 2012). Some of these proteins modulate the receptor and are referred as transmembrane regulatory proteins whereas others display transient interactions with the receptor. Many proteins determine exocytosis, endocytosis and synaptic targeting of AMPARs mediating transient interactions with these receptors. In addition, at synapses AMPARs or their auxiliary subunits are also anchored with scaffolding proteins binding the receptor to a complex structure. An important part of this work is the AMPAR modulation by TARPs (transmembrane proteins) and the possible effect of CPT1-C (transient interaction) over this modulation. For this reason, the next section will be focused in AMPARs possible interactions, focusing in transiently and transmembrane interactions as both are an important part of this thesis work.

5.1. AMPAR auxiliary proteins (transmembrane interactions)

A protein is classified as an auxiliary subunit of an ionic channel or receptor if it follows 4 main criteria:

- It should not constitute an integral component of channels pore forming subunits.
- It has to remain stable association with its partner receptor.
- It has to modulate receptor pharmacology.
- It has to be an important player for functional receptor assembly (Copits and Swanson 2012).

This section will be focalized in 4 families of AMPAR interacting proteins at transmembrane level, including Cornichons homologs (CNIHs), GSG1L (Germ cell-specific gene 1-like protein), CKAMPs (cysteine-knot AMPAR modulating proteins; aka Shisas) and TARPs (Transmembrane AMPAR Regulatory Proteins) which are one of the main parts of this thesis. It is worth mentioning that although TARPs and GSG1L have been traditionally classified in different families (mainly due to important functional dissimilarities), a very recent phylogenetic study indicates that GSG1L actually belongs to the TARP family of claudins (Ramos-vicente and Bayés 2020). The figure 9 shows a prototypical structure of each family of AMPAR auxiliary proteins.

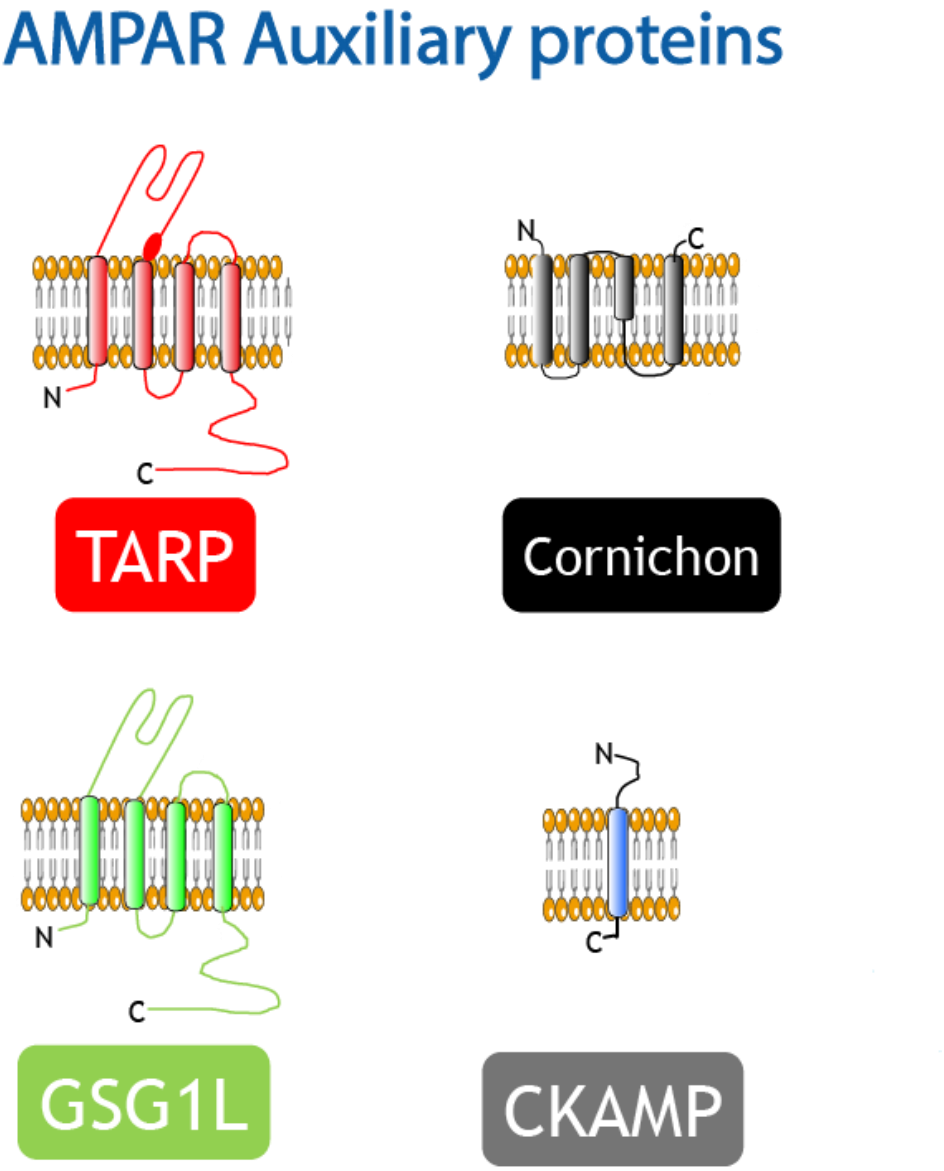


Figure 9. AMPAR auxiliary proteins. This figure is a very simplified version of the AMPAR auxiliary proteins where it is shown the transmembrane domains and the C and N-terminal domains of each one.

5.1.1. Cornichons homologs

Most of the AMPAR auxiliary subunits belong to two families of proteins. On the one hand the claudin homolog family that comprises TARPs and GSG1L, and on the other hand the cornichon homolog (CNIH). The first member of this family family was discovered in *Drosohpila* as a protein that participates in the ER export pathway and named Cornichon (CNI) (Bökel et al. 2006). Later, this protein was shown to not be an AMPAR auxiliary subunit although a posterior proteomic study identified two members

of this family, CNIH2 and CNIH3 (due to their homology with CNI named as CNI homolog, CNIH), as AMPAR auxiliary subunits (Schwenk et al. 2009). Since that, several studies have uncovered many aspects of the role of CNIHs and how interact with AMPARs.

Once these members of the CNIH family were identified as AMPAR auxiliary subunits, studies trying to correlate both proteins arose. One of these publications was focused in uncover how they were related during the development. In a rat model it was showed that CNIHs have high levels of mRNA and protein expression at cortex and hippocampus (only CNIH2 at cerebellum) in early stages after birth that were followed by a decrease at adulthood, contrary to AMPAR subunits expression pattern. However, at adult individuals the overall ratio of CNIHs (CNIH2 specifically) integrated with AMPAR at cell membrane did not change. This fact purposes the question about the meaning of the excess of CNIHs expressed early in the development. It was hypostatized that maybe they would be acting in other proteins trafficking at stages where neuron migration and development activity is still high (Mauric et al. 2013).

On the other hand, other works were focused in the CNIH structure. First of all, to elucidate CNIHs structure it was used the primary sequence of these proteins and it was predicted they had a structure with three transmembrane domains (Brockie et al. 2013). However, it seems that in this publication and others as well, a section in the second transmembrane domain was misinterpreted given an incorrect structure (Wudick et al. 2018; Shanks et al. 2012). Nevertheless, a very recent work using cryo-electron microscopy (cryo-EM) uncovered CNIH3 structure and redefined the previous concepts. At this publication, CNIH3 is presented with 4 transmembrane domains and extracellulars N and CTD (Nakagawa 2019). Moreover, it was described that CNIH3 is mostly embedded in cell membrane. This suggests that mechanistically, the interaction between this auxiliary subunit and GluA LBD at the extrasynaptic space is unlikely probable; different from other AMPAR auxiliary subunits TARPs and GSG1L (Twomey et al. 2017a; Beatriz Herguedas et al. 2019a).

Moreover, as CNIHs were purposed as AMPAR auxiliary subunits, it was (and it still is) primordial to study how these proteins modulate this receptor function. In terms of AMPAR biology, CNIH2 and 3 intervene in either AMPAR trafficking and gating modulation once at cell surface. In heterologous systems and cultured neurons, it has been shown that CNIH2 and 3 can enhance AMPAR surface expression when they are co-assembled. That means that these two members of the CNIH family still conserve their role as cargo exporters (Bökel et al. 2006; Harmel et al. 2012). However, a part from their role in AMPAR trafficking to cell membrane, CNIHs exerts modulation over receptor biophysical properties once it is delivered. These proteins act modulating receptor kinetics by slowing desensitization and deactivation independently of the GluA subunit composition (Schwenk et al. 2019; Yun Shi et al. 2010; Coombs et al. 2012). In addition, it has been shown that CNIHs can increase AMPAR conductance in a similar way as some members of the TARP family (Yun Shi et al. 2010; Coombs et al. 2012) and also relieve polyamine block (Coombs et al. 2012). Altogether shows that CNIH tend to modulate AMPARs stabilizing the channel in an open, active configuration (Schwenk et al. 2009).

However, their effects in AMPAR modulation in a more physiological model, as cultured neurons or glial cells, have been more controversial. Overexpression of CNIHs at oligodendrocyte precursor cells (OPC) have been reported to increase AMPAR single channel conductance but it does not affect kinetics or polyamine block relieve (Coombs et al. 2012). At hippocampal pyramidal neurons, overexpression of CNIH2 did not affect AMPAR kinetics when agonist was applied to outside-out patches when compared to non-transfected neurons (Yun Shi et al. 2010). On the other hand, CNIH2 knocked down mossy cells (cells from mossy fiber, a region at hippocampus) showed a decay in time constant kinetics when excitatory post-synaptic currents (EPSCs) where analysed. However, there was no effect when the knockdown was performed in aspiny interneurons (Boudkkazi et al. 2014). Altogether can imply a very sophisticated AMPAR modulation by CNIHs taking into account that these proteins can be present at AMPAR complex along with other auxiliary subunits (Y. Shi et al. 2010; Kato et al. 2010a). This fact sharply increases the complexity of AMPAR modulation by these auxiliary subunits as CNIHs modulation will change depending in how they interact not only with

AMPARs but also with the AMPAR complex (that includes other auxiliary subunits). However, it can maybe explain the divergence of results due to a different expression of GluA and auxiliary subunits at different cell types.

5.1.2. Germ cell-specific gene 1-like protein (GSG1L)

As mentioned before in this section, member of the claudin homolog family have demonstrated to be AMPAR auxiliary subunits. The most deeply studied of these members are members of the subfamily of TARPs. However, a few years ago another integrant of the claudin homolog family was proved to be an AMPAR auxiliary protein. First publications where this protein was mentioned (or at least the gene that codified for GSG1L) were in genome-wide association (GWA) and genome-wide expression assays (P. Liu et al. 2012; Becanovic et al. 2010). However, at these previous publications there was nothing about GSG1L as AMPAR regulatory proteins. Nevertheless, two parallel proteomic studies revealed that Germ cell-specific gene 1-like protein (GSG1L) could accomplish the role of an AMPAR auxiliary subunit (Shanks et al. 2014; Schwenk et al. 2012).

Different from TARPs and CNIHs, GSG1L is not as deeply studied but in the past years many works tried to bring light about this novel AMPAR auxiliary subunit. At this point, much more is known about GSG1L structure and function modulating AMPARs. Surprisingly, at the overall of the data provided by different researchers, it seems that GSG1L exerts a 'suppressing' modulation over AMPAR as it will see in the following lines.

Structurally, GSG1L shares important homology with TARPs as both are members of the claudin homolog family. GSG1L has also 4 transmembrane domains and it has been purposed to exert effects in AMPAR via his extracellular loops like TARPs. Differences at this part of the structure respect to TARPs seem to be key in how GSG1L modulates AMPAR (Twomey et al. 2017b; X. Gu et al. 2016). It is important because even sharing several similarities in terms of structure; GSG1L and TARPs contribute to biophysical properties of AMPARs in a very different way. Contrary to CNIH and TARPs GSG1L has a

role diminishing AMPAR activity.

On the other hand, in terms of AMPAR surface delivery, this protein demonstrated to negatively regulates the receptor trafficking at hippocampal granule neurons when it was overexpressed (Mao, Gu, and Lu 2017). Nevertheless, GSG1L acts also selectively chaperoning GluA4 in abducens motor neurons. These differences will maybe be explained by the expression of other AMPAR auxiliary proteins at these different neuronal populations.

Focusing AMPAR biophysics, GSG1L alters many properties of AMPAR once at cell membrane. In expression system electrophysiological recordings shown that GSG1L could increase AMPAR time to recover from the desensitization state and also diminish AMPAR single-channel conductance (Schwenk et al. 2012; McGee et al. 2015). In addition, also contrary to TARPs and CNIH, GSG1L enhanced polyamine block at CP-AMPARs (McGee et al. 2015).

Moving into more physiological models it was also tested the effect of GSG1L in native AMPARs. At cerebellar neurons expressing heterologously GSG1L miniature EPSCs (mEPSCs) mediated by CP-AMPARs are altered at some parameters. Frequency ratios of these mEPSCs events and rectification index were shown to be decreased by GSG1L effect. Moreover, in cultured hippocampal neurons GSG1L weakens single-channel conductance and peak amplitude of mEPSCs; as it was seen an increase on these parameters when GSG1L was knocked down at these cells (that previously expressed GSG1L at normal levels). On the other hand, GSG1L have shown to accelerate AMPAR deactivation and desensitization kinetics, meaning to promote an AMPAR closed state (X. Gu et al. 2016; McGee et al. 2015). In agree with this fact structural studies of AMPAR-GSG1L complex showed that GSG1L stabilizes AMPAR complex in an inactive or desensitized states, contrary to other auxiliary subunits (Twomey et al. 2016b; Zhao et al. 2016; Twomey et al. 2017a). By contrast, GSG1L seems to not modulate kinetics of synaptic events at hippocampal granule neurons (EPSCs) (X. Gu et al. 2016), giving strength to the idea of different AMPAR modulation at different cell types depending on AMPAR complex composition. Interestingly and related to this last point, in

expression systems, GSG1L have shown to modulate AMPAR properties in a complex with other auxiliary subunits. For example, this protein can suppress CNIH2 modulation in heterologous transfected cells but did not affect modulation exerted by TARP γ2 (X. Gu et al. 2016; Schwenk et al. 2012).

However, it is interesting to mention that in GSG1L KO rat model animals showed behavioral abnormalities in object recognition tests and also deficits in LTP formation. Altogether implies a key role for this protein in AMPAR function even when it seems to not enhance AMPAR activity. Furthermore, it is necessary to study in more detail how GSG1L can interact with AMPAR complexes in native receptors and the effects of this protein in receptor physiology and pathology.

5.1.3. Cysteine-knot AMPAR modulating protein (CKAMP)

At the time this thesis work is written (in 2020) a decade has passed since the first member of a new AMPAR auxiliary subunit was identified. The cysteine-knot AMPAR modulatory protein (CKAMP) family was identified in 2010 (von Engelhardt et al. 2010) and as others AMPAR auxiliary subunit, CKAMP discovery came from a proteomic study in research of novel proteins that could modulate AMPAR function. At this first work the first CKAMP member identified was named as CKAMP44 according with the molecular weight of the protein. Later, more members were added to the CKAMP family and named also by their molecular weight CKAMP39, CKAMP54 and CKAMP59 (Karataeva et al. 2014; Klaassen et al. 2016; L. J. M. Schmitz et al. 2018; Farrow et al. 2018). The four members of these family are also alternatively named as shisa9 (CKAMP44), shisa8 (CKAMP39), shisa6 (CKAMP52) and shisa7 (CKAMP59). However, across the last decade the knowledge about this family of proteins has sharply increased. As there is still more to discover, it seems that CKAMPs like GSG1L have a role diminishing AMPAR activity, contrary to TARPs or CNIH.

The different members of this family of auxiliary subunits show specific expression patterns along the brain. The first discovered, CKAMP44, is expressed in the majority of brain regions including hippocampus, cerebral cortex, striatum, thalamus, olfactory

bulb and cerebellum. On the other hand, CKAMP39 expression is restricted to cerebellum and olfactory bulb while CKAMP52 and 59 are together expressed at hippocampus. However, CKAMP52 is also expressed at cerebellum and septum while CKAMP52 is expressed also at cortex and olfactory bulb. Prenatally there is a high CKAMP59 expression contrary to CKAMP39 and 52 which are barely detectable. Postnatally little changes occur in the expression of these CKAMP members. CKAMP39 and 52 however suffer an upregulation at cerebellum and olfactory bulb and CKAMP59 is downregulated at thalamus and brainstem.

With an analysis of the nucleotide sequence via reverse transcription polymerase chain reaction (RT-PCR) the first member discovered of this family of proteins served to predict CKAMPs structure. At the moment, CKAMPs are considered type I transmembrane proteins and clearly differ from other AMPAR auxiliary subunits. CKAMPs possess only one transmembrane domain with an extracellular NTD. A predicted disulfide bond of cysteine at a region rich in this amino acid stabilizes the extracellular structure giving name to this family to CKAMPs. Finally, these auxiliary subunits have a large intracellular CTD. Indeed, the CTD of CKAMP44 is a key structure for this auxiliary subunit to exert modulation in AMPAR at both membrane and trafficking (Khodosevich et al. 2014).

In terms of AMPAR trafficking, CKAMP44 is necessary for receptor targeting to synapses in dentate gyrus granule cells and lateral geniculate nucleus relay neurons (Khodosevich et al. 2014; X. Chen et al. 2018). By contrast, CKAMP52 and 59 do not affect AMPAR synaptic expression in CA1 neurons. Surprisingly, these proteins showed to decrease AMPAR surface expression in expression systems what may means that in neurons AMPAR trafficking is rescued by other auxiliary subunits (Klaassen et al. 2016; L. J. M. Schmitz et al. 2018). On the other hand, the association of different members of CKAMP family with GluA specific subunits can also affect AMPAR trafficking.

The complexity of matching results in expression system with neuronal primary cultures is present also looking at biophysical properties of the channel. One reason is that CKAMP family members show to have differential modulation in a subunit-specific

manner. However, results match consistently (reviewed in von Engelhardt 2019). Just to make an idea of this complexity in the following lines there are pointed some of the results obtained in expression system. In transiently transfected *Xenopus* oocytes CKAMP39 and 52 can slow deactivation and speed desensitization kinetics when associated with GluA2 but not GluA1. Moreover, CKAMP39 can increase time for recovery to desensitization of GluA1 and GluA2 (not edited form) homomers (Farrow et al. 2015b). In HEK293 cells used as expression system CKAMP39, 52 and 59 modulate basically GluA2 AMPARs. All of these members of CKAMP family decrease AMPAR peak amplitude. In addition, CKAMP39 diminish peak amplitude in GluA1 homomers too. But surprisingly and to add more complexity to CKAMP specific-subunit modulation, CKAMP39 reduces steady state currents only in GluA2 homomers while CKAMP52 increases this parameter in both GluA1 and GluA2 homomers (Farrow et al. 2015a). On the other hand, CKAMP effect on single-channel conductance has not been tested deeply. However, it will be interesting to test as some members can reduce peak amplitude at some GluA subunits.

In neurons most of the work done has been performed with CKAMP44. At CA1 cells this protein diminishes deactivation and slows desensitization kinetics in CA1 cells. On the other hand, increases single-channel conductance and rectification in somatic AMPARs from granule cell from dentate gyrus. On the other hand, looking at EPSC kinetics, CKAMP44 does not seem to exert any modulation in dentate gyrus granule cells but CKAMP52 slows deactivation and rise time kinetics. More detailed data is summarized at von Engelhardt 2019.

In addition, CKAMPs can also form part of AMPAR complex with other auxiliary subunits exerting synergic modulation of receptors biophysical properties and trafficking. Altogether configures a very exciting scenario for research in order to study CKAMP modulation along with other auxiliary subunits in neurons (Khodosevich et al. 2014).

5.1.4. Transmembrane AMPAR Regulatory Proteins (TARPs)

Finally, last but not least, this section will cover the most studied AMPAR auxiliary subunit. Transmembrane AMPAR regulatory proteins (TARPs) are fundamental part of this thesis project that involves new insights into the AMPAR regulation by TARPs and how the ratio AMPAR-TARP can modify receptor biophysical properties.

TARPs are members of the claudin homolog family along with the other AMPAR auxiliary subunit GSG1L. These AMPAR regulatory proteins are the most widely expressed and extensively studied AMPAR auxiliary subunits (Jackson and Nicoll 2011; Haering et al. 2014; Schwenk et al. 2012). Indeed, studies showed that in vivo the majority of AMPARs are associated with TARPs (Nakagawa et al. 2005; Vandenberghe, Nicoll, and Bredt 2005). This fact implies that most of the AMPAR-mediated signaling is modulated by TARPs.

The first member identified from this family that regulates AMPAR function was γ2 also named stargazin (the reason to name it 'stargazin' will be explained later). However, when this protein was discovered, its predicted role was not related with AMPARs. First, γ2 was suggested to represent the first example of a neuronal voltage dependent calcium channel (VDCC) γ subunit based on its similarity in structure to the γ1 subunit. The first γ identified was γ1, a protein that was found to be solely expressed in skeletal muscle (Jay et al. 1990; Powers et al. 1993; Freise et al. 2000). The role of this protein was to modulate the calcium entry through the L-type VDCCs of skeletal myotubes (Letts et al. 1998). For that reason and for structurally similarities γ2 was thought to play a role related with VDCC. In addition, it was proved its ability to weakly modulate VDCC. Nevertheless, the main function of γ2 was later uncovered to be the modulation of other kind of ionic channels.

γ2 (also named stargazin) was discovered in the stargazer mutant mouse where the Cacng2 gene is disrupted by an insertion of a retrotransposon in an intron that results

in a premature transcriptional termination of inefficient splicing. The lack of functional γ2 protein conferred to stargazer mouse a characteristic behavior. In fact, the name stargazin comes from the mouse behavior who seems to be usually 'looking/gazing stars' (metaphorically speech). However, despite this 'funny' fact, γ2 disruption leads into several alteration like epilepsy, head tossing and cerebellar ataxia (Letts et al. 1998; Noebels et al. 1990).

Near a decade after it was reported stargazer mouse behavior and condition, two publications related γ2 with AMPARs giving a new perspective to study this protein. That was a fundamental milestone in the AMPAR-TARP research. There it was demonstrated that γ2 regulates AMPAR currents and also synaptic targeting of AMPARs (Hashimoto et al. 1999; L. Chen et al. 2000). The ataxic and epileptic mutant mouse Stargazer lacks functional AMPARs on cerebellar granule cells, where is fundamental to the surface trafficking of CI-AMPARs (Studniarczyk et al. 2013). In addition at Chen's work (in 2000) it was observed restored AMPAR-mediated currents when CGCs were transfected with Cacgn2 gene (codifies for γ2) (L. Chen et al. 2000). Surprisingly, the lack of γ2 does not affect AMPAR currents in forebrain and later was seen that γ2 role in other neuronal types is played by other γ subunits and sometimes the lack of one γ subunit can be rescued by another member of TARPs family (L. Chen et al. 2000; Menuz et al. 2008).

Over the past years, six other stargazing-like genes classified as cacng3-8, encoding proteins γ3- γ8, were discovered and they follow the established nomenclature of the VDCC γ (Klugbauer et al. 2000; Burgess et al. 2001; Chu, Robertson, and Best 2001; Moss et al. 2002). The following figure (figure 10), adapted from Haering et al. 2014 is a phylogenetic tree of all tetraspanin-resembling four transmembrane domain containing AMPAR auxiliary subunits.

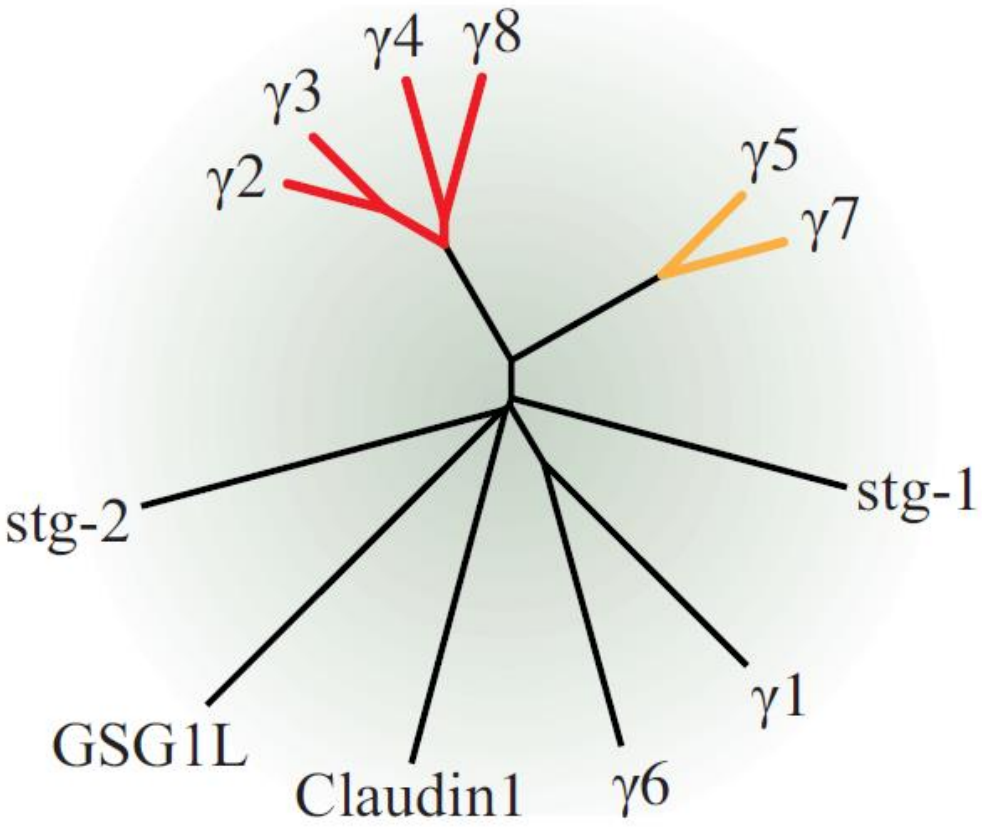


Figure 10. Phylogenetical tree of AMPAR auxiliary subunits with 4 TMD (adapted from Haering et al. 2014). This tree shows phylogenetical how the different members of TARP family are placed in the claudin homolog family. Note that CNIH 2 or 3 even if they have 4 TMD are not in the tree due to phylogenetical relationships.

Stargazin and other three of these proteins γ3, γ4 and γ8 are put together in a family of proteins whose principal characteristic is to be transmembrane AMPAR regulatory proteins. These members of the TARP family restore AMPAR-mediated currents in CGCs of the stargazer mice (Tomita et al. 2003). Nowadays, γ2, γ3, γ4 and γ8 are subdivided into two subfamilies as type Ia (γ2 and γ3) and type Ib (γ4 and γ8) due to the magnitude of their effects on AMPAR gating properties (Kato et al. 2010a). The other γ-like proteins γ5 and γ7 are classified in type II TARPs.

Structurally all members of TARP family show several similarities. In addition, TARPs share topological similarities with the tight junction protein claudins and GSG1L, for

this reason are members of the claudin homolog family. However, differences between TARP members that affect their function will be commented in following sections.

In terms of structure, TARPs are a group of proteins with 4 TMDs with intracellular N- and C-terminal domains as well as claudins (H. Suzuki et al. 2014; Nakamura et al. 2019). On the other hand, these AMPAR auxiliary subunits share with the cellular adhesion proteins two extracellular loops that in case of TARPs are important in receptor gating modulation. The two extracellular loops that form the extracellular region (ECR) differ considerably. First loop (L1) links first TMD (TMDI) with second TMD (TMDII) and is composed by 4 β strands (β1-4) named in order 1 to 4 from the most proximal to TMDI (β1) to the most distal (β4). Along these strands there are two loops. First loop is between β1 and β2 strand and another between β3 and β4. Moreover, β3 and β4 are connected by a disulphide bond. Finally, there is an additional loop after β4 strand and TMDII. Before TMDII starts there is an extracellular helix (ECH). This ECH lacks in GSG1L and specifically differences it from TARPs. The second loop (L2) connects TMDIII and TMDIV extracellularly and has a single β strand (β5) preceded by a loop (TMDIII- β5 loop), being this loop much simple and short. On the other hand, the TMDII and TMDIII domains are connected by a short intracellular segment.

Curiously, the whole structure of the TARPs looks like a hand where the extracellular resembles the finger and the transmembrane domains configure the forearm (Saitoh et al. 2015; Nakamura et al. 2019). The figure 11 illustrates how a prototypical TARP looks like.

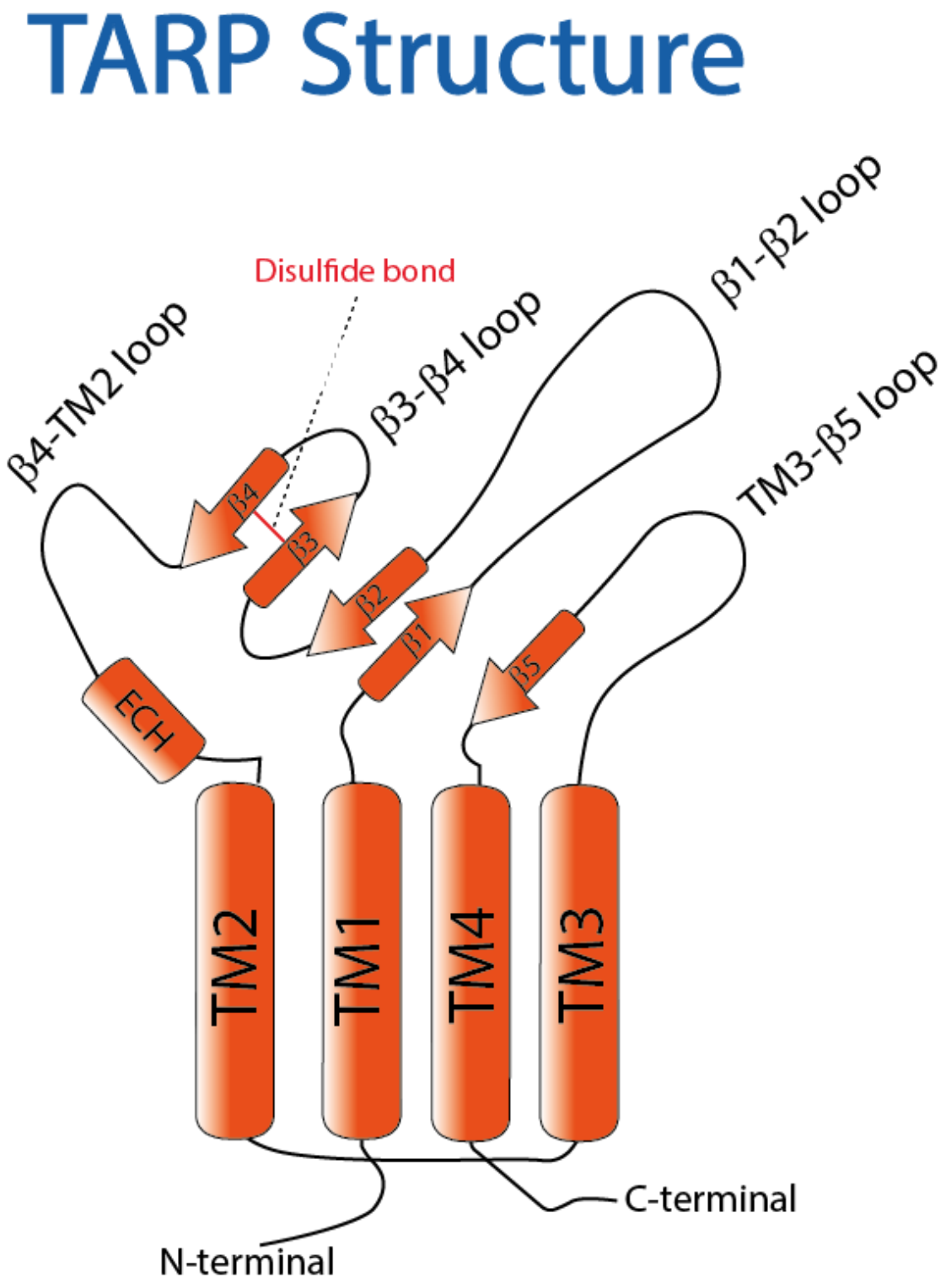


Figure 11. Prototypical structure of a TARP

Moving into a more functional aspects, but not forgetting about structure; this paragraph will focus a bit in AMPAR-TARP interaction. In this interaction the extracellular domain of TARPs plays an important role due to the effect that exerts in

AMPAR modulation. This part of TARPs affects receptor gaiting while the TMDs exert more structural and anchoring roles (Tomita et al. 2005; Riva et al. 2017). Structural studies using cryo-EM microscopy along with other techniques have shown that extracellular loops of TARPs interact with the LBD of the receptors to control gating, similarly as it happens in GSG1L (Beatriz Herguedas et al. 2019a; Twomey et al. 2017a). Indeed, as it will be explained later, differences between the extracellular loops can explain the different modulation exerted by type Ia and type Ib TARPs (Riva et al. 2017).

On the other hand, CTD also intervenes in AMPAR trafficking and modulates AMPAR gating as it has been shown that modulate complex assembly and also has a role in receptor desensitization and pore permeation (Tomita et al. 2005; Turetsky, Garringer, and Patneau 2005; Ben-Yaacov et al. 2017; Soto et al. 2014).

Another aspect that all members of TARP family share is the capacity to increase single-channel conductance of AMPAR. By contrast, their effect modulating the peak open probability of the channel is less clear as different results have been obtained till date (Soto et al. 2009; Suzuki et al 2008. Tomita et al. 2005; Shi et al 2010).

Finally, an important parameter that all members of TARP family modulates is the channel blocking by endogenous polyamines (like spermine, spermidine or putrescine). These molecules have shown to block many ligand-gated ion channels that are cationic selective (D Bowie and Mayer 1995; Haghighi and Cooper 1998; Z. Lu and Ding 1999). In this case, block by endogenous polyamines affects exclusively CP-AMPARs at depolarized membrane potentials and its caused by a single molecule that occludes the ion channel pore intracellularly (Rozov and Burnashev 1999). It has been extensively studied that TARPs (and CNIHs) can release this channel blocking. The mechanism underlying this attenuation is due to a modulation in receptor gaiting. In association with auxiliary subunits AMPAR permeation to the extracellular side is increased for polyamines. Then, there is a faster exit rate of the blocker that attenuates the effect of these compounds (D Bowie, Lange, and Mayer 1998; P. M. G. E. Brown, McGuire, and Bowie 2017).

However, it is very important to mention that TARPs do not act over and specific GluA subunit but in an intermediate region between AMPARs constituents. That fact will be essential to explain some of the results of this thesis because of the asymmetric arrangement of GluA subunits (Zhao et al. 2016) and then the different positions where TARPs can modulate the receptor (Beatriz Herguedas et al. 2019a).

Finally, even sharing structure, TARPs can modulate differently AMPARs depending on which subunit modulates the receptor. The following subsection will explain some of these differences.

5.1.4.1. Type Ia (γ2 and γ3) and type Ib (γ4 and γ8) TARPs

TARPs are divided in two subfamilies type I and type II TARPs. Type I subfamily is also split into type Ia and Ib TARPs. All members show very similar properties and structures in contrast with type II TARPs. However, these two subfamilies of TARPs differ mostly in terms of AMPAR modulation.

Type I TARPs affect AMPAR biophysical properties such as gating and pharmacology but have also a decisive role in AMPAR trafficking and synaptic targeting (Kato et al. 2016; J. F. Watson, Ho, and Greger 2017; L. Chen et al. 2000).

All type I members have shown to modulate AMPARs but there are some differences between subfamilies. In contrast to type Ia TARPs, type Ib TARPs (γ 4 and γ8) exert more pronounced effects in deactivation and desensitization of AMPAR. In terms of kinetics AMPARs modulated by type Ib TARPs produced slower responses in presence of agonist in either in neuron primary culture and expression systems (Cho et al. 2007; Milstein et al. 2007). On the other hand, type Ia TARP γ2 accelerates AMPAR recovery from desensitization (Coombs et al. 2017; Tomita et al. 2005) (that is the time needed for receptor to recover 2/3 of the maximum response) while γ8 slows this recovery (Schwenk et al. 2012; Beatriz Herguedas et al. 2019a). That point has important implications in a physiological context since desensitization and recovery from

desensitized states impacts on high-frequency transmission (A. Arai and Lynch 1998).

Interestingly, a recent published work demonstrated that differences in extracellular L1 (which is longer in γ8) and L2 loops between γ2 and γ8 can be the reason for the differential modulation. In expression systems exchanging L1 loop between γ8 to γ2 has showed that this segment can modify γ2 effects on desensitization kinetics and steady state. However, the opposite exchange has no effect modifying γ8 properties (Riva et al. 2017). According to this data, extracellular loops are also modulators of steady-state currents which is a parameter strongly related with AMPAR gating kinetics. Indeed, it is also reported that type I TARPs can enhance AMPAR steady-state currents which are dramatically reduced in the absence of these proteins or in the presence of type II TARP γ5 (Kato et al. 2008).

On the other hand, it has been also very well established that all members of type I TARP can increase single-channel conductance in receptors composed by different GluA subunits (Soto et al. 2007; Shelley, Farrant, and Cull-Candy 2012; Tomita et al. 2005).

Another important point that differentiates Type I TARPs from type II is their role in AMPAR trafficking. Type I TARPs are fundamental regulating AMPAR trafficking and synaptic targeting while type II TARPs seem not to play such a predominant role in function (nevertheless, see (Studniarczyk et al. 2013). With PDZ-binding motif (PBM) at their C-termini it is suggested that they can interact with MAGUK proteins for AMPAR anchoring. In addition, members of type Ia and Ib seem to be essential for synaptic establishment of AMPARs. γ2 tail is necessary for PSD-95 binding in synaptic assembly (Bats et al. 2007; Zeng et al. 2019). On the other hand, it has been also purposed a model where γ8 plays a role in receptor accumulation at PSD by PDZ interactions(J. Watson et al. 2020). Indeed, the disruption of the interaction mediated by the PBM implies an increase of AMPAR diffusion and prevents receptor accumulation at synaptic sites (Bats, Groc, and Choquet 2007; J. Watson et al. 2020). All together

shows that members of type Ia and Ib TARP family are key players in AMPARs establishment and trafficking to cell membrane.

Nevertheless, more research about AMPAR-TARP function and interaction needs to be done due to the vast number of combinations that are present in neurons, taking into account that not only type I TARPs are present in AMPAR complex but also other auxiliary subunits as CKAMPs, Cornichons or GSG1L. Moreover, it has been reported differences in AMPAR modulation depending on the model used. In expression systems respect neurons in primary cultures divergence in results are probably due to a differences in expression levels, as an example the effect in kainite efficiency seen in expression systems (Tomita et al. 2007; Cho et al. 2007) but not in CGCs (Milstein et al. 2007). On the other hand, and commented in previous sections, the presence of more than one type of other auxiliary subunits can imply different modulation of AMPARs. This fact made arise the issue of how works the AMPAR complex. At this point, an important question emerged in AMPAR-TARP modulation and it was how AMPAR-TARP stoichiometry will affect receptor properties. This interesting point will be explained in more detail at the end of this section.

5.1.4.2. Type II TARPs (γ5 and γ7)

Different from type I TARPs, the type II TARPs do not exert a strong influence in AMPAR trafficking and have variable effects on AMPAR gating and pharmacology depending on the GluA subunit composition (Tomita et al. 2003). Altogether has made that this subfamily was taken apart.

At the beginning the type II TARP γ5 was assumed to not act as a TARP as it failed rescuing AMPAR-mediated responses (Tomita et al. 2003). However, the role of γ5 modulating AMPARs has some controversy. However, it was later demonstrated thatγ5 has a TARP role in Bergmann Glia, a cell type that express high levels of γ5 along with only CP-AMPARs. This TARP has shown that can modulate GluA2-lacking AMPARs in this cell type. More specifically, it was suggested that γ5 has selective effects on long-form CP-AMPARs (Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-candy 2009)

although there is some controversy since another work claimed that γ5 was specifically modulating GluA2 subunit (Kato et al. 2008). In terms of AMPAR modulation γ5 can increase CP-AMPARs single-channel conductance and also release polyamine block but not modifies AMPAR kinetics (a modulation that type I TARPs mediate (Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-candy 2009). On the other hand, it was showed that γ5 has modulatory effects on GluA2-containing AMPARs in a selective way increasing peak and decreasing steady-state currents. Moreover, γ5 increases rates of deactivation and desensitization of GluA2-containing AMPARs (Kato et al. 2008; Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-candy 2009). As seen before in other AMPAR auxiliary subunits, this divergence in results obtained by different groups can be explained maybe by the nature of the AMPAR complex where the TARP is placed.

On the other hand, γ7 which is the other member of type II TARP subfamily appears to follow some of the same criteria of the other members of the TARP family. Nevertheless, at the very beginning γ7 was supposed to not act as TARP due to its similarity to γ5. However, it was revealed that this protein enhances AMPAR-mediated currents (Kato et al. 2007; Turetsky, Garringer, and Patneau 2005) and on the other hand, this TARP has shown to selectively enhance synaptic expression of CP-AMPARs targeting theme to synapses in CGCs (Studniarczyk et al. 2013). In addition, γ7 showed the capability to resensitize AMPAR currents. This property is shared along with γ4 and γ8 but that not all TARPs have (Kato et al. 2010a). The following figure (figure 12) is modified from Kato et al. 2010b and show the effects of different TARPs in this parameter called resensitization.

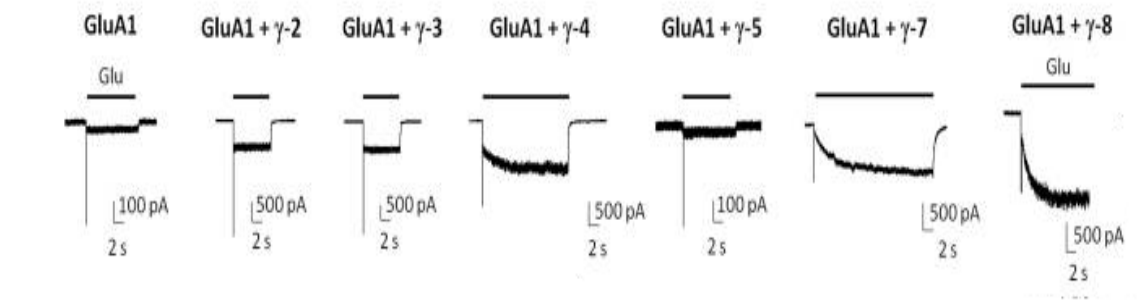


Figure 12. Resensitization currents mediated by type Ib and γ7. Members of type Ib and γ7 can modulate AMPAR resensitizing the receptor and allowing it to become active again if the agonist is applied prolonged during time.

5.1.4.3. AMPAR-TARP stoichiometry

As explained at the end of '**Type Ia (γ2 and γ3) and type Ib (γ4 and γ8) TARPs**', the number of TARPs per AMPAR complex differ from neuronal type and also can explain differences in AMPAR modulation that have been reported in many publications. An important technical improvement in the study of AMPAR-TARP modulation was the use of fusion proteins to try to establish fixed AMPAR-TARP stoichiometries. This technique has been relevant to undercover the role of AMPAR-TARP stoichiometry in AMPAR function (Yun Shi et al. 2009; Dawe et al. 2019; B Herguedas 2019). One of the reasons for this improvement was that the use of fusion proteins permits to limit the effect of differential expression levels that can occur in heterologous expression systems.

Nevertheless, even being TARPs the most studied AMPAR regulatory proteins, less is known about the number of TARP molecules that can be present in an AMPAR complex although AMPAR-TARP stoichiometry has been studied by different methodologies. On the one hand it was tried to bring some light in this topic by single-molecule subunit counting (Hastie et al. 2013) and with electrophysiological approaches (Yun Shi et al. 2009). On the other hand, recent studies characterized different stoichiometries using functional and high definition structural data (Beatriz Herguedas et al. 2019a; Dawe et al. 2019).

In addition, the number of TARPs per AMPAR, as well as elucidating better AMPAR complex composition, can bring new insights into pharmacological treatments due to the different structure of AMPAR complex depending on their auxiliary subunits. Not mentioned before to comment it here, the number and type of TARPs in AMPAR can modulate differently the pharmacological properties of this receptor. On the one hand, the affinity for the partial agonist kainate can vary depending on the number of TARPs per AMPAR (Yun Shi et al. 2009). On the other hand it has been proved that some drugs can block selectively AMPAR specific neuronal populations depending on their association with TARPs (Kato et al. 2016).

However, and mentioned in the previous section, native receptors possess different stoichiometries and auxiliary subunits acting in a complex manner that finally configures the physiological response of AMPARs and the integration of the signalling mediated by this receptor. All together configures a complex picture where each single part is fundamental to understand AMPAR function.

5.2. Transiently AMPAR interacting proteins

It has been widely demonstrated that AMPAR function is modulated by different auxiliary subunits at membrane level. Probably this is the reason for the vast degree of knowledge about AMPAR auxiliary subunits. However, it has been shown that AMPARs transiently interact with other proteins and this is also important for membrane trafficking (Carrasco et al. 2012; Gratacòs-Batlle et al. 2014, 2018b; Bissen, Foss, and Acker-Palmer 2019). It could be said that their role is perhaps underrated; however, the AMPAR availability to membrane insertion depends on the previously functional assembly at the ER and later in AMPAR anchoring.

At this section it will be covered some of the proteins that play also an important role in AMPAR physiology but do not modulate receptor biophysical properties (mainly due to their absence at the plasma membrane together with the receptor). These proteins are key in AMPAR assembly and trafficking (Porcupine, ABHD6, FRRS1L, CPT1C) and

work in a synergistic way.

5.2.1. Porcupine and ABHD6

AMPAR signalling is fundamental for the correct function of the entire nervous system. For this reason, it is important not only their biophysical behaviour but also their assembly and trafficking. The proteins explained before form part of the AMPAR auxiliary subunits. However, about 30 proteins interact with AMPAR at some stage of their cycle and most of themes are still not deeply studied.

That is the case of porcupine (PORCN) and ABDH6 (α/β-hydrolase domain-containing 6). Both proteins are widely expressed along many tissues and cells types (Schwenk and Fakler 2020) and very few articles have been published about these two proteins in relation with AMPARs. On the one hand, PORCN proteins are a group of transmembrane enzymes localized at ER that catalyzes O- Palmitoylation (a palmitoylation over a serine or threonine residue that should not to be confused with palmitoylation) over enzymes that intervene in wnt signaling (wnt proteins are codified by Wingless and Int genes giving the name wnt) (Caricasole et al. 2002). On the other hand, ABHD6 along with ABHD12 was firstly characterized as brain enzyme with 2-AG hydrolase activity which is the hydrolysis of the endocannabinoid of 2-arachidonoylglycerol (2-AG) (Blankman, Simon, and Cravatt 2007). However, in 2016 it was published that both enzymes intervene in AMPAR biology (Erlenhardt et al. 2016). Nevertheless, the catalytic activity of enzymes seems not to be the answer to how they modulate AMPAR traffic (Erlenhardt et al. 2016).

Both proteins were showed to participate in AMPAR surface expression in hippocampal neurons. First, PORCN KO mice neurons as well as the knockdown neurons showed reduced AMPAR expression at cell membrane (GluA1 subunit and GluA2/3 AMPARs). Interestingly PORCN knockdown in neurons showed also changes in the characteristics of glutamate-evoked currents in whole-cell recordings. This fact indicates that AMPAR complex composition may be altered. However, no changes in LTP experiments were seen in PORCN KO mice. Interestingly, when this protein is co-

transfected with GluA subunits in expression systems it causes a retention of AMPAR that cannot be solved even co-transfecting along with other auxiliary subunits like stargazin or CNIHs. These results probably mean that in neurons there is a much more complex machinery that is missing in expression systems.

On the other hand, ABHD6 in contrast to PORCN seems to trap GluA subunits at ER preventing their traffic (Schwenk et al. 2019). The overexpression of this protein in both expression systems and neurons produces a decrease of AMPAR mediated currents (Schwenk et al. 2019; Wei et al. 2016). In fact, the lack of ABDH6 has been reported to enhance AMPAR currents (Wei et al. 2016). However, ABHD6 is an important protein in the assembly chain of AMPAR complexes. In a very recent publication it is proposed that this protein associates with GluA monomers in a dimeric complex. This stable complex prevents GluA association with other AMPAR subunits forming dimers, by contrast of what occurs ins expression systems where GluA subunits interact by their high-affinity interactions via TMDs (K. Kim, Yan, and Tomita 2010). However, how ABHD6 stabilizes AMPAR monomers and inhibits their dimerization is still unclear.

However, despite the lack of knowledge about how exactly these two proteins modulate AMPAR trafficking, it seems clear that they participate in the first steps of AMPAR biogenesis once synthetized. Finally, it is purposed a model where the interactions of PORCN and ABHD6 are followed by the action of the FRRS1L and CPT1C proteins. The interaction of the FRRS1L-CPT1C complex with the AMPAR complex is explained in the following section

5.2.2. FRSS1L

One of the proteins that intervene in AMPAR assembly at ER is the ferric-chelate reductase 1-like (FRRS1L) also named C9orf4. However, first evidences of this protein came from a proteomic study that associated FRRS1L with AMPAR as a non-core associated protein but no further research was done (Schwenk et al. 2012). Later, a few studies reported that mutations in FRRS1L could lead into pathological conditions

such as epileptic encephalopathy, severe intellectual disabilities in humans and been related with Huntington disease (Madeo et al. 2016; Stewart et al. 2019; Schneider and Bird 2016). Moreover, in these studies was pointed that abnormalities in AMPAR signalling could be an important factor in pathogenesis.

