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Presynaptic release regulating metabotropic receptors: dimerization and receptor cross talk

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ABSTRACT

The Class C subfamily of the G protein coupled receptor (GPCR) family is one of the most important in the central nervous system (CNS), and includes the glutamatergic and GABAergic metabotropic receptors (mGlu and $GABA_B$). The mGlu receptors are fundamental in modulating the efficiency of chemical neurotransmission in the CNS and, as a consequence, they are also involved in several neurological and neurodegenerative disorders. Recently, dimerization of these receptors has become an important topic of investigation and it has been proposed to be crucial to physio-pathological processes in the CNS, as well as to the impact of therapeutics in patients.

In this thesis, I will discuss the basic properties of Class C GPCRs focusing on the first and second group of mGlu receptors (Group I and II) and their possible dimerization and/or cross talk with other receptors. I will focus on recent findings concerning these processes that have been mainly obtained by using purified isolated nerve endings (here referred to as synaptosomes), a subcellular preparation of choice for studying presynaptic release regulating receptors. Starting from the pharmacological characterization of Group I and II mGlu receptors, I will then discuss different examples of cross-talk linking these receptors to other ones (i.e. the $GABA_B$ and the $5\text{-}HT_{2A}$ receptors). I will also show results obtained using electrophysiology to study the role of these receptor subtypes in the modulation of synaptic transmission in hippocampal slices. The resulting picture is undoubtedly complex and highlights how the cross-talk and dimerization of these receptors represent a new frontier in neuropharmacological studies.

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ABBREVIATION

5-HT$_{2A}$ 5-hydroxy-tryptamine-2A receptor

7-TM 7-transmembrane domain

[^{3}H]D-ASP [^{3}H]-D-aspartate

A_o active orientation

AC adenyl cyclase

ACPD amino-cyclopentanedicarboxylic acid

ACPT-11S,3R,4S-1-aminocyclopentane-1,3,4-tricarboxylic acid

ADP adenosine disphosphate

AGS G protein signalling

APS1 autoimmune polyendocrine syndrome type 1

BBB blood brain barrier

BRET Bioluminescence Resonance Energy Transfer

c.f. final concentration

CA1 Cornu Ammonis-1

cAMP cyclic adenosine monophosphate

CaS Calcium-sensing receptor

CNS Central Nervous System

CRD Cysteine rich domain

Cs Close state

CSF cerebrospinal fluid

CXCR C-X-C chemokine receptor

DAG diacylglycerol

DG Dentate Gyrus

EDAM extracellular domain allosteric modulator

ER endoplasmatic reticulum

ERK-1/2 extracellular signal-regulated kinases

EVH-1 Enabled / Vasp homology-1

FRET Fluorescence Resonance Energy Transfer

GABA gamma-Aminobutyric acid

GDP Guanosine disphosphate

GFAP Glial Fibrillary Acidic Protein

GIRK G protein-gated inwardly rectifying potassium

GNAS

GPCR G Protein Coupled Receptor

GPRC6a G protein receptor Class C 6a

GTP Guanosine trisphospate

HPLC High Performance Liquid Chromatography

IMP inositol monophosphate

IP_3 inositol trisphosphate

MAP4 (S)-2-amini-2-methyl-4-phosphonobutanoic acid

MAPK mitogen-activated protein kinase

mGlu metabotropic glutamate receptor

MCPG amino-cyclopentanedicarboxylic acid

MHC melatonin-concentrating hormone

MPEP 2-methyl-6-(phenylethynyl) pyridine

MRI Magnetic Resonance Imaging

NAAG N-Acetylaspartylglutamic acid

NAM negative allosteric modulator

NF-κB nuclear factor- κB

NMDA N-metil-D-aspartate

Os open state

PAM positive allosteric modulator

PDZ post synaptic density protein (PSD95)/Drosophila disc large tumor suppressor (Dlg1) / zonula occludens-1 protein (zo-1).

PKC protein kinase C

PLC phospholipase C

PTx *Bordetella pertussis* toxin

R_o resting orientation

RAIG retinoic acid induced receptor

RAP1GAP Ras-proximate-1 GTPase activating protein

Rho-GEF Ras homologous protein - GDP/GTP exchange factor

RNA ribonucleic acid

TAS taste receptor

TBS Tris-Buffered Saline

TNFα Tumor Necrosis Factor α

TR taste receptor

VFT Venus Fly Trap

VIP-PACAP vasoactive intestinal peptide – Pituitary adenylate cyclase-activating polypeptide

vs versus

1 INTRODUCTION

1.1 G Protein-Coupled Receptors: the Class C

1.1.1 General considerations

While discussing on cell communication, signal mechanisms or neurotransmission in the organisms, we cannot ignore the centrality of one of the mostly recognized super family of proteins: the G protein coupled receptor (GPCR) family. GPCRs are among the ancient machines involved in the signal transduction in vertebrates and plants. They are typified by a widespread distribution, different characteristics and peculiar structural conformations. Owing to give an overall definition of the function of these proteins, I could mention these words written by Joel Bockaert:

"GPCRs are involved in the recognition and transduction of messages as diverse as light, Ca^{2+}, odorants, small molecules including amino-acid residues, nucleotides and peptides, as well as proteins. They control the activity of enzymes, ion channels and transport of vesicles via the catalysis of the GDP–GTP exchange on heterotrimeric G proteins (Gα–βγ)" (Bockaert J. and Philippe Pin J., 1999).

These few words unveil the greatness and the complexity of the subject. Despite the importance and the diffusion of GPCR proteins in the organisms, the research has been underdeveloped for many years because of technical limitation. For instance, until 2007, only few crystal structures were available, like bovine rhodopsin receptors, which limited the possibility to approach functional and pharmacological studies with computational analysis. This limitation has been partly overcome in the last decade opening the road to studies concerning their structures and conformations. Thousand of papers were then published about the ability of these receptors to control and modulate

signal mechanisms, and it is nowadays recognized their relevance as targets for drugs. Indeed, more than 30 % of drugs on the actual pharamcautical market has the GPCRs proteins as target.

Studies about the amino acid sequence allowed to classify the GPCR proteins and to further subclassify them in subfamilies. The first classification divided GPCRs in three main families:

- The first family contains most of the so far known GPCRs and it is further subdivided in three different groups: receptors for odorants and rhodopsin (Group A), peptides (Group B) and glycoprotein hormones (Group C).
- The second family contains GPCRs recognizing hormones like glucagon and secretine (the weight control hormones), different toxins (like Latrotoxin) and the VIP-PACAP complex. Curiously, the receptors belonging to this family have a morphology comparable to that of the subgroup C of the first family.
- The third family consists of the metabotropic glutamate and GABA receptors, in addition to the Ca^{2+} sensing receptors. This is a very important family for the neurotransmission in the CNS and represent the topic of this work.

Beside these families, three other minor families of GPCR proteins have been hypothesized to exist: the receptors for pheromones, the cAMP receptors (expressed only in some organisms) and the frizzled/smoothened receptors. Although these GPCRs share poor or no sequence similarity one each other, they belong to a common superfamily, the GPCR one, that would suggest a molecular convergence of the protein evolution to a common organization.

Starting from 2003, a new classification system was proposed based on crystal structures and human genome sequence (Fredriksson R. et al., 2003). According to this classification (Vsevolod K. et al., 2012), human GPCRs are divided in :

1. the GPCR Rhodopsin family (the so called Class A), which is subdivided in α, β, γ and δ groups;
2. the Secretin and Adhesion family or Class B;
3. the Glutamate family or Class C;
4. the Frizzled / TAS2 family.

The study I carried out during my PhD training focused on the functional and pharmacological characterization of GPCRs belonging to the Class C and specifically to the metabotropic glutamate receptors (mGlu) and the $GABA_B$ receptors.

1.1.2 Class C GPCR

The so called Class C or third family of GPCR proteins assumed an important role in the study of the control of chemical neurotransmission and, more in general, of CNS plasticity during the last decades. This class consist of metabotropic glutamate receptors (mGlu), GABAergic receptors ($GABA_B$), Ca^{2+} ions receptors (CaS), sweet and umami tastes receptors (T1Rs), basic amino acids receptors (GPRC6a) and some orphan receptors (Kniazeff J. et al., 2011).

Despite the difference in amino acid sequences, function and conformation, these receptors share a common structural feature, defined by the large extracellular domain, which is in part expressed also in bacterial periplasmic binding proteins and in some ligand-gated ion channels.

The main members of the third family of GPCRs and the first to be cloned were the metabotropic glutamate receptors (Masu et al., 1991). The term "metabotropic glutamate receptor" was firstly used by professor Sugyjama to indicate a quisqualate-sensitive receptors expressed in Xenopus oocytes injected with rat brain RNA (Sugyjama et al., 1987). In the history of this group, the mGlu1 protein was the first crystal structure of the extracellular domain described, which gave important advances in understanding the Class C proteins. Soon after, Muto and collaborators reported the structures of the mGlu3 and the mGlu7 receptor proteins (Muto et al., 2007).

The group of metabotropic glutamate (mGlu) receptors is activated by glutamate, the major excitatory neurotransmitter in the CNS. They participate to the synaptic transmission and to the integration of the information, beyond the cell excitability and signal modulation. This explain why they are so extensively studied and they are thought to be involved in almost all the neurological impairments. Today, the mGlu receptors have been subdivided into three different groups based on their sequence, localization and signal transduction:

1. The first mGlu group is composed by the mGlu1 and mGlu5 receptors. They are mainly coupled to Gαq proteins and they were firstly localized in the post synaptic compartment. They are also functionally present in the presynaptic elements (Moroni et al.,1998; Pittaluga, 2016) and in non neuronal cells (Biber K et al., 1999).
2. The second group refers to the mGlu2 and the mGlu3 receptors and, opposite to the first group, they couple to Gαi/o proteins. They are widely expressed in the CNS, at both the pre and the post synaptic compartments as well as in astrocytes/glia cells (Bruno V et al., 2001).
3. The third group consists of the mGlu4, the mGlu6, the mGlu7 and the mGlu8 receptor subtypes. As for the previous group, they couple to Gαi/o proteins and they are expressed in the nerve terminals where they contribute to the modulation of glutamate transmitter release. The mGlu6 receptors are expressed only in Bipolar cells of the retina (for the third group and previous groups see also the review of Conn & Pin, 1997; Cartmell and Schoepp, 2000 and Niswender & Conn, 2010).

The second actor of the Class C GPCRs correlates to the central inhibitory transmission. It is the $GABA_B$ receptor, which is activated by GABA, the major inhibitory neurotransmitter of the mature CNS. Most of the brain regions but also some peripheral areas express the $GABA_B$ receptors at both the pre and the post synaptic component of chemical synapse where they control neurotransmitter exocytosis and regulate the excitatory post-synaptic currents (Bettler & Tiao, 2006). Altered $GABA_B$ functions are involved in different kind of diseases and neurological disorders like depression, addiction and autism representing, therefore, a very interesting target for drugs. Even if the structure will be deeply analysed in the next chapters, I would like to remember that these receptors are obligatory heterodimers composed by the ligand binding $GABA_{B1}$

protein and the G protein coupling $GABA_{B2}$ protein, which is the main feature of the $GABA_B$ receptors (Kniazeff J. et al., 2004).

One of the most important ion for the homeostasis in the organism is calcium that, beyond its excitatory functions, is the ligand of the Calcium-sensing receptors (CaS). This group of receptors can be also modulated by different amino acids, polyamines and other divalent cations (Riccardi et al., 2009). They mainly locate in the periphery at the parathyroid gland where they participate to the control of Ca^{2+} metabolism, which is fundamental at least for kidney and bone. Mutations at these receptors are associated with the development of abnormal calcemic condition. In addition, CaS receptors have been found in the CNS where they were proposed to take part into the process of nerve outgrowth. Interestingly, these receptors can switch between the "on" and the "off" states with a very fine and high sensitivity (Riccardi et al., 2009).

