UNIVERSITAT DE BARCELONA

Novel regulation of AMPA receptor function by interacting protein CPT1C

Natalia Yefimenko Nosova

UNIVERSITAT DE BARCELONA

PhD Thesis

Novel regulation of AMPA receptor function by interacting protein CPT1C

Programa de Doctorat en Biomedicina
Departament de Patologia i Terapèutica experimental
Campus de Bellvitge
UNIVERSITAT DE BARCELONA

NATALIA YEFIMENKO NOSOVA

Barcelona, 2016

Director de tesis: David Soto del Cerro

Tutor de Tesis: Carles Solsona Sancho

Al meu fill, Ivan

Не трогать веру нельзя. Она — основа религии, а любая религия в ваши дни — грубое и примитивное суеверие. Суеверие есть уверенность, на знании не основанная. Наука борется с суевериями, как свет с потемками…Дмитрий Иванович Менделеев.

The most all penetrating spirit before which will open the possibility of tilting not tables, but planets, is the spirit of free human inquiry. Believe only in that. Dmitri Ivanovich Mendelev

This is it. To enrol as a PhD student was the best idea and the worst idea I ever had. Since I started my PhD I lost a lot and I found more. I learnt. I met wonderful people. I fulfilled a commitment I made when I was a little girl.

Primer de tot i sobre tot voldria agraïr al meu director de tesi, David Soto. Després de totes les peripècies, he tingut la sort de trobar-te en el meu camí professional i aprendre com alumne i persona. Em faltarien pàgines per dedicar-te, només vull que sàpigues que recordo totes i cada una de les alegries que hem viscut. També totes les dificultats que hem tingut i com les hem superat. Tu, l'Esther i jo. A l'Esther, la meva companya, moltes gràcies per ensenyar-me el que saps i per fer-me costat en els moments més difícils. El nostre petit grup ha experimentat tot tipus de vivencies però m'enduc un dels millors records de la meva vida – aquests anys amb vosaltres, la meva petita familia científica.

Als meus companys de laboratory – l'Anna, la Krys, la Eli, l'Helena, el Franico, en Joni, el Benja, el Pablo, la Ceci – sou genials. Tot el que he viscut amb vosaltes es pot resumir en una sola paraula – felicitat. Als companys de mojitades i activitats varies dels labs adjacents i el passadis de la 4º planta gràcies per tots els somriures i riures que hem compartit. May the fourth (floor) be with you!

I also would like to thank Professor Stuart Cull-Candy and Professor Mark Farrant. I had such amazing stay at your laboratory and I learnt from you and your colleagues. My fantastic labmates, Ian, Valentina, Dorota, Steve, Sarah, Maz, Karolina, Andrew, Rebecca, Cecile, Julia and Tommy, thank you for all your time and effort to make me feel so good.

Durant aquests tres anys he tingut uns moments genials amb les noietes de Novico, la Dolors, la Bego i la Blanca, embolicant-vos amb tot tipus d'activitats (i les que vindran!). Con la gente de Sant Joan Despí, Miguel i Ana, Juande y Mari Carmen, Maria y Àngel y los de SpiderBikes, haciendo rutas a distancias cortas desde el bar de la patata hasta el Jordi. Con la Maria i los chicos de la clase de fotografía, espero hacer muchas más i mejores fotos juntos. A mi reina inca, la Mari, muchísimas gracias por todo el apoyo que me has dado durante estos años. Si ti este camino no sería igual.

Hi ha un capítol especial en la meva vida els últims mesos – la colla de Synapse. Gràcies per tots els riures i els ànims durant la recta final de la tesi.

Спасибо моей дорогой и любимой Насте (и твоей семье) за всю любовь и поддержку. Ты часть моей семьи и моей души. Ты мой человек!

Моей любомой сестре Оле, за то что ты есть. Конечно же моей Кате за твою любовь. Жаль что не могу сказать папе спасибо, но где бы ты ни был, я всегда помню как ты хотел чтобы твоя дочка стала доктором. Медицинским доктором я не стала, но я все же думаю что ты бы гордился бы мной. Никаких слов не хватить чтобы выразить мою благодарность моей маме. Ты дала мне жизнь и все возможности чтобы я смогла вополотить в жизнь мои мечты. Спасибо тебе за твое терпение.

També voldria donar les gràcies al Pep, el meu amor. T'ha tocat la época més difícil de la tesi i m'has donat tot el suport que necessitava. Gràcies per la teva paciència.

A l'Ivan, el meu fill. Has crescut molt durant els anys de la tesi. Has vist que el resultat val la pena tot l'esforç. Espero que et serveixi en la teva vida. Saps que pots fer moltes coses (i una mica més) i sempre em tindràs al teu costat.

Natasha Yefimenko,

Barcelona, 19 de maig del 2016

Index

1. INTRODUCTION

1.1. Introduction: the glutamate story

Nowadays the non-essential amino acid glutamate (Glu) is widely recognized as the predominant excitatory neurotransmitter in the central nervous system (CNS). Studies of glutamate and its role in neuronal transmission, development and disease have a long history. Early research showed that brain tissue possesses high concentrations of glutamate together with a high glutamate uptake activity (Krebs, 1935; Stern et al., 1949). The first indication of the physiological role of glutamate was the observation by Hayashi where injections of glutamate into brain produced convulsions, hence proposing that glutamate might act as a neurotransmitter (Hayashi, 1954). Following this first observation, the excitatory action of L-glutamate on single cells *in vivo* was described (Curtis et al., 1960). However, it took several years and various studies to finally recognize glutamate as a synaptic transmitter.

Firstly, several years of experiments were needed for the recognition of glutamate receptors (GluRs) diversity. One important milestone for glutamate receptors distinction was the synthesis of specific antagonists related to glutamate (Figure 1.1). Among these, the first one was D-isomer of N-methyl-aspartate (NMDA), which was shown to be 10 times more potent than L-glutamate and served to name that glutamate receptor subclass (Curtis and Watkins, 1960). The next finding in NMDA receptor research was made by Evans in 1977 through the observation that Mg^{2+} and other divalent cations limited channel conductance in a voltage-dependent manner (reviewed by Watkins and Jane, 2006). Later, the glycine requirement as a co-agonist was demonstrated (Johnson and Ascher, 1987). In addition to NMDA, Shinozaki and Konishi showed the structural similarity of kainic acid with glutamate and identified an excitatory action of kainate in the mammalian CNS (Shinozaki and Konishi, 1970). Four years later, quisqualic acid was described (Shinosaki and Shibuya,

1974), which resulted in a potent excitatory agent, leading to the discovery of a subclass of glutamate receptors: quisqualate receptors. Years later this receptor was renamed to α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) (Krogsgaard-Larsen et al., 1980), after some experiments where AMPA was able to excite neurons through distinct receptors from those activated by kainate. Thus, in parallel with glutamate receptors functional determination their classification and nomenclature were changing based on the physiological responses as either sensitive or insensitive to these agonists. Thereby, these receptors were firstly stratified in two classes of ionotropic glutamate receptors termed NMDA and non-NMDA receptors, where non-NMDA receptors were sub-classified into quisqualate and kainate receptors. After Krogsgaard work published in 1980 three classes of glutamate receptors were established: NMDA, AMPA and kainate receptors, named after their prototypical agonists (Monaghan et al., 1989).

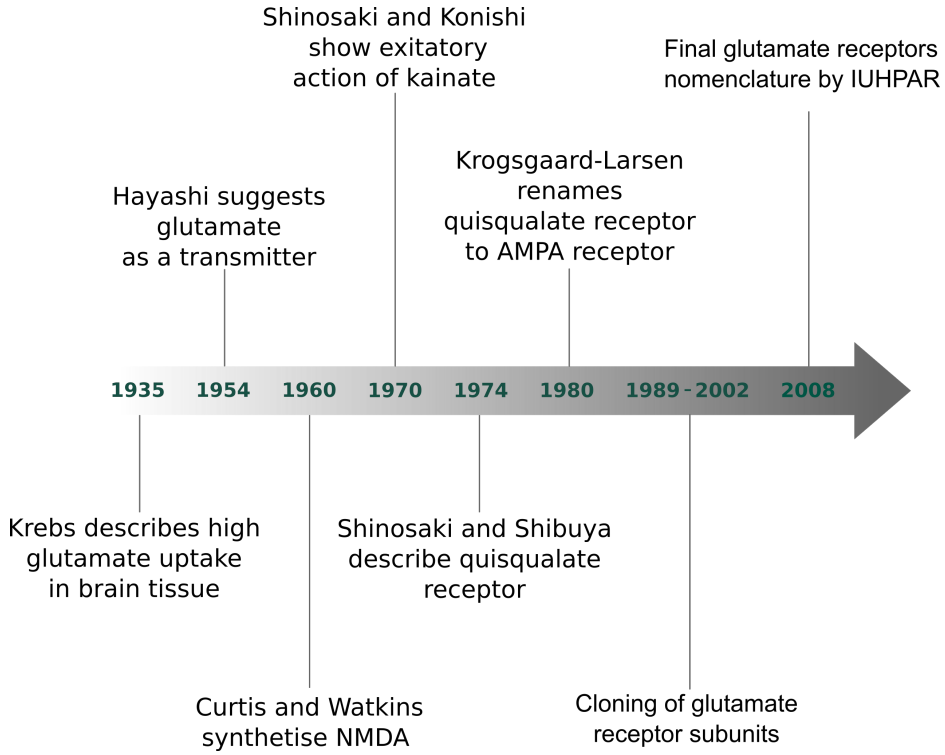

Figure 1.1. Glutamate research milestones.

Another crucial milestone in glutamate receptors investigation was the great development of molecular biology, in particular receptor cloning techniques, which revealed the structure and helped elucidating the function of multiple glutamate receptors throughout the 1990s. The earliest glutamate receptor was cloned and sequenced by Heinemann's group in 1989 (Hollmann et al., 1989). The subunit was named GluR-K1 based on its sensitivity to kainate. However, after performing binding studies it was shown that it was indeed an AMPA receptor (AMPAR) (Sommer et al., 1990). Following those works, further AMPAR subunits were cloned by distinct groups in a short period of time. Three more subunits were sequenced and named GluR5-7, showing kainate-preferring properties and about 70% homology between them. Also subunits with higher kainate affinity were cloned (KA1 and KA2), which could form a functional channel together with GluR5-6 only. In parallel with these works, NMDA subunits were cloned between 1991 and 2002 by different groups. Moreover, several proteins were cloned showing glutamate binding properties (δ1 and δ2), nevertheless these proteins could not form functional channel with other subunits (reviewed by Lodge, 2009). Finally, in 2008 the International Union of basic and clinical pharmacology (IUHPAR) agreed the new and final glutamate receptors nomenclature (Table 1.1).

The assumption that the neurophysiological effects of glutamate were mediated exclusively by ionotropic glutamate receptors was considered until Sladeczek and colleagues in 1985 reported that glutamate could induce the formation of molecules that belong to a major second messenger system (Sladeczek et al., 1985). After that report, Nakanishi laboratory cloned in 1992 the first metabotropic glutamate receptor. Subsequent studies, using molecular cloning techniques, unravelled up to eight mammalian metabotropic glutamate receptors, which share a common molecular morphology (Masu et al., 1991).

IUPHAR subunit nomenclature	Previous nomenclatures
AMPA receptors	
GluA1	GLUA1, GluR1, GluRA, GluR-A, GluR-K1, HBGR1
GluA2	GLUA2, GluR2, GluRB, GluR-B, GluR-K2, HBGR2
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, NR2A, GluRε1
GluN2B	GLUN2B, NMDA-R2B, NR2B, hNR3, GluRε2
GluN2C	GLUN2C, NMDA-R2C, NR2C, GluRε3
GluN2D	GLUN2D, NMDA-R2D, NR2D, GluRε4
GluN3A	GLUN3A, NMDA-R3A, NMDAR-L, chi-1
GluN3B	GLUN3B, NMDA-R3B
Delta receptors	
GluD1	GluRδ1
GluD2	GluRδ2

Table 1.1. Glutamate receptors nomenclature by IUHPAR (2008).

On-going studies revealed a wide range of GluR functions beyond excitatory post synaptic currents (EPSC) generator in neuron-to-neuron communication. In early stages they are key players in brain development: they are related to neuronal migration, differentiation, axonogenesis and neuronal survival (Balázs et al., 1988, Wilson and Keith, 1998, Komuro and Rakic, 1993). In the mature nervous system GluRs are involved in plasticity changes, where the alterations in synaptic structure and efficacy are responsible for many neurological functions, including cognition, memory, behaviour, movement and sensation. Finally, in a variety of pathological

conditions, persistent and overwhelming activation of GluRs mediate neuronal injury and death (reviewed by Cull-Candy et al., 2006; Isaac et al., 2007; Liu & Zukin, 2007).

1.2. Glutamate receptors: overview

Glutamate receptors are classified into two groups according to the functional properties of these channels: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).

iGluRs are a major class of ligand-gated ion channels, which mediates the majority of the excitatory neurotransmission in the CNS. They share a common tetrameric pore-forming modular structure (Rosenmund et al., 1998). Each of the four receptor forming subunits have several functional domains such as the large amino-terminal or N-terminal domain (NTD), the ligand-binding domain (LBD), the transmembrane domains (TMD) and the C-terminal domain (CTD) (Figure 1.2). The ionotropic glutamate receptors are divided into NMDA, AMPA and kainate receptors (KARs), based on their biophysical properties and molecular profile.

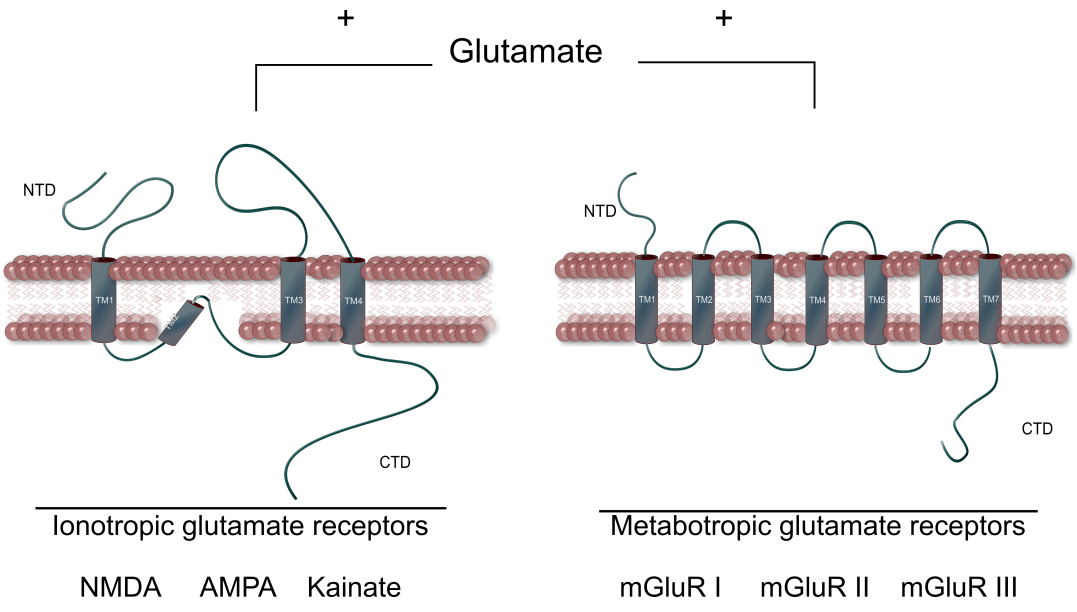

Figure 1.2. Glutamate receptors classification.

The second group encloses mGluRs belonging those to the G-protein-coupled receptor family and which are membrane-bound proteins activated by extracellular glutamate. The members of this glutamate receptors group consist of a single protein that has seven transmembrane spanning regions (Figure 1.2). Based on their pharmacology, physiological effects and sequence homology, mGluRs were subdivided in three groups. Thus, Group I (mGluR1 and mGluR5) activate phospholipase C and Group II (mGluR2 and mGluR3) and Group III (mGluR4, 6, 7 and 8) inhibit adenylyl cyclase. They are widely distributed throughout the CNS and in neurons can be found either at presynaptic or at postsynaptic level. Unlike iGluRs, metabotropic glutamate receptors have mainly a modulatory role and are responsible for fine-tuning of glutamatergic transmission (Niswender & Conn, 2010), although they are also involved in long term plasticity processes (Malenka and Bear 2004; Bellone et al., 2008; Gladding et al., 2009).

1.3. The ionotropic glutamate receptor family

1.3.1. Generalities

The biochemical and electrophysiological properties of the glutamate-gated channels have been well studied over the decades. These glutamate receptor ion channels are found in pre- and postsynaptic cell membranes of neurons as well in glial cells and are widely distributed through the CNS (Traynelis et al., 2010). As previously mentioned, it is generally accepted to divide all ionotropic receptors into three types, based on the agonist that activates them primarily. They are named AMPA, kainate, and NMDA receptors, based on their affinities for the synthetic agonists; however it is known that this classification is nominal due to the existence of the cross-reactivity between agonists and receptors.

There are remarkable sequence homologies within the iGluRs, suggesting they have arisen from common origins. iGluRs are comprised of individual

constituent subunits, forming ion channels with specific pharmacology properties and functional roles depending on their subunit composition. Generally, they are non-selective cationic channels with relative permeability to Na^+ and Ca^{2+}. All three types of iGluRs share a similar structure: four subunits are required to make a functional channel with a large extracellular N-terminal and a cytoplasmic C-terminal part (Figure 1.2. left panel). These tetrameric structures are formed by four identic (homomeric receptors) or 2 or 3 different (heteromeric receptors) subunits. Each subunit contains three transmembrane domains (TM1, TM3 and TM4) and the re-entrant loop on the cytoplasmic side without spanning the membrane (TM2). This loop connects TM1 with TM3 and forms the ion channel pore. The ligand-binding domain is formed by two separate extra cellular loops containing S1 and S2 domains. Finally, all different iGluR subunits have a variable-length cytoplasmic carboxyl terminus, which includes sites for palmitoylation and phosphorylation and is crucial for interaction with intracellular proteins as will be explained later.

The iGluR family displays a huge molecular and functional diversity. This is due to the fact that several glutamate ion channel subunits co-assemble within the family and produce wide variety of receptor combinations. Moreover, alternative splicing and RNA editing extend this variability by conferring a versatility of characteristics to the receptors. Additionally, different domains are subjected to several types' of post-translational modifications, such as phosphorylation and palmitoylation among others. In native iGluRs these modifications are dynamic and reversible and depend on the synaptic events (Zigmond et al., 1999; Kew and Kemp, 2005). These regulations lead to changes in biochemical properties of the receptor, such as synthesis, subunit assembling and protein-to-protein interactions. They also impact on receptor trafficking, endocytosis, synaptic delivery and clustering (Traynelis et al., 2010). Furthermore, these modifications are responsible for changes of electrophysiological properties of iGluRs.

Finally, these changes affect the receptor physiology along with changes in synaptic plasticity.

From a mechanistical point of view, there are distinct conformations for iGluRs: closed, open, and desensitized. The rapid switching between these conformations is referred to as gating. The opening of an ion pore results from a structural conformational change in the receptor due to glutamate association to the LBD and as a result there is a cation influx that changes the resting potential of the postsynaptic membrane. After the removal of the ligand from the synaptic cleft there is the deactivation of the receptor and subsequent closure of the ion pore. The time that receptors spend in their open state is limited by the process of desensitization, in which the ion pore of the receptor closes in the continued presence of glutamate. This characteristic probably serves as a protective mechanism against prolonged depolarization and too much calcium influx into the cells.

1.3.2. NMDA receptors

Thanks to the synthesis and study of NMDA along with various NMDA receptors' (NMDARs) antagonists by Jeff Watkins in 1981 (Watkins, 1981), a detailed understanding of the mechanisms underlying NMDA channel behavior was provided by different research groups.

The NMDA receptor subunit has similar structural characteristics as other members of the ionotropic glutamate receptor family and is one of the best characterized glutamate receptors. NMDAR is a heterotetrameric plasma membrane channel composed of four subunits derived from three related types: GluN1, GluN2, and GluN3 subunits (Nakanishi, 1992; Mori and Mishina, 1995; Seeburg et al., 1995). The variety of the functional NMDARs found at specific neuronal populations with different biophysical properties is due to different splice variants and diverse combinations (Chazot et al., 1994; Luo et al., 1997; Green and Gibb, 2001; Hansen et al., 2014). Different groups have shown that two obligatory GluN1 and two GluN2

subunits (Laube et al., 1998) or combinations of GluN2/3 subunits (Monyer et al., 1994; Ulbrich and Isacoff, 2008) are required to generate a fully functional receptor. GluN2 subunit determines the distinct physiological and pharmacological properties of NMDARs (Cull-Candy et al., 2001).

An important differential characteristic of NMDARs that makes them unique within iGluRs is their requirement for a co-agonist to gate. Thus, apart from L-glutamate, other amino acids like glycine and D-serine need to bind to NMDARs, as a co-agonist for channel openning. Different binding sites are found in different subunits - glycine and D-serine bind to the GluN1 and GluN3 subunits while glutamate binds to the GluN2 subunit (reviewed by Lee at al., 2014). Interestingly, GluN1 in complex with GluN3 forms a receptor that responds to glycine and does not require glutamate (Chatterton et al., 2002).

The activation and opening of NMDARs is both ligand-gated (agonist and co-agonist) and voltage-dependent (Nong et al., 2003; Papouin et al., 2012). This is to another distinct feature of NMDARs: they have voltage-sensitive Mg^{2+} block. This block is decisive for NMDAR function as synaptic coincidence detectors of presynaptic glutamate release and postsynaptic depolarizing potential in neurons (Mayer et al., 1984). Thus, NMDARs require binding of glycine and glutamate in combination with the relief of voltage-dependent Mg^{2+} block to open an ion conductive pore across the membrane bilayer and allow the subsequent Ca^{2+} influx that contribute to its main role in CNS: to trigger synaptic plasticity.

Modulation of NMDAR activity can be performed in positive and negative way. Positive modulators enhance agonist effect or its affinity by binding allosterically. There is a large list of compounds, endogenous or exogenous, acting as allosteric modulators. Some neurosteroids or endogen polyamines potentiate the activity of NMDA receptors by increasing the open channel probability or open frequency (Williams et al., 1990; Lerma, 1992).

NMDAR antagonists are classified accordingly to their action site. Competitive antagonists bind at the glutamate-binding site (as D-APV acting on the glutamate binding site) or glycine-binding site (7-Chlorokynurenic acid). Channels blockers such as MK801 or memantine (Johnson et al., 2015) act on the ion channel pore; while the non-competitive antagonists bind at specific modulation sites.

NMDA receptors are essential mediators of brain plasticity. These channels are capable to translate specific neuronal activity patterns into long-term changes in synapse structure, function and effectiveness, modifications that underlie higher cognitive functions. Importantly, the high Ca^{2+} permeability of NMDARs – compared to other iGluRs – governs its ability for such plasticity changes. Ca^{2+} influx through the NMDAR channel contributes to a cascade of intracellular events that trigger long-term potentiation (LTP) or long-term depression (LTD) (Paoletti et al., 2013). Additionally, these receptors are involved in various neurological and psychiatric disorders (Cull-Candy et al., 2001; Paoletti et al., 2013; Soto et al., 2014a). For example, it has been widely documented that intense NMDAR activation in ischemia (e.g. during stroke) results in a more extreme Ca^{2+} entry and cell death (Choi, 1992; Lai et al., 2014).

1.3.3. Kainate receptors

KARs together with AMPA receptors form part of the former non-NMDA receptor family. However, KARs constitutes a separate group from the NMDA and AMPA receptors. The studies on KARs remained behind due to the lack of specific compounds to block or activate these channels. This scenario changed after the discovery of GYKI53655 compound by Lerma and colleagues in 1995 (Paternain et al., 1995). Nowadays, it is known that KARs are widely expressed through the brain pre- and postsynaptically and are involved in the onset and specially the modulation of synaptic transmission.

KARs can assemble as homo- or heterotetramers, composed of various combinations of the five subunits from GluK1 through GluK5. Within KARs group there are low-affinity and high-affinity subunits. Nevertheless, the number of possible subunit combinations is limited due to the fact that only low-affinity subunits (GluK1 through GluK3) can form functional homomeric receptors. To form functional KARs composed by high-affinity subunits GluK4 and GluK5, these subunits might be co-assembled in a complex together with low-affinity subunits. In addition to these subunits, GluK1-3 has multiple splice variants owing to RNA editing at Q/R site at TM2. Additionally, more isoforms were arisen thanks to the alternative splicing of GluK1–GluK3 subunits (Gregor et al., 1993; Schiffer et al., 1997; Jamain et al., 2002). Apparently GluK4 and GluK5 are not subjected to this type of processing.

To modulate neural circuits in the CNS, KARs use dual signalling modes: ionotropic (canonical) and metabotropic (non-canonical). Typically, as happens with iGluRs, KARs are activated by glutamate or other agonists and can depolarize neuronal membranes. The particular characteristic of KARs is that they also display a second mode of signalling mediated by G protein-coupled activation of protein kinases (Rozas et al., 2003). Yet it remains unclear how the receptors couple to these metabotropic pathways.

KARs play significant roles in brain physiology. The modulation of the neural circuits is conducted through diverse mechanisms. Firstly, they mediate postsynaptic depolarization and also are responsible for KAR-mediated synaptic events with small amplitude and slow kinetics at some synapses. Additionally, KARs exhibit presynaptic modulation of both, glutamate and GABA release at different sites. Yet, they directly alter the neuronal excitability by acting on voltage-gated ion channels (Contractor et al., 2011). Moreover, they are a key player in the neural maturation during development. However, many properties and functions of these proteins remain elusive yet.

1.3.4. Delta receptors

Two delta subunits (GluD1 and GluD2) were described as putative ionotropic glutamate receptors based upon amino acid sequence homology with other iGluRs. Despite the fact that there are strong evidences that delta subunits can act as a pore channels (Schmid and Hollmann; 2008) it has been found that these receptors do not bind glutamate (Kakegawa et al., 2007). However, they can bind D-Serine and glycine but nevertheless this amino acids are not able to gate the channel (Naur et al., 2007).

A recent study showed widespread expression of GluD1 through the brain (Hepp et al., 2014) while GluD2 subunit is expressed prominently in the Purkinje cells of the cerebellum (Araki et al., 1993; Lomeli et al., 1993) where is critical for proper development of neuronal circuits and functions of the cerebellum (Kakegawa et al., 2008; Kashiwabuchi et al., 1995). Meanwhile, *in vitro* studies of recombinant GluD1 receptor showed its role in promotion of synapse formation (Orth et al., 2013; Ryu et al., 2012).

However, delta subunits function is still a mystery and several lines of evidence point towards a non-canonical function (as glutamate-gated ion channels) of these two “orphan” subunits. GluD2 extracellular interaction with Cerebellin 1 precursor protein (Cbln1) determines the normal structure/number of synaptic contacts and LTD processes in parallel fiber-Purkinje cell synapse (Matsuda and Yuzaki, 2012). Cbln1 clusters several proteins at the synaptic junction via the C-terminus of GluD2 indicating a role of GluD2 in synaptic organization. On the other hand, some late research indicates that GluD2 gating might depend on mGluRs activation (Kato et al., 2012; Ady et al., 2014).

1.4 AMPA receptors

1.4.1. Structure

As other iGluRs AMPARs can be found as homomeric or heteromeric receptors. They are dimer of dimers, assembled into tetramers and formed of four different subunits: GluA1, GluA2, GluA3 and GluA4 (Hollmann and Heinemann, 1994). The tetrameric structure, established by expression of the recombinant receptor in oocytes (Mano and Teichberg, 1998) and in human embryonic kidney 293 (HEK293) cells (Rosenmund et al., 1998) is composed of either homomers of the same subtype or heteromers of two differing subunits. Typically they are either GluA1/2 or GluA2/3 (Boulter et al., 1990; Nakanishi et al., 1990; Wenthold et al., 1996), although other combinations can be found as GluA2/4 in cerebellar granule cells (Swanson et al., 1997). The mature subunit protein is approximately 900 amino acids in length, with a molecular weight about 105 kD (Hollmann and Heinemann, 1994). GluA1 and 4 have long carboxy-terminal tails (see Figure 1.3), while GluA2 and 3 have short tails (Kohler et al., 1994). However, GluA2 and GluA4 can be infrequently found as well in specific neurons as long and short forms respectively due to alternative splicings (Kolleker et al., 2003; Kawahara et al., 2004a).

All four AMPAR subunits share the same membrane topology, a large amino-terminal extracellular domain (NTD), an intracellular carboxyl terminus (CTD), three full membrane spanning domains (TM1, TM3 and TM4) with the second hydrophobic sequence forming a re-entrant loop lining the channel pore (TM2) and an extracellular loop between TM3 and TM4 (Hollmann and Heinemann, 1994). Stern-Bach et al., in 1994 demonstrated that the membrane proximal region of the first extracellular domain, termed S1, and the initial portion of the extracellular loop (S2) form the agonist binding domain, also called ligand binding domain (see Figure 1.3).

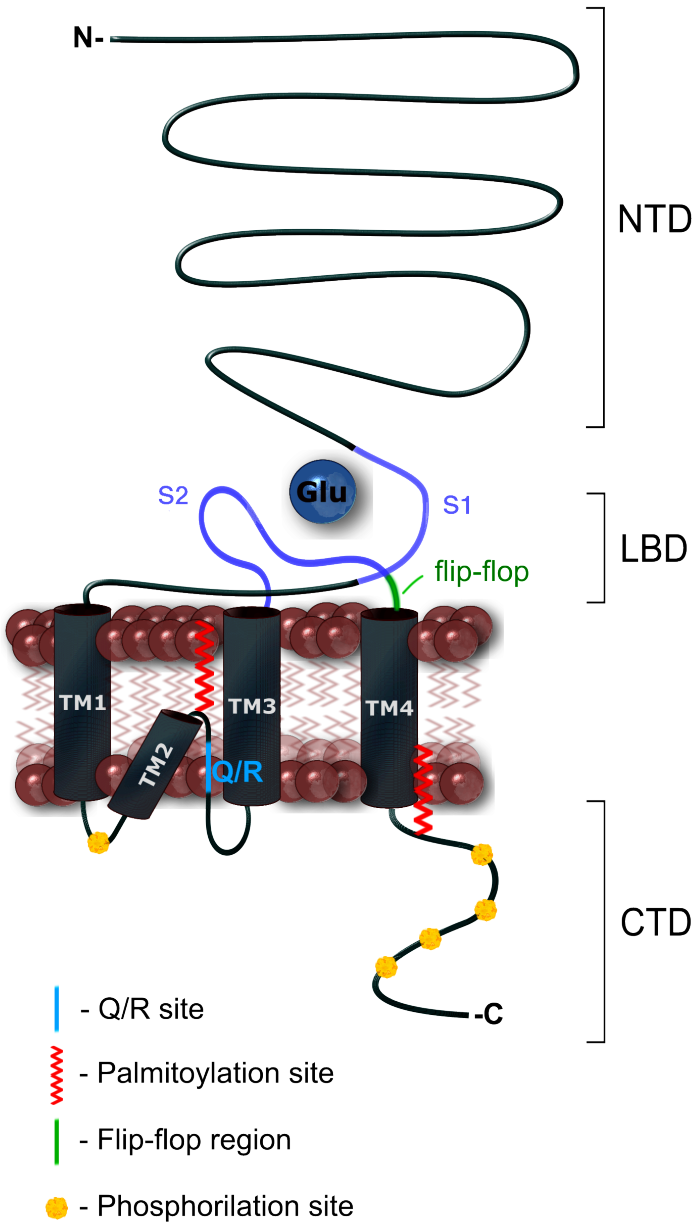

Figure 1.3. AMPAR subunit topology. Schematic representation of the different domains of AMPA receptor with a large amino-terminal extracellular domain (NTD), an intracellular carboxyl terminus (CTD), three full membrane-spanning domains (TM1, TM3 and TM4) and the re-entrant loop (TM2). The agonist binding domain comprising S1 and S2, so called ligand-binding domain (LBD), flip-flop region and Q/R site are shown. The residues that are palmitoylated (in red) and phosphorylated (for GluA1 in yellow and GluA2 in orange) are highlighted.

In eukaryotes, the N-terminus or so called N-terminal domain contains about 400 amino acids. Yet no clear function has been described to this large and most sequence-diverse domain (Hansen et al., 2010; Kumar and Mayer, 2013), although deletion of the NTD slows desensitization kinetics (Bedoukian et al., 2006; Möykkynen et al., 2014). Passafaro and colleagues (2003) described that the NTD of GluA2 is involved in dendritic spine morphogenesis, perhaps through a receptor-ligand complex.

Following a large extracellular NTD, comprising almost half of the molecule (400 out of 900 aas), there are four hydrophobic transmembrane domains (TM1–TM4), the second of which loops in and out of the cytosolic side of the membrane, forming the ion channel pore. Changes in a single amino acid within this region determine the major biophysics of the ion channel, including the calcium permeability (Hume et al., 1991; Cull-Candy et al., 2006). GluA2 subunit confers calcium impermeability to the channel (CI-AMPAR) due to a large and positively charged arginine residue, located in TM2 at amino acid 607 (the pore region). In the other AMPAR subunits the amino acid, presents at this position, is a fairly smaller and negatively charged glutamine.

The LBD is formed from a pocket, comprising S1 and S2 domains, is created by a combination of the proximal extracellular NTD and the initial segment of the loop between TM3 and TM4 (Mayer, 2005). After the third membrane domain there is a long extracellular loop containing an alternatively spliced exon, named the flip/flop region (Sommer et al.,1990). The flop versions generally desensitize much more rapidly than the flip forms in response to glutamate. Furthermore, the flop channels are less responsive to the pharmacological agent cyclothiazide, which blocks desensitization (Sommer et al., 1990).

The fourth transmembrane domain is followed by the CTD with 50–100 amino acids length. The intracellular AMPAR C-tail determines the binding of the subunits to specific interacting proteins, which determine AMPAR

functional properties by defining their specific cell location and trafficking characteristics. CTD contains as well most of the well-characterized phosphorylation sites, which are important in the regulation of the receptors by adding or removing of phosphate groups at serine, threonine, or tyrosine residues (Song and Huganir, 2002). All four types of subunits are also palmitoylated (see following sections) at two different cysteine residues (Figure 1.3), close to their TM2 and in the CTD (Hayashi et al., 2005).

1.4.2. Post-transcriptional modifications

There are two mechanisms of RNA processing events on GluA subunits, which are functionally relevant in the regulation of glutamatergic neurotransmission: editing and alternative splicing (Seeburg, 1996).

Q/R site

Of the four AMPAR subunits, only the GluA2 subunit is subjected to RNA editing process by which a primary codon is modified after transcription. In particular, this subunit is edited at a key amino acid residue located at the pore region – TM2 domain in the inner vestibule of the channel at the selectivity filter. This position in GluA1, GluA3 and GluA4 subunits encodes glutamine (Q) at position 607. In GluA2 it can be edited into arginine (R) at mRNA level, being this editing process catalysed by adenosine deaminases (ADARs) 1 and 2 (Bass, 2002). Editing at this site is nearly 100% efficient in neurons and it profoundly alters the properties of GluA2-containing AMPARs – namely Ca^{2+}-permeability, polyamine block and channel conductance.

It has been extensively studied that the single aminoacid change at the Q/R site determines the Ca^{2+} permeability of AMPARs (Sommer et al., 1991; Hollmann et al., 1991; Verdoorn et al., 1991; Seeburg, 1996). When the glutamine is replaced by a positively charged arginine, a negative electrostatic potential, formed with the N-terminus of the TM2 loop is neutralized, preventing the diffusion of cations through the channel pore,

giving rise to Ca^{2+}impermeability of AMPARs (CI-AMPARs) (Burnashev et al., 1992, Verdoorn, et al.1991, Geiger, et al., 1995, Hollmann, et al., 1991; Kuner et al., 2001). In the non-edited subunits (GluA1, 2, 3 and 4) the negatively charged glutamine attracts cations and confers Ca^{2+} permeability to AMPARs (CP-AMPARs).

Besides determining the Ca^{2+} permeability of AMPARs, Q/R editing of GluA2 also determines electrophysiological properties of the receptor. AMPARs with edited GluA2 exhibit a linear current-voltage relationship, whereas those with unedited GluA2 have an inwardly rectifying current-voltage relationship (Burnashev et al., 1992; Bowie and Mayer, 1995; Geiger et al., 1995; Kamboj et al., 1995; Koh et al., 1995; Swanson et al., 1997) (see Figure 1.4). This differential IV behaviour results from a differential block by endogenous polyamines (PA). GluA2-lacking AMPARs, are blocked by endogenous intracellular PAs, which are found in every type of cell and act as voltage-dependent ion channel blockers (Bowie et al., 1995; Kamboj et al., 1995; Koh et al., 1995) (See “AMPA receptor pharmacology”).

Finally, Q/R site also determines single channel conductance of GluA2-containing or -lacking AMPARs, exhibiting the later larger pore unitary conductance (Geiger et al., 1995; Swanson et al., 1997).

Besides changing dramatically the receptor intrinsic characteristics, the Q/R site also modulates AMPAR subunits tetramerization and retention in the ER, thus playing an essential role in AMPAR trafficking (Greger et al., 2003). Basically, the occurrence of a charged arginine residue at the Q/R site of GluA2 strongly influences subunit interactions during tetramerization, in a way that the juxtaposition of GluA2 subunits of different dimers is energetically unfavourable.

Most of the AMPARs in vivo are found in heterotetramer combination of GluA1/GluA2 or GluA2/GluA3 (Geiger et al., 1995; Wenthold et al., 1996;

Tsuzuki et al., 2001; Sans et al., 2003). In the mature CNS edited GluA2, thus impermeable to Ca^{2+}, are the dominant expressed form in synapses (Paschen and Djuricic, 1995; Carlson et al., 2000; Kawahara et al., 2003; Barbon et al., 2010). Under normal conditions only few CP-AMPARs are widely present in different types of neurons and glia. Different studies demonstrated that the unusual up-regulation of Ca2+permeable AMPARs results in different neuronal diseases and synaptic plasticity (Cull-Candy et al., 2006; Liu and Zukin, 2007).

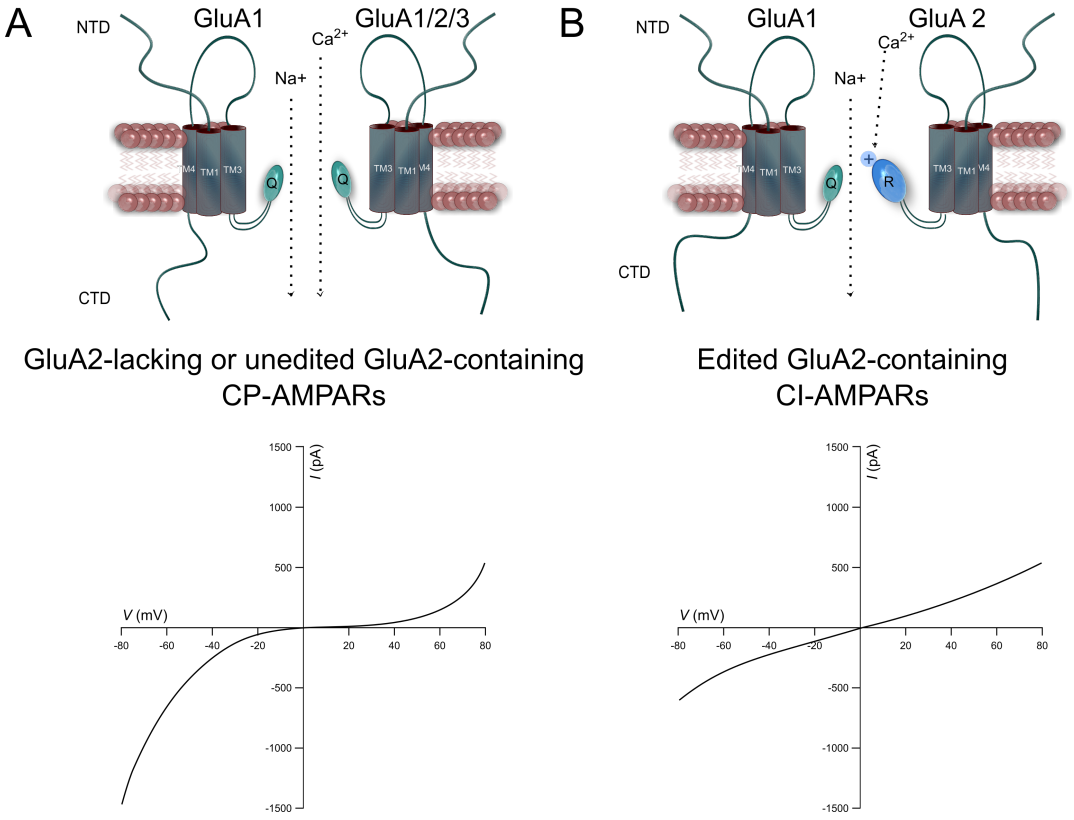

Figure 1.4. RNA editing of AMPAR subunits. A. GluA2-lacking or unedited GluA2-containing AMPARS are permeable to Ca^{2+}, sensitive to PA block, thus are inwardly rectifying and display high conductance. B. GluA2-containing AMPARs allow flux of monovalent cations only, having low single channel conductance and showing linear IV relationship due to lack of the intracellular polyamine block.

Alternative splicing

Another molecular mechanism aimed to increase the molecular diversity of AMPA receptors is AMPAR splicing variance. One of the region which undergo this editing is in the extracellular ligand binding domain at S2, a region preceding the fourth membrane domain (Figure 1.3). Alternative splicing at this region of AMPARs generates two variants, i.e., flip and flop (Sommer et al., 1990). This variance is generated when internal coding sequences of pre-mRNA exons are incorporated differently into the mature mRNA.

The flip-flop region regulates different electrophysiological properties of the AMPA channel. The flop variant of the GluA2 subunits is known to desensitize and deactivate more rapidly than the flip versions, but with slower recover (Sommer et al., 1990). Interestingly, GluA1 flip and flop variants exhibit the same rate of desensitization, depending on the glutamate concentration (Mosbacher et al., 1994).

An additional region subject to alternative splicing is the C-tail of the AMPAR subunits. GluA1, GluA4 subunits and a splice variant of GluA2 (GluA2L) have long cytoplasmic C-terminal tails. GluA2, GluA3 subunits and the alternative splice form of GluA4 (GluA4c) have shorter cytoplasmic domains (Gallo et al., 1992). The trafficking characteristics of long-tailed AMPAR subunits dominate over those of short-tailed subunits; these subunits containing receptors are rapidly mobilized from the receptor pool in the ER and inserted into the synapse during activity period. Short-tailed AMPARs subunits GluA2 and GluA3 without GluA1 are trafficked from the ER more slowly (Greger et al., 2003; Coleman et al., 2006; Mah et al., 2005).

Thus, AMPAR subunits composition as well as post-transcriptional modifications, especially RNA editing, endow AMPARs with substantial diversity and also determine the ion channel characteristics of the receptor.

1.4.3. Post-translational modifications

Phosphorylation

This type of post-translational modification is an important regulatory mechanism that controls many aspects of AMPA receptor function. All four AMPA subunits are phosphorylated by different protein kinases (essentially PKA, PKC and CAMKII) at serine, threonine or tyrosine residues of the intracellular C-terminal domain (Lee, 2006, Wang et al., 2005; Lussier et al., 2015).

GluA1 subunit is phosphorylated at serine 831 (Ser831) and serine 845 (Ser845) of the C-terminal domain (Mamen et al., 1997; Roche et al., 1996) (Figure 1.3). Phosphorylation of Ser831 by PKC and CaMKII increases the single channel conductance of AMPARs. It also has been found that phosphorylation at this residue is augmented following the induction of long-term potentiation (LTP) (Barria et al., 1997; Lee et al., 2000).

Another key residue of GluA1 subunit is Ser845, which is phosphorylated by PKA and this modification leads to the changes in the amplitude of single-channel currents, number of active channels, peak probability and receptor desensitization (Banke et al., 2000). Different studies showed that S845 is crucial in activity-dependent synaptic trafficking of GluA1 to the cell membrane (Derkach et al., 2007; Oh et al., 2006; Seol et al., 2007).