However, at the first time FRRS1L was reported to be associated with AMPARs it was not very clear how the relationship with the receptor was. FRRS1L was predicted to be a single-pass transmembrane protein that interestingly was widely expressed along brain regions in similar amounts as AMPAR subunits (Schwenk et al. 2012).

Now, much more is known about this AMPAR transiently interacting protein. First, it was tested that FRRS1L participates in AMPAR assembly at ER being part of priming complex along with CPT1C. Moreover, both proteins are exclusively localized at the ER being absent in AMPAR complex at cell membrane (Brechet et al. 2017b). Interestingly the priming complex formed by FRRS1L has shown to interact with AMPARs before the AMPAR complex is joined by other auxiliary subunits such as TARPs, CNIHs or GSG1L. Moreover, and related with this fact, it is proposed a model where this protein priming complex acts as a catalyst in AMPAR biogenesis. The priming consists in the association of 4 FRRS1L-CPT1C complexes with one AMPAR (a tetramer of 4 GluA subunits). Then, the catalytic process mediated by FRRS1L and CPT1C acts promoting the interaction and assembly of the receptor tetramer with TARPs, CNIHs or GSG1L. Then the AMPAR complex including auxiliary subunits exits from the ER while the priming complex stays at this organelle (Brechet et al. 2017b). The importance of this catalytic process relapses in the fact that AMPAR interaction with other auxiliary subunits is highly necessary for ER exit and finally synaptic delivery (Coleman et al. 2006; Penn, Williams, and Greger 2008). However, it is worth to mention that the catalytic action of the priming complex is reported to not be mandatory for AMPAR association with other auxiliary subunits but its absence sharply decreases AMPAR surface expression and alters receptor signalling (Brechet et al. 2017b; Schwenk et al. 2019).

Finally, in a more physiological aspect, the role of FRRS1L has implications not only in AMPAR exit from ER but also in the final composition of AMPAR complex. As

commented at the beginning of the section, this protein has been related with pathological conditions where AMPAR signalling is altered. In fact, in rat model the knockout of this protein leads into changes of the AMPAR complex composition. As seen in previous sections, these changes imply a modification in receptor properties what is at the end translated in different signal integration.

5.2.3. CPT1C

In the previous section it was mentioned the importance of a priming complex that catalyses AMPAR association with other auxiliary subunits. This priming complex is formed by FRRS1L and the carnitine palmitoyltransferase 1C (CPT1C) as it was previously mentioned. However, different from FRRS1L, CPT1C belongs to a previously known family, the CPT1 family.

In this protein family CPT1A and B have an important role in lipid metabolism at mitochondria and are the most widely studied CPT1 forms (N. F. Brown et al. 1997; Zammit 1999). These two enzymes catalyze the exchange of acyl groups between carnitine and CoA to allow the transport of long chain fatty acids from cytoplasm to mitochondria for β-oxidation (N. F. Brown et al. 1997). CPT1C is highly homologous with the other CPT1 members which are proteins formed by a 2 transmembrane domains; however, CPT1C has many particularities that make this protein differ from its family members. First it has a 100-fold lower catalytic activity compared to the other CPT1 members (Sierra et al. 2008). In addition, different from the other family members, CPT1C is located in the endoplasmic reticulum (ER) instead of the mitochondria (Carrasco et al. 2013) and is a specific CPT1 brain isoform highly expressed in hypothalamus, hippocampus, cortex and cerebellum (Price et al. 2002).

Later, as other AMPAR-interacting proteins, CPT1C appeared in the so mentioned proteomic study performed by Jochen Schwenk (Schwenk et al. 2012) as a protein that could interact with AMPARs. At this point it had more sense its expression in brain. Then, some publications tried to bring light about the relationship between this protein and the glutamate receptors.

Recent studies point that CPT1C is an ER resident protein with an important role priming the AMPAR complex (receptors plus auxiliary subunits) along with FRRS1L (Brechet et al. 2017b). There it is purposed that CPT1C in association with FRRS1L mediate catalysis of AMPAR association with other auxiliary subunits and then promote AMPAR exit from the ER. In addition, it was observed that the priming has no preference for any AMPAR subtype so this step not depends on the GluA subunits that form the receptor. However, other works found that CPT1C has preference for GluA1-containing AMPARs, promoting GluA1 homomers delivery till cell membrane (Gratacòs-Batlle et al. 2014). For this promotion of GluA1-containing AMPARs till cell membrane it was purposed that CPT1C acts as a depalmitoylating enzyme over GluA1 subunit (Gratacòs-Batlle et al. 2018). The reason for this purposed model relies in various aspects. On the one hand, CPT1C acts as a depalmitoylating enzyme as the other members of CPT1 family (Sierra et al. 2008). On the other hand, GluA1 has two palmitoylable sites at C585 and C811 that determines this subunit traffic (T. Hayashi, Rumbaugh, and Huganir 2005; Yang et al. 2009; Lin et al. 2009) . In fact, C585 residue is crucial for GluA1 membrane delivery enhancement mediated by CPT1C (Gratacòs-Batlle et al. 2014).

However, the GluA1 selectivity of CPT1C was tested mostly in expression systems. When moved to neuronal primary culture CPT1C transfection increased AMPAR surface trafficking in CPT1C KO neurons (Gratacòs-Batlle et al. 2014, 2018b). That probably indicates that in more physiological models the preference is not detectable. It is worth to mention that in CPT1C KO hippocampal pyramidal neurons AMPAR mediated currents and surface expression was diminished. However, AMPARs can still traffic to cell membrane and exhibit responses in presence of agonist. This fact matches with the idea that CPT1C and FRR1L role is important but not mandatory for AMPAR traffic. Nevertheless, it seems clear that even AMPAR can exit from ER and arrive to membrane; the receptor signaling is affected as it is seen in animal models. In rodents it has been reported that KO mice display poor performance in Morris water maze (D'Hooge and De Deyn 2001) and exhibits deficient motor coordination. Both indicate alteration in hippocampal and cerebellar function (Carrasco et al. 2012, 2013).

On the other hand, CPT1C gain of function has been related to severe growth retardation and reduction of brain weight in transgenic mice (Reamy and Wolfgang 2011). In addition, it has been related with hereditary spastic paraplegia and with a role in cell senescence (D. Hong et al. 2019; Y. Wang et al. 2018); altogether makes this protein an interesting subject of study.

The last examples of AMPAR impairment function due to the absence or alteration of CPT1C show that even a non-essential AMPAR-interacting protein is crucial for correct balance and correct signaling in the nervous system.

To end this section and to relate briefly a part of this thesis project with the before mentioned importance of the AMPAR-TARP stoichiometry, just highlight the importance of the ER resident proteins in AMPAR assembly with auxiliary subunits. As it was explained before, a model purposed that the complex FRRS1L-CPT1C acts promoting AMPAR association with auxiliary subunits.

5.3. AMPAR scaffolding proteins

As explained at the beginning of the section, in '**5. AMPAR interacting proteins**', synaptic AMPARs are forming a complex where the receptor is associated with auxiliary subunits but also mediates interactions with scaffolding proteins via receptors or auxiliary subunits domains. The following section will put the focus on the proteins that are present in the AMPAR complex but are not auxiliary subunits.

First to mention and to remember, AMPARs can freely diffuse in the plasma membrane of neurons; however, at synapses these receptors are clustered in a complex macromolecular machine positioned postsynaptically across from synaptic vesicle release sites at the presynaptic active zone called post synaptic density (PSD) (J. F. Watson, Ho, and Greger 2017).

At this site of the synapse AMPARs are anchored by interactions with other proteins. These interactions can be mediated directly by the GluA subunits or via auxiliary

subunits. For this reason, it will be covered proteins that interact both, directly and indirectly with AMPARs.

In terms of direct interactions, the GluA2 or GluA3 subunits AMPAR can interact directly with PDZ domain-containing proteins, which are a group of proteins that share PDZ-binding motif and mediate receptors anchoring. These GluA subunits mediated this interaction via C-tail where they have the –SVKI amino acid sequence (Dong et al. 1997). Among the proteins with the PDZ domain, in AMPAR physiology it should be highlighted the glutamate receptor-interaction proteins (GRIPs) family and the protein interacting with C kinase (PICK1) (Dong et al. 1997; Srivastava et al. 1998; J. Xia et al. 1999).

The members of the GRIP family have shown to mediate final steps of AMPAR delivering to cell membrane via interactions with members of the motor proteins kinesins. On the other hand, GRIPs seems to play a role in both, AMPAR insertion and internalization from the synapse (Osten et al. 2000; Chung et al. 2000; Braithwaite, Xia, and Malenka 2002; DeSouza et al. 2002). That functional regulation of receptor insertion at membrane is translated in plasticity processes like long term depression (LTD). Indeed, it has been shown that GRIP1 and GRIP2 double KO mice showed impairment forming LTD at Purkinje cells (Takamiya et al. 2008).

On the other hand, PICK1 also uses its PDZ-domain to bind to AMPARs GluA2 and/or GluA3 C-tails. The role of PICK1 in AMPAR trafficking and anchoring has not been extensively studied like members of GRIP family. Nevertheless, it has been demonstrated that PICK1 is required for synaptic plasticity that involves AMPARs as LTD formation is altered in diverse regions of the nervous system as hippocampus, cerebellum, cortex and ventral tegmental area (Terashima et al. 2008; Steinberg et al. 2006; Jo et al. 2008; Bellone and Lüscher 2006).

In addition, there is another large group of proteins that mediates interaction with AMPARs and it is formed by the membrane-associated guanylate kinase (MAGUK) family. This is a group of proteins with a vast number of members that are expressed

along different cell types and tissues including brain (Danielson et al. 2012; Butz, Okamoto, and Südhof 1998).

MAGUKs have all a guanylate kinase-like domain (GK-like domain) and are known to be scaffolding proteins that operate organizing densities and activities of both AMPA and NMDARs. This large family is subdivided in several subgroups where the most studied are the discs large homologs (DLGs), the calcium/calmoudulin-dependent serine protein kinase (CASK) and the plamitoylated membrane proteins (MPPs) (J. Zhu, Shang, and Zhang 2016).

These large family of proteins has been associated with AMPARs during many years but hardly ever in direct association with these receptors. However, members of MAGUK family have been reported to form part of the AMPAR complex by interacting with other proteins associated with AMPARs. For example, CASK binds to GRIP1, that can be associated with GluA2 or GluA3 subunits (C.-J. Hong and Hsueh 2006); and PSD-95 (postsynaptic density protein 95; member of DLGs subfamily) binds to γ2 TARP via PDZ binding motives (PBMs) of the auxiliary subunit (Chetkovich et al. 2002; Schnell et al. 2002; Hafner et al. 2015). However, it has been discovered that DLG-1, member of DLGs subfamily, has the capability associate directly and exclusively with the CTD of GluA1 subunit different to other members DLG subfamily as DLG2-4 (Leonard et al. 1998; Fukata et al. 2005; Coleman et al. 2006). In addition, other direct interactions from MAGUK members with GluA subunits cannot be discarded due to the large number of components of this family of proteins. Finally, it is worth to mention that in a more physiological context MAGUK proteins are linked to human pathologies as it has been related that some mutations in the genes that encode for members of this family can lead into many forms of cancer and neurological disorders (J. Zhu, Shang, and Zhang 2016; Grant 2012; Najm et al. 2008)

6. NMDARs

As already introduced, NMDARs are tetrameric ionotropic glutamate-gated channels like AMPA and Kainate receptors. These receptors are widely expressed in CNS and are highly permeable to Ca^{+2} but also to Na^{+} and K^{+} (Mayer and Westbrook 1987). The Ca^{+2} influx through these receptors triggers most of the NMDAR-dependent physiological and pathological effects. For this reason, NMDARs are of special interest due to their role in synaptogenesis, synaptic plasticity and learning and memory (Michael Hollmann and Heinemann 1994; Lau and Zukin 2007; Sanz-Clemente, Nicoll, and Roche 2013).

However, NMDAR downstream signaling pathways have strong implications in CNS disorders such as stroke, neuropathic pain and neurodegenerative disorders like Parkinson, Huntington and Alzheimer disease (see for reviews Mellone and Gardoni 2013; R. Wang and Reddy 2017). Because of their implication in this wide range of pathological and physiological conditions there is a strong interest to understand their function and the possibilities of pharmacological modulation.

6.1. Subunit diversity along the brain

The subunits that form the tetramer are named GluN1-3 and posttranscriptional mRNA processing raises the number of splice variants of the GluN1 subunit that can combine to form the receptor. GluN1 subunit has 8 splice variants (Sugihara et al. 1992; Zukin and Bennett 1995) and in addition, GluN2 and 3 are encoded by four and two genes respectively, codifying for the subunits GluN2A-D and GluN3A and B (Monyer et al. 1992). All together brings a high number of possible combinations to form an NMDAR. However and contrary to AMPARs, NMDARs are always heterotetramers containing obligatorily 2 GluN1 subunits in combination with 2 GluN2 or GluN3 subunits (Ulbrich and Isacoff 2008). Moreover, the co-existence of different GluN1 variants or even GluN2 types in a single receptor has been described (Blahos and Wenthold 1996; Chazot and Stephenson 1997; Sheng et al. 1994).

The obligatory subunit GluN1 is expressed ubiquitously in CNS in all life-stages. It exists a different developmental and regional variation depending on the GluN1 isoform (Laurie and Seeburg 1994; Paupard, Friedman, and Zukin 1997). The GluN1 subunit is encoded by a single gene but it has been reported eight isoforms named GluN1-1a – GluN1-4a and GluN1-1b – 4b. In the first set of GluN1 variants, GluN1-a, the differences between rely in CTD lengths that affect receptor trafficking. On the other hand GluN1-b variants differ in the extracellular region of the subunit and affect receptors gating and pharmacology (Rumbaugh et al. 2000; Vance, Hansen, and Traynelis 2012; Horak and Wenthold 2009). This subunit is widely expressed and abundantly distributed throughout the brain. Its expression starts at embryonic 14 (E14) and remains till adulthood. However, the distribution even ubiquitously differs in terms of splice variants along the nervous system (Pierre Paoletti 2011; Watanabe et al. 1992; Monyer et al. 1994). For example, GluN1-a isoforms is found in homogeneously throughout brain grey matter, whereas GluN1-b isoforms are mostly restricted to CA3-layer but also expressed in sensorimotor cortex, neonatal lateral caudate, thalamus, hippocampal CA3 field, and cerebellar granule cells (Monyer et al. 1994).

On the other hand, the GluN1-GluN3 combination is expressed at only very few levels (reviewed in Paoletti 2011).

Different from the GluN1 subunit, GluN2 subunit have expression patterns that differs during the development and regions (Watanabe et al. 1992; Akazawa et al. 1994; Monyer et al. 1994). These differences influence receptor biophysical properties (Akazawa et al. 1994; Monyer et al. 1994; Sheng et al. 1994).

In rodent models, GluN2 expression is under developmental control. GluN2A It is not expressed at embryonic stages like other GluN2 subunits and its expression starts after birth at very low levels. However, it rises to become widely and abundantly expressed along the CNS in adult (Monyer et al. 1994). On the other hand, GluN2A-containing NMDARs show characteristic biophysical properties. These receptors display faster postsynaptic currents that cells that not express this subunit. Moreover, in expression

systems GluN2-containing NMDARs mediate peak currents 4-fold larger than GluN2B-containing (Flint et al. 1997; N. Chen, Luo, and Raymond 1999).

By contrast the GluN2B expression starts in embryonic brain peak is around postnatal day 7-10 while GluN2A expression is rising sharply and GluN2B is progressively been restricted to forebrain areas, remaining at high levels in these regions. This variation of pattern in expression leads into changes from GluN1/GluN2B to GluN1/GluN2A receptors in many brain regions, for example in thalamic and cortical neuron during early postnatal development (McKay et al. 2018; Bar-Shira, Maor, and Chechik 2015). The early expression of GluN2B has been related to rapid cortical synaptogenesis and to have an important role in brain development and circuit formation (Cohen and Greenberg 2008; Hall, Ripley, and Ghosh 2007). Indeed, the global KO mice for GluN2B display neonate lethality whereas overexpression of GluN2B subunit has shown to enhance spatial memory performance and hippocampal LTP potentiation in mice (Kutsuwada et al. 1996; Y. P. Tang et al. 1999). In terms of the biophysical properties, the change from GluN2B-containing NMDARs to GluN1/GluN2A receptors influences receptor biophysics. In this aspect it has been reported NMDAR sensitivity to ifenprodil (a selective antagonist for GluN2B subunit) at very early stages of development while very low effect at more advanced phases (due to a major expression of GluN2A subunit) (Williams et al. 1993; Barth and Malenka 2001). On the other hand, GluN2B-containing NMDARs show less amplitude in peak current and also slow desensitization kinetics (that leads into high steady-state currents) (N. Chen, Luo, and Raymond 1999).

The other two GluN2 subunits have not the same predominant role as GluN2A and GluN2B. The GluN2C subunit appears late in the development (postnatal 7 approximately) and its expression remains mainly confined to the cerebellum (in CGCs) and the olfactory bulb. On the other hand, GluN2D expression is present in the embryonic brain at caudal regions. Then its expression rises markedly following the birth but remains weakly expressed in adult CNS at diencephalon and brainstem (reviewed in Paoletti, Bellone, and Zhou 2013). This switch, like the GluN1/GluN2B to GluN1/GluN2A change is manifested functionally usually in changes in kinetics and

sensitivity to allosteric NMDARs modulators that are subunit-specific (Fritschy et al. 1998; Watanabe et al. 1998).

Finally, as mentioned before, there is the GluN3 subunit with two different forms, GluN3A and GluN3B. The GluN3A expression has peaks in postnatal life and has low levels in adulthood in rodents. By contrast, GluN3B expression levels are low at first stages of postnatal life and increases progressively since reaching his maximum levels in adult animals. In adults, GluN3B is highly expressed in motor neuron (Pierre Paoletti, Bellone, and Zhou 2013). In addition, GluN3B is widely expressed in rat forebrain, cerebellum and lumbar sections of spinal cord, suggesting a role for this subunit in NMDAR function in adult (Wee et al. 2008).

6.2. Structure

Along with the other glutamate receptors, NMDARs share the typical modular organization with four transmembrane domains in each subunit and an extracellular region with a large globular clamshell-like domains and a prominent NTD that is involved in subunit assembly (Yao and Mayer 2006; Pierre Paoletti and Neyton 2007).

All of the NMDAR subunits have a significant homology level and are highly related in structure and show great similarities with the other members of the glutamate-gated channels (Traynelis, Wollmuth, Mcbain, et al. 2010). About organization, all subunits have an extracellular NTD linked to an extracellular clamshell-like ligand binding domain (LBD), which in turn is connected to the TMDs that form the ion channel. Same as other glutamate-gated ion channels, NMDARs have 3 transmembrane domains named TMDI, TMDIII and TMDIV and a reentrant loop named TMDII that forms the ion channel. Finally, the receptor have an intracellular CTD variable in length depending on the subunit and involved in NMDARs trafficking, anchoring and coupling to intracellular components of the signaling complexes (reviewed at (Dingledine et al. 1999; Pierre Paoletti 2011)). As in AMPARs, NMDARs have a split LBD into two discontinuous segments, S1 and S2, by insertion of the ion channel. However, distinct from AMPA and Kainate receptors, NMDARs needs two agonists binding to channel opening as

explained below. The GluN1 subunit binds to glycine whereas GluN2 and 3 are responsible of glutamate binding. In addition, the global structure of the receptor resembles a mushroom and different from AMPARs, the extracellular layers are more compact; with a NTD adopting a different conformation resembling an interdigitated structure within the crevices of the LBD layers. The TMD however, reassembles AMPAR topology like was mentioned from all iGluRs (C.-H. Lee et al. 2014).

6.3. Synthesis, trafficking and anchoring

At ER levels NMDARs subunits are synthetized with an excess of the obligatory subunit GluN1 relative to GluN2, what means that there is sufficient amount of GluN1 available for GluN2 or 3 which are not obligatory for tetramer formation (Huh and Wenthold 1999; Chazot and Stephenson 1997).

In terms of tetramer formation, it has been reported that the NTD promiscuity between GluN1 and 2 can explain the development of different working models of the assembly of NMDARs. In fact, there are three possible models to explain tetramer formation. These possible explanations where reviewed in 2014 and also in 2017 without discarding or confirming any model (Horak et al. 2014; Hansen et al. 2017).

In the first one, it is explored the idea that GluN1-GluN1 and GluN2-GluN2 homodimers are initially formed and are required for functional heterotetramer formation (Meddows et al. 2001; Schorge and Colquhoun 2003; Papadakis, Hawkins, and Stephenson 2004; Hansen, Furukawa, and Traynelis 2010).

The second model purposed explains that GluN1-GluN2 hetero dimers are required for formation of the heterotetrameric receptors. The heterodimers then are associated to in a dimer-of-dimers arrangement that conform finally the heterotetramer (Qiu et al. 2005).

Finally, the most recent model suggests that the NTDs of GluN1 subunits initially homodimerize and then the GluN2 subunits are added sequentially to form the

tetramer (Schüler et al. 2008; Atlason et al. 2007). However, in all models the NTD seems to be the key feature for receptors formation and it is indeed essential in the initial dimerization of GluN subunits (P Paoletti, Neyton, and Ascher 1995; Schorge and Colquhoun 2003; Schüler et al. 2008).

However, it is worth to mention that at least in expression systems NMDAR can also be formed by assembly of two GluN1 subunits and GluN2A plus GluN2B, forming a triheteromeric NMDAR with three different subunits (in total, (2)GluN1-GluN2-GluN2B) (Lü et al. 2017).

On the other hand, following their synthesis, the NMDARs subunits retention at ER level is important due to the fact that this glutamate receptor is obligatorily a heterotetramer, so subunits cannot traffic to cell membrane as homomers. After synthesis, it has been shown that GluN2 and some splice variants of GluN1 are retained at ER until they are assembled (McIlhinney et al. 1998). In addition, in GluN1 lacking mice it has been demonstrated that GluN2 subunits are accumulated in ER (Fukaya et al. 2003). To control this assembly or retention at ER it has been purposed different regions to play an important role. For example, the CTD of some GluN1 splice variants in the C1 cassette (Standley et al. 2000; Scott et al. 2001; Horak and Wenthold 2009), the phosphorylation serine residues of GluN1 target of PKA and PKC nearby the RRR motif (Scott et al. 2001) or the glycine binding site can also be necessary for the functional release of NMDARs (Kenny et al. 2009). Similarly, the glutamate binding site of the GluN2B subunit controls the early processing of NMDARs and it has been speculated that newly synthetized NMDARs activated by agonists at ER can be 'tested' by a specific quality control machinery like it happens in AMPARs (Penn, Williams, and Greger 2008).

After tetramerization and ER release NMDAR are processed in the somatic Golgi apparatus and distributed to the *trans* Golgi network and endosomes to reach at the end the membrane. Most of the NMDAR are processed in cell body and then transported to synapse but some of theme use dendritic ER or Golgi outposts (Wenthold et al. 2003; Jeyifous et al. 2009).

Finally, at the synapse, NMDARs are associated with scaffolding proteins of the PSD, especially the before mentioned MAGUKs. PDZ binding domain at CTD of GluN2A and B subunits bind to the first and second PDZ domains of MAGUKs (Kornau et al. 1995; Niethammer, Kim, and Sheng 1996) and NMDAR are also bound to these proteins by other domains(Cousins, Kenny, and Stephenson 2009; Bard et al. 2010; B.-S. Chen et al. 2011). The principle MAGUKs that interact with GluN2A and B are PSD-95 and SAP102 in most of the forebrain mature synapses (Sans et al. 2000). PSD-95 is almost immobile in PSD while SAP102 is more widespread in extra synaptic sites and in the postsynaptic density (Müller et al. 1996; Sans et al. 2000; Standley et al. 2000). In addition, in spines SAP102 mobility is dependent on actin and glutamate receptor activation (Zheng et al. 2010, 2011; Müller et al. 1996).

At synapses NMDARs can be modulated by modulating the function of individual NMDARs complexes or by changes in the composition or number of NMDARs in the synapse (see reviews (Rebola, Srikumar, and Mulle 2010; Pierre Paoletti 2011). Individual complexes are purposed to be modulated in many ways, including co-agonist activation, inhibition by extracellular zinc, effects of polyamines and redox modulators and also by CTD phosphorylation of receptors at many sites (reviewed at (Horak et al. 2014)).

6.4. NMDAR Pharmacology

As in AMPARs, many compounds have been studied as modulators of these glutamate receptors due to their importance in physiological and pathological conditions. They have been classified as agonists, competitive and non-competitive-antagonists, and positive or negative allosteric modulators.

1. Agonists: The most important agonist in NMDAR is in fact the compound that gives name to these receptors, the N-Methyl-D-Aspartic acid (NMDA). This agonist is an amino acid derivate from aspartic acid and acts mimicking the action of glutamate, the physiological NMDAR agonist.

2. Antagonists (Competitive): The first antagonists discovered for NMDARs were glutamate competitors which acted in the binding site in GluN2A and B subunits. (R)-2-amino-5-phosphonopentanoate (R-AP5 or AP5) was one of the first antagonists discovered and remains widely used because of the strong selectivity showed by NMDARs in front of other iGluRs. On the other hand, (R)-CPP and (R)-AP7 are more selective for subunit composition blocking in major grade GluN2A in front of GluN2D. By contrast, Merz Pharma have developed MDL 105-519 that is a competitive antagonist that bind to the glycine binding site in GluN1 (Furukawa and Gouaux 2003).

3. Antagonists (non-competitive): Different from the competitive antagonists these set of compounds do not compete with glycine or glutamate to bind with the ligand binding site of the receptor. In terms of non-competitive antagonists there is a great number of compounds that act as open-channel blockers occluding the channel pore (Kew and Kemp 2005). As they all may diverge structurally, they are all positively charged and depend in membrane potential as act in a voltage-dependent manner, similarly as AMPAR open-channel blockers. As these blockers act over the pore, there is a poor discrimination between NMDAR subtypes. Examples are phencyclidine (PCP), thienylcyclohexylpiperidine (TCP) and ketamine. However, there are non-competitive antagonists that can discriminate between NDMAR subunits. Dizolcipine (MK-801) shows stronger inhibition for GluN2A or 2B containing receptors over GluN2C and 2D. In addition, there are non-competitive antagonists that can act in different GluN subunits. 7-Chlorokynurenic acid (7-CKA) for example, is a non-competitive antagonist of the glycine site (Kemp et al. 1988). In addition, memantine is an approved non-competitive antagonist for NMDARs that is used in Alzheimer disease treatment (Jon W Johnson and Kotermanski 2006; S. Lu and Nasrallah 2018).

4. Positive modulators: These compounds can increase the response or agonist affinity binding to a different site than the ones that bind the agonists. For example, endogenous polyamines can increase the NMDARs sensitivity to glycine binding at the interface between ATDs of GluN1 and GluN2B subunits (Mony et al. 2011) in GluN2B-

containing receptors (Williams et al. 1990). By increasing open channel probability, neurosteroids as pregnelone sulfate (Horak et al. 2004) can potentiate responses from GluN2A or 2B-containing receptors (Wu, Gibbs, and Farb 1991; Malayev, Gibbs, and Farb 2002; Horak et al. 2004). On the other hand, GluN2C or 2D can be potentiated by increasing channel opening frequency by a highly selective allosteric enhancer (3-chlorophenyl)(6,7-dimethoxy-1-((4- methoxyphenoxy)methyl)3,4-dihydroisoquinolin-2(1H)-yl) methanone (CIQ) (Mullasseril et al. 2010).

6.5. NMDAR in pathological conditions

As an important part of this thesis project is to analyze how distinct *de novo* mutations impact in NMDARs biophysical properties, it is important to at least comment briefly the role of NMDARs in some pathological conditions. As mentioned before the calcium influx through these receptors is crucial for many intracellular signals.

For example, in Alzheimer disease it has been thought that NMDARs signaling plays a critical role in the effects of beta-amyloid peptide (Malinow 2012) and blocking these receptors prevent the depression in synaptic transmission and the structural changes induced by the overexpression of beta-amyloid peptide (Selkoe 2002). In Parkinson disease where the glutamatergic and dopaminergic signaling are known to interact to control motor function. In this disease, GluN1 and 2B but not 2A expression is diminished. Interestingly, L-DOPA treatment reverses these alterations (Dunah et al. 2000). On the other hand, in schizophrenia, which is a mental condition characterized by disintegration of thought processes and emotional responsiveness caused by alterations in brain connectivity, the glutamatergic hypothesis suggests that the observed symptoms are due to a NMDAR hypofunction in cortico-strial projections (Gaspar et al. 2009; D. J. Gerber et al. 2003; Hahn et al. 2006).

All together show that directly or indirectly, the NMDAR function is a key component in the nervous system function. For this reason, alterations in NMDAR properties due to a mutation that impacts directly in the receptor function can lead into a complex

pathological condition.

6.5.1. De novo mutations in NMDARs

As seen before NMDAR is a key element in glutamatergic neurotransmission. It can play a fundamental role in neurogenesis, synaptogenesis and synaptic plasticity processes and GluN subunits expression vary during the development. For this reason, mutations in GRIN genes, which encode for NMDARs, can lead into neurodevelopmental disorders. *De novo* GRIN mutations have been reported to produce infantile epileptic encephalopathy and autosomal dominant mental retardation (Lemke et al. 2014; Endele et al. 2010; de Ligt et al. 2012; O'Roak et al. 2011). Most of the pathological conditions associated with the GRIN genes have been arranged in group called GRIN-related disorders. These are rare paediatric encephalopathies due to pathogenic GRIN variants. By the time this work has been done about 500 individuals house a likely-pathogenic GRIN variant that has been reported worldwide (www.grin-database.de). However, the incidence of GRIN-related disorders is probably underestimated as they are manifested clinically in a wide spectrum of neurological and systemic alterations. In this range are included intellectual disability, hypotonia, communication impairment, epilepsy, movement and sleep disorders and gastrointestinal disturbances (Platzer et al. 2017; Swanger et al. 2016; Lemke et al. 2016). The following figure extracted from Santos-Gómez et al. 2020 is a visual resource to situate the incidence of pathological GRIN variants along receptor subunits (figure 13):

Figure 13. GRIN variants along different GluN subunits. GRIN-related disorders have more incidence when are associated to GluN2A or GluN2B subunits while the GRIN variations on the other GluN subunits show no association with pathological treats.

One of the most important parts to understand how GRIN variations affects NMDAR function is to know the impairment caused by a mutation in the receptor function. An amino acid change can modify receptor trafficking, formation, biophysical properties of the channel and/or subunit-to-subunit interactions. Altogether, these alterations can modify neuron-to-neuron transmission and finally lead into a pathological condition. The difficult to study the effect of any mutation in a patient relies in evaluate how a single mutation modifies NMDARs biology. On the one hand, GRIN mutations have been reported to produce a gain of function (GoF) in NMDARs that

carry some specific variations. On the other hand, the expression of a pathogenic allele can reduce channel function or trafficking (loss of function, LoF). Moreover, it has been reported GRIN-related disorders associated to all GluN subunits (Platzer and Lemke 1993; XiangWei et al. 2019; Endele et al. 2010; Lemke et al. 2014) what not only means the crucial role of the NMDARs for normal brain condition but also a complex scenario for clinical and basic research collaboration.

7. AMPAR and NMDAR in long term potentiation

To end with this introduction, it is worth to talk about the long-term potentiation (LTP) which is a basic process of neuronal function that involves both AMPA and NMDARs.

The most accepted definition for the long-term potentiation is a long-lasting enhancement strength that follows short high-frequency activity. This strengthen of the synapse can happen at two different levels, the presynaptic and the postsynaptic part. Indeed, it has been topic of discussion for many years whether this process implies mostly the presynaptic or the postsynaptic terminal. For example, there is a general concordance that in mossy fibre LTP is NMDAR independent and induced and expressed presynaptically (Roger A Nicoll and Schmitz 2005; R A Nicoll and Malenka 1995). On the other hand, LTP in the CA1 region is induced postsnaptically and has a strong postsynaptic component that directly involves AMPA and NMDARs (Roger A Nicoll 2003; Malinow and Malenka 2002). In addition, there are several variables that have been purposed to affect and modulate LTP. Among others, the frequency of stimulation, the pattern of this stimulation and the strength of the stimulus are the most widely accepted to be crucial in LTP formation. The debate between pre and postsynaptic mechanisms is more extensively reviewed in (Granger and Nicoll 2014).

As the present work is based in postsynaptic receptors, this section will be focused in the postsynaptic mechanisms that imply AMPA and NMDARs.

Explained before, it seems now that NMDARs are critical in many LTP learning and memory process but not all (NMDAR-independent LTP) (T. V. P. Bliss, Collingridge, and

Morris 2013). NMDARs are a deeply voltage dependent ionic channel and conduct little current at resting potentials due to a physiological Mg^{+2} block. However, when the Mg^{+2} is released (as it happens when there is an important postsynaptic depolarization during high activity) it has a high Ca^{+2} conductivity, unlike AMPARs. This was prove to be fundamental for NMDAR-dependent LTP as Ca^{+2} chelation prevents this process (Lynch et al. 1983). The Ca^{+2} inflow through NMDARs activates calcium-calmodulin-dependent kinase II (CaMKII) what seems to be necessary and sufficient for LTP formation (Lisman, Yasuda, and Raghavachari 2012). Then on, in a more long-lasting time-scale, CamKII engages the actin cytoskeleton resulting in spine enlargement a morphological variation (Bosch et al. 2014; Herring and Nicoll 2016; Lisman, Yasuda, and Raghavachari 2012).

However, to drive the Ca^{+2} pass into the spine, it is needed the NMDAR unblock due to a change in the membrane potential. The glutamate release at synapse activates the other glutamate receptor implied in this process. Then, ion flux through AMPARs (mostly Na^{+}) depolarizes cell membrane allowing Mg^{+2} skips from NMDAR and allowing the receptor to conduct Ca^{+2} into the spine region,

The last step in LTP formation is the rapid accumulation of AMPARs at synapses. Studies performed by GluA1 homomeric AMPARs overexpression showed that LTP drives these receptors into the synapse (Y. Hayashi et al. 2000). First by an activity dependent exocytosis and second by the pool of free diffused extrasynaptic AMPARs that can be captured by the PSD. However, the importance of these two mechanisms of increasing the number of AMPARs at synapses is still unclear (Jurado et al. 2013; Lledo et al. 1998; Patterson, Szatmari, and Yasuda 2010).

The steps and how AMPARs and NMDARs work in LTP formation are reviewed expensively in (Roger A. Nicoll 2017). However, the point is that both glutamate receptors function is deeply synchronized in this process of rearrangement of synaptic connection that is key in memory and learning; thus, in brain function. The figure 14 schematizes how increases Ca^{+2} entry at postsynaptical terminal due to the combined action of AMPA and NMDARs.

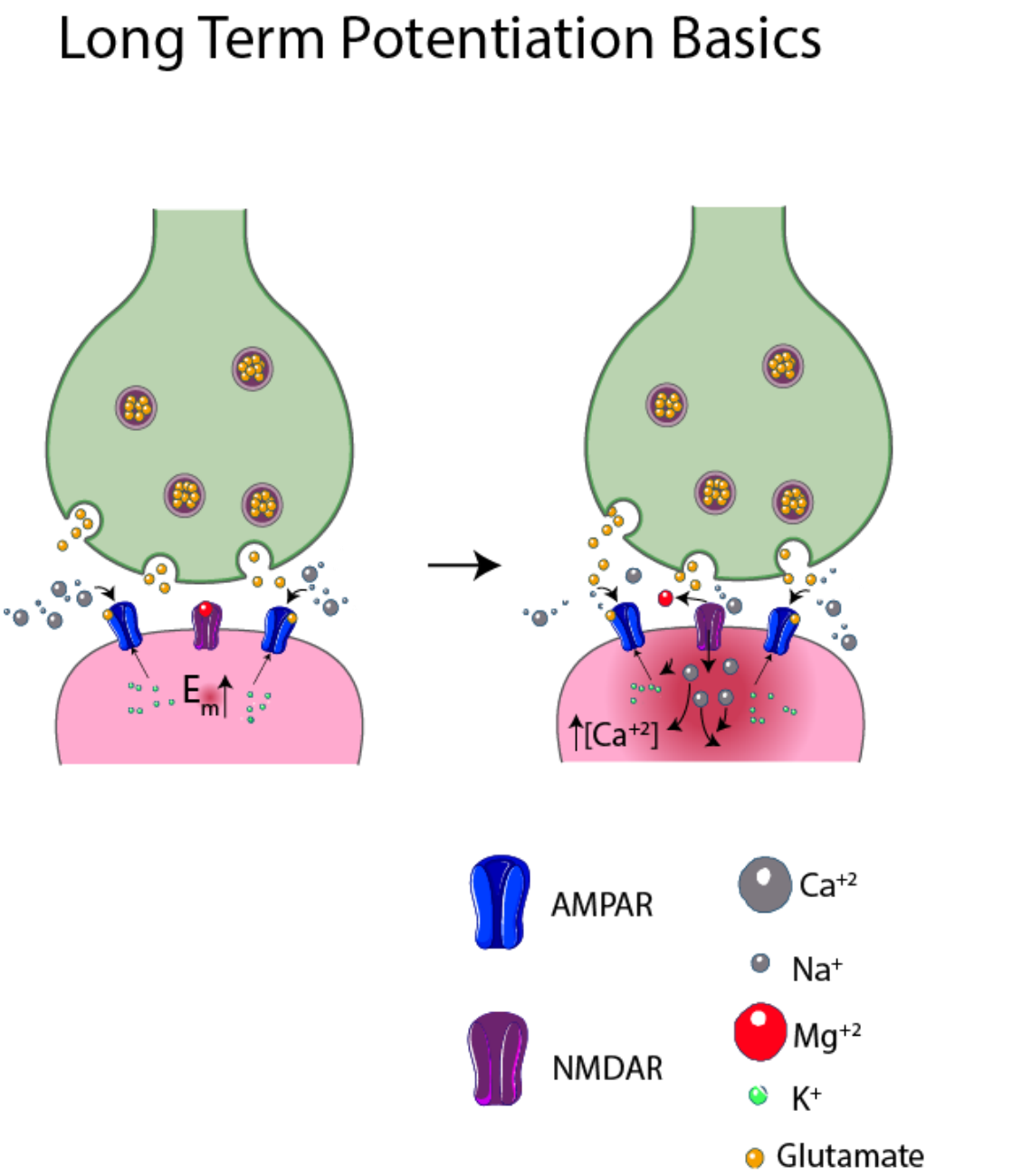


Figure 14. Increase in the Ca^{+2} inflow due to AMPA and NMDAR action. When glutamate is released it activates AMPARs while NMDARs are blocked by the divalent cation Mg^{+2}. AMPAR activation permits mostly Na^{+} inflow to postsynaptic terminal. The Na^{+} entry depolarized the membrane potential and makes Mg^{+2} drop unlocking NMDAR. Then NMDAR can be activated by glutamate and glycine and allows major entry of Ca^{+2} inside the presynaptic terminal. The Ca^{+2} entry activates many processes that will alter synaptic structure at postsynaptic termini. Depending on the frequency of the stimulation (neurotransmitter release) different kinds of synaptic plasticity will occur.

2. Objectives

2. Objectives

Since the first iGluRs were cloned at the late 80s much more is known about these ionotropic receptors whose natural agonist is glutamate. Thanks to the development of molecular biology techniques and genetic manipulation, iGluRs have been classified in 3 receptors families depending on their affinity to other agonists than glutamate. Later, the different members of this family of channels have been extensively studied in terms of, for example, biophysical properties, expression patterns or interaction with other proteins at different models (expression systems, primary cultures, brain slices …). Indeed, in the past 3 decades our knowledge about the glutamate transmission (including mGluRs) has sharply increase if we take into account that glutamate was not even known as a neurotransmitter since Hayashi's experiments suggested it in the middle of twentieth century.

However, much more is need to research to understand more precise how glutamatergic signalling works. For this reason this thesis project tries to bring new insights into iGluRs in physiological and pathological conditions.

For example, in terms of AMPAR research, since the early 2000s several publications have been focused not only in AMPAR itself but on the interaction of the receptors with other proteins. Till date, many AMPARs interacting-proteins have been discovered. Some of them are crucial for AMPAR biology as they act as auxiliary subunits. However, still less is known about how the AMPAR-complex is configured natively. As an example, the number of TARPs (the most studied AMPAR auxiliary subunit) per AMPAR in AMPAR complex have not been yet deeply studied. Not many publications have address this issue which is crucial to understand AMPAR signalling. For this reason, in this thesis project it was taken the challenge of bring more light about this topic. To study how the AMPAR-TARP stoichiometry plays a role in AMPAR function it were purposed the following objectives:

2. Objectives

1. Characterization of biophysical properties of CP and CI-AMPARs depending on AMPAR-TARP stoichiometry.

2. Study of differential modulation of TARP stoichiometry in CP- and CI-AMPARs.

3. Study of AMPAR-TARP stoichiometry effect on pharmacological properties of AMPARs.

4. Characterization of AMPAR-TARP stoichiometry with different TARPs (γ-2, γ-3, γ-4 and γ-8).

5. Determination of functional stoichiometries in neuronal populations.

6. Study the role of other proteins (CPT1C) in the regulation of AMPAR-TARP stoichiometry.

On the other hand, as a part of the new discoveries in glutamatergic signalling and pathology, the NMDARs are being related in the past years with a group of complex disorders related to the genes that codify for GluN subunits. These GRIN-related disorders been discovered in part due to the improvements made in genetic sequencing techniques. These techniques allow to perform genetic diagnosis and in the past years several clinical cases of patients with GRIN mutations have been reported. However, even being easy to establish a correlation between a GRIN mutation with a disorder related to glutamatergic signalling, it is more difficult to study the impact of each GRIN variant with the receptor function. For this reason, it is important to study how each mutation modifies NMDAR properties to bring to patients an appropriate treatment. As a clear explanation, if a modification in a GRIN gene implies disorder generated by a receptor gain of function, a possible treatment will be to inhibit the receptor. On the other hand, if it happens the opposite, it is needed to apply a different treatment (it this case enhancing receptor activity). To study NMDAR properties in the context of GRIN-related disorders in this thesis project the other principal objective was to study **the role of *de novo* mutations in NMDAR biophysical properties (7)**.

2. Objectives

Hypothesis

Mentioned in the objectives of this thesis, now our knowledge about glutamate receptors and their role in neurotransmission and nervous system physiology has grown sharply. At the moment there are several research lines working to uncover aspects still unknown about glutamatergic signalling. For this reason, this thesis project tried to expand the knowledge about iGluRs.

On the one hand, based on previous publications about AMPAR-TARP stoichiometry (Hastie et al. 2013; Yun Shi et al. 2009) and due to the high diversity of possible combinations that can be forming an AMPAR, here it is hypothesized that different AMPAR-TARP stoichiometry can modulate differently AMPAR properties and function.

On the other hand, it has been proved that alterations in GRIN genes leads into function impairment of NMDAR (Soto et al. 2019; Swanger et al. 2016; Platzer et al. 2017). That brings a complex scenario with a wide range of GRIN variants affecting glutamatergic signalling and therefore nervous system physiology. The work in this thesis wanted to check the hypothesis that GRIN variants affect by different means NMDAR properties giving varieties with a loss or a gain of receptor's function.

3. Methodology

1. Cell lines culture and maintenance

For this thesis, tsA201 cells have been used as a system to heterogeneously express AMPARs

or NMDARs. tsA201 cells is a cell line derived from HEK293 that is stably transfected with the temperature sensitive gene for SV40 T-antigen to allow plasmid replication using the SV40 origin and this way to produce high levels of recombinant proteins (Sigma catalogue #85120602). The cell line was a kind gift of Francisco Ciruela (University of Barcelona), who purchased them from the American Type Culture Collection (ATCC; Reference CRL-3216). The ATCC confirmed the identity of HEK293T by STR profiling (STR Profile; CSF1PO: 11,12; D13S317: 12,14; D16S539: 9,13; D5S818: 8,9; D7S820: 11; TH01: 7, 9.3; TPOX: 11; vWA: 16,19; Amelogenin: X). After the purchase of the cell line, mycoplasma tests have been performed in the laboratory on every new defrosted aliquot.

Cells were maintained in flasks of 25 cm^2 (or 50ml) with treated surface for better adherence of the cells (Sudelab catalogue #340CCFPV50200) in 5ml of Dulbecco's Modified Eagle's Medium Mix F12 (DMEM F12) supplemented with 10% of volume fetal bovine serum (FBS; Sigma Aldrich catalogue # F7524) and 1% volume penicillin-streptomycin (Sigma Aldrich catalogue # P4333-100ml). Cell split was performed depending on the requirements of the experimental schedule during the week. However, at least two splits were performed each week, one at the start and one at the end of the week. Flasks with an 80-90% of confluence were split with 400 µl of accutase (Sigma Aldrich catalogue # A6964-100ml) after discarding media and PBS wash (Dulbeco's Phosphate Buffered Saline **with Ca^{+2} and Mg^{+2}**; Sigma Aldrich catalogue # D8662-500). After 1 min with accutase in the incubator cells were split by pipetting and up to 5ml of DMEM F12 supplemented was added. Finally 5 or 10% (depending on the desired confluence for next flask) of the volume (5ml total) was added into a new flask and filled up to 5ml with DMEM F12 supplemented.

In figure 15 it is shown schematically the procedure of cell split for maintenance and seed in 24 wells plate (Sigma Aldrich catalogue # **M8812).**

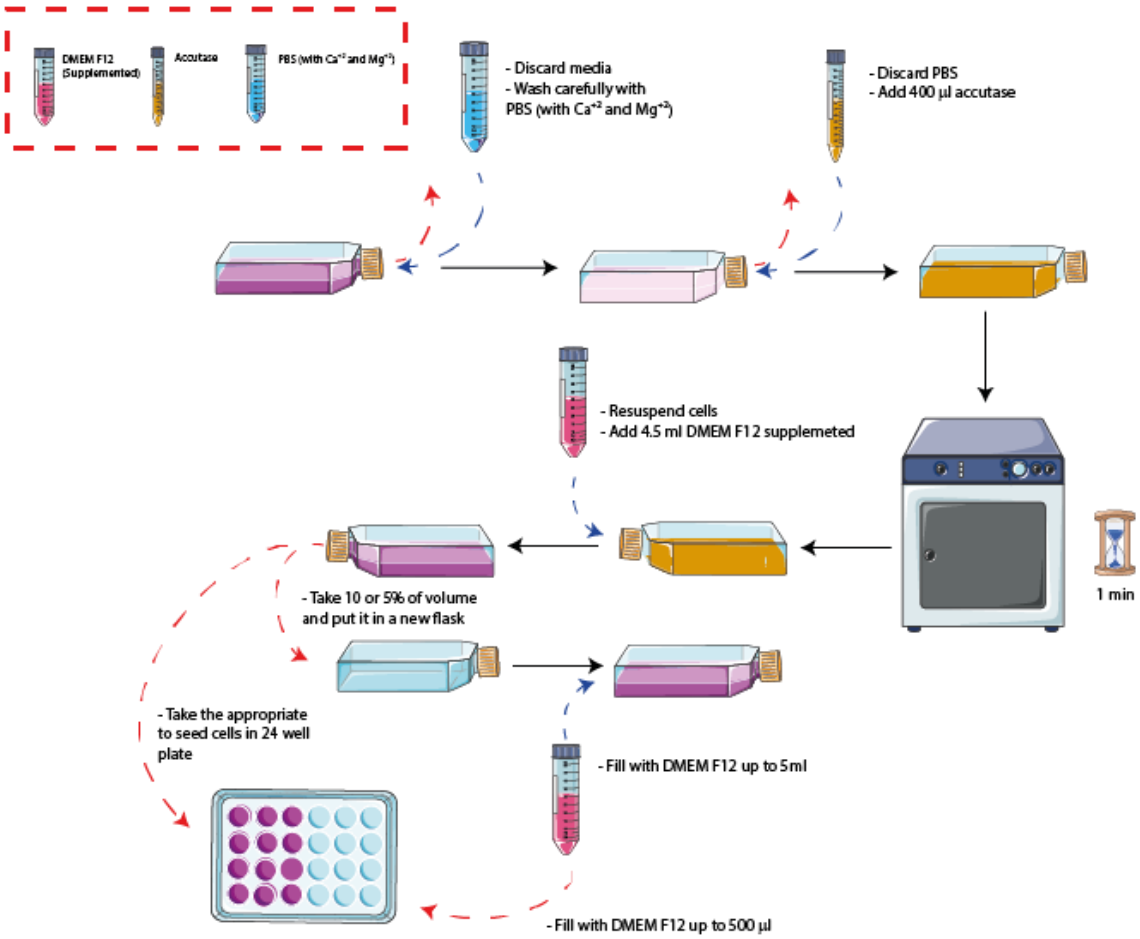


Figure 15. Scheme of cell split procedure. Cells are split at least twice a week and seeded at different confluence according the needs during the week for other experiments. For maintenance usually are split and seed at 5-10% of the volume of resuspension (5ml).

The schedule of electrophysiological experiments influenced when the cells were split. Usually at the end or start of the week cells were seed in a new flask and also seeded in 24 well plate at different density to transfect along the week or at the start of next week (usually on Mondays). Figure 16 illustrates the typical schedule of a normal week.