The Class C GPCR proteins also includes the umami and the sweet taste receptors, two groups of the family of the taste receptors. They are localized in the taste buds and they are displayed as heterodimeric receptors (T1R2 and T1R3 for sweet receptors, T1R1 and T1R3 for umami receptors) (Nelson et al., 2002). Sweet receptors are linked to the Gα-transducin for the transduction signal pathway and they do not only respond to sweet ligands but they are also modulated by sweetener, sweet protein and some D-amino-acids, which affinity is reported to change between species. The umami receptors are commonly associated to the Japanese and oriental foods. They are stimulated by L-amino acids but, only in the human phenotype, they are activated by sodium glutamate and L-aspartate. They are also potentiated by inositol monophosphate (IMP), guanosil monophosphate (GMP) and adenosil monophospate (AMP) molecules. Undoubtedly, these receptors have great interest in the field of the food and sweetener industry (Chandrashekar et al., 2006).

The last known member of this Class C is the GPRC6A receptor, which has been only recently described. In fact some recent studies proposed that the receptor activation is triggered by several basic L-amino-acid like arginine, lysine or ornithine. The structure of this receptor shares some homology with the CaS and the mGlu receptors and they usually form homodimers linked by disulphide bridge. The GPRC6A is expressed in different area of the organism such as in kidney, taste buds and CNS. We still do not know exactly the function of this receptor but some studies proposed its involvement in the modulation of nutrient-dependant process (Wellendorph et al., 2009).

Finally, some orphan receptors have been correlated to the Class C GPCRs. Specifically, the retinoic acid-induced (RAIG) and the GPR156/158/179 receptors. Briefly, the first one is particularly expressed in the retina while the second group has structure comparable to that of the $GABA_B$ receptors. Both receptors lack the extracellular domain and, consequently, they would be expected to have particular ligand-mediated mechanism of activation (Kniazeff et al., 2011).

In order to better achieve our aim, in this thesis, when I will talk about Class C, I will refer mainly to the mGlu receptors and the $GABA_B$ receptors, which are also the most known receptors of this class.

1.1.3 Structure and function

The organization and classification of the GPCR proteins rely on the proteins structures that strictly correlate to the mechanism of activation of the receptors and to the coupled mechanisms of transduction. X-crystallography or FRET techniques were pivotal to the description of GPCRs structure, as demonstrated by many studies performed in the last three decades.

Beyond the specific families and subfamilies, GPCR proteins share a general core structure composed of a N-terminal domain and a central core domain characterized by seven transmembrane helices (7-TM), linked by three intracellular and three extracellular loops and a C-terminal domain (Baldwin, 1993). The most of these GPCRs conserved a disulfide link made by two cysteine residues in the first and the third extracellular loops, fundamental for the stabilization and the organization of the typical conformation of the seven 7-TMs. Differences in the amino acid sequence affect the length, the function and the structural organization of the N-terminal extracellular domain as well as of the C-terminal intracellular domain and the intracellular loops.

Among all domains, the 7-TM domain represents the core domain of GPCR proteins, and its structure was mainly characterized with crystallization techniques. Specifically, based on the results obtained from these studies, we learned the crucial involvement of the 7-TM domain in the modulation and in the activation of these receptors. However, this domain as well as the other domains show some differences among the various families and subfamilies, carrying out some peculiarities in the GPCRs functions (Spengler et al., 1993; Bockaert J and Pin P.L., 1999).

The third family or Class C GPCRs is typified by the presence of a large extracellular domain characterized by two lobes separated by a “hinge region”. This domain is the so called Venus Fly Trap (VFT) domain. Another common feature of the Class C of GPCRs is the large transmembrane core composed of seven helices (7-TM) domain

linked to both N-terminal and C-terminal tail. The two main domains, the VFT and the 7-TM domains, communicate one to each other via an enriched cysteine domain (CRD), that is a rigid module characterized by 9 highly conserved cysteine residues. Further, CRD and VFT domains are stabilized by a conserved and rigid disulfide bond. The function of this enriched cysteine *"communicative bridge"* or CRD domain is associated to the communication of the conformational changes between the "*sensorial domain*" or VFT domain and "*transmitter domain*" or 7-TM domain (Kniazeff et al., 2011).

Sensorial domain or VFT domain

The large extracellular domain or the VFT domain is involved in the orthosteric binding momentum of the receptor, hence the name "*sensorial domain*". First studies on this domain begun with the description of the presence of VFT in bacterial periplasmic protein, where these receptor proteins are involved in the transport of various molecules (Bockaert J and Pin JP, 1999).

During the last 30 years, the study of GPCRs has become more and more important in the CNS field. Specifically, the most spread excitatory neurotransmitter, glutamate, and the central inhibitory neurotransmitter, GABA, played the role of main actors in most of these studies. This explained why most of the structural analysis of the Class C GPCRs were performed to describe the mGlu and the $GABA_B$ receptors. On the basis of the results from the X-ray crystallography, the metabotropic glutamate receptor type 1 (mGlu1) was reported to express the VFT domain. Soon later it was demonstrated that the presence of this domain is not restricted to the mGlu1 but that also other mGlu receptors possess it (Kunishima et al.,2000; Muto et al., 2007), so that this domain was proposed as a characteristic domain of the Class C GPCRs proteins.

The VFT domain is characterized by two lobes composed by α-helices around a large β-sheet in the metabotropic glutamate receptors, with the glutamate binding site in the cleft between the two lobes (Muto et al., 2007). This structure was demonstrated to be conserved in almost all the other receptors belonging to this class, underling a general convergence at this level in this family.

The structure VFT domain plays a central role in the protein because it is associated to the orthosteric binding site. In other words, changes in conformation of this domain lead to the first steps of the activation of Class C GPCRs by orthosteric agonists. Consequently, it became evident the link between the VFT domain characterization and the design of possible new orthosteric compounds acting at this level (Pin J.P. et al., 2004).

The study of the bacterial periplasmatic structures gave the firsts insights about the possible conformations of the VFT region. The domain can adopt two major state, an open state (Os) and a closed state (Cs). The Cs is stabilized by the ligand binding in the crevice located in a specific region of the lobe (Quiocho, 1990). Specifically, the orthosteric agonist binds the VFT adopting the Os and the occupancy of the binding site leads to the closure of lobe-II through specifically interactions. The structural modifications that follow the binding of the agonist stabilize the receptor protein in the close state (Cs). Orthosteric antagonists mainly act by preventing these interactions leading to a block of the conformational changes of the 7-TM domain.

These conformational changes underline the strict correlation linking the ligand profile and the VFT sequence. For instance, genetic alterations that impede the adoption of the Cs of the VFT sequence convert the antagonist-like interaction of (S)-2-amini-2-methyl-4-phosphonobutanoic acid (AP-4), a well known mGlu3 antagonist, and of 1S,3R,4S-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-1) another mGlu 3 antagonist, into full orthosteric agonists (Bessis et al., 2002). In addition, first studies on the three-

dimensional structure adopted by the mGlu1 receptor showed that the receptor can adopt the Os despite the absence of glutamate and the Cs conformation in the presence of glutamate (Kunishima et al., 2000). Although it is not the matter of discussion of this section, it should be taken in consideration that the mGlu and most of the Class C receptors exist in the dimeric form and that this form is pivotal to assure the activation of the receptors. The mGlu1 receptor was the first Class C receptor to display a "constitutive activity". This refers to the possibility to find the receptor in the active state even in the absence of the agonist (Kniazeff et al., 2004). Paralleled to the studies performed in the glutamatergic system, other analysis performed in the GABAergic system confirmed the same general mechanism for the VFT domain, showing also that the Cs is sufficient for the activation of the receptors.

The data so far available demonstrated that the binding of the agonist to the VFT region of the receptor leads to a large rearrangement of the conformation. Specifically, it has been suggested the presence of two general orientations adopted by the VFT domain: the resting orientation (here named R_o) and the active orientation (here named A_o). These two orientations are normally in an equilibrium state. In the R_o orientation, the lobe-I linked to the VFT is separated from the lobe-II while in the A_o orientation, the two lobes interact one each other, because of the reorganization of the conformational state. It has been proposed that this reorientation bridges the binding of the orthosteric ligand with the mechanism of activation of the 7-TM domain.

Recently, new advances in this field lead to propose the existence of six different possible orientations, here reported as: $R_{o/o}$, $R_{o/c}$, $R_{c/c}$ and $A_{o/o}$, $A_{o/c}$, $A_{c/c}$. It is assumed that when the orthosteric agonist binds the cleft of the lobe-I forces the VFT dimer to pass through the $R_{o/o}$, $R_{o/c}$, to the $A_{o/c}$, $A_{c/c}$ states, causing a large reorientation. In this complex picture, it has been already demonstrated the key role of lobe-II interaction. For instance, blocking the VFT dimer by for example N-glycosylation at the surface of

lobe-II result of the loss of the G-protein transduction signal in the $GABA_B$ receptors (Rondard et al., 2008). Also, these studies suggest that a large conformation changes through lobe-II is necessarily request to the G protein activation (Kniazeff et al., 2011). However, the exact orientation/conformation mechanisms are still not completely clear. For these reasons it is essential to continue to investigate about these structures in order to completely elucidate the activation mechanism of these receptors.

Transmitter domain or 7-TM domain

All the families of the GCPRs, including the Class C, share a general common component: the 7-TM core domain. It physically connects the extracellular domain with the intracellular one, acting as the "*Transmitter domain"* within the receptor protein. In other words, it links the "*sensorial domain*" to the associated G protein which activates intracellular second messenger cascade. The 7-TM domains are typified by the presence of a central helix 3 and an amphipathic helix 8 (Pin et al, 2003). In 2003 Pin and colleagues showed that the intracellular loops 2 and 3 as well as the helix 8 are fundamental for the G-protein coupling. There are several other peculiarities relevant to the 7-TM functions which are shared in this Class C of GPCRs proteins. First, the ionic lock between the conserved arginine (3.50) in TM3 and the conserved glutamate (3.60) in TM6 leads to the mechanism of activation. Second, there is a common conformational change which involves a conserved tyrosine (6.48) in TM7. Third, the so called "toggle switch" interests the rotamer conformation of the conserved tryptophan (6.48) in TM6. All these specific sequence similarities underline a main feature already observed for other domains: Class C receptors share common general regions in the mechanism of action and transduction signal in the 7-TM region (see Pin et al., 2003; Hofmann et al., 2009; Rosenbaum et al., 2009).

Another important step in the characterization of this domain is the discovery of the binding pockets located in the 7-TM. A very large family of compounds was drowned, called positive/negative allosteric modulators, that binds this pocket and that will be deepened in the next chapters.

The crucial role of the 7-TM domain in controlling the function of these receptors has been already described by different kind of experiments and works. For instance, the VFT and CRD domains were deleted and the C-terminal truncated in these experiments. This "minimalistic" version of the original receptor can be completely activated and inhibited by molecules acting at its 7-TM domain. In this case, these allosteric modulators perform the role of the full agonist/antagonist (Binet et al., 2004; Goudet et al., 2004). Indirectly, these experiments and the role of 7-TM domain were confirmed by some orphan receptors of Class C like RAG1 receptor, which presents only the 7-TM region.

Communicative bridge or CRD domain

The studies focused on the VFT and 7-TM domains and their dimeric activation unveiled another important region that allows the transduction of the signal from the "*sensorial domain"*(VFT) to the "*transmitter domain"*(7-TM).

This region is named rich-cysteine domain (CRD). As described in literature, this domain connects the VFT region and the 7-TM region and it is characterized by a highly conserved sequence consisting of 9 cysteine amino acids. Except for the $GABA_B$ receptors, this domain was found in the mGlu receptors, CaS receptors, T1R receptors and GPRC6A receptors. The structure was firstly elucidated after crystallography analysis of the mGlu3 structure (Muto et al., 2007). The domain is characterized by a rigid 40 Å long structure that acts like a fundamental gear between the "*sensorial domain"*(VFT) and the "*transmitter domain"*(7-TM). Experiments carried out in

different group of Class C receptors confirmed its important role as a "*communicative bridge"*. Either the deletion or the mutation of the CRD sequence abolished the orthosteric agonist-induced mechanism of activation (Hu et al., 2000; Jiang et al., 2004). In this gear, the specific disulphide covalent bond which links the CRD domain to the VFT core seems to play a key role. Studies focussing on the deletion of the covalent bond in this region showed the silencing of the receptor functions despite the binding of the agonist to the respective site (Kniazeff et al., 2011).