GluA2 subunit, present in the majority of AMPA receptors, is phosphorylated by PKC at Ser880 of the C-terminal PDZ motif (Figure 1.3). This well-studied phosphorylation site has been shown to play an important role in AMPA receptor trafficking (Matsuda et al., 1999; Chung et al., 2000; McDonald et al., 2001). GluA2 subunit interacts with several proteins, involved in the subunit-specific trafficking of AMPA receptors to the synapse: NSF, GRIP, ABP and PICK1 (see also “interacting proteins”) among others (Dong et al., 1997). This interaction is mediated by S880 phosphorylation: when GluA2 is phosphorylated at S880, the interaction

with PICK1 remains stable, while GRIP/ABP interaction is altered (Matsuda et al., 1999; Chung et al., 2000). Taking into account the differential pattern of interaction proteins behavioral, where GRIP/ABP are able to stabilize GluA2 at the cell surface and PICK1 is capable to promote receptor internalization, it has been suggested that phosphorylation at this residue is important for GluA2 removal during LTD (reviewed Palmer et al., 2005).

Palmitoylation

AMPA receptor S-palmitoylation is a dynamic and reversible protein-lipid modification that serves as a regulatory mechanism, modulating AMPAR trafficking properties. This modification consists in covalent attachment of palmitic acid (16-carbon containing fatty acid) to specific intracellular cysteine residues of AMPAR (Figure 1.3). Some of DHHC (Asp-His-His-Cys) proteins, identified as palmitoyl acyltransferases (PATs), catalyze AMPA receptor palmitoylation by transferring palmitoylCoA to cysteine through a thiol linkage. The opposite process, consisting in the removal of the thioester-linked palmitic acid from modified cysteine residues is regulated by palmitoyl thioesterases (PTE) (Shipston et al., 2011; Fukata et al., 2010), a group of depalmitoylation enzymes including palmitoyl-protein thioesterase-1 (PPT1), acyl-protein thioesterase-1 (APT1) and acyl-protein thioesterase-2 (APT2), but their substrat specificity and other characteristics remain unknown.

AMPA receptor GluA1 subunit is palmitoylated at two conserved sites: one within the pore domain (C585) and the other one in the C-tail juxtamembrane region (C811) (Hayashi et al., 2005). Palmitoylation at C585, mediated by the palmitoyl acyltransferase DHHC-3, occurs in the early secretory pathway at ER where it regulates AMPA receptors stability and protects them from degradation (Yang et al., 2009). Moreover, depalmitoylation at this residue helps trafficking GluA1 to plasma membrane (Hayashi et al., 2005). After transportation of the receptors to Golgi apparatus, these are accumulated due to suppression of AMPARs

trafficking by palmitoylation (Hayashi et al., 2005). Contrarily, GluA1 C811 palmitoylation inhibits GluA1-4.1N protein interaction, which is important for stabilizing AMPA receptors on the cell surface (Shen et al., 2000), thus increasing the endocytosis of GluA1 when it is palmitoylated. This is a synaptic activity dependent protein (Lin et al., 2009) and the DHHC catalyzing activity on this interaction has not been determinated yet.

Other modifications

AMPA receptors also undergo ubiquitination, another well studied post-translational modification that can affect AMPAR trafficking. This reaction consists in the reversible addition of a small 76 amino acid protein – ubiquitin – to lysine residue, via a covalent isopeptide bond between the C-terminal lysine and ubiquitin. Recent studies described the sites of ubiquitination, mapping to Lys868 in GluA1 and Lys870/Lys882 in GluA2 C-terminal. Ubiquitinization plays a crucial role in ubiquitin-mediated signalling in AMPA receptor trafficking modulation and turnover (Patrick et al., 2003).

Finally, it has been shown that AMPARs can also be subjected to S-nytrosilation – a post-translational modification consisting in a covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine (Hess et al., 2005) – at GluA1 C875 (Selvakumar et al., 2013). This modification is rare and more frequently affects to interacting proteins of AMPAR subunits (Wang et al., 2012). However, there exists a functional important interplay between S-nitrosylation of GluA1 C875 and other post-translational modifications since S831 phosphorilation depends on C875 S-nytrosilation and that determines endocytosis of AMPARs (Selvakumar et al., 2013).

1.4.4. Assembly and trafficking

Over the past years, the learning about AMPAR trafficking has been expanded elucidating AMPAR lifecycle from its synthesis and dendritic transportation to membrane insertion and removal. It has been shown that

all four AMPAR subunits folding and assembly into functional receptors takes place in the ER in a two-step process: subunit dimerization followed by tetramerization through dimer to dimer formation (Ayalon and Stern-Bach, 2001). AMPA receptor N-terminal domain mediates dimerization process and is not required for homomeric assembly (Ayalon et al., 2005). It is also known that heterodimers assembly dominates over homo-dimerization (Mansour et al., 2001). Another structural domain of AMPA receptor, LBD domain, plays a key role in transition from dimer to tetramer (Greger et al., 2007). Finally, the C-terminal domain controls trafficking properties of AMPARs by different “post-translational”-dependent interactions with a wide range of proteins (See “Post-transcriptional modifications” & “AMPAR interacting proteins” sections).

After its assembly, AMPA receptors leave the ER and travel through the Golgi system by vesicular transport towards the dendritic compartments. Endo- and exocytosis as well as lateral diffusion are involved in plasma delivery and removal of the receptor. Once the AMPAR is close to the cell membrane, SNARE protein family conducts the fusion of the vesicle, followed by the delivery of the receptor to the dendritic shaft plasma membrane or directly to the dendritic spine (Figure 1.5).

After delivery to the cell surface, AMPARs are highly mobile and tend to be diffused laterally along the cell surface. AMPA receptor represents a highly dynamic macromolecular complex, where a high amount of indirect and direct interacting proteins modulate receptor life cycle and properties (Song and Huganir, 2002). Extra-synaptic AMPARs are freely diffused whereas synaptic AMPA receptors' mobility is getting slower and it eventually get immobilized by the interacting partners. AMPA receptor retention at the plasma membrane is regulated by PSD95-like membrane-associated guanylatekinase (PSD-MAGUK) protein family, which comprises PSD93, PSD95, SAP97 and SAP102 proteins. The very first protein, which binds directly to AMPARs, is SAP97, although its function remains to be unclear

(Leonard et al., 1998; Howard et al., 2010). The best studied PSD-95 has been shown to play a crucial role in AMPARs accumulation at the synapse together with the transmembrane AMPA receptor regulatory proteins (TARPs) (Bats et al., 2007). Furthermore, AMPAR also binds to PICK1 (Xia et al., 1999) and GRIP/ABP (Dong et al., 1997), which both contain PDZ domains and compete for binding to the CTD of GluA2.

There are two modulation pathways for AMPAR exocytosis: constitutive and activity-dependent. The first one is determined by AMPAR subunit composition (see “Post-transcriptional modifications” section). In addition to constitutive cycling, the most extensively studied form of trafficking comprises the activity-dependent delivery of receptors to the synapse. This form of exocytosis has been identified as one of the mechanisms that underlies several forms of long-term synaptic plasticity (Carroll et al., 2001; Malinow and Malenka, 2002). GluA2/GluA3 short-tailed heteromers exhibit similar behaviour to GluA2 and mostly cycle in and out of the membrane, thus maintaining the pool of the receptors (Passafaro et al., 2001; Shi et al., 2001). Contrarily, long-tailed GluA1/GluA2 and GluA1/GluA4 require synaptic activity for the membrane insertion (Hayashi et al., 2000; Shi et al., 2001).

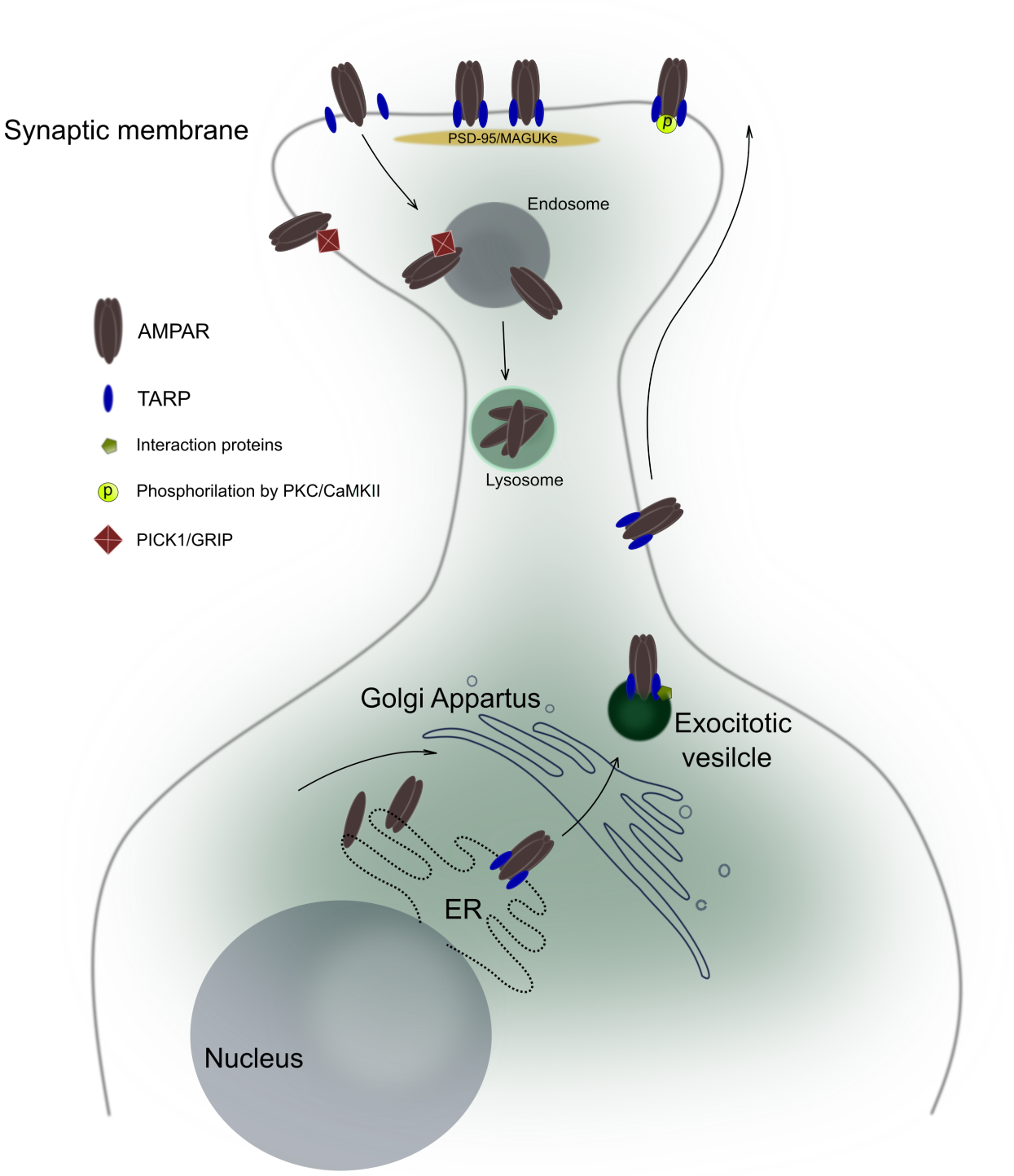

Figure 1.5. Schematic representation of AMPAR cell trafficking.

1.4.5. Gating and pharmacology

Gating

One of the primary features of AMPA receptor function as a ligand-gated ion channel is its gating behaviour (opening/closing) related to the binding and unbinding of the ligand. The very first step in AMPA receptor gating is glutamate binding at LBD site. This part of the channel is formed by the association of two polypeptide domains, D1 and D2. This dimeric interface is mainly composed by S1 and S2 segments (See "AMPA receptor structure" section). The glutamate binding leads to AMPAR activation through the rotation of D2 domain towards the D1 domain, followed by clamshell-like structure closure. Such conformational modification corresponds to the channel opening (Armstrong and Gouaux, 2000).

The activated conformation of the channel is unstable and hence it tends to return into a more stable state. There are two ways to recover the stability: the re-opening of LBD with the consequent unbinding of glutamate (deactivation) or the re-arrangement of the dimer interface (desensitization). During deactivation the receptor conformation changes leading to glutamate unbinding and channel closure. Extended exposure to glutamate drives to desensitization, a separate process with a different time course where the transmembrane helices reposition into a more relaxed conformation.

Contrarily to NMDA receptors, AMPARs do not require a co-agonist for activation. At least two molecules of glutamate are necessary for AMPA receptor activation. Different research groups have shown by the use of recombinant AMPARs that homomeric and heteromeric channels display three subconductance levels and that the amplitudes of each depends on the number of glutamate molecules bound to the receptor (Rosenmund et al., 1998). Auxiliary subunits can modulate the amplitude or the occurrence of this subconductance states (Shelley et al., 2012; Tomita et al., 2005)

In addition, different AMPAR subunits exhibit quantitative differences in the kinetics of deactivation, desensitization and recovery due to alternatively spliced subunits (flip/flop region) (Lomeli et al., 1994; Mosbacher et al., 1994; Sommer et al., 1990). The auxiliary subunits (TARPs) also control AMPAR kinetics through their first extracellular loop interacting with the ligand binding domain of AMPARs (Cho et al., 2007; Milstein et al., 2007; Tomita et al., 2005).

Pharmacology

Extensive research has been performed to describe different compounds that exert modulatory effects on AMPAR gating. They are classified as competitive agonists and antagonists, positive and negative allosteric modulators (or non-competitive antagonists) and channel pore blockers (uncompetitive antagonists).

Agonists. Glutamate acts on AMPARs as a full agonist, resulting in large peak currents with small steady-state current and full and quick desensitization in the presence of the agonist. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) is a specific compound for AMPAR with mimic effects of the glutamate that gave name to this subtype of receptors. Another important chemical, structurally related to glutamate is kainate, which also binds to AMPARs, generating smaller and non-desensitizing currents. Willardiine derivative compounds also act as AMPAR partial agonists (Patneau et al., 1992; Jin et al., 2003)

Antagonists. AMPA antagonists are able to block AMPARs and, in neurons, reduce or avoid the depolarization of the postsynaptic membrane. Several classes of AMPAR antagonists with different binding modes have been developed over the past decades.

Competitive antagonists. Widely used AMPAR competitive antagonists, indispensable in elucidating the cell biology and physiology of AMPARs, are the quinoxiline derivatives DNQX, CNQX and NBQX (Armstrong and

Gouaux, 2000). These compounds bind AMPA receptors with high affinity at the glutamate-binding site, thus preventing their activation by glutamate. It has been shown that NBQX exhibits higher selectivity for AMPARs (Wilding and Huettner, 1996). Interestingly, CNQX acts as a partial agonist of AMPA receptor channel when is associated with TARP auxiliary subunits (Menuz et al., 2007; Kott et al., 2009).

Non-competitive antagonists. The non-competitive antagonist family includes GYKI52466, which shows good selectivity for AMPA receptors over kainate receptors and is used for chronic blockade of neuronal AMPARs (Donevan and Rogawski, 1993).

Positive allosteric modulators. Positive allosteric AMPAR modulators allow to extend the open state of the receptor. One of the best described, cyclothiazide, which forms part benzothiadiazines family, is a commonly used modulator of AMPA/kainate receptors. This compound blocks receptor desensitisation, potentiating the current flow throughout the channels. Cyclothiazide binds AMPA receptors at flip/flop region and prevents the conformational change required for desensitization (Sun et al., 2002). It is widely used to increase steady-state currents to facilitate detection of surface AMPA receptors (Partin, et al., 1995). Other compounds from benzoylpiperidines family that also binds allosterically, CX-516 and CX-546, have been shown to potentiate AMPAR function (Sun et al., 2002; Jin et al., 2005).

Open channel blockers. A number of synthetic and natural polyamines (spermine and spermidine) have been shown to be AMPARs channel blockers. These compounds represent a group of small positively charged molecules with molecular weight less that 1kD which were isolated from spiders and wasps venom (Shaw, 1979; Bowie et al., 1995). These molecules, expressed in every cell type, are commonly used to measure rectification of synaptic currents to detect the presence of CP-AMPARs in neurons. PAs block CP-AMPAR channel in voltage-dependent manner,

generating the strong inward rectification of the unedited AMPARs (Bowie & Mayer, 1995; Donevan and Rogawski, 1995; Isa et al., 1995; Kamboj et al., 1995; Koh et al., 1995). Their channel blocker mechanism of consists in positioning of the PA head group 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. Thus, the more depolarized is the membrane, the stronger is the block of the pore by the positive PAs, translating into the characteristic inwardly rectifying current-voltage relationship (IV curve). Over +50 mV, outward currents tend to increase by pushing polyamines to pass through the pore and unblock the channel (Bowie & Mayer, 1995; Koh et al., 1995). Cull-Candy laboratory demonstrated that stargazin association with CP-AMPAR attenuated the polyamine block of these receptors (Soto et al., 2007). PAs serve as an important pharmacological tool to study native AMPARs because of their ability to selectively inhibit GluA2-lacking AMPARs.

1.4.6. AMPARs in physiological and pathological conditions

The most important function of AMPARs in the CNS is the fast excitatory neurotransmission (Traynelis et al., 2010). Virtually all glutamatergic excitatory transmission relies on the fast kinetic properties of AMPARs. Apart from their main role, AMPARs are crucial in synaptic strength modulation and support cellular forms of learning. Over the past years, one of the most active areas in neuroscience has been focused on the research of AMPARs involvement in learning and memory through regulation of LTP and LTD. Both processes require the activity-dependent trafficking of AMPARs to either strengthen the synapse in case of LTP or decreasing the total sensitivity of the synapse, in case of the latter (Earnshaw and Bressloff, 2006). During LTP, independently of the mechanism that triggers it, the number of AMPARs is rapidly increased at a given synapse. Some of them, namely silent synapses, contain NMDARs only at their resting state.

After LTP induction, AMPARs are rapidly trafficked into synapses and contribute to the depolarization of the postsynaptic neuron (Isaac et al., 1995; Liao et al., 1995). To date, it is known that CP-AMPARs give an alternative to NMDARs route for Ca^{2+} flux into cells, playing an important role in NMDA-independent LTP (Lu et al., 2007; Asrar et al., 2009; Wiltgen et al., 2010). The majority of AMPARs are GluA2-containing receptors and the role of GluA2 subunit has been demonstrated to be an important player in induction of LTP. Different studies demonstrated the crucial role of GluA2 in NMDAR-independent LTP by use of knock out or altered GluA2 (at Q/R site) animals (Feldmeyer et al., 1999; Jia et al., 1996). Interestingly, some studies confirmed that GluA2-lacking AMPARs are capable to switch to GluA2-containing receptors in high-frequency activity dependent manner (Liu and Cull-Candy, 2000; 2002) and require rapid subunit-specific trafficking of AMPARs where the interacting proteins PICK1, GRIP, and NSF play an important role (Gardner et al., 2005; Liu and Cull-Candy, 2005). Finally, AMPA receptors endocytosis is crucial in the induction of LTD triggered by NMDA receptor activation. LTD rely on AMPAR internalization (Henley et al., 2011), this process requires clathrin-mediated endocytosis and a GTPase dynamin (Anggono and Huganir, 2012).

On the other side, deficits and dysregulation of synaptic AMPARs and their abnormal function are involved in a variety of neurological disorders and neurodegenerative diseases. Alzheimer's disease (AD) is characterized by decreased in AMPAR activation and synapse loss. Thus, it has been demonstrated that dysregulated endocytosis of AMPARs at the synapse may contribute to the progressive loss of memory in AD (Tang, 2009). Moreover, β-amyloid, one of the key players in AD pathology, has been shown to disrupt activity-dependent AMPAR trafficking (Gu et al., 2009). Ischaemic processes also involve AMPARs. Cerebellar Purkinje cells and pyramidal hippocampal neurons of CA1, which express mostly CI-AMPARs, are vulnerable to ischemia, thus demonstrating the implication of AMPARs in excitotoxic events (Crepel et al., 1982; Cull-Candy et al., 1998). Ischemic

process leads to downregulation of CI-AMPARs, up-regulation of CP-AMPARs, selective GluA1 trafficking and consequently to neuronal cells death (Naylor et al., 1996; Rakhade et al., 2008; Friedman et al., 1999; Ying et al., 1998; Quintana et al., 2015). Also, in amyotrophic lateral sclerosis, AMPARs allow cytotoxic levels of Ca^{2+} into neurons, leading to motor neuron death due to changes in GluA2 editing (Kawahara et al., 2004b). AMPAR participation and the excitotoxic effect are also described in other neurodegenerative processes such as Parkinson's and Huntington's diseases as well as neurological disorders such as epilepsy and schizophrenia (Hollmann and Heinemann, 1994; Yamada, 1998; Lees, 2000; Goff et al., 2001; Danysz, 2002b; Johnson et al., 2009;). Finally, several inherited psychiatric and neurodevelopmental disorders have been correlated with AMPAR subunits or even with auxiliary proteins of AMPARs mutations (Soto et al., 2014a; Uzunova et al., 2014).

1.4.7. AMPARs interacting proteins

Native AMPARs in the mammalian brain associate with more than 30 different proteins forming macromolecular complexes (Schwenk et al., 2012; Figure 1.6). Some of them are referred to as transmembrane regulatory proteins whereas others display transient interactions with the receptor (See "AMPA receptor assembly and trafficking"). Transient interactions between AMPARs and several proteins determine principally exocytosis, endocytosis and synaptic targeting. There is an enormous variety of these proteins mentioned before (i.e. ABP/GRIP, 4.1N, PSD95, SAP97, PICK1, NSF, NARP, Mint1, Lyn – see Song and Huganir 2002 and Henley, 2003) and this section will focus on describing briefly those associating in a stable manner with AMPARs.

AMPA receptors auxiliary proteins

Four main criteria can be applied to classify a protein as an auxiliary subunit. It should not constitute an integral component of channel pore forming subunits, remain in stable association with its partner receptor,

modulate its pharmacology and be an important player for functional receptor assembly (Copits and Swanson, 2012). It was proposed by Schwenk et al., 2012, that the inner core of the AMPA receptor complex consists of the GluA tetramer and four auxiliary proteins comprising TARPs and cornichon homolog proteins (CNIHs), in fact the best-characterized two families of auxiliary proteins (Straub and Tomita, 2012; Kato et al., 2010; Haering et al., 2014).

Transmembrane AMPA receptor regulatory proteins (TARPs)

TARPs are crucial for AMPARs. Different studies confirmed indeed that AMPARs in the CNS are mostly associated with TARPs (Menuz et al., 2008; Schwenk et al., 2009) and only in special and determined rare situations AMPARs can be found TARP-less in neurons (Bats et al., 2012). TARPs are a family of non-pore-forming transmembrane proteins with direct AMPARs interaction. They are specific and essential auxiliary subunits for AMPA receptors and are differentially distributed in the brain (Fukata et al., 2005; Nicoll et al., 2006; Milstein & Nicoll, 2008; Coombs & Cull-Candy, 2009; Sager et al., 2009; Kato et al., 2010). Based on functional differences and sequence homologies, TARP family is classified into the type I TARPs comprising the subunits γ2 (stargazin), γ3, γ4, and γ8, and the type II TARPs, γ5 and γ7 (Haering et al., 2014). TARPs are composed of four transmembrane domains that interact with AMPARs at LBD site. It also has been shown that TARPs C-terminal tail interacts with a variety of proteins (Deng et al., 2006). Their intracellular PDZ binding domain (TTPV) can interact with synaptic anchoring protein PSD-95 (Dakoji et al., 2003; Schnell et al., 2002). Thus, TARPs C-terminal domain directs effective trafficking of AMPAR-TARP complexes (Bedoukian et al., 2008). In fact, PSD-95 binding motif is required to stabilize AMPAR at the synapse. Disruption of this interaction leads to an increase in AMPAR diffusion and prevents their accumulation at synaptic sites (Bats et al., 2007).

Stargazin, the prototypical TARP, expressed in almost every type of neuron, controls AMPAR trafficking at multiple points during the secretory process (Nicoll et al., 2006). Stargazin and its closely related γ-3, γ-4, and γ-8 paralogs interact directly with all AMPAR subunits promoting their transport to the cell surface (Chen et al., 2000, Tomita et al., 2003) and modulating the channel function (Tomita et al., 2004; Tomita et al., 2005, Soto et al., 2007). In presence of TARPs, AMPA receptor channel shows slower kinetics of deactivation and desensitization (Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005). Additionally, TARPs affect the efficacy of kainate and the affinity and efficacy of AMPAR enhancers, such as cyclothiazide (Tomita et al., 2005; Tomita et al., 2006; Turetsky et al., 2005). Moreover, TARPs also increase AMPAR single-channel conductance (Soto et al., 2007, 2009; Tomita et al., 2005) and decrease AMPAR block by polyamines (Jackson et al., 2011; Soto et al., 2007).

Cornichon homolog proteins

Two homologues of CNIHs - CNIH2 and CNIH3 - have also been described by proteomic study as a native AMPAR constituents (Schwenk et al., 2009). CNIHs structure comprises a short cytoplasmic NTD motif, three transmembrane domains and a short CTD domain. These proteins bind as part of the AMPAR complex at ER, affecting channel trafficking and its biophysical properties. Thus, CNIHs, expressed in a heterologous system can increase AMPAR surface expression (Schwenk et al., 2009). Additionally, these auxiliary subunits slow AMPAR deactivation and desensitization (Gill et al., 2012; Coombs et al., 2012). Different groups showed that cornichon family shares some of other properties with TARPs (Kato et al., 2010; Shi et al., 2010).

New incognita on interacting AMPAR proteins…

There are a set of other AMPAR interacting proteins, present in lower amount, forming the outer shell of the AMPAR complex with wide range of size and variability. One of the out core constituents capable to interact with

AMPAR subunits is Carnitine palmitoyltransferase 1C (CPT1C) (Schwenk et al., 2012; Figure 1.6).

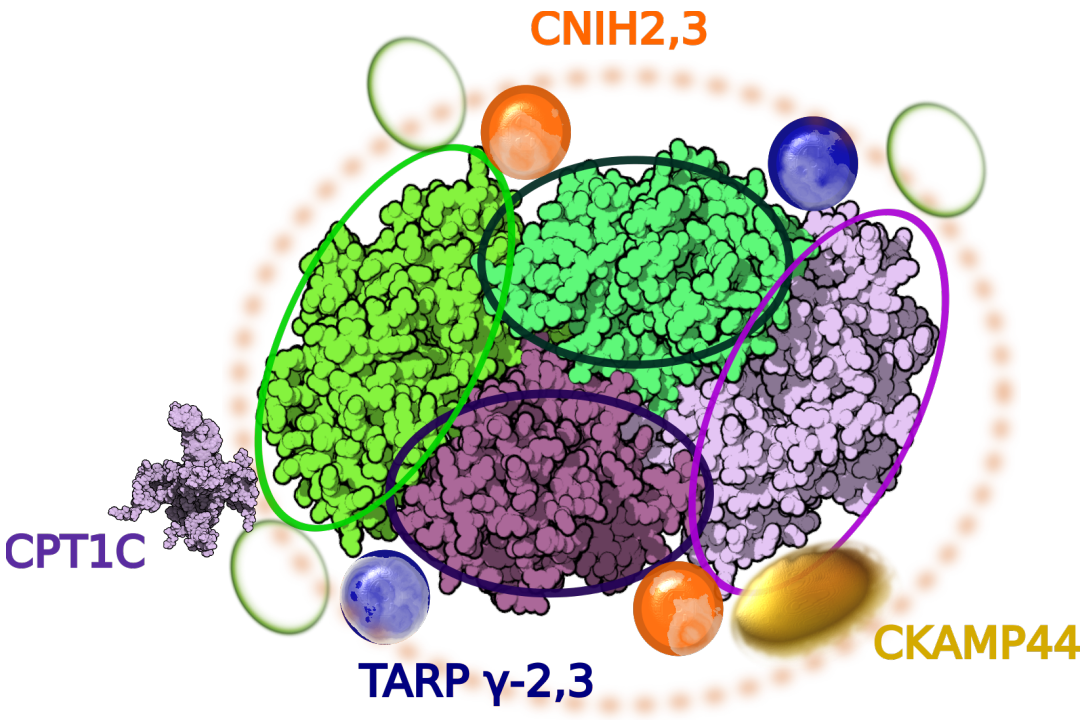

Figure 1.6. Model for the assembly of native AMPARs, as derived from the proteomic study by Schwenk et al., 2012. The tetrameric structure is shown from a cross-section of the crystal structure (light and dark green and purple). Solid circles in orange and blue represent the binding sites for inner core constituents; white circles show binding sites with unknown function for outer core components where CPT1C is included.

1.5. CPT1C

1.5.1. The CPT1 family and the new arrival: CPT1C

CPT1 enzymes belong to the carnitine acyltransferases family that catalyse the exchange of acyl groups between Coenzyme A (CoA) and carnitine to facilitate the transport of fatty acids through different intracellular membranes. Specifically, the most studied isoforms of CPT1, named CPT1A and B, transform long chain acyl-CoAs into acylcarnitines for their transport through the mitochondrial outer membrane (MOM) and the subsequent use of the transported fatty acids for β-oxidation in the mitochondrial matrix. These two widely-studies isoforms catalyse the rate-limiting step of fatty acid oxidation given their inhibition by malonyl-CoA – the first intermediate in fatty acid synthesis (McGarry and Brown 1997, Zammit 1999). This mechanism allows a fine regulation of cell metabolism inhibiting a catabolic pathway (β oxidation) when fatty acid biosynthesis is turned on (high levels of malonyl-CoA). The two isoforms are localised at the MOM but they have differential tissue distribution. CPT1A is expressed mainly in the liver but it is also the isoform most ubiquitously expressed (McGarry and Brown, 1997) being also expressed in brain (Lavrentyev et al., 2004) and it is crucial for the organism survival since knockout mice for this isoform are not viable from day 10 of gestation to term (Nyman et al., 2005). CPT1B is the isoform mainly expressed in skeletal and cardiac muscle and also in adipose tissue (Esser et al 1993; McGarry and Brown, 1995).

In 2002, when *in silico* studies from Human Genome databases became available, Zammit's group found a novel family member of CPT1s and they called this new isoform CPT1C (Price et al., 2002). CPT1C sequence contains all the motifs previously shown to be essential for CPT1 activity and malonylCoA binding. This work showed as well that CPT1C was expressed at the mRNA level in testis and brain (with especially high mRNA levels in hippocampus and some hypothalamic nuclei, but also in cortex

and cerebellum). At the protein level, CPT1C expression was clear in mouse and rat brain. Surprisingly, CPT1C did not exert any catalytic activity with different substrates in yeast extracts (Price et al., 2002) or with fatty acids of different lengths as substrates in extracts from HEK293 cells transiently expressing CPT1C, confirming the lack of CPT1 catalytic activity (Wolfgang et al., 2006).These two works performed the radiometric CPT1 catalytic activity assay which was later proved to be 400 times less sensitive than a new chromatographic methods (Jáuregui et al., 2007). This method showed greater sensitivity than the radiometric method and led subsequent studies determine that CPT1C could form palmitoylcarnitine from palmitoyl-CoA with 100 times lower specific activity than CPT1A (Sierra et al., 2008), suggesting that CPT1C could act as a carnitine palmitoyltransferase but with very low efficiency, or maybe suggesting that CPT1C had another activity. Moreover, it was described that CPT1C is localised at the ER and not in the mitochondria (Sierra et al., 2008), which points also to the hypothesis of a totally different function of this brain specific isoform, from the function described for canonical CPT1s.

1.5.2. CPT1C structure

CPT1C shares a high degree of primary amino acid identity and similarity to CPT1A and CPT1B (Wolfgang et al., 2006). Two transmembrane domains can be predicted and distinct regions can be identified: an N-terminal domain (NTD), a C-terminal domain (CTD) and a loop between the first and the second transmembrane (Figure 1.7). In CPT1A and CPT1B the NTD and CTD are both facing the cytosol and interact with each other (Fraser et al., 1997) and it has been shown that CPT1C adopts the same topology but in the ER membrane (results from Esther Gratacòs-Batlle thesis; Figure 1.8).

Recent data describing the 3D conformation of the NTD (50aa) of CPT1A identified two possible conformations: an inhibitory form and a non-inhibitory form. In this study the authors concluded that CPT1C is only

stable in a state similar to the inhibitory conformation, making CPT1C unable to catalyse the carnitine palmitoyltransferase activity with greater efficiency (Samanta et al., 2014). It had been previously described that within the NTD and the two transmembrane domains of CPT1A resides the amino acid sequences determining translocation of the newly synthesized protein from the endoplasmic reticulum to the MOM (Cohen et al., 2001).

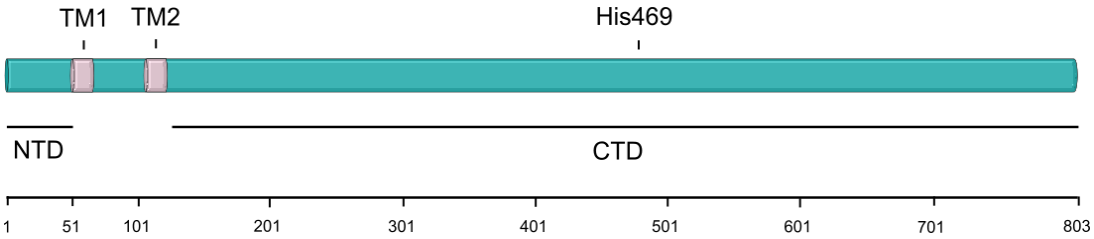

Figure 1.7. Schematic representation of CPT1C sequence with N-terminal (NTD) and C-terminal (CTD) segments; two transmembrane domains (TM1 and TM2) and the key catalytic residue Histidine 469 are also shown.

It was also characterized that the specific primary sequence of the first 150 amino acids of CPT1C targets this protein to the ER membrane and when exchanging this part of the protein between isoforms, the subcellular localization of each protein resulted shifted.

CTD region contains all the residues that had been identified to be important for substrate binding and for catalysis in CPT1A, and all of them could also be found in CPT1C primary amino acid sequence (Price et al 2002). Among all these important residues there is a crucial catalytic histidine residue that when mutated to alanine in CPT1A abolishes its catalytic activity (Morillas et al., 2001). This catalytic residue is Histidine 473 in CPT1A sequence and His 469 in CPT1C sequence. Remarkably, this catalytic histidine is conserved among all the members of Carnitine acyltransferase family (Jogl et al., 2004). The main difference between isoforms in this CTD is an extended C-terminal tail, of 30 amino acids, in CPT1C that is not present in CPT1A or in CPT1B (Price et al., 2002).

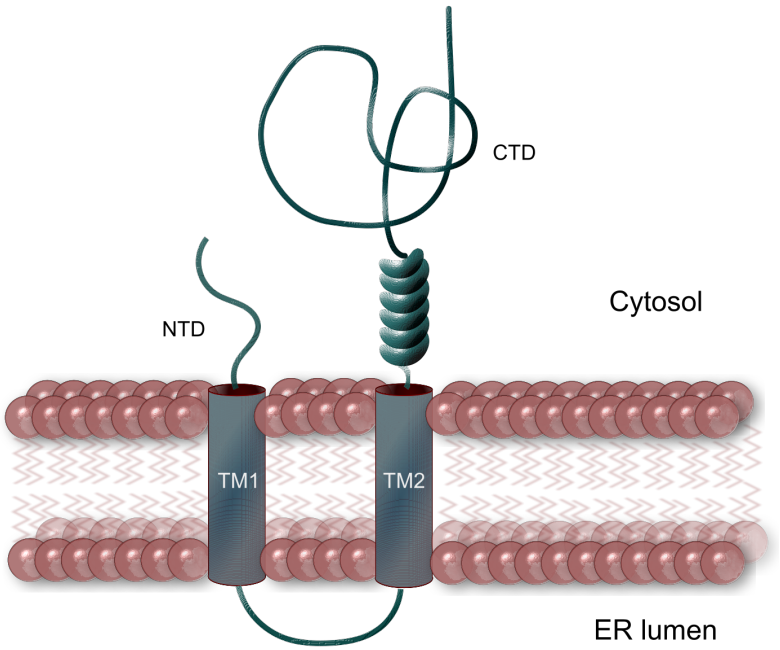

Figure 1.8. Schematic representation of CPT1C topology, showing the two transmembrane segments (TM1 and TM2). The loop connected the TM1 and TM2 is orientated to the ER lumen. The N-terminal regulatory domain (NTD) and C-terminal catalytic segment (CTD) are exposed on the cytosol.

1.5.3. Physiological role of CPT1C and beyond

To elucidate the physiological role of the CPT1C isoform different groups produced CPT1C knockout (CPT1C-KO) mice. All these KO animals are viable and fertile; furthermore they do not display remarkable histological abnormalities through the lifetime (Wolfgang et al., 2006; Gao et al., 2009; Carrasco et al., 2012).

The first phenotype described for CPT1C KO mice was related to the study of the feeding behaviour of this mice model. These studies derived from the high expression levels found in feeding control centres of the hypothalamus (Price et al., 2002). CPT1C loss resulted in suppression of food intake and a decrease in body weight following a normal or low-fat diet (Wolfgang et al., 2006; Gao et al., 2009). Interestingly, when the daily intake of these animals was replaced by a high fat diet, it was observed that their body

weight increased more than control animals, becoming resistant to insulin. These results suggested that CPT1C is involved in energy homeostasis and control of body weight (Wolfgang an Lane, 2006).

However, Casals' group could find other neurological phenotypes in CPT1C KO mice. These mice showed impairment in motor functions, in muscle strength and presented hypoactivity (Carrasco et al., 2013) and behavioural learning deficits (Carrasco et al., 2012). Moreover, they observed that CPT1C overexpression increased ceramide levels in cultured neurons while CPT1C deficiency reduced them. These changes in ceramide levels are correlated with dendritic spine morphology, therefore CPT1C-deficient neurons displayed more immature dendritic spine morphology and supplementation with ceramides in the media reversed this effect (Carrasco et al., 2012). On the other hand, it has also been described that a gain-of-function of CPT1C in the brain of transgenic mice results in severe growth retardation and in a reduction of brain weight (Reamy and Wolfgang, 2011).

A recent study found that a familial hereditary Arg37Cys mutation in CPT1C provokes autosomal dominant hereditary spastic paraplegia (Rinaldi et al., 2015), linking for the first time CPT1C to human disease.

Interestingly, CPT1C has been found to be expressed in some tumor cell lines that makes them resistant to glucose deprivation and hypoxia (Zaugg et al., 2011) and more recently aberrant expression of CPT1C mRNA have been found in human high grade glioblastomas (Cirilio et al., 2014) suggesting a possible implication of CPT1C in some other diseases.

2. OBJECTIVES

AMPARs are widely distributed in the brain and their physiological roles are crucial for the correct brain functioning. Their properties and functional outcome is determined by several factors as subunit composition, post-translational modifications and interacting partners (either auxiliary or transient interactors).

The aim of the work described in this thesis was designed to discover, understand and characterize AMPAR functional effect by CPT1C, a recently described protein interacting with AMPAR subunits in brain tissue. In order to accomplish the general goal three specific purposes were pursued:

1. To evaluate the putative CPT1C effect/modulation on AMPAR mediated responses over a range of physiologically relevant GluA subunits.

2. To study whether a putative modulation of AMPARs by CPT1C is happening in a more physiological system (cortical and hippocampal neuronal cultures)

3. To depict the molecular mechanism of AMPAR-CPT1C interaction and functional consequences.

3. EXPERIMENTAL PROCEDURES

3.1. Mutagenesis

3.1.1. Plasmid constructs

To obtain GluA1 cDNAs with mutations in the palmitoylation sites, site-directed mutagenesis was used to change specific base pairs. GluA1(C585S) and GluA1(C811S) mutant cDNAs resulted from changing the codon TGT to TCT and TGC to TCC, respectively. Both changes produce a cysteine to serine switch making these palmitoylation targets disappear. For the double mutant GluA1(C585,811S) GluA1(C585S) cDNA was used as a template and we introduced the C811S mutation to create the GluA1(C585,811S) product, in which both palmitoylation sites from GluA1 were eliminated. Primers containing the mutation/s were designed and then synthetized by Integrated DNA Technologies (IDT). Primers used were the following:

C585S: AA GGA T**C**T GAC ATT TCC CCC AGG TCC C

C811S: CCT TAA TCA GAG TTC T**C**C TAC AAA TCC CGT AGC G

where the codon of interest is underlined and the changed nitrogenised base is marked in bold.

To obtain CPT1C mutant, site-directed mutagenesis was used to replace histidine 469 to alanine. Primers containing the mutation was designed and then synthetized by Integrated DNA Technologies (IDT). The mutated fragment was obtained using the forward primer 5'- CCT CAG CGT GGA G**GC** CTC ATG GGC TGA CTG CC -3' and the reverse primer 5'- GG CAG TCA GCC CAT GAG **GC**C TCC ACG CTG AGG -3' to obtain the plasmid CPT1C(H469A).

The plasmids containing the fusion proteins: CPT1C with the N-terminus of CPT1A (A-CPT1C) and CPT1A with the N-terminus of CPT1C (C-CPT1A),

that were used in the mislocalization experiments were kindly provided by the laboratory of Núria Casals. Information about their cloning process can be found in Carrasco et al., 2008.

All constructs were fully sequenced to verify sequence integrity. All plasmid vectors are under the control of the same promoter (CMV promoter).

3.2. Cell lines and neuron cultures

3.2.1. tsA201 cell line

tsA201 is a transformed human kidney cell (HEK293) line stably expressing an SV40 temperature-sensitive T antigen. This cell line does not express CPT1C protein (Sierra et al., 2008) and was maintained in DMEM:F12 3.15 g/L, containing 10% fetal bovine serum and 1% penicillin–streptomycin solution in 5% CO_2 and 95% O_2 at 37°C. The maintenance culture was passaged once a week and the medium was changed every 2-3 days. To split tsA201 cells, grown in 25cm^2 flasks, first they were washed with PBS preheated at 37ºC and then detached by adding 400 µl Accutase® followed by a mechanical dissociation.

3.2.2. Cos-7 cell line

Cos-7 is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. The cells were maintained in complete DMEM:F12 containing 10% fetal bovine serum and 1% penicillin–streptomycin solution in 5% CO_2 and 95% air at 37°C. The maintenance was performed the same way as for tsA201 cell line.

3.2.3. Primary cortical neuron cultures

Cortical neuron cultures were prepared from mouse embryos (E18). All the experimental procedures were carried out according to European Union guidelines (Directive 2010/63/EU) and following protocols that were

approved by the Ethics Committee of the Bellvitge Biomedical Research Institute (IDIBELL). The cerebral cortex was isolated and maintained in cold Hank's Balanced Salt Solution (HBSS, Gibco) supplemented with 0.45% glucose. After removal of the meninges, the cortical tissue was digested mildly with trypsin for 17min at 37°C and mechanically dissociated. Cells were washed three times in HBSS and re-suspended in Neurobasal medium supplemented with 2mM Glutamax (Gibco) before filtering in 70µm mesh filters (BD Falcon). Cells were plated onto glass coverslips (5×10^4 cells/cm^2) coated with 0.1mg/ml poly-L-lysine (Sigma). Two hours after seeding, the plating medium was replaced by complete growth medium (Neurobasal medium supplemented with 2% B27 (Invitrogen) and 2mM Glutamax) and the coverslips were incubated at 37°C in a humidified 5% CO_2 atmosphere. Every 3–4 days, half of the conditioned medium was removed and replaced by fresh growth medium. All the experiments were performed with 10 DIV cultured cells.