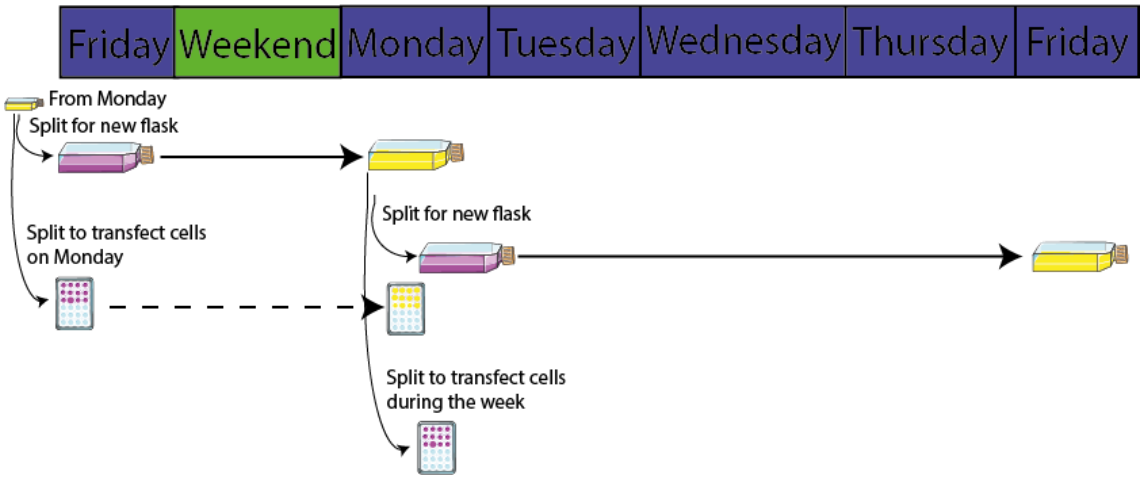


Figure 16. Schedule of cell maintenance. Cell were usually split on Friday and it was obtained a flask for maintenance and cells were also seed in a 24 well plate to transfect on Monday. After weekend cells were split also for maintenance and seeded at a density that allows to get an 80-90% confluence flask at the end of the week. On Monday cells split from previous Friday's flask were also seed at different densities in 24 well plate to transfect during the week.

1.1. Cell line frost and thaw protocol

tsA201 cells were used in this thesis to perform functional experiments to investigate new insights into iGluRs. However, these cells have a limited life time and they lose efficiency at each cell passage. Cells were maintained from approximately pass 20 to 40 and were discarded when they were performing badly at electrophysiological experiments (around pass 40). For this reason, it was usual to thaw frozen cells aliquots conserved in cryopreservation services at some point during the year (4-5 times). On the other hand, from each thaw aliquot it was prepared a flask to maintain cells from thaw aliquot and also two or three more flasks to freeze new cells and get more aliquots for future experiments. It permits to have a practically unlimited source of tsA201 cells to use as expression system.

To **thaw** cells the protocol used was the following:

1) Pre-warm two 15ml falcon with 9ml and 5ml of DMEM F12 supplemented media at 37ºC.
2) Take an aliquot of frozen cells from cryopreservation services and put it in ice to bring it to the laboratory.

3) Thaw rapidly the aliquot using bath at 37ºC. Check it continuously to avoid diminish cell viability due to DMSO (dimethyl sulfoxide; toxic to cells and apolar dissolvent) from freezing medium.
4) Recover the 1ml of the aliquot and put it in the pre-warmed falcon with 9ml media DMEM F12 supplemented.
5) Centrifuge at 1000rpm for 5 minutes.
6) Discard supernatant and resuspend with 5ml of pre-warmed media DMEM F12 supplemented.
7) Place the media with cells in a flask treated for cell adhesion (as explained before).
8) After few hours check if it possible the adhesion of cells. If it considered, not adhered cells can be removed by discarding media and carefully refiled with 5ml of new media.
9) After one day from it is suitable to check cell confluence and make a maintenance pass if it is needed.

On the other hand, to freeze an aliquot of cells it was used the following protocol to prepare 3 aliquots:

1) With a near of 90% confluence flask cells are split as explained in the previous section.
2) Split cells are recovered in a 15ml falcon and centrifuge for 5 minutes at 1500rpm.
3) After centrifuge media is discarded and cells are resuspended with 3ml of freezing media (DMEM F12 free of FBS and antibiotics + 20% in volume of FBS + 10% in volume of sterile DMSO).
4) Distribute rapidly the 3ml of resuspended cells in 1ml tube for cryopreservation.
5) Store at -80ºC the aliquots in a rack placed over isopropanol solution (permit a graded congelation) at least one day but not more than a week.
6) At least 24 hours later aliquots can be moved to cryopreservation services were they can be stored for years.

2. Constructs and construction

For transfection in cell lines we used cDNAs codifying for GluA subunits from rat (flip isoforms) and rat GluN subunits. GluAX:γ-2 tandem proteins were made from the GluAX and the γ-2 plasmid vectors using a 5 glycine linker between the two subunits.

GluN subunits were attached to GFP (green fluorescent protein) or HA (human influenza hemagglutinin) proteins to use the plasmids for molecular biology experiments in other projects. The attachment of these tags did not affect NMDAR functionality as it was seen in previous work (Soto et al. 2019).

To perform experiments with the GluA subunit present in GCGs it was need to obtain the GluA4c (short isoform of GluA4) from mRNA and finally subclone the sequence in a plasmid vector.

2.1. RNA extraction and cDNA obtaining

The RNA extraction was performed to obtain mRNA from rat cerebellum and later use this product to achieve cDNAs from all tissue sample. It was chosen an adult rat cerebellum because GluA4c for is highly expressed at this tissue at this life stage according with literature (Gallo et al. 1992).

After dissection cerebellum was rapidly frost using liquid nitrogen to avoid RNA degradation. Then, tissue sample was minced. The total RNA was extracted using the NucleoSpin RNA, mini kit for RNA purification (from Macherey-Nagel catalogue #740955.50) and following manufacturer's protocol.

Total RNA was quantified using a spectrophotometer NanoDrop™ (from Thermo Fischer Scientific, catalogue #ND-ONE-W). After quantification sample with sufficient amount of RNA (between 300-500 ng) was retrotranscripted into cDNA.

For retrotranscription (the inverse step of transcription using a retrotranscriptase, a common process in RNA viruses) it was used the kit from Thermo Fischer Scientific, SuperScript™ IV First-Strand Synthesis System (catalogue #18091050). The protocol used was according manufacturers indications and after cDNA obtaining it was performed a PCR to amplify GluA4c cDNA for later subclonning in plasmid vector. The primers used for PCR are detailed in the following section.

2.2. PCR fragment subcloning

After obtaining amplified fragments of GluA4 by PCR it was proceed to subclone the fragments in plasmid vector. First plasmid vector (pIRES mCherry) was digest using restriction enzymes (see **2.2. GluA subunits**) generating sticky ends and dephosphorylated using diphosphatase from New England Labs (catalogue #M0510S) kit. PCR products (GluA4c fragment) was also digested using the same enzymes used for plasmid vector.

PCR digested fragments and digested plasmid were purified using NucleoSpin®&PCR Clean Up kit from Macherey-Nagel (catalogue #740609.50) according manufacturers protocols.

Ligation was performed with T4 ligase from New England Labs (catalogue #M0202S) kit and insert:plasmid ratio was 1:3. Mass from each component was calculated using online calculator facility from New England Labs https://nebiocalculator.neb.com/#!/ligation.

Later, plasmid vectors containing the insert were transformed into DH5-α super competent bacteria (catalogue #18265017; *E. coli* cells engineered to maximize transformation efficiency). Bacteria was growth over-night in luria broth agar plates with 30μg/ml kanamycin to allow only transformed bacteria growth. 4-5 different bacterial colonies were tacked to grow in luria broth liquid media in 15ml falcons for bacteria culture (falcons that allow CO_2-O_2 exchange). Bacterial culture was put in a

shaker at 220rpm at 37ºC over-night. Finally plasmid DNA was extracted from bacteria using NucleoSpin Plasmid, Mini kit for plasmid DNA from Macherey-Nagel (catalogue #740588.50) and insert ligation was tested by enzyme digestion using restriction enzymes. At the end of the process, plasmid vector with the insert were sequenced by StabVida laboratories (https://www.stabvida.com/es).

2.3. GluA subunits

GluA1, GluA2 and **GluA4** cDNAs (rat, flip isoforms) used in this thesis project were old gifts from S. Heinemann (Salk Institute, La Jolla, CA, USA) and P. Seeburg (Max Planck Institute, Heidelberg, Germany).

For this work we used the short version of GluA4, named **GluA4c** (first described in Gallo et al., 1992) in its flip form. The GluA4c subunit was cloned from mRNA obtained from adult rat cerebellum (*Rattus norvegicus*) into a pIRES-mCherry plasmid vector as it is mentioned and detailed in the previous section. The primers used were the following:

Primer Forward (5'-3'): **GCGC GCT AGC ATG AGG ATT TGC AGG CAG ATT** (**GCGC GCT AGC** restriction site cloned using NheI-HF enzyme, catalog: NEB #R3131S).

Primer Reverse (5'-3'): **CGCGG CTC GAG ATT CTT AAT ACT TTC GGT TCC A** (**CGCGG CTC GAG** restriction site cloned using XhoI-HF enzyme, catalog: NEB #R0146S).

2.4. GluA fusion proteins and other AMPAR regulatory proteins

GluA1:γ2 cDNA was a generous gift from Ian Coombs (UCL, London, UK).
GluA2:γ2 cDNA was a generous gift from Ian Coombs (UCL, London, UK).
GluA4c:γ2 cDNA was made from the GluA4c and the γ2 plasmidic vectors.
GluA1:γ3 cDNA was a generous gift from Roger Nicoll (UCSF, San Francisco, CA, USA).
GluA1:γ4 cDNA was a generous gift from Roger Nicoll (UCSF, San Francisco, CA, USA).

GluA1:γ8 cDNA was a generous gift from Roger Nicoll (UCSF, San Francisco, CA, USA).

γ2 cDNA was a generous gift from Roger Nicoll (UCSF, San Francisco, CA, USA).

CPT1C cDNA was a generous gift from Núria Casals (Universitat Internacional de Catalunya)

2.5. Site directed mutagenesis

In this thesis project 5 site-directed mutagenesis were performed using the **QuickChange Multi Site-Directed Mutagenesis Kit** from Agilent Technologies (catalogue #200514).

GluA1 to **GluA1(R)**: it was changed a glutamine at position 600 to an arginine to make AMPARs containing GluA1(R) CI-AMPARs.

Primers used:

Forward **5' - GGGGGCCTTCATGCGGCAAGGATGTGACA - 3'**

Reverse **5' - TGTCACATCCTTGCCGGATGAAGGCCCCC - 3'**

GluA4c: it was performed 2 site directed mutagenesis to eliminate two EcoRI restriction targets without changing the codon. The EcoRI site was needed to subcloning GluA4c in γ2 vector to get GluA4c:γ2. For that reason it was mandatory to get rid of the EcoRI targets in the middle of GluA4c sequence.

Primers used:

First EcoRI: **5' - GAAGCAGGTTCGTATTCAAGGGCTGACTGG - 3'**

Second EcoRI: **5' - CGGGATCAACAAAAGAGTTCTTCAGAAGATCAAAAATAGC - 3'**

EcoRI target:

5'...G▼AATTC...3'
3'...CTTAA▲G...5'

GluA2:γ2 (C549L) and **GluA4c:γ2** (C550L): Change of a cysteine for a leucine at 549 and 550 positions in GluA2:γ2 and GluA4c:γ2 vectors respectively. These mutations were performed to abolish TARP effect over GluA2 or GluA4c subunits as it was expected

from Hawken, Zaika, & Nakagawa, 2017.

Primers used for **GluA2:γ2**:

Forward **5' - TAGCCTATGAGATCTGGATGTTAATTGTGTTTGCCTACATTGGG - 3'**

Reverse **5' - CCCAATGTAGGCAAACACAATTAACATCCAGATCTCATAGGCTA - 3'**

Primer used for **GluA4c:γ2**:

Forward **5' - CTGGCCTATGAGATCTGGATGTTAATAGTGTTTGCATACATTGGTG - 3'**

Reverse **5' - CACCAATGTATGCAAACACTATTAACATCCAGATCTCATAGGCCAG - 3'**

2.6. GluN subunits

To study by means of patch clamp technique the effect of different GRIN variations in NMDARs, different plasmid vectors were used codifying for WT subunits and GluN with specific site-directed mutations. The GluN mutations correspond to clinical-reported cases.

- For GluN1 subunits expression it was used cDNAs for **GluN1-HA** and **GluN1-GFP**. GluN1 was attached to HA (hemagglutinin*) or GFP (green fluorescent protein) as tag proteins.*

*- For GluN2A and GluN2B subunits expression were used cDNAs for **GluN2A-GFP** and **GluN2B-GFP**.*

In the following figure (figure 17) it is summarized and schematized the different types of transfection performed to record currents from different groups of AMPAR or NMDAR.

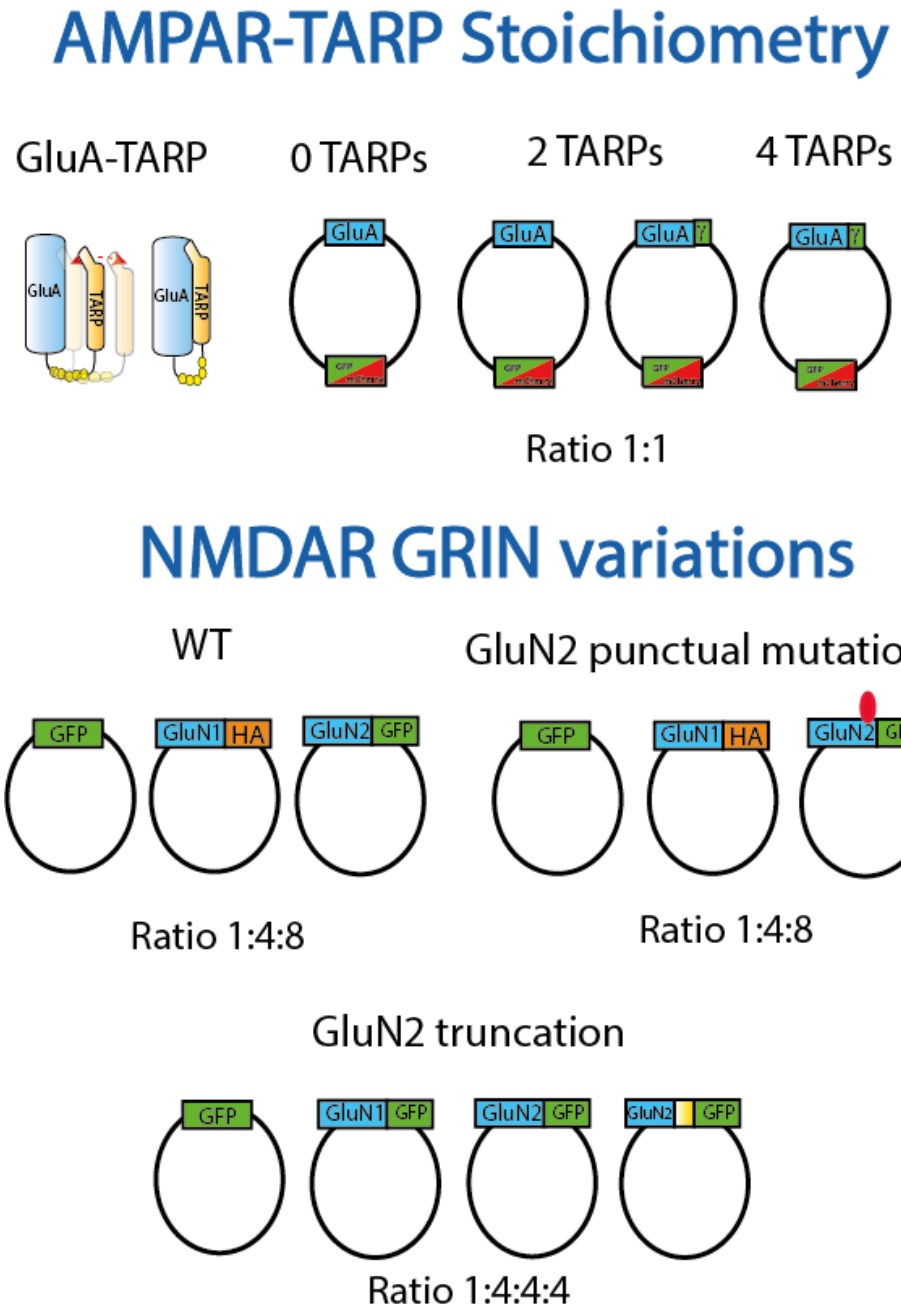


Figure 17. Constructs used during the development of this thesis in this thesis. At the top on the left side it is shown a scheme of the GluA-TARP fusion protein used for AMPAR-TARP stoichiometry experiments. This fusion protein permitted to fix stoichiometries of 0, 2 or 4 TARPs per AMPAR. To study receptor properties of GRIN variants three different transfection were performed. On the one hand the WT with GluN1 subunit plus GluN2A or GluN2B (GluN2 in the scheme) in a 1:2 ratio and a vector encoding for GFP to mark transfected cells. On the other hand, to analyse point mutations on NMDARs it was transfected the mutated subunit (GluN1, GluN2A or GluN2B; in the scheme marked with a red circle) along with the WT subunit of the heteromer. It was maintained the ratio of the WT group. Finally, to study the effect of GRIN truncated variants it was transfected half of subunit truncated plus half of WT subunit. Showed in the scheme, half of GluN2 and half of GluN2 truncated (yellow square). All GluN

subunits were attached to GFP to avoid differential expression by using different plasmid vectors. Just to mention, the GFP of GluN subunits attached to the fluorescent proteins is hardly visible with microscope fluorescence and for that reason GFP is added in a plasmid aside.

3. Transfection for patch clamp experiments

During this thesis two procedures for transfection were used. Despite both were very similar they have some differences. On the one hand it was used the polyethylenimine (PEI) as a transfection reagent. PEI is a polymer obtained from ethylamine. On the other hand, it was used the commercial reagent X-tremeGENE 9 from Sigma Aldrich (catalogue # 6365779001), named in this work as x-treme. Just subjectively, cells appeared healthier using PEI transfection but the efficiency of the process was lower. For this reason X-treme transfection was preferentially used in NMDAR experiments as GluN subunits were not expressed in iRES plasmid (permit to express separately but with same transcription the protein of interest and a fluorescent protein like GFP) that allow to detect cell transfection.

Transfected cells were seeded in treated coverslips with poly-D-lysine (Sigma Aldrich catalogue # A-003-E). The procedure to prepare the coverslips was the following:

1) Coverslips were treated with nitric acid for 2-3h and rinsed with PBS without Ca^{+2} and Mg^{+2}.

2) Coverslips were then maintained in ethanol 70% solution.
3) They were flamed and put in 24 or 4 well plate (same size of well, 1.5ml).
4) 240µl of poly-D-lysine was added. Poly-D-lysine was prepared with 1ml stock solution and 9ml miliq water filtered.
5) Plates were put in incubator for 30-45 minutes. Optimal time was 30 minutes and trying to not exceed 45 minutes.
6) Poly-D-lysine was recovered for another use (up to 5 uses, after the fifth it was discarded).

7) Coverslips were washed 3 times with PBS without Ca^{+2} and Mg^{+2}.

8) Finally, plates were covered with foil and kept at 4ºC.

At the following lines it will be described the different procedure to transfect tsA201 cells using both reagents.

3.1. Transfection procedure using PEI

PEI reagent was used to transfect tsA201 cells when they were seeded and adhered in wells from a 24 well plate described before (catalogue and reference).

Procedure:

1) Prepare transfection media with up to 1.4 μg of DNA and PEI [1μg/1μl] with a ratio in mass of 1:3 DNA:PEI.

2) Let the eppendorf with the mix incubate for 2-5 minutes at room temperature on the hood.

3) Add up to 500μl of DMEM F12 free (not supplemented with FBS and antibiotics).

4) Let the mix 20 minutes at room temperature on the hood.

5) Choose cells from the well at 70-80% of confluence. Remove media and carefully make a wash with PBS (**with Ca^{+2} and Mg^{+2}**) avoiding to add the PBS at centre of the well to minimize cell detachment.

6) Discard PBS and add the DNA mix carefully to avoid cell split.

7) Kept the cells at incubator for 2.5-4 hours. 3 hours is the optimal time (at least in this thesis) and try to not exceed 4.

8) Split the cells and seed in wells with treated coverslips to an optimal density for electrophysiology experiments.

9) Cells are ready to be patched 20-24 hours later with appropriate levels of expression.

The following figure, figure 18, schematize the procedure for transfection using PEI reagent.

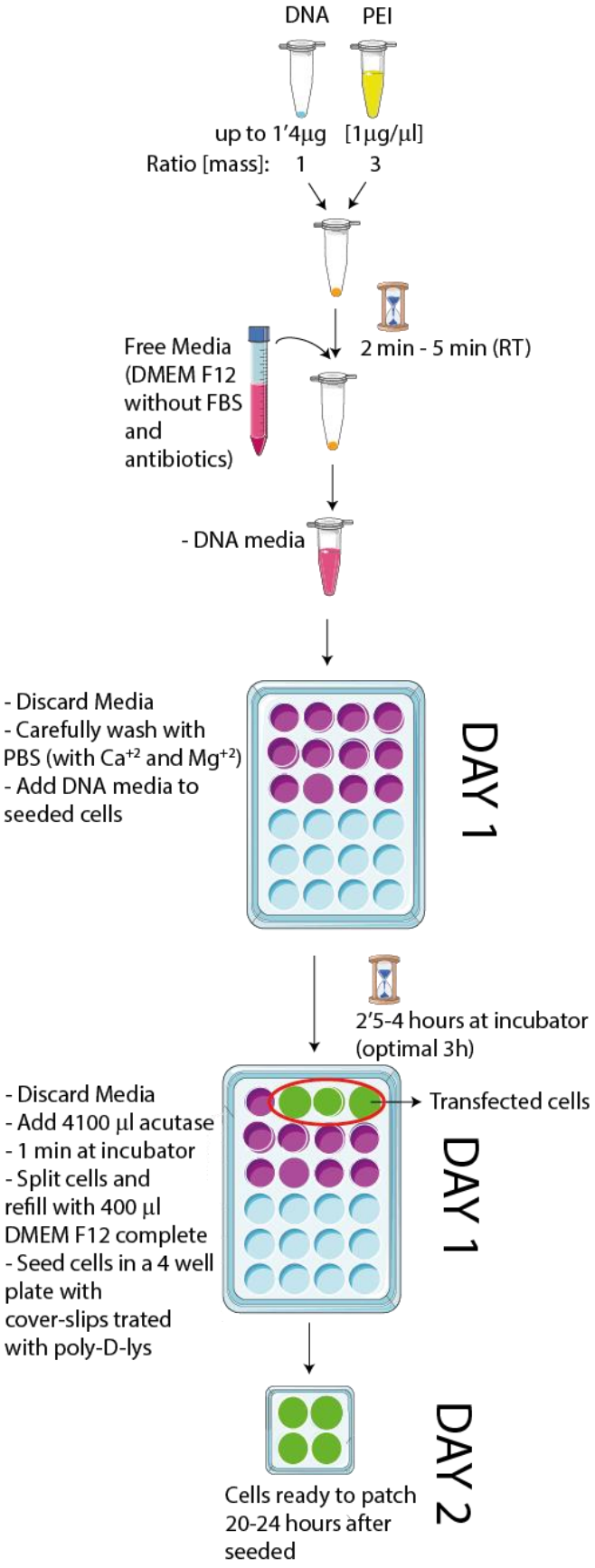


Figure 18. Scheme of the PEI transfection protocol. Steps using PEI transfection protocol. It can be used with cells adhered to wells in a plate and cells can be patched a day after transfection.

3.2. Transfection procedure using X-treme reagent

1) Prepare transfection media with 50µl DMEM F12 free and 1.8µl of x-treme for 1.3µg DNA and swirl.
2) Let the eppendorf 2-5 minutes at room temperature on the hood.
3) Add DNA and swirl.
4) Let the mix 20 minutes at room temperature on the hood.
5) Choose cells from the well at 40-60% of confluence or it can be used lifted cells. Add the DNA mix.
6) 24 hours after split the cells and seed in wells with coverslips treated with poly-D-lysine.

The next figure (figure 19) schematizes transfection steps using x-treme as DNA transfection reagent.

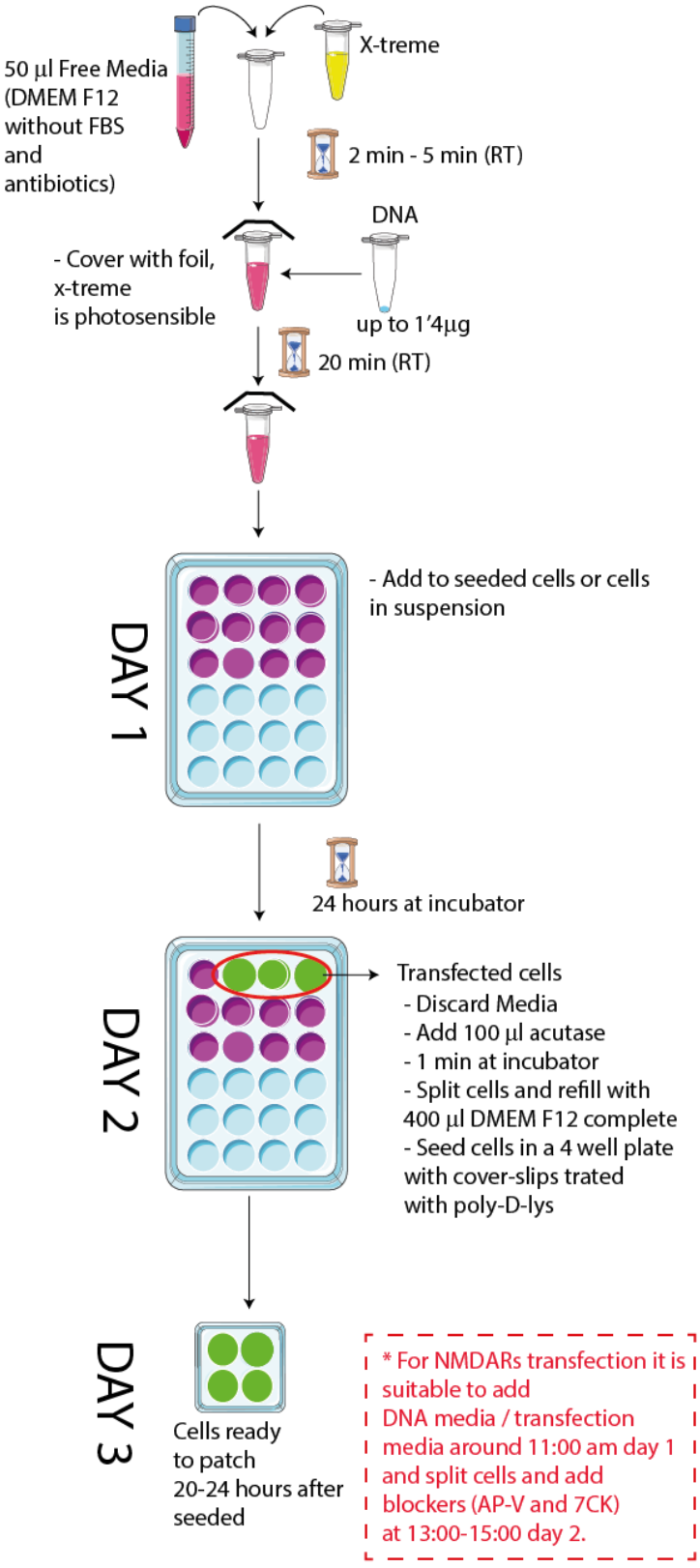


Figure 19. Transfection with x-treme reagent. Protocol for transfection using x-treme reagent to cells already adhered in wells but can be applied for cells in suspension. Cells are transfected in day 1 and split on day 2. Patch experiments can be performed on day 3.

4. Cerebellar granule cell culture

Primary cultures of cerebellar granule cells (CGCs) were performed in this thesis in order to determine the functional AMPAR-stoichiometry present in this cell type.

The CGC cultures were prepared from pups on Postnatal Day 7–8. The cerebella from 8 to 10 mice pups were collected in 9.5 ml buffer containing Krebs buffer supplemented with bovine serum albumin (BSA) and $MgSO_4$ (solution A). Thereafter, meninges are carefully removed and cerebella is dissected out, minced carefully with a blade, and dissociated at 37°C for 15 min with a solution containing 250 μg /ml trypsin. After 15 minutes, solution with 2.7 μg/ml DNAse and 8.32 μg/ml Soybean Trypsin Inhibitor (SBTI) is added. CGCs are separated from non-dissociated tissue by sedimentation and, finally, resuspended in basal medium Eagle's (BME) supplemented with 10% inactivated fetal calf serum, 25 mM KCl, and gentamicin (5mg/ml).

Finally, CGCs are plated onto poly-L-lysine-coated 24-well plates at a density of 300,000 cells/cm^2. After 16–18 h in culture, cytosine arabinose is added to a final concentration of 10 μM to inhibit glial cell proliferation.

Once neurons are plated electrophysiological experiments were performed at 6-8 days after dissection.

Krebs buffer and other solutions used before platting the cells are the following:

Krebs buffer (10X)

For 50 ml:

- NaCl 3.53 g
- KCl 180 mg
- KH_2PO_4 83 mg
- D-Glucose 1.285 g

- $NaHCO_3$ 1.07 g

- Phenol red (pH indicator) 5 mg

Solution A

- 5 mL **Krebs Buffer 10x** + 20 ml miliq water.

- 150 mg **BSA**

- 400µl **$MgSO_4$ stock solution** (3.82%)

- Up to 50 ml miliq water.

Solution B

3.12 mg **trypsin** in 12.5 mL de solution A.

Solution C

7.5mL de solution A add:

+ 1.25mg de **DNAse.**

+ 3.9 mg de **SBTI** (soybean trypsin inhibitor).

+ 75µl $MgSO_4$ stock solution (3.82%).

Solution D

2 ml de solution C in 10.5mL de solution A.

Solution E

6.25 mL de solution A + 50µl de **$MgSO_4$** stock solution (3.82%) + 50µl de **$CaCl_2$** stock solution (0.18%).

At figure 20 it is schematized the steps of the CGCs culture. More detailed information (however, maybe with some modifications) about CGCs culture is described at AU - Lee, AU - Greene, AU - Mason, & AU - Manzini, 2009.

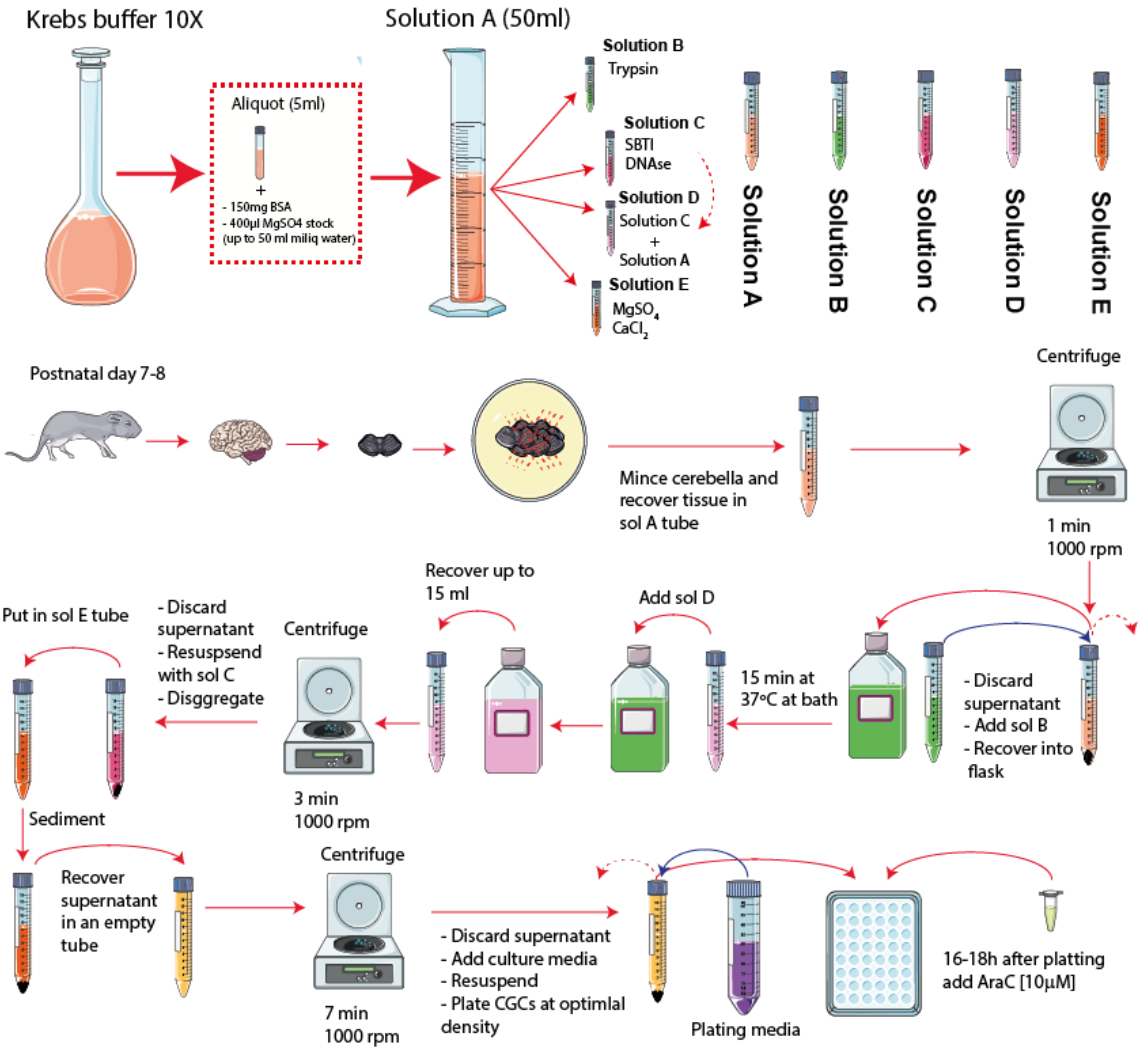


Figure 20. Scheme of CGC culture. The figure shows at the top the solutions used during dissection and before platting the CGCs. Next, it is shown in a summarized way the different steps to perform the primary culture of this neuronal type. In red straight arrow are indicated sequential steps while in curved arrows (red and blue) it is shown displacement of solutions or media. Dashed arrows indicate discard supernatant.

4.1. Coverslip preparation for CGCs culture

For CGCs primary culture coverslips with poly-L-lysine were used in a 24 well plate. Coverslips were prepared a day before the primary culture or even before the dissection with the following procedure:

1) Coverslips with any treatment were autoclaved.

2) 500µl Poly-L-lysine was added to the coverslips placed in wells from a 24 well plate (Sigma Aldrich catalogue #P6282). Poly-L-lysine was prepared with 1 ml of stock

solution in 9 ml of miliq water filtered.

3) After 30 minutes at RT in the hood the Poly-L-lysine is discarded (and not recovered) and the plate is kept at the incubator until CGCs are plated.

5. Electrophysiology experiments

5.1. General procedures

Recordings have been performed from isolated transfected cells or cerebellar granule cells (GGCs) visualized with an inverted epifluorescence microscope. Cells expressing EGFP and/or mCherry fluorescent proteins were selected for patch-clamp recordings (except CGCs that are not transfected).

To evoke receptors currents agonist solution was rapidly applied by switching at the cell/patch by a piezoelectric translation of a theta-barrel application tool made from borosilicate glass. The following figure (figure 21) shows an example of evoked-current using this device called ‘fast application tool (FAT)’.

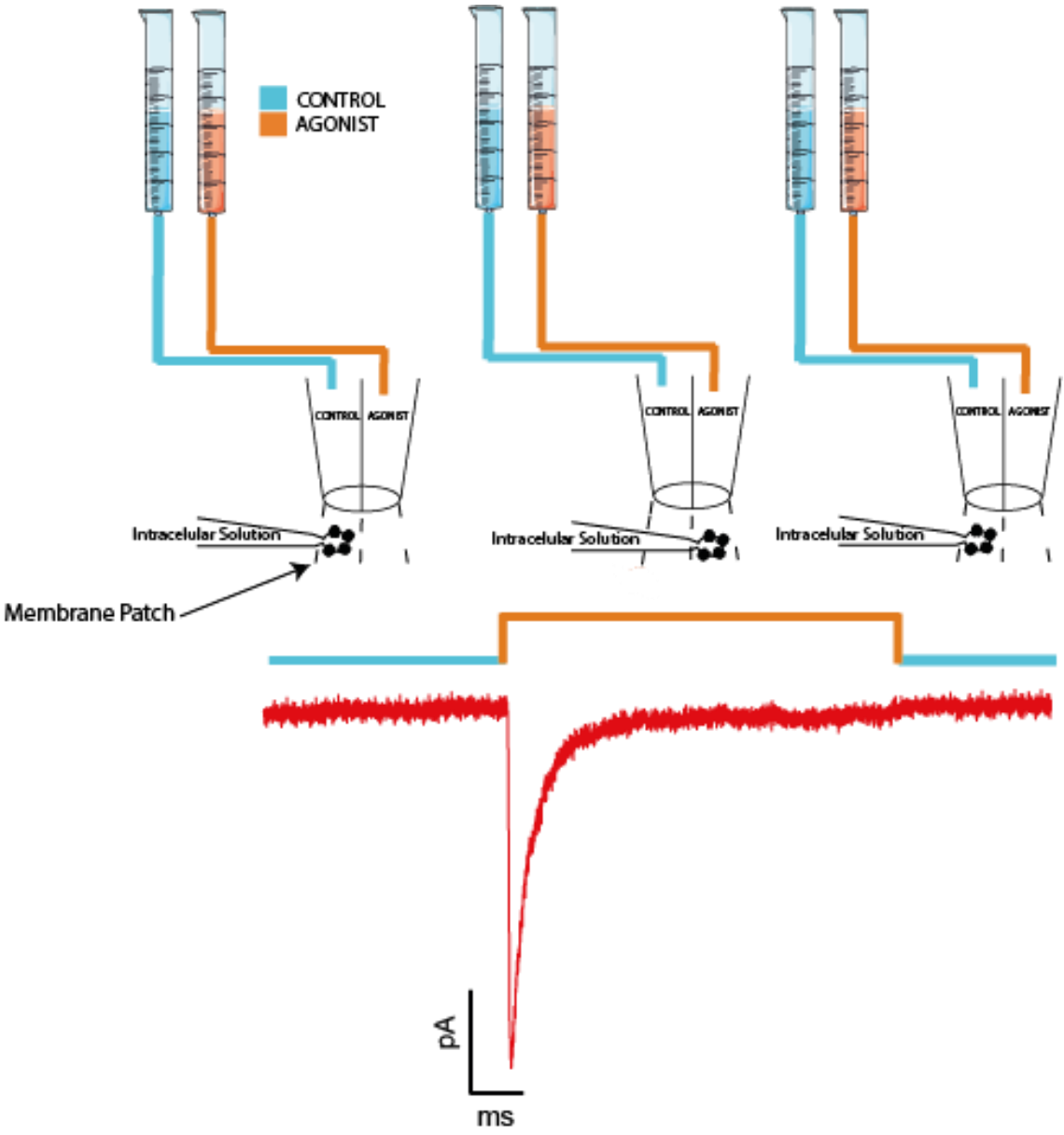


Figure 21. Fast application tool. Theta-barrel coupled to a piezoelectric device allows to apply control solution (blue) and rapidly switch to apply agonist solution (orange) to a membrane patch or cell and evoke currents like the one present at the bottom of the figure (red).

The recordings in this thesis project were done using the following solutions:

AMPAR extracellular solution (in mM): 145 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH to 7.42 with NaOH).

AMPAR intracellular solution (in mM): 145 CsCl, 2.5 NaCl, 1 Cs-EGTA, 4 MgATP, and 10 HEPES

(pH to 7.2 with CsOH) + The polyamine spermine tetrahydrochloride added to intracellular solution at 100 µM.

AMPAR recording solutions:

- **Control**: Extracellular solution diluted 5% with miliq water.

- Agonist (tsA201 patches recordings): Extracellular solution + Glutamate (10 mM).

- Agonist (tsA201 whole-cell recordings): Extracellular solution + AMPA 100 µM + Cyclothiazide 50 µM.

- Perampanel (tsA201 whole-cell recordings): Extracellular solution + AMPA (100 µM) + Cyclothiazide (50 µM) + Perampanel (5 µM). *Perampanel (Quimigen catalog #A12498-5) is strongly hydrophobic so it is need to be dissolved in a non-polar dissolvent. It was used DMSO preparing stock solution at 14.31mM.

Agonist (CGCs patches recordings): Extracellular solution + AMPA 100 µM.

NMDAR extracellular solution (in mM): 140 NaCl, 5 KCl, 1 CaCl2, 10 glucose, and 10 HEPES, adjusted to pH 7.42 with NaOH

NMDAR intracellular solution (in mM): 140 CsCl, 5 EGTA, 4 Na2ATP, 0.1 Na3GTP and 10 HEPES, adjusted to pH 7.25 with CsOH

NMDAR recording solutions:

- Control: Extracellular solution diluted 5% with miliq water.

- Agonist: Extracellular solution + Glutamate (1 mM) + glycine (50 µM)

- Agonist + Mg^{+2}: Agonist solution + Mg^{+2} (1 mM).

- Agonist low glycine concentration: Extracellular solution + Glutamate (1 mM) + glycine (1 µM)

- Agonist low glycine concentration + serine: Extracellular solution + Glutamate (1 mM) + glycine (1 µM) + glycine (3 µM)

5.2. Whole-cell recordings

Recordings using this configuration were performed to measure the current of the whole cell. The whole-cell recordings were used to assess receptors trafficking or to compare receptors currents taking into account the contribution from all the receptors to the maximum peak.

For whole-cell recordings from isolated cells thick-walled electrodes were used. These electrodes had a resistance of 3–5 MOhms to ensure good electrical access to the recorded cell. To compare responses from different cells to agonist it was used the current density. This parameter considers the capacitance of the membrane as a measure of the cell size. It allows to correct the error of comparing cells with different sizes and uses the following ratio for current density: –pA/pF; maximum current divided by input capacitance measured from the amplifier settings.

5.3. Fast agonist application into outside-out patches

Most of the experiments of this thesis have been performed using membrane patches in outside-out configuration. This configuration allows to record a few amount of channels present in a small piece of membrane. The agonist/s solution was applied to membrane patches solution by rapidly switching from control solution (without agonists) using the fast application tool. This permitted application of both agonist and control solution in an ultra-fast fashion. In these experiments, control and agonist solution flow continuously through the two barrels and solution exchange occurs when movement of the translator is triggered by a voltage step, which provoke response of receptors.

Outside-out patches recordings from isolated cells were performed using thick-walled electrodes with a resistance of 5–10 MOhms, higher resistances than the ones used in whole-cell recordings in order to get more stability of the membrane patch.

Figure 22 is a scheme of the fast application of agonist and also of different configurations of the patch clamp technique.

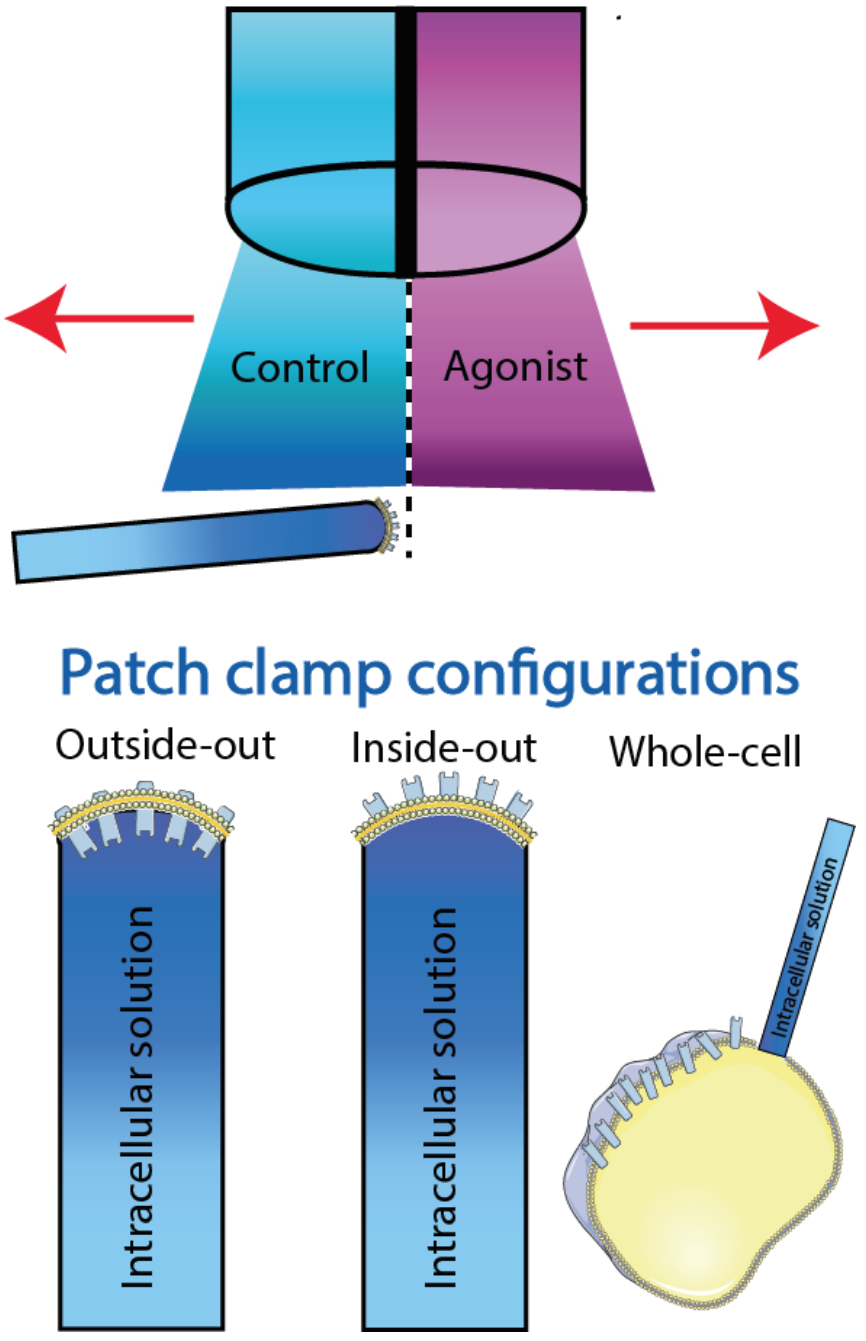


Figure 22. Fast agonist application and different patch clamp configurations. On the top it is shown how the fast application tool is used to apply control and agonist solution to a membrane patch. Red arrows symbolize the lateral movement of the theta-glass. At the bottom of the figure are shown different patch clamp configurations. The first one is an inside-out patch were the extracellular part of the receptor is exposed to intracellular solution. The second configuration is an outside-out patch and there the intracellular part of the receptor in exposed to intracellular solution like it happens in a cell. In the last configuration the whole-cell is patched and it allows to record the response from all channels/receptors of the cell.

5.4. Non-stationary fluctuation analysis (NSFA)

Single-channel properties of AMPARs can be deduced from macroscopic responses by means of the study of the variance of the current along time. To do so, glutamate (10 mM) is applied onto outside-out patches containing AMPARs during 100 ms and the ensemble variance of all successive pairs of current responses is calculated using specialized software. The variance analysed is compared to the background noise before agonist application. By using this analysis, the single-channel conductance, the total number of channels and the maximum open probability can be obtained.

This analysis is suitable to obtain values of single-channel conductance from low-conductance channels like AMPARs without making single channel recordings to avoid errors from background noise. On the other hand, single-channel conductance from receptors like NMDARs, that have high conductance values, is obtained in single-channel recordings and not using NSFA.

In the figure 23 it is illustrated this type of analysis with an average trace from a recording and the plot of the variance over the peak current.

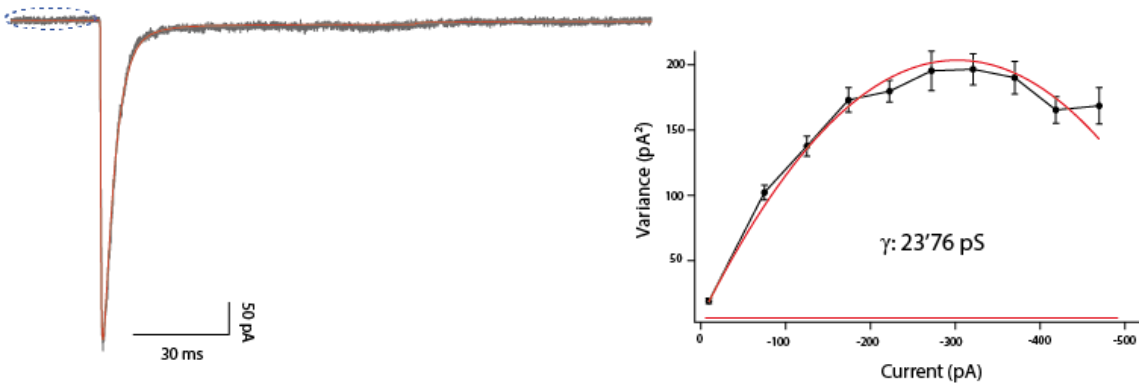


Figure 23. Example of NSFA. On the left an average trace in red overlapped with one of the sweeps used to calculate this average trace. In dashed circle it is shown the background variance that is used to analyse the variance after agonist application. On the right it is shown the plot of the variance over current. The slope of the curve that cuts the graphic in the origin (point 0,0) is used to calculate the intensity I (pA) and will give the value for single channel-conductance.