To resume, the binding of the orthosteric agonist causes changes in the orientation of the VFT domain, that in turn brings the lobe-I and the lobe-II closer one to each other. Subsequently, the respectively CRD domains, which are linked by a covalent bond to the VFT domains, move closer one to each other to complete the activation process. The result of this cascade of events is the continue transition of the "information" from the "*sensorial domain"*(VFT)" to the "*transmitter domain"*(7-TM) by means of the "*communicative bridge"*(CRD). To further investigate this process of activation, Rondard and his colleagues performed "engineered experiments" to modify the cysteine bond sequence. Their approach suggested that locking CRD dimer in specific conformation mimics the active state of the receptor, even in the absence of the agonist (Rondard et al., 2010).

These overall results confirmed: i) the onset of changes in the relative conformation through the involvement of the CRD domains; ii) that CRD modification and specifically the disruption of the covalent bond blocks the agonist mediated activation mechanism; iii) that CRD bridges the VFT domain and 7-TM domain.

Some words must be spent about the case of $GABA_B$ receptors due it is peculiar structure. This receptor does not show the typical CRD domain. In this context, we should take in mind the obligatory heterodimeric nature of $GABA_B$ receptors, composed by the two subunits, the $GABA_{B1}$ and the $GABA_{B2}$ receptors. In literature, it has been

suggested that the mechanism of activation for these receptors is mediated by a direct communication between the VFT domain and 7-TM domain. Specifically, two activation ways were proposed to take place:

i) a *trans*-activation based on the occupancy of the orthosteric agonist binding site and thus the VFT closure of $GABA_{B1}$ that would directly change the conformation of 7-TM of $GABA_{B1}$ in turn promoting the G protein activation by 7-TM of the $GABA_{B2}$ subunit.

ii) the *cis*-activation based on the closure of the VFT of the $GABA_{B1}$ receptor subunit by an orthosteric agonist which induces a conformational change of the VFT of the $GABA_{B2}$ subunit and consequently of the 7-TM domain of the $GABA_{B2}$ receptor (Monnier et al., 2010).

1.1.4 Ligands acting at Class C GPCR

In general, Class C GPCRs show a well-defined structure mainly represented by the VFT and 7-TM domains which are linked through the CRD domain, with some exceptions like the lack of the CRD domain in the $GABA_B$ receptors group. This structure accounts for the existence of different binding sites for the ligands (Rondard et al., 2011). Therefore, the Class C receptors display a potential variety for the development of drugs which can modulate receptor activity in different ways, depending on the sites and on the mechanisms of interaction. In theory, these drugs can bring many beneficial effects in different context: from the neurological to the psychiatric disorders, from hormonal to the calcemic diseases, to improve the food physiology and to face the obesity (Brauner-Osborne et al., 2007). Nonetheless, few drugs belonging to this group have been so far approved for the use in therapy. Some important examples are Baclofen and Cinacalcet in addition to the group of sweet and umani enhancer/modulator compounds.

In this chapter I am going to describe the general mechanism of action of the ligands with respective receptors. The aim is to introduce general concepts that are fundamental to understand the physiological and pathological consequences due to different sites of modulation of the Class C GPCRs. In other words, I am going to introduce ligand acting at VFT domain, at the 7-TM domain and other possible sites. Attention will be also dedicated to the new possible pharmacological tools like antibodies, to recombinant nanobodies and to "extracellular domain allosteric modulators" or EDAMs. Refer to the authors mentioned during the discussion for further information.

Ligands acting at VFT domain

As described in the previous section, endogenous agonists of Class C GPCRs bind in the VFT cleft, stabilizing its close state. This site of action is the so called "orthosteric"

binding site. Orthosteric agonists induce the VFT domain closure which is the first step in the mechanism of receptor activation. Conversely, the orthosteric or competitive antagonists stabilize the VFT domain in the open state, preventing the action of the agonists. In addition, compounds acting as "partial agonist" are also overtaken. The term "partial" refers to the capability of these molecules to give a partial closure of the VFT or, alternatively, to cause a less relevant stabilization of the close state when compared to the full agonists (Kunishima et al., 2000; Tsuchiya et al. 2002; Bessis et al., 2003; Kniazeff et al., 2004). The first receptor to be studied for its structural conformation was the mGlu1 receptor. These studies demonstrated that the receptor exists in the open conformation in the absence of glutamate and in the close conformation in the presence of glutamate (Kunishima et al., 2000). Another important demonstration came from the studies on the consequences elicited by the mutagenesis of the crevice of the VFT of mGlu3 receptors, that converts full antagonists like MAP4 and ACPT-II in full agonists (Bessis et al., 2003). These and other researches in literature investigated also how endogenous and/or exogenous synthetic compounds can bind with specific interaction in the VFT pocket.

As far as the mGlu and $GABA_B$ receptors are concerned, L-glutamate and γ-aminobutyric acid (GABA) are the endogenous agonists of these groups of receptors respectively. These amino acids interact with the orthosteric binding sites thanks to the presence of five residues in the VFT cleft that are involved in the binding process of the α-amino and α-carboxy group of these amino acids (Acher and Bertrand, 2005). In the same manner, other specific amino acids can act as ligand of other types of Class C receptors like CaS, T1R1, T1R3 and GPRC6 receptors.

The mGlu receptors are divided in three different subgroups (I, II and III) and consist of eight different receptor subtypes. Glutamate is the endogenous orthosteric agonist for all the metabotropic glutamate receptors and binds the highly conserved binding pocket.

The efficacy of glutamate to act as a full agonist at all the eight receptor subtypes indirectly indicates that the eight receptors are typified by an important sequence homology. The first challenge to pharmacologically characterize these receptors was to find compounds able to modulate mGlu receptors but devoid of affinity for the ionotropic glutamate receptors. In this sense, the first pharmacological breakout was the discovery of the trans-1-amino-cyclopentanedicarboxylic acid (trans-ACPD), a selective agonist of mGlu receptors with no activity at ionotropic glutamate receptors (Schoepp et al., 1999). Starting from this first molecule, other compounds were derived and typified by a different range of action towards the different mGlu subtypes (Cartmell and Schoepp, 2000). It was the case of the first selective competitive antagonist: the R,S,-α-methyl-4-carboxyphenylglycine (MCPG) (Birse et al 1993; Eaton et al., 1993). More recently, the impressive work by Jim Monn, David McKinzie and colleagues at Eli Lilly industries gave us an important pool of selective compounds for the mGlu2/3 receptors with nanomolar potencies. Here, I want just report two of the progenitors of this huge family: the (1S,2S,5R,6S) aminobicyclo [3.1.0] hexane-2,6-dicarboxylic acid (LY354740) and the (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) (see also the review of Schoepp et al., 1999). Interestingly, this example gives us the idea of the prolonged and huge involvement of the private sector in the study of these receptors.

A comparable story about the pharmacological design and creation of new compounds concerns the orthosteric agonists and antagonists at $GABA_B$ receptors. The story began from the fundamental study of the group led by Norman Bowery, which underlined the difference with the $GABA_A$ ion channel receptors and unveiled the $GABA_B$ involvement in many physiological processes (see next sections and Bowery et al., 1999; Fan Q.R. and Frangaj A., 2018). An important discovery for the study of $GABA_B$

receptors pharmacodynamics was the introduction of Baclofen, which still represents one of the most famous examples of GPCR drug approved for therapy.

The huge amount of studies focusing on these receptors gave also important insights about the receptor mechanisms and, as far as the $GABA_B$ receptors are concerned, at least two main characteristics should be taken into consideration.

Firstly, the competitive antagonists bind specific sites at the $GABA_B$ receptors by blocking the 7-TM sequence in the inactive state rather than directly interacting with the VFT domain (Grünewald et al., 2002). This is the reason why they are not defined "orthosteric" ligand, but rather "inverse" agonists.

Secondly, Ca^{2+} ions can modulate $GABA_B$ receptors as well as other Class C receptors. Specifically, it has been demonstrated that the affinity of agonists at some Class C GPCRs is increased by the presence of Ca^{2+}. It is the case for the GPCR6 receptors and the $GABA_B$ receptors, where the change in the affinity of some agonists for the GABA receptors elicited by Ca^{2+} is due to the interaction of agonist with a serine 269 that follows the binding of GABA at the $GABA_{B1}$ subunit (Galvez et al., 2000). Despite these first observations, the ionic modulation of $GABA_B$ receptors appear more complex and not completely disserted. In fact it has been demonstrated that some agonists like Baclofen as well as some antagonists are insensitive to the presence of Ca^{2+}.

Although they are not the subjects of this work, some words must be spent also for ligands acting at the VFT domain in the other types of Class C receptors:

- The sweet and umami taste receptors, T1R, show a impressive range of possible orthosteric agonists. These receptors are activated and modulated by i) sweet-tasting D-amino acids which a similar mechanism of other receptors. ii) sugars including glucose, fructose or sucrose present in food. The possibility that also carbohydrates can act as ligand is reported as unique characteristic in the Class C GPCRs, which is proposed to

be associated with a large crevice of the binding domain and specific interaction(s) in the hinge region of VFT. iii) protein detected in food, which mechanism of interaction is not completely understood, but it seems to be associate to the so called "sweet fingers" of these receptors. The target of the sweet and umami receptors is of interest in the food industry research, as the well known examples of some artificial sweetener like acelsulfame and aspartame that can act as agonists at these receptors (for more information see Nelson et al., 2001; Morini et al.,2005; Assadi-Porter et al., 2010).

- CaS receptors which are the only Class C receptors which can be activated by ions, in particular Ca^{2+}, without the presence of other ligands. Ca^{2+} binds the VFT cleft but also interacts with the 7-TM. As already said, these receptors are activated by a really narrow range of calcium concentration, which is fundamental in calcemic homeostasis (Ray et al., 2005).
- GPCR6 receptors which recognize basic L-amino acids like arginine, ornithine or lysine and to lesser extent other small amino acids like alanine and glycine. They are also modulated by divalent cations like Ca^{2+} and Mg^{2+}(Pi et al., 2005; Wellendorph et al., 2009).

Recently, it has been shown that closed to the VFT binding domain there is a small site where molecules can interact in order to modulate the affinity of the agonists. This new class of small molecules is called EDAM. In this context, first studies were performed on IMP and GMP which can interact with T1R receptors as extracellular allosteric modulators. In metabotropic glutamate receptors, the EDAM compounds can interact also with adjacent binding pocket which is differently built in the eight subtypes in contrast to the major VFT cleft. Therefore, EDAMs belong to a new important and selective class of compounds which may represent innovative devices to have a narrow modulation of the Class C GPCRs proteins.

Ligands acting at 7-TM domain

One of the first discoveries in studying the mGlu receptors is the possibility that they can reach the active state even in the absence of the agonist. This possibility was firstly showed for the Group I mGlu receptors. These receptors displayed a constitutive activity, firstly demonstrated by the high basal IP formation measured in cells expressing these receptors compared to mock-transfected cells (Joly et al., 1995; Prézeau et al., 1996). The constitutive activity was insensitive to the action of competitive antagonist which were associated to the prevention of the closure of VFT. This finding rose up the definition and characterization of a new class of compounds: the so called "inverse agonists" which prevent such constitutive activity (Lefkowitz et al., 1993).