3.2.4. Primary hippocampal neuron cultures

Hippocampi were obtained from fetal mice brains at 16 days of gestation, maintained in HBSS solution supplemented with 0.45% glucose. Hippocampi were chopped into small pieces and enzymatically digested with Trypsin solution from bovine pancreas (Type XI; Sigma) for 10min at 37°C followed by a mechanical dissociation. Dissociated cells were centrifuged at 1000xg for 10min, washed 3 times in HBSS solution and re-suspended at a density of 3×10^5 cells/cm^2 in Neurobasal media supplemented with 5% FCS, 5% HS, 50U/ml penicillin, 50µg/ml streptomycin, 2mM glutamine, and 10mM glucose, and plated onto poly-L-lysine pre-coated wells. Cultures were kept at 37°C in 95% O_2 / 5% CO_2 atmosphere until 7 days *in vitro* (DIV) when the plating medium was replaced by Neurobasal media supplemented with 10% HS, 50U/ml penicillin, 50µg/ml streptomycin, 2mM glutamine, 10mM glucose, and 10µM

cytosine arabinoside to stop non-neuronal cell proliferation. All the experiments were performed with mature cultures (15–16 DIV).

3.3. Transfection protocol

3.3.1. Cell lines

Twenty four hours before transfection, 1.5×10^6 cells were plated into T25 flasks for coimmunoprecipitation (Co-IP) and Acyl Biotin Exchange assays (ABE) or 0.5×10^5 cells onto poly-D-lysine-coated coverslips for immunofluorescence (IF) and electrophysiological (EP) experiments.

Cells were transiently co-transfected with 5.4μg total cDNA (for Co-IP and ABE) and 0.6μg total cDNA (for IF and EP) using X-tremeGENE transfection reagent (Roche) according to the manufacturers' directions (Figure 3.1). In all transfections the ratio used was 1:2 (GluA:CPT1C), except for the experiments where stoichiometry effect was assessed and the ratios used were 1:2 and 1:1(GluA:CPT1C). Media was replaced 24 hours after transfection with fresh media containing 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide 50μM (NBQX; Tocris-ABCam, Abcam) to prevent AMPAR-mediated toxicity. For EP experiments, cells were re-plated onto glass coverslips to allow optimal density. All experiments were performed 48 hours later.

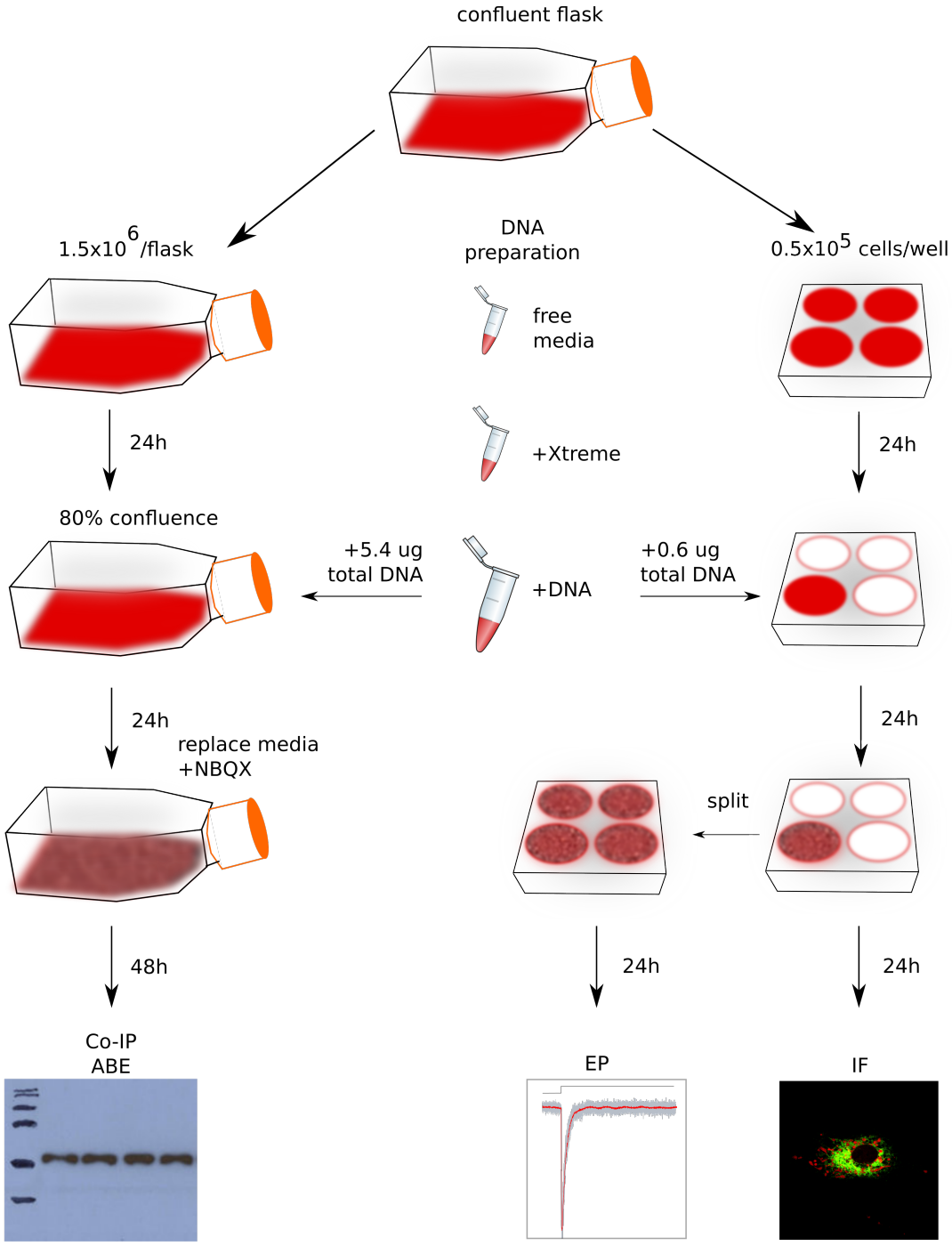

Figure 3.1. Transfection protocol scheme.

3.3.2. Primary cultures

The transfection was performed using Lipofectamine 2000 on day 7 in vitro (div7), according to the manufacturers instructions and the cells were fixed 72h after transfection.

3.4. Immunocytochemistry

Immunofluorescence was performed in tsA201 cells grown on poly-L-lysine treated coverslips, 48h after transfections. Washes were always performed by immersion of the coverslips in PBS or PBS-G (20mM Glycine in PBS). The composition of the different solutions used was: Fixation solution (2% PFA in PBS), permeabilisation solution (0.1% Triton X-100 in PBS-G), blocking solution (10% NGS, 2% BSA, 0.1% Triton X-100 in PBS-G), antibody incubation solution (4% normal goat serum and 0.1% BSA in PBS-G) and triton-antibody solution (antibody incubation solution containing 0.1% Triton X-100). Antibodies incubations were performed in a humid chamber at 37°C for 1h.

For co-localization of CPT1C with GluA1 or GluA2 and for determining the level of surface expression of GluA1 in tsA201, the following method was used: staining surface AMPARs was achieved by labeling live cells with mouse anti-GluA1-NT or mouse anti-GluA2 antibodies (both from Merck Millipore) in a 1:200 solution in DMEM:F12, for 7-10min at 37°C. In neuronal cultures, the surface staining step was performed for 1 hour at 37°C on fixed neurons (4% PFA + 4% sucrose). After washes at room temperature PBS, tsA201 cells were fixed for 15min at room temperature and incubated with goat anti-mouse Alexafluor 555 (Molecular Probes) at 1:250 in antibody incubation solution. After 2-3 washes in PBS-G, cells were fixed again to preserve the binding of the first secondary antibody. Cells were subsequently permeabilized for 5-10min and blocked for 30min. Next, and in order to label the intracellular pool of AMPARs in each cell, GluA1 or GluA2 were tagged, incubating the coverslips with the same mouse anti-GluA1-NT or mouse anti-GluA2 antibodies at 1:200 (in triton-

antibody incubation solution). Following washes in PBS-G, cells were incubated with goat anti-mouse Alexafluor 647 (Molecular Probes) at 1:500 (in triton-antibody incubation solution). Coverslips were then washed and then mounted with Fluoromount (Invitrogen).

For co-localization of CPT1C with GM-130 (Golgi apparatus marker), cells were transfected with CPT1C-EGFP and Golgi staining was performed on fixed, permeabilised and blocked tsA201 cells by incubating cells with mouse anti-GM-130 (BD-Biosciences) at 1:50 in triton-antibody incubation solution and subsequently with goat anti-mouse Alexafluor 555 (Molecular Probes) at 1:500.

For co-localization of CPT1C with an endoplasmic reticulum (ER) marker we co-transfected tsA201 cells with 600ng of total DNA at a ratio of 1:2 (ER-KDEL: CPT1C). 48h after transfection cells were fixed for 15min in 4% PFA, washed and mounted in Fluoromount.

3.5. Co-localization analysis by confocal microscopy

For co-localization analysis purpose all confocal images were acquired on a spectral confocal microscope (Leica TCS-SL, CCiTUB) with 40x or 63x oil immersion lenses. For all experiments the same conditions were used: zoom setting of 2.0, the z-step for cortical neurons, tsA201 and Cos-7 cells of 0.7μm was applied.

To prevent cross-talk from the data in multiple-colour imaging, a multi-tracking mode method was employed. Each image was acquired through laser excitation lines 488, 543 and 633 and Differential Interference Contrast (DIC).

Quantification of GluA1 surface expression was performed using Image J (open source, NIH). This digital analysis of co-localization of two or more fluorescent molecules provides the information about how many fluorescent signals occupy the same voxel of the analysed image. Each stack was Z-

projected to the maximum intensity. With the freehand selection tool individual cells or dendrites coexpressing the receptor and CPT1C-EGFP or GFP (expression verified by tracking fluorescence intensity in the green channel) were traced and fluorescence in each channel was measured. The fluorescence values of each cell/dendrite were then analysed, red integrated density (INTDEN) being the value corresponding to surface expression of the receptor in that cell/dendrite, and blue INTDEN being the value for intracellular expression of GluA1 in the same cell (tsA201 cells with low levels of blue fluorescence were not quantified). The mean background intensity was obtained from three different areas of each image and subtracted from each measurement using the following formula:

CTCF=INTDEN - (AREA* MEAN FLUORESC BKGD)

where CTCF stands for corrected total cell fluorescence. Then, the ratio surface to intracellular was obtained from the CTCF value from red fluorescence (surface) divided by the CTCF value from blue fluorescence (intracellular), and normalized to the reference condition (GluA1+GFP). Finally, column graphics including the mean ratio of each condition were plotted and the error bars (SEM) were obtained. A set of at least 3 different immunofluorescences for each condition was performed and 10-50 cells of each condition were analysed for each immunofluorescence. For neuronal experiments, three separate independent cultures (10DIV) were performed and 70 and 80 dendrites from 21 and 23 neurons for each condition were analysed.

Quantification of co-localization was performed using the Manders Overlap Coefficient (MOC) (Manders et al., 1993), calculated in Image J via the JACoP plugin from images of single cells. This coefficient ranges between 1 and 0 in a way that values close to 1 indicate high co-localization while values close to 0 indicate low co-localization values.

3.6. Coimmunoprecipitation

Coimmunoprecipitation (CoIP) is designed to detect protein-protein interactions. Thus CoIP consists on the binding of a target protein with a specific antibody and precipitation of the immune complexes with Protein G or Protein A immobilized onto beads such as agarose. The precipitated immune complexes are denatured and resolved for further analysis. The procedure can be divided into the following steps: sample preparation, immunoprecipitation procedure, and analysis by Western blot and/or other methods. In our specific conditions:

Cell lysate. tsA201 cells were transfected as described previously. Forty eight hours later, transfected cells were washed twice with room temperature PBS and collected in 1ml 50mM Tris-HCl (pH 7.4) with protease Inhibitor cocktail (Sigma) on ice. All subsequent steps were performed at 4ºC. Cells were lysed in a Polytron (VDI 12; VWR) at force 5, for 20sec, twice. Lysates were centrifuged at 1,000xg for 10min to pellet nuclei and unlysed cells. The supernatant was further centrifuged at 20,000xg for 30min, and the membrane fraction (pellet) was re-suspended in solubilisation buffer (1% Triton X-100, 150mM NaCl and 50mM Tris-HCl pH8, containing protease inhibitors) and homogenized with a Polytron for 20sec. After 20min on ice, insoluble material was pelleted with a 30min centrifugation at 20,000xg and the supernatant was quantified using the BCA method (Thermo Scientific).

Immunoprecipitation. 200-300µg of total protein was incubated with 4µg of antibody overnight at 4ºC with orbital agitation (antibodies: mouse anti-GluA1-NT [N-terminus], rabbit anti-GluA2 [cytoplasmic domain] both from Merck Millipore, rabbit serum anti-GFP from Invitrogen). Antibody-protein complexes were pulled down by incubating with 80µl of Protein-A sepharose beads (Sigma) pre-equilibrated with solubilisation buffer, for 2h at 4ºC using orbital shaker. Precipitated complexes were washed in

solubilisation buffer three times and eluted with 2x SDS/DTT sample buffer, heated 10min at 76ºC and separated on SDS/PAGE.

Before adding the antibodies, 10% of total protein (100µl) was removed as input samples. 500µl of pre-cooled acetone was added to the input samples to precipitate proteins; the mixture was vortexed and incubated overnight at −20ºC. The precipitated proteins were cleared by centrifugation at 20,000xg for 20min, supernatant was removed, and pellets were air-dried for 15-30min and re-suspended with appropriate volume of 2x SDS/DTT buffer.

Immunoblotting. Samples were separated by SDS/PAGE in 4-15% mini-protean TGX precast gels, transferred using Trans-Blot Turbo transfer system on nitrocellulose membranes (all from BioRad). Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 3% (wt/vol) BSA or 5% non-fat skim milk. Membranes were incubated with primary antibody at 4ºC overnight.

Peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibodies (Dako) diluted in blocking solution, were detected by using ECL reagent (Amersham) and a LAS3000 Intelligent Dark Box (Fujifilm). Quantification of the western blots was performed with Image J (NIH). Afterwards, stripping of the membranes was performed to remove the antibodies from the blot and later to incubate with the reverse antibodies.

3.7. Acyl-Biotin Exchange assay

Detection of palmitoylation levels of GluA1 subunits was performed following the methodology described in Brigidi and Bamji, 2013.

Cell lysate. All pH solutions were adjusted the same day of the experiment. 48 h after transfection, tsA201 cells coexpressing GluA1 and GFP or GluA1 and CPT1C-EGFP, were washed with ice-cold PBS, scrapped, collected and lysed with a 30G syringe 6 times in ice-cold lysis buffer (1% IGEPAL, 50 mM Tris-HCl pH7.5, 150 mM NaCl, 10% Glycerol, Protease Inhibitor

Cocktail (Roche) and PMSF containing 50mM N-ethylmaleimide (NEM) (Sigma). All steps where performed at 4ºC with sybsequent lysis buffer also contained protease inhibitors. Lysates were cleared by centrifugation at 16,000xg for 30min and the amount of protein in the supernatant was determined using the BCA method (Thermo Scientific).

Immunoprecipitation. 750µg – 1.5 mg of total protein were used for overnight immunoprecipitation of GluA1 (4µg of anti-GluA1-NT antibody (Merck Millipore)). Then, protein-antibody complexes were pulled-down with Protein-A sepharose beads (Sigma) for 1-2 h.

Hydroxylamine (HAM) Cleavage. The total immunoprecipitate was then resuspended in lysis buffer containing 10mM NEM and it was split into two equivalent samples: one sample for specific cleavage and unmasking of the palmitoylated cysteine's thiol group by 1M hydroxylamine treatment (+HAM sample) and a second equivalent sample but in the absence of HAM to control non-specific incorporation of biotin (-HAM sample). Before performing HAM treatment, samples were totally washed to avoid any presence of unbound NEM by performing a step in stringent buffer NEM LB containing 0,1% SDS. 1M HAM solution was prepared in pH7.2 lysis buffer and +/-HAM treatment was performed for 1h at room temperature.

Biotin-BMCC Labeling. After 2-3 washes with lysis buffer plus 1% Triton X- 100 pH 7.4, selective labelling of the palmitoylated cysteine using a thiol-reactive biotinylation reagent, biotin-BMCC (1µM) (Thermo Scientific) in pH6.2 lysis buffer was done for 1h at 4ºC in +/-HAM samples, a wash in pH 6.2 lysis buffer and three washes in pH 7.4 lysis buffer were performed. Agarose beads were eluted using 30µl of 2x SB/DTT 50mM sample was vortexed and heated at 75ºC for 10 min. Then, the thiol-biotinylated proteins following the ABE steps were resolved by SDS-PAGE and Western Blotting was performed. Membranes were blocked with 3% BSA in TBS-T and incubated with Streptavidin-HRP (Invitrogen) (1:5000 from a 1mg/ml stock in 0.3% BSA). After stripping, the same membrane was

incubated with an anti-GluA1-NT antibody (1:1000) to normalize palmitoylation levels to the amount of immunoprecipitated protein.

3.8. Electrophysiology

3.8.1. General procedures

Cells were visualized with an inverted microscope (IX50; Olympus). Electrodes were fabricated from borosilicate glass (1.5mm o.d., 0.86mm i.d., Harvard Apparatus) pulled with a PC-10 vertical puller (Narishige). Electrode resistance varied between configurations (see below). Macroscopic currents were recorded at room temperature (22-25˚C) in the whole-cell configuration (wc) or from outside-out patches (o) excised from GFP-positive cells. Currents were recorded with Axopatch 200B amplifier, filtered at 2kHz (wc) or 10kHz (o) and digitized at 5kHz (wc) or 50kHz (o) using Digidata 1440A interface with pClamp 10 software (Molecular Devices Corporation).

EXTRACELLULAR SOLUTION		INTRACELLULAR SOLUTION	
Compound	**Concentration (mM)**	**Compound**	**Concentration (mM)**
NaCl	145	CsCl	145
KCl	2.5	NaCl	2.5
$CaCl_2$	1	Cs-EGTA	1
$MgCl_2$	1	Mg-ATP	4
HEPES	10	HEPES	10
Glucose	10		

Table 3.1. Solution used for electrophysiological experiments with tsA201 cells.

Solutions applied to the extracellular and cytoplasmic surfaces of the membrane will be referred to as external and internal solutions, respectively. Compositions of typical external and internal solutions used in patch-clamp experiments in tsA201 cells are shown in Table 3.1 (solutions used in mammalian neurons are given in Table 3.2). The extracellular solution was set to pH to 7.3 with NaOH. The intracellular solution was adjusted to pH=7.2 adding CsOH. Spermine tetrahydrochloride (Sigma Aldrich) was added to intracellular solution at 100μM in all cases.

3.8.2. Whole-cell recordings

Whole-cell recording is one of the most suitable methods for intracellular investigations of ion channel behavior. A schematic representation of the whole-cell recording configuration is given in Figure 3.2.

Patch electrodes were made from thick walled borosilicate capillary tubing (GC-150F; Harvard Apparatus Ltd, Edenbridge, UK) using a two-step puller (Narishige, Japan). The pipette resistance was of 3-5MΩ, giving a final series (or access) resistance of 5-15MΩ.

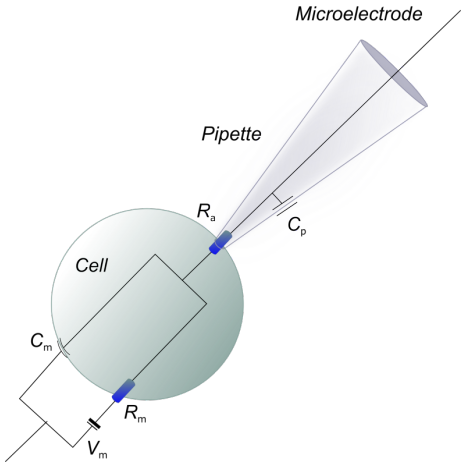

Figure 3.2. Schematic representation of whole-cell configuration. The seal is formed by the glass microelectrode and the cell membrane, thus isolating the membrane patch electrically. The pipette can access to the cell cytoplasm through the cell membrane rupture, produced by the brief but strong suction. Abbreviations used: Cp -pipette capacitance, Ra - access resistance, Cm- membrane capacitance, Rm- membrane resistance, Vm - membrane potential.

A voltage ramp protocol was used to change the holding potential: 0 to –80mV then to +80mV at a rate 160mV/s; with the voltage held at –80mV for 200ms previous to the ramp (Figure 3.3).

GFP-positive isolated cells were chosen for whole-cell recordings. Receptors were activated by a bath application of 1mM glutamate plus 25μM cyclothiazide to prevent receptor desensitization.

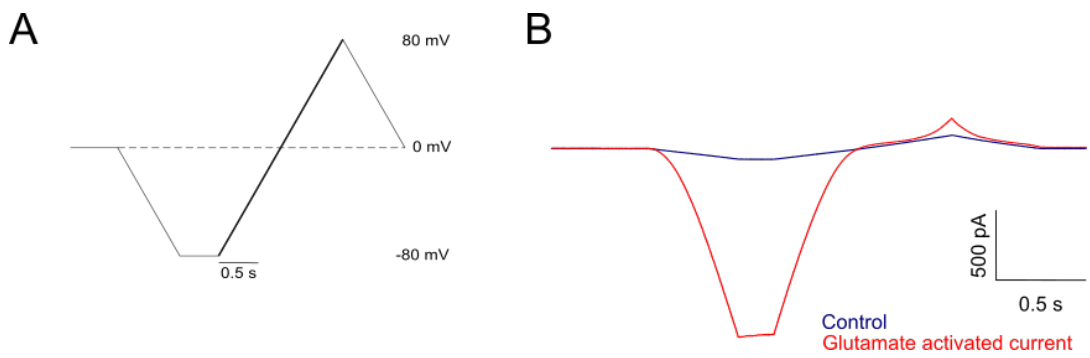

Figure 3.3. A: Whole cell recordings. Schematic depicture of the voltage ramps protocol for holding potential change (from 0 to -80mV and then to +80mV) using Digidata 1440A interface with pClamp 10 software from Molecular Devices Corporation. B: Representative traces obtained from cells expressing CP-AMPAR (GluA1): trace in the absence of glutamate (blue) and activated current (red) are shown.

For the whole cell recordings analysis control traces were subtracted from stable agonist-activated responses and the average current recorded at –80mV or –60mV was measured. In all recordings, to control for differences in cell surface area, the response to glutamate was expressed as current

density (–pA/pF; maximum current at a given potential divided by input capacitance as measured from the amplifier settings).

The rectification index (RI) was defined as the absolute value of glutamate-evoked current at +60mV divided by that at −60mV:

$$\mathrm{RI} + 60\mathrm{mV}/ - 60\mathrm{mV} = |\mathrm{I}_{+60\mathrm{mV}}|/|\mathrm{I}_{-60\mathrm{mV}}|$$

The glutamate current ramp was then plotted against voltage and normalized to generate the I/V plot (+80/-80) (see Figure 3.4).

Waveforms were imported into IGOR version 6.1 (Wavemetrics, Lake Oswego, OR) and recordings were analysed using Neuromatic (http://www.neuromatic.thinkrandom.com).

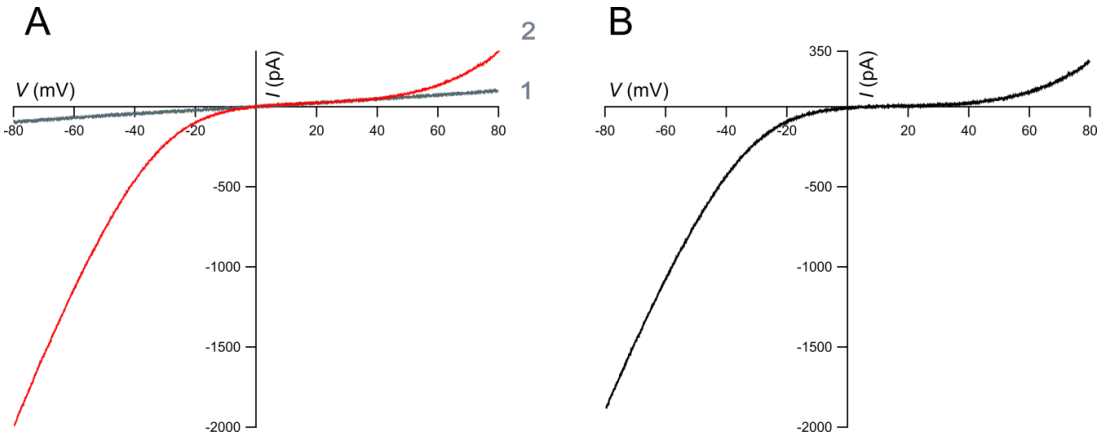

Figure 3.4. The IV relationship construction. A. The representative traces recorded by applying control solution (1) and agonist-activated responses to glutamate containing solution (2). B. IV relationship of subtracted current between before and during glutamate application representing glutamate-activated responses.

3.8.3. Agonist application to excised patches

Electrodes with a final resistance of 8-12MΩ were used for out-side out patches recordings. Rapid agonist application was achieved by switching

between a continuously flowing control solution (extracellular solution diluted by 4%) and a glutamate-containing solution (10mM glutamate and 2.5mg/ml of sucrose diluted in extracellular solution). Solution switching (see Figure 3.5) was achieved by piezoelectric translation of a theta-barrel application tool made from borosilicate glass (1.5 mm o.d.; Sutter Instruments) mounted on a piezoelectric translator (P-601.30; Physik Instrumente).

During each experiment 100ms jumps were applied to outside-out patches at a holding potential of –60mV (Figure 3.5). At some recordings jumps at +60mV were applied to calculate RI according to the formula shown previously. At the end of each experiment, the adequacy of the solution exchange was tested by destroying the patch and measuring the liquid-junction current at the open pipette (the 10-90% rise time was normally 200-400μs).

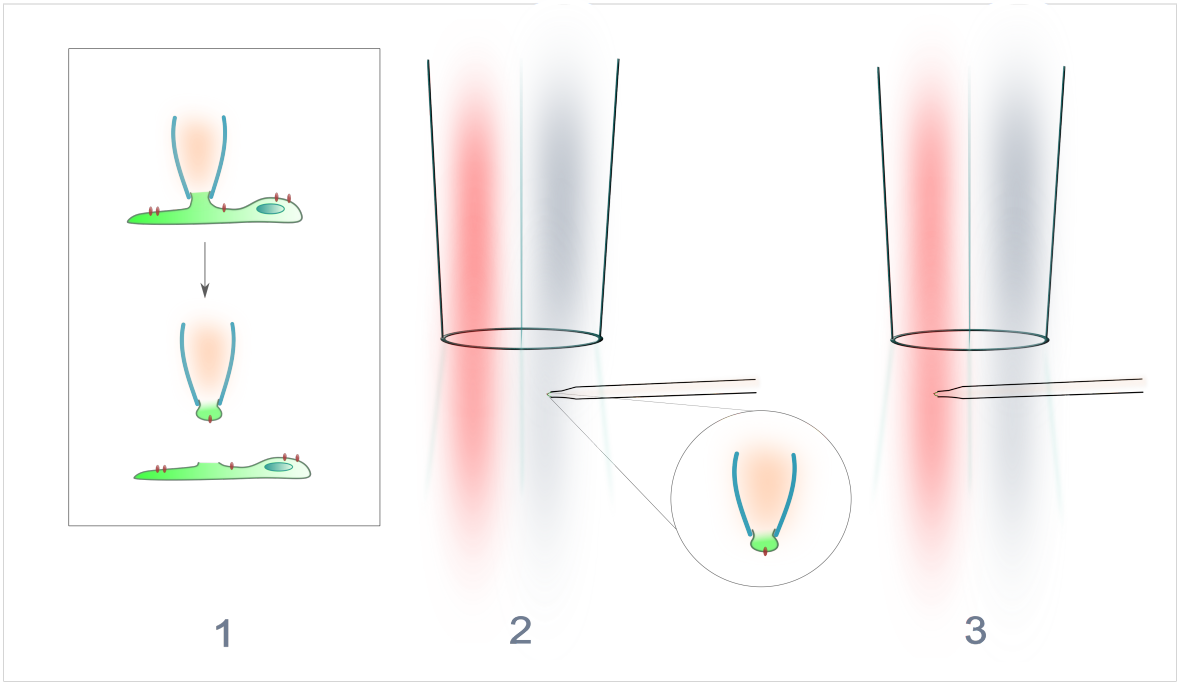

Figure 3.5. 1. Scheme of out-side out configuration: after reaching the whole-cell, the patch pipette is pulled back to excise a membrane patch containing receptors. 2 and 3. Representation of the out-side out patches switching between control (blue) and agonist (red) containing solution.

3.8.4. Non-stationary fluctuation analysis (NSFA)

To deduce the channel properties from macroscopic currents, several consecutive responses to glutamate (10mM) of outside-out patches (100ms duration, 1 Hz, V_{hold} –60mV) were analysed. The ensemble variance of all successive pairs of current responses was calculated. The single channel current (i) and the total number of channels in the patch (N) were calculated by plotting this ensemble variance against mean current ($\bar{I}$) and fitting with Sigworth parabolic function:

$$\sigma^2 = \sigma_B^2 + \left(i\bar{I} - \left(\frac{\bar{I}^2}{N} \right) \right)$$

where σ^2_B is the background variance. Along with normal peak-to-peak variation in the currents due to stochastic channel gating, some patches presented a gradual decline in peak amplitude. The mean response was calculated from the periods of the recordings showing stable responses that were identified using a Spearman rank-order correlation test (Igor Pro with Neuromatic). The weighted-mean single-channel conductance was determined from the single-channel current and the holding potential corrected for the calculated liquid-junction potential (+4.9mV; pClamp 10).

3.8.5. Miniature EPSC recordings

Whole cell recordings were obtained from cultured hippocampal neurons cultures 15 - 16 days after plating. Extracellular and intracellular solutions are shown in the Table 3.2 (pH values were adjusted to 7.3 with NaOH and 7.2 with KOH for extracellular and intracellular solutions respectively). To isolate AMPAR-mediated miniature EPSCs (mEPSC), the following blockers were added to the extracellular solution: 1μM TTX, 50 μM D-AP5, 25μM 7-CK, and 20μM SR95531/Gabazine (all from Abcam). Series resistance (Rs) was typically 15-22 MΩ, and was monitored at the beginning and at the end of the experiment. Cells recordings where the

series resistances varied by more than 20% were rejected. Events were detected using amplitude threshold crossing, with the threshold (typically ~7-8 pA) set according to the baseline current variance. For amplitude and kinetic analyses only events with monotonic fast rise (<1ms) and uncontaminated decay were included.

EXTRACELLULAR SOLUTION		INTRACELLULAR SOLUTION	
Compound	*Concentration (mM)*	*Compound*	*Concentration (mM)*
NaCl	115	K-gluconate	116
KCl	5	KCl	6
CaCl2	1.8	NaCl	2
MgCl2	1	EGTA	0.5
TEA-Cl	1	HEPES	20
HEPES	5	Mg-ATP	2
Glucose	20	Na-GTP	0.3

Table 3.2. Solution used for electrophysiological experiments with neurons.

3.9. Statistical analysis

Electrophysiological recordings were analysed using IGOR Pro (Wavemetrics Inc.) with NeuroMatic (Jason Rothman, UCL). Data are presented in the text as the mean ± SEM from n patches and in the figures as bar plots of the group mean, with error bars denoting the SEM.

Comparisons between two groups were performed using the non-parametric Mann-Whitney U test. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism version 5.0d for Mac OS X or Windows 8 (GraphPad Software, San Diego California USA, www.graphpad.com).

4. RESULTS

Several proteomic studies have recently showed that CPT1C is one of the numerous protein constituents of native AMPARs in adult rat brain tissue (Schwenk et al., 2012; Schwenk et al., 2014). Thus, the primary objective of this thesis has been focused on the study of AMPAR function regulation by CPT1C by using molecular biology and electrophysiological techniques in heterologous expression systems and neuronal cell cultures. Electrophysiological characterisation of CPT1C effect on AMPARs has unravelled the role of CPT1C in AMPAR trafficking and the mechanism involved in CPT1C modulation of AMPARs. In parallel, the use of molecular biology and immunofluorescence techniques has helped us to give some clues whether CPT1C interacts with AMPARs and has shed light on CPT1C modulation of AMPAR function. To sum up, the work performed in this thesis has shown for the first time a role for the enigmatic CPT1C protein, which we show here that plays a role on AMPA receptor surface expression.

4.1. AMPAR-CPT1C Interaction

4.1.1. AMPA receptor subunit GluA1 coimmunoprecipitates with CPT1C in heterologous systems

Since CPT1C has been demonstrated to interact with AMPAR subunits in brain tissue from rats (Schwenk et al., 2012; Schwenk et al., 2014), we first decided to determine if in our expression system model CPT1C was able to interact with AMPAR subunits.

To test the interaction between both proteins, we first performed a coimmunoprecipitation assay to assess whether GluA1 subunits interact with CPT1C. For this purpose tsA201 cells were transiently transfected with

GluA1 and CPT1C-EGFP. For negative controls cells were transfected with GluA1 together with an empty plasmid containing GFP (pEGFP) or CPT1C-EGFP along with an empty plasmid (pcDNA3.0). Following this step, solubilized membranes of the transfected cells were immunoprecipitated with anti-GFP or with anti-GluA1-NT antibody and subsequently analysed by Western blotting (n=3). The correct expression of both proteins was tested (Figure 4.1, left panel). The ~100 KDa band was not detected with GluA1 antibody in anti-GFP immunoprecipitates from cells transfected only with GluA1 or CPT1C-EGFP . Anti-GFP antibody could pull down GluA1 when expressed together with CPT1C-EGFP (Figure 4.1, upper middle panel). Additionally GluA1-NT antibody was able to pull down CPT1C-EGFP protein (Figure 4.1, lower right panel). This experiment revealed that recombinant GluA1 subunits interact with CPT1C in tsA201 cells when both proteins are co-expressed.

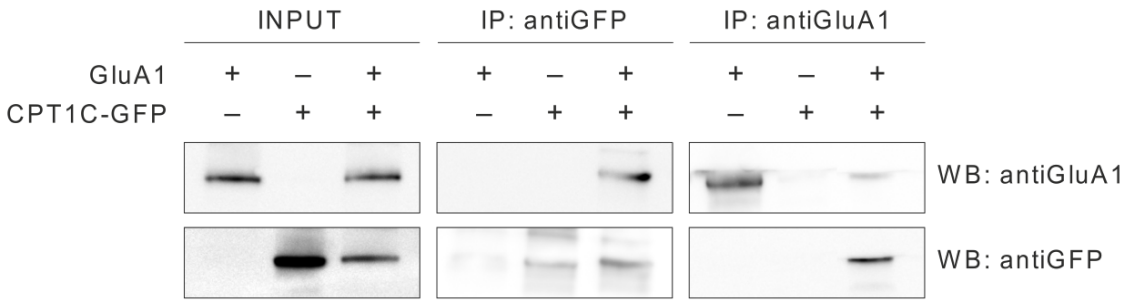

Figure 4.1. CPT1C interacts with recombinant GluA1 subunits. Western Blot analysis obtained from tsA201 membrane fraction expressing GluA1 alone or together with CPT1C-EGFP . This experiment was replicated three times. Solubilised membranes of the transfected cells were immunoprecipitated with antiGFP antibody (IP: antiGFP) or with anti-GluA1-NT antibody (IP: antiGluA1). Samples underwent SDS-PAGE separation followed by Western Blot analysis using anti-GluA1-NT (WB: antiGluA1) or anti-GFP (WB: antiGFP) antibodies. Input samples (INPUT) show correct expression for the used constructs (left panels). GFP recognizing antibodies could pull down GluA1 when expressed together with CPT1C-EGFP (upper middle panel). No band was detected by GluA1 antibodies in extracts from cells expressing only GluA1 or CPT1C in anti-GFP-immunoprecipitates.

4.1.2. AMPA receptor subunit GluA2 coimmunoprecipitates with CPT1C in heterologous systems

Among the four different types of AMPAR subunits, GluA2 is a unique and important subunit. It confers very distinct properties to AMPAR when compared with other GluA subunits. Besides, it is greatly expressed along a wide range of neurons in the CNS (Traynelis et al., 2010).

Thus, to confirm whether CPT1C also interacts with GluA2 subunits in tsA201 cells, identic conditions were provided for coimmunoprecipitation assays of GluA2 and CPT1C. The experiment was repeated three times. As negative controls, cells were transfected with GluA2 together with an empty plasmid containing GFP (pEGFP) or CPT1C-EGFP along with an empty plasmid pcDNA3.0. The correct expression of both proteins is observed (Figure 4.2, left panel). In this experiment GluA2 coimmunoprecipitated with CPT1C-EGFP (Figure 4.2, middle upper panel) in the membrane fraction of the cells expressing both proteins when it was pulled down with anti-GFP. Also CPT1C-EGFP was pulled down by anti GluA2 antibody (Figure 4.2, lower right panel). The absence of the band (Figure 4.2, upper middle panel), typical for GluA2, was observed in anti-GFP immunoprecipitates of membrane fraction of cells transfected with GluA2 or CPT1C-EGFP alone. Either no band was detected in anti-GluA2 immunoprecipitates in extracts expressing only one of the proteins of interest. Therefore we confirmed the GluA2-CPT1C interaction in tsA201 cells expressing both proteins.

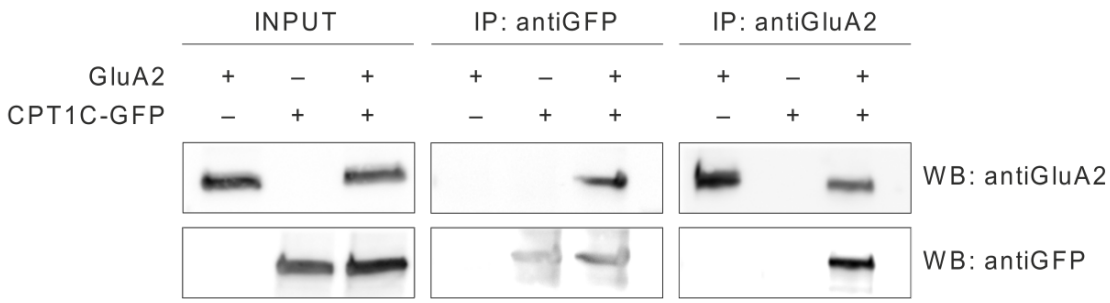

Figure 4.2. CPT1C interacts with recombinant GluA2 subunits. Representative immunoblot of tsA201 membrane fraction expressing either GluA2 alone of together with CPT1C-EGFP (this experiment was repeated 3 times). Solubilized membranes of the transfected cells were immunoprecipitated with anti-GFP antibody (IP: antiGFP) or with rabbit polyclonal anti-GluA2-CTD antibody (IP: antiGluA2). After SDS-PAGE separation Western Blot analysis was performed using mouse anti-GluA2 (WB: antiGluA2) or anti-GFP (WB: antiGFP) antibodies. Input samples show correct expression of used constructs (left panel). GFP recognizing antibodies could pull down GluA2 when expressed together with CPT1C-EGFP . No band was detected by GluA2 antibodies in extracts from cells expressing only GluA2 or CPT1C in anti-GFP-immunoprecipitates.

4.2. Modulation and characterization of AMPAR-mediated currents by CPT1C

4.2.1. CPT1C increases whole-cell currents mediated by GluA1 homomers

Given that our coimmunoprecipitation experiments confirmed the interaction between GluA1 or GluA2 and CPT1C in heterologous systems, the next step was to elucidate whether CPT1C was contributing to AMPAR function. In order to assess any functional consequences of CPT1C-AMPAR interaction, electrophysiological recordings were performed using tsA201 cells expressing GluA1 alone or in presence of CPT1C. We used a 1:2 ratio (GluA1:CPT1C) to ensure proper CPT1C levels in the transfected cells.

Whole-cell recordings were carried out to measure the glutamate-evoked currents at different holding potentials by applying a voltage ramp from −80 to +80mV. The recordings were performed in presence of 1mM glutamate into the bath where transfected cells were placed. The bath included 25μM of CTZ to block receptor desensitization. Two examples of currents recorded between −80 and +80mV in cells transfected with GluA1 or GluA1+CPT1C are shown in Figure 4.3. The differences between AMPAR responses in tsA201 cells transfected alone or together with CPT1C are highlighted in the Figure 4.4.

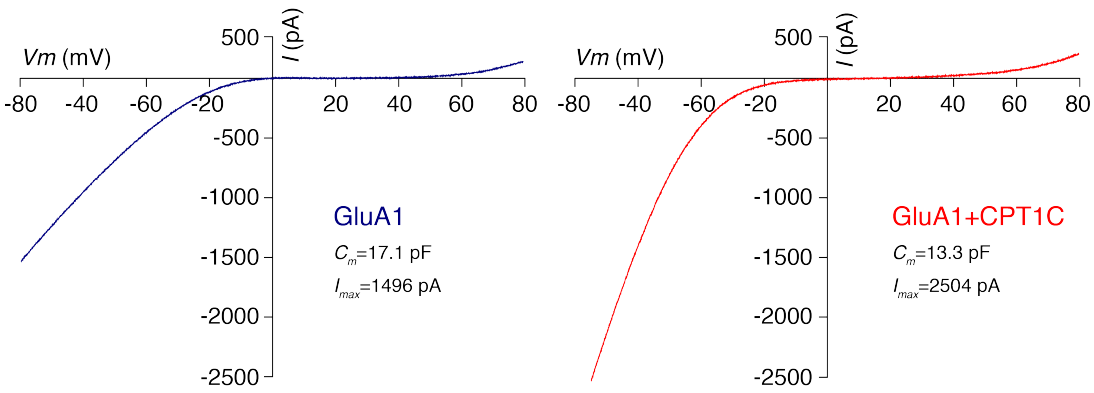

Figure 4.3. Representative AMPAR responses of tsA201 cells expressing homomeric GluA1 alone (left) or together with CPT1C (right) in the presence of 1mM glutamate and 25 μM CTZ, plotted as current at different membrane voltages (IV curve). Cell membrane capacitance and maximum current for these two cells are shown. The current voltage relationship IV is obtained from the whole-cell glutamate responses at membrane potentials between -80mV and +80mV.

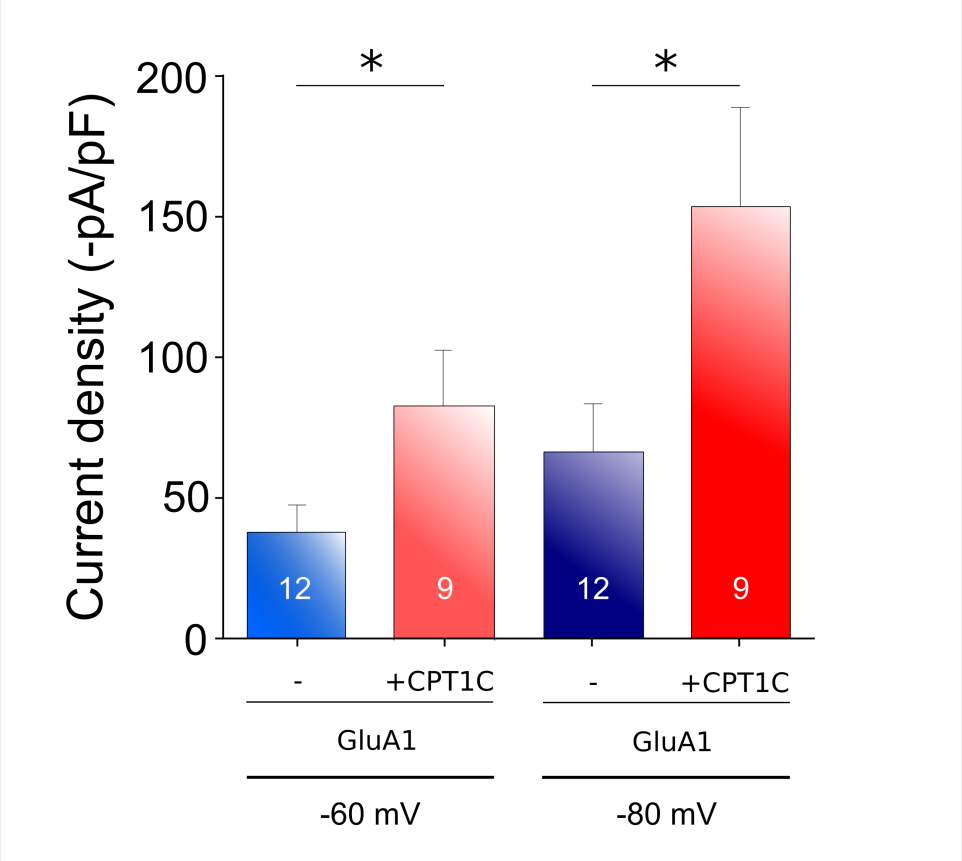

*Figure 4.4. Average of normalized currents at -60 and -80 mV for GluA1 alone (blue bars) or together with CPT1C (red bars). GluA1 current density (-pA/pF) was increased when co-expressed with CPT1C at both tested voltages (*p < 0.05; Mann–Whitney U-test) for both holding potential.*

The results obtained from these experiments showed that whole-cell current density measured at −60 mV from cells expressing GluA1 alone was lower than those currents recorded for GluA1 together with CPT1C (37.86 ± 9.636 pA/pF *vs.* 83.42 ± 19.78 pA/pF; p = 0.049; Mann–Whitney U-test; n = 12 and 9 respectively) (Figure 4.4). Additionally there was a significant difference between whole-cell current density measured at -80 mV for cells expressing GluA1 alone or together with CPT1C (66.35 ±

17.15 pA/pF *vs*. 153.6 ± 35.25 pA/pF; p = 0.023; Mann–Whitney U-test; n = 12 and 9 respectively).