5.5. Receptor kinetics (desensitization, deactivation and rise-time)

This analysis permits to evaluate the kinetics of deactivation and desensitization of AMPAR and NMDAR responses. It allows to study receptors pharmacology and gating dynamics. It was studied from each experiment the averaged currents describing recovery from desensitization state of the receptor fitted with a double-exponential function:

$$I = A_f \exp(\tau/\tau_f) + A_s \exp(\tau/\tau_s)$$

where A_f and τ_f are the amplitude and time constant of the fast component of recovery and
A_s and τ_s are the amplitude and time constant of the slow component. If the calculated τ_s was longer than 1s or within 10% of τ_f, a single exponential was used. For double exponential fits, the weighted time constant of recovery τ_w) was calculated according to:

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

All responses were recorded at -60mV of membrane potential.

On the other hand, the rise-time measured the lapse of time needed between agonist application and receptors maximum peak current or from 10 to 90% of current. Figure 24 illustrates the rise-time concept:

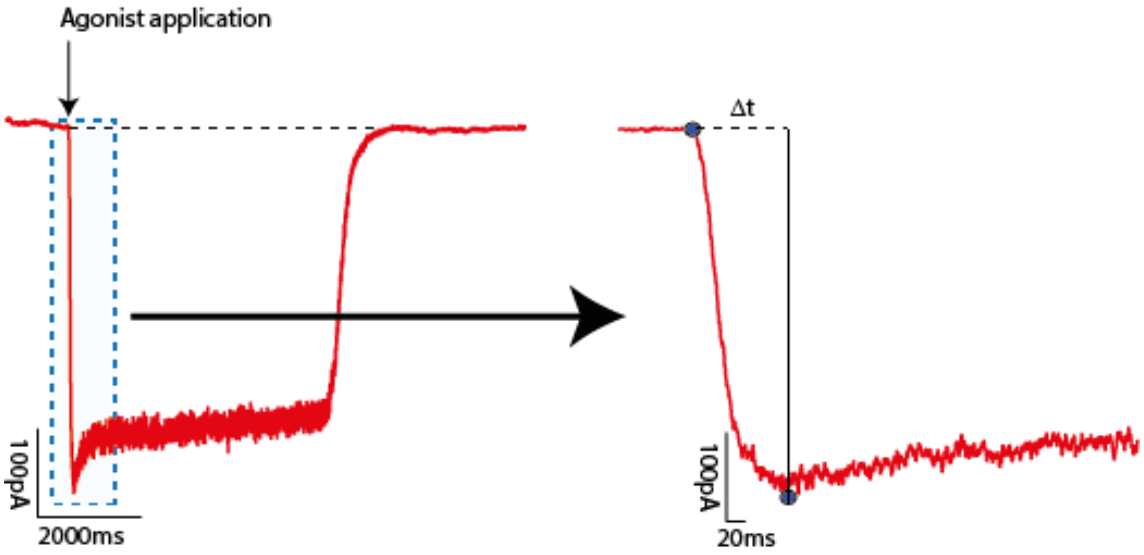


Figure 24. Rise-time illustration. On the left an average trace with a dashed square highlighting the peak evoked by agonist application (black arrow). On the right it is horizontal expanded the dashed square region and pointed with dashed line the time increment.

5.6. Recovery from desensitization

After desensitization, AMPARs remain desensitized for a brief period of time lasting for several milliseconds. This feature influences postsynaptic responses integration and the time AMPARs spend desensitized is modulated by several factors (as for example the presence of TARPs). To study recovery from desensitization, a two-pulse protocol (or paired pulse protocol) of 25-50 ms each will be used. After a first pulse a second pulse is applied at different time intervals (from 25 ms to 625 ms). The paired pulses are separated 1s to allow full recovery from desensitization. To estimate the percentage of recovery, the magnitude of peak current at the second pulse is compared with the first one. In figure 25 it is shown an average trace using this protocol. It is shown in the figure how the latest applications of agonist evoke responses with bigger peaks due to recovery from desensitization.

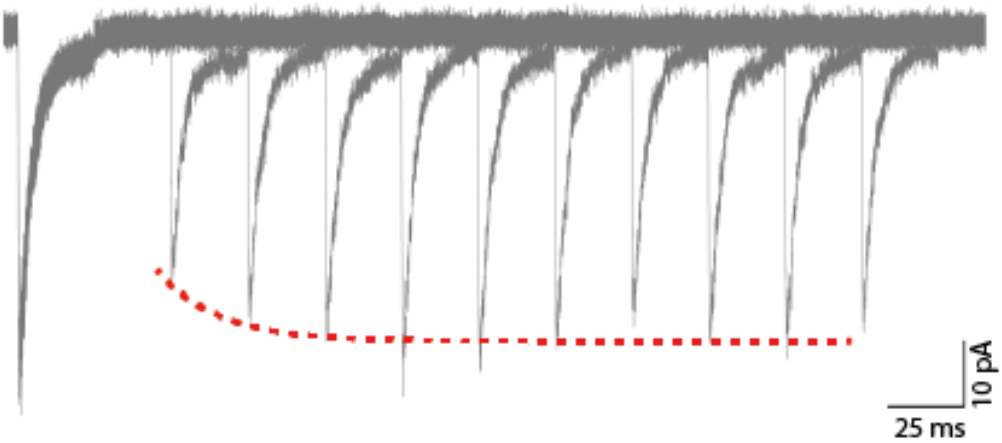


Figure 25. Recovery from desensitization protocol. This protocol is used to evaluate the recovery from desensitization of receptors by applying a two-pulse protocol where the lapse of agonist application between pulses is increased at each trace/sweep (in the figure are all sweeps overlapped). In the average sweep the first peak is compared with next peaks to calculate the percentage of recovery. In read dashed line it is shown a single exponential fit from second till last peak of the trace.

5.7. Current-Voltage relationships

CP-AMPARs are strongly blocked by intracellular polyamines (spermine) in a physiological manner causing a rectification of the currents. This block determines the degree of current flowing through AMPARs and hence influences postsynaptic depolarization time course in neurons. In order to study this spermine block of CP-AMPARs at different membrane potentials 10mM glutamate is applied onto outside-out membrane patches at different holding potentials (from −80 mV to +80 mV in 20 mV steps) and the peak current is used to construct the current-voltage relationship. The rectification index (RI) is defined as the absolute value of glutamate-evoked current at +60mV divided by that at −60mV: RI + 60mV/ − 60mV = |I+60mV|/|I−60mV|.

In addition, RI was used in experiments with CI-AMPARs to ensure the recordings were from an almost fully heteromeric population. Explained in the introduction, GluA2 subunit hardly ever form homotetramers and has high affinity to form dimers with other subunits. However, in an expression system AMPARs assembly is not regulated like in neurons and exists the possibility that other subunits traffic to cell membrane forming homotetramers without GluA2 subunit. To avoid record populations of CP-AMPARs in experiments with CI-AMPARs it was used the value of the RI. In CI-AMPARs the RI value is near 1 because they are not blocked by endogenous polyamines (peak at +60mV ≈ peak at -60mV membrane). To consider that a membrane patch has a majority of CI-AMPARs it was set a RI threshold of 0.7. Recordings of CI-AMPARs below 0.7 of RI were discarded. The following figure (figure 26) schematize the protocol used to record current-voltage curves.

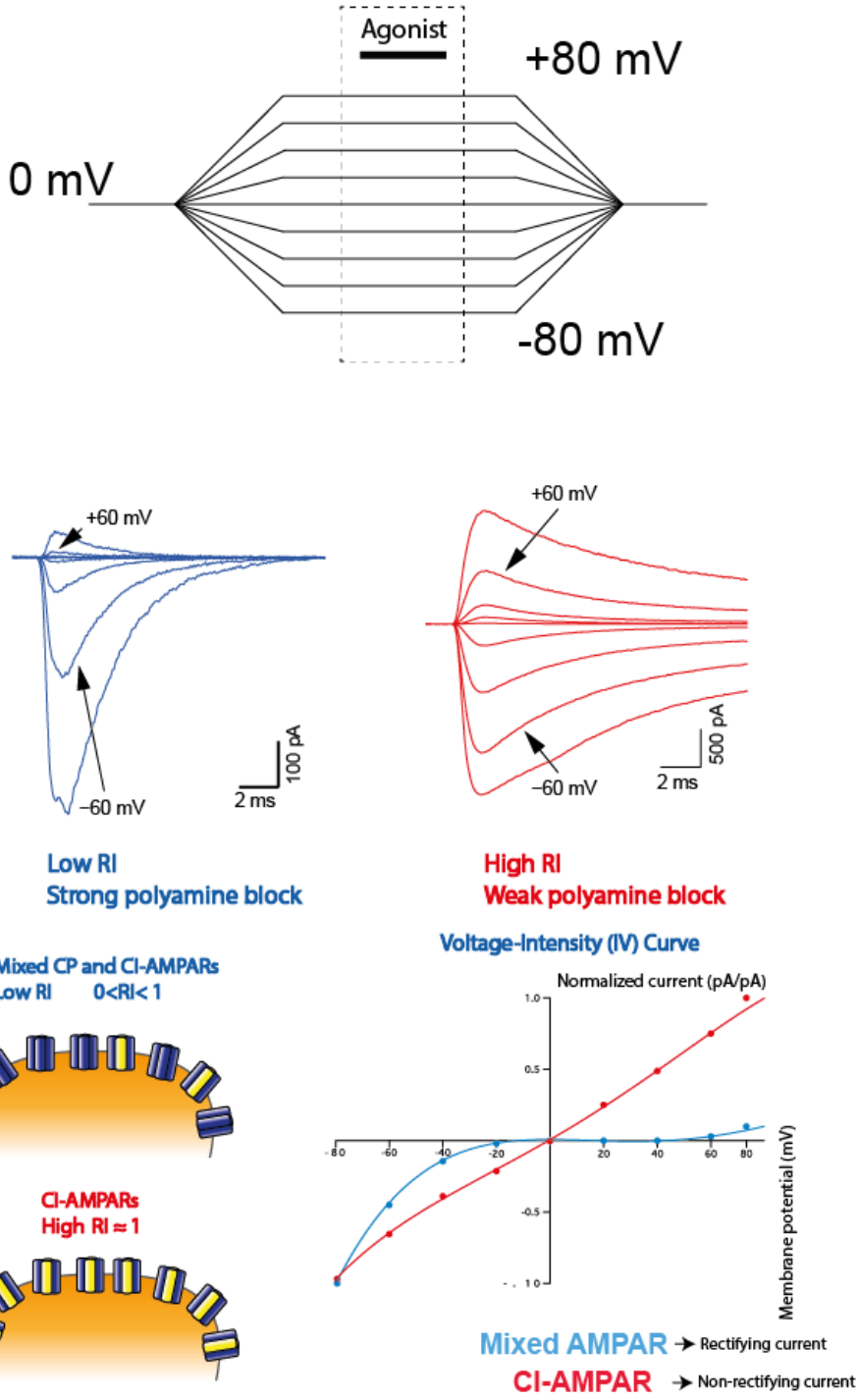


Figure 26. IV protocol and polyamine block. At the top of the figure it is shown the IV protocol where agonist is applied at different membrane potentials from -80mV to +80mV in steps of 20 mV.
In the middle, two examples of traces with different RI values. On the left, low RI value and strong polyamine block while on the right high RI value and weak polyamine block.
At the bottom two AMPAR populations with different RI values. The first example is a mixed population of CP and CI-AMPARs with low RI value and rectifying current. The second example is a population of CI-AMPARs with an RI value near 1 as the receptors are not blocked by polyamines (non-rectifying current).

6. Crosslinking assay

To test the possible heterogenity of AMPAR-TARP population in CP-AMPARs with 2 TARPs per receptor (transfection with GluA1 subunit and GluA1:γ2 fussion protein) it was performed a crosslinking assay. Different to CI-AMPARs, in CP-AMPARs it was much more complicated to ensure that the 2 TARPs group had indeed 2 auxiliary subunits per receptor. For this reason the electrophysiological experiments were complemented by this assay.

This protocol permits to maintain receptors subunits assembly (maintain the tetramer) for later detection by western-blot technique. It is based in the use of homobifunctional N-hydroxysuccinimide ester (NHS ester) and bis(sulfosuccinimidyl) suberate (water-soluble analog of NHS). This compounds react with $-NH_2$ free radicals at 7-9pH forming stable amide bonds. It allows to multimeric proteins maintain their subunits attached. The crosslinking assay was thought to test if 2 TARPed AMPARs were the major population on cell membrane in 2 TARPs condition.

The molecular weight estimated for populations of receptors with 0, 2 and 4 TARPs was:

- 548kDa for 4 TARPed AMPAR
- 474kDa for 2 TARPed AMPAR
- 400kDa for no TARPed AMPAR

To perform this assay it was used the commercial kit BS^3 from Thermo Fischer Scientific (catalogue # 21580). It was applied the protocol to cross-link membrane proteins so the cross-linkage was performed at the extracellular region. The following protocol was applied:

1) Cell transfection using same conditions of DNA ratio as used for patch clamp experiments.

2) Wash cells with HBSS and incubation with BS3 solution from the kit during 10 minutes at 37ºC in incubator.

3) Add 1M glycine solution (100mM final) to well to end cross-linking by quenching the reaction and incubate 10 minutes at 4ºC

4) Discard liquid and add ice-cold lysis buffer and maintain cells at 4ºC.

5) Split the cells by scraping in ice-cold lysis buffer transferring material to chilled eppendorfs.

6) Sonicate cells for about 5 seconds and centrifuge eppendorf 2 minutes at 20000rpm at 4ºC.

7) Take supernatant and store at -80ºC.

8) Protein quantification to ensure it is enough sample for western-blot procedure.

9) Detection of AMPAR complex by western-blot using antibodies against GluA1 and/or γ2 subunit C-terminal regions as the cross-linkage was done in the extracellular region what possibly alter antigen-antibody interaction at this region.

10) Gels for western-blot used were nuPAGE tris-acetate gels from Thermo Fisher Scientific (catalogue #EA03785BOX). Running buffer and protein transfer was made according manufacturers indications for these type of gels.

Lysis buffer preparation:

- 25 mM HEPES, pH 7.4
- 500 mM NaCl
- 2 mM EDTA
- 1 mM DTT
- 1 mM phenylmethylsulfonyl fluoride (PMSF)
- 20 mM NaF 1× protease inhibitor cocktail tablet (Calbiochem, cat. no. 539131)
- 0.1% of volume Nonidet P-40
- 1 µM okadaic acid (Calbiochem, cat. no. 459620)
- 1 µM Microsystin-LF (Calbiochem, cat. no. 475815)
- 1 mM sodium orthovanadate (NaOV)

BS^3 solution:

- 5mM Sodium citrate buffer (3ml of 5mM sodium citrate solution + 4.2ml of 5mM solution of citric acid to achieve pH 5).
- When the previous solution and everything else is ready BS^3 is reconstituted in the original vial by adding 1.68ml of sodium citrate buffer. It is vortex inmediately for aproximately 30 seconds.
- It is important to check the solubility of the cross-linker during the experiment.

7. Statistical analysis and software

Analysis of currents waveforms from patch clamp experiments and curve fitting was performed using the software IGOR Pro 6.06 (Wavemetrics) using NeuroMatic 2.03 (Rothman and Silver, 2018; http://www.neuromatic.thinkrandom.com).

On the other hand, the statistical analysis of the results obtained was performed using the software GraphPad Prism version 8.0.1 for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com). Comparisons between two groups were performed using the parametric Student' *t*-test for data following a normal distribution or using the non-parametric Mann-Whitney U test for comparisons between groups in which one of them did not follow a normal distribution. Normality of data distribution was tested by Shapiro-Wilk normality test. All statistical differences between more than two groups were examined by one-way ANOVA, followed by Newman-Keuls multiple comparisons test. P values < 0.05 were considered statistically significant as follows: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.000

4. Results

In the present thesis, it has been studied the function on ionotropic glutamate receptors (AMPARs and NMDARs). The main purpose was to investigate these iGluRs in both, their physiological function and also in the framework of neurological conditions. First, it will be presented the results obtained about the study of AMPAR-TARP stoichiometry in AMPAR function and next the effect of *de novo* GRIN mutations in NMDAR biophysical behaviour.

All along the results, P values shown have the following meaning: p< 0.05 are considered statistically significant as follows and were annotated * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001 and **** = p-value < 0.0001.

AMPAR-TARP stoichiometry in CP-AMPARs

1. γ2 graded effect over CP-AMPAR kinetics

As it has been presented in the introduction, TARPs can modulate AMPAR kinetics among other biophysical properties. The archetypical and most studied TARP γ2 (or stargazin) modifies AMPAR kinetics by slowing the desensitizing responses of this receptor (Priel et al. 2005). Nevertheless, the possible AMPAR-TARP stoichiometry modulation over AMPAR kinetics is quite unknown. In this thesis it was wondered how stoichiometries of 2 and 4 TARPs per receptor could modulate channel properties. This issue is still understudied and it is not clear whether an increasing number of TARPs per channel can exert an increasing effect in kinetics modulation or by contrast the maximum effect of this modulation is achieved by a low number of auxiliary subunits. To study between these two possibilities were used GluA1:γ2 fusion proteins (commented in **methodology 5.3.;** and previous validated in (Soto et al. 2014)) to obtain in tsA201 cells populations of 2 and 4 γ2 subunits per AMPAR. Results obtained were compared also with a 0 γ2 group where cells were transfected with only GluA1 plasmid vector. AMPAR currents from transfected cells were recorded using patch clamp technique using the outside-out configuration and applying 10mM glutamate steps during 100ms using a piezoelectric controller as explained in **methodology 5.3.**

Desensitization kinetics of GluA1 homomeric receptors with different AMPAR-TARP stoichiometry measured as weighted time constant (τ_w) showed a clear dependence of the number of γ2 per receptor. The increase in the amount of γ2 per AMPAR rise the values of τ_w (2.32 ± 0.16 ms, 3.77 ± 0.39 ms and 6.70 ± 0.41 ms for 0 TARPs, 2-TARPs and 4-TARPs respectively; one-way ANOVA; Figure 27A and B).

In contrast to this well-defined graded change observed in desensitization kinetics, there was not the similar clear pattern when looking at steady-state currents. Interestingly, both properties are usually correlated and a slow desensitization kinetics usually implies greater steady state currents. However, here it was not find significant differences in all group comparisons. A significant change was detected only when CP-AMPAR was fully saturated with 4 γ2. However, a graded effect cannot be completely discarded since 2 TARPs showed mean intermediate mean values between TARPless and 4 TARPed conditions (2.78 ± 1.04 for 0 TARPs; 5.58 ± 1.70 for 2 TARPs vs. 13.95 ± 1.85%for 4 TARPs; one-way ANOVA; figure 27C).

On the other hand, it was also analysed the effect of AMPAR-TARP stoichiometry in receptor activation (rise time) and it was not found significant differences between any group (0.46 ± 0.06 ms, 0.61 ± 0.12 ms and 0.67 ± 0.08 ms for 0 TARPs, 2 TARPs and 4 TARPs respectively; one-way ANOVA; Figure 27D).

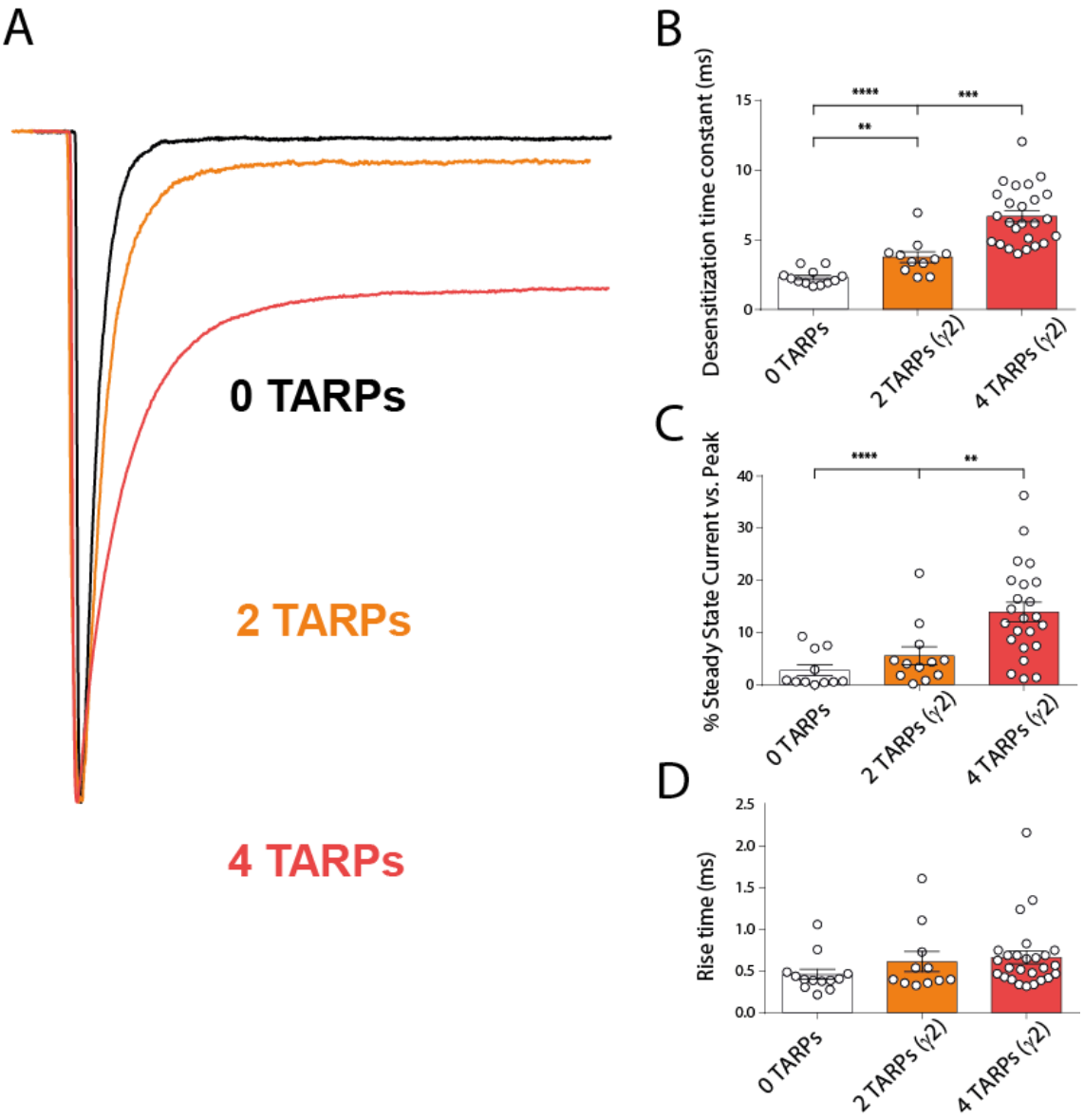


Figure 27 Kinetic properties of CP-AMPARs. CP-AMPAR (GluA1 homomers) kinetics are differentially modulated by AMPAR-TARP stoichiometry. (A) Overlapped traces of the 3 different condition evoked at -60 mV by rapid application of 10 mM glutamate onto outside-out patches from cells expressing 0, 2 or 4 TARPs per AMPAR. (B) Pooled data of the weighted time constant of desensitization (τ_w, des). Bar graph with standard error deviation of the mean (SEM) indicated and every individual experimental value plotted as an open circle. (C) Pooled data showing the increase in the steady state current only in 4 TARPed CP-AMPARs. (D) Rise time of glutamate-activated currents is not affected by TARPs.

2. Recovery from desensitization is modulated by AMPAR-TARP stoichiometry

It has been shown that TARPs can also speed AMPAR recovery from desensitization (Ruiz et al. 2005; Cais et al. 2014; Carbone and Plested 2016; Priel et al. 2005) contrary to not TARPed receptors. However, as in kinetics, how the number of TARPs per AMPAR can modulate this parameter is still not defined. In this set of experiments, we applied a paired pulsed of 10 mM glutamate applications separated by an increase of time intervals onto membrane patches from cells expressing different AMPAR-TARP stoichiometry combinations. The value from the recovery rate comparing first evoked current to the second was used to fit this rate with a single exponential function. The obtained recovery time constant (τ) was used to compare recovery from desensitization between the different groups. Results showed, as in steady state currents, only significant differences between 4 TARPs with 0 and 2 TARPs condition. However, the 2 TARPs group values are halfway between 0 and 4 TARPs although it were not found statistical differences (98.57 ± 7.35 ms for 0-TARPs, 68.91 ± 5.92 ms for 2-TARPs and 53.86 ± 4.78 ms for 4- TARPs; one-way ANOVA; Figure 28C)

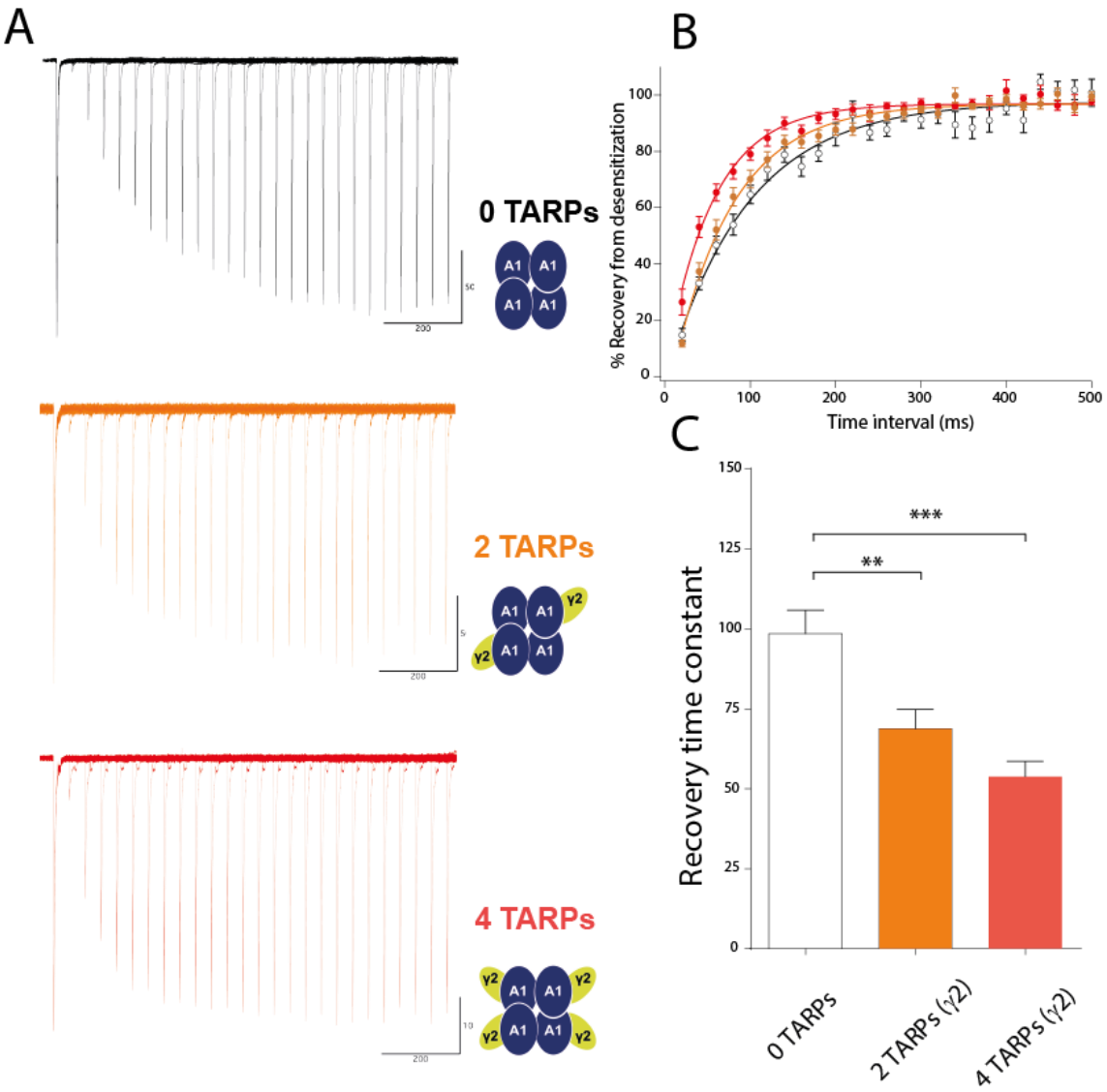


Figure 28. Recovery from desensitization of CP-AMPARs is enhanced in a graded manner with increased γ2. (A) Representative traces of a paired-pulse protocol with increasing time interval between pulses for CP-AMPAR with different AMPAR-TARP stoichiometries. Currents were recorded into outside-out patches (B) Recovery from desensitization dynamics where it can be observed a gradual diminishment in the time needed to recover as the number of γ2 increases. (C) Recovery time constant values for the experiments showed in A and B.

3. Polyamine block in CP-AMPARs is attenuated in stoichiometry-dependent manner by γ2

A canonical property of CP-AMPARs explained in the introduction, is the capability to be strongly blocked by intracellular polyamines at depolarized membrane potentials.

This blockage is attenuated by TARP modulation (Soto et al. 2007; Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-candy 2009). Here it was tested if this property can be stoichiometry dependent. The results obtained with RI calculated showed a crystal-clear graded effect depending on the number of γ2 per AMPAR (0.056 ± 0.004 for 0-TARPs; 0.128 ± 0.011 for 2-TARPs and 0.274 ± 0.021 for 4-TARPs; one-way ANOVA; figure 29).

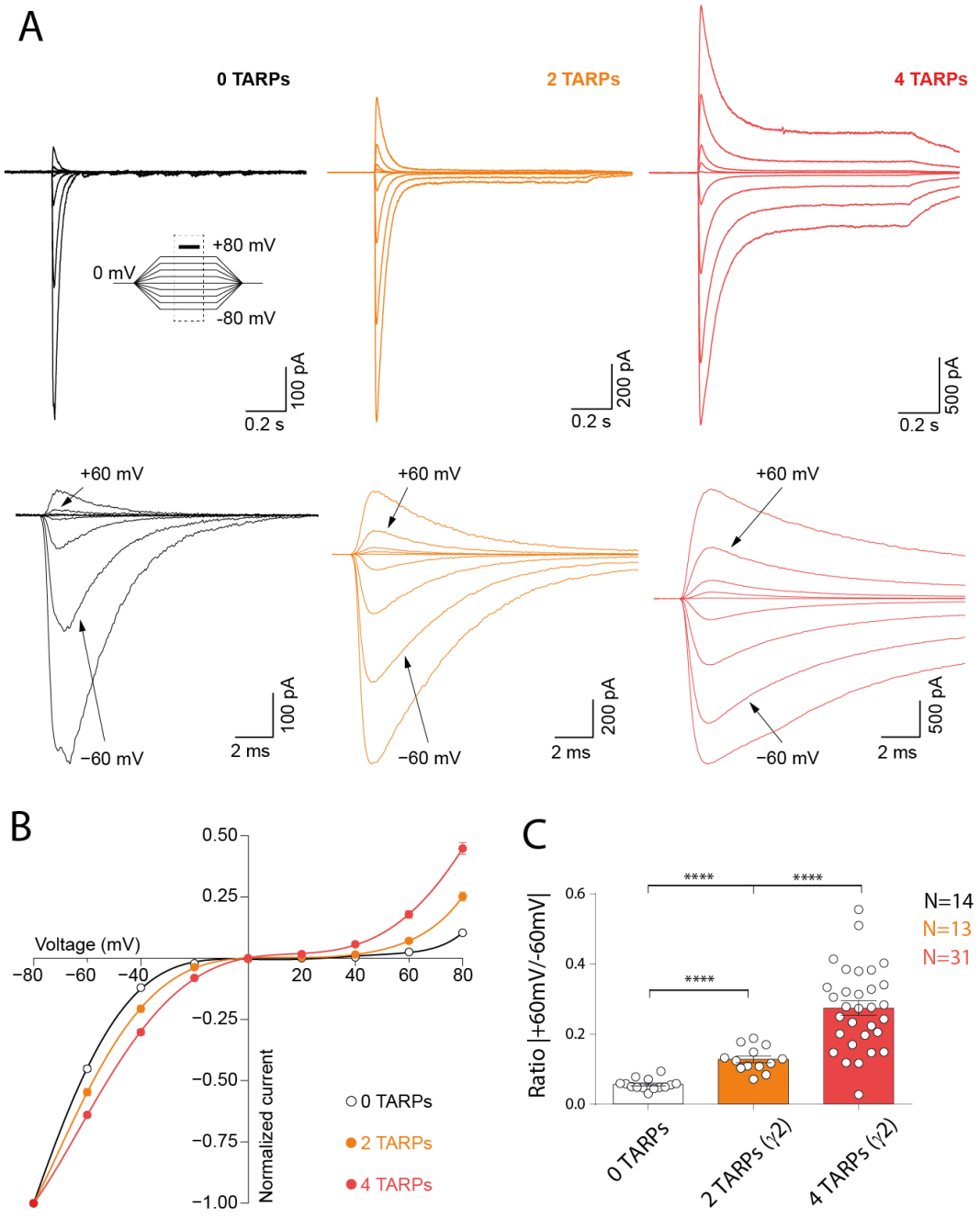


Figure 29. Attenuation CP-AMPAR block by spermine depends on the number of γ2 associated with

receptor. (A) Top: Representative glutamate-evoked currents from outside-out patches at different membrane potentials from -80 to +80 in 20 mV steps from cells expressing CP-AMPARs, Bottom: Time scale expansion of recordings in A where traces recorded at +60 mV and -60 mV membrane potentials are pointed with black arrows. (B) I-V relationships constructed from glutamate-evoked peak currents of patches held at different membrane potentials in different AMPAR-TARP stoichiometries. (C) Pooled data showing an increase in the RI as the number of TARPs per CP-AMPAR increases. The RI in 2 and 4 γ2 per CP-AMPAR complex is higher compared with 0 TARPs condition (TARPless). Bar graphs meaning as in Figure 29.

4. 2-TARPed AMPAR population verification

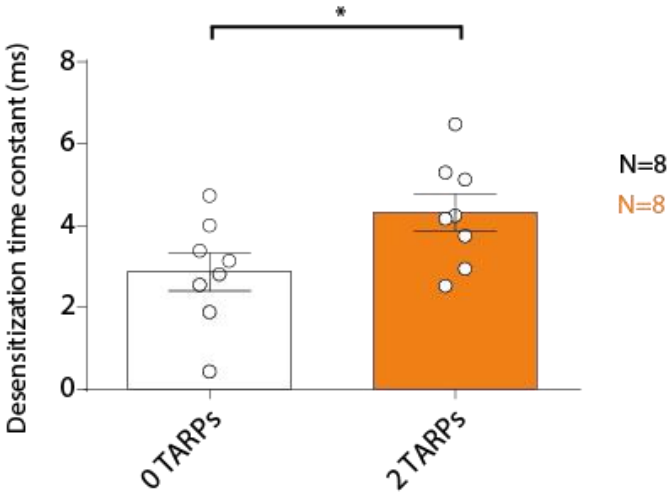
The results obtained with γ2 stoichiometry in AMPAR support the idea of a graded modulation of GluA1 homotetrameric receptors, increasing the effect of the auxiliary subunit as its number per receptor also increases. However, it exists the possibility of the presence in our recordings of a mixture of AMPAR-TARP stoichiometries in the 2 TARP condition with 0 and 4 TARPed receptors instead of a pure population of GluA1-GluA1:γ2 heteromers. This would account for the intermediate phenotype observed in most of the parameters analysed above. Therefore, to discard that possibility, it was decided to create a GluA1 subunit with an arginine at the Q/R site (GluA1(R)) to co-transfect GluA1(Q) and GluA1(R) to form heterotetramers. Using the polyamine block to check the formation of a pure population of heterotetramers it was tested if desensitization kinetics was also slowed by 2 TARPs condition in front of 0 TARPs condition using GluA1(R) - GluA1(Q):γ2 and GluA1(R)-GluA1(Q) heteromers. For these experiments, RIs below 0.7 were not considered an AMPAR receptor population of mostly heteromers.

Results showed that the 80% of patches recorded with the co-transfection GluA1(R)-GluA1(Q) subunits were over 0.7 (8 over 10) and 100% of RIs over 0.7 in GluA1(R)-GluA1(Q):γ2 transfection (n=9). However, this can be explained by the tendency of GluA1(R) to not form homotetramers (as it happens with GluA2, look at **introduction 4.2.1. RNA editing**). Nevertheless, desensitization kinetics of these recordings confirmed the effect of γ2 in the complex. As detected with GluA1(Q) forms, in the

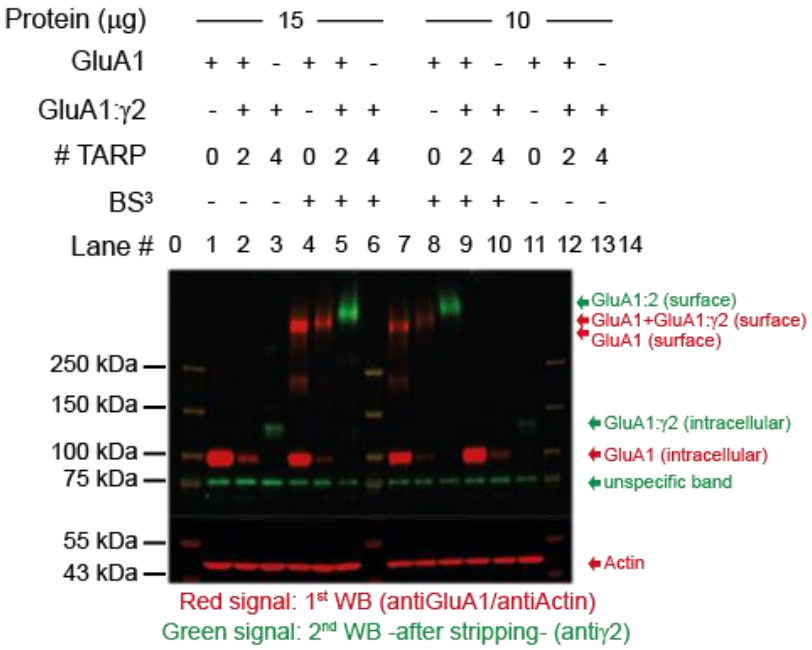
GluA1(R)-containing receptors the weighted time constant (τ_w) of the TARPed receptor was slowed significantly compared with the TARPless receptor (2.87 ± 0.47 for 0-TARPs vs. 4.32 ± 0.46 for 2-TARPs; student's t-test, figure 30). This suggests that the results obtained in the 2-TARPed condition with GluA1(Q) from were acquired putatively from a major 2-TARPed population, although it cannot be completely ruled out the presence of a small amount of 'contaminating' homomeric AMPARs. In addition, it should be mention that other properties like polyamine block or conductance were not tested in these experiments because of the intrinsic properties conferred by GluA1(R) subunit: on one hand the arginine residue does not permit polyamines to block AMPARs as it happens in GluA1 subunit and on the other hand, single-channel conductance levels cannot be compared as GluA1(R)-containing receptors are not permeable to Ca^{+2} like also GluA2-containing AMPARs.

Furthermore, to perform a different approach to rule out a mixed population, it was used a cross-linking assay to detect by molecular weight whether the 2 TARP group was integrated by a majority of receptors with 2 γ2 auxiliary subunits. As explained in the **methodology** section, this assay allows to detect by western-blot different proteins conserving the subunit association (the AMPAR tetramer). For this reason, GluA1 subunits forming AMPAR tetramers can be measured by their molecular weight and differences due to association with γ2 can be detected. That implies different molecular weights to 0, 2 or 4 TARPed AMPARs. The results suggest, like in the electrophysiological approach, that a 2 TARPed AMPAR population in 2 TARPs condition. However, a small portion of other AMPAR-TARP stoichiometries cannot be discarded. 2 TARPed AMPAR where detected with a slightly higher molecular weight than TARPless receptors but less than full TARPed AMPARs.

A



B



Red signal: 1st WB (antiGluA1/antiActin)
Green signal: 2nd WB -after stripping- (antiγ2)

C

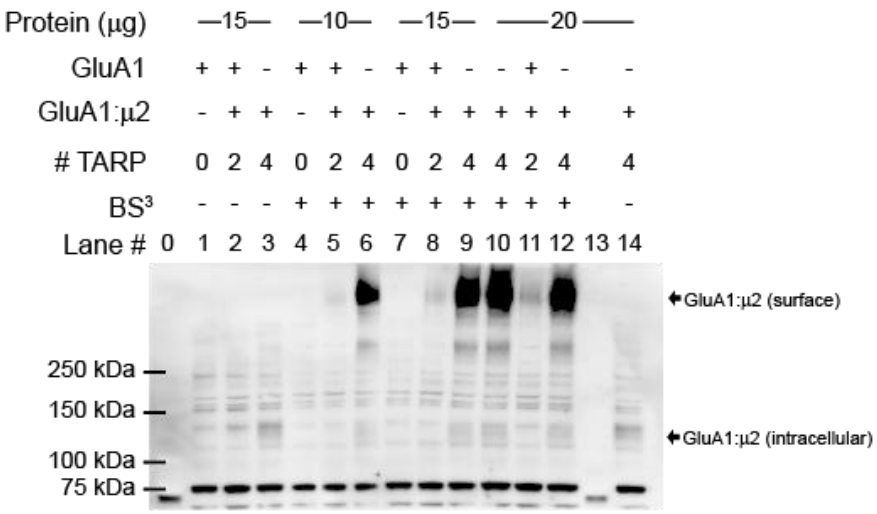


Figure 30. AMPAR population in 1:2 AMPAR-TARP stoichiometry. (A) Pooled data of AMPARs recordings in 0 and 2 TARPs per receptor condition using GluA1(R) to ensure heteromeric AMPAR formation. By comparing desensitization time constant it can be observed than in 2 TARPs condition the effect slowing kinetics is maintained like in previous results with GluA1(Q). In (B), it was first probed (red signal) the bottom part of the membrane with anti-Actin antibody to confirm that equal protein amounts where loaded in each lane. The upper part of the membrane was probed with an anti-GluA1 antibody against a C-terminus epitope. Tetrameric GluA1 surface receptors were detected in different molecular weights in extracts from cells expressing different condition (0, 2 or 4 TARPs condition). Detection suggested a higher molecular weight for 2 TARPs condition in front of 0 TARPs condition. GluA1:γ2 tetramers were not detected with anti-GluA1 antibody probably due to some epitope masking in the tandem form (C-terminus of GluA1 is linked to γ2). Monomeric/intracellular GluA1 (100 kDa) is also detected with this antibody in 0 TARPs and 2 TARPs extracts, although much less GluA1 seems to be expressed in the 2 TARPs extracts. After stripping, it was probed the same membrane with an anti-γ2 antibody recognising an intracellular epitope (green signal). Tetramers were observed in high molecular weight bands only in 4 TARPs condition, and this band had a higher molecular weight than that observed for the tetramers detected with the anti-GluA1 antibody in the 2 TARPs condition. The intracellular signal corresponding to the monomeric tandem GluA1:γ2 (137 kDa) is observed in the 4 TARPs condition but not in the 2 TARPs condition suggesting that this form has not been expressed. Altogether suggests that in the 2 TARPs condition receptors contained indeed two γ2 subunits as they showed a slightly higher molecular weight than tetramers from the 0 TARPs but less than in 4 TARPs condition.
In another membrane (C) where it was first probed with anti-γ2 antibody it was detectable a small amount of tetrameric GluA1:γ2 in the 2 TARPs condition when long exposures were performed. The faint high molecular weight band in 2 TARPs extracts was mainly detected when 20μg of protein was loaded. The observed band had the same molecular weight as GluA1:γ2 tetramers from the 4 TARPs condition, suggesting that 2 TARPs extracts express mainly at the surface GluA1 containing 2 TARPs tetramers and a very small amount of GluA1:γ2 tetramers.

5. 4 γ2 TARPed AMPAR is required to increase receptor conductance

It was decided next to study whether single channel conductance was modulated by different stoichiometries since this is a parameter clearly modulated by TARPs (Soto et al. 2007; Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-candy 2009; Soto et al. 2014; E. Suzuki, Kessler, and Arai 2008; Y. Shi et al. 2010) by means of NSNA, which permits the measure of both channel conductance and open probability. By performing NSNA,

values for single-channel conductance and peak open probability from macroscopic responses were obtained. The results obtained showed similar values for TARPless GluA1 homomeric receptors and 2 γ2 TARPed AMPAR with no indications of a graded effect for TARP stoichiometries. However, single-channel conductance of 4 γ2 TARPed AMPAR showed significant differences with the groups mentioned before, being the conductance for fully saturated AMPARs higher than 0 and 2 TARPs groups (16.58 ± 0.69 pS for 0 TARP; 17.03 ± 2.06 pS for 2 TARP; 24.34 ± 1.69 pS for 4 TARP; one-way ANOVA; Figure 31C).

On the other hand, different from the well-known effect of TARP increasing AMPAR single-channel conductance (Soto et al. 2007; Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-candy 2009), their modulation of the peak open probability it has been more controversial. It has been published that γ2 can increase the peak open probability (E. Suzuki, Kessler, and Arai 2008) in AMPAR but also that this TARP has no effect for this parameter (Soto et al. 2007; Yun Shi et al. 2010).
The NSNA performed in this experiments allowed to determine the number of channels contributing to each response besides of the unitary conductance. Henceforth, peak open probability ($P_{o,peak}$) can be easily deduced from the experimental mean peak current analysed. The results obtained in this thesis project showed no difference between different AMPAR-TARP stoichiometries or even between TARPed and TARPless AMPARs (0.54 ± 0.06, 0.47 ± 0.06 and 0.62 ± 0.05 for 0 TARPs, 2 TARPs and 4 TARPs; One-way ANOVA; Figure 31D).

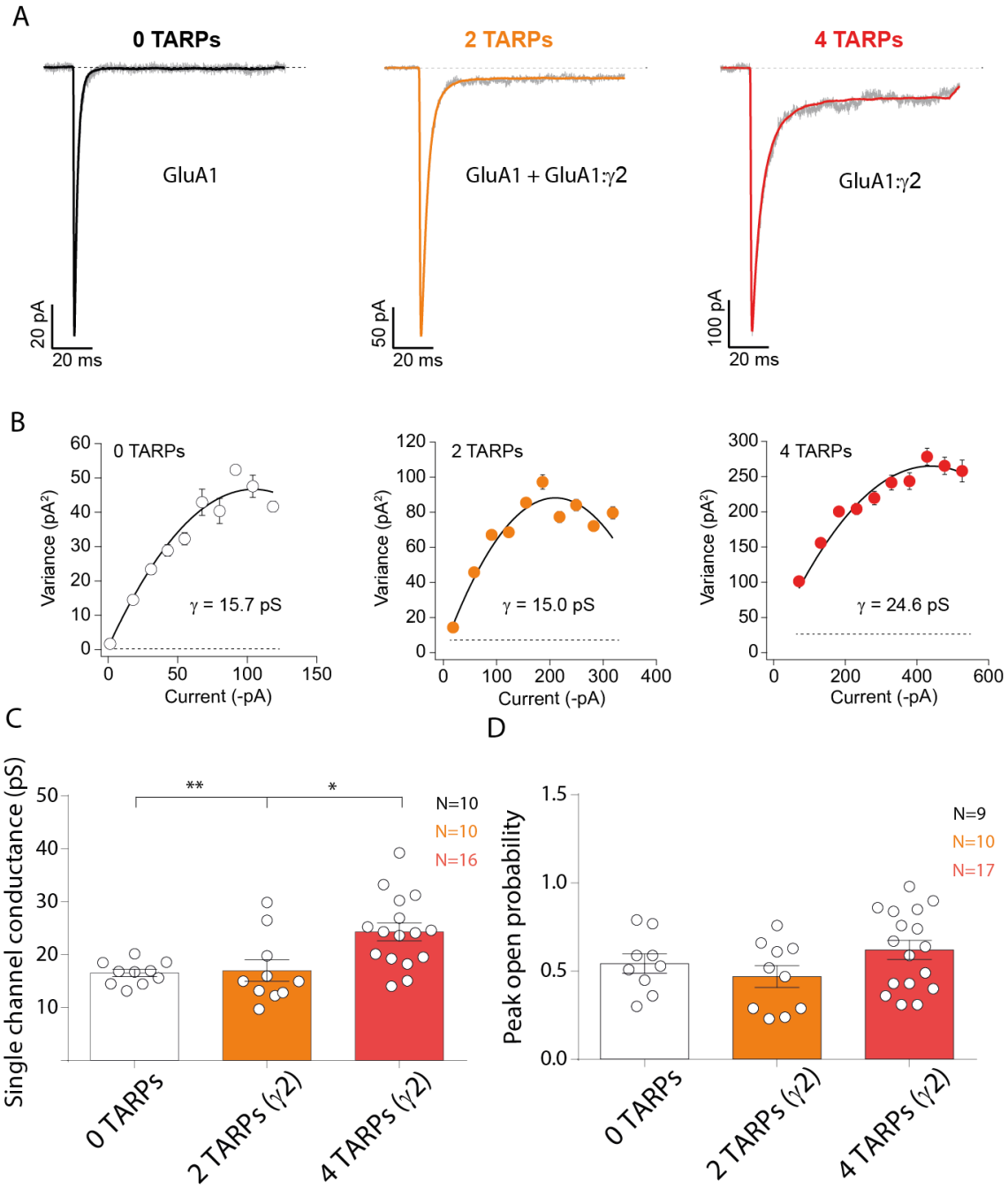


Figure 31. Four TARPs are essential to increase CP-AMPAR channel conductance. (A) Typical responses at a holding potential of -60 mV to rapid application of 10 mM glutamate onto excised patches from cells expressing homomeric GluA1 alone or together with 2 or 4 γ2 subunits. A single trace is shown in grey overlaid with the mean response. (B) Current-variance plots for the traces shown in A, the slope of which gave the weighted single-channel conductance indicated. Dashed lines show the baseline variance and error bars denote SEM. Single channel conductance values for these recordings are presented. (C) Pooled data showing an increase of the single channel conductance only in a full-TARPed CP-AMPAR. (D) Pooled data for peak open probability of CP-AMPARs were no differences were seen.