This class of compound has been demonstrated to interact with a specific pocket in the heptahelical 7-TM domain. Interestingly, the mechanism remembers what happens when we use antagonists for $GABA_B$ receptors. Several studies from Pin J.P. and colleagues (2004, 2011) elucidated the mechanism of activation, which also indirectly confirms the possibility that the 7-TM domain can reach the active state even in the absence of ligands at the orthosteric binding site. In addition, these studies opened the discussion about the real mechanism of action of several synthetic compounds considered orthosteric antagonists. In fact, it was demonstrated that some of them interact with the 7-TM domain and, for this reason, they should be associated to the inverse agonists class. As far as the mGlu receptors are concerned, this is the case of 2-methyl-6-(phenylethynyl) pyridine (MPEP), a mGlu5 selective antagonist (Pagano et al., 2000). Finally, it has been already demonstrated that the constitutive activity has important physiological consequences, especially in the cerebellum, as the Group I metabotropic glutamate receptors is concerned. However, the closure of the VFT

domain by this event still represents the necessary condition to reach the full activity of the receptor.

An important step in the study of Class C GPCRs and, in particular, of the 7-TM domain is the identification of a crevice within the 7-TM. This binding pocket can be a target for synthetic compounds studied for these receptors. This crevice is located between TM3, 5, 6 and 7 (Pagano et al., 2000; Pin et al., 2003; Brauner-Osborne et al., 2007; Conn et al., 2009), and in some cases it is typified by two distinct sites, as for example mGlu5 receptors (Chen et al., 2008). Compounds acting at this level are called allosteric modulators and, specifically, they are referred to as positive allosteric modulator or PAM if they enhance agonist activation and they are referred to as negative allosteric modulator or NAM if they reduce the agonist activity. PAMs facilitate the activation mediated by the binding of orthosteric agonists which results in an increase of the receptors and G protein coupled activation. NAMs block the activation of G proteins by stabilizing the inactive form of 7-TM (Rondard et al., 2011). Based on their mechanism of action, PAMs and NAMs possess high therapeutical potential because they can show a high selectivity on subtype receptors as well as they can modulate the endogenous activity of the receptors without forcing a constant activation or block of the receptors (Kniazeff et al., 2011). Cinacalcet, a PAM of CaS receptors, is a drug already approved and a good example of this class (Tfelt-Hansen and Brown, 2005).

Interestingly, endogenous PAMs or NAMs that can bind the 7-TM pocket were not described so far (for the discussion see Kniazeff et al., 2011). On the other side, PAMs and NAMs do not show necessarily a completely overlapping in the binding target (Miedlich et al., 2004). This is caused by differences between the interactions developed by the amino acids residues of NAMs or PAMs with the amino acids sequence of the crevice in the 7-TM domains of the receptors.

PAM compounds offer the possibility to positively modulate the receptors without forcing the constant induction of the active state of the receptor considered mediated by the agonist. Moreover, they could induce less pronounced down-regulation through desensitization of these receptors (Conn et al., 2009). However, some limitations have been reported. For instance, it has already been reported that some PAMs could act as partial agonists. Concomitantly, in truncated receptors or when the 7-TM domain of mGlu and $GABA_B$ receptors are expressed alone, they become full agonists (Binet et al., 2004; Goudet et al., 2004). Parallel to these limitations, NAM compounds showed in some cases the characteristic of inverse agonist and or full antagonist at truncated or mutate receptors.

Finally, many PAMs and NAMs are under study for the modulation of taste group GPCRs. Also some compounds are under deeply investigation to overcome problems offered by pure agonist by a pharmacodynamis and pharmacokinetic point of view. In this sense, a great example is the hope reserved for the development of PAMs for $GABA_B$ receptors.

Other ligands for the Class C GPCRs

Beyond the classical pharmacological tools, other possible targets to modulate these receptors are under investigation. One of these possibilities is the so called "*communicative bridge"* or CRD. This domain has a fundamental role in the activation mechanisms, as illustrated in the previous chapter. However, only prototype molecules have been designed to interact with the CRD domain. It is also difficult to predict the range of action and the selectivity of these compounds.

Recently, the study of central diseases, especially autoimmune and neurological disorders, leads to define a new class of compound acting on GPCRs: the antibodies, specifically those interacting with the extracellular domain of Class C receptors which

have been demonstrated to possess a modulator activity on the target receptor. It is not clear which is the specific site of action of this new pharmacological tool and how they impact the receptor-mediated functions. Moreover, the production of auto-antibodies recognizing the GPCRs were observed in different kind of pathologies.

One evidence originates from the studies clarifying the existence and the role of auto antibodies against the extracellular domains of mGlu1 receptors. These antibodies have been proposed to cause paraneoplastic cerebellar ataxia in adult patient suffering from Hodgkin's disease (Coemans et al., 2003; Marignier et al., 2010). Accordingly, when injected into the subarachnoid area in mice, these antibodies produce reversible ataxia. Although it is not still completely clarified, the mechanism seems to be associate to the block of the activity of mGlu1 receptor protein, which leads to reduction of the G protein-mediated inositol phosphate messenger formation, as observed when administering mGlu1 antagonists.

A second evidence came from the study of the CaS receptors. It was found that antibodies recognizing these receptors can interact with the VFT domain of this structures and can interfere with the function of the calcium ions. Antibodies lead to a block of the closure of the VFT domain mimicking the antagonist action. This idea has been associated with a familiar hypocalciuric/hypercalcemia pathology because this kind of antibodies has been found in the serum of patients suffering from this genetic disease (Kifor et al., 2003). On the other hand, autoantibodies were discovered in patient suffering from autoimmune polyendocrine syndrome type 1 (APS1), an autosomal recessive disorder often associated with hypoparathyroidism (Kemp et al., 2009). These antibodies mediate the activation of the related receptor by inducing the inositol phosphate and calcium-dependent ERK1/2 kinase signalling pathways. Finally, Makita and collaborators hypothesized that antibodies can indirectly interfere with the

action of CaS receptors by modulating the balance between Ca^{2+}/Gq and Ca^{2+}/Gi-dependent phosphorylation mechanisms (Makita et al., 2007).

Another study described the identification of auto antibodies recognizing the $GABA_{B1}$ subunit of the $GABA_B$ receptors in the sera of patients suffering of limbic encephalitis with seizures (Lancaster et al., 2010). In this contest, authors proposed that antibodies are likely pathogenic, as they induce syndromes similar to experimental phenotypes in which the receptor does not function properly, compatible with the conclusion that the auto antibodies behave like antagonist(s).

To briefly resume all these studies, autoantibodies can affect the receptor activity in different ways, for instance by acting as agonists or antagonists or allosteric modulators. This idea rose up the possibility not only to use antibodies as marker in the diagnostic field but, importantly, to use them in pharmacological studies, as a valid tool to characterize receptors. This idea will be discussed further in the next sections.

Further advances in the comprehension of the link between antibodies and GPCRs were achieved by recent studies that introduced the idea of using recombinant nanobodies. They are defined as the smallest antigen binding fragment derivative from the "heavy chain-only" antibodies. Two type of nanobodies raised against the 7-TM of the Class-A GPCR CXCR4 chemokine receptors are successfully achieved by the study of Smit and colleagues (Jähnicken et al., 2010). These high affinity nanobodies competitively inhibited the receptor activity by binding two distinct but overlapping sites in the extracellular loops of the receptor, one having a neutral antagonist activity and the other one an inverse agonist action. Although no or less other nanobodies have been described yet (Scholler et al., 2017), the method illustrated could be proposed also to Class C GPCRs to generate powerful pharmacological devices to modulate the function of these receptors.

1.1.5 G protein

In the GPCR family, G proteins play the fundamental role to translate the signal coming from the extracellular domains to the cytosol. Therefore, the activity of G proteins strictly correlates to the receptor activation. The G proteins promote the impulse for the activation of the second messenger cascades. As other proteins, they are involved in a cycle of activation and deactivation that mainly depends on the enzymatic GTPase activity. In the discussion of the G protein function and structure, some critical points must be considered: i) G-coupling intracellular signalling depends on differences among families and subfamilies of G proteins and on the respective coupled receptors; ii) there is a huge number of intra/extracellular factors, like enzymes, channels and proteins, which are correlated to the GPCRs activation/inactivation; iii) Class C GPCRs members can bind different kind of G proteins when they heterodimerize with other receptors, activating consequently different intracellular pathways; iiii) the activity of second messengers can reverberate in the G protein life metabolism.

For these reasons, the detailed discussion of the G proteins is not matter of this work but I am going to try to resume the key points in order to have a general picture. For further details see references reported in the discussion.

The binding of ligands causes conformational changes within the receptor protein which in turn leads to the activation of the receptor(s). These changes reflect the activation of the G protein gear and, as a consequence, the mobilization of selected intracellular pathways and second messenger production.

The G proteins are heterotrimeric proteins composed by α-, β- and γ-subunits localized in the inner side of the cell membrane (Hamm, 1998). G proteins are divided into four families based on the similarity of their subunits: $G_{i/o}$, G_s, $G_{q/11}$, and $G_{12/13}$. Each family consists of different members which can offer specific activation patterns. However,

members of the same family usually share similar intracellular pathways. Every subunits which composes the G protein is encoded by different genes, that determine the characteristics of the single subunit and the overall function of the G protein.

The Gα-subunit is characterized by two domains: one is the GTPase domain, crucial for the activation and the consequent induction of intracellular pathways, and the second is an helical domain masking the GTP within the core domain (Cabrera-Vera et al., 2003). The helical domain of Gα-subunit is the most divergent among the G protein families.

The Gβ-subunit has a typical β-helical-like structure, which plays an important role in the specific interaction with the other subunits and activation mechanism.

The Gγ-subunit interacts with the Gβ-subunit through the N-terminal coil and other extensive contacts along the amino acid sequence (Lambright et al., 1996). These two last subunits assemble to produce the well-known Gβγ-dimer that is linked to the hydrophobic pocket in the Gα-subunit. Specifically, it is proposed that GTP binds the Gα in this pocket, then reducing the affinity between α and βγ units and favoring their dissociation after the activation (Lambright et al., 1994).

The crystal structure studies had shown that the G protein can adopt at least three different conformational states: the inactive (GDP-bound), the active (GTP-bound) and the transition state (GDP-GTP). However, only the active and inactive states are so far well characterized (Cabrera-Vera et al., 2013).

The general mechanism of G proteins is based on an activation/inactivation life cycle. Generally, the functional process can be resumed as follow: the Gβγ-complex and the Gα-subunit in GDP-bound state are associated one each other. In this conformation G protein is not activated. The coupled receptor induces the heterotrimer G protein to promote the exchange of GDP in GTP on the Gα-subunit site. The Gα-subunit in the GTP-bound state dissociates from the activated receptor and from the Gβγ-complex, and both the Gα-subunit and the Gβγ-dimer modulate the activity of a variety of

effectors. The deactivation process starts when the signalling is hydrolysed by the GTPase enzymatic process, which is inherent to the G protein Gα-subunit site. The resulting GDP-bound Gα-subunit gathers again with the βγ-dimer to start a new cycle if activated receptors are present (Cabrera-Vera et al., 2003; Birnbaumer L., 2004; Wettschureck and Offermanns, 2005).

The impressive number of studies carried out in the last decades gives new insights in the functional mechanism of the G protein. First, at the beginning, the core function was only associated to the Gα component, giving only a support role to the other domains, including Gβγ-subunits. Nowadays, we know that both the activation and deactivation processes involve the Gα as well as Gβγ domains, indicating a general major complex scenario (Birnbaumer L., 2004; Wettschureck and Offermanns, 2005). The study performed on the structure of G proteins unveiled some characteristics which are worth to be remembered in the context of GPCRs proteins.