Although the current density takes into account the size of the cell and normalize the responses (–pA/pF) to avoid for example bigger current responses from bigger cells with more membrane surface, no differences were found between the capacitances of the tested cells expressing GluA1 alone or together with CPT1C (23.81 ± 3.39 pF *vs*. 18.67 ±1.82 pF; p= 0.667; Mann–Whitney U-test; n = 12 and 9 respectively) (Figure 4.5).

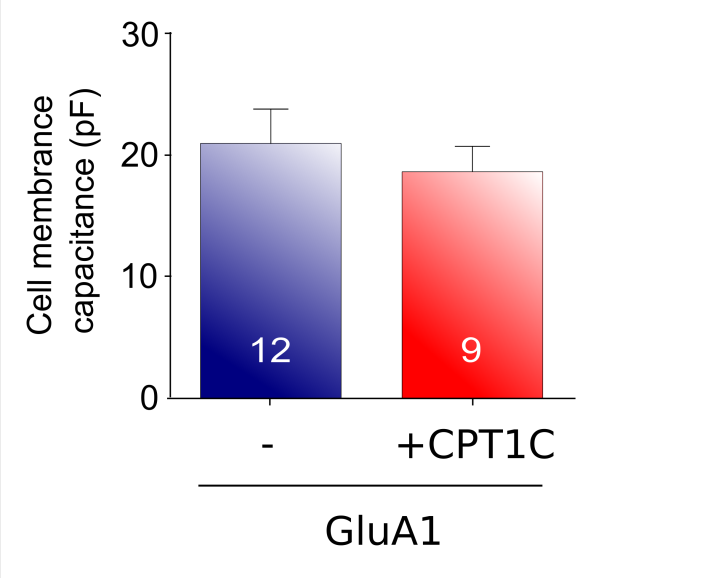

Figure 4.5. No significant differences were found between cell membrane capacitance in tested cells expressing GluA1 alone or together with CPT1C (p> 0.05; Mann–Whitney U-test; n = 12 and 9 respectively).

4.2.2. GluA1-CPT1C stoichiometry is important in the magnitude of the current increase

There is a large volume of published studies describing the role of AMPARs and accessory subunits – in example TARPs (Nicoll and Bredt, 2006; Milstein and Nicoll 2008; Straub and Tomita, 2012). Some studies have considered TARP/AMPAR stoichiometry (Shi et al., 2009; Kim et al., 2010) and have found that this is variable and depending on the number of TARP

molecules present in an AMPAR complex, the behavior of the receptor is changed. Thus to go further in understanding the modulation of CPT1C on AMPARs we decided to investigate whether AMPAR-CPT1C stoichiometry shapes AMPAR-mediated responses. For this purpose tsA201 cells were transfected with GluA1 alone and together with CPT1C at 1:1 ratio respectively. As control the same conditions were used but at 1:2 transfection ratio. Interestingly, no effect was found in cells transfected at 1:1 ratio. Figure 4.6 shows current densities at -80 mV for both 1:2 and 1:1 ratio respectively (52.24±12.81pA/pF for GluA1 *vs*. 146.2 ±22.79pA/pF for GluA1+CPT1C at 1:2 ratio with n= 10 for both conditions and p=0.0052; 165.2±21.30pA/pF for GluA1 *vs*. 209.1±35.17pA/pF for GluA1+CPT1C at 1:1 ratio with n= 13 for both conditions and p>0.05). Hence, these data indicate that CPT1C likely needs a minimum stoichiometry to functionally affect AMPARs.

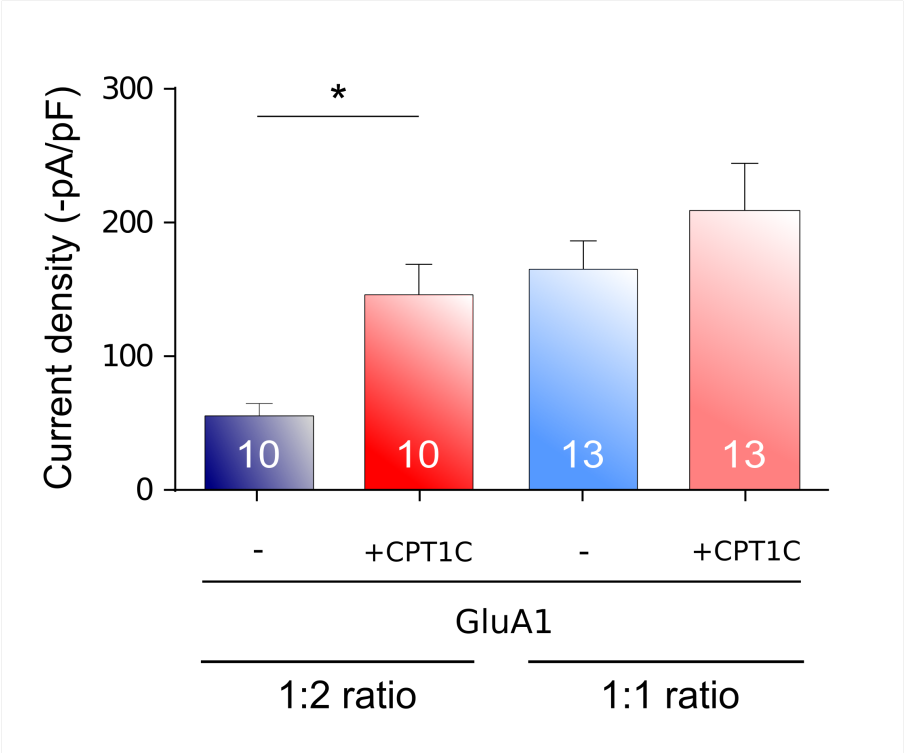

*Figure 4.6. Average of normalized current density at -80 mV for GluA1 alone or together with CPT1C at ratio 1:2 and 1:1 respectively. GluA1 current density (-pA/pF) was not increased when co-expressed with CPT1C at 1:1 ratio despite a clear effect at 1:2 ratio (*p < 0.05; Mann–Whitney U-test).*

4.2.3. CPT1A does not alter GluA1 induced whole-cell currents

Since CPT1A is also expressed in neurons as CPT1C (McGarry and Brown, 1997), we evaluated whether CPT1A could have a similar effect on AMPAR function. To that end, electrophysiological whole-cell recordings were carried out using tsA201 cells expressing GluA1 alone or in presence of CPT1A. No significant differences were found between current densities from cells expressing GluA1 alone or together with CPT1A (37.86 ± 9.64pA/pF *vs*. 49.9 ± 19.32pA/pF; p = 0.796; Mann–Whitney U-test; n = 12 and 5 respectively) where cells tested had similar cell membrane capacitance (Figure 4.7; right panel). These results suggest that CPT1C protein effect on GluA1 induced currents is specific of this isoform.

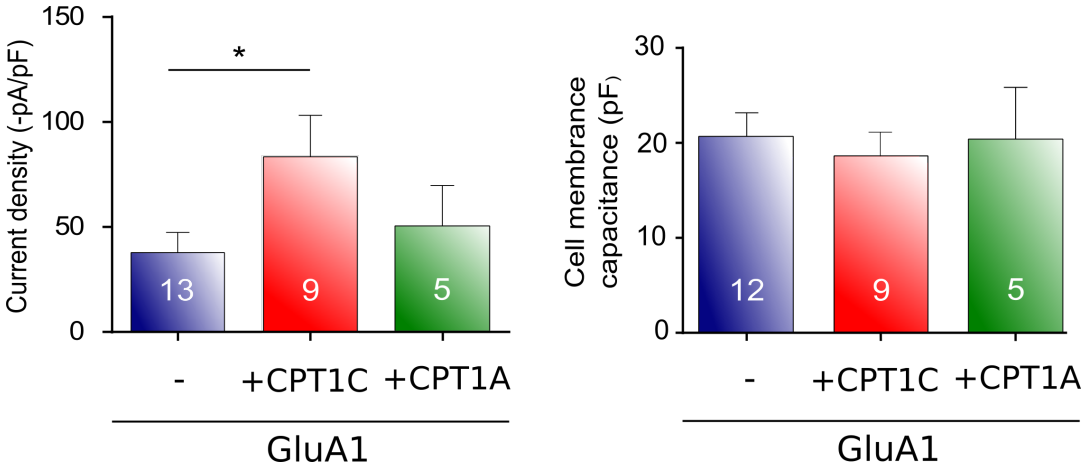

Figure 4.7. Average normalized current density at –60 mV for GluA1 alone or together with CPT1A. GluA1 current density (–pA/pF) was not increased when co-expressed with CPT1A (p > 0.05; Mann–Whitney U-test). No significant differences were found between cell membrane capacitance (right panel) in tested groups.

4.2.4. CPT1C subcellular localitzation determines its effect on AMPAR currents

The N-terminal of CPT1s is responsible for CPT1C and CPT1A specific and different location (Sierra et al., 2007). Concretely, while CPT1C N-terminal domain determines its ER targeting, CPT1A N-terminal domain is responsible for its mitochondrial location. Thus, we decided to explore whether the effect of CPT1C on AMPARs is caused by ER localization and if CPT1A could exert a similar effect on AMPA mediated currents than CPT1C when targeted to ER. For this purpose, two chimeric proteins were used, where N-terminal of CPT1C was cloned into the CPT1A-EGFP plasmid (named hereafter: C-CPT1A) and N-terminal of CPT1A was replaced into CPT1C-EGFP plasmid (named hereafter: A-CPT1C). First, to confirm CPT1A and CPT1C mislocalization, these constructs were transfected in COS-7 cells. Co-localization imaging experiments were performed with these constructs and MitoTracker (a potential-sensitive dye that accumulates in mitochondria) or KDEL (ER marker).

As it can be observed in the confocal photo-micrographs in the Figure 4.8 the CPT1C with the localization motif of CPT1A (A-CPT1C) shows a clear mitochondrial pattern, meanwhile CPT1A with the localization domain of CPT1C (C-CPT1A) localises at the ER. Quantification of these localizations was performed by using Mander's Overlap coefficient (MOC) as it is shown in the Figure 4.9. Hence, we confirmed the change in the subcellular location for CPT1C and CPT1A depending on the switch of their location motifs.

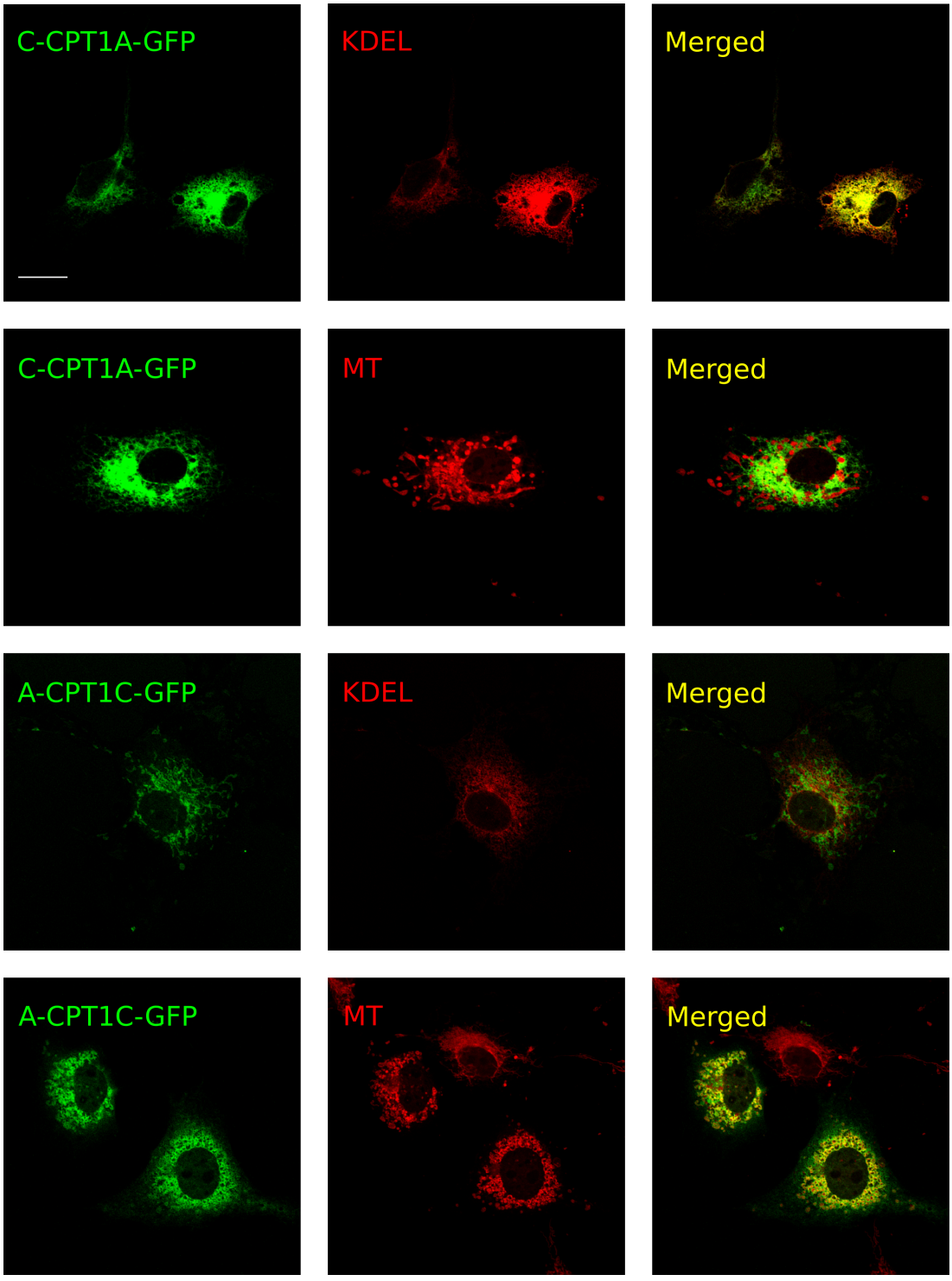

Figure 4.8. Representative confocal images of COS7 cells expressing C-CPT1A or A-CPT1C constructs, co-tranfected with KDEL plasmid (KDEL) or treated with MitoTracker (MT). The constructs (green signal) and ER o mitochondria (red signal) are shown. Scale bar: 20 µm.

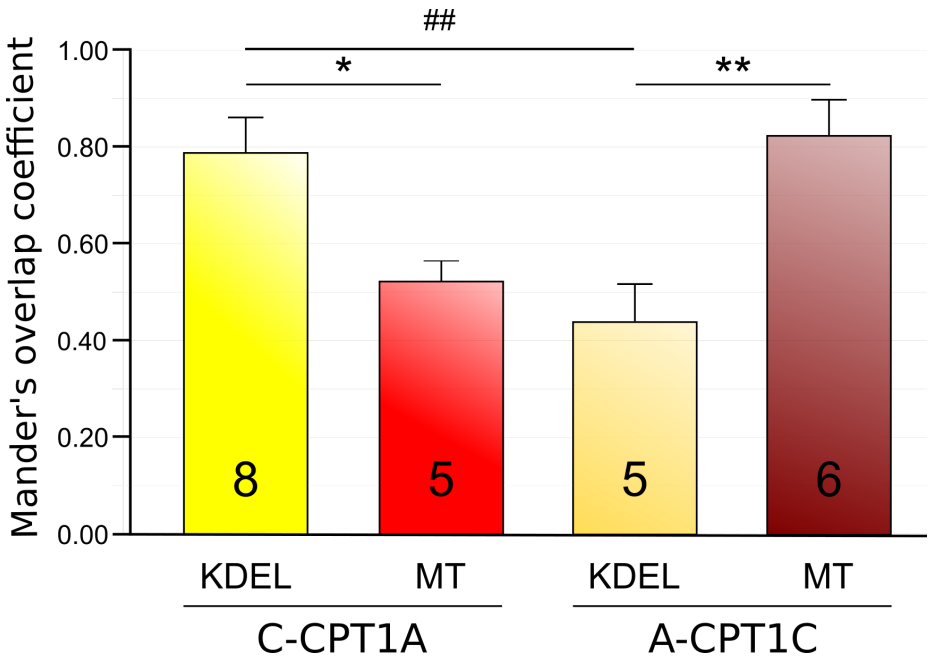

*Figure 4.9. Bar graph indicating co-localization values by Mander's coefficient expressed as mean value. Error bars represent SEM. MOC values for C-CPT1A co-transfected with KDEL were statistically different from A-CPT1C transfected with KDEL (bright yellow and pale yellow columns respectively); ##p=0.0031; Mann–Whitney U-test; n = 8 for C-CPT1A and n=5 for A-CPT1C). MOC values for A-CPT1C in MitoTraker (MT) treated cells show strong co-localization (dark red column) when compared with A-CPT1C co-transfected with KDEL (**p= 0.0043). MOC values for C-CPT1A (bright yellow column) co-transfected with KDEL were statistically different compared with the cells expressing this construct but treated with MT (*p=0.0109).*

Next, in order to assess the functional consequences of CPT1A and CPT1C on AMPAR function when they are in a different subcellular location from their usual one, electrophysiological whole-cell recordings were carried out using tsA201 cells expressing GluA1 alone, in presence of CPT1C or with chimeric C-CPT1A and A-CPT1C. As seen before, Figure 4.10 shows that GluA1 current density was lower in cells expressing GluA1 alone when compared with cells expressing GluA1 together with CPT1C (80.83 ± 11.75 pA/pF *vs*. 133.9 ± 16.29 pA/pF; p = 0.0062; Mann–Whitney

U-test; n = 23 and 31 respectively). GluA1 current density of the cells expressing GluA1 alone was similar to the current density of the cells co-expressing A-CPT1C due to the mislocalization of CPT1C (80.83 ± 11.75 pA/pF *vs*. 102.7± 16.29 pA/pF; p = 0.1571; Mann–Whitney U-test; n = 23 and 16 respectively) indicating that the effect of CPT1C depends on its subcellular location. No differences were found when compared the cells expressing GluA1 alone or with GluA1 co-expressed with CPT1A at ER location (C-CPT1A) (80.83 ± 11.75 pA/pF *vs*. 97.24± 16.97 pA/pF; p=0.1571; Mann–Whitney U-test; n = 23 and 14 respectively). Finally, GluA1 current density of the cells co-expressing A-CPT1C was similar to those when co-expressed with C-CPT1A (102.7± 16.29 pA/pF *vs*. 97.24± 16.97 pA/pF; p = 0.8419; Mann–Whitney U-test; n = 16 and 14 respectively).

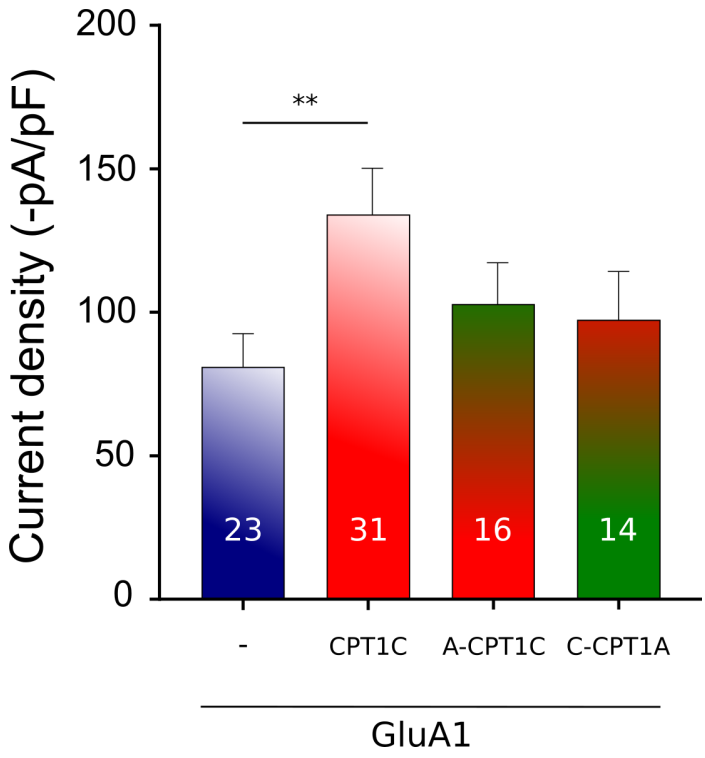

*Figure 4.10. Average normalized current density at –80 mV for GluA1 alone or together with CPT1C or chimeric proteins. No significant differences were found between GluA1 current density (–pA/pF) in cells co-expressing A-CPT1C or C-CPT1A (*p > 0.05; Mann–Whitney U-test).*

These results show that A-CPT1C protein is not able to modulate AMPAR-induced currents in the same extent as CPT1C wild type form. However it looks like there is a residual effect probably due to its pass through the ER before its traslocation to the mitochondria but it is not significantly different for GluA1 group and definitively this effect is lower when compared with CPT1C. Additionally, when CPT1A localises at ER together with GluA1 is not modulating AMPAR-induced currents, confirming the specificity of CPT1C member of the CPT1 family.

4.2.5. CPT1C has no effect on GluA2 homomers mediated whole-cell currents

A large and growing body of literature has investigated the role of GluA2 subunit in AMPAR function. This subunit is widely expressed in the brain (Lu et al., 2009), forming predominantly AMPAR heteromers together with GluA1 (Greger et al., 2003) and determining many of the major biophysical properties of the native receptor (reviewed by Isaac et al., 2007). Since the study performed by Schwenk and colleagues in 2012 determined that CPT1C is also forming part of GluA2-containing AMPAR macromolecular complex and our results confirmed that CPT1C also interacts with GluA2 subunit in heterologous systems, the next step of the present thesis was to determine whether CPT1C could also modulate GluA2 homomeric AMPARs using whole-cell density current experiments.

As can be seen from the Figures 4.11-12, no increase in current density was detected at neither –60mV nor –80mV in cells expressing GluA2 homomers alone or together with CPT1C. The quantification of the current densities at –60 mV did not show differences between groups: 43.44 ± 11.99pA/pF for GluA2 alone *vs*. 36.99 ± 14.33pA/pF for GluA2+CPT1C, p= 0.3481; Mann–Whitney U-test; n= 7 and 9 respectively; for current densities at -80 mV: 61.85 ± 16.48pA/pF for GluA2 alone *vs*. 52.28 ± 20.27pA/pF for GluA2+CPT1C; p= 0.2991; Mann–Whitney U-test; n= 7 and 9 respectively)

(see Figure 4.12). No differences were found between the capacitances of the tested cells expressing GluA2 alone or together with CPT1C (25.79 ± 4.03pF vs. 26.93 ± 3.89pF; p= 0.8371; Mann–Whitney U-test; n = 7 and 9 respectively).

The results obtained from these experiments revealed a subunit specificity of CPT1C modulation.

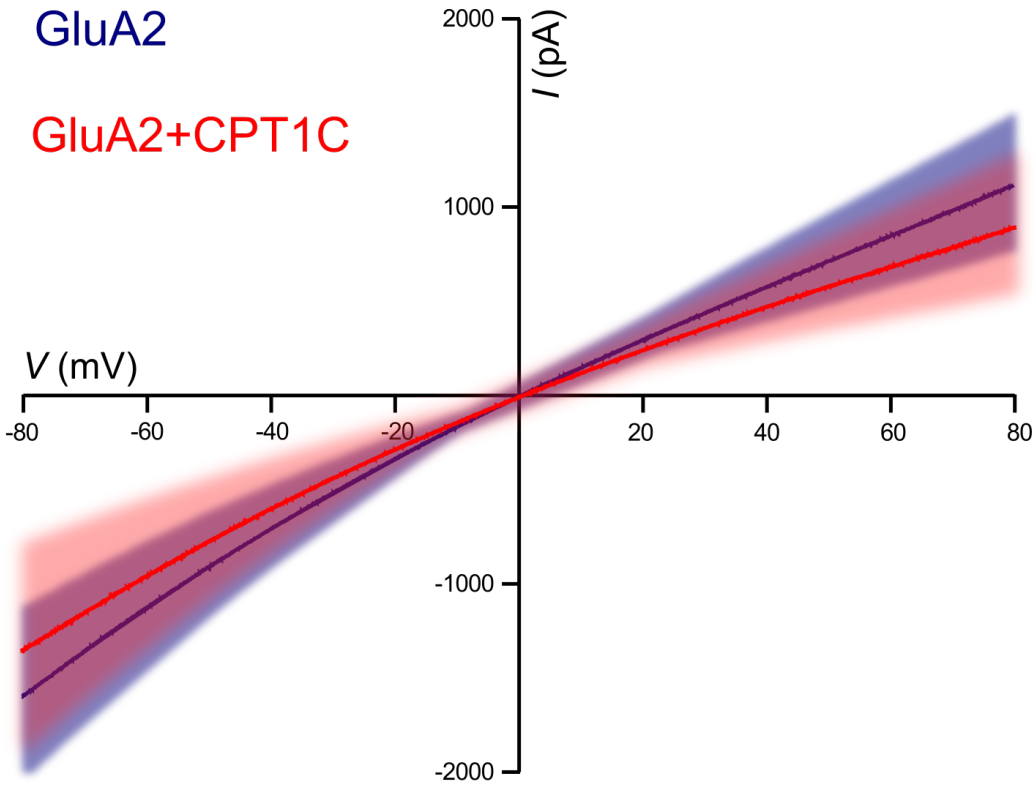

Figure 4.11. Average of non-normalized AMPAR responses of tsA201 cells expressing either homomeric GluA2 alone (blue) or together with CPT1C (red) in the presence of 1mM glutamate and 25µM CTZ, plotted as IV relationship. The current vs. voltage relationship IV is obtained from the whole cell glutamate response at membrane potentials between –80 and +80mV.

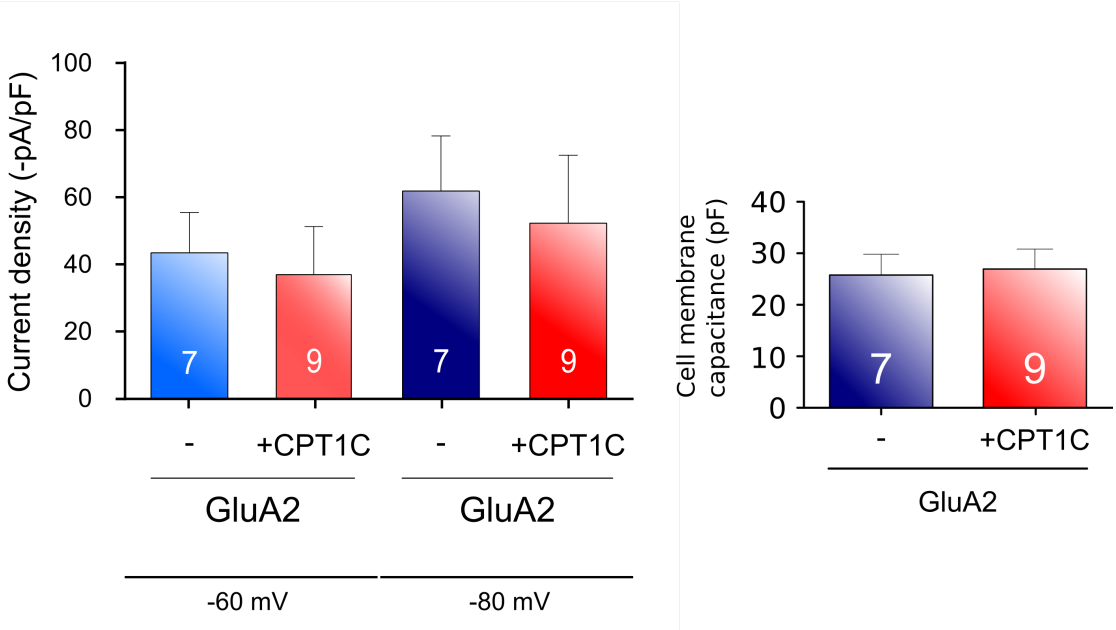

Figure 4.12. Average normalized currents at -60 and -80 mV for GluA2 alone or together with CPT1C. GluA2 current density (-pA/pF) was not increased when co-expressed with CPT1C (p> 0.05; Mann–Whitney U-test). Numbers in bars denote the number of recordings. No significant differences were found between cell membrane capacitance (right panel) in tested groups.

4.2.6. CPT1C increases whole-cell currents of GluA1/GluA2 heteromeric AMPARs

As extensively shown in the literature, GluA2-containing (heteromeric) AMPARs prevail in the glutamatergic neurons in CNS, being expressed along with GluA1, GluA3 or GluA4 (Gallo et al., 1992; Kondo et al., 1997; Lu et al., 2009; Reimers et al., 2011) and subunit heteromerization gives AMPARs a wide range of functional and trafficking properties.

Thus the next step of the present study was to evaluate whether GluA1/GluA2 heteromeric receptors currents were similarly affected by CPT1C as GluA1 homomeric receptors. To do so tsA201 cells were transfected with GluA1 along with the GluA2 plasmid in presence or

absence of CPT1C. In these experiments the GluA1 plasmid was expressing mCherry protein and GluA2 plasmid was not expressing any fluorescent protein. GluA2 expression was confirmed by the linearity of the IV relationship. Records with RI<0.7 were rejected assuming too much contribution of GluA1 subunit. The ratio of the receptor:CPT1C was 1:1:4 (GluA1:GluA2:CPT1C) to favor the presence of CPT1C on the AMPAR complex. This way it was possible to patch mCherry and GFP positive cells allowing the recording of GluA1-containing receptors with GluA2 present in the receptor (checked by recordings linearity).

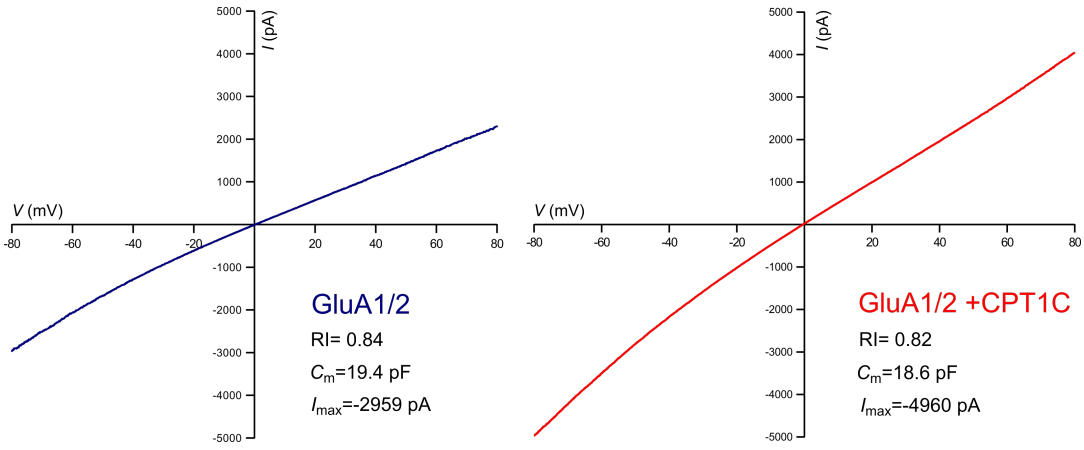

Figure 4.13. Whole cell IV relationship for two cells with similar membrane capacitance and rectification index, expressing GluA1/GluA2 heteromeric AMPARs alone or together with CPT1C. The current voltage relationship IV was obtained from the whole cell glutamate response at membrane potentials between –80 and +80 in the presence of 1mM glutamate and 25µM CTZ.

Recordings in Figure 4.13 show two representative responses for heteromeric GluA1/GluA2 (with or without CPT1C), and it is apparent that for both conditions, the patched cells displayed linear responses, ensuring the presence of GluA2 subunits into the complex. As it can be observed, no differences were found between groups as for the rectification index (RI) (Figure 4.14, right panel), thus confirming that GluA2 subunit was present in the same proportion in both groups ($RI_{+60/-60}$ = 0.84 ± 0.04 without CPT1C

vs. 0.82 ± 0.04 with CPT1C; p = 0.8910; Mann–Whitney U-test; n = 19 and 18 respectively).

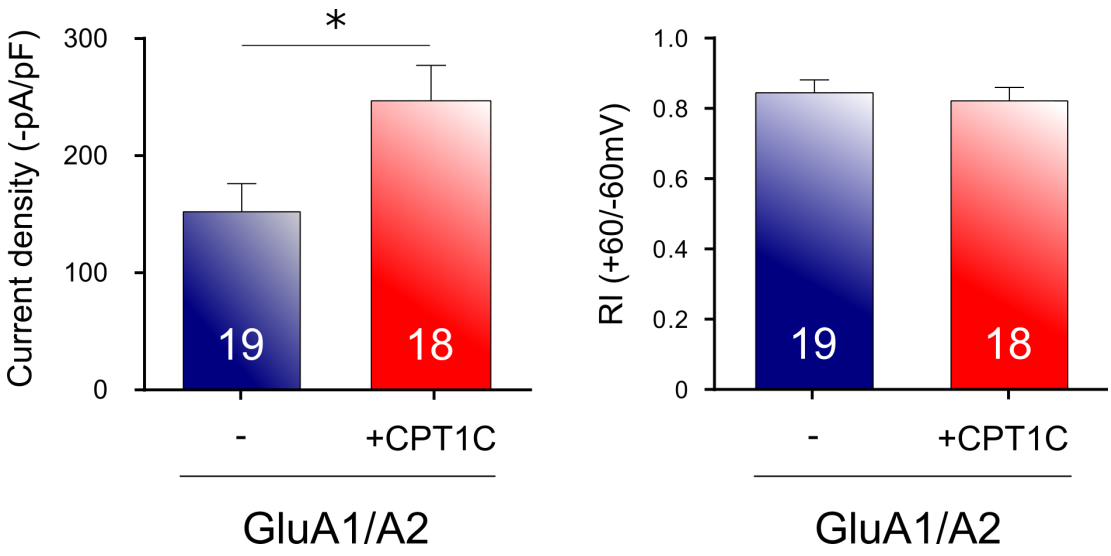

*Figure 4.14. Average normalized currents at −80 mV for GluA2 alone or together with CPT1C (left panel). GluA1/A2 current density (−pA/pF) was increased by co-expression with CPT1C (*p < 0.05; Mann–Whitney U-test). Rectification index (RI; $I_{+60mV/I-60mV}$) gives a read-out of GluA2 incorporation (right panel). Numbers in bars denote the number of recordings. Average RI for the cells recorded in both conditions showing no differences between groups.*

As shown in Figure 4.14 (left panel), CPT1C increased current density of GluA1/A2 heteromeric receptors (152.1 ± 23.94 pA/pF for GluA1/GluA2 *vs*. 246.9 ± 30.26 pA/pF for GluA1/GluA2 + CPT1C; p = 0.0222; Mann–Whitney U-test; n = 19 and 18 respectively). These experiments showed that the main combination of AMPARs found typically in neurons is also modulated by CPT1C.

4.2.7. CPT1C gain of function in low glucose conditions

Historically, research investigating the factors associated with CPT1C function has focused on its role in brain metabolism due to the role of CPT1A&B in FA oxidation.

It has been long known that glucose is the most important energy source for the adult brain under normal conditions. A decrease in glucose levels decreases malonyl-CoA levels and thus releases its inhibition of CPT1 system (McGary et al., 1997). Hence, in order to assess whether changes in the energy-dependent state of CPT1C affect AMPARs function, tsA201 cells were maintained and transfected in low glucose conditions (1g/L, instead of 3.15 g/L).

We found that current density of tsA201 cells expressing GluA1 alone was similar in low and high glucose conditions (49.2 ± 6.77 pA/pF for GluA1 in low glucose conditions *vs*. 43.56 ± 8.32 pA/pF for GluA1 in high glucose media; p = 0.1528; Mann–Whitney U-test; n = 12 and 25 respectively)(see Figure 4.15).

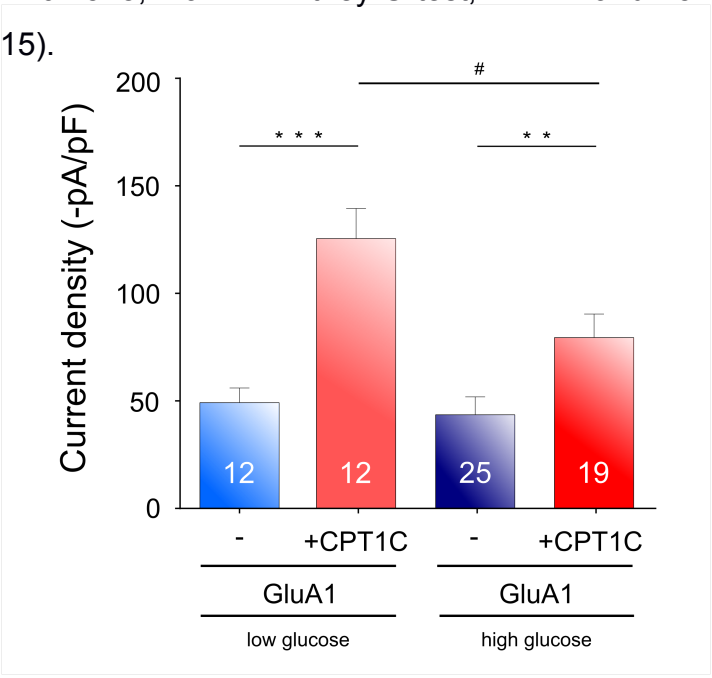

Figure 4.15. Average normalized currents at -60 for GluA1 alone or together with CPT1C. Current density of tsA201 cells maintained in low glucose conditions expressing GluA1 together with CPT1C is increased comparing with the same conditions but kept in high glucose medium (p=0.0215).

Furthermore, current density was increased in cells expressing GluA1 together with CPT1C in high glucose conditions (43.56 ± 8.32pA/pF for

GluA1 alone *vs*. 79.47 ± 10.93pA/pF for GluA1+CPT1C; p = 0.006; Mann–Whitney U-test; n = 25 and 19 respectively) as seen before.

In low glucose conditions GluA1+CPT1C expressing cells showed increased current density when compared with cells expressing GluA1 alone (49.2 ± 6.77pA/pF for GluA1 *vs*. 125.4 ± 14.03pA/pF for GluA1+CPT1C; p = 0.0003; Mann–Whitney U-test; n = 12 for both).

Interestingly, this increment in current density was more pronounced than the one observed for high glucose conditions. Statistical comparison showed significant differences between current densities from tsA201 cells expressing GluA1+CPT1C maintained in low glucose conditions when compared with GluA1+CPT1C maintained in high glucose conditions (p=0.0215; Mann–Whitney U-test; n = 12 and n=19 respectively).

It seems then that CPT1C functional effect on GluA1 is dependent on the metabolic state of the cell, a factor that is affecting CPT1C function.

4.2.8. CPT1C does not modify gating properties of AMPARs

The experiments carried out so far have clearly found a significant increase in the glutamate-evoked GluA1-mediated currents in presence of CPT1C. The total amount of current carried by a given population of receptors depends on several factors, which include the single channel conductance, the kinetics, the open probability of the receptor and the number of receptors contributing to the current. Any alteration in these parameters might result in changes in the current magnitude. So, given that CPT1C is an interacting protein of the AMPAR complex, one possibility was that CPT1C could modulate the single channel conductance or open probability of AMPARs either by a direct interaction (Soto et al., 2007, Soto et al., 2014b; Suzuki et al., 2008; Coombs et al., 2012; Shelley et al., 2012) or indirectly by phosphorylation (Derkach et al., 1999; Banke et al., 2000; Kristensen et al., 2011) as it has been extensively shown for other proteins that interact with AMPAR subunits. To evaluate this possible scenario, the

next step of the present work was to investigate the mechanisms contributing to the current increase observed in AMPARs together with CPT1C. To determine whether AMPAR single channel conductance was altered by CPT1C tsA201 cells were transfected with GluA1 either alone or together with CPT1C. Then, fast applications of glutamate (10 mM; 100 ms duration; 1 Hz) were applied onto out-side out patches followed by non-stationary fluctuation analysis of the glutamate-evoked responses. Figure 4.16 shows typical responses for GluA1 homomers alone or together with CPT1C. Figure 4.17 shows the noise analysis for the cells displayed in Figure 4.16, analysis by which the single channel conductance is inferred.

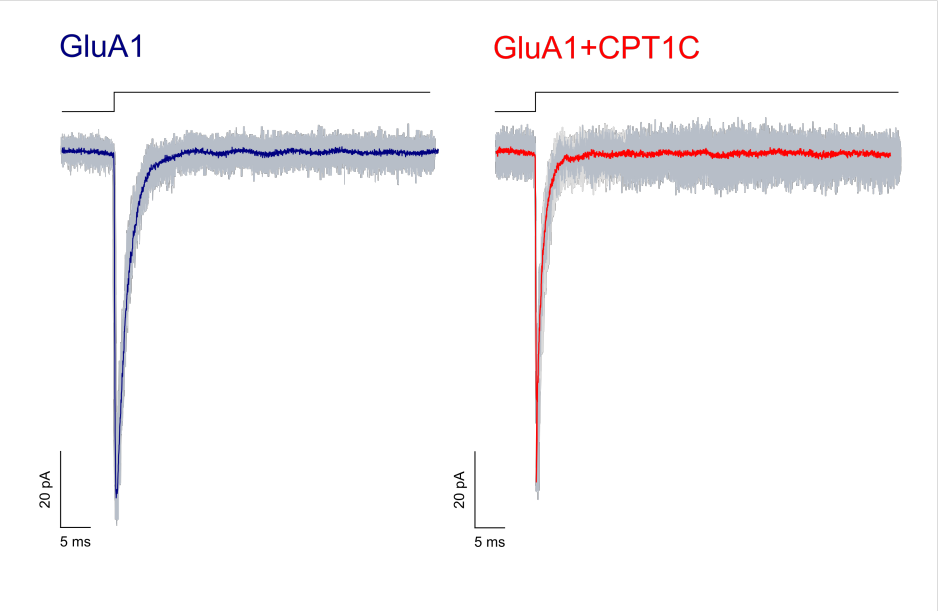

Figure 4.16. Current activated by rapid application of 10mM glutamate (100 ms, −60mV) into an outside-out patch from a tsA201 cell expressing GluA1 alone (left panel). Trace in grey represents a single response and the average of 37 responses is shown in red. Right panel shows current activated in the same conditions as GluA1 but for an outside-out patch from a tsA201 cell expressing GluA1 plus CPT1C-EGFP . The average of 38 responses is shown in red and a single response is in grey color.