6. Single-channel conductance behaves equally in AMPAR:TARP stoichiometry with type Ia TARPs

Despite the fact that γ2 is the prototypical TARP and the more widely studied, other members of the family are also important and their spatiotemporal distribution though the nervous system is very important. So, to test if different members of TARP family exerts the same modulation of AMPARs in terms of AMPAR-TARP stoichiometry, recordings with other members of the TARP family were performed. Type Ia TARP is a subfamily of this auxiliary subunits that comprises γ2 and γ3 proteins. Both proteins have been arranged together and separately from type Ib TARPs due to similarities in modulating AMPARs. To maintain the same conditions used with γ2, these experiments were performed also using tsA201 cells as expression systems and expressing GluA1 homotetramers with combinations of 0, 2 or 4 TARPs per AMPAR. All the results obtained with different TARPs are dissected in the following sections.

As seen with γ2, single-channel conductance is increased only with 4 γ3 TARPed GluA1 homomers and no graded effect is seen in 2 γ3 TARPed condition (16.58 ± 0.69pS, 19.96 ± 2.93pS and 24.07 ± 2.65pS for 0 TARPs, 2 TARPs and 4 TARPs; Kruskal-Wallis multiple comparison test; Figure 32A). In addition, peak open probability was analysed and, as seen for γ2, there were no differences between groups (0.54 ± 0.06, 0.58 ± 0.06 and 0.48 ± 0.06 for 0 TARPs, 2 TARPs and 4 TARPs; One-way ANOVA; Figure 32B).

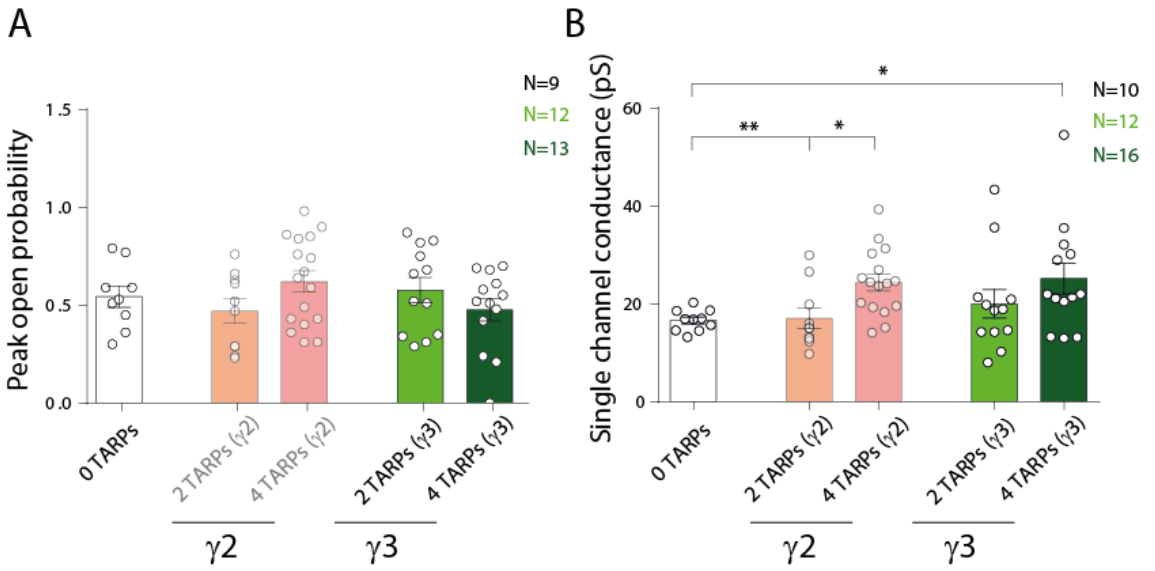


Figure 32. γ3 TARP increases single channel conductance only when the receptor is full-TARPed. In bold, 2 TARPs and 4 TARPs γ3 and in dim colours the same conditions but using γ2. γ2 and γ3 conditions are grouped separately. (A) Peak open probability did not show differences depending on AMPAR-TARP stoichiometry. Attenuated bars of the bar graph show previous results obtained with γ2 TARP. (B) Only 4 TARPed AMPAR with γ3 show a significant increase in single-channel conductance.

7. The graded effect in AMPAR-TARP stoichiometry disappears with γ3 TARP

Looking at the other parameters studied in AMPAR-TARP stoichiometry with γ2 auxiliary subunit to compare with γ3, the graded effect seen before completely disappears. The following results suggest that 2 γ3 per AMPAR are enough to elicit the maximal effect in AMPAR modulation.

In terms of receptors kinetics, 2 TARPs and 4 TARPs condition showed no significant differences between theme. However, as logical, these conditions presented desensitization time constant values that significantly differ from the TARPless condition (2.32 ± 0.16ms, 4.85 ± 2.12ms and 5 ± 1.18ms for 0-TARPs, 2-TARPs and 4-TARPs; One-way ANOVA; Figure 33A). Subsequent with these results, steady state currents measured did not differ in conditions with TARPed AMPARs but did it significantly when compared to TARPless receptors (2.78 ± 1.38, 14.04 ± 2.91 and 8.15 ± 1.48 for 0-TARPs, 2-TARPs and

4-TARPs; One-way ANOVA; Figure 33B). Interestingly, currents from desensitized receptors from 2 TARP condition presents a mean value higher than in 4 TARPed AMPAR; nevertheless, there are no significant differences. This pattern can be explained by the heterogeneity of possible AMPAR-TARP stoichiometry in 2 TARP condition however, it is clear that values between 2 and 4 TARPed condition do not significantly differ. Similarly, measuring receptors time activation (rise time), 2 TARPed condition shows higher values than 4 TARPed AMPARs. Nevertheless, no differences were seen comparing 0, 2 and 4 TARPs condition with γ3 auxiliary subunit, as it occurred when was tested with γ2 (0.46 ± 0.06ms, 0.6 ± 0.08ms and 0.44 ± 0.06ms for 0-TARPs, 2-TARPs and 4-TARPs; Kruskal-Wallis multiple comparison test; Figure 33C). In the same line, looking at polyamine block, RIs values with a graded pattern observed with γ2 TARP did not recapitulate when γ3 was the auxiliary subunit for AMPARs. When analysing AMPAR-TARP stoichiometry with γ3, 2 and 4 TARPs condition display similar values with no significant differences between both groups. Here as expected, polyamine block in TARPed AMPARs conditions was significantly released compared to TARPless AMPARs (0.03 ± 0.004, 0.37 ± 0.05 and 0.45 ± 0.04 for 0 TARPs, 2 TARPs and 4 TARPs; One-way ANOVA; Figure 33D).

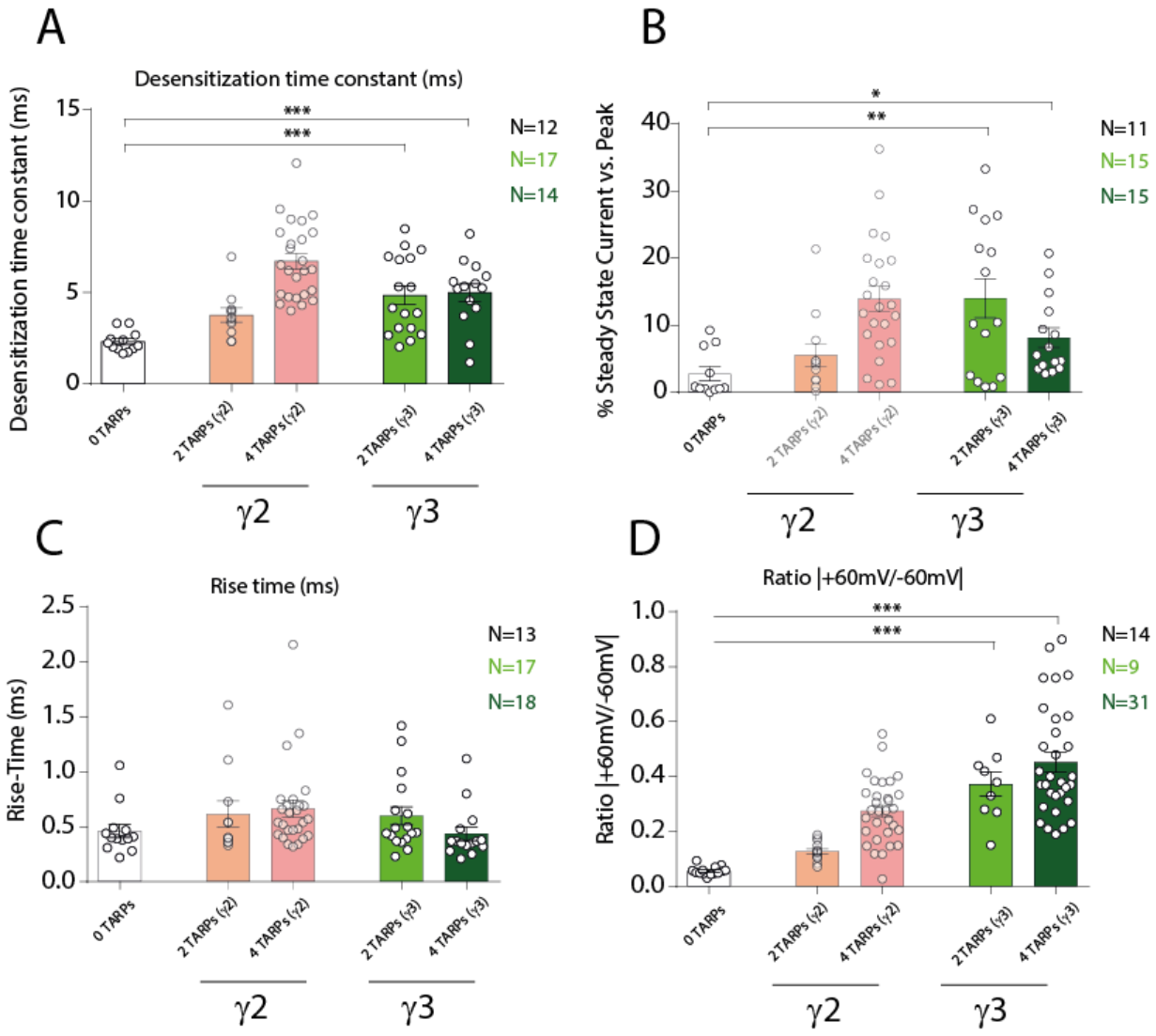


Figure 33. AMPAR-TARP stoichiometry with γ3 behaves different than with γ2. Pooled data (A-D) with 2 and 4 TARPs conditions using γ2 (dim bar graph) and γ3 as AMPAR auxiliary subunits. In A, B and D the graded effect exhibited by γ2 is not repeated by γ3 where 2 and 4 TARPs conditions show no differences between both conditions but with TARPless condition. In C, rise time values using γ3 in different AMPAR-TARP stoichiometries did not show significant differences with 0 TARPs condition or between each other.

8. Recovery from desensitization is speeded in 4 ?3 TARPed AMPAR

As in the previous results with the type Ia TARP γ3, the graded effects seen with γ2 are not repeated. While the recovery from desensitization showed a not crystal-clear but highly suggested graded pattern with γ2, where more TARPs per AMPARs mean faster recovery, only with γ3 4-TARPed AMPARs recovers significantly faster than TARPless AMPARs. In addition, 4-TARPed receptors with γ3 recovers also faster than 2-TARPed AMPAR whose recovery from desensitization is surprisingly slower than TARPless AMPARs but not significantly different (251.98 ± 22.7ms, 354.91 ± 74ms and 168.53 ± 11ms for 0 TARPs, 2 TARPs and 4 TARPs; One-way ANOVA; Figure 34A). This striking result (0 vs 2 TARPs conditions) can be due to the high dispersion of values in 2 TARPs condition.

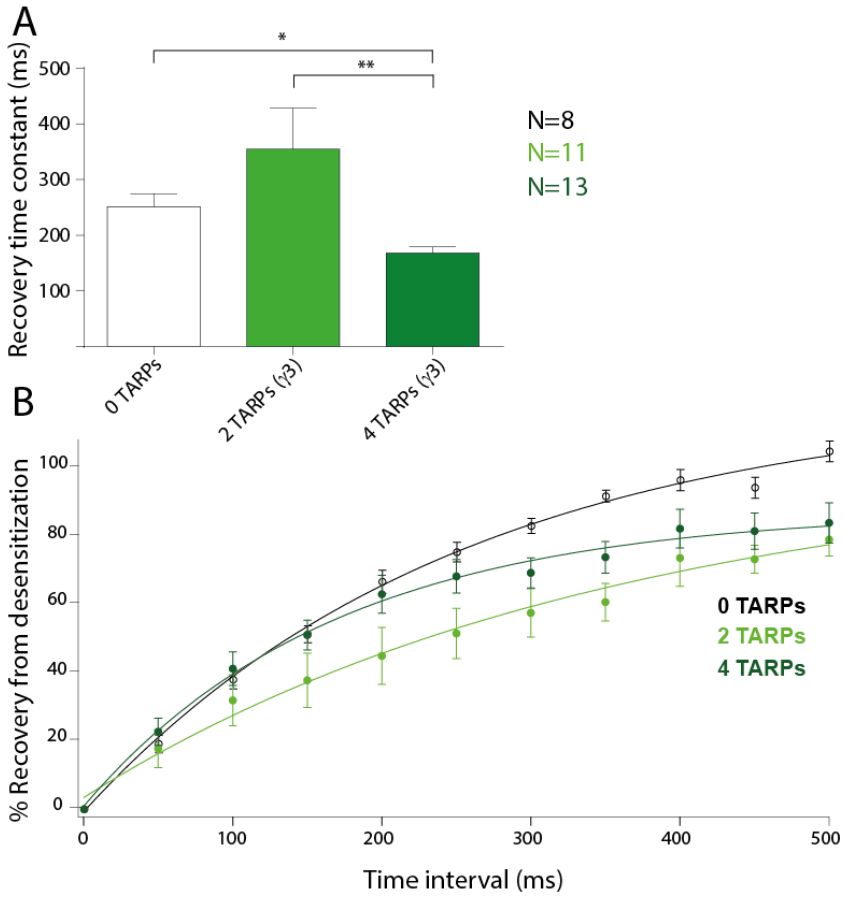


Figure 34. Recovery from desensitization is speeded only in 4 γ3 TARPs per AMPAR stoichiometry. (A) Bar graph showing a significant minor recovery time constant from 4-TARPed compared to 0 and 2 TARPs conditions. The full-saturated AMPAR recovery is the fastest from all conditions. (B) Recovery from desensitization dynamics with a diminishment in the time needed

to recover only when AMPARs are full saturated with 4 γ3 TARP.

9. Type Ib TARPs (γ4 and γ8) increases AMPAR conductance in 2 TARPs per AMPAR condition

The effect of AMPAR-TARP stoichiometry in receptors biophysical properties that has been showed till now was focused on type Ia TARPs (γ2 and γ3). However, as mentioned in the **introduction** (**5.1.4.1. Type Ia (γ2 and γ3) and type Ib (γ4 and γ8) TARPs**), type Ib TARPs show slightly different behaviour in terms of AMPAR modulation. They exert a more pronounced effect in AMPAR kinetics (Cho et al. 2007; Milstein et al. 2007) and it is due to differences in the extracellular loops of both families of TARPs (Riva et al. 2017). Surprisingly, with type Ib TARPs was not possible to record evoked currents from full TARPed AMPAR (4 TARPs condition). A possible reason for the lack of current will be commented in the discussion. However, when GluA1:γ4/8 were co-transfected with GluA1 subunit in tsA201 cell macroscopic responses were possible to record. The possibility that in co-transfection the responses recorded belonged just to GluA1 homotetrameric receptors was discarded as RI significantly differ from TARPless AMPAR (figure 10D). On the other hand, different from type Ia TARPs, 2 γ4 or γ8 TARPed AMPARs showed significant increase in single-channel conductance compared to TARPless receptors. Indeed, conductance values from 4 γ2 TARPed AMPARs were similar to the ones achieved in 2 TARPed AMPARs with type Ib TARPs (16.58 ± 0.69pS, 25.06 ± 2.77pS and 27.72 ± 3.69pS for 0 TARPs, 2 TARPs (γ4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4) and 0 vs 2 TARPs (γ8); figure 35A). However, peak open probability with type Ib TARPs behaves equally as type Ia TARPs since it did not differ depending on AMPAR-TARP stoichiometry or TARP type (0.54 ± 0.05, 0.58 ± 0.06 and 0.48 ± 0.06 for 0 TARPs, 2 TARPs (γ 4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4) and 0 vs 2 TARPs (γ8); figure 35B).

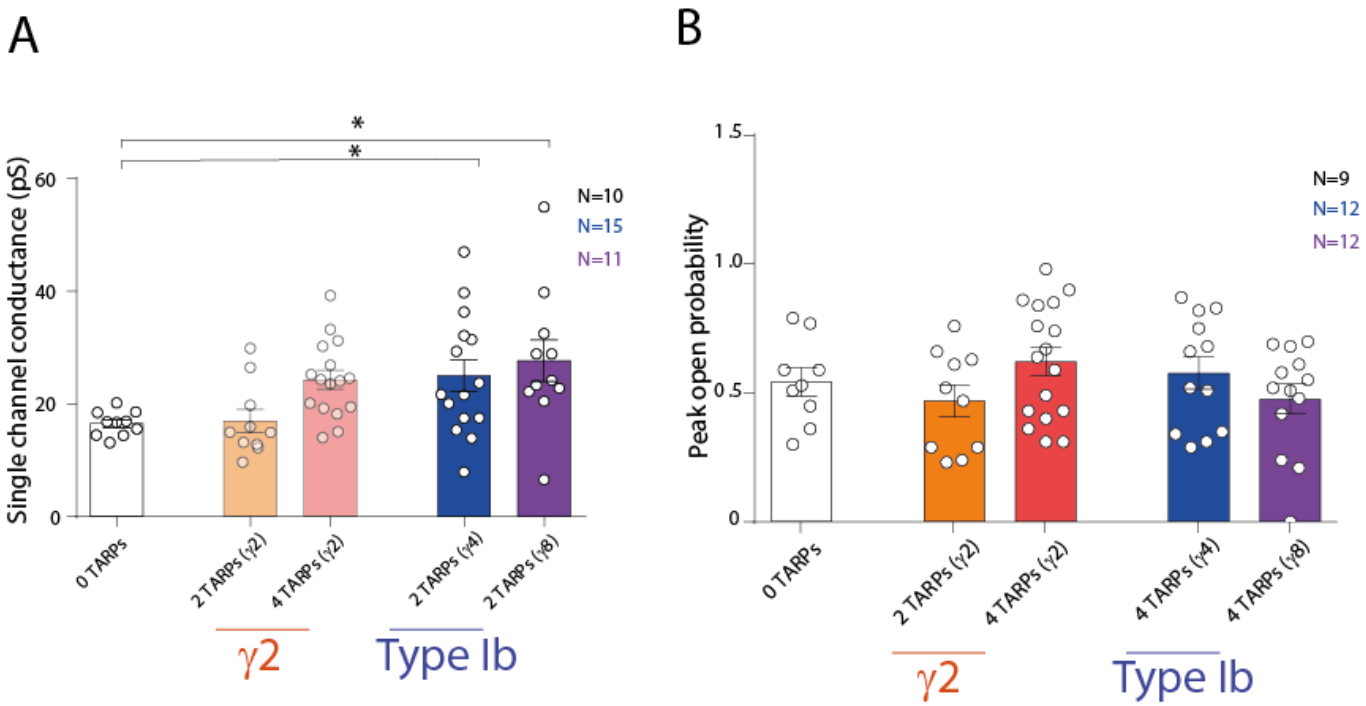


Figure 35. Type Ib TARPs increase single-channel conductance in 2 TARPed AMPARs. (A) Pooled data showing the increase in single-channel conductance mediated by the presence of 2 type Ib TARPs in AMPAR. In dim colours 2 and 4 TARPs per AMPAR conditions using γ2 to study AMPAR-TARP stoichiometry. Single-channel conductance modulated by type Ib TARPs shows similar values to 4 γ2 TARPed AMPARs seen at the 5th point of results section. (B) Peak open probability does not show significant changes with type Ib TARPs in 2:1 TARP:AMPAR stoichiometry.

10. AMPAR kinetics and polyamine block is also modulated by 2-TARPed receptor with type Ib TARPs

As mentioned before, type Ib TARPs have been reported to have strong effect on AMPAR kinetics. However, it has not been deeply studied how AMPAR-TARP stoichiometry can modulate this parameter with type Ib TARPs. The results obtained in this thesis shown that desensitization kinetics in 2-TARPed receptors with type Ib TARPs is significant slower compared to TARPless AMPARs (2.32 ± 0.16ms, 4.16 ± 0.41ms and 3.1 ± 0.23ms for 0 TARPs, 2 TARPs (γ 4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4) and 0 vs 2 TARPs (γ8); figure 36A). According to these results, steady state currents are also significantly enhanced in 2 TARPs condition (2.78 ± 1.38, 7.56 ± 1.06 and 16.14 ± 3.98 for 0 TARPs, 2 TARPs (γ 4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4)

and 0 vs 2 TARPs (γ8); figure 36B), contrary to 2 γ2 TARPed receptors (however it cannot be completely discarded). On the other hand, rise time kinetics show no significant differences when 2 type Ib TARPs are modulating AMPARs (0.46 ± 0.06ms, 0.43 ± 0.03ms and 0.44 ± 0.07ms for 0 TARPs, 2 TARPs (γ 4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4) and 0 vs 2 TARPs (γ8); figure 36C).

Besides, like in type Ia TARPs, the polyamine block is also released when 2 γ4 or γ8 TARPs are associated to the receptor. Interestingly, the values for RI are similar to 4 TARPed AMPARs with γ2 TARP (0.06 ± 0.004, 0.26 ± 0.05 and 0.27 ± 0.05 for 0 TARPs, 2 TARPs (γ 4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4) and 0 vs 2 TARPs (γ8); figure 36D). This modulation of polyamine block by TARPs seems to be a sensible parameter as is always modulated by the presence of this auxiliary subunit.

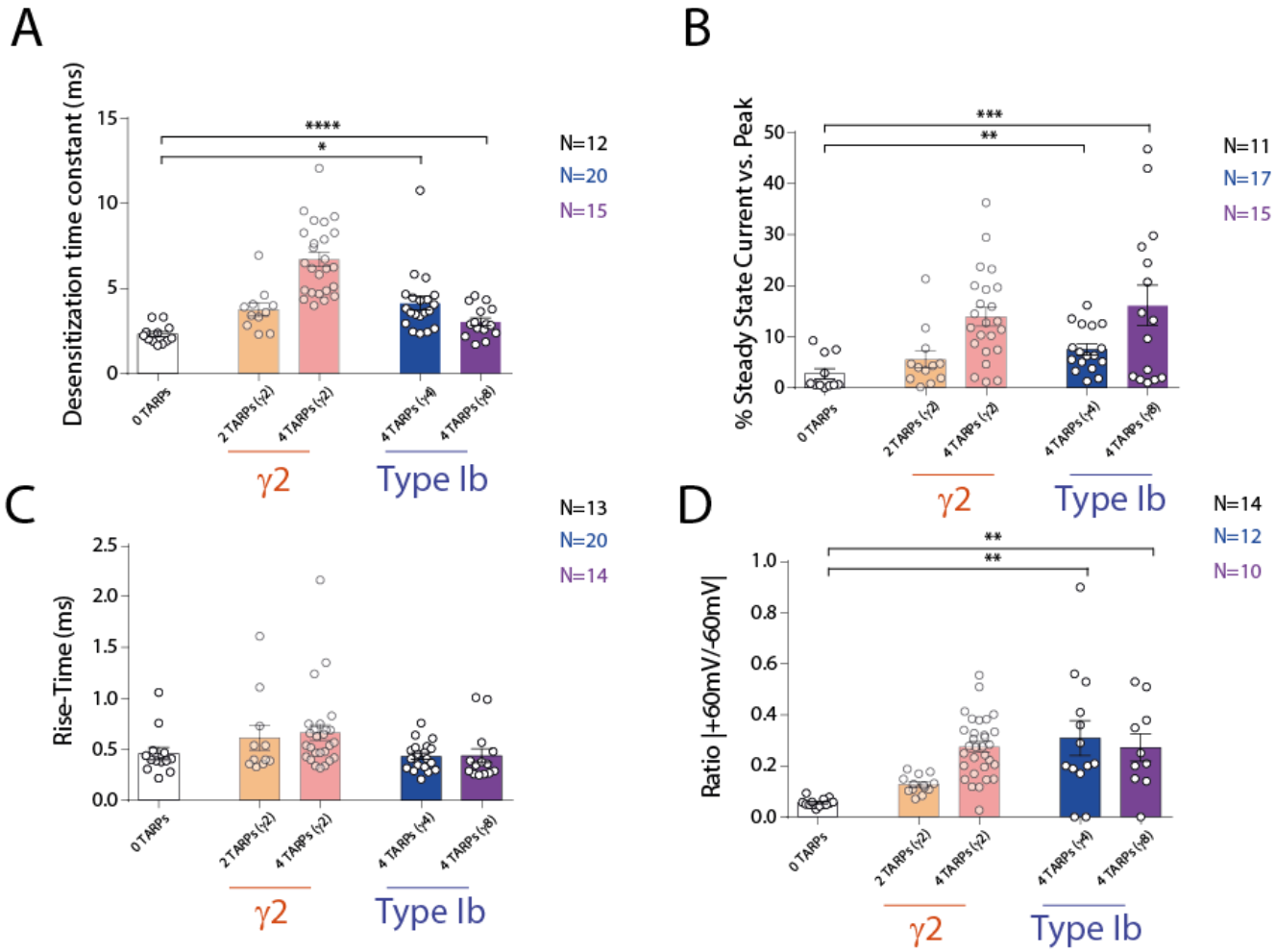


Figure 36. AMPAR-TARP-stoichiometry with type Ib TARPs modulates AMPAR kinetics and polyamine block. (A-B) AMPARs with 2 type Ib TARPs show slow desensitization kinetics and higher steady state currents than TARPless AMPARs. (C) Rise time kinetics are not modulated by any AMPAR-TARP stoichiometry or TARP type. (D) Polyamine block is also released by the presence of 2 γ4 or γ8 TARPs in AMPAR.

11. Type Ib TARPs speed recovery from desensitization

As the other TARP combinations, γ4 and γ8 TARPs speed AMPAR recovery from desensitization. However, different from type Ia TARPs, type Ib TARPs modulate AMPAR recovery with 2 TARPs per AMPAR in a clear manner with statistical significance (251.98 ± 22.70ms, 171.93 ± 17.1ms and 65.99± 6.97ms for 0 TARPs, 2 TARPs (γ 4) and 2 TARPs (γ8) respectively; unpaired T-test comparison for comparison 0 vs 2 TARPs (γ4) and 0 vs 2 TARPs (γ8); figure 37A). Indeed, in terms of mean values, 2 γ8 TARPed AMPARs shows the fastest recovery from desensitization recorded in these experiments with CP-AMPARs.

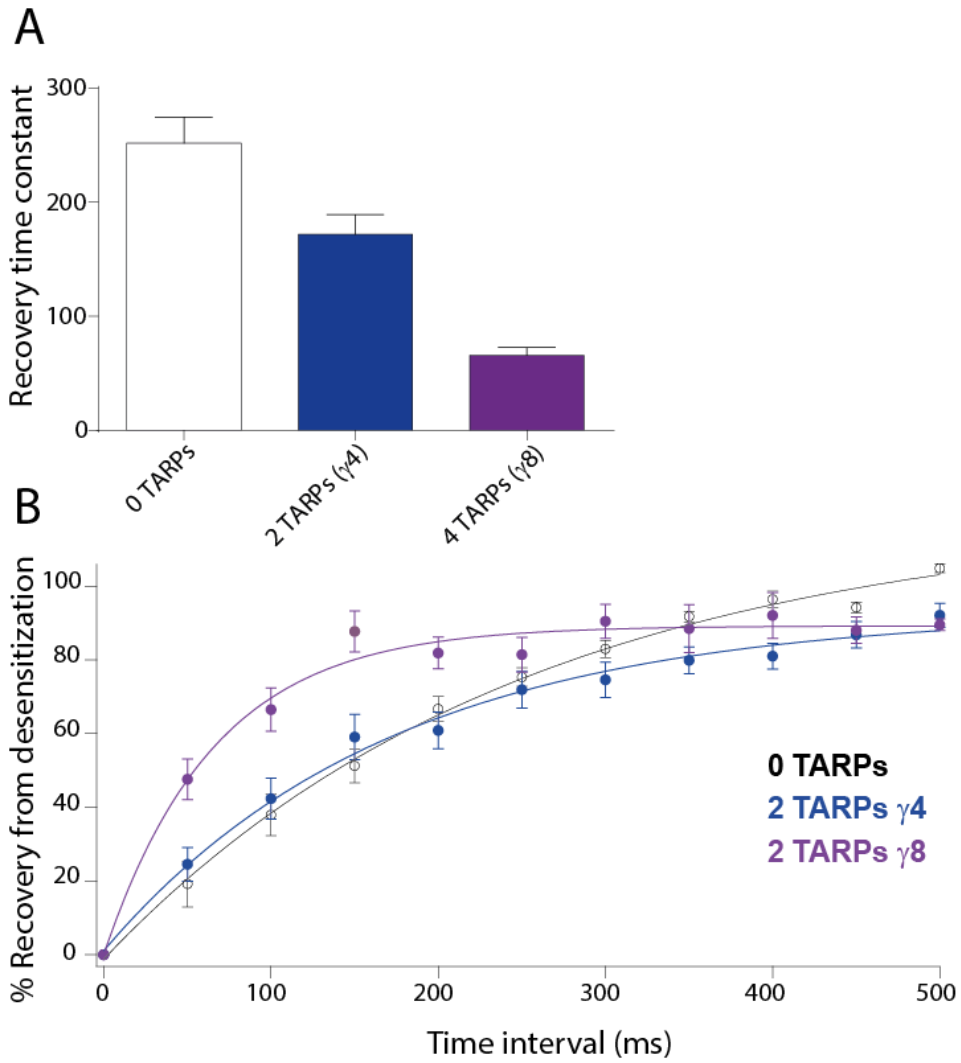


Figure 37. Type Ib TARPs speed recovery from desensitization. (A) Type Ib TARPs accelerate AMPAR recovery from desensitization in 1:2 AMPAR:TARP stoichiometry. In addition, TARP γ8 speeds this recovery more intensely than other TARPs. (B) Recovery from desensitization dynamics with a diminishment in the time needed to recover peak current with both type Ib TARPs.

AMPAR-TARP stoichiometry in CI-AMPARs

As explained, AMPARs display a great variety receptors depending on the subunits that

conform the channel, and CI-AMPARs (GluA2-containing AMPARs) constitute indeed the majority of AMPARs expressed in central nervous system. This AMPAR subtype is formed by a heteromeric combination of at least 2 different subunits and particularly exhibit low single-channel conductance besides the Ca^{+2} unpermeability. In terms of subunit structuration to form the receptor, GluA subunits are arranged in a particular form in CI-AMPARs, with GluA2 subunit positioned at pore distal region also called BD positions (Herguedas et al. 2019). At this receptors, auxiliary subunits are positioned at preferentially different places in the tetramer structure (Zhao et al. 2016). This fact implies differential organization of auxiliary subunits and therefore variable effect on their modulation over the AMPAR (reviewed at Chen and Gouaux 2019). On the other hand, another distinctive property of CI-AMPARs is that they are not blocked by endogenous polyamines at depolarized membrane potentials. In this set of experiments, it was taken advantage of this property to check stoichiometries of 2 TARPs per AMPAR measuring the RI. If an AMPAR population from a recording showed at least an RI of 0.7, the population of receptors recorded was considered as mainly CI-AMPARs. This would means that AMPARs present at membrane had GluA2-GluA1/3/4; however, lower RI means a significant mixture population with GluA2-GluA1/3/4 heteromers and GluA1 (for example) homomers.

The idea to explore CI-AMPARs was due to a two major reasons. First, to record responses and analyse biophysical properties in structurally different AMPARs type different as the GluA1 homotetrameric AMPARs studied (Beatriz Herguedas et al. 2016; Zhao et al. 2016). Second, to compare these results obtained in tsA201 cells with a more physiological system as a neuronal type later on. The neuronal type chosen for that purpose was the CGCs. This cell type offered a suitable model to study AMPAR-TARP stoichiometry due to their limited variety of GluA subunits and AMPAR auxiliary subunits expressed. CGCs express mostly GluA2 and GluA4c (short form of GluA4) and γ2 but not CNIHs (CGCs express also γ7 but is not determinant in CI-AMPARs expression (Studniarczyk et al. 2013)).

To analyse AMPAR-TARP stoichiometries in CI-AMPARs, the different stoichiometries stablished were also 0, 2 and 4 TARPs per AMPAR. However, in the 2 TARPs per AMPAR

it was distinguished if γ2 was linked to GluA2 or to GluA4c (figure 38).

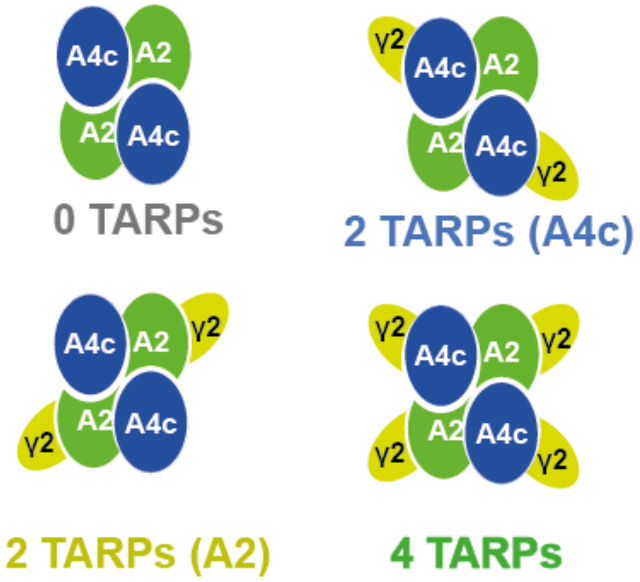


Figure 38. Different AMPAR-TARP combinations used for AMPAR-TARP stoichiometry experiments in CI-AMPARs.

12. GluA4c characterization

As mentioned, the combination of AMPAR subunits chosen for experiments in CI-AMPARs was GluA2-GluA4c since these two are the major AMPAR subunits in CGCs. As GluA4c is a short isoform from GluA4 that has not been deeply studied, after its cloning from rat cerebellum, it was decided to explore the possible differences in biophysical properties compared with GluA4. TsA201 cells were transfected with plasmid vector codifying for GluA4 or GluA4c to record from homotetrameric assemblies. None of the biophysical properties studied showed significant differences. In terms of kinetics it was not seen differences between homomeric GluA4 or GluA4c AMPARs (3.74 ± 0.49ms, 3.97 ± 1.33ms for GluA4 and GluA4c respectively; student's t-test; figure 39A). On the other hand, both CP-AMPARs showed no differences when measuring polyamine block (0.06 ± 0.03, 0.08 ±0.02 for GluA4 and GluA4c respectively; Mann-Whitney test; figure 39B). In addition, using NSNA, values for single-channel conductance and peak open probability obtained showed also no differences between both types of AMPARs (for single-channel conductance 16.63 ± 1.06pS, 17.54 ± 1.21pS for GluA4 and GluA4c

respectively; Mann Whitney test; figure 39C and for peak open probability 0.55 ± 0.1; 0.38 ± 0.09 for GluA4 and GluA4c respectively; student's t-test; figure 39D).

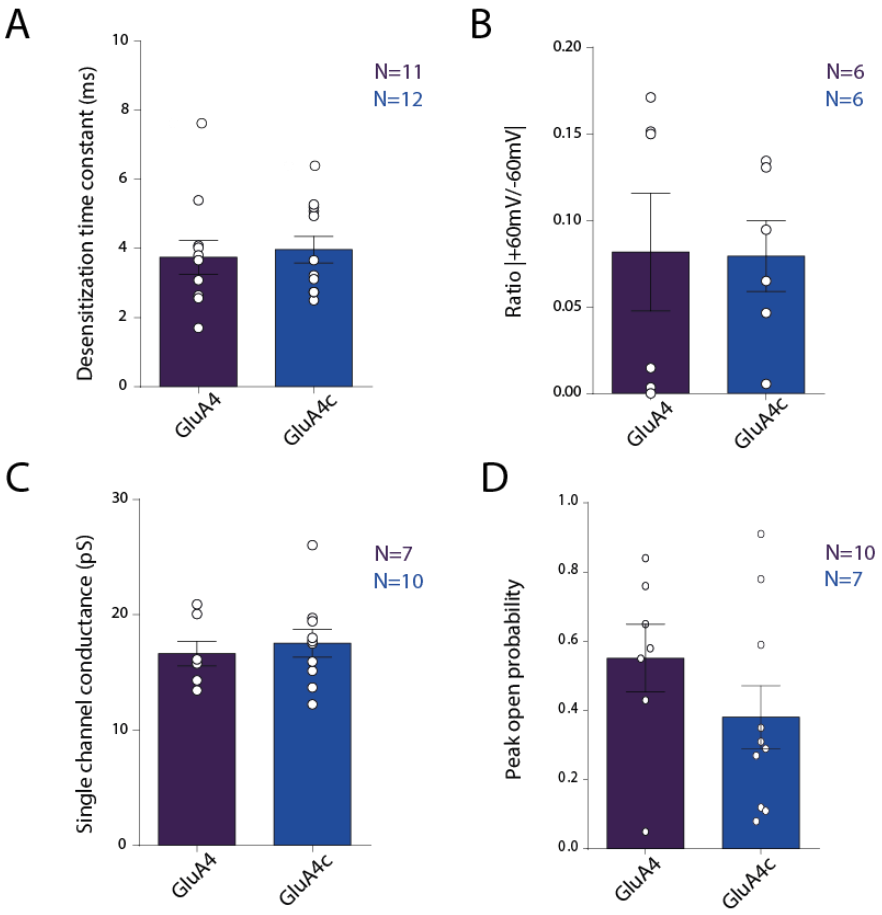


Figure 39. GluA4 and GluA4c homotetramers shows no differences in the biophysical properties analysed. (A-D) Pooled data showing results obtained from analysing 4 different biophysical properties of AMPARs. No significant differences were detected between these two different homotetramers.

13. The importance of TARP location rather than TARP quantity

Different from CP-AMPARs, the results obtained with CI-AMPARs (using GluA2-GluA4c) showed that some biophysical properties of these subtype of AMPARs are conditioned mostly by the subunit where γ2 is linked instead of the amount of TARP per receptor. Mentioned in the introduction, a very well-known property of TARP auxiliary subunits is to slow AMPARs kinetics what implies promoting open states of receptor. However, in these CI-AMPARs made by the combination of GluA2 and GluA4c subunits, receptors desensitization kinetics have been maintained unmodified, with no significant differences, between TARPless AMPAR and 2 TARP condition with γ2 linked to GluA2. By

contrast, when γ2 was linked to GluA4c subunit, desensitization time constant increased in 2 TARPs (A4c) and 4 TARPs condition (GluA2:γ2-GluA4c:γ2; 4T) (4.76 ± 0.28ms, 7.23 ± 0.43ms, 5.42 ± 1.31ms, 8.43 ± 0.61ms for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2) and 4 TARPs respectively; one-way ANOVA; figure 40B). Thus, desensitization kinetics behaviour in these CI-AMPARs is apparently not changed by γ2 unless the TARP is attached to GluA4c subunit. Concerning the activation time to reach the peak current (rise time), it was not noticed any variation amongst the different combinations tested (0.47 ± 0.05ms, 0.49 ± 0.07ms, 0.46 ± 0.1ms, 0.39 ± 0.04ms for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2) and 4 TARPs respectively; Kruskal-Wallis multiple comparison test; figure 40C).

In addition, other kinetics properties of the receptors as their recovery from desensitization were analysed. Strikingly, when γ2 was linked to GluA2 subunit the recovery from desensitization was differentially slowed in comparison to other conditions. 4 TARPed CI-AMPARs also performed slow recovery from desensitization when compared to 0 ant 2 TARPs (A4c); however, the effect was more pronounced in 2 TARPs (A2) whose recovery was the slowest (51.35 ± 8.18ms, 60.62 ± 4.73ms, 143.75 ± 2.72ms, 107.19 ± 9.29ms for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2) and 4 TARPs respectively; Kruskal-Wallis multiple comparison test; figure 40G). That significant effect in slow recovery when γ2 is linked to GluA2 is probably countered when the auxiliary subunit is linked to GluA4c as 4 TARPs condition showed intermediated values.

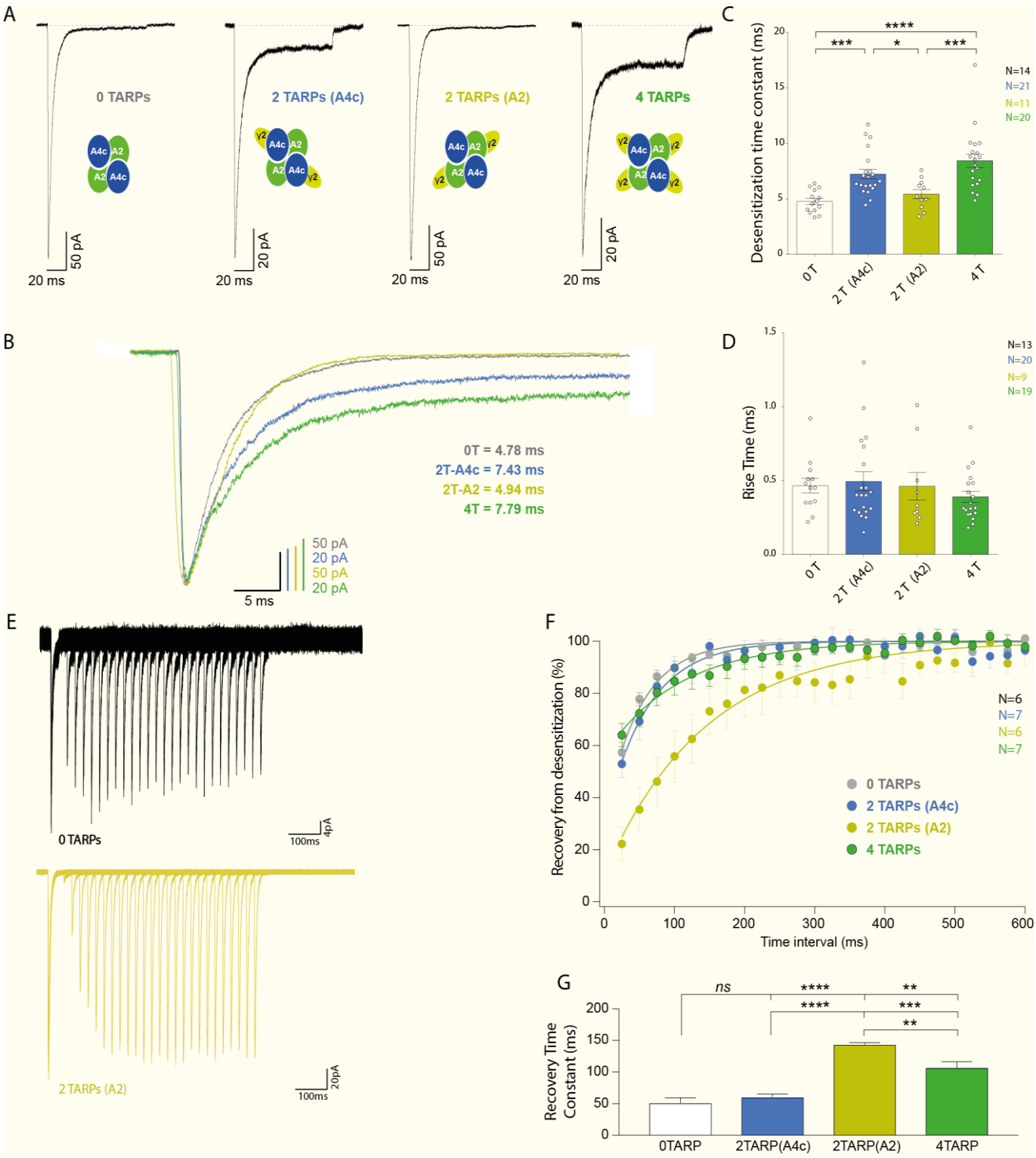


Figure 40. CI-AMPAR kinetics differentially modulated by γ2 linkages to GluA subunits. (A) Representative traces of currents at −60 mV from cells expressing CI-AMPARs without or with TARP γ2 linked to GluA subunits. Under the traces a scheme of the subunits forming the receptors with γ2 associated to different AMPAR subunits is shown. (B) Peak-scaled normalization from traces shown in A for a better comparison of desensitization kinetics. (C) Weighted time constant of desensitization (τw,des) where is clear that desensitization is slowed only when γ2 is linked to GluA4c subunit. (D) Rise time of the current activation is not changed by the AMPAR-TARP stoichiometry. (E) Representative traces monitoring recovery from desensitization for CI-AMPAR in cells expressing 0 TARPs or 2 TARPs linked to GluA2 subunit

where it is manifest the difference between the two conditions. (F) Recovery of desensitization kinetics showing a relatively slow recovery only in 2-TARPed (located in GluA2) CI-AMPARs. (G) Recovery time constant values for the experiments showed in E and F.

Moving to other properties analysed, by means of NSNA we obtained values for single-channel conductance and peak open probability and results that reinforce the idea that γ2 modulated differentially depending on the subunit it was linked to. In terms of peak open probability as seen before in other experiments, no differences were seen at any condition (0.48 ± 0.05, 0.46 ± 0.05, 0.52 ± 0.08, 0.58 ± 0.04 for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2) and 4 TARPs respectively; one-way ANOVA; figure 41D). However, looking at single-channel conductance it is observed once again that γ2 exerts different modulation depending on the subunit where it is linked. At all different stoichiometries tested γ2 significantly increases single-channel conductance compared to TARPless AMPARs; nevertheless, when the auxiliary protein is linked to GluA2 subunit this parameter sharply increases compared to TARPless and 2 TARPed (A4c) conditions. Interestingly, both stoichiometries of 2 TARPed AMPAR present an increase in single-channel conductance but there is not a summative effect in 4 TARPs condition. Indeed, 4 TARPed AMPARs show slightly lower single-channel conductance than 2 TARPs (A2) condition, although there is not significantly difference (5.13 ± 0.05pS, 9.72 ± 1.09pS, 15.85 ± 1.62pS, 12.57 ± 1.12pS for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2) and 4 TARPs respectively; one-way ANOVA; figure 41E).

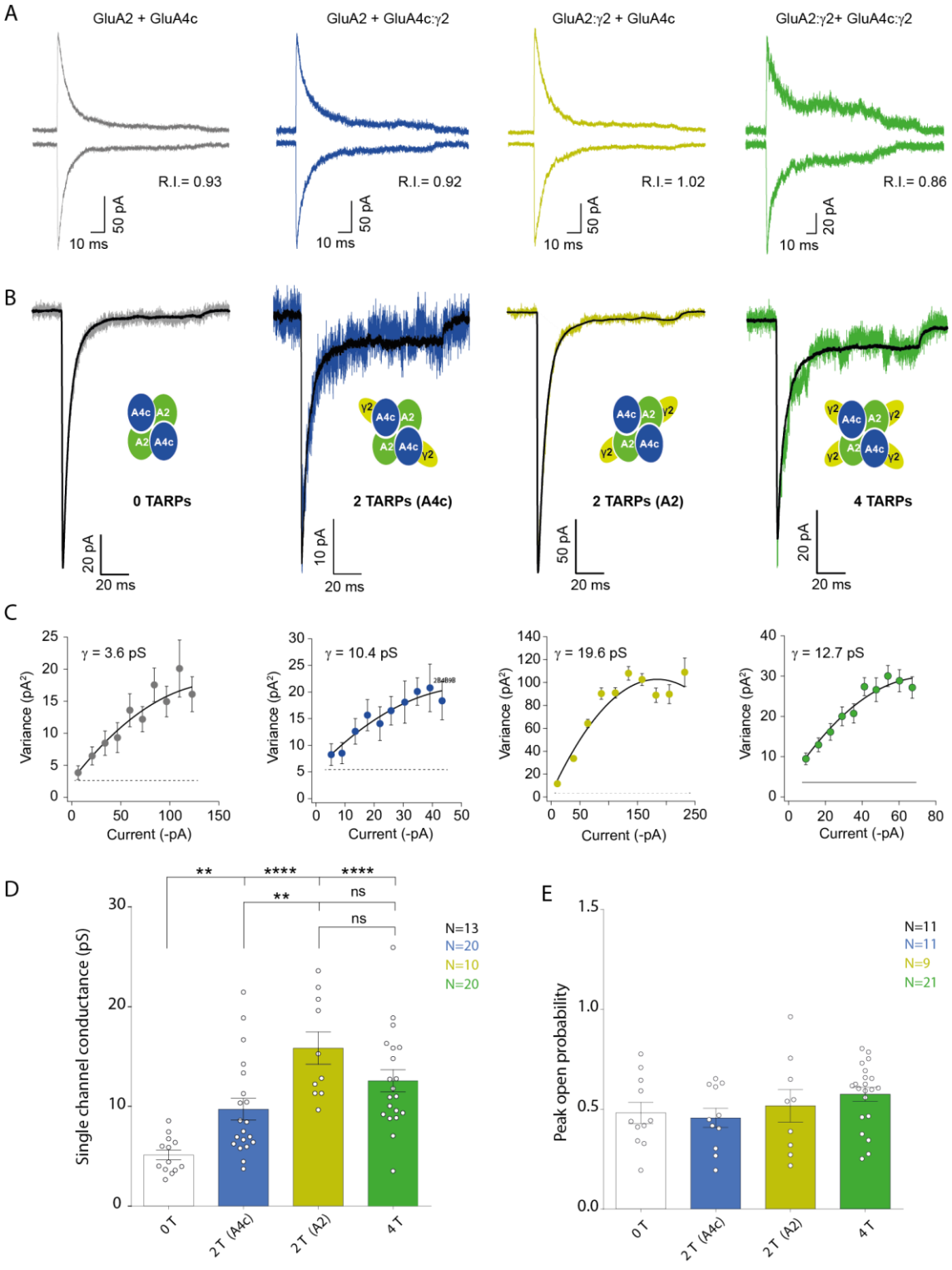


Figure 41. γ2 differentially modulates single-channel conductance depending on their position in AMPAR complex. (A) Evoked currents by rapid application of 10 mM glutamate from membrane patches at +60 mV (upward traces) and −60 mV (downward traces) with their corresponding RI. (B) Average traces of current responses evoked at −60 mV used for NSNA shown in black overlaid with a representative single response. Insets show the studied combination. (C) Current-variance plots for the recordings shown

in B, with the weighted single-channel conductance for the single recordings. (D) Pooled data showing a distinct degree in single channel conductance increase when γ2 is present into the AMPAR complex. (E) Pooled data for peak open probability of CI-AMPARs, where no effect of TARP stoichiometry was evident.