One crucial step in the activation of the G protein component is the interaction with the coupled receptor. This interaction is mediated by specific sequences and regions of the proteins. For instance, a high conserved region of the C terminus sequence of the Gα subunit has been established, specifically defined by four residues. In addition modifications, like phosphorylation, of these four residues can be requested for the correct interaction between proteins, for example in the mGlu receptors. Opposite, the *Pertussis Toxin* (PTx) carries out an abnormal ADP-ribosylation of these four residues which completely blocks the activation process of G_i/G_o proteins (Van Dop et al., 1984; Unemorl et al., 1997). The so mentioned role of the C terminal region in the interaction system can be also observed with antibodies and peptides acting at this level. Both antibodies and peptides can bind this terminal region by specific interactions with the amino acid sequences. The results could be quite different. For example, it has been already reported that antibodies recognizing the C terminal domains in the Gα subunits

can abolish receptor G protein signalling (McFadzean et al., 1989). Synthetic peptides can stabilize the mechanism of the activation or, in opposite, they can have an antagonist like behaviour by binding the C terminal domain (Rasenick et al., 1994). In addition to the C terminal region, also some area of the Gα-subunits region are involved in the successful interaction between G protein and coupled receptor. However, we should remember that the mechanism of communication between G proteins and receptors depends on complex interactions, which could differ among the G protein families (Cabrera-Vera et al., 2003).

In order to induce the G protein activation, the coupled receptors need to be activated by the proper ligands, like orthosteric agonists. The induction promotes the conversion from GDP to GTP within the Gα-subunits. GDP is also spontaneously released from the heterotrimeric G protein at a rate that varies depending on the Gα-subunit (Cabrera-Vera et al., 2003). This rate is mainly based on the characteristics of C terminal domain and Gα-subunit. This process is linked to the idea of a "constitutive activation/deactivation" process that can be influenced by many factors such as Gβγ subunit and the presence of Ca^{2+} or Mg^{2+} ions (Higashijima et al., 1987). Furthermore, it has been observed that the spontaneous release have physiological consequences. In fact, alterations or mutations affecting this spontaneous activity have been already linked to pathologies like pseudohypo-parathyroidism and gonadotropin-independent precocious puberty.

It has been demonstrated the crucial role of the Gβγ-subunits in the GDP/GTP conversion. Specifically, Gβγ-subunits take an active role in GDP release creating a sort of "exit path" for the guanine nucleotide to move out the G protein. The G proteins posses a cavity between Gα and Gβγ, oriented to the plasma membrane. The modification of this crevice during the activation process seems to induce the separation of Gβγ-dimers from the G protein concomitantly to the GDP/GTP conversion

(Lambright et al., 1994; Bohm et al., 1997; Cabrera-Vera et al., 2003). These overall characteristics offer another demonstration of the important role of the Gβγ subunits and of the complexity of its activation process.

As far as the G proteins are concerned, the class of "receptor-independent activators of G protein signalling "(AGS) requires some comments. The AGS family can activate the G proteins without coupling with a specific receptor. Unfortunately, few information are available about their physiological role and mechanism of interaction (see Jamora et al., 1997; Cismowski et al., 1999).

The two effectors of the G protein: Gα and Gβγ subunits

The Gα-subunit activity depends essentially on the belonging family. At least four different families are recognized. A huge amount of effector proteins is described to be specifically linked to a family of Gα-subunit. Despite of this, a relative number of other subtypes are discovered every day as well as enzymes/proteins/channels/second messengers that could not be linked to the families already described. Therefore, the overall picture could become quite confused and complex.

The G proteins of the Gi ($Gi_{1,2,3}$ specifically)/Go family are widely expressed in the CNS, and they can induce receptor-dependent inhibition of several subtypes of adenylyl cyclases (AC) (Sunahara et al., 1996). Similarly to the Gα pathway, these G proteins also release Gβγ-dimers, that are almost completely transduce the effects of Go subtype while the Gα-subunit controls the opening of the Ca^{2+} and K^+ channels, modulates cytoscheletric fibres and the cellular growth. In the modulation of synaptic transmission, attention was paid to the capability of $G\alpha_i/G\alpha_o$ to control the N- and P/Q type calcium channels as well as the K^+ channels (GIRKs) which lead to a hyperpolarization, relevant at the postsynaptic sites. This second mechanism is also robustly mediated by the Gβγ subunits. In addition, it is worth reminding the peculiar Gα subtype called $G\alpha_z$. Even if

the $G\alpha_z$ mechanism of activation is not completely clear, Chen and colleagues illustrated a possible way of interaction with the RAP1GAP and GRIN family, correlating these proteins to the neuritis outgrowth (Chen et al., 1999).

The $G\alpha_s$ subgroup of the Gα proteins is strongly coupled to the activation of AC pathway which carries out the increase of the cAMP activity. Notably, all these proteins are derived from the GNAS gene families which have high numbers of promoters and splice variants (Wettschureck and Offermanns, 2005). In this group, the $G\alpha_{olf}$ is the major subtype coupled to the AC pattern involved in olfactory system modulation. These G proteins can be also found in other central regions, like the nucleus accumbens and the striatum. Here, they are critically involved in the function of some receptors, like the dopamine (D_1) and the adenosine (A_2) ones (Zhuang et al., 2000).

The $G\alpha_q$ family is linked to the activation of phospholipase C (PLC) and of a great number of kinases like Bruton's tyrosine kinases. Mice bearing the genetic deletion of Gq develop severe central and peripheral defects and antagonists able to discriminate the specific subtypes of this G family proteins are not available. Differently from the $G\alpha_i$, the $G\alpha_q$ subtypes are involved in the activation of the synaptic transmission in the CNS, particularly in the LTP and LTD development. Moreover, the $G\alpha_q$ and the respective Gβγ dimer induce the mobilization of intracellular calcium. Furthermore, there are also evidence that activation of $G\alpha_q$ or $G\alpha_{11}$ are involved in the induction of endocannabinoid system. Specifically, GPCRs like Group I mGlu receptors can also lead to the activation of the retrograde function of cannabinoid system by favouring the activation of endocannabinoids pathway (Levenes et al., 2001; De Petrocellis et al., 2004).

The $G\alpha_{12/13}$ are also involved in the activation of intraterminal pathways, like Rho-GEF complex, which modulate the metabolism of GTP/GDP system. Moreover, this class of protein also control various other effectors including phospholipase A_2, Na^+/H^+

exchanger and c-*jun* NH_2-terminal kinase (see Wettschureck and Offermanns, 2005 and relative references).

While Gα subuntis are classified in different subfamilies, the subclassification of the Gβγ subunits is still lacking. In literature, the structure of five Gβ-subunits and twelve Gγ-subunits have been characterized. In theory, if Gβ and Gγ combined each other, there would be over sixty possible results (Downes et al., 1999). Despite the old idea that the dimerization between different β and γ subtypes could be a random event, recent studies demonstrated some selectivity and limitation to the dimers formation (Wettschureck and Offermanns, 2005). To note, it is proposed that the Gβγ-dimers composition can affect the quality and efficacy of effectors activation and, consequently, it can mediate receptor G-coupling specificity.

The Gβγ-dimers do not present great conformational changes between the active and inactive states (Gaudet et al., 1996). Once the Gβγ dissociates from Gα, it can interact with a great number of effectors, including ion channels, PLC families, kinases and intraterminal messenger like Calmodulin and Dynamin I (Cabrera-Vera et al., 2003). As far as the CNS is concerned, the Gβγ is reported to be linked to particular effectors controlling synaptic vesicle machinery, beyond the Ca^{2+} and K^+ ions channels and the PLC pathway. Interestingly, it has been demonstrated that this dimer can directly control the SNARE complex by binding syntaxin, SNAP25 and cysteine string domain (CSP) (Blackmer et al., 2000, 2001). The complex involving G protein domains, SNARE complex and Ca^{2+} channels suggested a possible unique way to control synaptic release of vesicle (Betke et al., 2012).

Therefore, Gβγ subunits can stimulate or block the induction of many effectors, mainly dependent on the β and γ subunits as well as on the Gα subunits influence. Recent finding have established that Gβγ dimer can directly modulate some important

intracellular complexes linked to the genes modulation and protein synthesis, like ERK and MAPK families (Bell et al., 1995; Cassier et al., 2017).

G protein inactivation and cycle modulation

The G protein inactivation process strictly depends on the GTP hydrolysis to GDP (Cabrera-Vera et al., 2003).

The GTPase activity and the inactivating pathway are mediated by a feedback system of control triggered by intracellular pathways. The protein that directly interacts with the GTPase site is the so called "regulator of G protein signal" or RGS, that is strictly linked to GTPase activating protein (GAP). It is not matter of discussion of this work but RGS is a large family of proteins which can interact with different affinity among the Gα-subunits (see Hollinger et al., 2002 and Cabrera-Vera et al., 2003). In general, RGSs enhance the GTPase activity of Gα-subunits, thereby reducing the duration and amplitude of the Gα/Gβγ system (Hollinger et al., 2002).

Covalent modifications, including the phosphorylation and/or acylation of protein processes as well as the "lipidation" process are important in the regulation of G protein cycle and expression (Cabrera-Vera et al., 2013). Phosphorylation and acylation are controlled by different intracellular patterns and represent general mechanism(s) of feedback control of the G proteins.

As far as the phosphorylation is concerned, protein kinase C enhances the phosphorylation of the Gα subunits, inhibiting them. In addition, the same group of proteins is involved in the regulation of RGS and GAP as well as of the Gβγ activity. The acylation processes act similarly and they influence the stabilization of G proteins in cell membranes (Cabrera-Vera et al., 2003). In conclusion, phosphorylation/acylation reactions can both indirectly catalyze the dissociation of the components of the G protein involved but also directly modulate some Gβγ-dependent effectors.

The so called lipid modification involves processes of myristoylation, palmitoylation and also carboxymethylation/isoprenylation reactions at the N terminal domain. Interestingly, the most important function of the covalent reaction is to modulate and to control the surface expression of G proteins, promoting or inhibiting it. Most of these reactions are selective for the Gα subfamily. For instance, the $G\alpha_i$ proteins are myristoylated while $G\alpha_{q,11,\ 13}$ proteins are mainly palmitoylated (Chen et al., 2001). Moreover, some studies suggested that carboxymetilation of the C terminus of Gγ-subunit can modulate the membrane expression of some G proteins (Fukada et al., 1994).

Two are the main consequences of the lipid modifications. One, already mentioned, relies on the role that lipid plays as central hydrophobic membrane anchor. The $G\alpha_i$-subunits are both myristoylated and palmitoylated, and the "lipidification" contribute to the membrane stabilization. In fact, it has been already demonstrated that the lack of one of the two mentioned reactions can cause a partial internalization of the G proteins (Wilson et al., 1995; Wise et al., 1997). On the other hand, lipids also regulates interactions between proteins and second messengers. For example, N-myristoylation of Gα modulates the interaction between Gβγ and the relative effectors, while palmitoylation increases the affinity of $G\alpha_s$ for Gβγ. In conclusion, lipid modification can be associated to a indirect feedback mechanism of control of the receptor activity (see Cabrera-Vera et al., 2003 and relative references).

1.2 DIMERIZATION

The GPCRs dimerization process between two monomeric proteins is one of the crucial step in the mechanism of activation of the Class C receptors (Kniazeff et al., 2014). Dimerization between receptors can modify many characteristics of the receptors involved, including the pharmacological profile. Moreover, dimerization, especially between receptors of different groups, can influence the impact of these receptors on neurotransmitters release and intracellular pathways (Yin et al., 2014; Olivero et al., 2018).

Definition

The term "dimer" indicates the interaction linking two receptor subunits or monomers. This process carries out functional and structural consequences. The monomers engaged in the receptor can either be the same ("homodimers") or can differ one to each other ("heterodimers"). In addition, more than two subunits can interact to create "oligomeric" assemblies (Chabre et al., 2009).

The knowledge of the Class C GPCRs dimerization started early. Romano and collaborators first observed the dimerization of mGlu5 receptors in the brain (Romano et al., 1996). Then several other studies, either by in vitro or in vivo experiments, described the dimerization of GPCRs, including mGlu receptors (Copani et al., 2000, Muto et al., 2000), CaS receptors (Ray et al., 1999), taste receptors (Nelson et al., 2001) and $GABA_B$ receptors (Marshall et al., 1999).