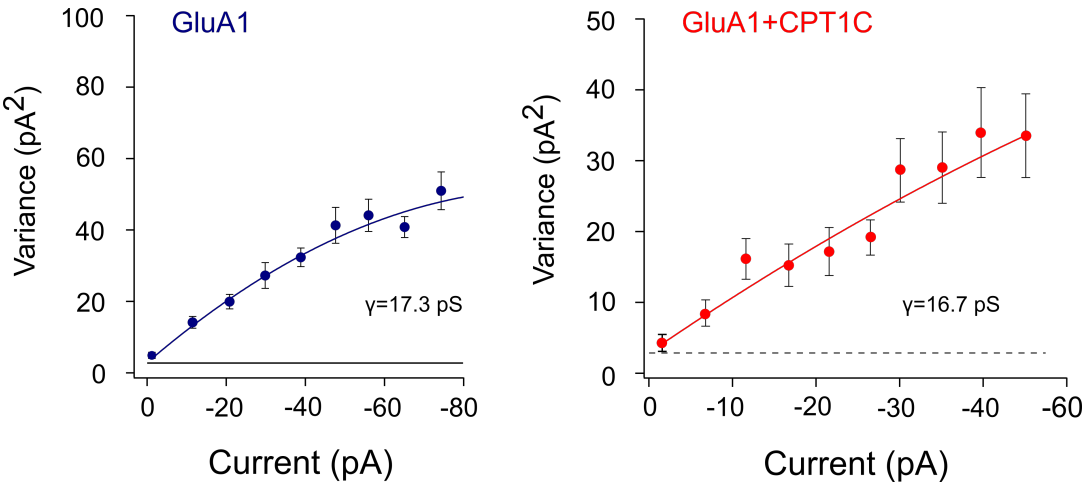

Figure 4.17. Current variance vs. mean current plotted for the patches shown in Figure 4.16 giving a weighted mean single-channel conductance of 17.3 pS for GluA1 alone and 16.7 pS for GluA1+CPT1C.

As it can be observed from Figure 4.18-A, the single channel conductance of GluA1 homomers was not modified when co-expressed with CPT1C (16.53 ± 1.07pS for GluA1 alone *vs*. 17.07 ± 1.31pS for GluA1+CPT1C; p = 0.8095, Mann–Whitney U-test; n = 17 for both). CPT1C presence neither affect peak open probability ($P_{o,peak}$) of AMPARs (0.43 ± 0.04 for GluA1 *vs*. 0.40 ± 0.05 for GluA1+CPT1C; p = 0.6052, Mann–Whitney U-test; n = 17 for both) (Figure 4.18-B). Furthermore, the AMPARs kinetics measured as the desensitization decay time constant were not changed (2.32 ± 0.18ms for cells expressing GluA1 alone *vs*. 2.53 ± 0.17ms for cells expressing GluA1+CPT1C; p = 0.3112, Mann–Whitney U-test; n = 18 for both) (Figure 4.18-C).

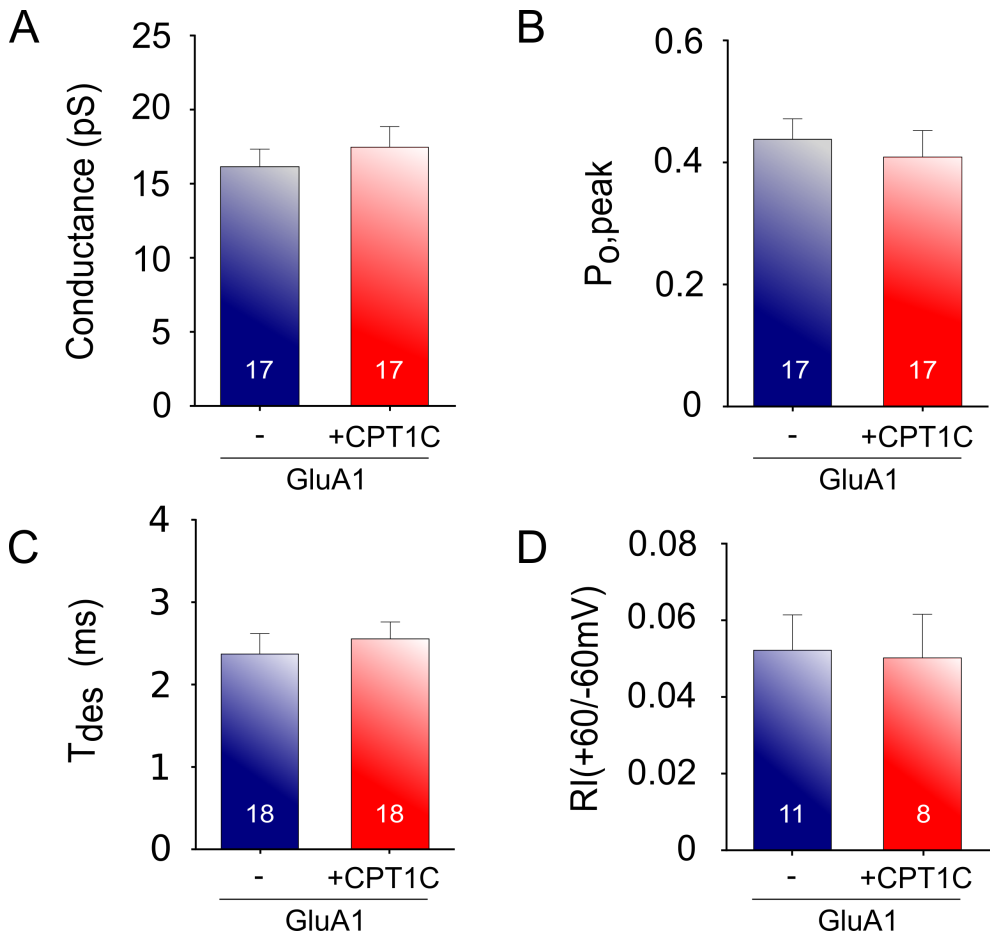

Figure 4.18. CPT1C interaction does not alter AMPA receptor gating properties. No effect was observed when GluA1 was expressed with CPT1C on single channel conductance (A), peak open probability (B), desensitization kinetics (C) or rectification index (D) of GluA1 homomeric AMPARs; p> 0.05, Mann–Whitney U-test for all groups.

Additionally, rectification index (RI) was measured to evaluate if any differences in CP-AMPARs block by polyamine in the presence of CPT1C was occurring, taking into account findings by Cull-Candy's laboratory who showed that several auxiliary proteins interacting with CP-AMPARs attenuate polyamine block of the channel (Soto et al., 2007; Soto et al., 2009; Coombs et al., 2012). Thus, RI was determined at +60/–60 mV as described in "experimental procedures", however no alteration was seen in the strong inwardly rectifying IV relationship of CP-AMPARs (Figure 4.18D; 0.051 ± 0.009 for cells expressing GluA1 alone *vs*. 0.049 ± 0.011 for cells

expressing GluA1+CPT1C; p = 0.8554, Mann–Whitney U-test; n = 11 and 8 respectively).

To sum up, the results so far show that glutamate-evoked current density of the recombinant GluA1 subunits is increased when CPT1C is present and this effect is specific of the CPT1 isoform CPT1C. However no changes in current density were found in cells expressing GluA2 subunit along with CPT1C indicating CPT1C subunit specificity. Additionally, electrophysiological experiments with outside-out patches from cells expressing GluA1 determined that channel properties (single channel conductance, peak open probability and desensitization kinetics) are not altered. Thus, these results suggest that the increased current density is probably due to an increase in AMPAR number at the cell surface. Moreover, these findings can further indicate that both proteins do not associate directly at the plasma membrane despite a larger amount of current when CPT1C is co-expressed with GluA1.

4.3. Co-localization studies of AMPARs and CPT1C

4.3.1. GluA1 subunits co-localise with CPT1C

The next step within the present thesis was to assess the exact cellular location where CPT1C interacts with GluA1. For this purpose, we studied the presence or absence of CPT1C at the cell surface. Thus, an immunofluorescence assay was performed using tsA201 cells co-transfected with GluA1 and CPT1C-EGFP . The aim of this experiment was to visualize the interaction between CPT1C and GluA1 subunits at the surface and internal compartments by using confocal microscopy (Figure 4.19). Confocal photo-micrographs in Fig 4.19 show GluA1 and CPT1C-EGFP (green in both panels) co-localization at intracellular compartments (light blue; left panel). The total pool of GluA1 subunits is shown in blue. No co-localization was found at the cell surface (right panel) where surface GluA1 is shown in red.

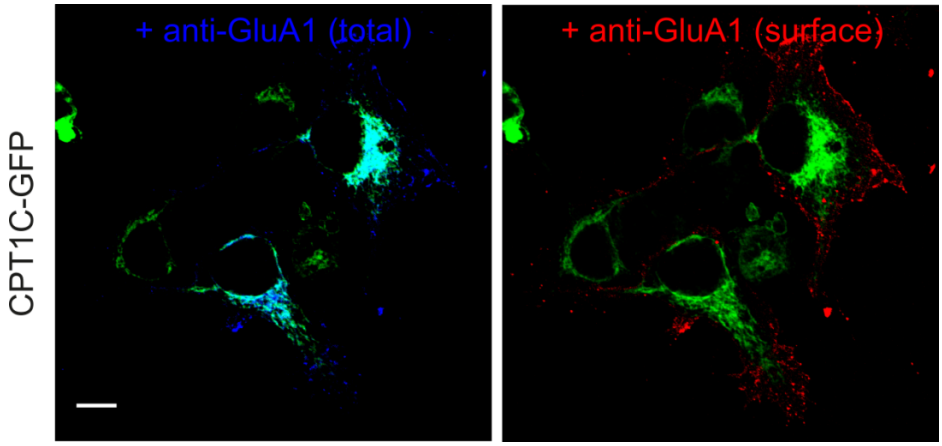

Figure 4.19. Confocal images showing GluA1 (dark blue) and CPT1C-EGFP (green) co-localization (light blue) at intracellular compartments (left panel) but not at the cell surface (right panel) where GluA1 was tagged in red. Scale bar 10µm.

4.3.2. GluA2 subunits co-localise with CPT1C

Since CPT1C also interacts with GluA2 although there is no apparent functional effect on AMPAR subunits, we wanted to study the co-localization of GluA2 and CPT1C as well. tsA201 cells were transfected with CPT1C-EGFP and GluA2 for immunofluorescence assay. Confocal images showed that CPT1C-EGFP co-localised with intracellular GluA2. Similarly, no co-localization was found with surface GluA2 (Figure 4.20).

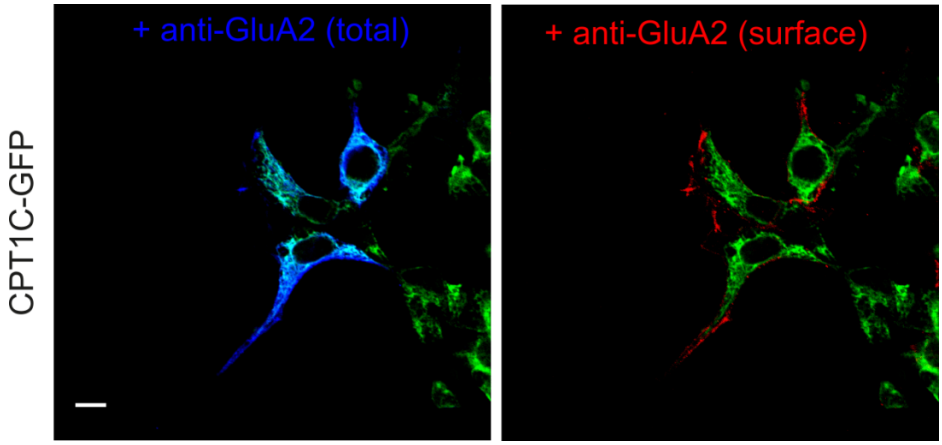

Figure 4.20 Confocal images showing GluA2 (dark blue) and CPT1C-EGFP (green) co-localization (light blue) in tsA201 co-transfected cells. Scale bar 10µm.

4.3.3. CPT1C co-localise with AMPAR subunits at the ER but not at Golgi

Results from co-localization studies with Mito-tracker in heterologous systems and neurons performed by Sierra et al., 2008 and Carrasco et al., 2012 showed that CPT1C is localised at ER membrane. Thus, co-localization experiments were performed in tsA201 cell lines to confirm the subcellular localization of CPT1C in the ER (Figure 4.21, left panel). Since GluA subunits dwell in the Golgi Apparatus during posttranscriptional modifications (Greger et al., 2002), the next step was to check whether CPT1C was present at Golgi level, which might be indicative that CPT1C and AMPARs might interact at this level. Using Golgi Apparatus marker (GM-130) we found that CPT1C showed no co-localization with GM-130 as seen in Figure 4.21 (right panel). Quantification of the co-localizations shown in the Figure 4.19, 4.20 and 4.21 was performed using Mander's Overlap coefficient (MOC) as shown in Figure 4.22. Both GluA1 and GluA2 subunits showed good co-localization with CPT1C (0.67 ± 0.14 for GluA1 and 0.59 ± 0.12 for GluA2; blue columns; n = 10).

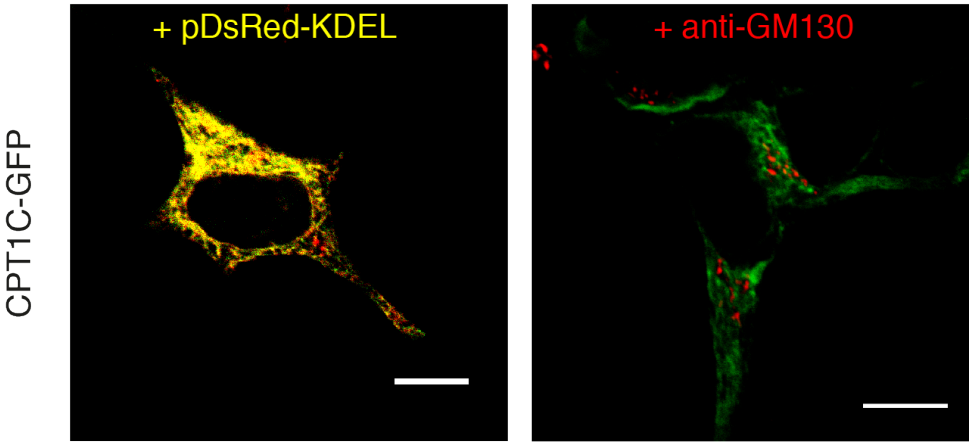

Figure 4.21. Confocal photomicrograph showing co-localization of CPT1C-EGFP with ER marker pDsRed-KDEL (yellow signal in left panel). Right panel depict lack of co-localization (no yellow signal) between CPT1C-EGFP (green) and GM-130 (red) in tsA201 transfected cells. Scale bar 10µm.

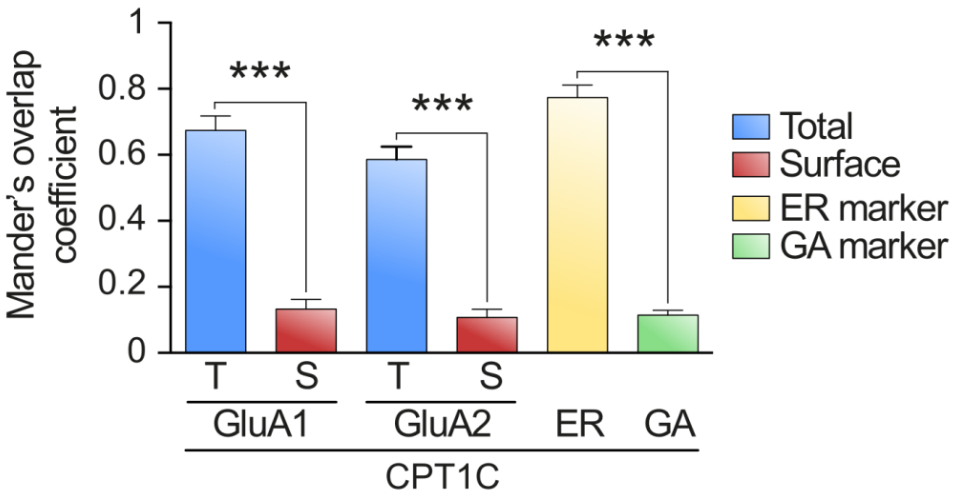

*Figure 4.22. Bar graph indicating co-localization values by Mander's coefficient expressed as mean value. Error bars represent SEM. MOC values for total GluA1 and GluA2 and CPT1C (blue columns) were statistically different from surface AMPARs and CPT1C (red columns; ***p < 0.001 for both comparisons; Mann–Whitney U-test; n = 10 for all conditions). MOC values for CPT1C-pDsRed-KDEL show strong co-localization (yellow bar). MOC values for CPT1C-GM130 (green bar) were similar to those of surface GluAs-CPT1C.*

Interestingly, these data demonstrate that there is no co-localization of CPT1C with surface receptors confirming that the interaction does not take place at the cell-surface. The MOC values for CPT1C and surface GluA1 (0.13 ± 0.09; n = 10) or surface GluA2 (0.11 ± 0.08; n = 10) are also shown in the Figure 4.22. Finally MOC analysis clearly shows that CPT1C is present into ER (yellow bar, Figure 4.22) but it is not trafficked to Golgi apparatus (green bar, Figure 4.22).

As it can be observed in the Figures 4.19-4.21, CPT1C expression seems to be restricted to areas close to the nucleus and with a reticular pattern. Results from these experiments indicate that CPT1C co-localises with intracellular GluA1 when co-expressed in tsA201 cell lines. Furthermore, CPT1C also co-localise with intracellular GluA2 subunits in cells expressing both proteins.

So, data obtained from these experiments demonstrates that AMPAR subunits are together with CPT1C at the ER level but not at the plasma membrane.

4.4. Effect of CPT1C on AMPAR trafficking

4.4.1. Surface expression of GluA1 is increased in the presence of CPT1C in heterologous systems

Since our previous experiments in the present thesis demonstrated that AMPAR gating properties were not altered by CPT1C, even though the whole-cell currents were increased when tsA201 cells were co-transfected with GluA1 and CPT1C, we hypothesized that such increase in whole-cell currents might be driven by an increased number of receptors present at the cell surface.

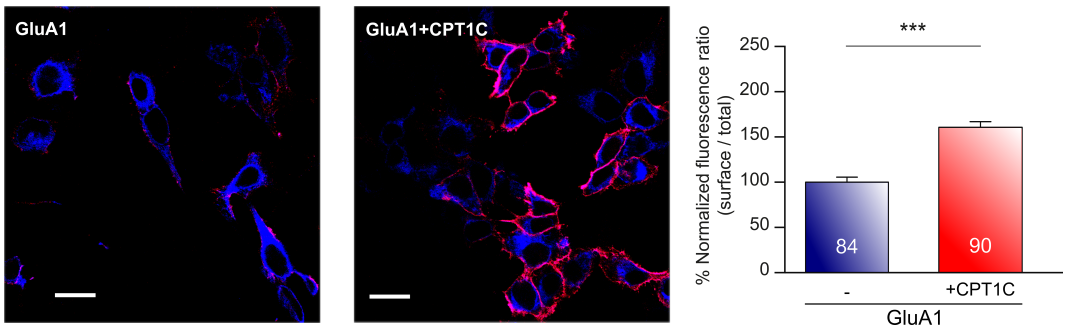

Figure 4.23. Representative confocal photomicrographs of tsA201 cells co-expressing GluA1 and GFP (left panel) or GluA1 and CPT1C-EGFP (middle panel). Surface GluA1 was labeled in live cells with anti-GluA1-NT and Alexafluor 555 (red signal). Subsequently cells were permeabilized and total GluA1 expression level was labeled with the same primary antibody but with Alexafluor 647 (blue signal). Scale bars: 20µm. Right panel shows immunocytochemistry quantification.

To test this hypothesis we determined AMPAR subunits surface and intracellular expression by means of immunofluorescence quantification. Surface GluA1 in live transfected tsA201 cells were immunostained (Figure 4.23, red signal), followed by cell permeabilization and staining of the total GluA1 pool (Figure 4.23, blue signal). Quantification was performed calculating the ratio of the surface expression of GluA1 (red signal) *vs*. the total expression level of GluA1 (blue signal) for the same cell due to the variability in expression levels.

As shown in Figure 4.23 (right panel) the normalized ratio surface to total GluA1 was increased in cells co-expressing GluA1 and CPT1C-EGFP (100 ± 5.53% for GluA1 alone *vs*. 160.6 ± 6.24% for GluA1+CPT1C expressing cells; $p < 0.0001$, Mann–Whitney U-test; n = 84 and 90 cells respectively from four immunocytochemistry experiments for each condition). These findings indicate a role of CPT1C in increasing GluA1 trafficking to the cell surface.

The same experiment was performed using tsA201 cells transfected with GluA2 subunits alone or together with CPT1C (Figure 4.24). Quantification analysis revealed that surface ratio was not increased in cells co-transfected with GluA2 and CPT1C-EGFP (100 ± 9.13% for GluA2 alone *vs*. 80.97 ± 9.95% for GluA2+CPT1C expressing cells; $p = 0.0759$, Mann–Whitney U-test; n = 37 and 38 cells respectively from three immunocytochemistry experiments for each condition) confirming the lack of effect on GluA2 in previous electrophysiology experiments.

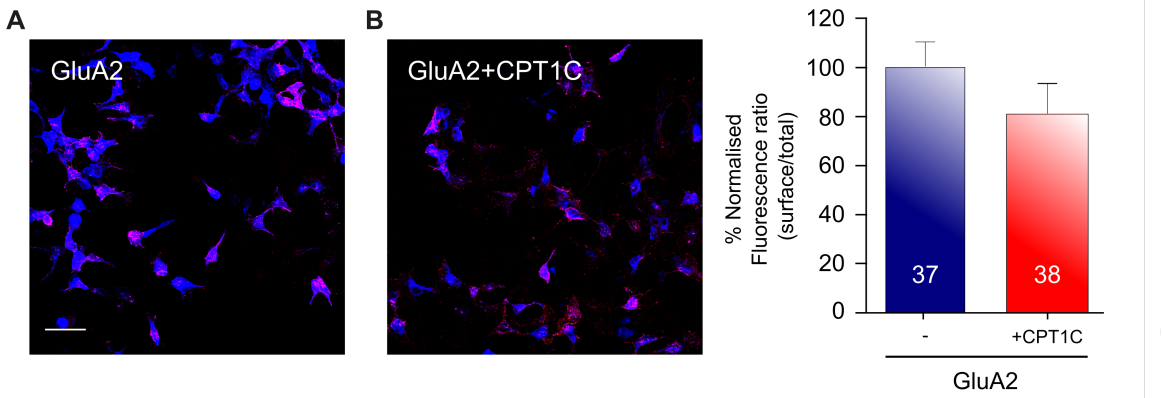

Figure 4.24. Representative confocal photomicrographs of the tsA201 cells co-expressing GluA2 and GFP (A) or GluA2 and CPT1C-EGFP (B). Scale bars: 20µm. Left panel: quantification of fluorescence ratio normalized to GluA2 ratios.

4.4.2. Surface expression of GluA1 is increased in the presence of CPT1C in cortical neurons

In order to study CPT1C role in surface expression of native AMPARs, neuronal cultures were used for immunofluorescence assay. For this purpose immunofluorescence experiments were carried out in primary cortical neurons cultures at 10 DIV (Figure 4.25). Following the methodology used with tsA201 cells, the GluA1 surface to total ratio was measured in dendrites from GFP transfected neurons compared with CPT1C-EGFP overexpressed neurons. Figure 4.25 (panels A2-4, B2-4) shows examples of the analyzed dendrites. The results obtained showed that neurons overexpressing CPT1C increased the GluA1 surface to total ratio (100 ± 3.58 % for control GFP transfected neurons *vs*. 118.8 ± 5.65 % for CPT1C-EGFP transfected neurons; p = 0.0226; n = 70 and 80 dendrites respectively from 3 different cultures each).

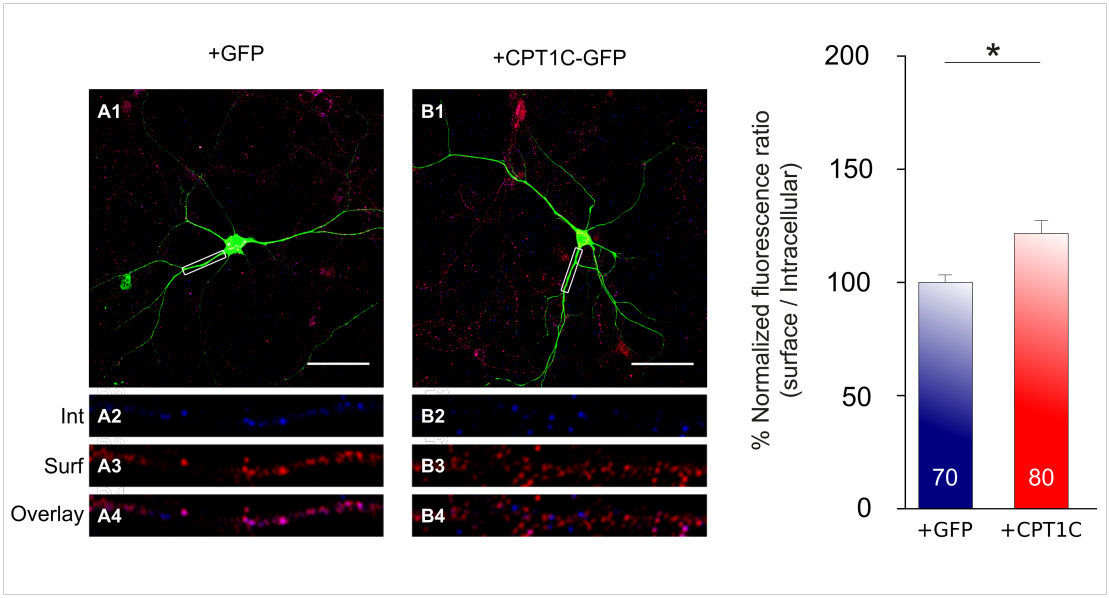

*Figure 4.25. Quantification of endogenous GluA1 surface to total ratio normalized to GFP transfected neurons, expressed as a percentage. Left: two examples of cortical neurons and some of dendrites amplified. Intracellular GluA1 is showed in blue; surface GluA1 is showed in red. GluA1 surface to total ratio was increased by overexpression of CPT1C-EGFP (*p = 0.0226; Mann–Whitney U-test). Data represent means. Error bars represent SEM.*

The results obtained from these experiments demonstrate that GluA1-containing AMPARs trafficking is enhanced by CPT1C in neurons as well as it happens in heterologous systems.

4.5. Molecular mechanisms of AMPAR modulation by CPT1C

4.5.1. GluA1 C585 mediates surface expression enhancement of AMPARs by CPT1C

Data obtained so far demonstrate an effect of CPT1C on the trafficking of GluA1. This effect could be performed by a chaperone-like activity of CPT1C or by some post-translational modification mediated by this protein directly on GluA1.

Different studies have demonstrated that AMPAR trafficking is regulated by post-translational modifications. Thus, it has been described that palmitoylation affects AMPAR trafficking (Hayashi et al., 2005; Lin et al., 2009; Yang et al., 2009). This modification consists in the reversible introduction of a lipid palmitate at some specific cysteine residues present in proteins. Sierra and colleagues described in 2008 that CPT1C can bind palmitoyl-CoA. Given that CPT1s can catalyze the breakdown of palmitoyl groups from CoA, it exists the possibility that CPT1C could bind palmitate groups from AMPARs and depalmitoylate them. Thus the next question addressed within the frame of the present work was whether the observed increase in surface expression of GluA1 could be mediated by changes in the palmitoylation state of GluA1 due to CPT1C.

To check this possibility first we created three mutant forms of GluA1 that cannot be palmitoylated at previously described palmitoylable cysteine residues 585 and 811 (Hayashi et al., 2005). We did that by changing the cysteine for a serine in two single mutant forms of GluA1 (C585S or C811S) and we obtained the double mutant form GluA1(C585,811S) as well, also mentioned as GluA1(DM). These experiments were designed to study the effect of these mutations on CPT1C effect in cell lines firstly using the immunofluorescence quantification of surface receptors.

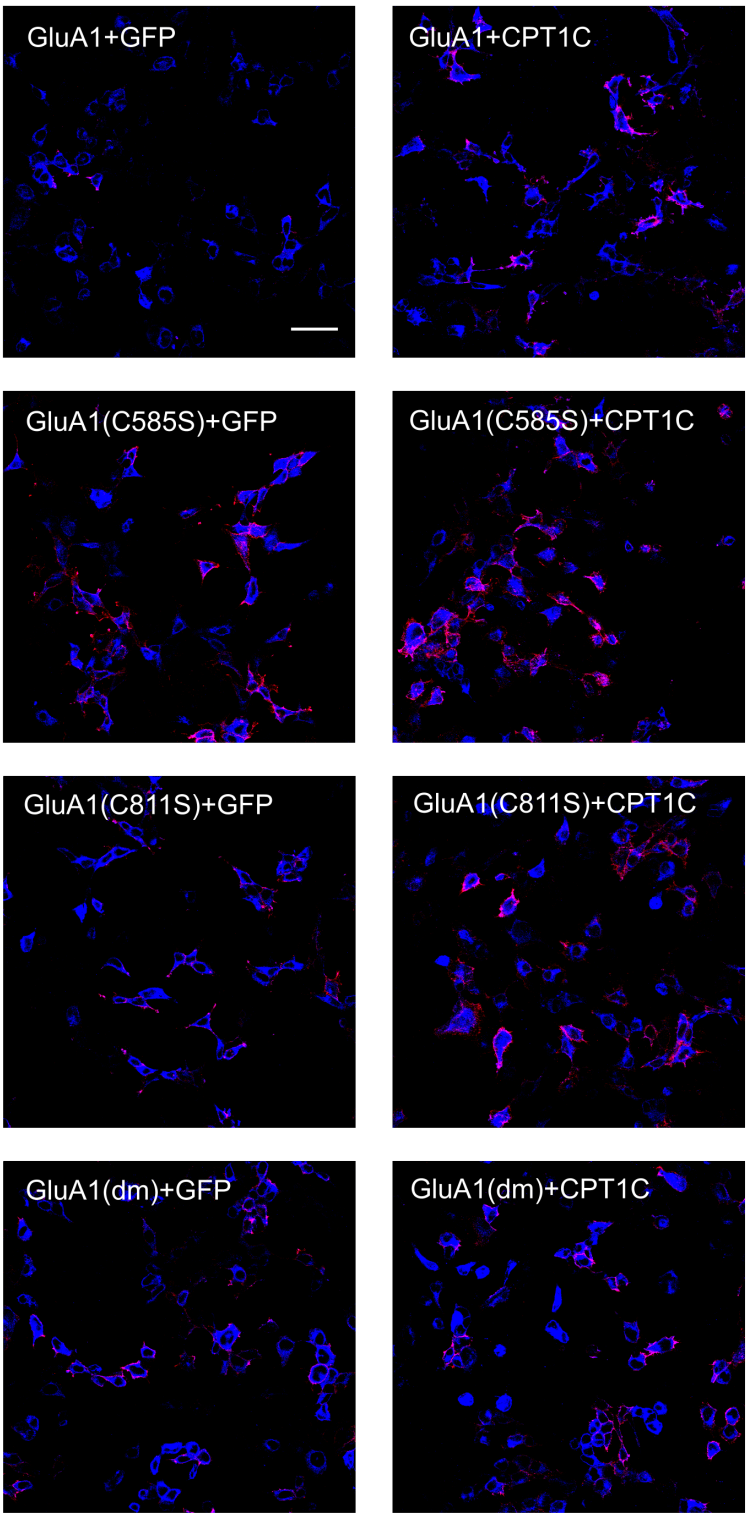

Figure 4.26. Representative confocal photomicrographs of tsA201 cells expressing different GluA1 constructs with GFP or CPT1C-EGFP . The surface GluA1 (red signal) and total GluA1 (blue signal) are shown. Scale bar: 50µm.

Figure 4.26 shows confocal images of surface GluA1 quantification experiments for GluA1 constructs alone (left panels) and together with CPT1C (right images).

As previously found, CPT1C increased the surface/total ratio of GluA1 (100.0 ± 4.86% for GluA1 *vs*. 182.6 ± 10.25% for GluA1+CPT1C; $p < 0.001$, n=88 and 91 respectively) (see Figure 4.27).

We observed that in the absence of CPT1C, GluA1(C585S) expression was enhanced at the cell surface by ~2-fold compared to native GluA1 ($p < 0.001$). These results are in keeping with previous findings (Hayashi et al., 2005). Interestingly, the effects of CPT1C co-expression and the GluA1(C585S) mutation were not additive. Specifically, CPT1C did not further increase the surface expression of GluA1(C585S) ($p= 0.4876$). Likewise CPT1C did not vary the high surface expression of the double mutant GluA1(C585,811S) ($p = 0.9014$). This suggests that C585 might be crucial in the CPT1C effect on trafficking of GluA1.

Interestingly, although surface expression of GluA1(C811S) was enhanced, CPT1C was still able to increase GluA1 surface expression, ruling out a possible involvement of C811 residue in CPT1C modulation of GluA1 trafficking (see Table 4.1 for more details).

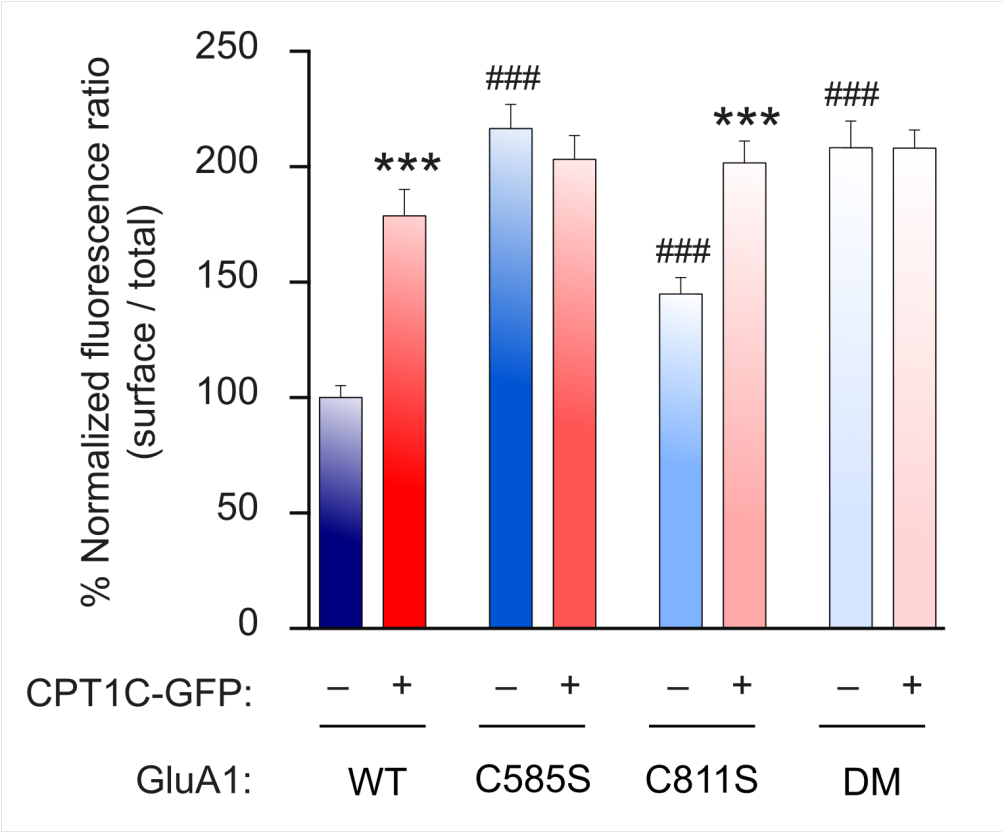

*Figure 4.27. Bars representing quantification of the GluA1 surface to total ratio normalized to GluA1, expressed as a percentage. GluA1 surface expression was increased by co-expression of CPT1C for both GluA1 and GluA1(C811S) (***p < 0.001; Mann–Whitney U-test) but not for GluA1(C585S) or the double mutant (DM). Non-palmitoylable forms of GluA1 increased surface expression of the receptors when compared to wild-type GluA1 (WT) (###p < 0.001; Mann–Whitney U-test).*

4.5.2. GluA1 C585 is critical for the enhancement of current density by CPT1C

Additionally whole-cell experiments were performed with cells expressing the equal constructs used for the immunofluorescence assay. Similarly to our previous immunofluorescence experiments, the current density of homomeric GluA1 was increased when the cells were expressing CPT1C as well (85.86 ± 21.95pA/pF for GluA1 *vs*. 155.5 ± 24.22 pA/pF for GluA1+CPT1C; p = 0.0334; n= 25 and 19 respectively).

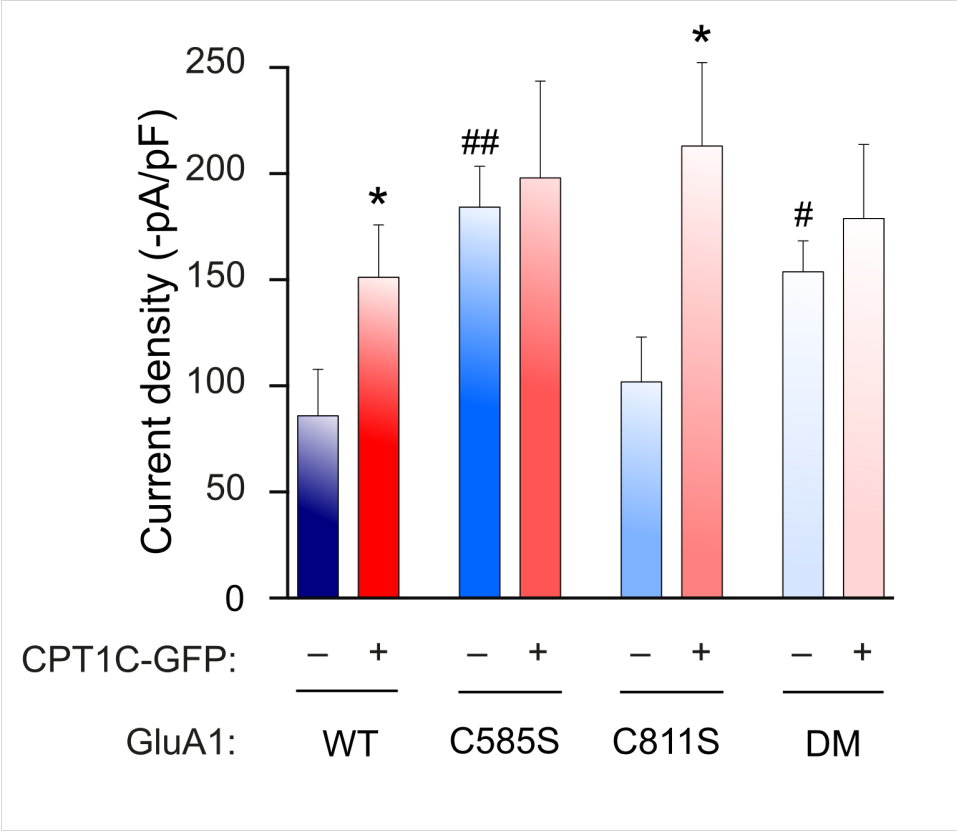

*Figure 4.28. Graph represents averaged normalized currents at −80 mV for different GluA1 constructs expressed alone or plus CPT1C in tsA201 cells. Current density (-pA/pF) was increased by co-expression of CPT1C with native GluA1 (WT) or mutant GluA1(C811S) (*p < 0.05; Mann–Whitney U-test) but not for GluA1(C585S). GluA1(C585S) increased the current density when compared to native GluA1 (##p < 0.01; Mann–Whitney U-test).*

Receptor		Normalized fluorescence ratio (Surface/intracellular) (%)	*n*	*p* value (*vs.* no CPT1C)	*p* value (*vs.* GluA1)
GluA1	-	100.0 ± 4.86	88		
	+ CPT1C	182.6 ± 10.25	91	<0.001	
GluA1(C585S)	-	196.9 ± 9.41	63		<0.001
	+ CPT1C	191.3 ± 9.47	67	0.4876	
GluA1(C811S)	-	146.5 ± 7.08	71		<0.001
	+ CPT1C	198.2 ± 8.99	70	<0.001	
GluA1(C585,811S)	-	208.2 ± 11.55	35		<0.001
	+ CPT1C	208.1 ± 7.87	37	0.9014	

Table 4.1. Normalized fluorescence ratio values (% of surface vs. intracellular). Number of cells analysed from tree different IF (n) is shown. Mann-Whitney U-test comparing values for GluA1 mutants with or without CPT1C (vs. no CPT1C) or values of mutant versions of GluA1 against GluA1 (vs. GluA1).

Receptor		Current density (pA/pF)	*n*	*p* value (*vs.* no CPT1C)	*p* value (*vs.* GluA1)
GluA1	-	85.86 ± 21.95	16		
	+ CPT1C	155.5 ± 24.22	13	0.0334	
GluA1(C585S)	-	184.3 ± 19.19	13		0.0041
	+ CPT1C	198.0 ± 41.25	11	0.7721	
GluA1(C811S)	-	97.32 ± 20.26	12		0.4166
	+ CPT1C	213.0 ± 36.12	11	0.021	
GluA1(C585,811S)	-	153.8 ± 14.58	10		0.0143
	+ CPT1C	178.9 ± 34.89	10	0.8534	

Table 4.2. Current density values for GluA1 mutants with or without CPT1C. Current density values at −80mV (−pA/pF) are shown. Number of recorded cells is shown (n). Mann-Whitney U-test comparing values for each GluA1 receptor in the absence or presence of CPT1C (vs. no CPT1C) or current density values of mutant versions of GluA1 against wild type GluA1 ("vs. GluA1" column).

As it is shown in the Figure 4.28, it was found that the glutamate-evoked current carried by cells expressing GluA1(C585S) alone was increased (p= 0.0041; n= 13) when compared with GluA1 WT (85.86 ± 21.95pA/pF for GluA1 *vs*. 184.3 ± 19.19pA/pF for GluA1(C585S); n= 16 and 13 respectively). Analogously to immunofluorescence assay, no cumulative effects was found when GluA1(C585S) was co-expressed with CPT1C. Concretely, CPT1C did not further increase GluA1(C585S) current density (184.3 ± 19.19pA/pF for GluA1(C585S) *vs*. 198.0 ± 41.25pA/pF for GluA1(C585S)+CPT1C; p = 0.77; n = 13 and 11 respectively).

Furthermore, as it can be observed from the Figure 4.28, CPT1C did not increase the current density of the double mutant GluA1(dm) (153.8 ± 14.58pA/pF for GluA1(DM) alone *vs*. 178.9 ± 34.89pA/pF for GluA1(DM) expressed together with CPT1C); p=0.85; n= 10 for each group). On the other hand, as it happened in the immunocytochemistry experiments shown in Figure 4.27, CPT1C was able to increase current density from the GluA1(C811S), indicating that CPT1C modulation was not mediated by affecting this residue.

These findings together with the results from the immunofluorescence assay, showed in Figure 4.27 indicate a role of C585 on GluA1 trafficking and CPT1C modulation.

4.5.3. CPT1C and GluA1 interaction does not dependent on palmitoylation of C585

Since C585 seems to be crucial in the GluA1 enhancement on trafficking effect mediated by CPT1C, the next step of the present work was to test whether the lack of palmitate group could alter the interaction between GluA1 and CPT1C, which could explain thus the lack of effect in the GluA1(C585S) mutant. In order to check if CPT1C ability to interact with GluA1 was abolished when residue 585 was non-palmitoylated, co-IP assays were performed using the GluA1(C585S) or other mutant forms and

CPT1C. Figure 4.29 shows the ability of GluA1 to interact with CPT1C despite the lack of palmitate residue in the non-palmitoylated forms of cysteines 585 and 811, as well as the double mutant.