14. γ2 position in AMPAR complex account for changes in receptor pharmacology

A previous study by Roger Nicoll's group revealed that AMPAR efficiency to the partial agonist kainate is enhanced as the number of TARPs in the AMPAR complex increase (Yun Shi et al. 2010). For this reason, in this thesis work we wondered whether perampanel, a non-competitive inhibitor of AMPARs used in the clinic for the treatment of seizures (indeed the first AMPAR antagonist to receive regulatory approval (Hanada et al. 2011), would vary its blocking effect depending on the number of TARPs present on AMPARs. To address this question, it was rapidly applied the antagonist in a set of experiments where whole-cell currents were activated in transfected tsA201 held at −60 mV with 100 µM AMPA plus 50 µM cyclothiazide to avoid desensitization. The outcome of those experiments did show the same pattern as the one found for desensitization: TARP γ2 modified the percentage of block only when it was attached to GluA4c subunit. CI-AMPARs with 2 TARPs at the GluA2 subunit displayed a similar block as TARPless CI-AMPARs. However, the block by perampanel in a 2-TARPed at GluA4c and in a 4-TARPed CI-AMPARs was higher than for TARPless receptors (47.33 ± 5.95; 70.65 ± 8.06; 46.13 ± 5.47; 71.64 ± 6.76; for 0T, 2T(A4c), 2T(A2) and 4T conditions respectively, Student's test; figure 42B).

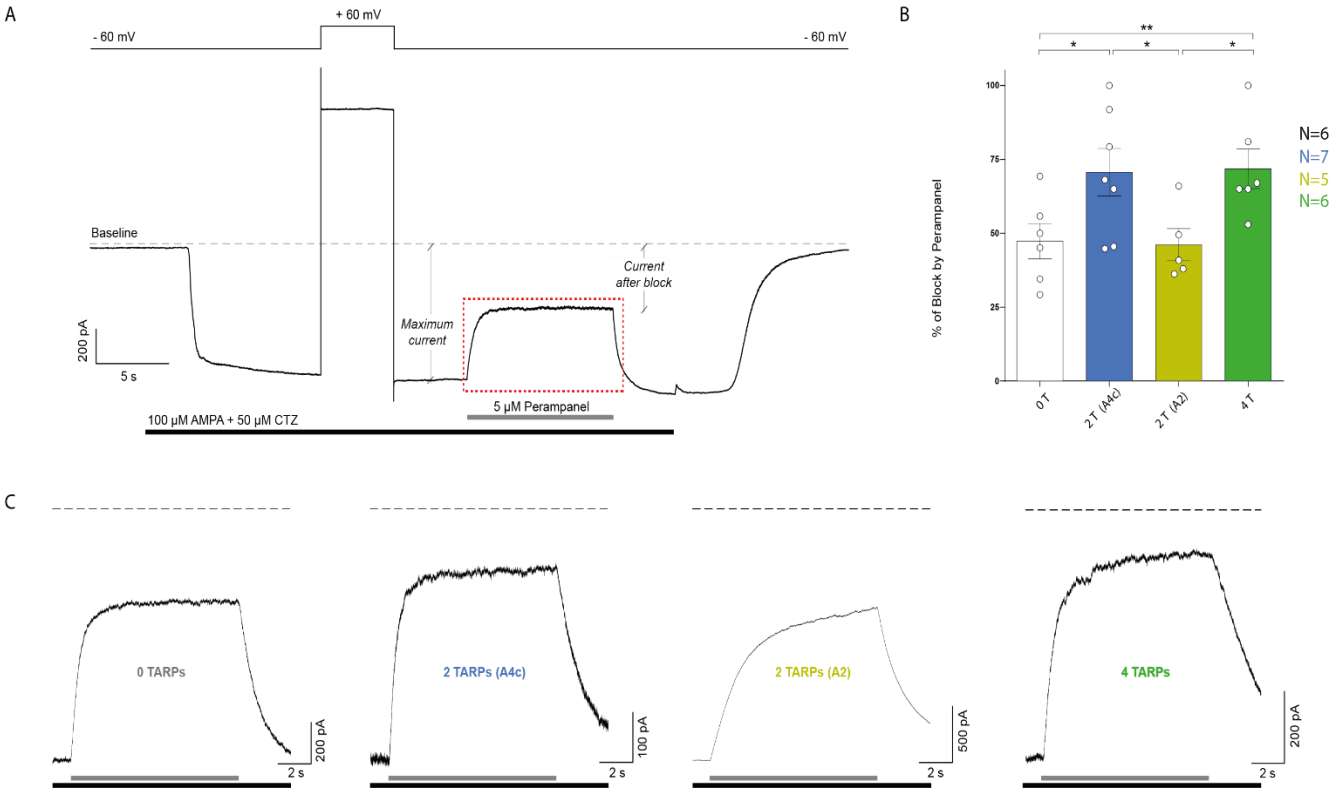


Figure 42. Perampanel block depends on AMPAR-TARP combination. (A). Representative whole-cell recording showing the protocol used to determine the percentage of perampanel block. This trace corresponds to a cell expressing GluA2 and GluA4c. Cells were clamped at -60mV during the recording with a 5s pulse duration at +60mV to assess for GluA2 presence. Black bar shows application of AMPA 100mM + Cyclothiazide 50µM to tsA201 cells expressing different AMPAR-TARP combinations. Grey bar shows application of Perampanel at 5µM, dashed line shows baseline current. Maximum current after block are displayed. The dashed red frame represents the magnification part shown in panel C. (**B**) Pooled data for the percentage of blocking by Perampanel. (**C**) Representative traces of Perampanel blocking from each condition. Under each trace the black shows AMPA + CTZ and grey bar shows application of Perampanel.

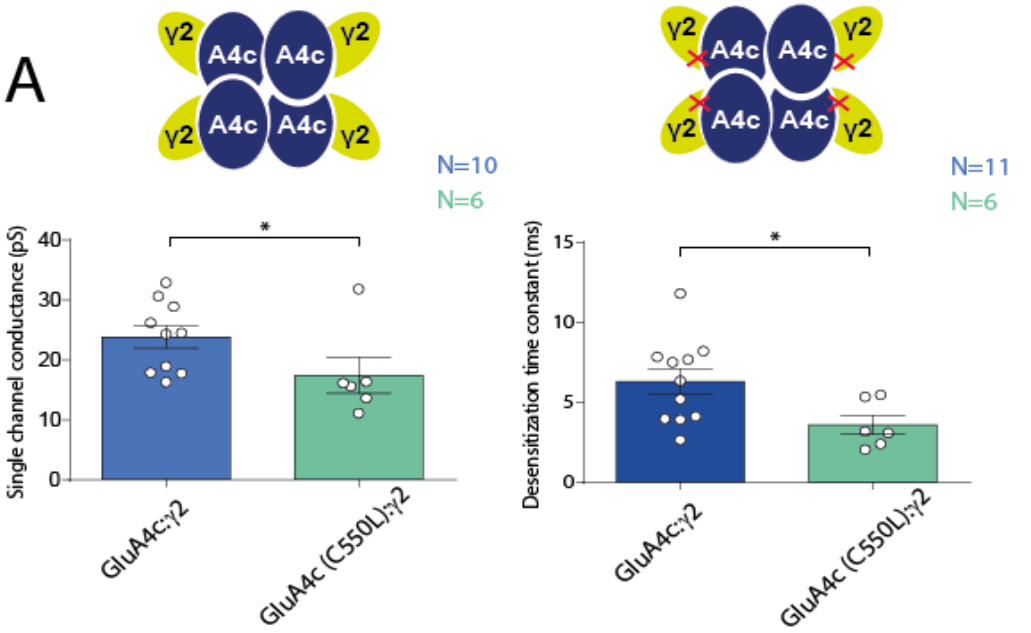
15. γ2 position in the AMPAR complex

Results obtained with CI-AMPARs shows that γ2 differentially modulated AMPAR properties depending on the subunit where it was linked; potentially exerting its modulation at different regions of the AMPAR complex. Indeed, it was mentioned before that in CI-AMPARs there is an asymmetry of GluA subunits positioning (Beatriz Herguedas et al. 2016; Zhao et al. 2016) and that TARP the modulation is not limited to concrete GluA subunit but to intermediate region between subunits (Beatriz Herguedas

et al. 2019a). For these reasons, it was tried to analyse were potentially γ2 was modulating. To study that, we decided to perform the cysteine to lysine acid change at 549 and 550 positions, for GluA2 and GluA4c subunits respectively, which is known that abolishes TARP effect over AMPAR desensitization kinetics as it has been described for CP-AMPARs (Hawken, Zaika, and Nakagawa 2017). Importantly, this amino acid change at TMI region of GluA subunits allows to maintain TARP physical interaction with AMPAR subunit as it does not split AMPAR-TARP complex. The same type of experiments performed by Natalie Hawken and colleagues analysing AMPAR were done to check if it was possibly to replicate the same effect it was used GluA4c to form homotetramers instead of GluA2(Q) (not edited form of GluA2) as in Hawken's work. The same abolishment of γ2 effect in AMPAR desensitization kinetics was observed as time constant values for GluA4c:γ2 tetramers with C550L amino acid change (GluA4c (C550L):γ2) were significantly lowered (6.31 ± 0.8ms, 3.6 ± 0.6ms, for GluA4c:γ2 and GluA4c (C550L):γ2 respectively; Mann Whitney test; figure 43A). Strikingly, it was observed that single-channel conductance was also diminished in GluA4c (C550L):γ2 homotetramers being significantly lower than in GluA4c tetramers (23.84 ± 1.87pS and 17.46 ± 2.99pS, for GluA4c:γ2 and GluA4c (C550L):γ2 respectively; Mann Whitney test; figure 43A). This indicates that C550L amino acid change speeds desensitization kinetics and lows single-channel conductance as γ2 exerts some modulation for these properties acting over TMI of CP-AMPARs.

Once confirmed the abolishing effect of CL mutation, it was tested the same idea in CI-AMPARs to investigate the putative subunit in which the TARP was acting. To assess this question site directed mutagenesis were performed on plasmids codifying for GluA2:γ2 and GluA4c:γ2 (C549L and C550L amino acid changes respectively). The results obtained in CI- AMPAR were different as the obtained in CP-AMPARs. Recordings in 2 TARPs stoichiometry with γ2 linked to GluA2 (C549L) showed no changes in desensitization kinetics (which was not modified in 2T A2 condition compared to TARPles AMPAR) (5.42 ± 0.4ms, 5.65 ± 0.57ms, for 2T A2 and 2T A2 (C549L) respectively; student t-test; figure 43B) or single-channel conductance (15.85 ± 1.62ms, 15.29 ± 2.89ms, for 2T A2 and 2T A2 (C549L) respectively; student t-test; figure 43B). On the other hand, when γ2 was

linked to GluA4c in 2 TARP stoichiometry there was no significant differences looking at desensitization kinetics between the presence of GluA4c or GluA4c (C550L) in the AMPAR (7.23 ± 0.43ms, 7.16 ± 0.44ms, for 2T A4c and 2T A4c (C550L) respectively; student t-test; figure 43C). However, surprisingly, single-channel conductance was diminished like in TARPless AMPAR when GluA4c (550L) was present in the receptor linked to γ2 (9.72 ± 1.09pS, 5.94 ± 0.71pS, for 2T A4c and 2T A4c (C550L) respectively; student t-test; figure 43C). The obtained results show that in CI-AMPARs the C550L amino acid change abolishes γ2 effect over single-channel conductance by eliminating TARP interaction with TMI of GluA4c. Surprisingly it is not observed the same with GluA2 subunit reinforcing the idea of the asymmetric disposition of GluA subunits and the differential TARP modulation depending on their position in the receptor complex.


A
γ2
A4c
A4c
γ2
γ2
A4c
A4c
γ2
N=10
N=6
Single channel conductance (pS)
40
30
20
10
0
*
GluA4c:γ2
GluA4c (C550L):γ2
N=11
N=6
Desensitization time constant (ms)
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10
5
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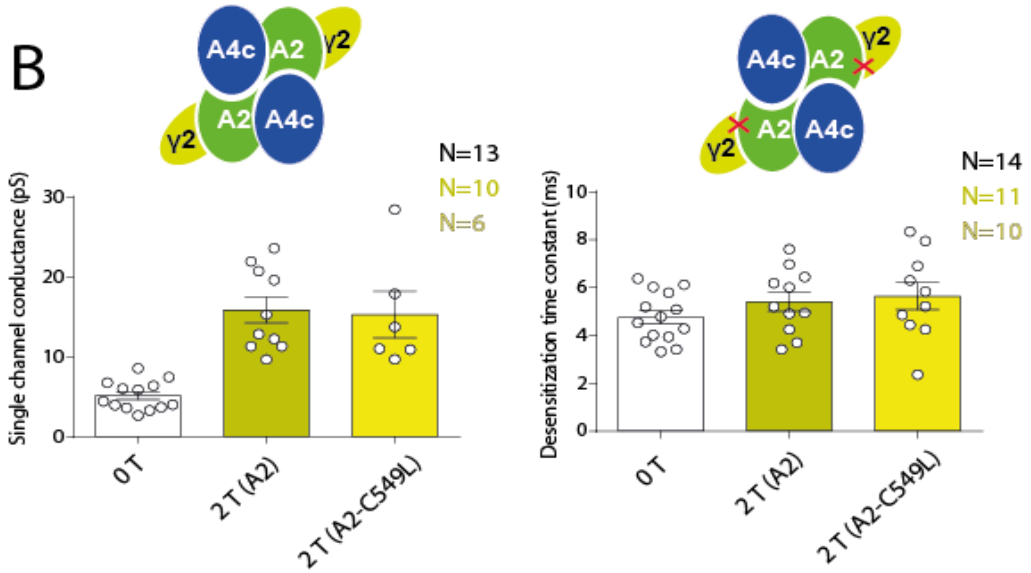
GluA4c:γ2
GluA4c (C550L):γ2


B
A4c
A2
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N=13
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N=6
Single channel conductance (pS)
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2 T (A2)
2 T (A2-C549L)
N=14
N=11
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Desensitization time constant (ms)
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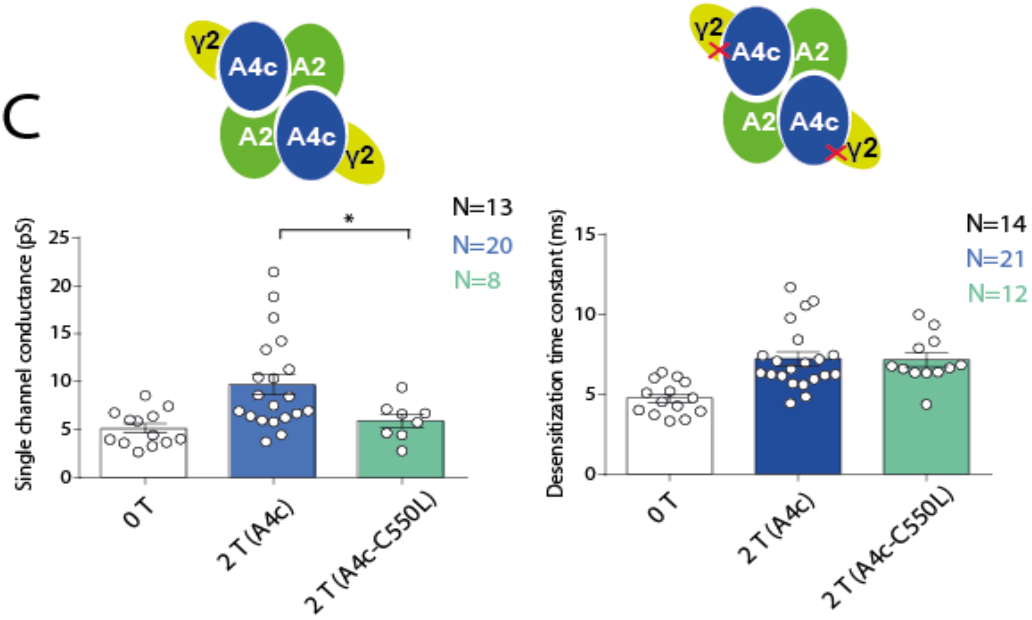
2 T (A2-C549L)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γ2
A4c
A2
A2
A4c
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N=13
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N=8
Single channel conductance (pS)
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2 T (A4c-C550L)
N=14
N=21
N=12
Desensitization time constant (ms)
15
10
5
0
0 T
2 T (A4c)
2 T (A4c-C550L)

43. Effect of C549L and C550L AMPAR amino acid changes over AMPAR-TARP modulation. (A) Pooled data showing the effect C550L change in GluA4c homotetramers in decreasing the weighted time constant of desensitization (τw, des) and single channel conductance. (B-C) C549L and C550L in GluA2 and GluA4c respectively subunits have no effect in time constant of desensitization (τw, des) in 2 TARPed CI-AMPARs. In addition, C549L change in GluA2 subunit in CI-AMPARs shows no differences in single-channel conductance compared with 2T(A2) CI-AMPARs. However, C550L in GluA4c (2T A4c (C550L) shows a decrease in single-channel conductance compared with 2T (A4c) CI-AMPARs.

16. AMPAR TARP stoichiometry in CGCs soma

One of the principal ideas of this thesis project was to study AMPAR-TARP stoichiometry in a neuronal type. For this reason, it was tried to correlate results obtained in expression systems with a more physiological model. Using expression systems and fusion proteins it was possible to fix stoichiometries of 2 and 4 TARPs per AMPAR as it was mentioned before. To do that correlation, first it was chosen a neuronal type with a limited range of GluA and AMPAR auxiliary subunits expression, to diminish the number of possible combinations. At this aspect, mentioned in the previous sections, CGCs offered a suitable model to perform this correlation as they express mostly GluA2/4c AMPARs and TARP γ2 and γ7 (Fukaya et al. 2005). Interesting, while γ2 have been demonstrated to be essential for CI-AMPAR expression at this cell type (L. Chen et al. 2000), the role of γ7 TARPs does not seem to be crucial (indeed the opposite) in CI-AMPARs expression at CGCs (Studniarczyk et al. 2013). In addition, at this neuronal type auxiliary proteins CNIHs have not been reported to be present.

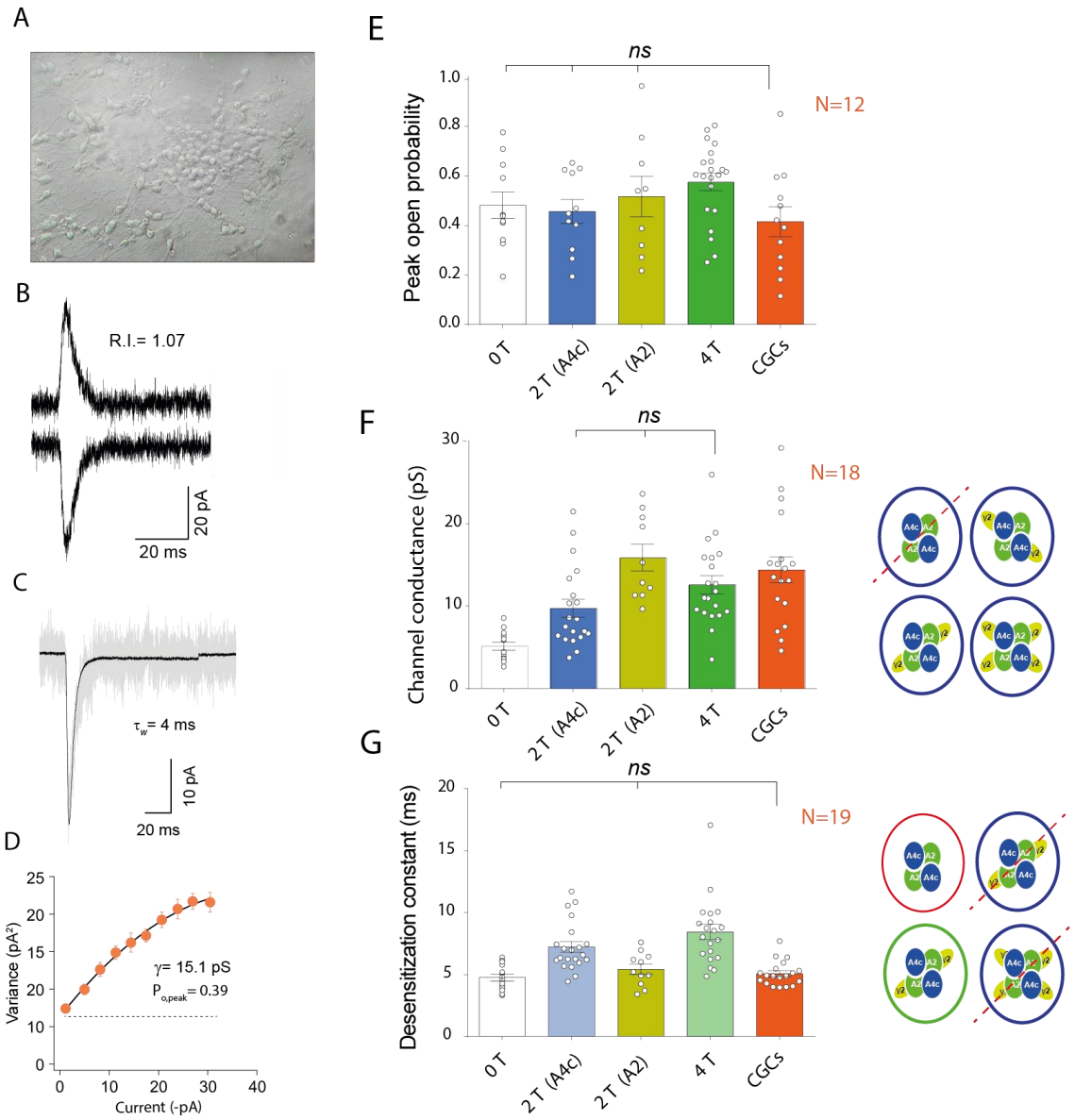
In this set of experiments currents from somatic outside-out patches of CGCs were recorded using AMPA 100μM as agonist instead of glutamate to avoid activate KARs (in addition NMDARs but they were mostly blocked by Mg^{+2} present in recordings solution). Desensitization kinetics and single-channels conductance were the most differing parameters in the different AMPAR-TARP combinations recorded in expression systems and these parameters were used to compare recordings in CGCs with tsA201 cells. However, looking at peak open probability first as this parameter did not shown

significant differences in previous experiments, no differences were seen between CGCs and the other conditions recorded in tsA201 cells expressing CI-AMPARs (0.48 ± 0.05, 0.46 ± 0.05, 0.52 ± 0.08, 0.58 ± 0.04, 0.42 ± 0.06 for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2), 4 TARPs and CGCs respectively; one-way ANOVA; figure 44E). In terms of single-channel conductance CGCs showed a significant increment compared to TARPless condition. In addition, values were slightly higher than in 4T condition but significantly different as it occurred with 2T (A2) condition (5.13 ± 0.05pS, 9.72 ± 1.09pS, 15.85 ± 1.62pS, 12.57 ± 1.12pS, 14.39 ± 1.56pS for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2), 4 TARPs and CGCs respectively; one-way ANOVA; figure 44F).

When we looked at desensitization kinetics of CGCs, in this neuronal type kinetics recorded showed low desensitization time constants. The values obtained exhibit fast kinetics and results did not differ significantly from the ones obtained for 0T and 2T (A2) (4.76 ± 0.28ms, 7.23 ± 0.43ms, 5.42 ± 1.31ms, 8.43 ± 0.61ms, 5.07 ± 0.23ms for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2), 4 TARPs and CGCs respectively; one-way ANOVA; figure 44G).

Summarizing, in CGCs AMPAR-evoked responses showed a CI-AMPAR with high single-channel conductance and fast desensitization kinetics as in 2T (A2) condition. Altogether indicates that the condition with 2 γ2 linked to GluA2 is the one that better replicates

the AMPAR behaviour in CGCs for the parameter analysed.



44. Currents from somatic CGCs exhibit properties of GluA2:γ2 + CluA4c CI-AMPARs. (A) CGCs in culture after 7 days in vitro. (B) Traces at +60 mV and −60 mV evoked with 100 µM AMPA from a CGC somatic patch showing the typical lineal response of a CI-AMPAR. (C) Representative response of current evoked at −60 mV by rapid application of 100 µM AMPA to somatic patches from CGCs. Grey: representative single response; black: average response. (D) NSNA from the recording in C. (E) Comparison of peak open probability values from CGCs were no significant differences from recordings in cell lines was observed. (F) Data showing comparison of single channel conductance values obtained in CGCs (orange bar) with recordings from transfected cell lines. The conductance values obtained resembled (without significant difference) to the ones seen with 2T or 4T conditions (marked in bold). Under the panel it is shown the possible AMPAR-TARP combinations surrounded by blue circle. Dashed red line shows the combination discarded (0T) because exhibits significant differences compared with CGCs. (G) Comparison of data from desensitization time constant (ms) in CGCs with recordings in tsA201 cells. The results are no significantly

different from conditions with 0T or 2T(A2). Under the panel the possible combination A2 (2T) circled in green as the one that did not differ significantly from CGCs recordings in both comparisons. (H) Representative trace from two-pulse protocol monitoring recovery from desensitization for CGCs somatic patches to 100 µM AMPA application.

To end the comparison between expression systems and CGCs it was studied the response in the recovery from desensitization. Here, AMPAR currents in CGCs exhibit a significant exceptional slow recovery compared to other with responses recorded in tsA201 cells. Indeed, recovery from desensitization recorded in CGCs was slower significantly than in 2T (A2) condition which was the slowest recorded in expression systems (51.35 ± 8.18ms, 60.62 ± 4.73ms, 143.75 ± 2.72ms, 107.19 ± 9.29ms, 397.38 ± 127ms for 0 TARPs, 2 TARPs (A4c), 2 TARPs (A2), 4 TARPs and CGCs respectively; Kruskal-Wallis multiple comparison test; figure 45B).

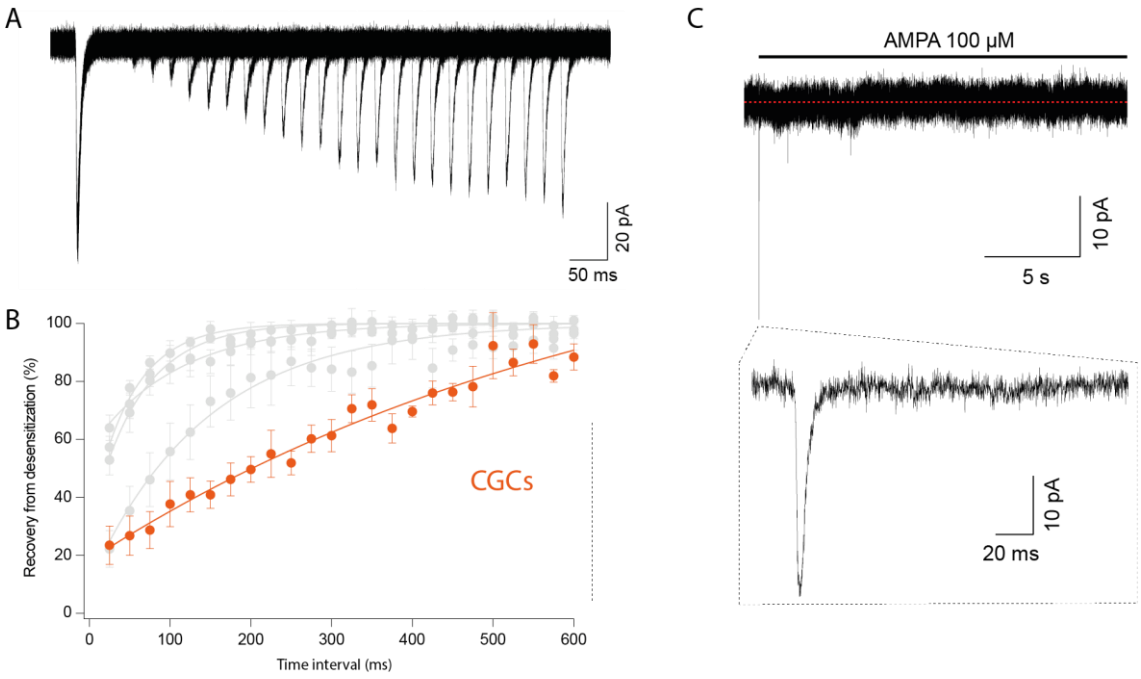


Figure 45. Recovery from desensitization in CGC is slower than in 2T(A2) condition. (A) Representative trace from two-pulse protocol monitoring recovery from desensitization for CGCs somatic patches to 100 µM AMPA application. (B) Recovery from desensitization kinetics of CGS somatic AMPARs compared with recoveries of GluA2:GluA4c combinations shown in Figure 6F (in grey). (C) Representative response to a 100 µM AMPA application for 20 s in a somatic patch from CGCs to test for the presence of γ7. No re-sensitization of the receptors is observed in the trace. Inset: magnification of 200 ms showing the initial fast desensitizing response.

That arise the question if this response can be an artefact due to the use of AMPA as agonist instead of glutamate (*see discussion section*). However, using AMPA as agonist to repeat recordings over 2T (A2) condition there were no significant differences with the responses recorded using glutamate as agonist ((143.75 ± 2.72ms 141.93 ± 43.6 ms, using glutamate and AMPA as agonist in 2T (A2) condition; Mann-Whitney test; figure 46). Discarding the possibility that AMPA was the cause of the slower recovery in CGCs it was thought that another AMPAR auxiliary subunit might be present in the complex. Long exposure to agonist (AMPA) in CGCs somatic patches were performed to test the possibility for γ7 presence in the receptor as this TARP confers the receptor the capacity to resensitisize after long agonist exposure. However, no resensitizing effect was observed as the steady state current did not arise when agonist was applied along 20s (steady state current was near 1% of the peak current during long agonist application, n=4; figure 17J). In addition, the presence of γ7 in CGCs CI-AMPARs seems to be not favoured (Studniarczyk et al. 2013). On the other hand, the presence of a member of CKAMP family, CKAMP 39, was tested in expression system. It is known that this AMPAR auxiliary subunit is highly expressed in cerebellum and that can slow recovery from desensitization (Farrow et al. 2015b). For this reason, it was tried to record currents from outside-out patches of tsA201 cells co-transfected with GluA2:γ2 + GluA4c (2T (A2) condition) + CKAMP 39. However, it was not possible to record currents in outside-out patches or even whole-cell recordings with this combination of AMPAR-TARP-CKAMP co-expression. The possible reason for the lack of currents are discussed in the discussion part of the present thesis.

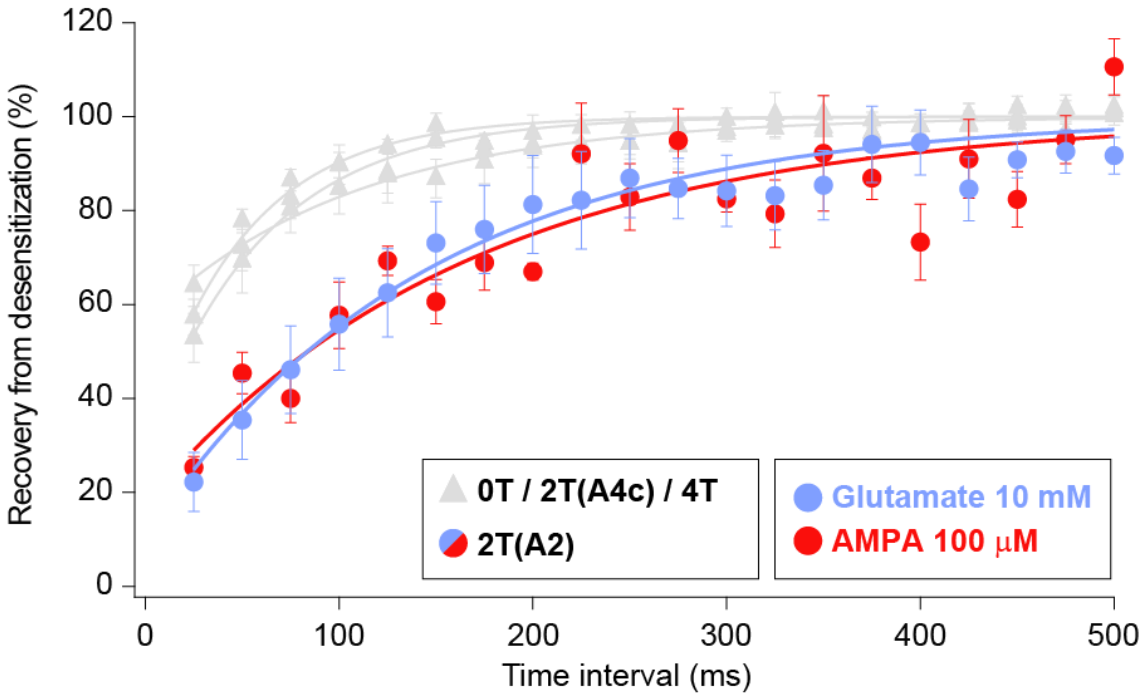


Figure 46. Recovery from desensitization using AMPA as agonist in 2T (A2) condition. Responses from membrane patches from tsA201 cells expressing the 2T (A2) AMPAR-TARP combination it was using AMPA as agonist did not differ from the ones obtained using glutamate. In grey 0T, 2T (A4c) and 4T conditions, coloured 2T (A2) using different agonists.

Exploring the role of CPT1C AMPAR-TARP stoichiometry

17. CPT1C and γ2 in AMPAR trafficking

As explained in the introduction, there are AMPAR interacting proteins that intervene AMPAR traffic to cell membrane (**5.2. Transiently AMPAR interacting proteins**) but do not modulate the receptors properties once it is delivered (different from auxiliary subunits). One of these proteins is CPT1C, a protein that selective enhances GluA1-containing AMPARs membrane traffic (Gratacòs-Batlle et al. 2014, 2018b). In addition, CPT1C along with FRSS1L display an important role priming AMPAR-TARP complexes before the receptor is able to exit from ER (Brechet et al. 2017b). For this reason, it was wondered whether CPT1C can display a synergic effect with γ2 enhancing AMPAR traffic when GluA1 subunit is already linked to the auxiliary protein. Recordings from

whole-cell responses expressing GluA1:γ2 + CPT1C and GluA1:γ2 + GFP (control plasmid that codifies only for GFP) where performed to compare maximum responses (normalized for membrane capacitance) from both conditions (figure 47A). The total current normalized for the membrane capacitance indicates indirectly the amount of receptors at cell membrane.

In this experiment no differences were observed in the magnitude of responses with or without co-expression with CPT1C (125.1 ± 23.99pA/pF, 124.9 ± 21.12pA/pF, for co-transfection with GFP and CPT1C respectively; Mann-Whitney test; figure 47B), meaning that this protein does not enhance AMPAR trafficking when GluA1 subunit is already saturated with γ2 (at least in transfected tsA201 cells in absence of other proteins as FRRS1L).

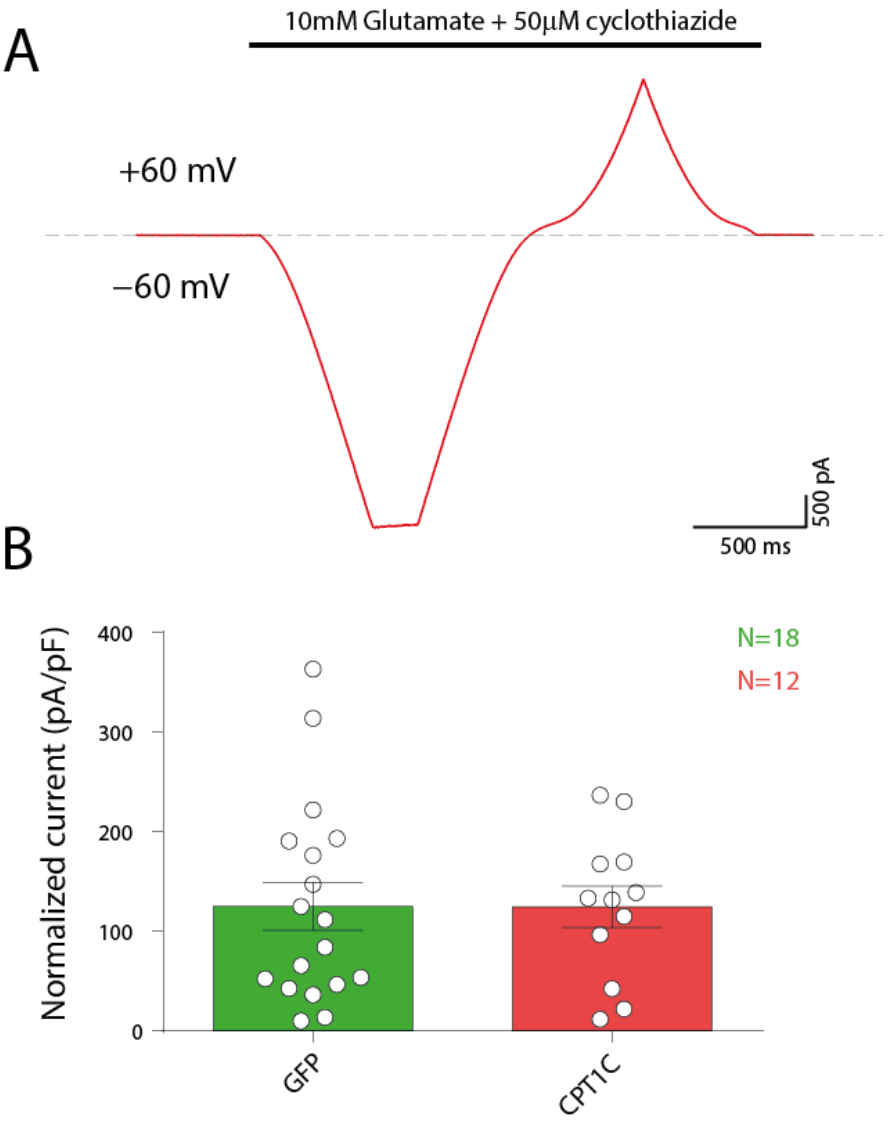


Figure 47. Normalized peak currents of GluA1:γ2 homotetramers are not enhanced by CPT1C. (A) Example of an average trace from whole-cell recording (red) applying 10mM glutamate and 50mM cyclothiazide to avoid AMPAR desensitization. Dashed line in black marks the baseline, over this line the membrane potential is +60mV and -60mV under the line. (B) Pooled date from whole-cell recordings showing normalized maximum currents by membrane capacitance. No differences were seen in absence or presence of CPT1C.

18. CPT1C in determining AMPAR-TARP stoichiometry

Taking advantage from the results obtained analysing AMPAR-TARP stoichiometry combining GluA1 homotetramers and γ2 it was decided to test if CPT1C favoured a particular AMPAR-TARP stoichiometry. Fixing AMPAR-TARP stoichiometries, it was seen a clear graded modulation in desensitization kinetics and polyamine block

attenuation but an 'all-or-nothing' effect looking at single-channel conductance, in previous results. For this reason, it was thought to detect, by analysing this parameter, whether CPT1C promotes an AMPAR-TARP stoichiometry of 2 or 4 TARPs per AMPAR.

tsA201 cells where co-transfected with GluA1 AMPAR subunit and γ2 (separate, not in tandem protein) and in addition CPT1C or GFP as control. The transfection ratio used was 2:1:4 GluA1:γ2:CPT1c/GFP respectively. Currents from membrane patches were recorded like in the first set of experiments, applying 10mM glutamate in an ultra-fast manner using a piezoelectric device. No significant were observed at any of the biophysical properties tested. Responses from cells co-transfected with CPT1C showed slightly higher values for desensitization kinetics (5.26 ± 0.63ms, 5.6 ± 0.43ms, for co-transfection with GFP and CPT1C respectively; Mann-Whitney test; figure 48A), single-channel conductance (14.59 ± 1.42pS, 15.81 ± 1.6pS, for co-transfection with GFP and CPT1C respectively; Mann-Whitney test; figure 48B) and RI (0.23 ± 0.06, 0.29 ± 0.03, for co-transfection with GFP and CPT1C respectively; Mann-Whitney test; figure 48C); nevertheless, far away from be significant to indicate a different AMPAR-TARP stoichiometry. In addition, looking at the values obtained at both groups, responses from AMPAR matched with the 2 TARPed AMPARs when stoichiometries where fixed using fusion proteins.

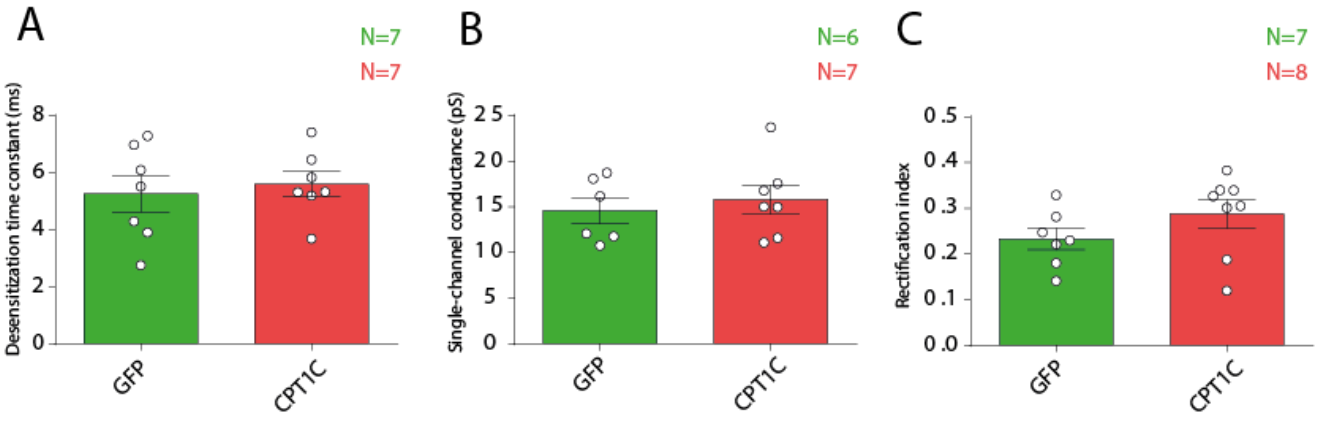


Figure 48. CPT1C does not modify AMPAR-TARP stoichiometry heterologously expressed in tsA201 cells. (A-C) Pooled data from membrane patches presenting GluA1:γ2 homotetramers from cells expressing the receptors and GFP or CPT1C. No significant differences indicating different AMPAR-TARP stoichiometry were found analysing desensitization kinetics, single-channel conductance or rectification index (to analyse polyamine block) (A-C respectively).

19. AMPAR-TARP stoichiometry in CGCs from CPT1C KO mice

Results from figure 21 seem to indicate that CPT1C is not involved in determining a given AMPAR:TARP stoichiometry. However, and taking into account the limitations of expression systems where the normal neuronal machinery involved in AMPAR biogenesis is absent, it was decided to study if in a neuronal model, CPT1C was somehow an important determinant of AMPAR:TARP stoichiometry. Thus, moving into a more physiological model taking advantage from the AMPAR-TARP stoichiometry characterization done in CGCs from WT mice in which a 2-TARPed AMPAR seemed to be present and also on the fact that we had the CPT1C KO mice. Therefore, it was assumed that if CPT1C was important for AMPAR:TARP stoichiometry, it would be able to see differences in such stoichiometry by measuring currents from wild type and KO animals. For this reason were recorded currents from outside-out patches of CGCs from control and CPT1C KO mice.

Different from the other experiments performed with CPT1C, GluA1 and γ2; the context is more physiological in CGCs where there is no lack of other AMPAR-interacting proteins that are not expressed in tsA201 cells. For this reason, it is expected that a change occurred in AMPAR-TARP combination can be essentially due to the lack of CPT1C in CGCs from CPT1C KO mouse.

The results obtained showed that responses in somatic membrane patches from CGCs of CPT1C KO mouse did not differ significantly from the ones in WT mice in terms of desensitization kinetics (5.07 ± 0.23ms, 4.7 ± 0.32ms, for CGCs from WT mouse and CGCs from CPT1C KO mouse respectively; Student's t-test; figure 49A) or single channel conductance (14.39 ± 1.56pS, 12.8 ± 1.52pS, for CGCs from WT mouse and CGCs from CPT1C KO mouse respectively; Student's t-test; figure 49B). However, looking at peak amplitude from the membrane patches, it was observed that in primary cultures from CPT1C KO mouse the responses were significantly lower than in

WT animal (18.71 ± 2.8pA, 11.17 ± 1.93pA, for CGCs from WT mouse and CGCs from CPT1C KO mouse respectively; Student's t-test; figure 49C). Taking into account the small dimension of the CGCs, the responses obtained from a membrane patch in these neurons are an accurate sample from a whole-cell response. Therefore, these data can indicate that CPT1C probably does not favour one AMPAR-TARP combination in concrete but enhances AMPAR trafficking to cell membrane as it was reported before (Gratacòs-Batlle et al. 2014, 2018b), but here in GluA1-lacking AMPARs. In addition, as since single-channel conductance was found to be not significantly increased in WT CGCs, the differences in peak amplitude can be explained due to differential receptor traffic to cell membrane.

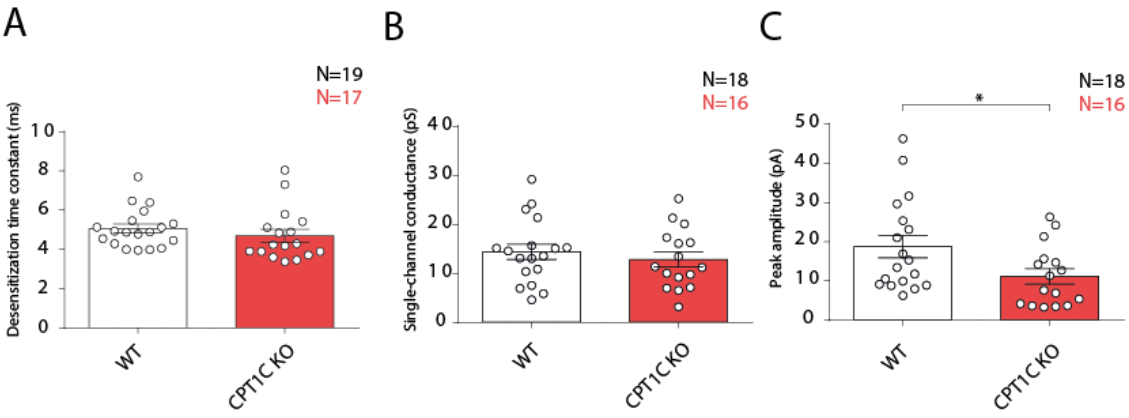


Figure 49. AMPAR-TARP stoichiometry in CGCs primary culture from CPT1C KO mouse. (A-B) Pooled data from different biophysical properties of AMPAR analysed in somatic patches from CGCs. No significant differences were observed in AMPAR desensitization kinetics or single-channel conductance from WT CGCs vs CPT1C KO CGCs. (C) Peak amplitude was significantly higher in CGCs from WT mouse compared to CGCs from CPT1C KO mouse indicating at least that AMPAR traffic to cell membrane is diminished in CPT1C KO mouse.

GRIN-related disorders are a group of pathologies linked to malfunction of NMDARs. In the following part of the results obtained during this thesis project, it will be presented the characterization of different GRIN mutations in terms of their impact in the biophysical properties over NMDARs. The effect that a specific mutation can exert in receptor function can affect many properties of NMDAR physiology. Here, the different variants analysed are classified in terms of loss of function (LoF) of gain of function (GoF) depending on how is modified the receptor's function. A variant that causes a reduction of the current area recorded compared to the WT condition is considered a LoF. It can be due to a reduction in the normalized peak current, steady-state current or an acceleration of desensitization or deactivation kinetics. The opposite happens with these parameters in the GoF variants. To stablish if a variant is a LoF or GoF it is important to look several of these parameters as a same mutation can confer to NMDAR a LoF in a discrete parameter but a GoF for another. Then, the alteration in the receptor function considers all the parameters modified in receptor biophysical properties to stablish whether the NMDAR is affected by the mutation due to a LoF or GoF. However, the normalized peak current usually determines the classification into a LoF or GoF as it has more pronounced effect over the total current area. In figure 50 it is schematized the dichotomy of LoF or GoF to classify GRIN variants.