Experimental approaches

Western Blot analysis is the simplest approach used to demonstrate the presence of receptor dimers. In Western blot analysis, the presence of a dimeric association of

receptors proteins can be usually suggested by the presence of immunopositivities having a mass consistent with the sum of the weight of the two proteins involved. In this case, the monomeric form of the protein involved became detectable only when applying reducing conditions during the protein extraction. To substantiate the conclusion, immunoprecipitation studies are usually carried out to unveil the presence of homo or heterodimeric association linking the receptor proteins (Olivero et al., 2018). However, these observations suggest but do not definitively prove the existence of dimers.

The study of dimerization events recently improved thanks to the introduction of fluorescence techniques (Phizicky et al., 1995). Specifically, Fluorescence Resonance Energy Transfer (FRET) permits to monitor the proximity and the orientation of dimers of GPCRs proteins. In FRET, one fluorophore (the ‘‘donor’’) transfers its excited-state energy to another fluorophore (the ‘‘acceptor’’), which usually emits fluorescence in a different wavelength. This technique offers the possibility to observe the interactions between receptors in the native organism, but it is limited by the possible tissue damages elicited by the introduction of the fluorophores or the exposure to fluorescence excitation. Owing to overcome these issues, another technique, the so called Bioluminescence Resonance Energy Transfer (BRET) was introduced, which takes the advantage of using natural fluorescence phenomena (Xu et al., 1999). Starting from these techniques, other fluorescence analysis and microscope detection (i.e. the time resolving FRET or TR-FRET, see Pin et al., 2009) are now currently in use to deeply investigate dimers and oligodimers in the Class C receptors.

Structural and functional consequences

The Class C of the GPCRs form constitutive dimers, which are fundamental for their function in cells. Dimerization implies the association of the VFT domains as detected

in crystallography studies (Kunishima et al., 2000; Pin et al., 2004). Specifically, the two VFT domains interact at the level of lobe-I, favouring the convergence of lobe-II. These processes catalyze the change in orientation of VFT structure and, consequently, the activation of the receptor (see chapter "Structure and activation mechanism" for further details and references).

The mechanism of activation of Class C of GPCRs requires the presence of dimers, even if the closure of only one VFT domain is sufficient to activate the receptors (Kniazeff et al., 2011). This conclusion relied on results from the study of metabotropic glutamate and GABA receptors. As far as the mGlu receptors are concerning, Kniazeff and collaborators demonstrated that the presence of one molecule of the orthosteric agonist is sufficient to promote the closure of one VFT domain, reaching the $A_{o/c}$ state, and, consequently, to induce the activation of mGlu receptors (Kniazeff et al., 2004, 2011). However, the presence and the activation of both VFT units in the dimeric assembly is necessary to obtain the full activation of the receptors and to complete the transduction of the intracellular second messengers. Similarly, the $GABA_{B1}$ and the $GABA_{B2}$ proteins are required to express the $GABA_B$ receptors (Bowery et al., 2002; White et al., 2009), but the occupancy only of the VFT domain of the $GABA_{B1}$ receptor with the orthosteric agonist is required to cause receptor activation (Pin et al., 2004).

Nowadays, five main characteristics of the Class C GPCR proteins were proposed to strictly depend on receptor dimerization (Terrillon & Bouvier, 2004).

1. The mechanism of exit of receptor proteins from the endoplasmic reticulum (ER). The dimeric association can mask the retention signals, favouring the correct escape of the protein from the ER. One example of this process is the $GABA_B$ receptors (Marshall et al., 1999). Specifically, the $GABA_{B1}$ receptor is normally retained in the ER when express alone because of a retention carboxy-terminal ER motif. The association with the $GABA_{B2}$ receptor subunit masks the

site and favours the passage from ER to the cell surface (Margeta-Mitrovic et al., 2000).

2. The binding momentum of the orthosteric ligands (see Terrillon & Bouvier, 2004 and relative references). Dimerization preferentially is favoured by orthosteric ligands acting at the relative binding site, although some few evidence also suggest that some receptors of the Class C can adopt the dimeric association despite the absence of the agonist/antagonist ligands (it is the case of mGlu 1 receptors, see Kunishima et al., 2000).
3. The pharmacological profile of the receptors. Heterodimerization of the δ and κ receptor subtypes increases the affinity to the orthosteric agonists (Jordan & Devi, 1999).
4. The signal transduction process that follows the activation of Class C GPCRs is mediated by the G proteins, which in turn control the intracellular pathways. Generally, a group of metabotropic receptors is associated to certain G proteins that couple specific patterns activation of enzymatic pathways. Dimerization can favors the association of the receptors to different G proteins. This is the case of μ and δ receptors that when in the dimeric association do not bind Gi protein but associate to Gs/Go proteins (Charles et al., 2003).
5. The internalization processes. Internalization is usually triggered by the occupancy of one orthosteric binding site, although also dimerization was proved to control or, at least, modulate the endocytosis processes of metabotropic receptors (Jordan & Devi, 1999)

To conclude, dimerization is a process that can influence at different level the life cycle of the receptor and reverberate on colocalize receptors.

1.2.2 Homo, hetero and oligodimers

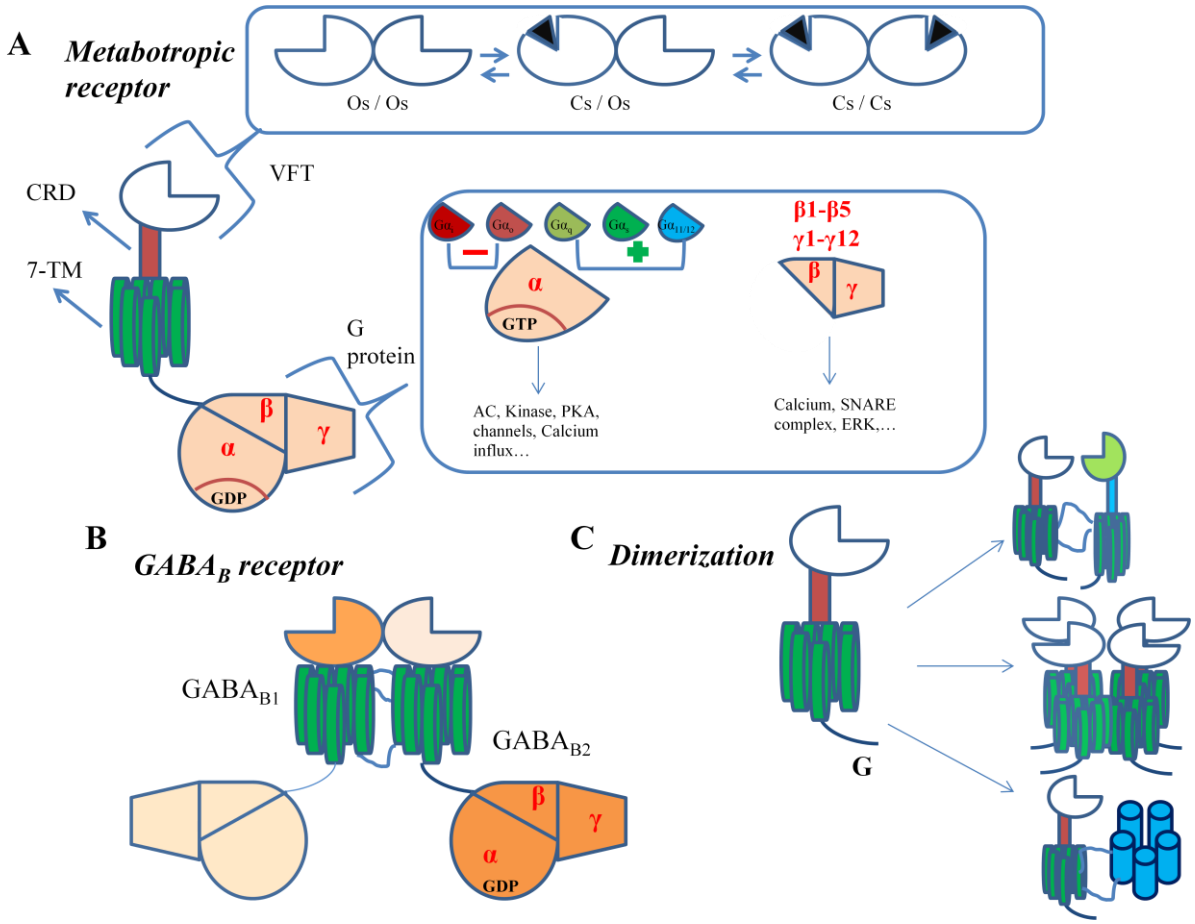
The association of two proteins of the same receptor subunit is called "homodimerization", and it was described for the first time for the mGlu5 receptors (Romano et al., 1996).

Differently, heterodimerization relies on the association of different receptors. Heterodimers are the consequence of a multi sited, non covalent amino acid interactions (Møller et al., 2017). The term heterodimers refers to a receptor complex: i) composed of monomers from the same groups; ii) composed of monomers from different receptor groups; iii) composed of receptors from different families.

A classic examples of heterodimerization between receptors belonging to the same group are the association of the mGlu1 and the mGlu5 receptors and of the mGlu2 and the mGlu3 receptors (Nicoletti et al., 2011). Differently, the heterodimerization between the mGlu2 and the mGlu4 is an example of dimerization of receptors belonging to different group within the same family, i.e. the mGlu receptor family (Yin et al., 2014; Niswender et al., 2016). The complexity of the heterodimerization largely increased because of the demonstration that also receptors belonging to different families can associate in heterodimeric assemblies. It is the case of heterodimers composed of the $GABA_B$ receptors and the CaS receptors, as well as heterodimeric assembly involving Class A and Class C receptors, such as the mGlu1 and the Adenosine-1 receptors (Kniazeff et al., 2011). These observations are deeply relevant by a therapeutical point of view. For instance, a possible involvement of the mGlu1/A1 heterodimers in pathological conditions has been already described (see Ciruela et al., 2001).

Recent studies with FRET and TM-FRET suggested the possibility that receptors subunits can form complexes built by more than two units, so called "oligodimers". This has been already well documented for Class A GPCR proteins (Milligan, 2004). The mGlu receptors did not show a marked trend to form oligodimers, even among

different groups of mGlu receptors (Brock et al., 2007; Doumazane et al. 2011). In opposite, oligodimers of $GABA_B$ receptors has been found, composed in some case of more than tetramers (Maurel et al., 2008). These oligomers were described to exist in native tissue and to strictly depend on the involvement of the $GABA_{B1}$ subunits, as supported by data from transgenic mice lacking $GABA_{B1}$ genes (Maurel et al., 2008; Comps-Agrar et al., 2011). The functional consequences of these oligodimers are still not clear. For instance, it was found that $GABA_B$ receptors oligodimers can modulate the receptor signalling. Indeed, it was observed that inhibiting $GABA_{B1}$-$GABA_{B2}$ interactions using either a non-functional $GABA_{B1}$ subunit as competitor or introducing a mutation in the $GABA_{B1}$ VFT domain increased the signal efficacy by approximately 50% when compared to control (Maurel et al., 2008; Comps-Agrar et al., 2011).



Schematic representation of metabotropic glutamatergic and GABAergic receptor. A) A schematic representation of the structure and the mechanism of function of the metabotropic receptors. The VFT domain can assume the close state or the open state, but, since the metabotropic receptor is present as dimer it is proposed that it can assume three main states (Os/Os, Cs/Os and Cs/Cs), that correlate to the absence of ligands, to a partial activation and to a full activation of the receptors respectively. The CRD, 7-TM and the G protein represent the other core regions of the metabotropic receptors. When ligands bind the receptor, GDP is converted in GTP to activate the G protein which in turn induces the separation of the α subunit and βγ subunits. These subunits present different isoforms and influence different intracellular pathways after their activation. **B)** $GABA_B$ receptor is composed by the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits, with the peculiar lack of the CRD bridge domain. Notably, the figure underlines the main role of $GABA_{B1}$ for the external binding site, and the role of the $GABA_{B2}$ for the activation of the G protein. **C)** Beyond the binding to another same receptor (homodimerization, i.e. mGlu1), dimerization can be realized with another metabotropic receptor of the same group (heterodimerization intra group, i.e mGlu2/3), with many same metabotropic receptor (oligomerization, i.e $GABA_B$ receptor oligomer) and with different receptors or channel groups/families (heterodimerization inter group, i.e. mGlu2/3 and 5-HT_{2A} or mGlu5 and NMDA).