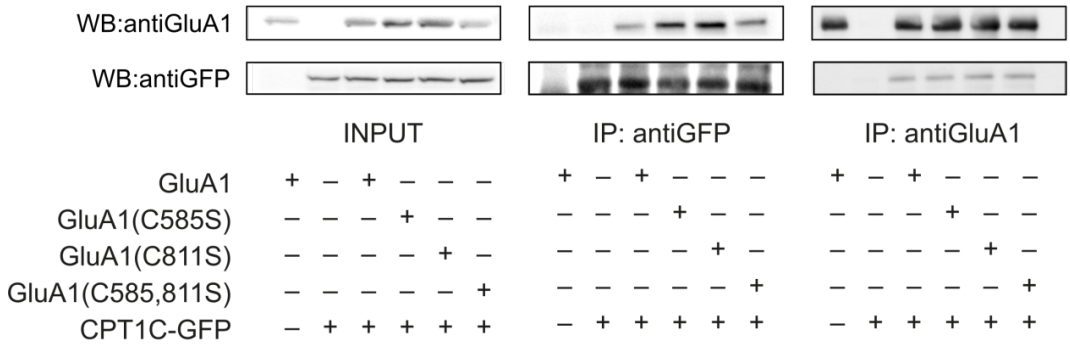

Figure 4.29. Co-IP of the tsA201 cells membrane fraction trasfected with GluA1 wild type or GluA1(C585S), GluA1(C811S), and GluA1(C585,811S) non-palmitoylable mutants together with CPT1C-EGFP . As negative controls GluA1 was co-expressed with an empty plasmid expressing GFP alone (first column) and CPT1C-EGFP was co-expressed with an empty pDsRed (second column). INPUT - input sample collected prior to immunoprecipitation of these extracts.

This result indicates then that the binding of both proteins depends on other domains/residues and that palmitoylable C585 does not determine this interaction despite its importance in CPT1C effect on trafficking properties of AMPARs.

4.5.4. CPT1C does not affect GluA1 subunit palmitoylation state

A previous study performed in Richard Huganir's laboratory (Hayashi et al., 2005), showed that palmitoylation of GluA1 at the C585 residue retains AMPARs in the Golgi apparatus. Depalmitoylation of GluA1 at C585 leads to more efficient trafficking of the GluA1 to the plasma membrane. Previous experiments of this thesis support these findings since the number of receptors at the surface in the mutant C585S, which cannot be

palmitoylated, are increased. Interestingly, the surface expression of GluA1(C585S) is approximately the same as GluA1(C811S) expressed with CPT1C (where CPT1C effect can only be on the intact cysteine 585). This result together with the fact that CPT1C has no effect on GluA1(C585S) suggested that CPT1C could be a potential GluA1 depalmitoylating enzyme.

So we evaluated the palmitoylation level of GluA1 when expressed alone or together with CPT1C. For this purpose an Acyl Biotin Exchange assay was performed by using the same methodology as in Brigidi and Bamji (2013). This assay allows the replacement of a pre-existing palmitate bound to cysteines of a given protein with a biotin group. The biotin is subsequently detected with streptavidin to give a read-out of palmitoylation levels. Therefore tsA201 cells were transfected with GluA1 alone or together with CPT1C-EGFP and palmitoylation levels were assessed by this methodology.

Figure 4.30 represents an SDS-PAGE of the thiol-biotinylated immunoprecipitates of GluA1 followed by ABE assay for both transfected conditions. Palmitoylation of GluA1 can only be detected in plus-hydroxilamine (+HAM) samples since HAM cleaves the palmitate groups, permitting the further biotin insertion in the free cysteines. The control sample was without the presence of HAM (-HAM). GluA1 palmitoylation levels (top) were detected by Western blotting with streptavidin-HRP (palmitoylation). After stripping the membranes the total amount of immunoprecipitated GluA1 was detected by Western blotting with anti-GluA1-NT antibody (anti-GluA1, bottom).

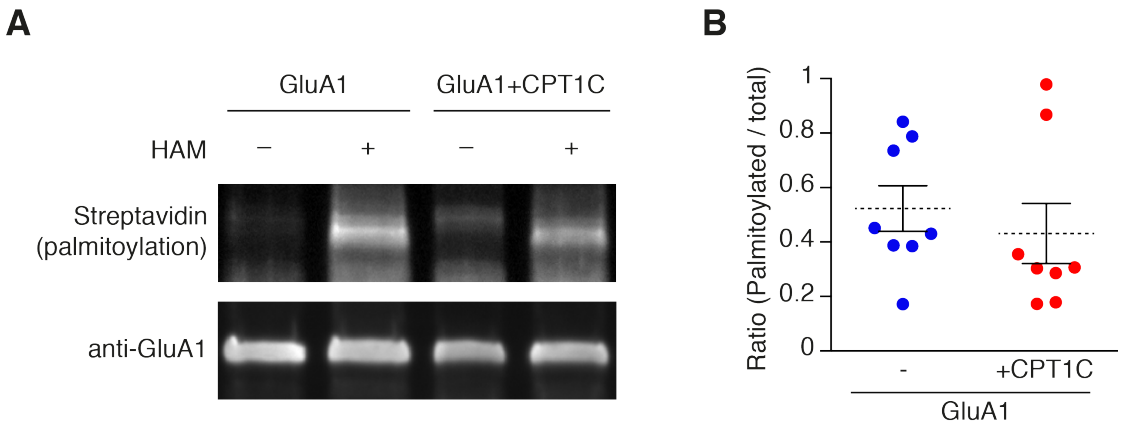

Figure 4.30. (A) Palmitoylation levels of GluA1 alone (GluA1) and together with CPT1C-EGFP (GluA1+CPT1C), in transfected tsA201 cells, detected by means of Acyl-Biotin Exchange (ABE). (B)Quantification of palmitoylation levels for GluA1 alone (blue circles) or GluA1 plus CPT1C (red circles) in tsA201 cells.

Ratio of palmitoylated GluA1 to total GluA1 for each single experiment is shown (Figure 4.30, B) together with mean (discontinuous horizontal lines) and SEM (continuous vertical lines) ($p > 0.05$; Mann–Whitney U-test; $n = 8$ for both). Palmitoylation levels of GluA1 in the absence of CPT1C were equivalent to GluA1 palmitoylation levels in the presence of CPT1C. Immunoprecipitated GluA1 was quantified to normalize palmitoylation levels. From the results it can be observed there is no significant difference (0.523 ± 0.084 for GluA1 *vs*. 0.431 ± 0.11 for GluA1+CPT1C; $p= 0.3228$; $n = 8$ for both).

4.5.5. GluA1 coimmunoprecipitates with CPT1C(H469A) in heterologous system

The palmitoylation assays did not show any difference in palmitoylation state of GluA1 due to the presence of CPT1C. However, when CPT1C was present there was a trend in diminishment of palmitoylation in GluA1; raising the possibility that ABE assay was not sensitive enough. Alternatively, other palmitoylation and/or depalmitoylation processes

independent of CPT1C might be masking CPT1C depalmitoylation effect. So, we decided to check functionally if CPT1C could depalmitoylate GluA1. The next step therefore was to investigate the role of CPT1C C-terminal domain which contains the residue His469 with catalytic activity, equivalent to a critical HIS residue described for CPT1A (Morillas et al., 2001). For this purpose a mutant version of CPT1C was created by changing histidine to arginine (H469A) using site-directed mutagenesis. Then, a co-immunoprecipitation assay was performed and subsequently analysed by Western blotting (n=3) to examine whether both proteins were still together. As it can be observed in Figure 4.31 (left panel), the correct expression of both proteins is shown and GFP recognizing antibody could pull down GluA1 when expressed together with CPT1C-EGFP or CPT1C(H469A) (upper middle panel). Additionally GluA1-NT antibody could pull down CPT1C-EGFP and CPT1C(H469A) proteins. This experiment revealed that recombinant GluA1 subunits interact with CPT1C(H469A) in tsA201 cells when these proteins are co-expressed.

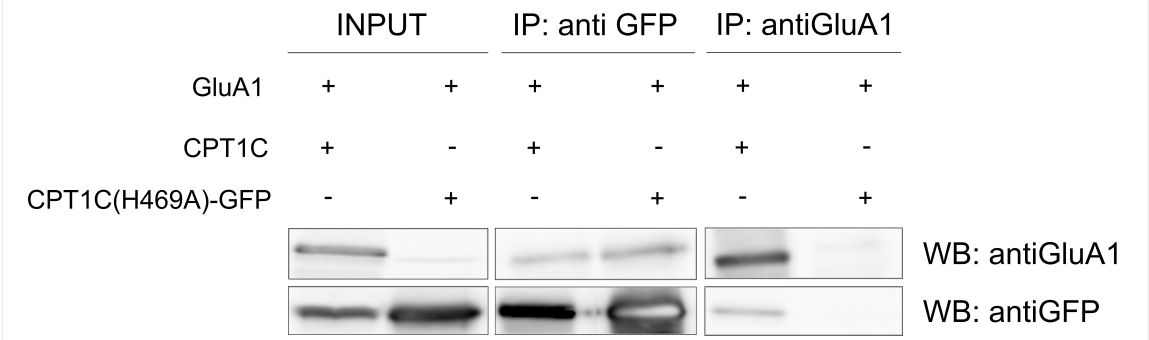

Figure 4.31. CPT1C(H469A) interacts with recombinant GluA1 subunits. Western Blot analysis obtained from tsA201 membrane fraction expressing GluA1 alone or together with CPT1C-EGFP . This analysis was replicated three times. Solubilised membranes of the transfected cells were immunoprecipitated with anti-GFP antibody (IP: antiGFP) or with anti-GluA1-NT antibody (IP: antiGluA1). Samples underwent SDS-PAGE separation followed by Western Blot analysis using anti-GluA1-NT (WB: antiGluA1) or anti-GFP (WB: antiGFP) antibodies. Input samples (INPUT) show correct expression of used constructs (left panel). GFP recognizing antibodies could pull down GluA1 when expressed together with CPT1C or CPT1C(H469A)-GFP (upper middle panel).

4.5.6. CPT1C(H469A) does not alter GluA1 induced whole cell currents

Following co-immunoprecipitation assays the electrophysiological whole-cell recordings were performed to assess whether the catalytic residue modulated AMPAR-induced currents. Figure 4.32 shows that the average normalised current density of GluA1 was enhanced when co-expressed with CPT1C compared with the cells expressing GluA1 alone (82.39 ± 15.83 pA/pF for GluA1 alone *vs*. 149.7 ± 19.98 pA/pF GluA1+CPT1C; p = 0.0051; Mann–Whitney U-test; n = 25 and 19 respectively). Interestingly, there was no statistical differences between current densities in cells expressing GluA1 alone or together with CPT1C(H469A) (82.39 ± 15.83 *vs*. 78.98 ± 18.26 pA/pF; p = 0.6209; Mann–Whitney U-test; n = 25 and 20 respectively.

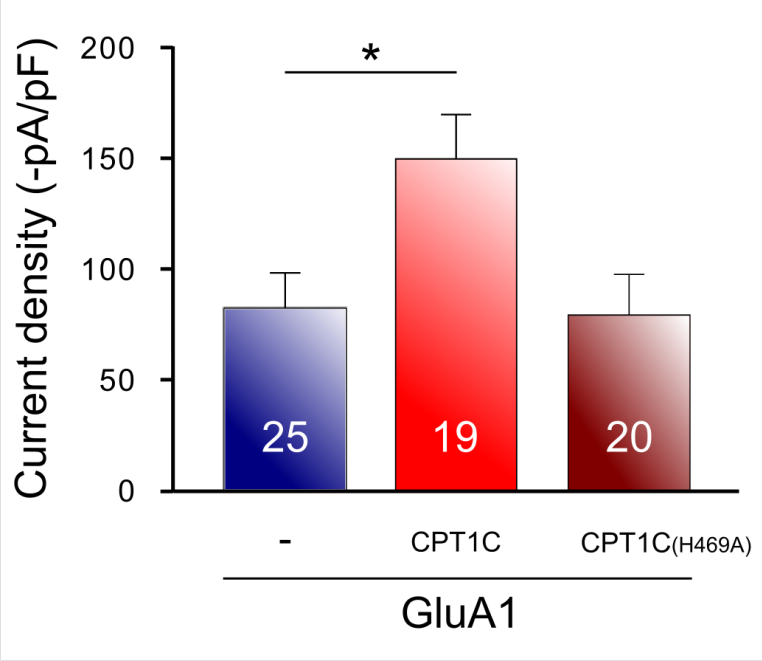

Figure 4.32. Average normalized current density at -80 mV for GluA1 alone, together with CPT1C or CPT1C(H469A). GluA1 current density (-pA/pF) was not increased when co-expressed with CPT1C(H469A) (p=0.6209; Mann–Whitney U-test).

These results show that the C-terminal catalytic domain with Histidine 469 residue plays a crucial role in GluA1 subunit modulation.

4.5.7. GluA1 coimmunoprecipitates with CPT1C after Palmostatin B treatment

Electrophysiological experiments with CPT1C(H469A) (Figure 4.32) suggested that CPT1C was exerting its trafficking effects on AMPARs by depalmitoylation although we could not observe changes in palmitoylation levels of GluA1 by CPT1C (Figure 4.30) (See discussion). So, we decided to further study if CPT1C could be a depalmitoylating enzyme of AMPARs. Depalmytoilation is regulated by palmitoyl thioesterase (PTE) (Shipston et al., 2011; Fukata and Fukata, 2010) allowing some proteins to leave GA and further trafficking to the cell membrane. Depalmitoylation has been shown to be performed by the thioesterases acyl protein thioesterase 1 and 2 (APT1/2), cytosolic enzymes that catalyse depalmitoylation of membrane anchored proteins, palmitoylated H-Ras and growth associated protein 43 (GAP43), respectively (Dekker et al., 2010). Inhibitors of APT1 activity, such as Palmostatin B (PB) have recently been described by Dekker and colleagues in 2010. Therefore we decided to use this compound as a tool to investigate the role of palmitoylation in AMPAR modulation by CPT1C protein.

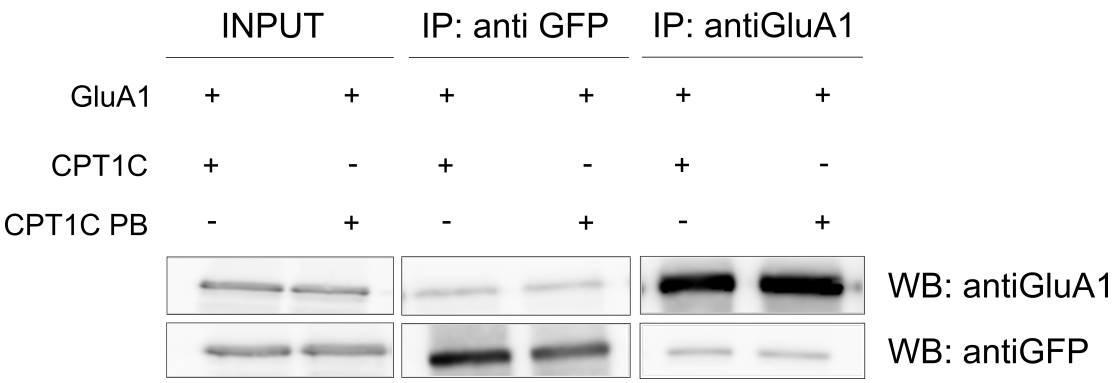

Figure 4.33. CPT1C interacts with recombinant GluA1 subunits when treated with Palmostatin B (PB). Western Blot analysis from tsA201 membrane fraction expressing GluA1+CPT1C-EGFP with or without PB treatment. This analysis was done 3 times. Solubilized membranes of the transfected cells were immunoprecipitated with anti-GFP antibody (IP: antiGFP) or with anti-GluA1-NT antibody (IP: antiGluA1). Samples underwent SDS-PAGE separation followed by WB analysis using ant-GluA1-NT (WB: antiGluA1) or anti-GFP (WB: antiGFP) antibodies. Input samples (INPUT) show correct expression of used plasmids (left panel). GFP recognizing antibodies could pull down GluA1 when expressed together with CPT1C-EGFP with and without PB treatment (upper middle panel).

To do so, first we did a co-immunoprecipitation assay to check if this compound affects AMPA-CPT1C interaction. For this purpose tsA201 cells were transiently transfected with GluA1 and CPT1C-EGFP and 24 h later were treated with Palmostatin B or DMSO during 24 h. Afterwards, the compound was removed and the membranes of the transfected cells were immunoprecipitated with anti-GFP or with anti-GluA1-NT antibody and subsequently analysed by Western blotting (n=3). The correct expression of both proteins is shown (Figure 4.33, left panel). GFP recognizing antibody could pull down GluA1 when expressed together with CPT1C-EGFP (upper middle panel) treated with PB. The obtained results show that treatment with Palmostatin B does not alter GluA1 and CPT1C interaction.

4.5.8. Palmostatin B decreases the CPT1C effect on GluA1 induced currents

After confirming the interaction of GluA1 and CPT1C with Co-IP experiments, electrophysiological recordings were performed to evaluate the possible effect of Palmostatin B on the GluA1 mediated currents when co-expressed with CPT1C (Figure 4.34). As control, in non PB treated cells, CPT1C increased GluA1 current density (73.21± 15.56pA/pF for GluA1 *vs*. 112 ± 10.55pA/pF for GluA1+CPT1C; p=0.0054; Mann–Whitney U-test; n = 22 and 19 respectively).

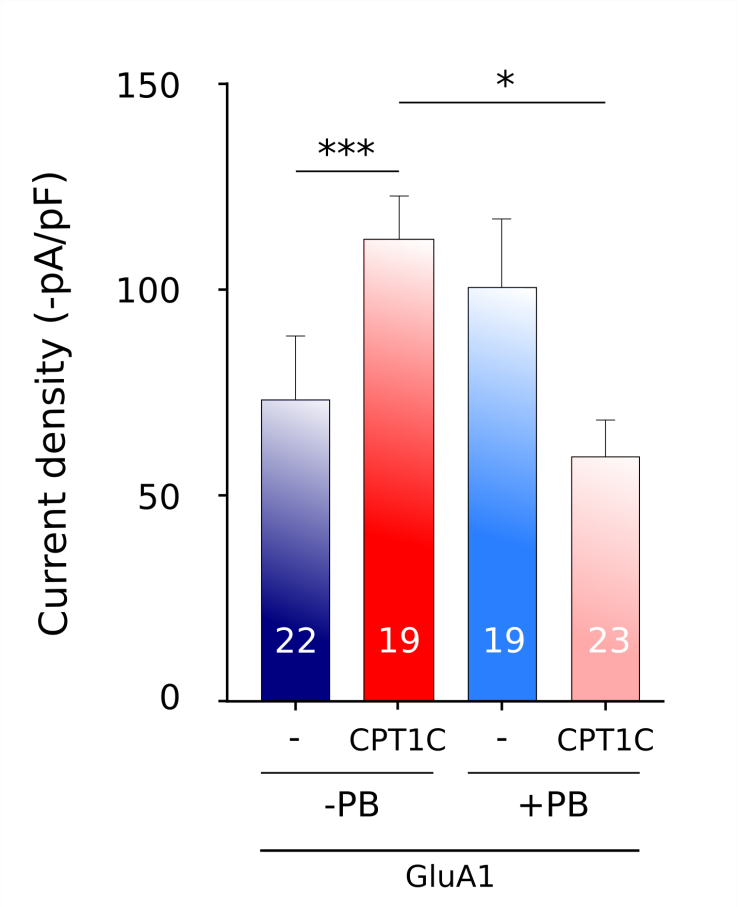

*Figure 4.34. Average normalized current density at –80 mV for GluA1 alone or together with CPT1C with and without treatment with Palmostatin B (PB). GluA1 current density (–pA/pF) was decreased when co-expressed with CPT1C in cells treated with PB (*p=0.0054; Mann–Whitney U-test). In non PB treated cells, CPT1C increased GluA1 current density (***p=0.0005; Mann–Whitney U-test).*

No statistical difference was found in GluA1 current density when expressed alone compared with the same condition but treated with PB (73.21 ± 15.56pA/pF *vs*. 100.5 ± 16.68pA/pF; p = 0.1401; Mann–Whitney U-test; n = 22 and 19 respectively). Interestingly, GluA1 current density was decreased when co-expressed with CPT1C in cells treated with PB when compared with GluA1 expressed together with CPT1C without PB treatment (59.35 ± 8.983pA/pF in Palm B treated cells *vs*. 112 ± 10.55pA/pF for GluA1+CPT1C in non-treated cells; p = 0.0005; Mann–Whitney U-test; n = 19 and 23 respectively). This experiment indicates that Palmostatin B may alter GluA1 enhancing properties of CPT1C most probably by inhibiting its PTE activity.

4.6. Functional studies of CPT1C on AMPARs in neurons

4.6.1. Synaptic transmission is reduced in CPT1C KO animals

Data from the present work demonstrate that AMPAR function is modulated by CPT1C. On the other hand study performed by Carrasco and colleagues in 2012 showed that dendritic spine maturation was weakened in CPT1C KO animals. Large mature spines have been shown to express AMPA receptors abundantly (Matsuzaki et al., 2001) while small and immature spines express a small number of AMPA receptors (Matsuzaki et al., 2004). Given that, the next step within the present framework was to check if the excitatory synaptic transmission was altered in animals lacking CPT1C protein. To this end whole-cell voltage-clamp recordings were performed using cultured hippocampal neurons to measure the AMPAR–mediated miniature EPSCs (mEPSCs). Cells were recorded in solution containing TTX to block synaptic transmission and avoid recording of spontaneous EPSCs (sEPSCs). D- AP5, 7-CK, and SR95531 were also added in the recording solution to block NMDA and $GABA_A$ receptors. The mEPSC

recordings were performed accordingly to the settings described in the Experimental procedures.

As it can be observed from the Figure 4.35 (and also in Figure 4.38), representative traces reveal the decrease in the amplitude of AMPAR mEPSCs.

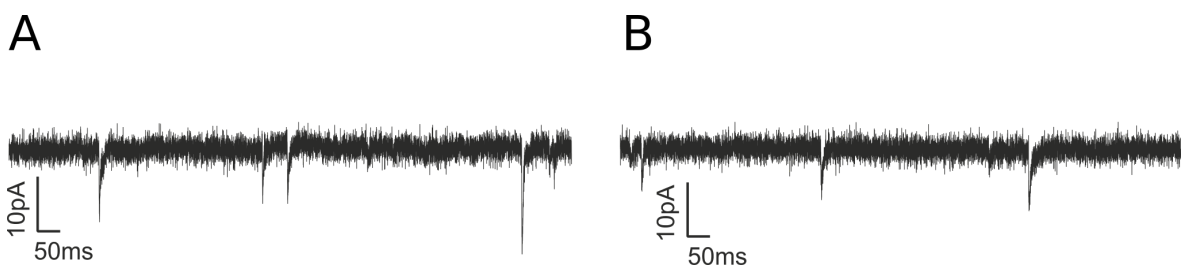

Figure 4.35. Representative traces of AMPA miniature EPSC recorded from the hippocampal neurons from WT (A) and from CPT1C-lacking animals (B).

Statistical analysis showed that mEPSC amplitude was decreased in neurons from CPT1C KO animals (Figure 4.36) when compared to WT neurons (–18.96 ± 0.95 pA for WT *vs.* –15.46 ± 0.99 pA for KO; n=26 and n=18, respectively; p=0.0175; t-test).

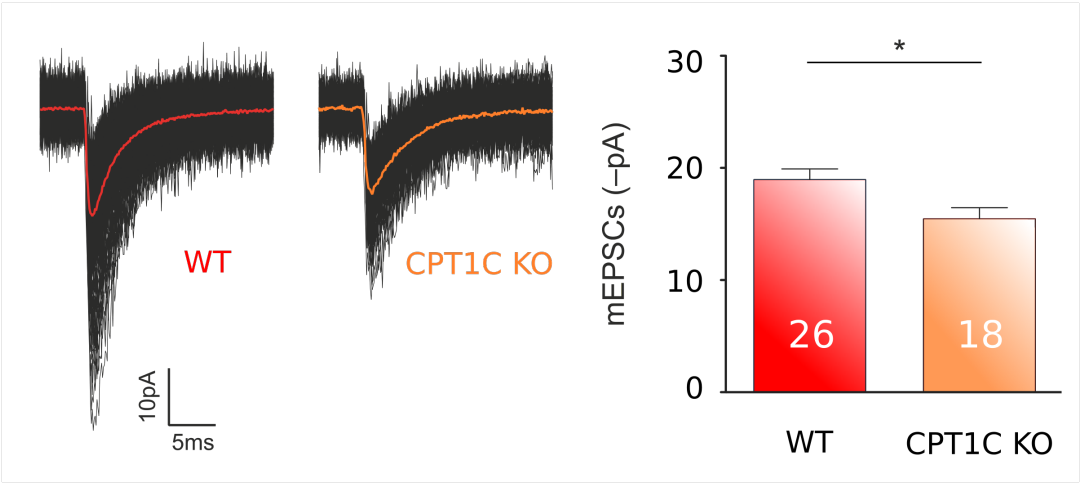

Figure 4.36. Representative individual (black) and average mEPSCs (colored traces), recorded from wild type (WT; red) and CPT1C lacking (CPT1C KO; orange) neurons (left panel). Bar graph for average values for both conditions is shown (right panel). Columns represent the mean ± SEM.

Cumulative amplitude histograms for mEPSCs (Figure 4.37) revealed that the distribution of amplitudes in CPT1C KO neurons shifted towards smaller values along the whole range, indicating a general decrease in the amplitude of postsynaptic AMPARs.

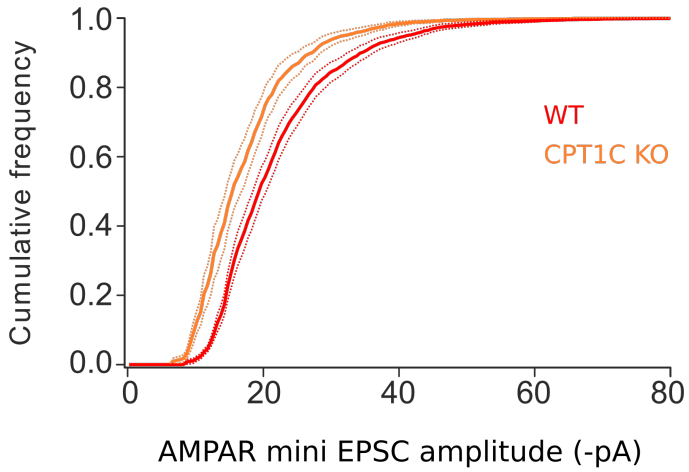

Figure 4.37. Cumulative probability distribution in CPT1C lacking animals (orange) shows a shift when compared with the wild type group (red). Discontinuous thin lines denote SEM.

When we examined the frequency of AMPA-mediated mEPSCs we did not find any significant differences (4.12±0.84 Hz for WT *vs.* 3.83±1.24 Hz for KO; p=0.3519; Mann-Whitney test; Figure 4.38), indicating that no apparent presynaptic alteration was present in CPT1C KO cells.

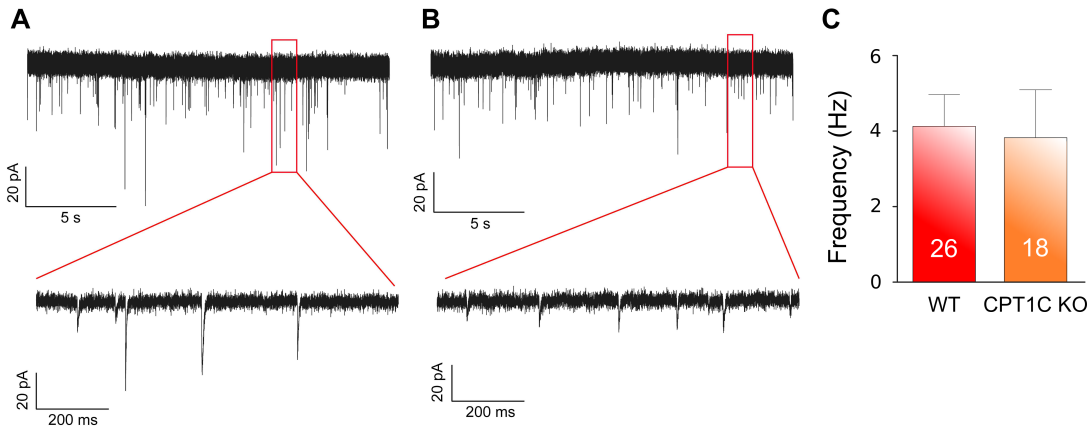

Figure 4.38. AMPA-mediated mEPSCs frequency was not altered on CPTC KO neurons when compared with the controls. A-B. Representative traces for a 20 seconds duration recording from a wild type (A) and CPT1C KO (B) animals. Lower traces are expansion of 1 second for theses two cells where it can be observed that despite the amplitude is decreased in KO animals, the frequency remains the same. C. Bars represent Average and SEM of 25 and 18 cells respectively; p>0.05.

We also measured the decay time constant for the recorded AMPAR-mediated mEPSCs in WT and KO animals since a change in the kinetics of AMPAR responses is indicative of alterations in their subunit composition (Lomeli et al., 1994; Moscbacher et al., 1994). The results showed that there were no changes in the decay time constant (3.67±0.20ms for WT *vs*. 3.69±0.16ms for KO; p=0.9256; t-test; Figure 4.39), suggesting that no alteration in AMPAR subunit composition takes place in CPT1C-deficient neurons.

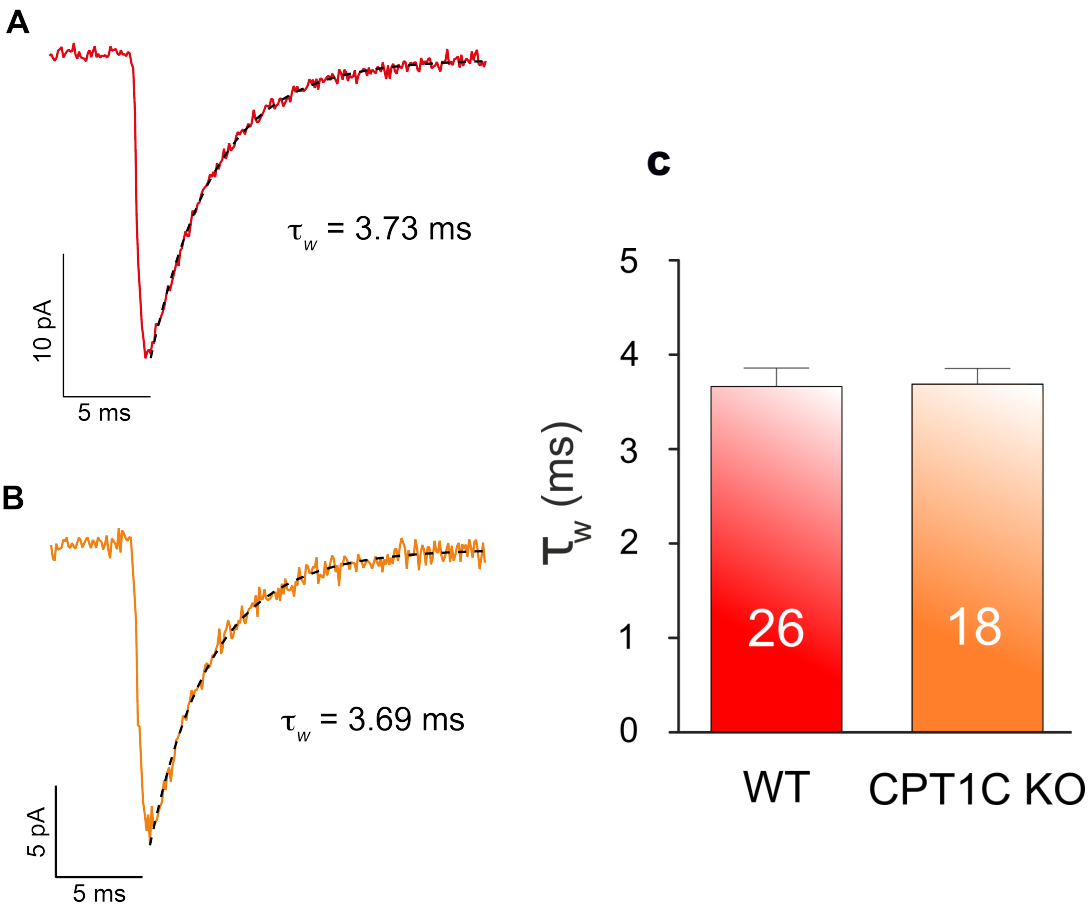

Figure 4.39. A. Average of 160 mEPSCs events from a WT neuron recording giving an amplitude of -21.25 pA and a weighted time constant (τ) of 3.73ms (black discontinuous line: fit to a double exponential) B. Same as A for a KO neuron (153 events; -16.31pA and τ=3.69ms) C. Kinetics of the recorded AMPAR mEPSCs measured as the decay time constant with a double exponential (τ_w) p>0.05.

In summary, the results obtained from these experiments demonstrated that the number of AMPARs in the synapses is clearly reduced and that basal synaptic activity is compromised in CPT1C KO animals.

5. DISCUSSION

AMPARs are key determinants in brain function since they mediate the majority of neuron-to-neuron communication in the central nervous system. Furthermore, they are important players in development, in glia-to-neuron communication, in plasticity processes and they have been implicated in neurological conditions. During the last 10 years, AMPAR field has been transfigured by the gradual discovery of transmembrane proteins interacting with AMPARs that regulate and determine their function in the brain. Since the finding in 2005 of the protein stargazin, which was shown to be crucial in the gating and trafficking of AMPARs, dozens of papers have been published describing how several of these proteins belonging to different families modulate AMPAR function and plasticity in neurons and glia (for review see Haering et al., 2014). More recently in 2012, a couple of works analyzed in parallel for the first time the proteome of the AMPAR complex and defined a new bunch of proteins that were able to interact with AMPAR subunits thus increasing the array of possible modulatory partners of this important class of glutamate receptor (Schwenk et al., 2012; Shanks et al., 2012).

During the development of this thesis work we have described a novel role of one of these defined proteins (carnitine palmitoyltransferase 1C – CPT1C) in AMPA receptor function regulation. CPT1C increases the surface expression of this ionotropic glutamate receptor subtype. Our results show that glutamate-evoked currents of recombinant GluA1 receptors are augmented by CPT1C co-expression and this effect is specific of the CPT1C isoform, since CPT1A does not share the same effect pattern, revealing a previously unknown physiological role for CPT1C. Moreover, CPT1C shows an AMPA subunit preference, not affecting GluA2 homomeric AMPARs but acting however on heteromeric GluA1/GluA2 receptors in a dominant fashion. The ER location of CPT1C seems to be crucial in modulating AMPAR surface expression as miss-

localization of CPT1C avoids this AMPAR modulation. Interestingly, our co-localization studies confirmed that GluA1-CPT1C interaction happens at ER level but not at the cell surface. The later, together with the fact that gating properties of GluA1 are not altered by CPT1C, indicates that this interacting protein is not acting as a “bona fide” auxiliary subunit and its role is focused on modulating AMPAR number at the cell surface. Indeed, this hypothesis is corroborated by our findings from the immunofluorescence experiments, where surface expression of GluA1 is increased in the presence of CPT1C in heterologous expression systems and cortical neurons. In agreement with a putative role of CPT1C in determining the AMPAR content at synapse level, we have demonstrated that synaptic transmission is diminished in CPT1C KO neurons. In parallel, we have shown that the palmitoylable cysteine 585 of the GluA1 subunit is crucial for the CPT1C enhancement of AMPA receptors trafficking although the palmitoylation state of this residue does not determine AMPAR-CPT1C interaction. The involvement of a depalmitoylation process in the increase of AMPAR trafficking is supported by our data on the catalytic residue His469 from the C-terminus of CPT1C and Palmostatin B – a palmitoyl thioesterase inhibitor – where it is evident that CPT1C effect on AMPAR trafficking is avoided.

In summary, the work performed on this thesis unravels a novel regulation of AMPA receptor function by the interacting protein CPT1C, which modulates AMPA receptor trafficking and this effect depends on the catalytic domain of C-terminal CPT1C acting on the cysteine 585 of GluA1 AMPAR subunits.

5.1. Role of CPT1C in normal synaptic transmission and synaptic plasticity

AMPARs are the principal mediators in rapid synaptic transmission in the nervous system, using the 90% of our brain synapses glutamate acting on AMPARs. Hence, any alteration in the normal AMPAR function and trafficking would potentially translate in dysfunctions at synaptic level. Here we have shown that CPT1C plays a role in modulating AMPAR trafficking by enhancing its surface expression and probably acting as a "chaperone-like" protein by depalmitoylating the channel at cysteine 585, a residue previously reported as crucial in AMPAR trafficking (Hayashi et al., 2005).
An important role of CPT1C in synaptic transmission was evident from previous studies where CPT1C knock-out mice had motor impairment, coordination problems, and hypoactivity (Carrasco et al., 2012; Carrasco et al., 2013). These motor impairments observed in the CPT1C KO mice reflect a synaptic dysfunction at cerebellar level, a fact that makes sense due to the high expression of CPT1C in cerebellum (Schwenk et al., 2014). Interestingly, it has been shown that cerebelar Bergmann glia, known to express only CP-AMPARs formed by GluA1 and GluA4 subunits (Iino et al., 2001; Matsui et al., 2005), is required for fine motor coordination (Saab et al., 2012). An impairment of GluA1-containing AMPARs trafficking in the CPT1C KO mouse at cerebellar level thus, might affect proper motor function. However, CPT1C expression seems to be restricted to neurons and it is not present in astrocyte glial cells (Sierra et al., 2007), although it cannot be discarded that lower amounts of CPT1C are expressed and might have not been detected by available techniques.

Another evidence for altered synaptic transmission in CPT1C deficient mice is that these animals show poor dendritic spine maturation in hippocampal neurons (Carrasco et al., 2012). It has been described that immature spines possess a smaller number of AMPARs compared with larger spines, which

have higher numbers of AMPARs (Matsuzaki et al., 2001; Matsuzaki et al., 2004). Indeed, silence synapses were AMPARs are not present at all correlate well with immature synapses (Petralia et al., 1999). Accordingly, it has been shown that lack of CPT1C diminishes AMPARs at synapses in hippocampal neurons (Fadó et al., 2015). In agreement with low AMPAR content at synapse level, our electrophysiological data with KO animals has proved that synaptic transmission is reduced in CPT1C deficient animals (Figure 4.35-4.37). Since AMPAR-mediated mEPSCs in pyramidal hippocampal neurons are diminished, in these animals the lack of CPT1C probably translates into a less efficient synaptic trafficking. However, it has been shown that the lack of CPT1C in neurons impairs AMPAR synthesis as well (Fadó et al., 2015). That would affect the amount of receptors at the synapse as well. Anyhow, both mechanisms (impaired synthesis and reduced trafficking) in the absence of CPT1C in the KO mice are not mutually exclusive and are rather complementary. Conversely to an absence of CPT1C, overexpression of CPT1C in cortical neurons increases AMPAR content at dendrites in cortical neurons (Figure 4.25). All these findings reveal an important relationship between CPT1C and AMPARs in determining normal synaptic transmission. However these changes are not dramatic since they suppose a ~20% decrease in mEPSCs amplitude (Figure 4.36), which supports the fact that the viability of the KO mice is not compromised (Wolfgang et al., 2006). This highlights a modulatory rather than a mandatory role of CPT1C in contrast with other AMPAR interacting proteins as for example stargazin, whose absence lead to a total lack of AMPAR-mediated currents in cerebellar granule cells (Chen et al., 2000) due to the fact that in these cells, stargazin is the only TARP expressed (Tomita et al., 2003).

Regardless of the mechanisms, it is clear that CPT1C enhances surface expression of AMPARs in neurons (this thesis work and Fadó et al., 2015). These data showing that CPT1C modulates AMPAR trafficking might be

relevant for synaptic plasticity processes, specially taking into account the specificity of CPT1C over GluA1. GluA1-containing receptors are maintained in reserve at the dendritic shaft and can be delivered to synapses during LTP (Zamanillo et al., 1999). Normally, in dendritic spines, there is an excess of GluA1 readily available for generating LTP (Mack et al., 2001). Thus any impairment in normal GluA1 trafficking will translate in a potential deficient learning. Indeed, the crucial role for GluA1 in LTP is evident by studies with mice lacking GluA1, which show no LTP (Zamanillo et al., 1999). Besides, point mutations in the PDZ-binding region of GluA1 or blocking the trafficking of GluA1 prevent its synaptic delivery in LTP (Piccini & Malinow 2002; Shi et al., 2001). CPT1C KO mice show poor performance in the water morris maze (Carrasco et al., 2012), implying that LTP might be compromised. Thus hippocampal plasticity is most probably altered in CPT1C KO mice as basal synaptic transmission is. Future studies of the physiological role of CPT1C in synaptic plasticity either in the hippocampus or the cerebellum would be of great interest to better clarify the role of CPT1C in the brain.

5.2. Subunit specificity of CPT1C modulation

Despite the clear effect of CPT1C on GluA1 homomeric receptors, GluA2 homomeric AMPARs seem not to be regulated by CPT1C (Figure 4.11, 4.12 and 4.24), while GluA2-containing AMPARs, which are the most abundant form of AMPARs in neurons, are also sensitive to CPT1C. It is very interesting that our coimmunoprecipitation experiments showed direct interaction of CPT1C with GluA2 subunits as well (Figure 4.2) regardless the lack of any evident effect. Perhaps, one possibility is that we have not been able to detect CPT1C effect on GluA2. We have just studied if the trafficking of GluA2 homomers was affected; yet we did not perform experiments of whether biophysical properties of GluA2 were somehow changed by CPT1C. Nonetheless, the lack of modulation of the gating properties of GluA1 by CPT1C (Figure 4.18) and the absence of co-

localization of GluA2 and CPT1C at plasma membrane (Figure 4.20) makes that possibility pretty unlikely.

Why is then GluA1 affected by CPT1C and GluA2 is not? Despite that GluA1 and GluA2 subunits have a great homology they have distinct key features in their structure, which translate into important functional differences (Traynelis et al., 2010). The first and most important one is the editing of GluA2 at the Q/R site. While GluA1,3 and 4 have a neutral glutamine, GluA2 has a bigger and positively charged arginine at the Q/R site in the entrance of the pore. Although it seems improbable that the functional consequences of CPT1C depends on the presence or absence of this residue, it is of note that the important C585 palmitoylable residue in GluA1 (C610 in GluA2) in the CPT1C effect is located just +3 aminoacids from the crucial Q/R site (Traynelis et al., 2010). Whether editing of GluA2 may avoid a putative depalmitoylation mediated by CPT1C is an incognita but future experiments with non-edited versions of GluA2 – GluA2(Q) rather than GluA2(R) – might give clues about that issue.

Another significant difference between both GluA subunits is the C-terminal domain. While GluA1 is 81 aminoacids long, GluA2 has a C-tail of 50 aminoacids and both tails differ considerably in their sequences (Malinow and Malenka, 2002). Interestingly, variations in the C-tails (and also the Q/R site) between both isoforms determine different trafficking properties of GluA1 and GluA2 (Greger et al., 2002; Henley & Wilkinson, 2013), as well as different type of interactions with other proteins and a differential regulation of their delivery to the membrane, stability and endocytosis (Malinow and Malenka, 2002). It is worth noting about the existence of a long form of GluA2 generated by alternative splicing (GluA2L) retaining a long C-tail that resembles those of GluA1 and GluA4 (Hollmann and Heinemann, 1994; Wisden and Seeburg, 1993). C-tail deletions of long-tailed AMPAR subunits and the use of GluA2L might help elucidating the

molecular mechanisms related with the subunit specificity of CPT1C effect on trafficking. This way it could be tested if the differential C-tail is an important determinant of CPT1C modulation as the different C-tail does not seem critical in CPT1C interaction from our results (GluA1 & 2 co-immunoprecipitation with CPT1C). These future works should also allow unmasking if the GluA-CPT1C interaction depends on a common motif located in that C-terminal part of the GluA subunits. Thus, it could be possible that the differential modulation by CPT1C on GluA1 and GluA2 was dependent on the C-tail length, on the specific C-terminal aminoacid composition, on Q/R site editing state of AMPAR subunits or a combination of any of these factors. To gain insight into CPT1C selective subunit modulation of AMPAR it would be important to elucidate also the domains of CPT1C participating in the interaction. The topology of CPT1A shows that N- and C-terminal domains face the cytoplasm (Fraser et al., 1997). Presumably CPT1C displays the same topology thus restricting the interaction with AMPAR subunits to their C-terminal tail or transmembrane domains (Thesis Esther Gratacós-Batlle). But independently of the molecular determinants, it is quite surprising that despite CPT1C interacts with GluA2, no evident effect has been found in this thesis.