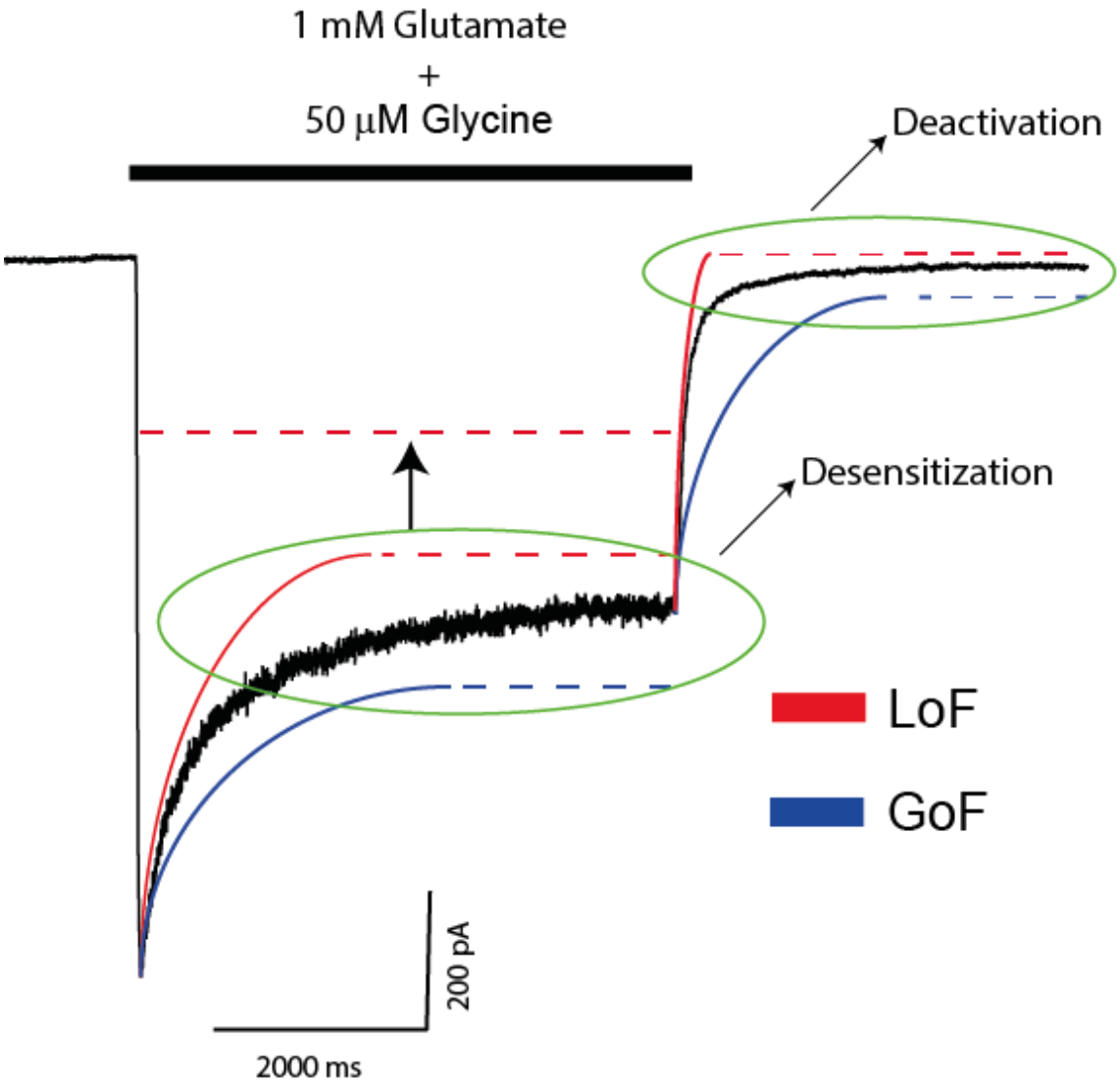


Figure 50. Scheme of LoF and GoF GRIN variant response respect WT NMDAR. The figure shows in black a response from whole-current of WT NMDAR when applying 1mM glutamate and 50mM glycine (black bar over the trace). In red lines it is schematized the loss of function characteristics like less steady-state current (black arrow pointing a red dashed line) or faster desensitization and deactivation kinetics. In blue it is shown slower desensitization and deactivation kinetics which implies an increase of the current area.

In addition, it is considered a GoF when the characteristic Mg^{+2} blocking that affects NMDARs is attenuated by a GRIN variant. This consideration is due to the fact that *in vivo* will imply that NMDARs will not need membrane depolarization to be able to respond to agonists and will be activate instantly by agonists release.

Finally, despite the different classifications that can be made to present the results

obtained in different GRIN variants (LoF and GoF, affecting GluN1, N2A or N2B, …) it was decided to classify them in truncations (a premature stop codon altering the final synthesis of NMDAR resulting in an incomplete form) or punctual mutations (one amino acid change). The truncated variants will be classified also by the subunit that are affected by the truncation. On the other hand, all punctual mutations will be arranged depending on the GluN subunit that are affecting and depending on their effect (LoF or GoF).

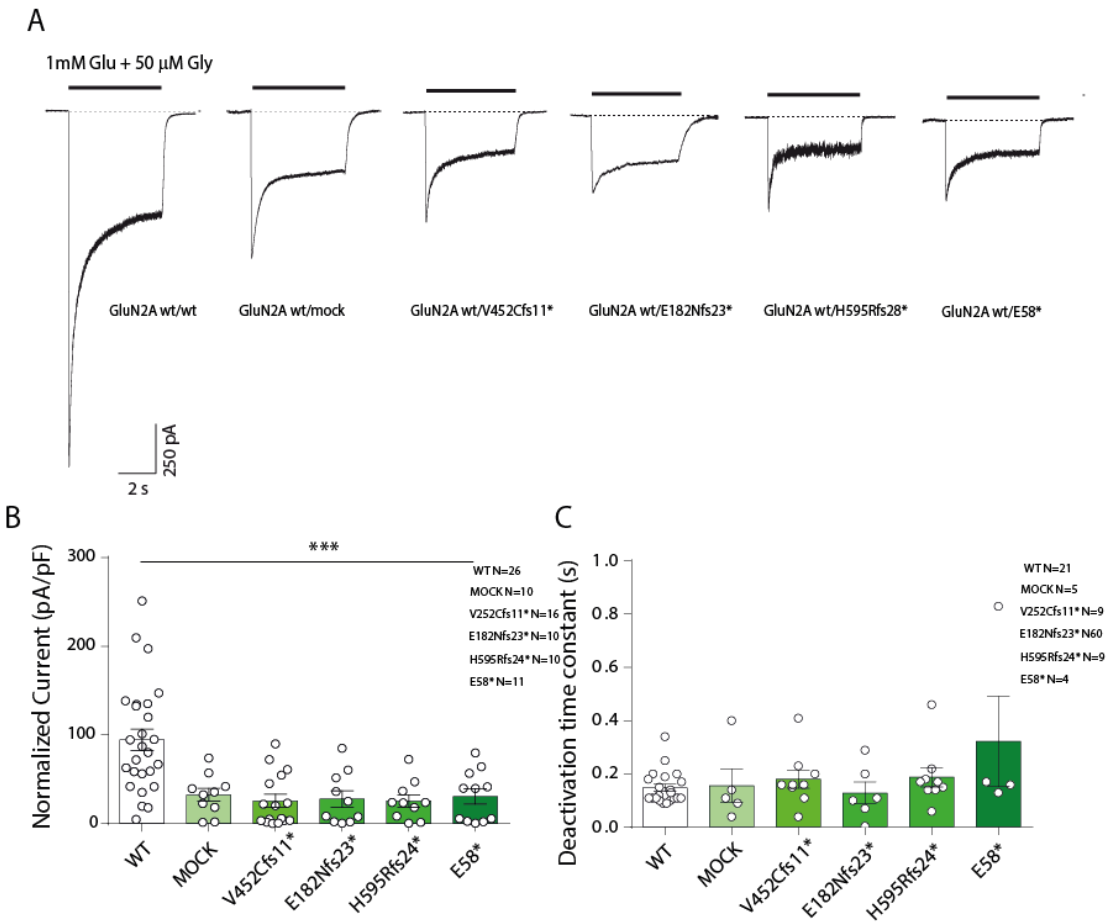
20. Premature stop codons reduce peak currents in GluN2A and GluN2B-containing NMDARs

Premature stop codons produce the end of synthesis process in a protein before it is completely synthesised. Proteins resulting from this interrupted process are usually aberrant variations that are not functional and then are eliminated by the cell. In NMDARs two distinct subunits are needed to form the functional receptor as it was explained in the introduction. 2 GluN1 subunits together with 2 GluN2A or GluN2B subunits form functional receptors (Traynelis, Wollmuth, Mcbain, et al. 2010) and the GluN1 subunit is mandatory to form functional receptors. However, the total amount of GluN subunits available can affect the total number of NMDARs. The results obtained in this thesis showed that GluN2A and GluN2B truncated forms expressed in heterologous systems along with GluN WT forms, reduce peak currents without altering receptor biophysical properties. For this experiments, whole-cell currents normalized by membrane capacitance were compared to investigate the effect of truncated forms in NMDAR properties. tsA201 cells expressing truncated forms were transiently transfected with a ratio of 1:1:1 GluN1:GluN2-WT:GluN2-truncated form respectively. WT condition used a ratio of 1:2 GluN1:GluN2 for transfection while an additional condition that simulated an haploinsuficience phenotype was performed by transfecting GluN1:GluN2:MOCK (plasmid that did not express any protein in mammalian cells) in a ratio 1:1:1 respectively. Cells transfected with a ratio of 1:2 GluN1:GluN2-truncated form did not show currents.

The results obtained for truncated forms of both GluN2A and GluN2B showed that isoforms with a premature stop codon significantly reduced peak amplitude currents normalized for membrane capacitance compared to WT condition. In addition, normalized currents recorded in truncated GluN2 conditions did not significantly differ from MOCK condition (94.76±11.99pF/pA; 32.65±7.2pF/pA; 25.95±7.34pF/pA; 28.11±9.33pF/pA; 30.94±8.54pF/pA; 25.82±6.96pF/pA for WT, MOCK, V452Cfs11*, E185Nfs23* , H595Rfs24* ; E58* respectively for GluN2A truncated forms; 25.98±4.23pF/pA; 8.23±7.2.09pF/pA; 10.25±1.74 pF/pA; 6.63±1.78pF/pA; 10.92±3.47pF/pA for WT, MOCK, R519*, D786Mfs23*, E839* respectively for GluN2B truncated forms; fs indicates a frameshift and * means stop codon, the number between fs and * indicates the amino acids till the premature stop; one-way ANOVA; figure 51B and E).

On the other hand, analysing deactivation kinetics it was found no significant difference between groups (0.53±0.08s; 0.4±0.06; 0.48±0.1s; 0.62±0.1s; 0.56±0.16s for WT, MOCK, R519*, D786Mfs23*, E839*; E58* for WT, MOCK, R519*, D786Mfs23*, E839*; E58* respectively for GluN2A truncated forms; 0.6±0.1s; 0.65±0.13; 0.71±0.09s; 0.55±0.07s; 0.79±0.12s; 0.26±0.19s respectively for GluN2B truncated forms; Kruskal-Wallis multiple comparison test; figure 51C and F). This implies that probably receptors at cell membrane are only NMDARs with WT subunits. By contrast, the effect of GRIN truncated variants probably leads into reduction of total receptors that traffic to cell membrane as co-transfection with only GluN2-truncated forms (without GluN2-WT) did not show currents.

GluN2A Truncations



GluN2B Truncations

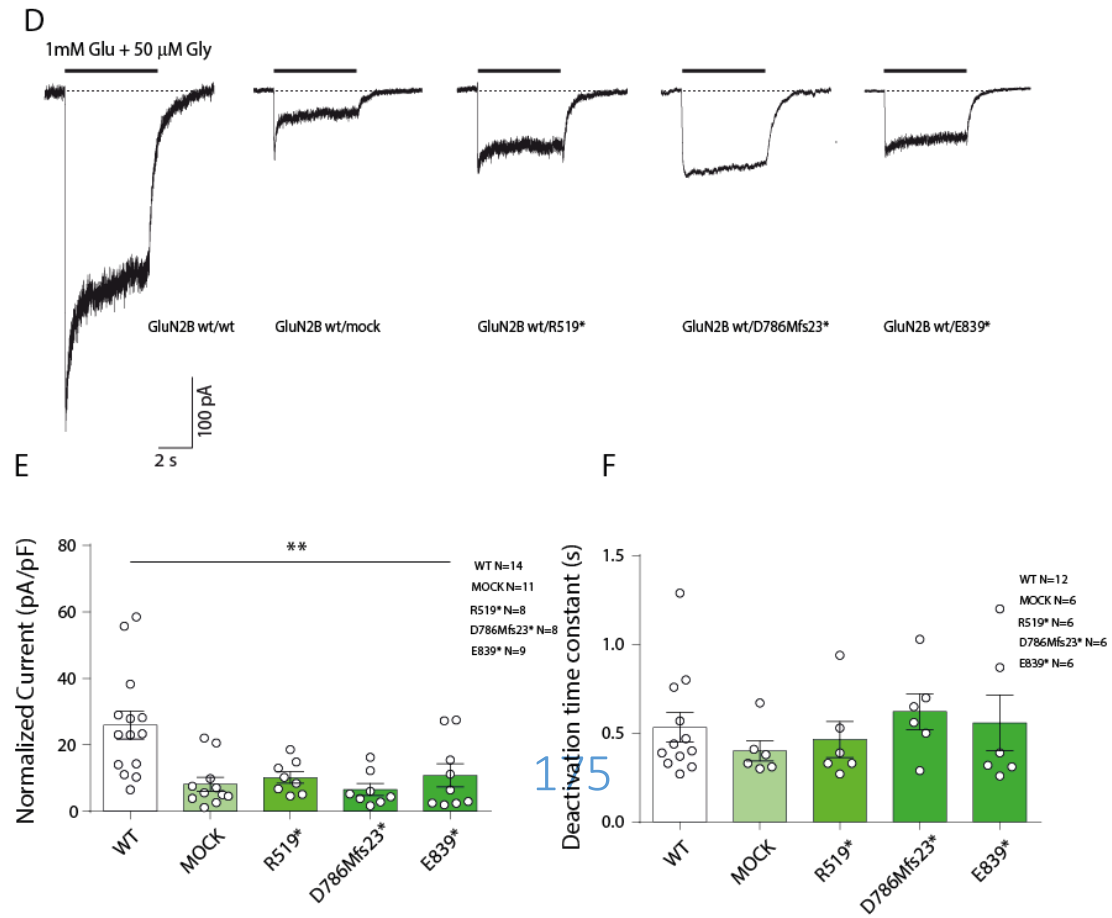


Figure 51. GluN2A and GluN2B truncations diminish normalized peak current. (A and D) Example average traces of every condition. (B and E) Pooled data of normalized peak current of WT, MOCK and truncated forms. Normalized currents in WT condition are significant higher compared to other conditions. (C and F) Pooled data of deactivation time constant with no significant differences between any conditions.

21. GluN1 truncated variants have no effect in NMDAR-mediated currents

Different from the effect of premature stop codons in GluN2 subunits, truncated isoforms of GluN1 mandatory subunit did not affect total current recorded in tsA201 cells expressing heterologously GluN1 subunits. For these experiments the same transfection ratio of GluN subunits was used being GluN1 total amount reduced in a half to transfect with GluN1 WT and GluN1 truncated form. GluN1 subunit was co-transfected with GluN2A for all conditions. In this case, neither, total current normalized by membrane capacitance or deactivation kinetics did not show significant differences between groups (76.37±9.69pA/pF; 74.68±16.44pA/pF; 55.55±14.20pA/pF and 0.15±0.69s; 0.75±0.32s; 0.14±0.04s for WT, W247* and Q556* respectively; one-way ANOAVA and Kruskal-Wallis multiple comparison test respectively; figure 52). Here it is shown that truncated variants from GluN1 subunits do not affect the magnitude of whole cell currents as GluN2 truncated variants. On the other hand, membrane receptors deactivation kinetics are not altered. It is correlated with the fact that GluN1 truncated forms are not related with GRIN-related disorders like it happens with GluN2 truncations (Santos-Gómez et al. 2020).

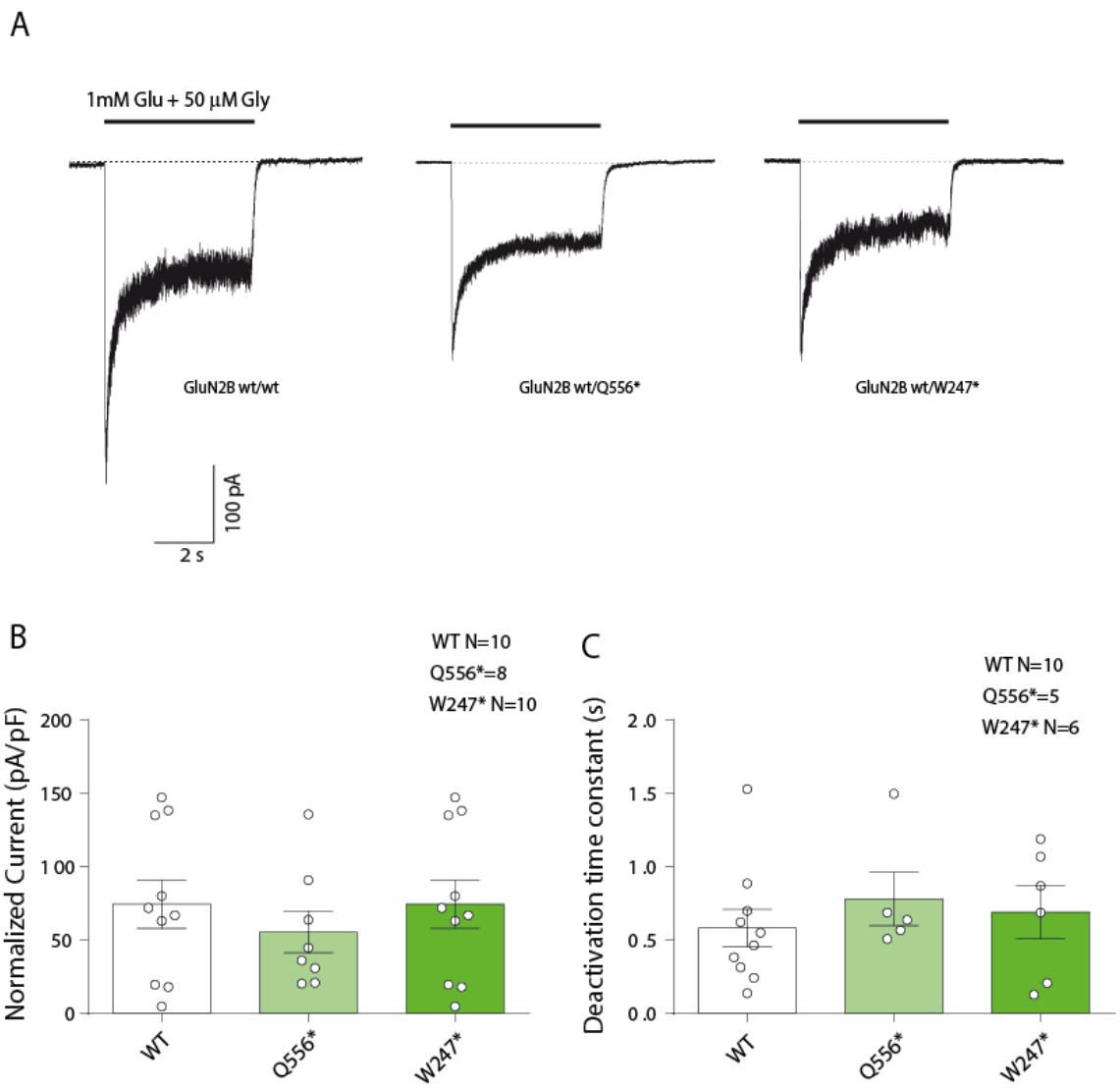


Figure 52. GluN1 truncations did not altered normalized peak current. (A) Example average traces of every condition. (B) Pooled data of normalized peak current of WT and truncated forms. Normalized currents in WT condition are not significant higher compared to other conditions. (C) Pooled data of deactivation time constant with also no significant differences between any condition.

22. GRIN punctual mutations can induce NMDAR LoF

A single amino acid change may seem an innocuous change in a protein structure as they formed by hundreds of theme. However, this small change can easily lead into a huge change in the protein function. For this reason, the present part of the thesis will present results obtained analysing different *de novo* mutations in GRIN genes that affect NMDAR modifying its biophysical properties.

Here are dissected many of these variants that were analysed in this thesis project by

patch clamp technique to assess the effect of particular mutations over NMDAR functionality. For all the experiments performed, recordings from NMDAR altered forms were compared with WT recordings from the same day. The number of recordings from some of the variations analysed is low due to the fact that in some cases the punctual mutation caused a drastic reduction in NMDAR currents so most of the transfected cells patched did not exhibit currents. Due to the impossibility to check if a non-current whole-cell recording was due to an effect of the mutation or due to the lack of one of the GluN codifying vectors (GluN subunits were not tags were not visible with microscope for patch clamp recordings), these attempts with no current were not considered. In addition, in these experiments the GluN subunit affected by a punctual mutation completely substituted the GluN WT form in the transfection; different from experiments with truncated isoforms.

The first set of GRIN variants analysed that are presented include GluN1 forms that drastically reduced peak current amplitude. The GluN1 variations A814D, P805L and G620R reduced peak currents normalized by membrane capacitance compared to WT condition using GluN2B to form the NMDAR (44.63±7.3pA/pF;22.1±3.25pA/pF; 10.49±3.29pA/pF; 11.84±4.26pA/pF for WT, A814D, P805L and G620R respectively; Kruskal-Wallis multiple comparison test; figure 53A).

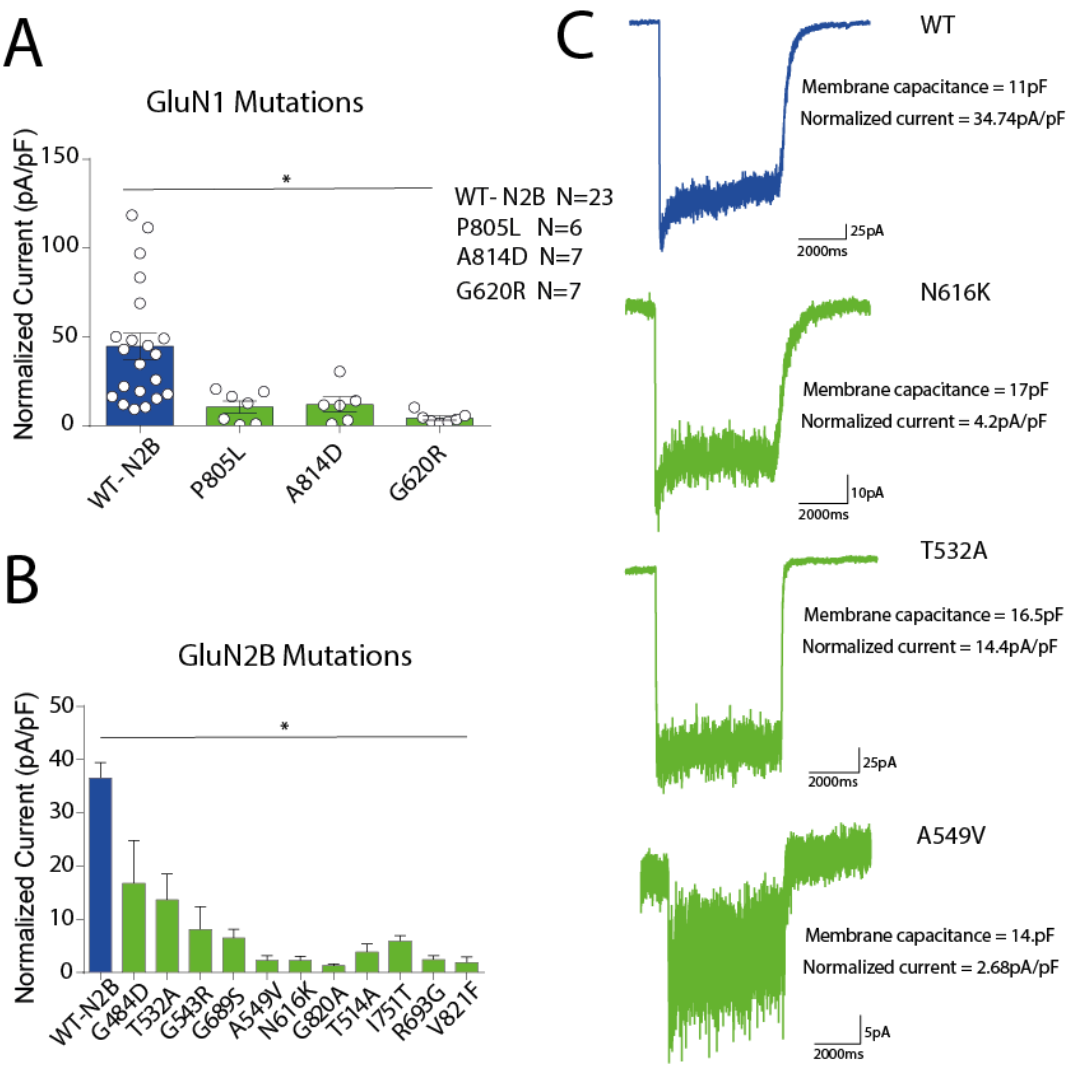


Figure 53. GRIN punctual mutations can sharply reduce NMDAR peak currents. (A-B) Normalized peak currents from GRIN punctual mutations. Mutations presented shown are present in GluN1 (A) and GluN2B (B) subunits. (C) Traces of NMDAR WT (GluN1-GluN2B; blue) and some of the punctual mutations analysed in GluN2B subunit (green).

The second set of GRIN variants analysed covered GluN2B variation that were classified as a LoF due to the considerable effect in this parameter and no further analysis was performed in other receptor properties as in most of cases the reduction of peak current made not possible to measure NMDARs kinetics or steady-state currents. At the following table it is summarized (variant, mean, SEM, N and statistical test the comparison in terms of normalized peak current between WT condition and the different GluN2B forms; figure 53B).

	Normalized peak current (pA/pF)				
GluN2B Variations	Mean	SEM	N	Significance	Test applyed
WT	35.00	8.576	8	*	Mann-Whitney test
G484D	9.77	2.552	6		
WT	35.12	7.214	5	*	Mann-Whitney test
T532A	13.63	4.913	12		
WT	17.9	5.011	5	*	Mann-Whitney test
G689S	6.48	1.615	5		
WT	45.36	6.186	11	*	Student's t-test
M824V	21.74	6.94	7		
WT	39.8	9.231	6	**	Mann-Whitney test
A549V	3.553	0.6542	7		
WT	55.63	16.68	6	**	Mann-Whitney test
N616K	2.25	0.8332	4		
WT	55.05	16.69	6	*	Mann-Whitney test
G820A	1.267	0.318	3		
WT	30.8	8.548	7	**	Mann-Whitney test
I751T	5.92	1.026	10		
WT	44.52	6.389	5	**	Mann-Whitney test
T514A	3.845	1.518	6		
WT	60.33	12.85	5	**	Mann-Whitney test
R693G	2.423	0.7241	6		
WT	25.38	10.66	7	*	Mann-Whitney test
V821F	1.89	1.007	3		
WT	46.42	12.93	6	**	Mann-Whitney test
R696H	8.493	4.193	6		

Table 3. GluN2B punctual mutations summary.

However, not all the mutations analysed in GluN2B subunit leaded into a LoF due to a decrease of peak current. The change of a methionine for a valine at 824 position in the GluN2B (M824V) produced a significant decrease in stead-state current (47±7.17%; 23.17±3.02% for WT and M824V respectively; Mann-Whitney test; figure 54D) and

faster desensitization kinetics (1.59±0.4s; 0.29±0.06% for WT and M824V respectively; Mann-Whitney test; figure 54C) but no significant differences in normalized peak current (51.07±10.14pA/pF; 29.38±011.41pA/pF for WT and M824V respectively; Mann-Whitney test; figure 54A). Both changes in these biophysical properties made that the current area recorded in the M824V was less than in WT receptors and this mutation was classified as a LoF (figure 54B).

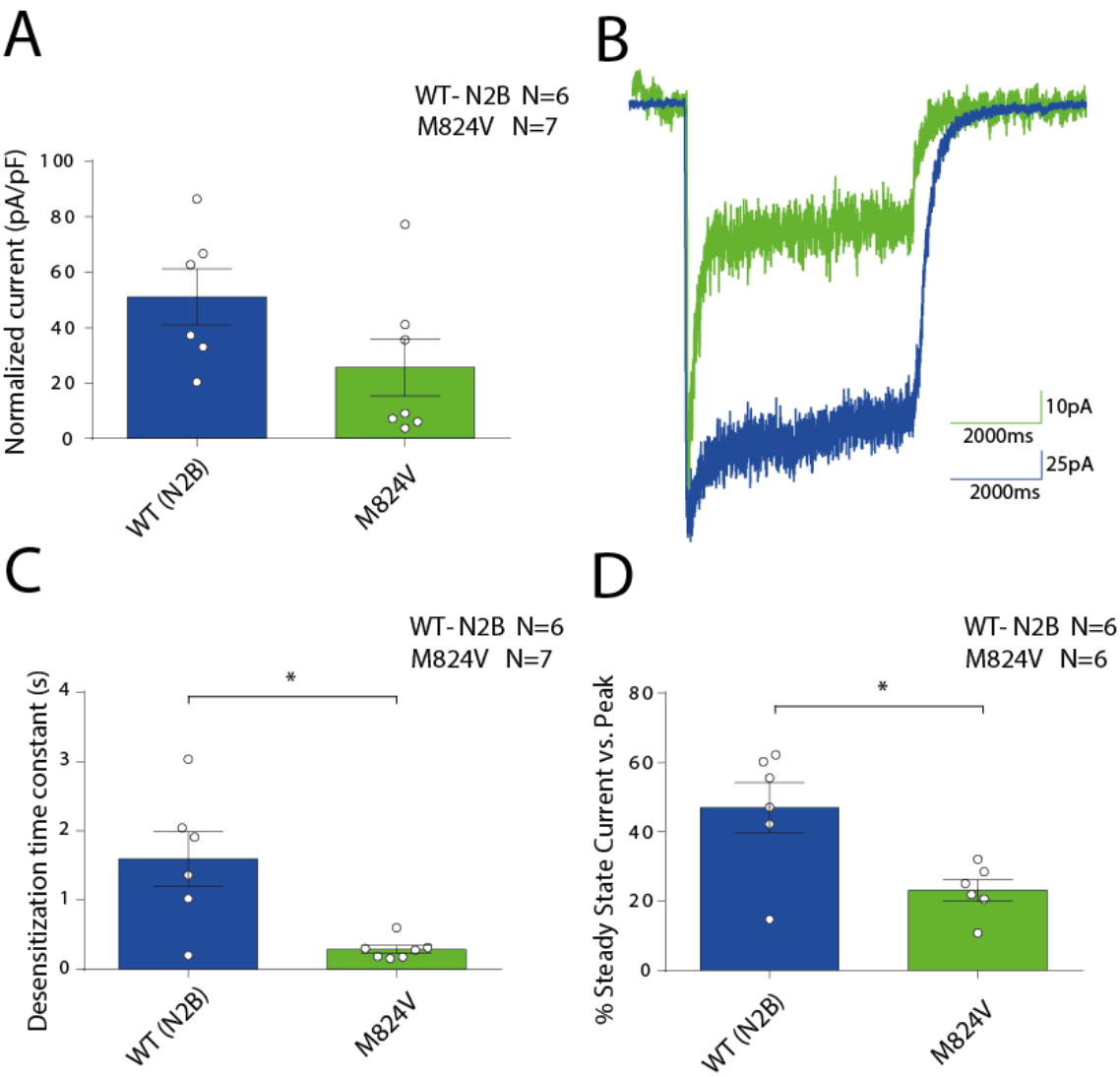


Figure 54. M824V mutation in GluN2B subunit. (A)Pooled data of normalized peak current with no significant differences between WT and M824V mutation. (B) Average traces of both conditions. It is seen the decrement in steady-state current in M824V (green) respect WT (blue). (C-D) Pooled data from desensitization time constant and the percentage of steady-state current respectively. Significant differences were seen at both parameters what lead into a diminishment of the total current area in M824V mutant compared to WT.

On the other hand, just one GluN2A punctual mutation was analysed. Curiously, this variation (V820I) presented reduced peak amplitude (34.88±8.96pA/pF; 8.26±1.65pA/pF for WT and V820I respectively; Student's t-test; figure 55A) and steady-state currents (51.43±2.12%; 40.05±4.25% for WT and V820I respectively; Student's t-test; figure 55C) and faster desensitization kinetics (0.77±0.11s; 0.28±0.04s for WT and V820I respectively; Mann Whitney test; figure 56C) (steady-state and desensitization kinetics were possible to measure contrary to GluN2B LoF; figure 55B).

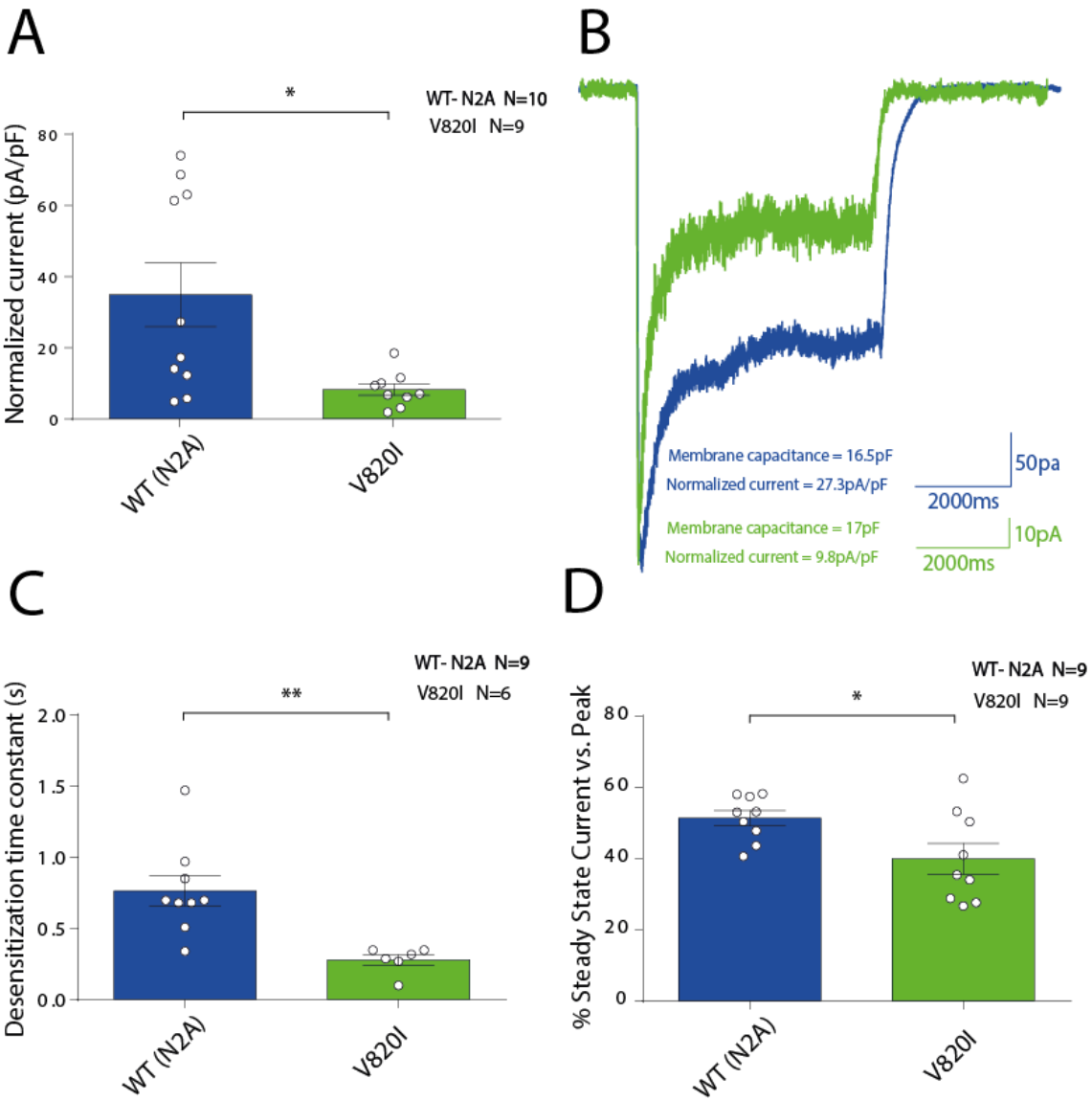


Figure 55. V820I mutation in GluN2A subunit multiple LoF. (A)Pooled data of normalized peak current with significant decrease in V820I mutation. (B) Average traces of both conditions with decrement in the steady-state current and faster desensitization kinetics in V820I (green) respect WT (blue). (C-D) Pooled data from desensitization time constant and the percentage of steady-state current respectively.

Significant differences were seen also these parameters.

23. GoF in NMDARs due to a GRIN variations

Among the different variants analysed in this thesis project, 2 of them in GluN1 were classified as GoF. On the one hand M641V in GluN1 showed no significant differences in normalized peak currents when compared to WT condition (39.01±8.66pA/pF; 22.10±3.25pA/pF for WT and M641V respectively; Student's t-test; figure 56B) but a slower deactivation kinetics that imply an increase in the current area (0.5±0.08s; 0.8±0.08s for WT and M641V respectively; Student's t-test; figure 56C). No other significant differences were seen comparing other parameters.

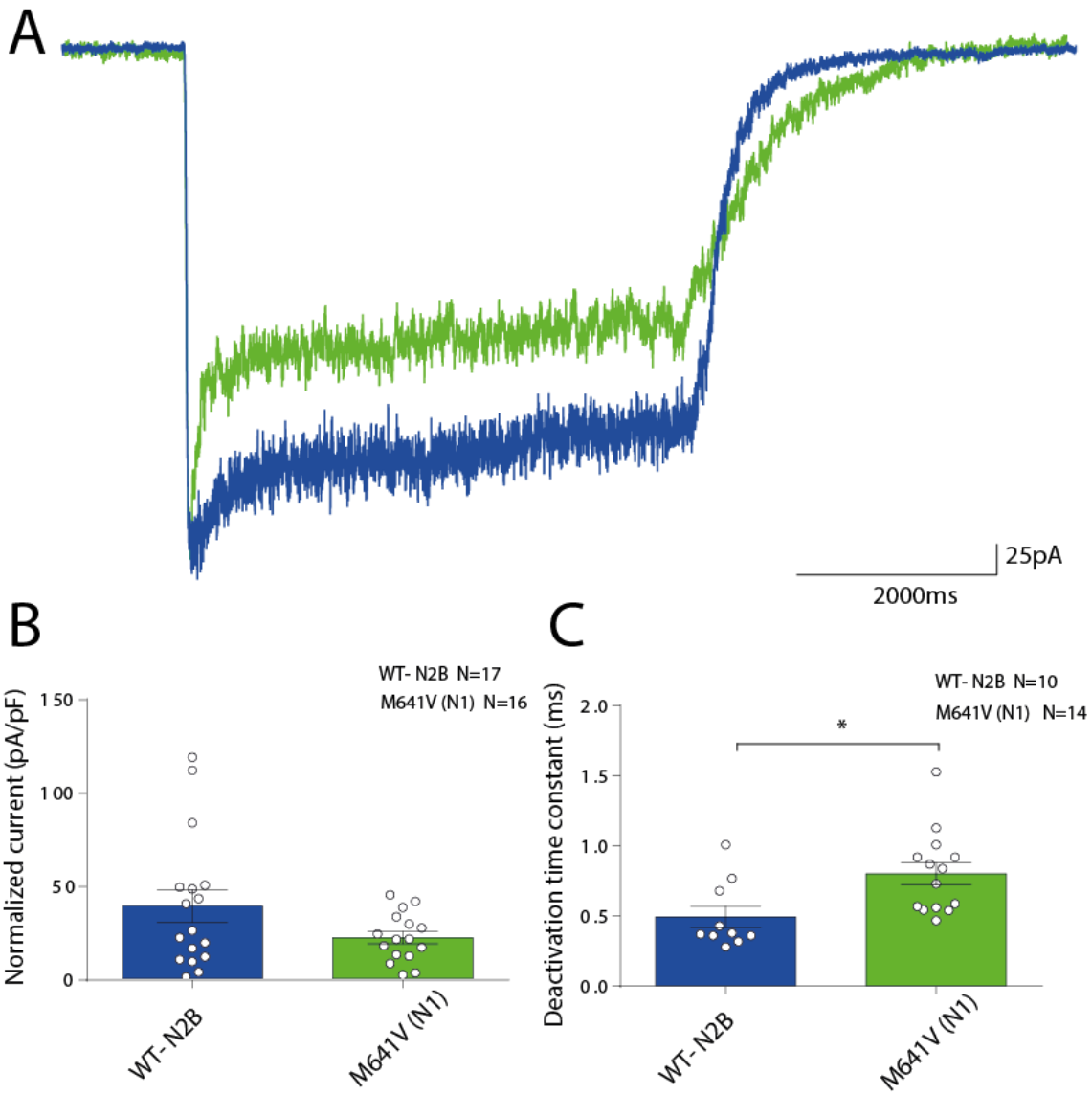


Figure 56. GluN1 mutation M641V increases total current area. (A) Overlapped representative average traces from both conditions. In green the M641V mutation that shows slower deactivation kinetics. (B-C) Pooled data from normalized peak current and deactivation kinetics. No significant differences were observed in peak currents but significant slower deactivation kinetics were seen in this GRIN variation of the GluN1 subunit.

Finally, the S617C variation of GluN1 subunit exhibited also no significant differences in normalized peak currents (32.3±4.7pA/pF; 42.87±13.75pA/pF for WT and S617C respectively; Mann-Whitney; figure 57B) compared to WT but a sharp decrement in Mg^{+2} block. Applying agonist solution with 10mM Mg^{+2} in whole-cell recordings it was seen in WT near 95% of blockage of the steady-state currents (agonist vs agonist+Mg^{+2} application). In the S617C variation the blockage was near 30%, suggesting that in a

more physiological condition the NMDAR receptor is over-activated due to the lack of block for Mg^{+2} present in physiological condition (94.02±2.83%; 31.27±8.33% for WT and S617C respectively; Mann-Whitney test; figure 57B).

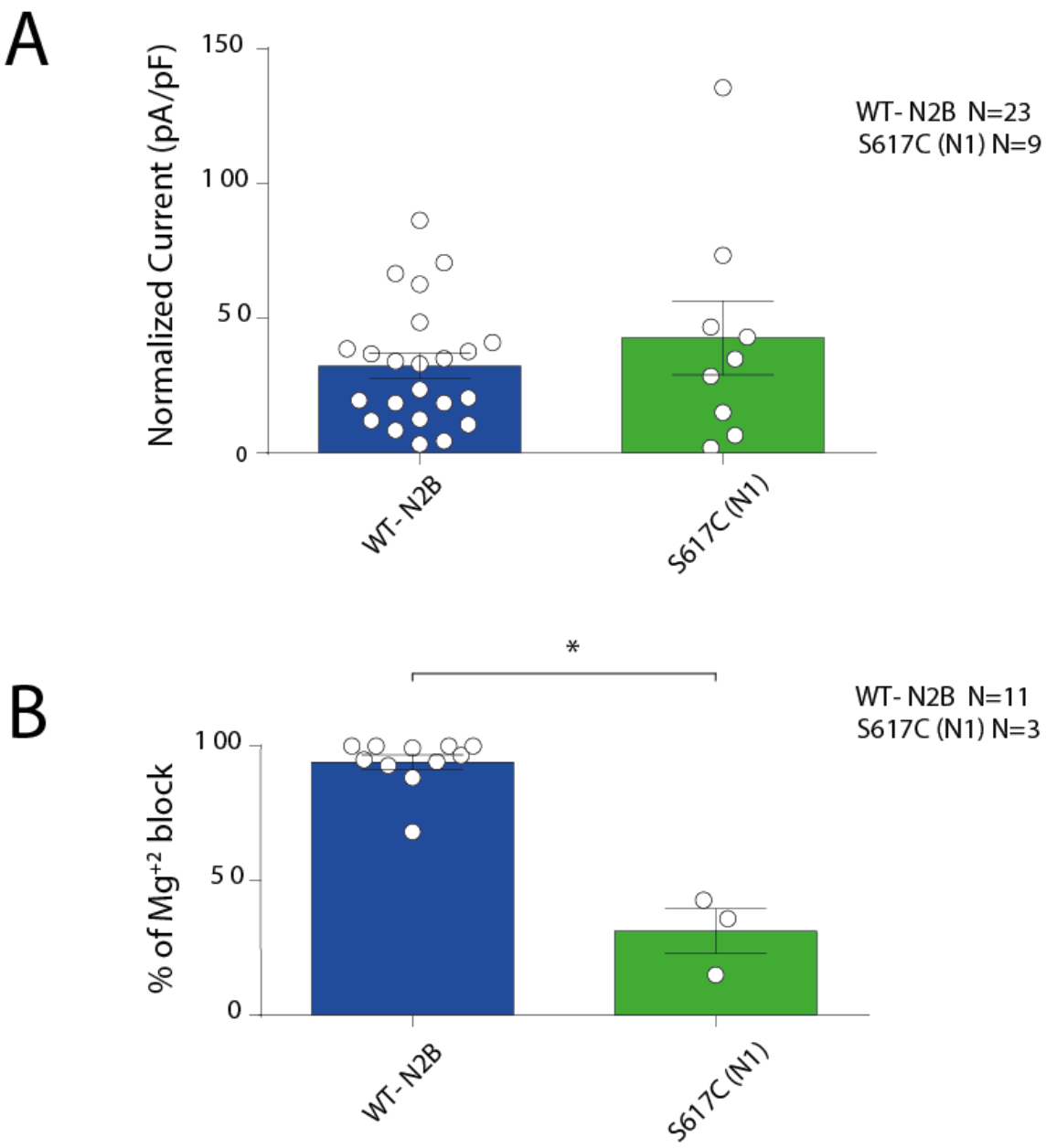


Figure 57. GoF in S617C mutation 8GluN1) due to a decrement in Mg^{+2} block. (A) No significant differences were seen in normalized peak currents when comparing the WT condition to S617C mutation but a significant decrease in Mg^{+2} block in the mutated GluN1 subunit (B).

Altogether, all variants analysed, GoF or LoF, imply a NMDAR malfunction meaning an affection in neuronal communication and signalling integration. The study of these

variants in a functional way could give in a future a better comprehension of glutamatergic signalling and in addition help to aboard new treatments in benefit of patients and families.

1. Discussion

At the beginning of this work it was explained that glutamate is the most abundant excitatory neurotransmitter in CNS. For this reason, the study of iGluRs is important to achieve a better understanding of how our nervous system works. This thesis project tried to contribute to a better comprehension over glutamate receptors. Focusing in iGluRs, specifically in AMPARs and NMDARs, this project has studied different aspects about their functionality.

One the hand, the study of AMPARs has been focused in relation with the AMPAR-TARP stoichiometry and how this can modulate AMPAR biophysical properties. In addition, it was explored the possible modulation of AMPAR-TARP stoichiometry by the interacting protein CPT1C. On the other hand, NMDARs where studied in the context of *de novo* GRIN mutations that affect receptors properties and induce GRIN-related disorders.

1. AMPARs in AMPAR-TARP stoichiometry

The study of the AMPAR-TARP stoichiometry in AMPAR function is not a completely unknown topic but definitively a not deeply studied one in AMPAR biology. One of the first evidences of a functionally variable AMPAR-TARP stoichiometry came from an observation made in neurons that mEPSCs were differentially altered depending on the expression levels of TARPs in CA1 pyramidal neurons and dentate gyrus pyramidal neurons (Yun Shi et al. 2009). In a brief period of time, another work from the same research group demonstrated that the pharmacology of recombinant AMPARs was TARP stoichiometry-dependent (Yun Shi et al. 2009). In this work the authors tested the kainate-mediated responses in AMPARs with different AMPAR-TARP fixed stoichiometries. In the same publication it was suggested that AMPARs from hippocampal pyramidal and dentate gyrus granule neurons were 4 and 2-TARPed, respectively. More recently, another work has provided evidence for the presence of different stoichiometries in cerebellar cells with 2 TARPed AMPARs in stellate cells and 4 TARPed AMPARs in Purkinje cells(Dawe et al. 2019). In the present thesis we have

expanded these previous findings by carefully dissecting the effect of different stoichiometries on basic AMPAR properties and we have observed a sophisticated modulation of TARPs either in CP- and CI-AMPARs.

2. Graded vs. all-or-nothing modulation of CP-AMPARs by γ2

Investigating the effect of AMPAR-TARP stoichiometry in AMPAR function we have found two clear patterns in stoichiometry modulation. On the one hand, biophysical properties like desensitization time or polyamine block attenuation, in GluA1 homotetramers (CP-AMPAR), followed a graded pattern where the AMPAR modulation was stronger as the number of TARPs per receptor increased. By contrast, single-channel conductance needed a full TARPed configuration to be significantly increased in comparison to TARPless receptors. Interestingly, this channel property has been repeatedly reported to be increased by TARP presence in AMPAR complex when both proteins where co-transfected in expression systems (Yun Shi et al. 2010; E. Suzuki, Kessler, and Arai 2008; Soto, Coombs, Renzi, Zonouzi, Farrant, and Cull-Candy 2009); however, without controlling the AMPAR-TARP stoichiometry, which indicates that in these works fully TARPed AMPARs were present in those recordings. On the other hand, other characteristics are not altered by the number of γ2 acting on AMPARs (peak-open probability or rise time). On the one hand, peak-open probability showed several disperse values that made not possible to stablish even a clear pattern or a tendency indicating that AMPAR-TARP stoichiometry with γ2 could modulate this parameter. On the other hand, TARP modulation over this parameter has been controversial as it has been reported both possibilities, first γ2 increasing peak open probability (Tomita et al. 2005) and contrary not modifying this channel property (Soto et al. 2007). Interestingly, slow deactivation kinetic and high steady-state current may imply an increased peak-open probability but here with 2 and 4 TARPed (which slowed receptor kinetics and increased steady-steady state currents in 4 TARPed AMPAR) receptors no changes are seen over this parameter.