1.3 METABOTROPIC GLUTAMATE RECEPTORS IN THE CNS

Glutamate is the major excitatory neurotransmitter in the CNS and the glutamate receptors represent one of the crucial machines to control CNS functions and activities. However, the few drugs approved in therapy and the lack of information of many physiological and pathological mechanisms of the CNS involving glutamate explain why we need to continue to investigate glutamate transmission and glutamate receptors.

The first receptors found to be activated by glutamate were the so called ionotropic receptors. Ionotropic receptors are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor, or quisqualate receptor), Kainate receptor and N-methyl-D-aspartate receptor (also known as the NMDA receptor). Beyond them, the first observation that quisqualate and glutamate can stimulate inositol phosphate production gave us a proof of the presence of non ionotropic glutamate receptors (Sladeczek et al., 1985). Few years later Sugiyiama introduced the term "metabotropic glutamate receptor" or mGlu receptor (Sugiyiama et al., 1987) From these first steps, the knowledge of these receptors hugely increased and the metabotropic glutamate receptors are nowadays classified as Class C GPCRs (Nicoletti et al., 2011). These receptors are subdivided in three different groups: Group I metabotropic glutamate receptors including mGlu1 and mGlu5 receptors, Group II metabotropic glutamate receptors consisting of mGlu2 and mGlu3 receptors and Group III metabotropic glutamate receptors, including mGlu4, mGlu6, mGlu7 and mGlu8 receptors. It is worth to remember that these receptors possess a high sequence homology, especially within the same group, so that the development of selective ligands targeting these receptors is still an open field of the pharmacological and pharmaceutical researches.

Beyond the Group III, the Group I and II of mGlu receptors are the most studies mGlu entities and there are a relative high number of evidence that both these groups of receptors are involved in many physiological and pathological conditions, like memory and learning or psychiatric and neurodegenerative disorders. However, their great expression in all CNS, the lack of selective compounds and the possible sides effects explain, at least in part, the relative lack of drugs approved in the therapy for these receptors. In this picture, the evidence of dimerization of these receptors largely improved the complexity of the scenario, at the same time offering an important challenge for the future researchers.

1.3.1 *Group I mGlu receptors*

Definition, structure and pharmacology

The cloning of the first metabotropic glutamate receptors in the laboratory of Nakanishi (Masu et al. 1991) can be consider the ancient milestone that opened the way to the mGlu receptors research, especially that concerning the Group I metabotropic glutamate receptors. Group I mGlu receptors includes the mGlu1 and the mGlu5 receptor subtypes, which are coupled to Gq proteins and that activates a common intracellular signalling (Ferraguti et al., 2008; Niswender and Conn, 2010). As described in previous chapters, the structure and the pharmacological profile of the mGlu1 were the first to be described in the literature (see for further details Kunishima et al., 2000; Nicoletti et al., 2011). In addition, the research on mGlu5 receptor firstly elucidated the dimerization processes (Romano et al., 1996). Indirectly, these studies confirmed that the mGlu1 and the mGlu5 receptors share most of the mechanism of activation and of signal transduction (Nicoletti et al., 2011).

Both the mGlu1 and the mGlu5 receptor proteins exist in different isoforms. For instance, mGlu1 exists in the $mGlu1_{\alpha}$ and the $mGlu1_{\beta}$ forms. The $mGlu1_{\alpha}$ isoform was proposed to be implicated in some important events requiring receptor heterodimerization. It is notably to observe that isoforms and splice variants mainly differ in the C-terminus domain. This fact has important pharmacological consequences in the regulation of the cell surface expression and internalization of these receptors. Nonetheless, this characteristic was found to be quite distinctive of the Group I mGlu receptors (Nisweender and Conn, 2010; Nicoletti et al., 2011).

Group I mGlu receptors possess a large extracellular VFT domain linked to the 7TM domain and C-terminal tail that includes a cysteine sequence or CRD domain (see the chapter of structure for further details and references). These receptors form constitutively dimers via disulfide bond and other specific interactions at the VFT

domain (Romano et al., 2001; Muto et al., 2007). The binding of one molecule of glutamate is sufficient to partially change the structure of one of the VFT domains and to induce the activation of the receptor but two molecules are necessary to achieve the full activity (Kniazeff et al., 2004; Muto et al., 2007). In particular, orthosteric agonists induce the closure of the VFT domain activating the receptors. It is the case of the ACPD and then of the derivate *S*)-3,5-Dihydroxyphenylglycine [(*S*)-3,5-DHPG], i.e. the first selective agonist of the Group I mGlu receptors.

Differently, orthosteric antagonists, i.e. R,S *a*- methyl-4-carboxyphenylglycine (MCPG) and more recently the mGlu1 antagonist (*S*)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), prevent the closure of the VFT domain. Interestingly, the Group I mGlu receptors can adopt an active state even in the absence of the ortosteric agonist and, in this case, the receptor activation is not prevented by competitive antagonist but can be blocked by inverse agonists like MPEP derivates (Prézeau et al., 1996; Pin et al., 2004). In addition to the orthosteric binding site, Group I glutamatergic receptor induced effects can be modulated by compounds acting at the 7-TM domains, the so called "positive or negative allosteric modulator" (PAM or NAM). One example is the 7-(Hydroxyimino) cyclopropa[*b*]chromen-1a-carboxylate ethyl ester (CPCOOEt), a high selective mGlu1 NAM compound (Annoura et l., 1996).

Signal transduction, receptor-mediated effects and clinical implications

Group I mGlu receptors mainly couple Gq/G_{11} proteins leading to the activation of the coupled intracellular pathways. Activation of the mGlu1 and 5 receptors leads to the mobilization of intracellular Calcium, overproduction of diacylglycerol (DAG) and inositol phosphate (IP) and activation of protein kinase C. The receptors also can cause the formation of cAMP and trigger mitogen-activated protein kinase (MAPK) mediated pathways (Bruno et al., 2001; Ferraguti et al., 2008;). Meanwhile it is reported that these

receptors negatively modulate the K^+ channels and the "tandem voltage gated K^+ channels" (Ikeda et al., 1995). In term of neurotransmission, the activation of phosphorylative mechanisms and the increase of intracellular calcium due to the activation of these receptors elicits the stimulation of exocytosis then controlling positively synaptic transmission (Bruno et al., 2001). As to the latter functions, the activation of both mGlu1 and mGlu5 receptors facilitate the glutamate exocytosis from nerve terminals isolated from different regions in CNS (Musante et al., 2008). Also, these receptors are crucially involved in important synaptic events like LTP and LTD (Nicoletti et al., 2010).

On the other hand, the activation of second messengers modulates a variety of cytosolic pathways that in turn control the protein expression in plasma membranes and the function of Group I mGlu receptors themselves. For instance, the G protein coupled receptors kinases (GRKs) family can induce the desensitization of these receptors (Iacovelli et al., 2003). In the context of the control of receptors expression, the C-terminal domain plays a pivotal role. This was well demonstrated by the studies performed with Homer proteins, which contains both PDZ and EVH-1 domains within the C-terminal tail of both mGlu1 and mGlu5 receptors (Kato et al., 1998; Nicoletti et al., 2011). By interacting with the C-terminal coiled-coil domains, Homer proteins family can modulate a cascade of events which leads to the modification of cell surface expression and signal transductions of Group I mGlu receptors. In addition, other Calcium dependent proteins like calmodulin or protein kinases can influence the receptors functions (Bortolotto and Collingridge, 1998 and 2000).

From a clinical point of view, the involvement of mGlu1 and mGlu5 receptors in pathological processes in the CNS is quite impressive. In particular, altered expression or functions of Group I receptors were proposed to play a role in schizophrenia, Fragile X syndrome, ataxia, Parkinson's disease, chronic pain, drug addiction, anxiety and

stress (Nicoletti et al., 2011). In addition, evidences were also provided indicating the involvement of mGlu1 and mGlu5 receptors in others diseases including cancer, Malignant Melanomas and Gastroesophageal reflux disorders (Nicoletti et al., 2011). In addition, the mGlu1 subunits play a key role in many neuroprotective and synaptic events, i.e. in "post ischemic" degeneration (Pellegrini-Giampietro, 2003). On the other side, mGlu5 receptor is strictly involved in many pathological processes modulating the deregulations of other receptors, including the NMDA ones (Nicoletti et al., 1999, 2011).

Localization, synaptic distribution and dimerization

Group I mGlu receptors possess a widespread distribution in the CNS. The mGlu1 receptor subtype is extensively detected in the cerebellum at the level of Purkinje Cells where it plays a key role in controlling glutamatergic synapses. In addition, it is also strongly expressed in the substantia nigra, globus pallidum, thalamic nuclei and in the hippocampus, cortex and hypothalamus (Ferraguti et al., 2008; Nicoletti et al., 2011). The mGlu5 expression is high in cortex, corpus striatum and hippocampus (Romano et al., 1996). In general, a diffuse overlapping in the expression of these two receptor subunits can be observed in the CNS. Interestingly, these two receptors can reciprocally modulate one each other their functions and expression. This was already observed for the cerebellum, where the epigenetic down regulation of mGlu5 receptors mediated by mGlu1 receptors activation in the post natal days of mice cerebellum was disserted (Notartomaso et al., 2018). In addition, the expression of mGlu1 and mGlu5 receptors can be altered in different pathological conditions, like multiple sclerosis (Fazio et al., 2008) or ataxia (Rossi et al., 2013). Altogether these studies underline a thin balance between these two receptors, that could bring important pharmacological consequences.

It is well recognized that these receptors are highly localized at the postsynaptic site where mGlu1 receptors mainly controls LTD and mGlu5 receptors mainly controls LTP in the hippocampus circuits (Nicoletti et al., 2011). They also control postsynaptic effects that rebound onto the presynaptic compartment like the modulation of the endocannabinoid system (Kano et al., 2008; Chiarlone et al., 2014). However, it is now recognized that these receptors have a strong and functional expression also at the presynaptic site where they control the release of several transmitters (Sánchez-Prieto et al., 1996; Pittaluga, 2016). Knocked out mouse model for the mGlu1 or mGlu5 receptors show general defects in the control of intracellular pathways, and develop different neurological and defects as well as locomotors impairments (Rossi et al., 2008; Bossi et al., 2018).

The Group I mGlu receptors represent a good model to study the impact of dimerization in the receptor activities. As described before, these receptors, as other mGlu receptors, dimerize after activation by agonists. The presence of homodimers is necessary for the fully activation of the receptors (Kniazeff et al., 2011; Møller et al., 2017). However, Group I receptors also form heterodimers involving the Group I mGlu receptors or other receptors, but not with Group II and III mGlu receptors (Doumazane et al., 2011), even if nowadays there are some evidence which put in discussion this dogma (Di Menna et al., 2018; Joffe et al., 2019). Specifically, the heterodimers mGlu1/5 has been observed in the mouse cortex even if the pharmacological consequences are not completely elucidated (Sevastyanova and Kammermier, 2014). In addition, it is worth to remember some of the heterodimerization of functional receptor-receptor interactions linking Group I and other type of receptors: NMDA (Bruno et al., 2001; Perroy et al., 2008), A_1 (Vázquez et al., 1995; Ciruela et al., 2001) and $GABA_B$ receptors (Tabata et al., 2004; Vergassola et al., 2018).

1.3.3 Group II mGlu receptors

Definition, structure and pharmacology

The second group of mGlu receptors consists of mGlu2 and mGlu3 receptors coupled to Gi/Go. The high sequence homology, about 70% of the amino acid sequence, and the overlapping of the receptors expression in the CNS made hard to distinguish the respective pharmacological contribution of the two receptors (Niswender and Conn, 2010). However, in the last decade new compounds and innovative approaches started to elucidate the respectively contribution of the mGlu2 and the mGlu3 receptors in receptor expression also permitting their pharmacological characterization (Hanna et al., 2013; Di Prisco et al., 2016; Olivero et al., 2017).