Notably, the majority of AMPARs in the CNS are heteromeric combinations (Lu et al., 2009; Traynelis et al., 2010) and this prompted us to study these AMPAR forms as part of this thesis. In general, the gating and trafficking properties of the subunits are dependent on their C-terminal domains, with the long form GluA1 subunit dominant over the short form GluA2 (Passafaro et al., 2001; Barry and Ziff, 2002; Henley & Wilkinson, 2013). For example, phosphorylation of the C-tail of native GluA1 subunits at different serine residues (S831 by PKC or CamKII and S845 by PKA) directly modulates the receptors properties by increasing single channel conductance and mean open probability (Mammen et al., 1997; Barria et al., 1997; Roche et al., 1996; Banke et al., 2000). However, there is an

exception: heteromerization of GluA1 with GluA2 abolishes channel-conductance increase of GluA1 S831 phosphorilation (Oh and Dekarch, 2005). However, the dominant effect of GluA1 S831 phosphorilation is restored in the presence of TARPs (Kristensen et al., 2011). Since the majority of AMPARs in neurons are always together with some member of the TARP family (Jackson and Nicoll, 2011), the work by Oh and Deckarch does not rule out the long form dominant effect hypothesis. More clearly, phosphorylation of GluA1 has been shown to regulate GluA1-containing receptors incorporation in synapses (Hayashi et al., 2000). Our results are in line with this dominant effect of long forms since CPT1C modulates GluA1/GluA2 in a similar direction as GluA1 homomers.

Our observations show that the effect of CPT1C on GluA2-containing heteromeric AMPARs and native AMPARs – mostly heteromeric combinations containing GluA2 – is less pronounced than the one observed for GluA1 homomeric AMPARs (i.e. Figure 4.4 and 4.14). Perhaps the 2-fold enhancement of GluA1 density at the cell membrane could be partially occluded in GluA2-containing receptors due to generally better trafficking properties of heteromeric combinations. Indeed, the current density values we obtained for heteromeric receptors were higher than for homomeric receptors (either GluA1 or GluA2) despite they have lower channel conductance (~5pS; Jackson et al., 2011) than GluA1 homomers (~20pS; Soto et al., 2014b). This reflects the fact that heteromeric combinations of AMPARs are favored at the expense of homomeric receptors when both subunits are present during the synthesis process at the ER (Cull-Candy et al., 2006). The enhanced trafficking of heteromeric combinations might translate into a less evident CPT1C influence on GluA2-containing receptors. Alternatively, stoichiometry might be an important determinant in CPT1C effect. We have seen in this thesis that this is an important parameter (Figure 4.6). Maybe different AMPAR combinations need different amounts of CPT1C on their complex to have a comparable effect.

It has been demonstrated that depending on the stoichiometry of AMPARs and other interacting proteins the modulatory outcome is different. TARPs for example can be present from 1 up to 4 molecules per AMPAR complex conferring distinct effects on AMPARs (Shi et al., 2009; Kim et al., 2010; Hastie et al., 2013).

Regardless a minor effect of CPT1C on heteromeric AMPARs, the fact that CPT1C mouse have some motor coordination problems (Carrasco et al., 2013) strengths the role of CPT1C on heteromeric combinations since GluA2/GluA4 and GluA2/GluA3 are the main AMPAR combination in granule and Purkinje cells cerebellar neurons respectively (Lambolez et al., 1992; Martin et al., 1993; Breese et al., 1996; Day et al., 1995). These cells are essential in cerebellar function: granule cells are the predominant type of neuron in the cerebellum while Purkinje cells are the sole output from the cerebellar cortex (Purves et al., 2001). Alterations in AMPAR function in these cells lead to motor problems, being as a clear example the severe ataxic problems of *stargazer* mouse were granule cells are devoid of AMPARs at all (Chen et al., 2000). Although at present the effect of CPT1C over these specific combinations of heteromeric AMPARs is unknown, the motor coordination problems of CPT1C-KO mice point to a possible regulation of the trafficking of cerebellar AMPARs by CPT1C.

5.3. CPT1C is not a genuine auxiliary subunit of AMPARs

Globally from our data, it looks like that this new AMPAR interactor has a precise role in the delivery of AMPAR subunits to the cell surface without affecting any gating characteristic. It has been described that many other AMPAR interacting proteins control AMPAR trafficking (Palmer et al., 2005; Anggono & Huganir 2012; Lu & Roche, 2012). This is the case as well for auxiliary AMPAR subunits such as TARPs that play an important role in surface trafficking while also altering the channel properties of AMPARs (Nicoll et al., 2006). CNIH proteins also increase AMPARs surface

expression (Schwenk et al., 2009) and modify the behavior of AMPARs both in expression systems and in neurons (Coombs et al., 2012; Kato et al., 2010). But this is not the case for CPT1C, as we have shown that this protein does not alter GluA1 channel properties. This fact is supported by the confocal imaging experiments where we did not see any co-localization between CPT1C and surface GluA1. Consequently, CPT1C cannot be considered a TARP-like real auxiliary subunit and it seems that its role is restricted to controlling AMPARs trafficking.

5.4. Glucose dependence on CPT1C modulatory effect on AMPARs

In Figure 4.15, we show that the increase in GluA1 glutamate-mediated currents was more evident in cells treated in low-glucose conditions. This indicates clearly that the metabolic energetic state of the cell determines a major effect of CPT1C. What could be the link between energetic balance, CPT1C and AMPARs? Conditions of glucose deprivation (as for example ischemia) have been shown to generate among others, an increase in CP-AMPARs expression in neurons (Pellegrini-Giampietro et al., 1992; Opitz et al., 2000; Tanaka et al., 2002;Noh et al., 2005; Blanco-Suarez and Hanley, 2014;). However, these mechanisms clearly rely on NMDAR, ASICs and VGCCs and most probably are a combination of all of them (Quintana et al., 2015). Moreover, the protocols used in literature are a combination of oxygen-glucose deprivation. In our experiments, a moderate decrease in glucose (form 3.15g/L to 1g/L) did not change basal GluA1 current density of HEK293 cells ruling out a direct link between low glucose and enhanced expression. However, CPT1C-dependent current increase of GluA1 was more evident in low glucose conditions. Low glucose inhibits FA biosynthesis, thus decreasing malonyl-CoA levels (López et al., 2007), a potent endogenous inhibitor of CPT1A and CPT1B (Swanson et al., 1998; Zammit, 1999). Although it is unknown if CPT1C is inhibited by malonyl-CoA principally due to a lack of CPT1 activity, CPT1C can bind malonyl-

CoA (Price et al., 2002; Wolfgang et al., 2006; Sierra et al., 2008), hence potentially inhibiting its function. Low glucose levels would translate then into a putative dis-inhibition of CPT1C and an enhanced effect of this protein. Despite a more evident effect of CPT1C on AMPARs surface expression in low glucose conditions, in this thesis we decided to carry all the experiments in 3.15 g/L glucose since cells grown in low glucose were very unhealthy (showing a high amount of vacuoles).

5.5. What about other subunits?

From this work, two questions arise regarding the functional interaction of CPT1C and other subunits of glutamate receptors, either pore-forming (GluK and GluN subunits) or auxiliary proteins (e.g. TARPs): firstly, does CPT1C modulate other glutamate receptors – namely KARs or NMDARs? And secondly, what happens when CPT1C exert its effect on an AMPAR that contain well-known auxiliary subunits – as for example TARPs or CNIHs?

The proteomic studies where the array of partners for AMPARs were described provided information for all four GluA pore-forming subunits and all of them were able to interact with CPT1C (Schwenk et al., 2012; Chen et al., 2014). However, a previous proteomic study focused on GluA4 subunit interacting proteins in rat cerebellum that found 17 subunit partners – most of which were novel interactors – did not show CPT1C as a protein capable of interacting with GluA4 (Santos et al., 2010). Still, this work did neither show GluA2 as a companion of GluA4 and it is well-known that cerebellar granule cells express mainly that combination of AMPAR subunits (Day et al., 2005). It is almost certain that CPT1C can bind GluA4 subunit (as shown in Schwenck et al) and specially taking into account that the cerebellum is very rich in both GluA4 and CPT1C protein expression (Schwenk et al., 2014).

The great homology between iGluR subunits opens the question of whether CPT1C could be conferring a similar trafficking properties to different ionotropic glutamate subunits others than GluAs. A proteomic analysis similar than the ones performed for GluA subunits (Santos et al., 2010; Schwenk et al., 2012; Chen et al., 2014; Schwenk et al., 2014) has not been done for the constituents of KAR or NMDAR subunits. In general interacting proteins of glutamate receptors seem to be mildly subunit specific. PICK1 or GRIP/ABP interact with GluA2 and GluA3 (Srivastava et al., 1999; Dev et al., 1999) or TARP γ-5 with interacts with long forms of AMPAR subunits (Soto et al., 2009). Some proteins, as the majority of TARPs and CNIHs can be together and modulate all AMPAR subunits (Díaz, 2010). But there are proteins capable of interacting with different subfamily subunits. This is the case for Neto-1, a protein that clearly act as auxiliary subunit of KARs (Straub and Tomita, 2012) that it has also been shown to interact with NMDAR subunit GluN2A and 2B (Ng et al., 2009). SAP97 bind specifically GluA1 (Leonard et al., 1998) and not other GluAs but can interact with GluK2 (García et al., 1998). NSF binds GluA2 and GluK2 (Nishimune et al., 1998; Coussen et al., 2005), Another example is 4.1N, which interacts with GluA1 and GluK2 (Shen et al., 2000; Coptis and Swanson, 2013). So the possibility that CPT1C might be interacting and thus modulating KARs could not be discarded at all. Determining the motifs of AMPAR and CPT1C important for the interaction would help to elucidate if CPT1C can interact with other iGluR subunits. We have studied in this thesis whether the absence of palmitate groups in the AMPAR subunits prevents the interaction (Figure 4.29) and our results indicate that apparently, more motifs are involved in GluA-CPT1C “bridal”. But, on the other hand, some evidences from our studies with miniature events does not support a role of CPT1C in KARs trafficking. KARs are expressed preferentially at presynaptic level were they modulate synaptic release. When we studied miniature events (mEPSCs) we did not observe a change in the frequency of such events (Figure 4.38). It is well established that

presynaptic alterations can be tracked by means of mEPSCs frequency changes. However, mEPSCs experiments in KO mice did not show any change in frequency. It could exist the possibility that presynaptic KARs are depicted of some modulatory effects of proteins when present at presynaptic compared with postsynaptic level, but it has been shown that even presynaptic AMPARs are subjected to modulatory effect of auxiliary subunits (Rigby et al., 2015) as postsynaptic AMPARs are. More experiments overexpressing CPT1C in neurons would give clues about a putative effect of CPT1C on KAR trafficking.

Regarding the effect of CPT1C together with TARPs at the same AMPAR complex, during the realization of this thesis, although not been shown, the effect of CPT1C on GluA1 homomeric receptors together with the prototypical TARP γ-2 (stargazin) was tested. The aim of such experiments was to determine if the trafficking enhancement effect seen by CPT1C was additive to that of γ-2 since it has been thoroughly demonstrated that this TARP enhances the trafficking of AMPARs by ~ 3 to 10-fold (Chen et al., 2003; Turetsky et al., 2005; Tomita et al., 2005). The proteomic work describing for the first time AMPAR subunits-CPT1C interaction gave information about the putative location of CPT1C into the complex. Specifically, CPT1C would interact in the periphery of the complex while TARPs would be present in the core of such complex (Figure 1.6; Schwenk et al., 2012). Given that TARPs and CPT1C do not compete for the same site it exist the possibility of a combined effect of both interacting proteins once both are bound to GluA subunits. However, when we performed whole-cell patch clamp experiments to measure glutamate-evoked currents we could not achieved good recordings from cells transfected with GluA1 plus CPT1C (in a CPT1C-EGFP vector) and γ-2 (in a mCherry pIRES vector). The principal problem we faced was that cells were too unhealthy, possibly due to an excessive activation from a higher number of AMPARs expressed on those cells. We tried to circumvent that problem with an

AMPAR blocker (NBQX) added to the media. Indeed, as stated in "Experimental Procedures", along this thesis, we have tried to minimize excitotoxicity of transiently transfected cells by using 50μM NBQX on the media to avoid receptor activation and cytotoxic effects before recordings (Coombs et al., 2012). However, notwithstanding NBQX presence, this group of cells was specially damaged and even when the cells could be patched, they died before the end of the recording. Whether too much Ca^{2+} entry through GluA2-lacking CP-AMPARs (we used GluA1 homomeric receptors for these experiments) or another unidentified factor was responsible for the detrimental of the cells is not known at this stage. Supporting the latest hypothesis, it has been described that some specific combinations of AMPARs are lethal to heterologous HEK293 cells, as for example GluA2flip/GluA4flip despite this combination is Ca^{2+} impermeable (Iizuka et al., 2000). Future experiments in the presence of CPT1C and TARPs could be performed with CI-AMPARs (GluA1/GluA2) to elude that possibility but taking into account the lethal expression issues (Izaki et al., 2000) by using flop forms.

5.6. CPT1C palmitoyl thioesterase activity on C585 at ER level

This thesis data has shed light on a role for the enigmatic CPT1C protein: enhancing plasma membrane delivery of GluA1-containing AMPARs most probably by depalmitoylating cysteine residue 585 (in GluA1) at endoplasmic reticulum level.

Even though CPT1C does not associate with AMPAR subunits at the plasma membrane level, it is clear that both proteins interact at some stages during AMPAR synthesis and trafficking pathway (Schwenk et al., 2012 and Figure 1 and 4). The fact that CPT1C shows a clear ER pattern (Figure 4.8; Sierra et al., 2008; Carrasco et al., 2012) makes this organelle a meeting point for both proteins where CPT1C seems to post-

translationally modify AMPARs accounting for the increased traffic to plasma membrane. Additionally, CPT1C does not seem to interact with GluA1 outside of the ER at all, as CPT1C does not co-localise with the Golgi Apparatus marker GM-130 (Figure 4.21) or at the plasma membrane level. Thus, the effect of the interaction of CPT1C/GluA1 takes place exclusively at the ER level and it seems that CPT1C dissociates from the AMPAR complex at some stage before AMPARs subunits move forward to the Golgi during their biosynthesis.

Amongst the several post-translational modifications that AMPAR subunits undergo during biosynthesis and that affect the trafficking of the receptors to the cell surface, an important one is the reversible palmitoylation of AMPARs. Each AMPA receptor subunit is palmitoylated at two conserved sites, (i.e. C585 and C811 in GluA1) and palmitoylation/depalmitoylation of these two residues determine AMPARs trafficking properties (Hayashi et al., 2005, Yang et al., 2009, Lin et al., 2009). More evidence from the literature supporting that depalmitoylation raises AMPARs surface expression comes from some study where it was shown that cocaine induced an increment of GluA1 and GluA3 palmitoylation in the nucleus accumbens from rats, increasing the intracellular location and decreasing surface expression of these AMPAR subunits (Van Dolah et al., 2011). Given that CPT1C can bind palmitoyl-CoA and can form palmitoylcarnitine (Sierra et al., 2008) it seemed plausible to consider whether CPT1C could bind a palmitoylated protein and form palmitoylcarnitine thus depalmitoylating the protein, which would explain AMPARs increase in surface expression. In this thesis we have explored that possibility. Our data by using a catalytic deprived version of CPT1C – CPT1C(H469A) – and a putative inhibitor of CPT1C depalmitoylating activity – palmostatin B – seems to confirm this depalmitoylating role of CPT1C.

When studying the role of AMPAR cysteine residues in CPT1C effect, we found that C585S mutation alone increased whole-cell currents and surface expression of GluA1 by ~2-fold. These results are in accordance with previous ones demonstrating that depalmitoylation of AMPARs at C585 acts as a triggering signal for receptor forward trafficking (Hayashi et al., 2005). Interestingly, in the presence of CPT1C, GluA1(C585S) no longer increased receptor trafficking. This points towards a crucial role of C585 residue in the CPT1C effect. This was confirmed by the fact that the GluA1(C811S) mutant is modulated by CPT1C to the same degree as GluA1 ruling out the involvement of C811 residue. Moreover the fact that CPT1C increases GluA1(C811S) trafficking to the same extent as GluA1(C585S) alone or with CPT1C suggests that the effect of CPT1C is dependent solely on the C585 residue. Our findings show that GluA1(C811S) increases surface expression when detected by immunofluorescence but current density is not increased in the same extent. This might be explained due to a different number of cells analyzed with each technique. Despite that discrepancy, the significant increment in both parameters when CPT1C is together with GluA1(C811S) indicates that GluA1 C811 is not crucial for the CPT1C effect.

It is striking that no changes in palmitoylation could be detected (Figure 4.30) when indirect experiments are pointing towards a clear depalmitoylation of GluA1 accounting for the surface expression enhancement. The fact that CPT1C produces an increase in GluA1 surface expression to the same extent as the non-palmitoylable form of GluA1(C585S), indicates that the effect of CPT1C on GluA1 subunits might be via depalmitoylation of C585. Moreover, the mutation of the catalytic histidine residue 469 of CPT1C, avoided the increase in AMPAR surface expression mediated by CPT1C. This specific residue is crucial in CPT1A and B for catalysis (Morillas et al., 2001). It seems plausible that the CPT1C homologue histidine could be related in the unbinding of palmitate groups

from AMPARs. Besides, Palmostatin B, a compound known by its specific inhibition of thioesterase activity of APT1 (Dekker et al., 2010) was able to preclude the increment in AMPAR-mediated responses (Figure 4.34) strongly suggesting that CPT1C acts as a depalmitoylating enzyme. However, when detecting palmitoylation levels with the ABE assay, we could not observe changes in GluA1 palmitoylation levels by co-expression with CPT1C. A possible issue with this methodology might be that the ABE assay not only detects palmitoylation but also other S-acylation modifications of GluA1, which might have not yet been described. Another possibility is that C811 might be depalmitoylated by CPT1C regardless of a functional effect. In neurons palmitoylation in that cysteine prevents the binding of GluA1 to 4.1N protein thus increasing its internalization (Lin et al., 2009). Since we have carried out most of the work in expression systems (presumably lacking neuronal form of 4.1 protein), it is plausible that a depalmitoylation can occur by CPT1C in C811 without any functional effect. Therefore other techniques might be necessary to detect the palmitoylation levels of GluA1 C585 unambiguously. Alternatively, important depalmitoylation process performed in the ER by CPT1C could be counteracted by additional palmitoylation of the receptor at other cell locations, thus making it difficult to detect changes in the final palmitoylation state. Therefore, this hypothesis should be closely examined with future specific refinements of palmitoylation assays. Another possibility to take into account is that other cysteines in the GluA1 subunit could be palmitoylated (by APTs) or depalmitoylated (by CPT1C or PTEs) and masking the depalmitoylation of AMPARs thus making ABE assay difficult to interpret. In this regard, there has been shown the existence of another cysteine in GluA1 tail (C875) that it can be S-nitrosilated (Selvakumar et al., 2013). Since C811 is susceptible of S-nitrosilation as well, we cannot rule out a putative C875 palmitoylation thus affecting a reliable read out on ABE assays.

Beyond AMPARs, and expanding the hypothetic depalmitoylation effect of CPT1C to other iGluRs, it is worth mentioning that GluK2 KAR subunit can be palmitoylated at cysteine residues in the C-tail (Pickering et al., 1995). It has been shown that the palmitoylation state determines its association with 4.1N protein and forward trafficking to surface membrane (Coptis and Swanson, 2013). However this regulatory mechanism works in the opposite direction than in AMPARs, where GluA1 palmitoylation at C811 prevents its association with 4.1N and decreases AMPAR surface expression by an enhanced activity-dependent endocytosis (Hayashi et al., 2005; Lin et al.; 2009). This might be explained by the fact that different cysteine residues are involved in this regulation (no analogous C811 exists in GluK2). In any case, we have found that C811 is not important in CPT1C on AMPAR trafficking effect, probably because that modification occurs at plasma membrane of neurons in an activity manner, and we have demonstrated that CPT1C is not localised at plasma membrane (Figure 4.19). However, these data by Coptis and Swanson reveals the importance of KARs regulation by palmitoylation processes and suggest that AMPA and kainate receptors share common signaling pathways resulting from this transient modification that differentially regulates their insertion and stabilization at plasma membrane. The fact that our data shows that CPT1C exerts its modulatory effect on trafficking by depalmitoylating C585 points to a possible similar regulation in KARs although no indications have been found (see *"*What about other subunits?*"* section of the present discussion). Finally, NMDA receptor subunits GluN2A and GluN2B are also regulated by palmitoylation at their C-terminal region leading to enhanced or reduced NMDAR surface expression depending the residue that is palmitoylated (Hayashi et al., 2009) and making them potential depalmitoylating targets of CPT1C.

5.7. AMPARs and CPT1C in neurological diseases

Many studies have demonstrated the implication of AMPARs in neurological diseases (Cull-Candy et al., 2006; Zarate et al., 2008; Chang et al., 2012; Henley and Wilkinson 2013). On the other hand, some studies show the implication of CPT1C in conditions that affect neurons and glia (Rinaldi et al., 2015; Cirilio et al., 2014).

Age related deterioration in cognitive faculties is one of the most challenging aspects of research. This decline is related in part to a reduced synaptic plasticity due to changes in postsynaptic membrane constituents, such as AMPA receptor number and function. The aberrant AMPAR trafficking and consequent abnormal changes in synapses are a core feature in age-dependent cognitive decline (Henley and Wilkinson, 2013). Interestingly this correlates with a decline in CPT1C expression with age (Carrasco et al., 2013). A recent study showed that AMPA receptor synthesis is regulated by CPT1C in the hippocampus (Fadó et al., 2015). In this thesis we showed that AMPA receptor trafficking is increased in the presence of CPT1C and also that CPT1C deficiency translates into a less efficient synaptic transmission. CPT1C would be a part of the normal AMPAR cycle and lack of CPT1C might be associated to aging-related problems although this idea has not been tested. Interconnected with ageing problems, another challenging condition that affects an important percentage of the population is Alzheimer disease (AD; Alzehimer´s association, 2014). One of the first symptoms of AD is spatial-learning problems, a function that clearly depends on the hippocampus (Burgess and O'Keefe, 2014; Bannerman et al., 2014). Remarkably, CPT1C is highly expressed in that area (Schwenk et al., 2014), where it seems to be involved in learning processes (Carrasco et al., 2012) suggesting a possible relationship between CPT1C, AMPARs and AD related symptoms.

On the other hand, an important neurological condition is Amyotrophic lateral sclerosis (ALS), a disease that affects motorneurons. The correlation between ALS and a deficient editing at the Q/R site has been demonstrated (Takuma et al., 1999; Kawahara et al., 2004b). Specifically, ALS patients have virtually no editing at the Q/R site and GluA2-containing AMPARs render permeable to Ca^{2+} ions. Thus an increment of Ca^{2+} permeability seems to be the source of motorneuron death in ALS disease. We have shown in this thesis that Ca^{2+}-permeable AMPARs are trafficked more efficiently to plasma membrane in the presence of CPT1C than those Ca^{2+}-impermeable (GluA2-containing). Since the molecular determinant of that differential trafficking has not yet been unraveled, one could hypothesized that this increment in CP-AMPARs would be more susceptible of enhanced trafficking in a neuron containing CPT1C thus increasing the degree of cytotoxicity in ALS patients motorneurons regardless of the intrinsic elevation in Ca^{2+} entry to the cells. Indeed, CPT1C play an important role in motorneurons as recently explained in a paper describing for the first time a direct correlation of de novo CPT1C mutation with a disease (Rinaldi et al., 2015). Specifically, CPT1C R37C mutation resulted in hereditary autosomal dominant spastic paraplegia (SP), a disease affecting corticospinal motorneurons and resulting in lower-extremity spasticity. This mutation is located in the regulatory domain of CPT1C. Although this study by Rinaldi *et al.* shows that CPT1C interacts with atlastin-1 (a frequently mutated protein in SP), a possible role in AMPAR malfunction cannot be discarded given the role of CPT1C on these glutamate receptors.

Even though CPT1C expression is restricted to the brain in healthy individuals, it functions as a stress-responsive gene under a variety of conditions, as its mRNA is up-regulated in cell lines from several different tissues as well as in mice following exposure to any one of a number of p53-activating stresses (Zaugg et al., 2008; Reilly and Mak, 2012). Therefore, it is suggested that CPT1C promotes cancer cell survival and

tumour growth and it has been proposed as a new therapeutic target in cancer treatment. It is noteworthy that recent studies report an aberrant expression of CPT1C in gliomas (Cirillo et al., 2014; Wakamiya et al., 2014) where CP-AMPARs are expressed (Ishiuchi et al., 2002). These findings highlight the importance of unravelling the molecular mechanisms of CPT1C, which could be of great interest across a range of fields, for example in the study of new anticancer therapies and new diagnostic or prognostic markers. Further understanding of the mechanisms that underlie the regulation of AMPA receptors by CPT1C may bring new therapeutic strategies for different diseases associated with both AMPARs and CPT1C.

6. CONCLUSIONS

The design of this thesis work was set out to explore the consequences of the CPT1C induced modulation on AMPAR mediated responses. The experiments in the present thesis framework have also sought to characterise the molecular mechanism of AMPAR-CPT1C interaction.

The main conclusions are:

1. GluA1 and GluA2 coimmunoprecipitate with CPT1C in heterologous systems.
2. CPT1C increases GluA1 homomers whole-cell currents and this effect is stoichiometry dependent.
3. The effect of this interacting protein on GluA1 induced currents is specific of CPT1C isoform and depends on H469 residue located in the catalytic domain of the C-terminal.
4. CPT1C increases whole-cell currents of GluA1/GluA2 heteromeric AMPARs and has no effect on GluA2 homomers-mediated whole cell currents.
5. AMPARs gating properties are not modified by CPT1C.
6. CPT1C co-localises with AMPAR subunits at the ER and the subcellular localization of CPT1C determines its effect on AMPAR currents.
7. The functional effect of CPT1C on GluA1 is dependent on the metabolic state of the cell.
8. Surface expression of GluA1 is increased in the presence of CPT1C in heterologous systems and neuronal cultures.
9. GluA1 C585 mediates surface expression enhancement by CPT1C and this residue is critical for the enhancement of current density by CPT1C. GluA1 and CPT1C interaction does not dependent on palmitoylation of C585.
10. CPT1C does not affect GluA1 subunit palmitoylation state although inhibition of depalmitoylation activity by Palmostatin B prevents CPT1C enhancement of GluA1 currents.
11. Synaptic transmission is reduced in CPT1C deficient animals and the effect seems to be only postsynaptic.

7. ABBREVIATIONS

4.1N	neuronal protein 4.1
kD	kiloDalton
ng	nanogram
µg	microgram
µm	micrometer
µM	micromolar
µl	microliter
ABE	Acyl Biotin Exchange assay
ABP	actin-binding protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP-2	adaptor protein-2
BSA	Bovine serum albumin
CAMKII	Ca^{2+} /calmodulin-dependent protein kinase II
cDNA	complementary DNA
CI-AMPAR	Ca^{2+} impermeable AMPAR
CMV	cytomegalovirus
CNIH	cornichon homolog proteins
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CoA	coenzyme A
Co-IP	coimmunoprecipitation
CP-AMPAR	Ca^{2+} permeable AMPAR
CPT1A	carnitine palmitoyltransferase 1A
CPT1B	carnitine palmitoyltransferase 1B
CPT1C	carnitine palmitoyltransferase 1C
CTCF	corrected total cell fluorescence
CTD	C-terminal domain
CX-516	6-(piperidin-1-ylcarbonyl)quinoxaline
CX-546	2,3-dihydro-1,4-benzodioxin-7-yl-(1-

	piperidyl)methanone
D-APV	D-amino-5-phosphonovaleric acid
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
ER	endoplasmic reticulum
EPSC	excitatory post synaptic current
GA	Golgi apparatus
GFP	green fluorescent protein
Glu	glutamate
GluR	glutamate receptor
GRIP	glutamate receptor-interacting protein
HAM	hydroxylamine
HEK293	human embryonic kidney 293 cells
iGluR	ionotropic glutamate receptor
IF	immunofluorescence
INTDEN	integrated density
IP	immunoprecipitation
iGluRs	ionotropic glutamate receptors
KAR	kainate receptor
mGluR	metabotropic glutamate receptor
mRNA	messenger RNA
mini-EPSC	miniature excitatory post synaptic currents
LBD	ligand-binding domain
LTD	long-term depression
LTP	long-term potentiation
Narp	neuro activity regulated petaxin
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartate
NTD	N-terminal domain
NSF	N-ethylmaleimide sensitive fusion protein

RNA	ribonucleic acid
PAT	palmitoyl acyltransferase
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PICK	protein interacting with protein kinase C,
Po	peak open probability
PSD-95	postsynaptic density protein 95
PKA	Protein kinase A
PKC	protein kinase C
PSD	post synaptic density
PTE	palmitoyl thioesterase
SAP-97	synapse-associated protein 97
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE	**SNA**P (Soluble NSF Attachment Protein) **RE**ceptor
TARP	transmembrane AMPA receptor regulatory protein
TBS	Tris Buffered Saline
TMD	transmembrane domain
tsA201	transformed human 201 kidney cells

8. BIBLIOGRAPHY

Ady V, Perroy J, Tricoire L, Piochon C, Dadak S, Chen X, Dusart I, Fagni L, Lambolez B, Levenes C (2014) Type 1 metabotropic glutamate receptors (mGlu1) trigger the gating of GluD2 delta glutamate receptors. EMBO Rep. (1):103-9.

Alzheimer association(2014) Alzheimer's disease facts and figures.

Anggono V, Huganir RL (2012) Regulation of AMPA receptor trafficking and synaptic plasticity. Curr Opin Neurobiol. 22(3): 461-9.

Araki K, Meguro H, Kushiya E, Takayama C, Inoue Y, Mishina M (1993) Selective expression of the glutamate receptor channel delta 2 subunit in cerebellar Purkinje cells. Biochem Biophys Res Commun 197(3): 1267–1276.

Armstrong N, Gouaux E (2000) Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand-binding core. Neuron 28(1): 165-81.

Asrar S, Zhou Z, Ren W, Jia Z (2009) Ca^{2+} permeable AMPA receptor induced long-term potentiation requires PI3/MAP kinases but not Ca/CaM-dependent kinase II. PLoS One 4(2):e4339.

Ayalon G, Stern-Bach Y (2003) Functional assembly of AMPA and kainate receptors is mediated by several discrete protein-protein interactions. Neuron 31:103–113.

Ayalon G, Segev E, Elgavish S, Stern-Bach Y (2005) Two regions in the N-terminal domain of ionotropic glutamate receptor 3 form the subunit oligomerization interfaces that control subtype-specific receptor assembly. J Biol Chem. 280(15):15053-60.

Balázs R, Jørgensen OS, Hack N. (1988) N-methyl-d-aspartate promotes the survival of cerebellar granule cells in culture. Neuroscience 27: Issue 2, 437–451.

Banke TG, Bowie D, Lee H, Huganir RL, Schousboe A, et al., (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. J Neurosci 20: 89–102.

Bannerman DM, Sprengel R, Sanderson DJ, McHugh SB, Rawlins JN, Monyer H, Seeburg PH (2014) Hippocampal synaptic plasticity, spatial memory and anxiety. Nat Rev Neurosci. 15(3):181-92.

Barbon A, Fumagalli F, Caracciolo L, Madaschi L, Lesma E, Mora C, Carelli S, Slotkin TA, Racagni G, Di Giulio AM, Gorio A, Barlati S (2010) Acute spinal cord injury persistently reduces R/G RNA editing of AMPA receptors. J Neurochem. 114(2):397-407.

Barry MF, Ziff EB (2002) Receptor trafficking and the plasticity of excitatory synapses. Curr Opin Neurobiol. 12(3):279-86.

Barria A, Muller D, Derkach V, Griffith LC, Soderling TR (1997) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276(5321):2042-5.

Bass BL (2002) RNA editing by adenosine deaminases that act on RNA. Annu Rev Biochem. 71:817-46.

Bats C, Groc L, Choquet D. (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. Neuron 53, 719–734.

Bats C, Soto D, Studniarczyk D, Farrant M, Cull-Candy SG (2012) Channel properties reveal differential expression of TARPed and TARPless AMPARs in stargazer neurons. Nat Neurosci. 15(6):853-61.

Bedoukian MA, Weeks AM, Partin KM (2006) Different domains of the AMPA receptor direct stargazin-mediated trafficking and stargazin-mediated modulation of kinetics. J Biol Chem. 281(33):23908-21.

Bedoukian MA, Whitesell JD, Peterson EJ, Clay CM, Partin KM (2008) The stargazin C terminus encodes an intrinsic and transferable membrane sorting signal. J Biol Chem. 283(3): 1597-600.

Bellone C, Lüscher C, Mameli M (2008) Mechanisms of synaptic depression triggered by metabotropic glutamate receptors. Cell. Mol. Life Sci. 65, 2913–292.

Blanco-Suarez E, Hanley JG (2014) Distinct subunit-specific α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking mechanisms in cultured cortical and hippocampal neurons in response to oxygen and glucose deprivation. J Biol Chem. 289(8):4644-51.

Breese CR, Logel J, Adams C, Leonard SS (1996) Regional gene expression of the glutamate receptor subtypes GluR1, GluR2, and GluR3 in human postmortem brain. J Mol Neurosci. 7(4):277-89.

Brigidi GS1, Bamji SX (2013) Detection of protein palmitoylation in cultured hippocampal neurons by immunoprecipitation and acyl-biotin exchange (ABE). J Vis Exp. (72).

Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. Science 249(4972):1033-7.

Bowie D, Mayer ML (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. Neuron 15 (2):453-62.

Burgess N, O'Keefe J (2011) Models of place and grid cell firing and theta rhythmicity. Curr Opin Neurobiol. 21(5):734-44.

Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron 8, 189–198.

Carlson NG, Howard J, Gahring LC, Rogers SW (2001) RNA editing (Q/R site) and flop/flip splicing of AMPA receptor transcripts in young and old brains. Neurobiol Aging. 21(4):599-606.

Carrasco P, Sahún I, McDonald J, Ramírez S, Jacas J, Gratacós E, Sierra AY, Serra D, Herrero L, Acker-Palmer A, Hegardt FG, Dierssen M, Casals N (2012) Ceramide levels regulated by carnitine palmitoyltransferase 1C control dendritic spine maturation and cognition. J Biol Chem. (25):21224-32.

Carrasco P, Jacas J, Sahún I, Muley H, Ramírez S, Puisac B, Mezquita P, Pié J, Dierssen M, Casals N (2013) Carnitine palmitoyltransferase 1C deficiency causes motor impairment and hypoactivity. Behav Brain Res. 256:291-7.

Carroll RC, Beattie EC, von Zastrow M, Malenka RC (2001) Role of AMPA receptor endocytosis in synaptic plasticity. Nat Rev Neurosci. 2(5):315-24.

Cirillo A, Di Salle A, Petillo O, Melone MA, Grimaldi G, Bellotti A, Torelli G, De' Santi MS, Cantatore G, Marinelli A, Galderisi U, Peluso G (2014) High grade glioblastoma is associated with aberrant expression of ZFP57, a protein involved in gene imprinting, and of CPT1A and CPT1C that regulate fatty acid metabolism. Cancer Biol Ther. 15(6):735-41.

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, Zhang D (2002) Excitatory glycine receptors containing the NR3 family of NMDAR subunits. Nature 415:793.

Chang PK, Verbich D, McKinney RA (2012) AMPA receptors as drug targets in neurological disease--advantages, caveats, and future outlook. Eur J Neurosci. 35(12):1908-16.

Chazot PL, Coleman SK, Cik M, Stephenson FA (1994) Molecular characterization of N-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. J Biol Chem 269:24403–24409.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature. 408(6815): 936-43.

Chen L, El-Husseini A, Tomita S, Bredt DS, Nicoll RA (2003) Stargazin differentially controls the trafficking of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate and kainate receptors. Mol. Pharmacol. 64:703–706.

Chen N, Pandya NJ, Koopmans F, Castelo-Székelv V, van der Schors RC, Smit AB, Li KW (2014) Interaction proteomics reveals brain region-specific AMPA receptor complexes. J Proteome Res. 13(12):5695-706.

Cho CH, St-Gelais F, Zhang W, Tomita S, Howe JR (2007) Two families of TARP isoforms that have distinct effects on the kinetic properties of AMPA receptors and synaptic currents. Neuron. 55(6):890-904.

Choi DW. (1992) Excitotoxic cell death. J Neurobiol. 23(9):1261-76.

Chung HJ, Xia J, Scannevin RH, Zhang X, Huganir RL (2000) Phosphorylation of the AMPA receptor subunit GluA2 differentially regulates its interaction with PDZ domain-containing proteins. J Neurosci. 20:7258–7267.

Cohen I, Guillerault F, Girard J, Prip Buus C (2001) The N-terminal domain of rat liver carnitine palmitoyltransferase 1 contains an internal mitochondrial import signal and residues essential for folding of its C-terminal catalytic domain. J Biol Chem. 16: 5403-11.

Coleman SK, Möykkynen T, Cai C, von Ossowski L, Kuismanen E, Korpi ER, Keinänen K (2006) Isoform-specific early trafficking of AMPA receptor flip and flop variants. J Neurosci. 26(43):11220-9.

Contractor A, Swanson G, Heinemann SF (2001) Kainate receptors are involved in short- and long-term plasticity at mossy fiber synapses in the hippocampus. Neuron 29:209–216.

Coombs ID, Cull-Candy SG (2009) Transmembrane AMPA receptor regulatory proteins and AMPA receptor function in the cerebellum. Neuroscience 162(3): 656-65.

Coombs ID, Soto D, Zonouzi M, Renzi M, Shelley C, Farrant M, Cull-Candy SG (2012) Cornichons modify channel properties of recombinant and glial AMPA receptors. J Neurosci. 32(29): 9796-804.

Copits BA, Swanson GT (2013) Kainate receptor post-translational modifications differentially regulate association with 4.1N to control activity-dependent receptor endocytosis. J Biol Chem. 288(13):8952-8965.

Coussen F, Perrais D, Jaskolski F, Sachidhanandam S, Normand E, Bockaert J, Marin P, Mulle C (2005) Co-assembly of two GluR6 kainate receptor splice variants within a functional protein complex. Neuron 47(4):555-66.

Crepel F, Dhanjal SS, Sears TA (1982) Effect of glutamate, aspartate and related derivatives on cerebellar purkinje cell dendrites in the rat: an in vitro study. J Physiol 329:297-317.

Cull-Candy SG, Brickley SG, Misra C, Feldmeyer D, Momiyama A, Farrant M (1998) NMDA receptor diversity in the cerebellum: identification of subunits contributing to functional receptors. Neuropharmacology 37:1369-1380.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol. 11:327–335.

Cull-Candy S, Kelly L, & Farrant, M (2006). Regulation of Ca^{2+}-permeable AMPA receptors: Synaptic plasticity and beyond. Current Opinion in Neurobiology 16, 288–297.

Curtis DR, Phillis JW, Watkins JC (1960) The chemical excitation of spinal neurones by certain acidic amino acids. J. Physiol. 50:656–682.

Curtis DR, Watkins JC. (1960) The excitation and depression of spinal neurones by structurally related amino acids. J. Neurochem. 1960;6:117–141.

Dakoji S, Tomita S, Karimzadegan S, Nicoll RA, Bredt DS (2003) Interaction of transmembrane AMPA receptor regulatory proteins with multiple membrane associated guanylate kinases. Neuropharmacology. 45(6):849-56.

Danysz W, Parsons CG (2002) Neuroprotective potential of ionotropic glutamate receptor antagonists. Neurotox Res. 4(2):119-26.

Dekker, FJ; Rocks, O; Vartak, N; Menninger, S; Hedberg, C; Balamurugan, R; Wetzel, S; Renner, S; Gerauer, M; Schölermann, B; Rusch, M; Kramer, J W; Rauh, D; Coates, GW; Brunsveld, L; Bastiaens, P I; Waldmann, H (2010) Small-molecule inhibition of APT1 affects Ras localization and signaling. Nat. Chem. Biol. 6, 449.

Deng F, Price MG, Davis CF, Mori M, Burgess DL (2006) Stargazin and other transmembrane AMPA receptor regulating proteins interact with synaptic scaffolding protein MAGI-2 in brain. J Neurosci. 26(30): 7875-84.

Derkach V, Barria A, Soderling TR (1999) Ca^{2+}/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc Natl Acad Sci U S A. 96(6):3269-74.

Derkach VA, Oh MC, Guire ES, Soderling TR (2007) Regulatory mechanisms of AMPA receptors in synaptic plasticity. Nat Rev Neurosci. 8 (2):101-13.

Díaz E (2010) Regulation of AMPA receptors by transmembrane accessory proteins. Eur J Neurosci. 32(2):261-8.

Dev KK, Nishimune A, Henley JM, Nakanishi S (1999) The protein kinase C alpha binding protein PICK1 interacts with short but not long form alternative splice variants of AMPA receptor subunits. Neuropharmacology 38(5):635-44.

Donevan SD, Rogawski MA (1993) GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. Neuron 10(1):51-59.

Donevan SD, Rogawski MA (1995) Intracellular polyamines mediate inward rectification of Ca(2+)-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Proc Natl Acad Sci U S A. 92(20):9298-9302.

Dong H, O'Brian RJ, Fung ET, Lanahan AA, Worley PF, Huganir, RL (1997) GRIP: A synaptic PDZ domain-containing protein that interacts with AMPA receptors. Nature 386:279–284.

Earnshaw BA, Bressloff PC (2006) Biophysical Model of AMPA Receptor Trafficking and Its Regulation during Long-Term Potentiation/Long-Term Depression. J Neurosci 26(47):12362-12373.

Esser V, Britton CH, Weis BC, Foster DW, McGarry JD (1993) Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. Direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function. J Biol Chem. 15:5817–5822.

Esser V, Brown NF, Cowan AT, Foster DW, McGarry JD (1996) Expression of a cDNA isolated from rat brown adipose tissue and heart identifies the product as the muscle isoform of carnitine palmitoyltransferase I (M-CPT I). M-CPT I is the predominant CPT I isoform expressed in both white (epididymal) and brown adipocytes. J Biol Chem. 71(12):6972-7.

Feldmeyer, D, Kask K, Brusa R, Kornau HC, Kolhekar R, Rozov A, Burnashev N, Jensen V, Hvalby O, Sprengel R, Seeburg PH (1999) Neurological dysfunctions in mice expressing different levels of the Q/R site-unedited AMPAR subunit GluR-B. Nat. Neurosci. 2, 57–64.

Fukata Y, Tzingounis AV, Trinidad JC, Fukata M, Burlingame AL, Nicoll RA, Bredt DS (2005) Molecular constituents of neuronal AMPA receptors. J Cell Biol. 169(3): 399-404.

Fukata Y, Fukata M (2010) Protein palmitoylation in neuronal development and synaptic plasticity. Nat Rev Neurosci. 11:161–175.

Fraser F, Corstorphine CG, Zammit VA (1997) Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane. Biochem J 1:711-718

Friedman LK, Koudinov AR (1999) Unilateral GluR2(B) hippocampal knockdown: A novel partial seizure model in the developing rat. J Neurosci. 19:9412–9425.

Gallo V, Upson LM, Hayes WP, Vyklicky L, Jr, Winters CA, Buonanno A (1992) Molecular cloning and development analysis of a new glutamate receptor subunit isoform in cerebellum. J Neurosci.12:1010–1023.

Gao XF, Chen W, Kong XP, Xu AM, Wang ZG, Sweeney G, Wu D (2009) Enhanced susceptibility of Cpt1c knockout mice to glucose intolerance induced by a high-fat diet involves elevated hepatic gluconeogenesis and decreased skeletal muscle glucose uptake. Diabetologia 52(5): 912-920.

Garcia EP, Mehta S, Blair LA, Wells DG, Shang J, Fukushima T, Fallon JR, Garner CC, Marshall J (1998) SAP90 binds and clusters kainate receptors causing incomplete desensitization. Neuron. 21(4):727-739.

Gardner SM, Takamiya K, Xia J, Suh JG, Johnson R, Yu S, Huganir RL (2005) Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF. Neuron 45, 903–915.

Geiger JR, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron 15, 193–204.