The results obtained for γ2 and GluA1 homotetramers arose the question whether the 2 TARPed condition (transfection of GluA1 and GluA1:γ2 plasmids) was a heterogeneous population of 0 and 4 TARPed CP-AMPARs. However, there is some evidence from this work in favour of a major heteromeric population when both constructs are co-transfected. The vast majority of the responses when recording GluA1(Q) + GluA1(R) were linear since in those conditions it is difficult that GluA1(R) homomers contribute significantly (when co-expressed with GluA1(Q)). In that situation, we observed the same effect in kinetic desensitization for 0 *vs.* 2 TARPed AMPARs as the ones seen with GluA1(Q), which supports the view of a predominant heteromeric population *vs.* two homomeric ones. Moreover, the fact that some intrinsic properties are undoubtedly affected in the 2-TARP condition (i.e. polyamine block attenuation or desensitization) while others are clearly not (channel conductance or rise time), in the same patches argues in favour of the assembling of mainly heteromeric receptors. Finally, it is worth mentioning that in crosslinking experiments we have observed an intermediate molecular weight of surface AMPARs in GluA1 + GluA1:γ2 transfection compared with GluA1 transfection (with a band at a lower weight) or with GluA1:γ2 (with a band at a higher molecular weight) suggesting that the degree of homomeric contamination in a heteromeric condition is minimal. Unluckily, it is not possible to discard a small presence of a mixed population despite these indications that a major 2-TARPed population exists in the GluA1+GluA1:γ2 condition.

3. AMPAR-TARP stoichiometry in CP-AMPARs with other members of TARP family

Following the experiments with the prototypical TARP γ2 It was decided to test how AMPAR-TARP stoichiometry behaved with members of TARP family. First of all, it was interesting to see that γ3, the other type Ia TARP, behaved similarly to γ2 in terms of single-channel conductance. 4 γ3 TARPed AMPAR showed an increase of channel-conductance while the 2 TARPed condition did no differ significantly from TARPless receptor. Hence, both type Ia TARPs behaved similarly related with this biophysical

parameter. These two TARPs were included in type Ia by structure similarities, which diverge from type IIb TARPs ((Kato et al. 2010a)). Other studies demonstrated that same separation based on similarities on attenuation of polyamine block (Jackson et al, 2011). By contrast, type Ib TARPs, γ4 and γ8, enhanced single-channel conductance in 2 TARPed condition. As mentioned before, it was not possible to record macroscopic responses in membrane patches from cells expressing heterologously the 4 TARPed condition with type GluA1:γ4/8 constructs. It was thought that with the constructs used the 4 TARPed condition probably was not favoured in expression systems as it seems that is not the most optimal configuration (Beatriz Herguedas et al. 2019a). However, it is worth to mention that in the so cited work from Herguedas et al. 2019 there was possible to achieve 4 TARPed configuration with γ8 despites it was in a CI-AMPAR, different from the GluA1 homotetramer used here.

On the other hand, the graded effect seen in γ2 seems to be exclusive for this TARP since it is not observed in γ3. Properties like desensitization kinetics or polyamine block are modulated indistinctly in CP-AMPARs by γ3 in heterologous systems. However, γ2 and γ3 have been traditionally included in the same "box" as they were classified different from type Ib TARPs due to their biophysical properties (Cho et al. 2007; Milstein et al. 2007). In the present results it is shown that type Ia TARPs may differ in AMPAR modulation when looking the effect of AMPAR-TARP stoichiometry. This probably has implications in neuronal transmission and integration since 2 TARPed AMPAR (taking into account only TARPs as auxiliary subunit) behave similar in terms of single-channel conductance but γ3 TARPed receptors seems to have slower desensitization kinetics and are less blocked by endogenous polyamines. Altogether could imply an increase in the total charge transfer that pass through the receptors meaning a different signalling at the postsynaptic neuron. In addition, it means that probably in a physiological context, with a sophisticated regulation of the AMPAR-TARP stoichiometry, there is not a simple TARP redundancy (Tomita et al. 2003; Menuz et al. 2008). In other words, in AMPAR biology, probably γ2 and γ3 are not totally equivalent and although one could supply in principle the function of the other one, the synaptic signals won't be equivalent despite that it was assumed that this happens

(Menuz et al. 2008).

Furthermore, recovery from desensitization differs when comparing γ2 and γ3 auxiliary subunits. When γ3 is modulating the GluA1 homotetramer it emerges an 'all-or-nothing' effect as the one seen for single-channel conductance, where only 4 TARPed receptors accelerate peak amplitude after receptor is desensitized. By contrast, recovery with type Ib TARPs was faster at 2 TARPed receptors respect TARPless AMPARs. Following this line, a very recent publication reported that all type I TARPs (γ2, γ3, γ4 and γ8) are able to speed recovery from desensitization when expressed heterologously along with GluA1 (Devi et al. 2020). In contrast, it has been published that γ8 slows recovery from desensitization in association with CI-AMPARs (Beatriz Herguedas et al. 2019b). Both results along with the ones presented in this thesis indicate that AMPAR modulation by auxiliary subunits strongly depends on AMPAR subunit composition. Indeed, as it was shown in **figure 42**, γ2 can slow recovery from desensitization when it is linked to the GluA2 subunit in CI-AMPARs when compared with TARPless AMPAR.

Finally, as seen with γ2, peak-open probability did not differ in different AMPAR-TARP stoichiometries with other members of the TARP family. The differences observed in other works with γ2 are extended to other members of the TARP family. One the one hand it has been published that γ3, γ4 and γ8 can increase peak-open probability expressed heterologously (Zhang et al. 2014). On the other hand, no differences were seen on Soto et al. 2009 looking at this property with prototypical TARPs. Interestingly, in this publication γ5 (which is a type II TARP) was found to decrease peak-open probability. Finally, it was also reported that γ2 increased this parameter but neither γ4 or γ8 in the same work (E. Suzuki, Kessler, and Arai 2008). Altogether makes TARP modulation over peak-open probability a complex issue to address. The data presented in this thesis project with other members of TARP family is in the same line with publications pointing that type I TARPs do not modulate peak-open probability in AMPAR. However, it cannot be discarded a TARP modulation over this parameter but the effect of this auxiliary subunit over the receptor seems to be complex. The

evidence come from the fact that peak-open probability seems to behave differently from work to work, contrary to other like desensitization kinetics or polyamine block which are have been reported several times to be modulated by TARPs.

Altogether implies that this biophysical property of AMPAR probably depends not only in the auxiliary subunit but also in receptor composition and AMPAR-TARP stoichiometry. Such a fine modulation of AMPARs is an exquisite example of the nervous system functioning.

4. AMPAR-TARP stoichiometry in CI-AMPARs

To study AMPAR-TARP stoichiometry in another AMPAR subtype we studied CI-AMPARs, and more precisely the receptor formed by GluA2-GluA4c. This subunit combination along with γ2 is the one present in CGCs (the most abundant neuronal cell in cerebellum, comprising almost 50% of all brain neurons). As mentioned in the introduction, GluA2 is the most expressed AMPAR subunit in the nervous system; however, GluA4c is a short rare isoform of GluA4 that is the most expressed CP-AMPAR subunit in CGCs but is specific of this cell type. Despite this isoform was first described at the beginning of the 90's (Gallo et al. 1992) not much is known about GluA4c. For this reason, it was decided to compare GluA4c biophysical properties with those of GluA4 homotetramers in heterologous expression systems. We found that in terms of the parameters analysed GluA4 and GluA4c homotetramers did not differ significantly at any property compared. However, in a more physiological model it cannot be discarded to observe other differences between this subunits as the long and short forms of GluA4 differ in the CTD which is important interacting with other AMPAR-interacting proteins and in AMPAR trafficking (Song and Huganir 2002; J. F. Watson, Ho, and Greger 2017).

In the experiments performed to study AMPAR-TARP stoichiometry in CI-AMPARs, we used the fusion proteins GluA2:γ2 and A4c:γ2 and it was observed that, in this type of AMPAR (that is structurally different from GluA1 homotetramers (Beatriz Herguedas et

al. 2016; Zhao et al. 2016)), γ2 exerts stronger and different modulation depending on the subunit where the auxiliary subunit is linked. Different from GluA1 homotetramers, there is not a graded or accumulative effect in AMPAR modulation or even an 'all-or-nothing' effect. The results obtained with this subunit combination show that when γ2 is linked to GluA2, which preferentially occupies the 'BD' pore distal positions in the AMPAR (He et al. 2016a; Beatriz Herguedas et al. 2019a), the auxiliary subunit intensively modulates recovery from desensitization and single-channel conductance but does not modify receptor kinetics compared to TARPless AMPAR. Interestingly, it has been shown that γ2 at the 'X' site of the AMPAR complex modulates receptor kinetics by electrostatic interactions (Twomey et al. 2016a; Zhao et al. 2016). The 'X' site positioning of γ2 in the receptor interacts predominantly with GluA subunits at 'BD' positions as it is illustrated in figure 58. Taking all together, in theory γ2 should modulate AMPAR kinetics when acting over GluA2 in AMPAR complex. However, kinetics were unaltered when γ2 was linked to GluA2 in 2T (A2) condition. On the other hand, γ2 increased single-channel conductance when linked to GluA4c but the increment was minor compared when was linked to GluA2. In addition, only linked to the short isoform of GluA4, γ2 slows AMPAR desensitization kinetics and increases AMPAR blockage by the non-competitive antagonist perampanel. These parameters are not affected when γ2 is translated in tandem with GluA2. Altogether shows that γ2 linkage it is determinant in AMPAR modulation as it drastically varies depending on the position that the auxiliary subunit occupies. The results obtained; however, expand more questions about GluA subunit arrangement and γ2 modulation over heteromeric AMPARs. Here it is shown that γ2 do not exert modulation over desensitization kinetics when is linked to GluA2 subunit which preferentially occupies 'BD' pore-distal positions. As mentioned before, the expected result would be a modulation of AMPAR desensitization kinetics if γ2 is acting over pore-distal subunit which seems to be potentially GluA2 position However, it cannot be discarded a different arrangement for GluA2 and GluA4c subunits over AMPAR in the experiments performed in this thesis, contrary to previous publications where GluA2 occupies 'BD' positions (He et al. 2016b; Beatriz Herguedas et al. 2019a). Discussed below, an additional set of experiments were performed to try to uncover in more detail how γ2 was modulating the receptor.

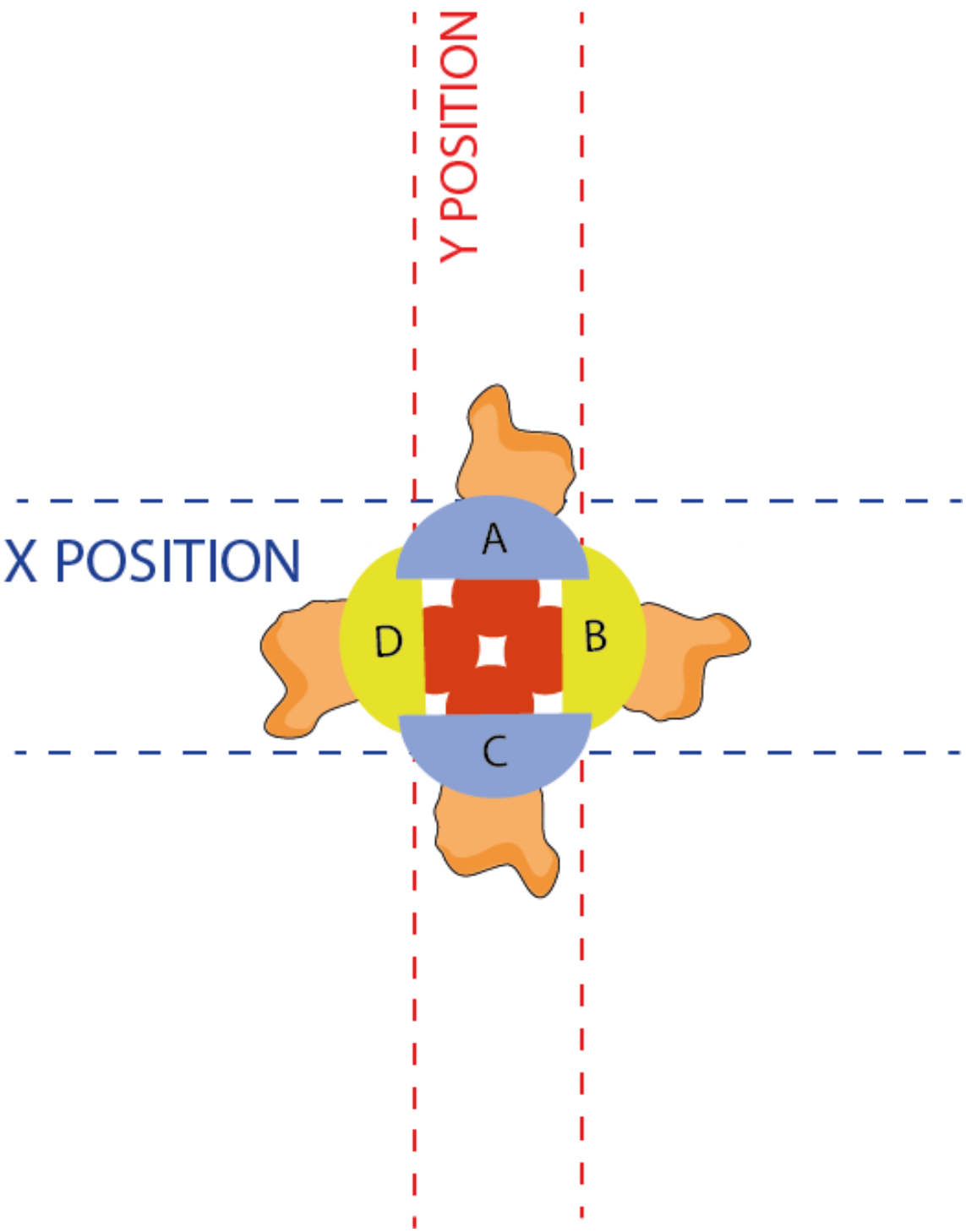


Figure 58. 'X' and 'Y' positions of TARP auxiliary subunits. The figure shows how TARPs can be positioned in 'X' and 'Y' positions depending on the axis positioning across the receptor. In yellow and blue TMDI, III and IV from GluA subunits positioned at 'AC' and 'BD' positions repetitively. In red the TMDII of each subunit that are forming the receptor pore. In orange is represented the auxiliary subunit.

In addition, it is worth to mention that the results obtained studying AMPAR-TARP stoichiometry effect over AMPAR pharmacology are in the same line as the ones obtained with AMPAR-TARP stoichiometry in CI-AMPAR. Here it is proved a differential receptor pharmacology depending on the number and/or position of TARPs in receptor complex. The differential response of AMPARs to positive or negative allosteric modulators (PAMs or NAMs) that are selective for AMPAR-auxiliary subunits complexes can be key to a better comprehension of AMPAR-related disorders and AMPAR function. In addition, the selectiveness for AMPAR-complex modulation can be important in future research due to the different AMPAR-complex expression along the nervous system. For example and before mentioned in the introduction, GluA subunit expression varies depending on brain region as well as the auxiliary subunits that modulate the receptor. That is the case of γ2 that is enriched in cerebellum and by contrast γ8 is more expressed in forebrain (Tomita et al. 2003). For this reason, specific targeting of the receptors complex can be a powerful tool in the study of brain region-selective studies. In terms of the drugs that target AMPAR complexes with auxiliary subunits, different compounds have come to light recently and target γ8-containing AMPARs (Gardinier et al. 2016; Maher et al. 2016; M. R. Lee et al. 2017). At this aspect it needs more research in order to find new compounds that targets selectively AMPAR complexes. However, some studies have yet found some possible candidates that can act as PAMs o NAMs in AMPAR complexes with γ2, CNIH3 or GSG1L in GluA2 homotetramers (Azumaya et al. 2017). Back to the compounds tested to be selective for AMPAR- γ8 complex , it was designed the antagonist (LY3130481/CERC-611) that selective act in forebrain AMPARs guided by AMPAR-γ8 interaction. This molecule can act as an antiepileptic drug different than perampanel which has a broad blockage spectrum and block similarly AMPARs from cerebellum and forebrain (with the consequences that arise this non-selective blockage as motor impairment) (French et al. 2012; Zwart et al. 2014). On the other hand, another group developed the 5-[2-chloro-6-(trifluoromethoxy)phenyl]-1,3-dihydrobenzimidazol-2-one (JNJ-55511118) and the 2-(3-chloro-2-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-5-yl)phenyl)acetonitrile

(JNJ-56022486) compounds that selective and reversely over AMPAR-γ8 complexes as NAMs (Maher et al. 2016). Then, in the same line with 5-aryl benzimidazolone and oxindole-based AMPAR modulators they designed another modulator to target AMPAR-γ8 complexes (Ravula et al. 2018; Savall et al. 2019). Altogether shows the importance of understand and discriminate AMPAR population by their auxiliary subunit association.

The results obtained in the present work in terms of AMPAR pharmacology showed that another possible target that can regulate AMPAR blockage by an antagonist is the position of the auxiliary subunit or its number rather than the TARP itself. The γ8 TARP-dependent AMPAR antagonists (TDAA) blocks preferentially AMPARs associated with this TARP as it acts over specific sites in the AMPAR-TARP union. This interaction seems to not be based on AMPAR-TARP stoichiometry but strongly depends in two residues at TMDIII and TMDIV in TARP structure (a valine at 177 position and a glycine at 210 position respectively) (M. R. Lee et al. 2017). However, both approximations are complementary and can be key for developing new treatments in the future in order to act over specific AMPAR populations and diminish possible secondary effects of the pre-existing drugs.

Summarizing, data obtained with CI-AMPARs implies that AMPAR-TARP modulation in this heteromeric receptor is ruled mostly by TARP position in the channel rather than by the number of TARPs per receptor. This arises the question about how AMPARs, in a more physiological “ambient” are regulated depending where the TARP is placed since a different location would mean different receptor properties and then distinct signal integration.

In this work we have observed that γ2 modulation in this CI-AMPAR was exerted predominantly by γ2 position in the receptor. However, this does not necessary mean that the TARP is solely acting on the GluA subunit where is attached. That question arose considering that the linker binding GluA2 or GluA4c to γ2 was flexible and movable. Thus, to try to uncover where the auxiliary subunit was acting at the AMPAR

complex, we used an amino acid change over TMDI that allowed to maintain γ2 union to receptor but cancelling its modulation over receptor kinetics. The amino acid change that produced this effect was a cysteine for a glycine in 549 position for GluA2 and 550 position for GluA4c as it was previously reported (Hawken, Zaika, and Nakagawa 2017; Beatriz Herguedas et al. 2019b). First we replicated the results published by Hawken, Zaika and Nakagawa in 2017 with CP-AMPARs (GluA2 (Q)). In homotetrameric GluA4c:γ2 with the C550L modification it was not only observed that γ2 modulation over receptor desensitization kinetics was abolished but also single-channel conductance was diminished, adding some extra information to the knowledge of this specific mutation. However, in CI-AMPAR conformed by GluA2-GluA4c subunits, the results obtained were far from symmetric. It was expected that if 'CL' mutation could modify desensitization kinetics and single-channel conductance in GluA4c:γ2, this amino acid change would have some effect over both subunits in CI-AMPARs. Nevertheless, when 'CL' amino acid modification was performed in GluA2:γ2 tandem, no differences were seen respect GluA2:γ2-GluA4c receptor. Since γ2 did not modify kinetics in GluA2:γ2-GluA4c receptor, it was not surprising to not observe any modification in this biophysical property of AMPAR. However, it was striking that single-channel conductance remained equal. This result probably indicates that in this context the increment single-channel conductance when the auxiliary subunit is linked to GluA2 do not need γ2 interaction with TMDI of this AMPAR subunit. On the other hand, when 'CL' modification was performed in GluA4c:γ2 tandem it was not observed an abolishment of γ2 modulation over receptor kinetics (which was the most distinctive feature of GluA4c:γ2 tandem). However, single-channel conductance modulation of γ2 linked to GluA4c was cancelled when 'CL' modification was performed in GluA4c:γ2 (a biophysical property that was not strongly enhanced by γ2). Altogether, does not clarify at which position of the receptor the prototypical TARP γ2 is acting. Nevertheless, still lacking a clearer mechanistically understanding, these results obtained permit to conclude that AMPAR-TARP interaction in this CI-AMPARs strongly depends in γ2 position and that this positioning clearly modifies receptor biophysical properties.

5. AMPAR-TARP stoichiometry in CGCs

Results obtained in the previous section allowed to investigate AMPAR-TARP stoichiometry in CGCs by comparing responses from somatic patches from this neuronal type to evoked currents in heterologous expression system. The data presented in this thesis project indicates that, in terms of TARP presence, 2-TARPed rather than 4-TARPed AMPARs are responsible for somatic responses in CGCs. This data is according to other publication since the overexpression of γ2 in this cell type increased kainate affinity (Milstein et al. 2007), indicating that CGCs are not totally saturated by TARPs, as the efficiency to kainate as agonist is strongly dependent on the number of TARPs in the complex (Yun Shi et al. 2009). When comparing the data from GluA2/GluA4c heteromeric receptors using fusion proteins with those from CGCs, it is evident that neither zero TARPs (low conductance) nor four TARPs (slow desensitization kinetics) are modulating somatic AMPARs in CGCs. Importantly, the findings in CGCs closely recapitulated those on expression systems only when 2 TARPs were attached to GluA2 subunit. In addition, it has been suggested that TARP subtypes might have different binding sites in the AMPAR complex (Greger, Watson, and Cull-Candy 2017) on the basis that, for example, only two γ4 can co-assemble with AMPARs as seen with single-molecule photobleaching in live cells (Hastie et al. 2013).

On the other hand, at hippocampal CA1 neurons there is almost exclusively expression of heteromeric CI-AMPARs (Wenthold et al. 1996; W. Lu et al. 2009) and present a 2-TARPs stoichiometry together with 2 CNIHs (Gill et al. 2012). However, this stoichiometry might not be possible in CGCs due to the lack of cornichon homolog proteins (Schwenk et al. 2009). The other auxiliary subunit that could modulate TARP that is expressed in CGCs is γ7, which could potentially be playing a role at somatic AMPARs although it has been shown to selectively suppress somatic CI-AMPARs in CGCs (Studniarczyk et al. 2013) and not to have an important involvement in excitatory transmission (Yamazaki et al. 2015). In our experiments, the absence of resensitization, a γ7 hallmark (Kato et al. 2007), in CGC somatic patches rules out γ7 functional presence (Results, figure 16). In addition, recent reports show evidence that this

recovery of the current upon prolonged agonist application might be a common feature of all TARPs (Carbone and Plested 2016) including γ2. However, resensitization is a characteristic feature that indicates the presence of 4-TARPed AMPARs (Kato et al. 2010c). Indeed, Purkinje cells from stargazer mice, with low γ7 stoichiometry lack this feature (Gill et al. 2012). In summary, absence of resensitization, a characteristic signature of a fully TARPed receptor, reinforces the view of a 2-TARPed conformation in CGCs.

Nevertheless, comparing the results in expression systems with recordings in CGCs, it was found a stinking difference when comparing the recovery from desensitization from 2-TARPed (A2) AMPARs with somatic AMPAR from CGCs. In tsA201 cells it was observed that the 2-TARPed stoichiometry (with γ2 fused to GluA2) displayed the slowest recovery from desensitization (results, figure 42) but somatic CGC responses displayed even a slower recovery rate. This parameter has an important role neuronal biology has it has been reported to have implications in a physiological context along with desensitization due to their impact on high-frequency transmission (A. Arai and Lynch 1998). The use of AMPA as agonist in CGC experiments to avoid activate other glutamate receptors (especially kainate receptors present in CGCs (Bahn, Volk, and Wisden 1994; Belcher and Howe 1997)) might account for the slow recovery since this agonist is known to speed entry into desensitization and to slow recovery, relative to glutamate (Zhang et al. 2006). However, the use of AMPA as agonist in the 2T(A2) condition did not slow recovery from desensitization compared with glutamate (results, figure 47). A possible explanation can be the presence of γ2 into the AMPAR. In the absence of TARPs, it has been demonstrated that an inverse relationship between the affinity of the agonist and recovery from AMPAR desensitization exist (Zhang et al. 2006). As seen in other publications, γ2 causes a drastic reorganization of the complex and a consequence of the presence of γ2 is the increased affinity of the receptor for glutamate (Priel et al. 2005; Tomita et al. 2005). Therefore, the change in the affinity for glutamate induced by γ2 that might potentially explain that recovery from desensitization in these experiments' conditions, was not changed when stimulating with AMPA or glutamate.

On the other hand, the use of AMPA *vs.* glutamate may also potentially alter the outcome of other properties. When we checked both agonists on GluA4c, single-channel conductance was not changed (16.63 ± 1.06pS, 17.54 ± 1.21pS; 14.40 ± 1.87pS for GluA4 and GluA4c using glutamate and GluA4c using AMPA; Kruskal-Wallis multiple comparison test; figure 60A) . Similar channel conductance estimates were reported, regardless of the use of AMPA or glutamate as agonist (Swanson, Kamboj, and Cull-candy 1997). Moreover, for GluA2/GluA4, similar conductance values have been described for both agonists (5.5–6 pS) (Swanson, Kamboj, and Cull-candy 1997), matching the values obtained in the results of this thesis and other studies (Jackson and Nicoll 2011) using glutamate as agonist. Conversely, in experiments performed with GluA4c subunit, AMPAR desensitization kinetics seemed to be significantly slower when AMPA was used as agonist (3.74 ± 0.49ms, 3.97 ± 0.38ms; 8.07±0.99ms for GluA4 and GluA4c using glutamate and GluA4c using AMPA; Kruskal-Wallis multiple comparison test; figure 60B below)despite previous reports indicating that the kinetic properties of AMPA-activated GluA4 homomers were comparable to those activated by glutamate (Swanson, Kamboj, and Cull-candy 1997). In principle, this might confuse the interpretation when comparing recordings in expression systems (glutamate used as agonist) with neurons (AMPA used as agonist). However, the kinetics of the currents evoked with AMPA in CGCs were fast; indeed, as rapid as the quicker responses observed in expression systems with glutamate. Therefore, it would be expected that the AMPAR-mediated responses in CGCs using the agonist glutamate would be even faster than using AMPA as agonist (in any case kinetics would be overestimated) which still rules out that the possible TARP stoichiometry present in CGCs were any of the slow combinations: 4 TARPs or 2 TARPs attached to GluA4c.

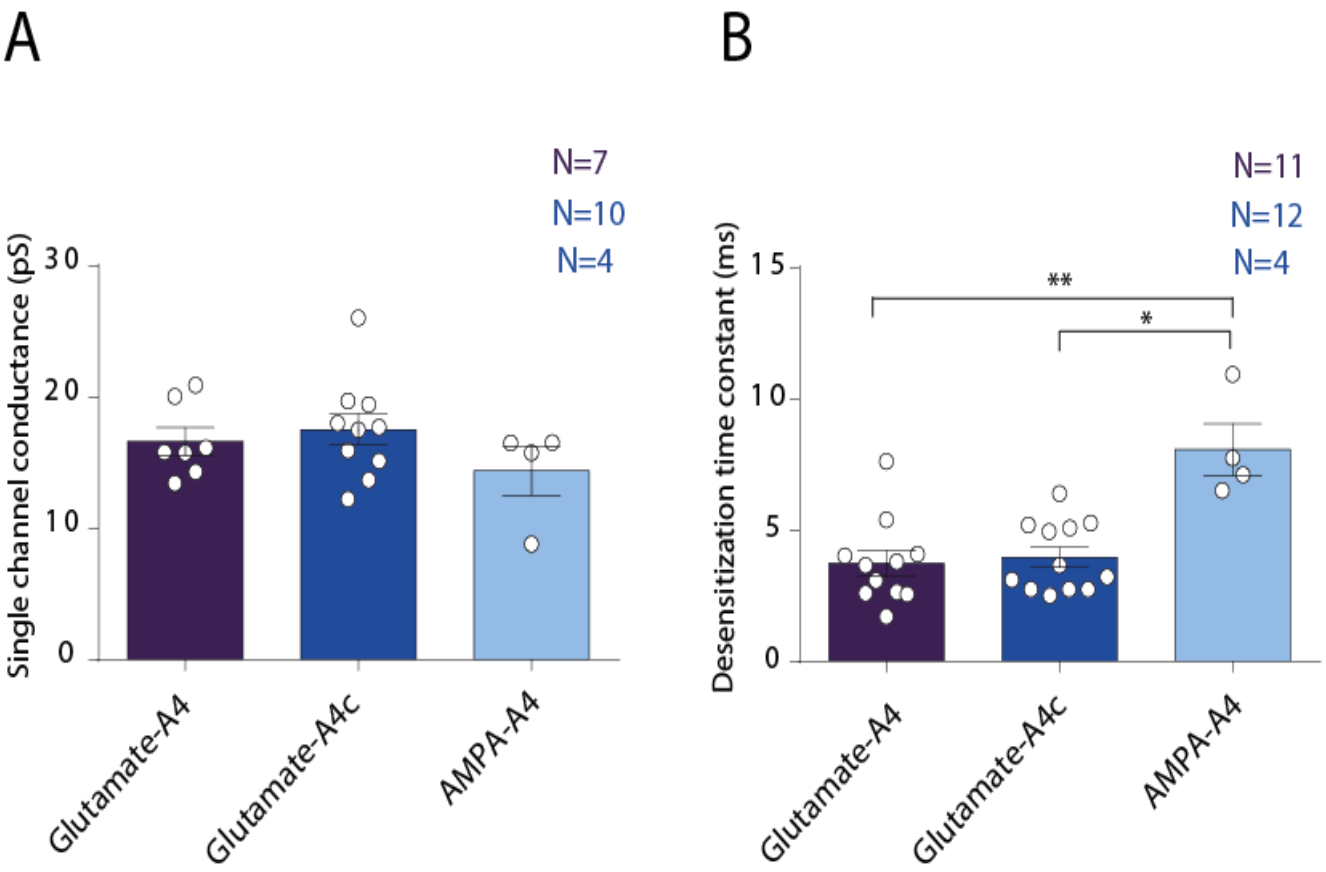


Figure 59. Comparison of evoked responses in GluA4 using glutamate or AMPA as agonist. (A-B) Pooled data comparing single-channel conductance and desensitization kinetics using glutamate and AMPA as agonists. Desensitization kinetics is slowed using AMPA as agonist (B) but no significant differences were found in single-channel conductance using both agonists.

On the other hand, the strikingly slow recovery of the currents in CGCs could be also attributed to the presence of CKAMPs in the native AMPAR complex since this biophysical property has been reported to be strongly reduced either in neurons and heteromeric recombinant AMPARs by the members of the CKAMP family (Farrow et al. 2015a; von Engelhardt et al. 2010; L. J. M. Schmitz et al. 2018). In cerebellum there is expressed one of the members of the CKAMP family, CKAMP39, (Farrow et al. 2015a; von Engelhardt 2019). In addition, CKAMP39 has been reported to reduce AMPAR peak amplitude expressed heterologously (Farrow et al. 2015a). Considering the reported absence of other members of the TARP family in CGCs, the functional absence of CNIH2 and the really low expression of other AMPAR auxiliary proteins as GSG1L and CKAMP44 (Zeisel et al. 2018), CKAMP39 was an excellent potential candidate. However, in our hands, when expressing GluA2:γ2 + GluA4c + CKAMP39 in tsA201 cells it was not possible to record AMPAR responses in neither patches nor whole-cell

configuration. This fact agrees with the previous mentioned work from Farrow et al. 2015 where it was described that CKAMP39 drastically decreases AMPAR currents when co-expressed in heterologous system. Indeed, with other group with more experience working with this family of auxiliary subunits it was confirmed the difficulty to record currents in such experiments. In addition, the lack of a visible tag in the CKAMP39 vector did not allow to ensure whether tsA201 cells were transfected with the 3 plasmids (GluA2:γ2 + GluA4c + CKAMP39). For this reason, it was not possible to discriminate if there was an effect of CKAMP39 in the rarerly occasional currents found in these experiments (2 over 12 in whole-cell, 1 over 11 in outside-out patches). Thus, in future works it will be suitable to knockdown CKAMP39 expression in CGCs to observe whether recovery from desensitization is altered without modifying other channel biophysical properties.

6. CPT1C role in AMPAR-TARP stoichiometry

To understand AMPAR biology, we must understand not only their biophysical properties once the receptor is at membrane but also their trafficking and assembly, which will ultimately determine their role. AMPAR biogenesis is a complex mechanism (Greger and Esteban 2007; Pick and Ziff 2018) where several proteins interact with AMPARs during their synthesis and release from the ER (Y. Hayashi et al. 2000; S.-H. Shi et al. 2001a; Ziff 2007; Brechet et al. 2017a; Gratacòs-Batlle et al. 2018a; Schwenk et al. 2019). In this line, proteins such as CPT1C or ABHD6 may play key role as they intervene forming an AMPAR-TARP priming complex (Schwenk et al. 2019). Moreover, the implication of this ER resident proteins seems not to be essential for basic AMPAR function but important for nervous system physiology. Once analysed AMPAR-TARP stoichiometry effect in receptors biophysical properties it was explored the effect of CPT1C in this context in heterologous systems to try to understand whether CPT1C could determine AMPAR:TARP stoichiometry due to its role in priming AMPARs with TARPs. CPT1C has been reported previously to selectively interact with GluA1-containing AMPARs and enhance their delivery to cell membrane (Gratacòs-Batlle et al. 2014, 2018a) but these works did not explore that possibility . In the experiments

carried out here to test that, no differences were seen in AMPAR biophysical properties when co-transfecting GluA1 and γ2 in presence or absence of CPT1C in tsA201 cells, indicating a putative same composition in terms of number of TARPs accompanying the AMPAR. These set of experiments allowed to infer that CPT1C has a role not a prominent role determining AMPAR-TARP stoichiometry at least at the conditions applied. Nevertheless, taking into account that CPT1C forms a priming complex for GluA subunits in association to other proteins it is possible that the lack of these proteins in heterologous systems did not permit CPT1C exerts its complete role. In addition, in expression systems it was not possible to see a synergic action of GluA1:γ2 homotetramers with CPT1C in terms of AMPAR trafficking to cell membrane. However, moving to more physiological model, where there is no lack of CPT1C partners at ER, in CGCs from CPT1C KO mouse, AMPAR peak responses were diminished in somatic patches (which proportionally are a considerable sample of the whole cell). Here arise the question whether this ER resident protein interacts preferentially with long GluA subunits (GluA1 or GluA4) since till date it has been published to enhance AMPAR traffic in GluA1-containing receptors (Gratacòs-Batlle et al. 2014, 2018a). On the other hand, comparing responses from somatic patches at this neuronal type from WT or CPT1C KO mouse, no differences in biophysical properties were found when desensitization kinetics or single-channel conductance. This fact probably implies a predominant role of CPT1C enhancing AMPAR traffic instead of modulating AMPAR-TARP configuration. To completely rule out CPT1C involvement in AMPAR:TARP stoichiometry, and due to the preference of CPT1C for GluA1, this possibility might be tested in other neuronal type as for example hippocampal pyramidal neurons, where GluA1 is an importantly expressed AMPAR subunit. However, AMPAR-mediated mEPSCs recorded in CPT1C deficient animals did not shown differences in deactivation kinetics (Fadó et al, 2015), which is in favour of CPT1C not been an important player in AMPAR:TARP stoichiometry. Studies of single channel conductance of these mEPSCs (by using peak-scaled non-stationary noise analysis) would help to completely corroborate previous findings.

7. De novo GRIN mutations in NMDAR function

NMDAR receptors are widely expressed glutamate receptors along CNS and are highly permeable to Ca^{+2} but also to Na^{+} and K^{+} (Mayer and Westbrook 1987). However, the Ca^{+2} influx through these receptors is one of the characteristic features of this ligand-gated channels. The Ca^{+2} influx through NMDARs triggers most of the NMDAR-dependent physiological and pathological effects. This property made NMDARs of special interest due to their role in synaptogenesis, synaptic plasticity and learning and memory (Michael Hollmann and Heinemann 1994; Lau and Zukin 2007; Sanz-Clemente, Nicoll, and Roche 2013). For this reason, abnormal function of these receptors is implied in several pathologies that involve the CNS.

Apart from other commonly known pathologies such as Alzheimer or Parkinson disease where NMDARs can be involved (see for reviews **Mellone and Gardoni 2013; R. Wang and Reddy 2017)**, the GRIN-related disorders are a "perfect stranger" for the common population. That is not surprising since disorders associated with GRIN genes are a group of genetic pathologies considered as a rare disease. Indeed, till date, about 500 individuals have been reported to have a GRIN-related disorder (www.grin-database.de). However, the number of cases can be easily underestimated as the phenotypes present a wide range of clinical symptoms. Interestingly, the new techniques for genome sequencing allowed to further investigate these cases and report it when a GRIN variant is potentially the cause of the whole symptomatology of the patient. At this aspect the new approaches in cellular and molecular biology permit to study functionally the different pathological GRIN variants that affect NMDAR. The main objective during this thesis was to analyse how biophysical properties where modified in different GRIN variants. The study of these clinical-reported variants from a functional approach permit a better understanding of the pathology in terms of NMDAR signalling and then to investigate for future pharmacological treatments (Soto et al. 2019).

8. GluN truncated isoforms

Some of the GRIN variants analysed that were reported as clinically pathological were caused by a premature stop in GluN subunit synthesis. These pathological variants affected GluN2 subunits but not GluN1. In the results presented here it was found that truncated forms in GluN2 subunits reduced peak amplitude in heterologous system when co-expressed with WT forms, similarly when the amount of cDNA transfected for GluN2 subunits was reduced in a 50%. These condition simulate an haploinsuficience phenotype and in addition the truncated variants seems to not be functional when form a tetramer with GluN1 subunit seen by other approaches different than patch clamp recordings (Santos-Gómez et al. 2020). The idea that truncated GluN2 forms do not affect NMDAR biophysical properties, apart from the diminishment of peak currents, is supported by the analysis of deactivation kinetics where no differences were observed comparing WT and truncated conditions. This suggests that the receptors recorded when WT and truncated forms were co-transfected were indeed WT NMDARs. Altogether, the GluN2 truncated forms can be classified as LoF variants due to their effect on receptors peak currents (even not being present in the channel complex). The reduction of the peak amplitude implies a diminishment in the current area generated evoked by the receptor with a consequent alteration of the transmission signalling.

On the other hand, different results were obtained with the obligatory subunit GluN1. Truncated variants at this subunit did not show a diminishment in peak amplitude currents or variation in deactivation kinetics. This agrees with the fact that truncated GluN1 forms are associated with non-pathological phenotypes described for this variants (Santos-Gómez et al. 2020).

9. GRIN missense mutations

The other group of GRIN variants analysed correspond to a set of mutations caused by single amino acid modification. Different from GluN2 truncations, the missense variants can confer a gain or a loss of function to the receptor depending on the amino acid change and also depending on the concrete position affected by this modification (Li et al. 2019). Indeed, in GluN1 and GluN2A near the 40% of the annotated pathological variants are a GoF (García-Recio et al. 2020). In the present work, various GRIN missense mutations were studied to uncover how NMDAR function was altered. Most of the variants studied confer to NMDAR a loss of function of the receptor when it has 2 copies of the mutated subunit. 3 of the mutations analysed that affected GluN1 and 11 from GluN2B were classified as a LoF due to the dramatic effect over normalized peak currents. In addition, the M824V mutant from GluN2B did not reduced NMDAR peak current but showed faster desensitization kinetics and reduced steady-state currents. Both characteristics diminished total current area altering NMDAR signalling. In this cases it is suggested to use as treatment that enhance NMDAR activity of both WT receptors and/or receptors with mutated subunits (Soto et al. 2019). Indeed, positive allosteric modulators for NMDAR can be suitable candidates to restore partially receptor function (W. Tang et al. 2020). A treatment that could improve receptors function can be key to improve patient's life quality. For this reason, it is important to analyse how a missense mutation affects receptor properties. In addition, in case of GluN1 and GluN2B the need for a quick diagnostic can be important to improve patient's condition during early stages of life as GluN1 is expressed throughout life and GluN2B seems to be widely expressed during early life stages (Cohen and Greenberg 2008; Hall, Ripley, and Ghosh 2007).

On the other hand, related to the importance of a correct diagnostic, some variants can induce a GoF in NMDAR function resulting in pathologic condition. In this case, enhancing receptors function with some treatments can be counterproductive. In the results obtained it were found that 2 variants were classified as GoF. On the one hand, the M641V from the GluN1 subunit was considered as a GoF due to an increase in the total area of current when the receptor was activated. This increment was produced by a slow deactivation time constant. The other variant analysed that produced a GoF

was also found in GluN1 subunit and the gain was correlated to a dramatic diminishment in the Mg^{+2} block. This change implies an over activation of the receptor which in physiological conditions is blocked by this divalent ion until the membrane potential is not depolarized by AMPAR activation. Altogether, the release of Mg^{+2} block can alter considerably neuronal signalling. In cases of GoF it will be suitable to block or inhibit NMDAR response. Indeed, there are drugs that are already approved to use to inhibit NMDAR activity like memantine (Jon W Johnson and Kotermanski 2006). Other memantine-like compounds tested in our laboratory might be potential candidates in the future to treat GoF GRINpathies (Leiva, Phillips, Turcu et al, 2018).

The final summary in the analysis of the GRIN variants associated to GRIN disorders can be that most of the variants commonly lead into a receptor LoF, however, the GoF cannot be discarded but it seems that, at least in NMDARs, amino acid changes in the sequence preferentially induce a LoF than a GoF. However, a recent published work summarizing the annotated variants analysed till date indicates that a considerable number of variants in GluN1 and GluN2A are a GoF. By contrast near 75% of GluN2B variants lead into a LoF. Moreover, most the missense mutations analysed compromises amino acids from TMDs or LBD what may indicates that these are especially sensitive domains for NMDAR correct function.In addition, the variations analysed did not show a complex pattern with a GoF in a parameter but a LoF in another with a same amino acid change. This unprovable but not disposable case would imply to analyse the whole current area to give a conclusion whether this hypothetic case is a GoF or LoF.

10. Final remarks

The overall results given in thesis project show in a small scale how complex and regulated is the nervous system function. Even studying a very basic but fundamental components of the synaptic transmission it is still needed further research to understand how the different AMPAR interacting proteins intervene in function and how can the study of GRIN-related disorders can improve NMDAR function. Indeed, is the complexity of our nervous system what makes it attractive and sometimes surprising. For this reason, the understanding its single components is mandatory to know how it works.

6. Conclusions

6. Conclusions

This thesis work explored new insights into ionotropic glutamate receptors exploring biophysical properties of AMPARs and NMDARs. AMPARs were studied in relation with TARPs, their prototypical auxiliary subunits, and their relation in a stoichiometry dependent manner. Moreover, it was explored the possible role of another AMPAR interacting protein, CPT1C, in AMPAR-TARP relationship. Differently, NMDARs were studied in the frame of *de novo* GRIN variants that affect receptor biophysical properties and are reported to cause GRIN related disorders. All the experiments and work performed allowed to conclude:

1. The prototypical type Ia TARPs modulate GluA1 homotetramers in stoichiometry dependent manner in heterologous system.
2. Type Ia TARPs modulates biophysical properties in GluA1 homotetramers in a graded fashion or in a 'all-or-nothing' manner depending on the intrinsic property of the receptor modulated.
3. GluA1 homotetramers modulation by AMPAR-TARP stoichiometry shows different behaviour depending on type Ia TARP present in AMPAR complex.
4. Type Ib TARPs can modulate CP-AMPAR properties in GluA1 homotetramers with a 1:2 AMPAR-TARP stoichiometry at all biophysical parameters studied (except peak open-probability and rise-time) different from type Ia TARPs.
5. Peak-open probability or rise-time are not modified depending on AMPAR-TARP stoichiometry with any TARP in GluA1 homotetramers.
6. Biophysical properties of GluA4 and GluA4c homomers do not differ.
7. In GluA2-GluA4c heteromers (CI-AMPARs) expressed in tsA201 cells, γ2 differentially modulates AMPAR biophysical properties and pharmacological response to perampanel depending on TARP position at the AMPAR complex.
8. Somatic AMPARs from CGCs in primary culture behaves like 2 TARPed CI-AMPARs (γ2 linked to GluA2) studied in expression systems in terms of single-channel conductance and desensitization kinetics.
9. Recovery from desensitization behaves differently in somatic CI-AMPARs from CGCs than CI-AMPARs expressed in heterologous system.

10. The change of a cysteine for a lysine in position 550 annulated γ2 effect over desensitization kinetics and single channel conductance in GluA4c homotetramers.
11. γ2 does not modulate CI-AMPARs interacting with TMDI when linked to GluA2 but the interaction with the same region modulates single-channel conductance when linked to GluA4c in 2 TARPed CI-AMPARs (C550L amino acid change).
12. CPT1C does not enhance membrane trafficking of fully γ2 TARPed GluA1 homotetramers in expression systems.
13. In heterologous system AMPAR-TARP stoichiometry is not affected by presence of CPT1C when cells are co-transfected with GluA1, γ2 and CPT1C.
14. Somatic CI-AMPARs in CGCs from WT mouse show no significant differences in biophysical properties analysed compared the ones expressed in CGCs from CPT1C KO mouse.
15. CPT1C enhances AMPAR membrane traffic from GluA2-GluA4c heterotetramers in CGCs from WT mouse compared to CGCs from CPT1C KO mouse.
16. GluN2A and GluN2B truncated isoforms cannot form functional receptors expressed heterologously.
17. GluN2A and GluN2B diminish normalized peak currents when co-expressed with WT forms.
18. GluN1 truncated forms do not diminish normalized peak currents when co-expressed with WT GluN1.
19. GRIN missense variations at different GluN subunits usually lead into loss of function of NMDARs; however, the gain of function of the receptor even though unprovable is feasible.

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Acronym list

2-AG	2- arachidonoylglycerol
7-CK	7-Chlorokynurenic acid
ABHD6	α/β-hydrolase domain-containing 6
AD	Alzheimer's disease
ADAR	Adenosine deaminases
AMPA	α-amino-3-hidroxi-5-metilo-4-isoxazolpropiónico
AMPAR	α-amino-3-hidroxi-5-metilo-4-isoxazolpropiónico receptor
AP5	Amino-5-phosphonopentanoate
BSA	Bovine serum albumin
CA1	cornu Ammonis 1
CaMKII	Calmodulin kinase II
CASK	Calcium/calmoudulin-dependent serine protein kinase
CGC	Cerebellar granule cell
CI-AMPAR	Ca+2-impermeable AMPAR
CKAMP	Cysteine-knot AMPAR modulatory protein
CNI	Cornichon
CNIH	Cornichon homolog
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CP-AMPAR	Ca+2-permeable AMPAR
CPT1A-C	Carnitine palmitoyltransferase 1A-C
cryo-EM	Cryo-electron microscopy
CTD	Carboxi-terminal domain
CTZ	Cyclothiazide
DLG	Cryo-electron microscopy
DMEM F12	Dulbecco's Modified Eagle's Medium Mix F12
DMSO	Dimethyl sulfoxide
E14	Embrionic 14
EPSC	Excitatory post synaptic currents
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FRRS1L	Ferric-chelate reductase 1-like
GABA	Gamma-aminobutyric acid
GCG1L	Germ cell-specific gene 1-like protein
GFP	Green fluorescent protein
GlcNA	N-acetylglucosamine
GluA	Glutamate AMPA
GluK	Glutamate Kainate
GluN	Glutamate NMDAR

GluR	Glutamate receptor
GoF	Gain of function
GPCR	G protein-coupled receptors
GRIN	
GRIP	Glutamate receptor-interaction proteins
GWA	Genome-wide association
HA	Hemagglutinin
HEK293	Human embryonic kidney 293
iGluR	Ionotropic glutamate receptors
IV curve	Current-voltage curve
JSTX	Joro spider toxin
KAR	Kainate receptor
KO	Knockout
LBD	Ligand binding domain
LoF	Loss of function
LTD	Long-term depresion
LTP	Long-term potentiation
MAGUK	Membrane-associated guanylate kinase
mEPSCs	Miniature excitatory post synaptic currents
mGluR	Metabotropic glutamate receptors
MPP	plamitoylated membrane protein
mRNA	messenger RNA
NAM	Negative allosteric modulator
NASPM	1-Naphthylacetyl spermine
NBQX	2,3-dioxo-6-nitro-7-sulfamoilbenzo(f)quinoxaline
NMDA	N-metil-D-aspartic acid
NMDAR	N-metil-D-aspartic acid receptor
NTD	Amino-terminal domain
OPC	Oligodendrocyte progenitor cells
P14	Postnatal 14
PA	Polyamine
PAM	Positive allosteric modulators
PAT	Palmitoyl acyl transferases
PBM	PDZ-binding motif
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PICK1	Protein interacting with C kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PORCN	Porcupine
PSD	Post synaptic density
PSD-95	Post synaptic density - 95
RT-PCR	Reverse transcriptase - polimerase chaint reaction
SBTI	Soybean trypsin inhibitor

TARP	Transmembrane AMPAR regulatory protein
TCP	thienylcyclohexylpiperidine
TDAA	TARP-dependent AMPAR antagonist
TMDI-IV	Transmembrane domain I-IV
VDCC	Voltage dependend calcium channel
wnt	Wingless Int
WT	Wild type