Gill MB, Kato AS, Wang H, Bredt DS (2012) AMPA receptor modulation by cornichon-2 dictated by transmembrane AMPA receptor regulatory protein isoform. Eur J Neurosci. 35(2):182-94.

Gladding CM1, Fitzjohn SM, Molnár E (2009) Metabotropic glutamate receptor-mediated long-term depression: molecular mechanisms. Pharmacol Rev. 61(4):395-412.

Gratacòs-Batlle E (2010) Molecular characterization of carnitine palmitoyltransferase 1C. Diss. Universitat de Barcelona.

Greger IH, Khatri L, Ziff EB (2002) RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum. Neuron 34(5):759-72.

Greger IH, Khatri L, Kong X, Ziff E (2003) AMPA receptor tetramerization is mediated by Q/R editing. Neuron 40:763-774.

Greger, IH, Ziff, EB, Penn, AC (2007) Molecular determinants of AMPA receptor subunit assembly. Trends in Neurosciences, 30, 407–416.

Gregor, P, O'Hara, BF, Yang, X., Uhl, GR (1993) Expression and novel subunit isoforms of glutamate receptor genes GluR5 and GluR6. NeuroReport 4, 1343–1346.

Green, G.M. & Gibb, A.J (2001) Characterization of the single-channel properties of NMDA receptors in laminae I and II of the dorsal horn of neonatal rat spinal cord. European Journal of Neuroscience, 14:1590-1602.

Goff DC, Freudenreich O, Evins AE (2001) Augmentation strategies in the treatment of schizophrenia. CNS Spectr. 6(11):904, 907-11.

Gu Z, Liu W, Yan Z (2009) {beta}-Amyloid impairs AMPA receptor trafficking and function by reducing Ca^{2+}/calmodulin-dependent protein kinase II synaptic distribution. J Biol Chem. 284(16):10639-49.

Haering SC, Tapken D, Pahl S, Hollmann M (2014) Auxiliary subunits: shepherding AMPA receptors to the plasma membrane. Membranes (Basel). 4(3):469-90.

Hansen KB, Furukawa H, Traynelis SF (2010) Control of assembly and function of glutamate receptors by the amino-terminal domain. Mol Pharmacol. 78(4):535-49.

Hansen KB, Ogden KK, Yuan H, Traynelis SF (2014) Distinct functional and pharmacological properties of Triheteromeric GluN1/GluN2A/GluN2B NMDA receptors. Neuron 81 1084–1096.

Hastie P, Ulbrich MH, Wang HL, Arant RJ, Lau AG, Zhang Z, Isacoff EY, Chen L (2013) AMPA receptor/TARP stoichiometry visualized by single-molecule subunit counting. Proc Natl Acad Sci U S A. 110(13):5163-8.

Hayashi T (1954) Effects of sodium glutamate on the nervous system. Keio J. Med.3:192–193

Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 24;287(5461):2262-7.

Hayashi T, Rumbaugh G, Huganir RL (2005) Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. Neuron 47:709–723.

Hayashi T, Thomas GM, Huganir RL (2009) Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. Neuron 29;64(2):213-26.

Henley JM (2003) Proteins interactions implicated in AMPA receptor trafficking: a clear destination and an improving route map. Neurosci Res. 45(3):243-54.

Henley JM, Barker EA, Glebov OO (2011) Routes, destinations and delays: recent advances in AMPA receptor trafficking. Trends Neurosci. 34(5):258-68.

Henley JM, Wilkinson KA (2013) AMPA receptor trafficking and the mechanisms underlying synaptic plasticity and cognitive aging. Dialogues Clin Neurosci. 15(1):11-27.

Hepp R, Audrey Hay , Aguado C, Lujan R, Dauphinot L, Potier MC, Nomura S, Poirel O, Mestikawy S, Lambolez B, Tricoire L (2014) Glutamate receptors of the delta family are widely expressed in the adult brain. Brain Structure & Function. 220, 2797-2815.

Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS (2005) Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol. 6(2):150-66.

Hollmann M, O'Shea-Greenfield A, Rogers SW, and Heinemann S (1989) Cloning by functional expression of a member of the glutamate receptor family. Nature 342, 643-648

Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} permeability of KA–AMPA-gated glutamate receptor channels depends on subunit composition. Science 252, 851–853.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci. 17:31-108.

Howard MA, Elias GM, Elias LA, Swat W, Nicoll RA (2010) The role of SAP97 in synaptic glutamate receptor dynamics. Proc Natl Acad Sci U S A. 107(8):3805-10.

Hume RI, Dingledine R, Heinemann SF (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 253(5023): 1028-31.

Jackson AC, Milstein AD, Soto D, Farrant M, Cull-Candy SG, Nicoll RA (2011) Probing TARP modulation of AMPA receptor conductance with polyamine toxins. J Neurosci. 31(20): 7511-20.

Jackson AC, Nicoll RA (2011) Stargazin from a new vantage-TARP modulation of AMPA receptor pharmacology. J Physiol. 589(Pt 24):5909-10.

Jamain S, Betancur C, Quach H, Philippe A, Fellous M, Giros B, Gillberg C, Leboyer M, Bourgeron T (2002) Linkage and association of the glutamate receptor 6 gene with autism. Mol Psychiatry 7:302–310.

Jáuregui O, Sierra AY, Carrasco P, Gratacós E, Hegardt FG, Casals N (2007) A new LC-ESI-MS/MS method to measure long-chain acylcarnitine levels in cultured cells. Anal Chim Acta. 599(1):1-6.

Jia Z, Agopyan N, Miu P, Xiong Z, Henderson J, Gerlai R, Taverna FA, Velumian A, MacDonald J, Carlen P, et al (1996) Enhanced LTP in mice deficient in the AMPA receptor GluR2. Neuron 17, 945–956.

Jin R, Banke TG, Mayer ML, Traynelis SF, Gouaux E (2003) Structural basis for partial agonist action at ionotropic glutamate receptors. Nat Neurosci. 6(8):803-810.

Jin R, Clark S, Weeks AM, Dudman JT, Gouaux E, Partin KM (2005) Mechanism of positive allosteric modulators acting on AMPA receptors. J Neurosci. 25(39): 9027-36.

Jogl G, Hsiao YS, Tong L (2004) Structure and function of carnitine acyltransferases. Ann N Y Acad Sci. 1033:17-29.

Johnson JW, Asher P (1987) Glycine potentates the NMDA response in cultured mouse brain neurons. Nature 323:529–531.

Johnson KA, Conn PJ, Niswender CM (2009) Glutamate receptors as therapeutic targets for Parkinson's disease. CNS Neurol Disord Drug Targets. 8(6):475-91.

Johnson JW, Glasgow NG, Povysheva NV (2015) Recent insights into the mode of action of memantine and ketamine. Curr Opin Pharmacol 20: 54–63.

Iino M, Goto K, Kakegawa W, Okado H, Sudo M, Ishiuchi S, Miwa A, Takayasu Y, Saito I, Tsuzuki K, Ozawa S (2001) Glia-synapse interaction through Ca^{2+}-permeable AMPA receptors in Bergmann glia. Science 292(5518):926-9.

Iizuka M, Nishimura S, Wakamori M, Akiba I, Imoto K, Barsoumian EL The lethal expression of the GluR2flip/GluR4flip AMPA receptor in HEK293 cells. Eur J Neurosci. 12(11):3900-8.

Isa T, Iino M, Itazawa S, Ozawa S (1995) Spermine mediates inward rectification of Ca^{2+}-permeable AMPA receptor channels. Neuroreport 6(15):2045-8.

Isaac JT, Nicoll RA, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. Neuron 15, 427–434.

Isaac JT, Ashby M, McBain CJ (2007) The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. Neuron, 54, 859–871.

Ishiuchi S, Tsuzuki K, Yoshida Y, Yamada N, Hagimura N, Okado H, Miwa A, Kurihara H, Nakazato Y, Tamura M, Sasaki T, Ozawa S (2002) Blockage of Ca^{2+}-permeable AMPA receptors suppresses migration and induces apoptosis in human glioblastoma cells. Nat Med. 8(9): 971-978.

Kakegawa W, Kohda K, Yuzaki M (2007) The delta2 'ionotropic' glutamate receptor functions as a non-ionotropic receptor to control cerebellar synaptic plasticity. J Physiol. 584(Pt 1):89-96.

Kakegawa W, Miyazaki T, Emi K, Matsuda K, Kohda K, Motohashi J, Mishina M, Kawahara S, Watanabe M, Yuzaki M (2008) Differential regulation of synaptic plasticity and cerebellar motor learning by the C-terminal PDZ-binding motif of GluRdelta2. J Neurosci 28(6):1460–1468.

Kamboj SK, Swanson GT, Cull-Candy SG (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainate receptors. J Physiol. 486 (Pt 2):297-303.

Kashiwabuchi N, Ikeda K, Araki K, Hirano T, Shibuki K, Takayama C, Inoue Y, Kutsuwada T, Yagi T, Kang Y et al (1995) Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR delta 2 mutant mice. Cell 81(2):245–252.

Kato AS, Gill MB, Ho MT, Yu H, Tu Y, Siuda ER, Wang H, Qian YW, Nisenbaum ES, Tomita S, Bredt DS (2010) Hippocampal AMPA receptor gating controlled by both TARP and cornichon proteins. Neuron 68(6):1082-96.

Kato AS, Knierman MD, Siuda ER, Isaac JTR, Nisenbaum ES, Bredt DS (2012) Glutamate Receptor δ2 Associates with Metabotropic Glutamate Receptor 1 (mGluR1), Protein Kinase Cγ, and Canonical Transient Receptor Potential 3 and Regulates mGluR1-Mediated Synaptic Transmission in Cerebellar Purkinje Neurons. The Journal of Neuroscience 32(44): 15296-15308.

Kawahara Y, Ito K, Sun H, Kanazawa I, Kwak S (2003) Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. Eur J Neurosci. 18(1):23-33.

Kawahara Y, Ito K, Sun H, Ito M, Kanazawa I, Kwak S (2004a) GluR4c, an alternative splicing isoform of GluR4, is abundantly expressed in the adult human brain. Brain Res Mol Brain Res. 127(1-2):150-5.

Kawahara Y, Ito K, Sun H, Aizawa H, Kanazawa I, Kwak S (2004b) Glutamate receptors: RNA editing and death of motor neurons. Nature 427(6977):801.

Kew JN, Kemp JA (2005) Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl) 179: 4–29.

Kim KS, Yan D, Tomita S (2010) Assembly and stoichiometry of the AMPA receptor and transmembrane AMPA receptor regulatory protein complex. Journal of Neuroscience, 30(3), 1064–1072.

Koh DS, Burnashev N, Jonas P (1995) Block of native Ca^{2+}-permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. J Physiol. 486 (Pt 2):305-12.

Köhler M, Kornau HC, Seeburg PH (1994) The organization of the gene for the functionally dominant alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor subunit GluR-B. J Biol Chem. 269(26):17367-70.

Kolleker A, Zhu JJ, Schupp BJ, Qin Y, Mack V, Borchardt T, Köhr G, Malinow R, Seeburg PH, Osten P (2003) Glutamatergic plasticity by synaptic delivery of GluR-B(long)-containing AMPA receptors. Neuron. 18;40(6):1199-212.

Komuro HI, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. Science 2; 260:95-7.

Kondo M, Sumino R, Okado H (1997) Combinations of AMPA receptor subunit expression in individual cortical neurons correlate with expression of specific calcium-binding proteins. J Neurosci. 17(5):1570-81.

Kott S, Sager C, Tapken D, Werner M, Hollmann M (2009) Comparative analysis of the pharmacology of GluR1 in complex with transmembrane AMPA receptor regulatory proteins γ2, γ3, γ4, and γ8. Neuroscience;158:78–88

Krebs HA (1935) Metabolism of amino acids. Biochem. J. 29: 1951-1969.

Kristensen AS, Jenkins MA, Banke TG, Schousboe A, Makino Y, Johnson RC, Huganir R, Traynelis SF (2011) Mechanism of Ca^{2+}/calmodulin-dependent kinase II regulation of AMPA receptor gating. Nat Neurosci. 14(6):727-35.

Krogsgaard-Larsen P, Honoré T, Hansen JJ, Curtis DR, Lodge D (1980) New class of glutamate agonist structurally related to ibotenic acid. Nature 284(5751):64–66.

Kumar J, Mayer ML (2013) Functional insights from glutamate receptor ion channel structures. Annu Rev Physiol. 75:313-37.

Kuner T, Beck C, Sakmann B, Seeburg PH (2001) Channel-lining residues of the AMPA receptor M2 segment: structural environment of the Q/R site and identification of the selectivity filter. J Neurosci. 21(12):4162-72.

Lai TW, Zhang S, Wang YT (2014) Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog. Neurobiol. 115 157–188 10.1016.

Lambolez B, Audinat E, Bochet P, Crépel F, Rossier J (1992) AMPA receptor subunits expressed by single Purkinje cells. Neuron 1992 Aug;9(2):247-58.

Laube B, Kuhse J, Betz H (1998) Evidence for a tetrameric structure of recombinant NMDARs. J Neurosci.18:2954.

Lavrentyev EN, Matta SG, Cook GA (2004) Expression of three carnitine palmitoyltransferase-I isoforms in 10 regions of the rat brain during feeding, fasting, and diabetes. Biochem Biophys Res Commun 315,:174–178.

Lee CH, Lü W, Michel JC, Goehring A, Du J, Song X, et al (2014) NMDA receptor structures reveal subunit arrangement and pore architecture. Nature 511, 191–197.

Lee HK, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature 405(6789):955-9.

Lee HK (2006) Synaptic plasticity and phosphorylation. Pharmacol Ther. 112:810–832.

Lees GJ (2000) Pharmacology of AMPA/kainate receptor ligands and their therapeutic potential in neurological and psychiatric disorders. Drugs 59(1):33-78.

Leonard AS, Davare MA, Horne MC, Garner CC, Hell JW (1998) SAP97 is associated with the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit. J Biol Chem. 273(31):19518-24.

Lerma, J (1992) Spermine regulates N-methyl--aspartate receptor desensitization. Neuron 8, 343-352.

Liao D, Hessler, NA, Malinow R (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature 375, 400–404.

Lin DT, Makino Y, Sharma K, Hayashi T, Neve R, Takamiya K, Huganir RL (2009) Regulation of AMPA receptor extrasynaptic insertion by 4.1N, phosphorylation and palmitoylation. Nat Neurosci. 12:879–887.

Liu SJ, Cull-Candy SG (2002) Activity-dependent change in AMPA receptor properties in cerebellar stellate cells. J. Neurosci. 22, 3881–3889.

Liu SJ, Cull-Candy SG (2005). Subunit interaction with PICK and GRIP controls Ca^{2+} permeability of AMPARs at cerebellar synapses. Nat. Neurosci. 8, 768–775.

Liu SJ, Zukin RS (2007) Ca^{2+}permeable AMPA receptors in synaptic plasticity and neuronal death. Trends in Neurosciences, 30, 126–134.

Liu SQ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Nature 405, 454–458.

Lodge D (2009) The history of the pharmacology and cloning of ionotropic glutamate receptors and the development of idiosyncratic nomenclature. Neuropharmacology 56: 6–21.

Lomeli H, Sprengel R, Laurie DJ, Kohr G, Herb A, Seeburg PH, Wisden W (1993) The rat delta-1 and delta-2 subunits extend the excitatory amino acid receptor family. FEBS Lett 315(3): 318–322.

Lomeli H, Mosbacher J, Melcher T, Höger T, Geiger JR, Kuner T, Monyer H, Higuchi M, Bach A, Seeburg PH (1994) Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. Science. 266(5191):1709-13.

López M, Lelliott CJ, Vidal-Puig A (2007) Hypothalamic fatty acid metabolism: a housekeeping pathway that regulates food intake. Bioessays 29: 248-261.

Lu J, Helton TD, Blanpied TA, Rácz B, Newpher TM, Weinberg RJ, Ehlers MD (2007) Postsynaptic positioning of endocytic zones and AMPA receptor cycling by physical coupling of dynamin-3 to Homer. Neuron 55(6):874-89.

Lu W, Shi Y, Jackson AC, Bjorgan K, During MJ, Sprengel R, Seeburg PH, Nicoll RA (2009) Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. Neuron 62:254-268.

Lu W, Roche KW (2012) Post-translational regulation of AMPA receptor trafficking and function. Curr Opin Neurobiol. 22(3):470-9.

Luo J, Wang Y, Yasuda RP, Dunah AW, Wolfe BB (1997) The majority of N-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). Mol. Pharmacol. 51, 79 – 86.

Lussier MP, Sanz-Clemente A, Roche KW (2015) Dynamic Regulation of N-Methyl-d-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors by Post-translational Modifications. J Biol Chem. 290(48):28596-603.

Mah SJ, Cornell E, Mitchell NA, Fleck MW (2005) Glutamate receptor trafficking: endoplasmic reticulum quality control involves ligand binding and receptor function. J Neurosci. 25:2215-2225.

Mack V, Burnashev N, Kaiser KM, Rozov A, Jensen V, Hvalby O, Seeburg PH, Sakmann B, Sprengel R (2001) Conditional restoration of hippocampal synaptic potentiation in Glur-A-deficient mice. Science 292(5526): 2501-4.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 30;44(1):5-21.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci. 25:103-26.

Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. J. Biol. Chem. 272, 32528-32533

Manders EEM, Verbeek FJ, Aten JA (1993) Measurement of colocalisation of objects in dual-colour confocal images. J. Microsc 169: 375–382.

Mansour M, Nagarajan N, Nehring RB, Clements JD, Rosenmund C (2001) Heteromeric AMPA receptors assemble with a preferred subunit stoichiometry and spatial arrangement. Neuron 2(5):841-53.

Martin LJ, Blackstone CD, Levey AI, Huganir RL, Price DL (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. Neuroscience 53(2):327-58.

Mano I, Teichberg VI (1998) A tetrameric subunit stoichiometry for a glutamate receptor-channel complex. Neuroreport 9(2):327-31.

Masu M, Tanabe Y, Tsuchida K, Shigemoto R, Nakanishi S (1991) Sequence and expression of a metabotropic glutamate receptor. Nature 349:760–765.

Matsuda S, Mikawa S, Hirai H (1999) Phosphorylation of serine-880 in GluA2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. J Neurochem. 73:1765–1768.

Matsuda K, Yuzaki M (2012) Cbln1 and the δ2 glutamate receptor--an orphan ligand and an orphan receptor find their partners. Cerebellum 11(1): 78-84.

Matsui K, Jahr CE, Rubio ME (2005) High-concentration rapid transients of glutamate mediate neural-glial communication via ectopic release. J Neurosci. 25(33): 7538-47.

Mayer GL, Westbrook PB, Guthrie P (1984) Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurones. Nature 309, 261–263.

Mayer ML (2005) Crystal structures of the GluR5 and GluR6 ligand binding cores: molecular mechanisms underlying kainate receptor selectivity. Neuron 17;45(4):539-52.

McGarry JD, Brown NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem. 244(1):1-14.

Menuz K, Stroud RM, Nicoll RA, Hays FA (2007) TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists. Science 318:815–817.

Menuz K, O'Brien JL, Karmizadegan S, Bredt DS, Nicoll RA (2008) TARP redundancy is critical for maintaining AMPA receptor function. J Neurosci. 28(35):8740-6.

Milstein AD, Zhou W, Karimzadegan S, Bredt DS, Nicoll RA (2007) TARP subtypes differentially and dose-dependently control synaptic AMPA receptor gating. Neuron 5(6): 905-18.

Milstein AD, Nicoll RA (2008) Regulation of AMPA receptor gating and pharmacology by TARP auxiliary subunits. Trends Pharmacol Sci. 29(7): 333-9.

Monaghan DT, Bridges RJ, and Cotman CW (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 29, 365-402

Mori H, Mishina M (1995) Structure and function of the NMDAR channel. Neuropharmacology 34:1219.

Morillas M, Gómez-Puertas P, Roca R, Serra D, Asins G, Valencia A, Hegardt FG (2001) Structural model of the catalytic core of carnitine palmitoyltransferase I and carnitine octanoyltransferase (COT): mutation of CPT I histidine 473 and alanine 381 and COT alanine 238 impairs the catalytic activity. J Biol Chem. 276(48):45001-45008.

Mosbacher, J, Schoepfer, R, Monyer, H, Burnashev, N, Seeburg, PH, Ruppersberg, JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. Science 266, 1059–62.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12 529–540 10.1016/0896-6273(94)90210.

Möykkynen T, Coleman SK, Semenov A, Keinänen K (2014) The N-terminal domain modulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor desensitization. J Biol Chem. 289(19):13197-205.

McDonald BJ, Chung HJ, Huganir RL (2001) Identification of protein kinase C phosphorylation sites within the AMPA receptor GluA2 subunit. Neuro-pharmacology 41:672–679.

McGarry, JD, Brown, NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur. J. Biochem. 244:1–14.

Nakanishi N, Shneidet NA, Axel R (1990) A family of glutamate receptor genes: evidence for the formation of hetetomultimetic receptors with distinct channel properties. Neuron 5, 569-581.

Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain. Science 23; 258:597–603.

Naur P, Hansen KB, Kristensen AS, Dravid SM, Pickering DS, Olsen L, Vestergaard B, Egebjerg J, Gajhede M, Traynelis SF et al (2007) Ionotropic glutamate-like receptor delta 2 binds d-serine and glycine. Proc Natl Acad Sci USA 104:14116–14121

Naylor P, Stewart CA, Wright SR, Pearson RC, Reid IC (1996) Repeated ECS induces GluR1 mRNA but not NMDAR1A-G mRNA in the rat hippocampus. Brain Res.35:349–353.

Nelson JK, Frølund SU, Tikhonov DB, Kristensen AS, Strømgaard K (2009) Synthesis and biological activity of argiotoxin 636 and analogues: selective antagonists for ionotropic glutamate receptors. Angew Chem Int Ed Engl. 48(17):3087-91.

Nicoll RA, Tomita S, Bredt DS (2006) Auxiliary subunits assist AMPA-type glutamate receptors.
Science. 311(5765):1253-6.

Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M, Collingridge GL, Nakanishi S, Henley JM (1998) NSF binding to GluR2 regulates synaptic transmission. Neuron 21(1):87-97.

Niswender CM, Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol.; 50:295-322.

Ng D, Pitcher GM, Szilard RK, Sertié A, Kanisek M, Clapcote SJ, Lipina T, Kalia LV, Joo D, McKerlie C, Cortez M, Roder JC, Salter MW, McInnes RR (2009) Neto1 is a novel CUB-domain NMDA receptor-interacting protein required for synaptic plasticity and learning. PLoS Biol. 7(2):e41.

Noh KM, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MV (2005) Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death. Proc Natl Acad Sci U S A. 102(34):12230-5.

Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, Salter MW (2003) Glycine binding primes NMDA receptor internalization. Nature 20;422(6929):302-7.

Nyman LR, Cox KB, Hoppel CL, Kerner J, Barnoski BL, Hamm DA, Tian L, Schoeb TR, Wood PA (2005) Homozygous carnitine palmitoyltransferase 1a (liver isoform) deficiency is lethal in the mouse. Mol Genet Metab. 2005 Sep-Oct; 86(1-2):179-87.

Oh MC, Derkach VA (2005) Dominant role of the GluR2 subunit in regulation of AMPA receptors by CaMKII. Nat Neurosci. 8(7):853-854.

Oh MC, Derkach VA, Guire ES, Soderling TR (2006) Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. J Biol Chem. 281(2):752-8.

Opitz T, Grooms SY, Bennett MV, Zukin RS (2000) Remodeling of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit composition in hippocampal neurons after global ischemia. Proc Natl Acad Sci U S A. 97(24):13360-5.

Orth A, Tapken D, Hollmann M (2013) The delta subfamily of glutamate receptors: characterization of receptor chimeras and mutants. Eur J Neurosci 37(10):1620–1630.

Palmer CL, Cotton L, Henley JM (2005) The molecular pharmacology and cell biology of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Pharmacol Rev. 57(2):253-77.

Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nature Reviews Neuroscience 14, 383–400.

Papouin T, Ladépêche L, Ruel J, Sacchi S, Labasque M, Hanini M, Groc L, Pollegioni L, Mothet JP, Oliet SH (2012) Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. Cell 3;150(3):633-46.

Partin KM, Bowie D, Mayer ML (1995) Structural determinants of allosteric regulation in alternatively spliced AMPA receptors. Neuron 14:833–843.

Paschen W, Djuricic B (1995) Regional differences in the extent of RNA editing of the glutamate receptor subunits GluR2 and GluR6 in rat brain. J Neurosci Methods. 56(1):21-9.

Passafaro M, Piëch V, Sheng M (2001) Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. Nat Neurosci. 4(9):917-26.

Passafaro M, Nakagawa T, Sala C, Sheng M (2003) Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. Nature 424(6949):677-81.

Paternain AV, Morales M, Lerma J (1995) Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. Neuron 1995;14:185–189.

Patneau DK, Mayer ML, Jane DE, Watkins JC (1992) Activation and Desensitization of AMPA / Kainate Receptors by Novel Derivatives of Willardiine". J Neurosci 12(2): 595–606.

Patrick GN, Bingol B, Weld HA, Schuman EM (2003) Ubiquitin-mediated proteasome activity is required for agonist-induced endocytosis of GluRs. Curr Biol. 13:2073–2081.

Pellegrini-Giampietro DE, Bennett MV, Zukin RS (1992) Are Ca^{2+}-permeable kainate/AMPA receptors more abundant in immature brain? Neurosci Lett. 144(1-2):65-9.

Piccini A, Malinow R (2002) Critical postsynaptic density 95/disc large/zonula occludens-1 interactions by glutamate receptor 1 (GluR1) and GluR2 required at different subcellular sites. J Neurosci. 22(13):5387-92.

Pickering DS, Taverna FA, Salter MW, Hampson DR (1995) Palmitoylation of the GluR6 kainate receptor. Proc Natl Acad Sci U S A. 92(26):12090-4.

Price N, van der Leij F, Jackson V, Corstorphine, C, Thomson R, Sorensen A, Zammit V (2002) A novel brain-expressed protein related to carnitine palmitoyltransferase I. Genomics 80, 433–442.

Priel A, Kolleker A, Ayalon G, Gillor M, Osten P, Stern-Bach Y (2005) Stargazin reduces desensitization and slows deactivation of the AMPA-type glutamate receptors. J. Neurosci. 25:2682–2686.

Purves D, Augustine GJ, Fitzpatrick D, et al (2001) Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; Circuits within the Cerebellum.

Quintana P, Soto D, Poirot O, Zonouzi M, Kellenberger S, Muller D, Chrast R, Cull-Candy SG (2015) Acid-sensing ion channel 1a drives AMPA receptor plasticity following ischaemia and acidosis in hippocampal CA1 neurons. J Physiol. 593(19):4373-4386.

Rakhade SN, Zhou C, Aujla PK, Fishman R, Sucher NJ, Jensen FE (2008) Early alterations of AMPA receptors mediate synaptic potentiation induced by neonatal seizures. J Neurosci. 28:7979–7990.

Reamy AA, Wolfgang MJ (2011) Carnitine palmitoyltransferase-1c gain-of-function in the brain results in postnatal microencephaly. J Neurochem. 118(3):388-98.

Reilly PT, Mak TW (2015) Molecular pathways: tumor cells Co-opt the brain-specific metabolism gene CPT1C to promote survival. Clin Cancer Res. 18(21):5850-5855.

Reimers JM1, Milovanovic M, Wolf ME (2011) Quantitative analysis of AMPA receptor subunit composition in addiction-related brain regions. Brain Res. 1367:223-33.

Rigby M, Cull-Candy SG2, Farrant M (2015) Transmembrane AMPAR regulatory protein γ-2 is required for the modulation of GABA release by presynaptic AMPARs. J Neurosci. 35(10):4203-14.

Rinaldi C, Schmidt T2, Situ AJ, Johnson JO, Lee PR, Chen KL, Bott LC, Fadó R, Harmison GH, Parodi S, Grunseich C, Renvoisé B, Biesecker LG, De Michele G, Santorelli FM, Filla A, Stevanin G, Dürr A, Brice A, Casals N, Traynor BJ, Blackstone C,

Ulmer TS, Fischbeck KH (2015) Mutation in CPT1C Associated With Pure Autosomal Dominant Spastic Paraplegia. JAMA Neurol. 72(5):561-70.

Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron 16:1179–1188

Rosenmund C, Stern-Bach Y, Stevens CF (1998) The tetrameric structure of a glutamate receptor channel. Science 280: 1596–1599.

Rozas JL, Paternain AV, Lerma J (2003) Noncanonical signaling by ionotropic kainate receptors. Neuron 39:543–553.

Ryu K, Yokoyama M, Yamashita M, Hirano T (2012) Induction of excitatory and inhibitory presynaptic differentiation by GluD1. Biochem Biophys Res Commun 417(1):157–161.

Saab AS, Neumeyer A, Jahn HM, Cupido A, Šimek AA, Boele HJ, Scheller A, Le Meur K, Götz M, Monyer H, Sprengel R, Rubio ME, Deitmer JW, De Zeeuw CI, Kirchhoff F (2012) Bergmann glial AMPA receptors are required for fine motor coordination. Science 337(6095):749-53.

Sager C, Terhag J, Kott S, Hollmann M (2009) C-terminal domains of transmembrane α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor regulatory proteins not only facilitate trafficking but are major modulators of AMPA receptor function. J. Biol. Chem. 284:32413–32424.

Samanta S, Situ AJ, Ulmer TS (2014) Structural characterization of the regulatory domain of brain carnitinepalmitoyltransferase1. Biopolymers 101:398-405.

Sans N, Vissel B, Petralia RS, Wang YX, Chang K, Royle GA, Wang CY, O'Gorman S, Heinemann SF, Wenthold RJ (2003) Aberrant formation of glutamate receptor complexes in hippocampal neurons of mice lacking the GluR2 AMPA receptor subunit. J Neurosci. 23(28):9367-73.

Santos SD, Manadas B, Duarte CB, Carvalho AL (2010) Proteomic analysis of an interactome for long-form AMPA receptor subunits. J Proteome Res. 9(4):1670-82.

Schiffer, HH, Swanson, GT, Heinemann, SF (1997) Rat GluR7 and acarboxy-terminal splice variant, GluR7b, are functional kainate receptor subunits with a low sensitivity to glutamate. Neuron 19, 1141–1146.

Schmid SM and Hollmann M (2008) To gate or not to gate: Are the delta subunits in the glutamate receptor family functional ion channels? Molecular Neurobiology 37(2-3): 126-141.

Schnell E, Sizemore M, Karimzadegan S, Chen L, Bredt DS, Nicoll RA (2002) Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. Proc Natl Acad Sci U S A. 99(21): 13902-7.

Schwenk J, Harmel N, Zolles G, Bildl W, Kulik A, Heimrich B, Chisaka O, Jonas P, Schulte U, Fakler B, Klöcker N (2009) Functional proteomics identify cornichon proteins as auxiliary subunits of AMPA receptors. Science 323 (5919):1313-1319.

Schwenk J, Harmel N, Brechet A, Zolles G, Berkefeld H, Müller CS, Bildl W, Baehrens D, Hüber B, Kulik A, Klöcker N, Schulte U, Fakler B (2012) High-resolution proteomics unravel architecture and molecular diversity of native AMPA receptor complexes. Neuron 74(4):621-33.

Schwenk J, Baehrens D, Haupt A, Bildl W, Boudkkazi S, Roeper J, Fakler B, Schulte U (2014) Regional diversity and developmental dynamics of the AMPA-receptor proteome in the mammalian brain. Neuron 84(1): 41-54.

Seeburg PH, Burnashev N, Kohr G, Kuner T, Sprengel R, Monyer H. (1995) The NMDAR channel: molecular design of a coincidence detector. Recent Prog Horm Res. 50:19.

Seeburg PH (1996) The role of RNA editing in controlling glutamate receptor channel properties. J Neurochem. 66(1):1-5. Review.

Selvakumar B, Jenkins MA, Hussain NK, Huganir RL, Traynelis SF, Snyder SH (2013) S-nitrosylation of AMPA receptor GluA1 regulates phosphorylation, single-channel conductance, and endocytosis. Proc Natl Acad Sci U S A. 110(3):1077-82.

Seol GH, Ziburkus J, Huang S, Song L, Kim IT, Takamiya K, Huganir RL, Lee HK, Kirkwood A (2007) Neuromodulators control the polarity of spike-timing-dependent synaptic plasticity. Neuron. 55(6):919-29.

Shaw GG (1979) The polyamines in the central nervous system. Biochem Pharmacol. 28(1):1-6.

Shelley C, Farrant M, Cull-Candy SG (2012) TARP-associated AMPA receptors display an increased maximum channel conductance and multiple kinetically distinct open states. J Physiol. 590(22): 5723-38.

Shen L, Liang F, Walensky LD, Huganir RL (2000) Regulation of AMPA receptor GluR1 subunit surface expression by a 4. 1N-linked actin cytoskeletal association. J Neurosci. 20(21):7932-40.

Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell;105(3):331-43.

Shi Y, Lu W, Milstein AD, Nicoll RA (2009) The stoichiometry of AMPA receptors and TARPs varies by neuronal cell type. Neuron 62(5), 633–640.

Shi Y, Suh YH, Milstein AD, Isozaki K, Schmid SM, Roche KW, Nicoll RA (2010) Functional comparison of the effects of TARPs and cornichons on AMPA receptor trafficking and gating. Proc Natl Acad Sci U S A. 107(37):16315-9.

Shinosaki H and Konishi S (1970) Actions of several anthelmintics and insecticides on rat cortical neurones. Brain Res.,24, 368-37 1.

Shinosaki, H and Shibuya I (1974) Potentiation of glutamate-induced depolarization by kainic acid in the crayfish opener muscle. Neuropharmacology 13, 1057-1065.

Shipston MJ (2011) Ion channel regulation by protein palmitoylation. J Biol Chem. 286: 8709–8716.

Sierra AY, Gratacós E, Carrasco P, Clotet J, Ureña J, Serra D, Asins G, Hegardt FG, Casals N (2008) CPT1c is localized in endoplasmic reticulum of neurons and has carnitine palmitoyltransferase activity. J Biol Chem. 283(11):6878-85.

Sladeczek F, Pin JP, Recasens M, Bockaert J, Weiss S (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. Nature 317: 717–719.

Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Köhler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science 249:1580–1585.

Sommer B, Köhler M, Sprengel R, Seeburg PH (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. Cell 67(1):11-9.

Song I, Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. Trends Neurosci. Nov;25(11):578-88.

Soto D, Coombs ID, Kelly L, Farrant M, Cull-Candy SG (2007) Stargazin attenuates intracellular polyamine block of calcium-permeable AMPA receptors. Nat Neurosci.; 10(10):1260-7.

Soto D, Coombs ID, Renzi M, Zonouzi M, Farrant M, Cull-Candy SG (2009) Selective regulation of long-form calcium-permeable AMPA receptors by an atypical TARP, gamma-5 Nat Neurosci. 12(3):277-85.

Soto D, Altafaj X, Sindreu C, Bayés A (2014a) Glutamate receptor mutations in psychiatric and neurodevelopmental disorders. Commun Integr Biol. 7: e27887.

Soto D, Coombs ID, Gratacòs-Batlle E, Farrant M, Cull-Candy SG (2014b) Molecular mechanisms contributing to TARP regulation of channel conductance and polyamine block of calcium-permeable AMPA receptors. J Neurosci. 27; 34(35):11673-83.

Srivastava S, Ziff EB (1999) ABP: a novel AMPA receptor binding protein. Ann N Y Acad Sci. 868:561-4.

Stern JR, Eggleston LV, Hems R, Krebs HA (1949) Accumulation of glutamic acid in isolated brain tissue. Biochem. J. 410-418.

Stern-Bach Y, Bettler B, Hartley M, Sheppard PO, O'Hara PJ, Heinemann SF (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. Neuron 13(6):1345-57.

Straub C, Tomita S (2012) The regulation of glutamate receptor trafficking and function by TARPs and other transmembrane auxiliary subunits. Curr Opin Neurobiol. 22(3):488-95.

Sun Y, Olson R, Horning M, Armstrong N, Mayer M, Gouaux E (2002) Mechanism of glutamate receptor desensitization. Nature 417:245–253.

Suzuki E, Kessler M, Arai AC (2008) The fast kinetics of AMPA GluR3 receptors is selectively modulated by the TARPs gamma 4 and gamma 8. Mol Cell Neurosci. 38(1):117-23.

Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. J Neurosci. 17(1): 58-69.

Swanson ST, Foster DW, McGarry JD, Brown NF (1998) Roles of the N- and C-terminal domains of carnitine palmitoyltransferase I isoforms in malonyl-CoA sensitivity of the enzymes: insights from expression of chimaeric proteins and mutation of conserved histidine residues. Biochem J. 335 (Pt 3):513-9.

Takuma H, Kwak S, Yoshizawa T, Kanazawa I. (1999) Reduction of GluR2 RNA editing, a molecular change that increases calcium influx through AMPA receptors, selective in the spinal ventral gray of patients with amyotrophic lateral sclerosis. Ann Neurol. 46(6):806-815.

Tanaka H, Calderone A, Jover T, Grooms SY, Yokota H, Zukin RS, Bennett MV (2002) Ischemic preconditioning acts upstream of GluR2 down-regulation to afford neuroprotection in the hippocampal CA1. Proc Natl Acad Sci U S A. 99(4):2362-7.

Tang BL (2009) Neuronal protein trafficking associated with Alzheimer disease: from APP and BACE1 to glutamate receptors. Cell Adh Migr. 3(1): 118-28.

Tomita S, Chen L, Kawasaki Y, Petralia RS, Wenthold RJ, Nicoll RA, Bredt DS (2003) Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. J Cell Biol. 161(4): 805-16.

Tomita S, Fukata M, Nicoll RA, Bredt DS (2004) Dynamic interaction of stargazin-like TARPs with cycling AMPA receptors at synapses. Science. 303(5663): 1508-11.

Tomita S, Adesnik H, Sekiguchi M, Zhang W, Wada K, Howe JR, Nicoll RA, Bredt DS (2005) Stargazin modulates AMPA receptor gating and trafficking by distinct domains. Nature 435: 1052–1058.

Tomita S, Sekiguchi M, Wada K, Nicoll RA, Bredt DS (2006) Stargazin controls the pharmacology of AMPA receptor potentiators. Proc Natl Acad Sci U S A. 103(26): 10064-7.

Tsuzuki K, Lambolez B, Rossier J, Ozawa S (2001) Absolute quantification of AMPA receptor subunit mRNAs in single hippocampal neurons. J Neurochem. 77(6):1650-9.

Turetsky D, Garringer E, Patneau DK (2005) Stargazin modulates native AMPA receptor functional properties by two distinct mechanisms. J. Neurosci. 25:7438–7448

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R, Sibley D (2010) Glutamate receptor channels: Structure, regulation, and function. Pharmacol Rev. 62(3), 405–496.

Ulbrich MH, Isacoff EY (2008) Rules of engagement for NMDA receptor subunits. Proc Natl Acad Sci USA 105:14163–14168.

Uzunova G, Hollander E, Shepherd J (2014) The role of ionotropic glutamate receptors in childhood neurodevelopmental disorders: autism spectrum disorders and fragile x syndrome. Curr Neuropharmacol. 12(1):71-98.

Van Dolah DK, Mao LM, Shaffer C, Guo ML, Fibuch EE, Chu XP, Buch S, Wang JQ (2011) Reversible palmitoylation regulates surface stability of AMPA receptors in the nucleus accumbens in response to cocaine in vivo. Biol Psychiatry. 69(11):1035-42. doi: 10.1016/j.biopsych.2010.11.025.

Verdoorn TA. Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. Science 252, 1715–1718

Wang JQ, Arora A, Yang L, Parelkar NK, Zhang G, Liu X, Choe ES, Mao L (2005) Phosphorylation of AMPA receptors: mechanisms and synaptic plasticity. Mol Neurobiol. 32:237–249.

Wang R, Mellem JE, Jensen M, Brockie PJ, Walker CS, Hoerndli FJ, Hauth L, Madsen DM, Maricq AV (2012) The SOL-2/Neto auxiliary protein modulates the function of AMPA-subtype ionotropic glutamate receptors. Neuron 75(5):838-50.

Watkins JC (1981) Pharmacology of excitatory amino acid transmitters. Adv Biochem Psychopharmacol. 29:205.

Watkins E, Jane D (2006) The Glutamate Story. Brit J Pharmacol 47: S100-S108.

Wenthold RJ, Petralia RS, Blahos J II, Niedzielski AS (1996) Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. J Neurosci.16(6):1982-9.

Wilding TJ, Huettner JE (1996) Antagonist pharmacology of kainate- and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-preferring receptors. Mol Pharmacol. 49(3):540-6.

Williams K, Dawson VL, Romano C, Dichter MA and Molinoff PB (1990) Characterization of polyamines having agonist, antagonist, and inverse agonist effects at the polyamine recognition site of the NMDA receptor. Neuron 5:199–208.

Wiltgen BJ, Royle GA, Gray EE, Abdipranoto A, Thangthaeng N, Jacobs N, Saab F, Tonegawa S, Heinemann SF, O'Dell TJ, Fanselow MS, Vissel B (2010) A role for calcium-permeable AMPA receptors in synaptic plasticity and learning. PLoS One 5(9).

Wilson MT1, Keith CH (1998) Glutamate modulation of dendrite outgrowth: alterations in the distribution of dendritic microtubules. J Neurosci Res. 52(5):599-611.

Wisden W, Seeburg PH (1993) Mammalian ionotropic glutamate receptors. Curr Opin Neurobiol. 3(3):291-8.

Wolfgang MJ, Lane MD (2006) The role of hypothalamic malonyl-CoA in energy homeostasis. J. Biol. Chem. 281: 37265–37269.

Wolfgang MJ, Kurama T, Dai Y, Suwa A, Asaumi M, Matsumoto S, Cha S H, Shimokawa T, Lane M D (2006) The brain-specific carnitine palmitoyltransferase-1c regulates energy homeostasis. Proc. Natl. Acad. Sci. U. S. A. 103, 7282–7287.

Xia J, Zhang X, Staudinger J, Huganir RL (1999) Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22:179–187.

Yamada KA (1998) Modulating excitatory synaptic neurotransmission: potential treatment for neurological disease? Neurobiol Dis. 5(2):67-80.

Yang G, Xiong W, Kojic L, Cynader MS (2009) Subunit-selective palmitoylation regulates the intracellular trafficking of AMPA receptor. Eur J Neurosci. 30:35–46.

Ying Z, Babb TL, Comair YG, Bushey M, Touhalisky K (1998) Increased densities of AMPA GluR1 subunit proteins and presynaptic mossy fiber sprouting in the fascia dentata of human hippocampal epilepsy. Brain Res. 798:239–246.

Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, Kaiser KM, Köster HJ, Borchardt T, Worley P, Lübke J, Frotscher M, Kelly PH, Sommer B, Andersen P, Seeburg PH, Sakmann B (1999) Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science 284(5421):1805-11.

Zammit VA (1999) Carnitine acyltransferases: functional significance of subcellular distribution and membrane topology. Prog Lipid Res. 38(3):199-224.

Zarate CA Jr, Manji HK (2008) The role of AMPA receptor modulation in the treatment of neuropsychiatric diseases. Exp Neurol. 211(1):7-10.

Zaugg K, Yao Y, Reilly PT, Kannan K, Kiarash R, Mason J, Huang P, Sawyer SK, Fuerth B, Faubert B, Kalliomäki T, Elia A, Luo X, Nadeem V, Bungard D, Yalavarthi S, Growney JD, Wakeham A, Moolani Y, Silvester J, Ten AY, Bakker W, Tsuchihara K, Berger SL, Hill RP, Jones RG, Tsao M, Robinson MO, Thompson CB, Pan G, Mak TW (2011) Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress. Genes Dev.

Zaugg K, Yao Y, Reilly PT, Kannan K, Kiarash R, Mason J, Huang P, Sawyer SK, Fuerth B, Faubert B. et al., (2011) Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress. Genes & Develop 25:10; 1041–1051.

Zigmond, MJ, Bloom, FE, Landis, SC,Roberts, JL, Squire, LR (1999) Fundamental Neuroscience. London: Academic Press.