As stated, the Group II mGlu receptors show the common structure of the Class C GPCRs (Muto et al., 2007). Nowadays, there are no full selective ligands acting as orthosteric agonists and antagonists that can distinguish for the mGlu2 and the mGlu3 receptors with the exception of N-acetyl-aspartyl-glutamate (NAAG), which has been demonstrated to selectively activate mGlu3 receptors (Neale et al., 2011; Di Prisco et al., 2016).

The orthosteric agonists of the Group II mGlu receptors include 2R,4R-APDC, and the carboxycyclopropylglycine derivatives, DGC-IV. However, these drugs also show an activity at NMDA receptors (Schoepp et al., 1999). More recently, Jim Monn at Eli Lilly laboratories described two potent and selective mGlu2/3 orthosteric agonists: LY 354740 and LY 379268 (Cartmell and Schoepp, 2000). For instance, a pharmacological analogous of LY 379268, the (-)-(1R,4S,5S,6S) -4-Amino-2-sulfonylbicyclo[3.1.0]hexane- 4,6- dicarboxylic acid (LY 404039) is under clinical evaluation for the treatment of schizophrenia. Moreover, a number of other compounds were undergone to clinical trials like LY 2140023 and LY 544344, two mGlu2/3 agonists.

From the same laboratory, LY 341495 and other derivates were synthesized as potent orthosteric antagonists. Recently, other ligands for the mGlu2/3 receptors were introduced that include allosteric modulators which show different selectivity for mGlu2 and mGlu3 receptors. It is the case of 2,2,2-Trifluoro-N-[4-(2-methoxyphenoxy)phenyl]-N-(3-pyridinylmethyl) ethanesulfonamide hydrochloride (LY 487379) that is a selective mGlu2 PAM and of [2-Fluoro-4-[2-(4-methoxyphenyl)ethynyl]phenyl][(3*R*)-3-hydroxy-1-piperidinyl]methanone (ML-337) that is a selective NAM mGlu3 (Niswender and Conn, 2010).

Signal transduction, receptor-mediated effects and clinical implications

Differently from the Group I mGlu receptors, mGlu2 and mGlu3 receptors are coupled to Gi/Go proteins, which in turn negatively modulate the intracellular pathways involved in neurotransmitter exocytosis (Tanabe et al., 1992). The activation of mGlu2/3 receptors is linked to the inhibition of cAMP production, of the voltage-sensitive Ca^{2+} channels and PI hydrolysis. These receptors also induce the activation of K^{+} channels, MAPK proteins and PtdIns-3-K pathway (Pin and Duvoisin, 1995). Like the other metabotropic receptors, their surface expression, internalization and desensitation is finely tuned. For instance, one of the most studied desensitation pathway controlling mGlu2/3 insertion in the plasma membrane is the GRK2/β-arrestin complex. Interestingly, it has been demonstrated that mGlu2 receptors could be resistant to this mechanism of desensitization (Iacovelli et al., 2009). In addition, both mGlu2 and mGlu3 receptors can interact with calmodulin, with protein phosphatase 2C and Ran binding protein which regulate their trafficking (Niswender and Conn, 2010).

Group II mGlu receptors inhibit the neurotransmission and favour membrane hyperpolarization. Specifically, mGlu2/3 receptors can be activated by an excess of synaptic glutamate released from neurons and from astrocytes. The mGlu2/3 receptors

can also modulate glutamate availability by controlling the expression of the cysteine-glutamate antiporter, which is critically involved in drug addiction. This event strictly correlates to the observation that mGlu2 negatively regulate the reward pathway activity in the mesolimbic dopaminergic system and that mGlu2 receptors knockout mice show increased reinforcing properties towards cocaine and derivates (Nicoletti et al., 2011).

The development of mGlu2/3 receptor agonists is attractive for their ability to inhibit the glutamate release and, consequently, the excitotossicity due to hyperglutamatergic events. There are also evidence that mGlu2/3 receptor agonists reduce the side effects of drug abuse like phencycline on working memory and locomotion (Moghaddam and Adams, 1998). As already said, the agonist LY 404039, a derivate of the orthosteric agonist LY 379268, is under clinical studies as antipsychotic. It was observed that it did not cause the side effects elicited by olanzapine, an approved antipsychotic drug, including the increase of body weight and blood triglyceride levels.

Beyond schizophrenia and drug addiction, mGlu2/3 receptors are involved in different kind of central disorders and neurodegenerative diseases. Specifically, these receptors have been implicated in chronic pain, anxiety and depression. For example the agonist LY 354740 has potent effects in several models of anxiety and pain without showing the classical desensitation processes, an event that is explained by considering the role of mGlu2/3 receptors in amygdala circuits, where these receptors strictly control GABAergic transmission (Swanson et al., 2005). mGlu2/3 antagonists are under evaluation for their antidepressive activity, because of their modulation of dopaminergic release in hippocampal network (Pilc et al., 2008). Finally, Group II mGlu receptors were found to play a role in controlling the impact of different type of stress in preclinical studies, including prenatal stress (Zuena et al., 2008; Marrocco et al., 2012).

The neuroprotective effect of these receptors is intensively studied in different neurodegenerative disorders. It has been shown that in a mouse model of Multiple

Sclerosis these receptors could be critically altered and that their activation carries out beneficial effects (Besong et al., 2002; Di Prisco et al., 2016). Alzheimer's disease, taupathologies and dementia are other important examples of diseases where mGlu2/3 receptors can have an active role. However, the available studies in literature show contrasting results about these diseases (Nicoletti et al., 2011).

Localization, synaptic distribution and dimerization

Group II mGlu receptors are widely expressed in all the regions of the CNS without significant differences between mGlu2 and mGlu3 receptors. The mGlu3 receptors were also found in embryonic stem cells and glioma-initiating cells, where their activation decreases cell differentiation by acting on type-4 bone-morphogenetic protein (BMP-4) receptor signalling (Melchiorri et al., 2007).

These receptors mainly show a presynaptic localization where they can be activated by glutamate. However, the presence of these receptors at the postsynaptic level in some CNS regions has been also demonstrated (Muly et al., 2007). Group II mGlu receptors, especially mGlu3 receptor subtype, were observed also in non neuronal cell like astrocytes and glial cells. The glial mGlu3 receptors are neuroprotective in Alzheimer's disease, because of the activation of TGF-β proteins which in turn protect from the β-amyloids toxicity (Caraci et al., 2009). The mGlu3/TFG-β axis was found to be also involved in the physio-pathology of Huntington's disease (Battaglia et al., 2010).

Both the mGlu2 and mGlu3 receptors have an important role in the modulation of synaptic events like LTD, neuroprotection and synaptic plasticity (Nicoletti et al., 2011). For instance, the crucial role of mGlu2 receptor in synaptic plasticity is well demonstrated in the olfactory system where it is accepted that this receptor can directly control a kind of specific olfactory memory linked to pheromones activity (Kaba et al., 1994).

Like the Group I mGlu receptors, also Group II mGlu receptors show an extensive homodimerization of their single receptor subunits. Dimers of mGlu2 and mGlu3 receptors were detected in different CNS areas and their engagement after activation by agonists is already demonstrated. Moreover, it has been observed the presence of mGlu2/3 heterodimers in different regions, especially in the cortex (Olivero et al., 2017). Differently from the first group, the Group II mGlu receptors can heterodimerize with mGlu receptors from the Group III (Doumazane et al., 2011). It is the case of the heterodimer between mGlu2 and mGlu4 receptors which, surprisingly, show a peculiar response to the orthosteric/allosteric agonists. For instance, the orthosteric mGlu2/3 agonist LY 379268 is less potent to activate this heterodimer than mGlu2/3 receptors (Yin et al., 2014).

It has been also demonstrated that mGlu2/3 receptor can also heterodimerize with non glutamatergic receptors. In particular, one of the most characterized receptor heterodimer is the complex between mGlu2 and 5-HT_{2a} receptors (Prezeau et al., 2010; Murat et al., 2018; Hideshima et al., 2018) that was proposed to play a main role in schizophrenia (Benneyworth et al., 2007; Gonzales Maeso et al., 2008).

1.4. $GABA_B$ RECEPTORS IN THE CNS

The γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS, ensuring the normal brain function and mediating a contrasting activity to the excitatory processes. GABA mediates its effects by acting at two different classes of receptors: $GABA_A$ receptors channels and $GABA_B$ metabotropic receptors. $GABA_A$ receptors are heteropentameric GABA gated chloride channels and they are generally associated to a fast inhibitory tone on neurotransmission (Benke et al., 2012). This channel has a widespread distribution in the CNS and it is the target of many drugs approved for anxiety, depression and other neurological diseases (Chua et al., 2017).

$GABA_B$ receptors are obligatory heterodimeric G protein coupled receptors belonging to the Class C GPCRs. The $GABA_B$ receptor carries out the slow and prolonged inhibitory transmission, showing different and peculiar characteristics compared to other metabotropic receptors. It is largely studied, and it represents a model for its structure when referring to the dimerization processes.

1.4.1 $GABA_B$ heterodimeric receptor

Definition, structure and pharmacology

The $GABA_B$ receptor is an inhibitory receptor that still represent an interesting target for drug discovery. It shares many characteristics with other Class C GPCRs but it also has peculiar features.

The $GABA_B$ receptor was first discovered in the 1979 by Dr. Norman Bowery (Bowery et al., 1979), who opened the way to further researches leading to the publication of thousands of papers and to the development of many compounds, underlining its undoubted role as mediator of the slow and prolonged inhibitory tone of central transmission (Trist G et al., 2010).

The $GABA_B$ receptor belongs to the Class C GPCR proteins and it shares with other members of this class some classical structural features like the presence of the large VFT domain and the 7-TM domain. However, it possesses some unique characteristics in the structure and function. The first important characteristic is that $GABA_B$ receptor is an obligatory heterodimeric receptor composed of the $GABA_{B1}$ and the $GABA_{B2}$ receptor subunits. Both these subunits are required to transmit the signal from the VFT domain to the coupled G protein. Specifically, $GABA_{B1}$ contains the active VFT domain which binds the orthosteric agonist and the $GABA_{B2}$ is correlated to the induction of the G coupled protein (Pin and Bettler, 2016). The second important feature of $GABA_B$ receptor is the lack of CRD domain: the activation mechanism mediated by the agonist goes directly from the VFT domain to the 7-TM because of specific interactions linking the two receptors subunits involved (Kniazeff et al., 2011).

Notably, the evidence that both $GABA_{B1}$ and $GABA_{B2}$ receptors subunits show the structure of an entire receptor appears to be conflicting with the mechanism of activation of the $GABA_B$ receptor. In fact $GABA_{B1}$ receptor possesses the 7-TM domain and $GABA_{B2}$ also has the VFT domain. Even if these domains share a good percent of

sequence homology with the respective sequence of the other receptor subunits, they are not strictly necessary for the activation of the $GABA_B$ receptor. During the activation, orthosteric ligands bind the VFT domain of $GABA_{B1}$ receptor subunit. Then, the change of conformation mediated by the agonist influences the 7-TM domain of $GABA_{B2}$ receptor subunit through specific interactions. For this reason, this process is named *trans-activation* and it is present also in other heterodimers receptors (Galvez et al., 2001). This event seems to be quite different from the mechanism of homodimerization of mGlu receptors. In mGlu receptors, despite the presence of dimers is necessary for the full activation of the receptors, the monomeric structure is sufficient to trigger a partial activation of the mGlu receptor (Kniazeff et al., 2011). In contrast, the missing of the specific domains, i.e. the VFT domain for $GABA_{B1}$ and the 7-TM domain for $GABA_{B2}$, results in an non functional $GABA_B$ receptor. Indeed, it has been already observed that the deletion of $GABA_{B2}$ VFT domain, which is not involved in the mechanism described, results in a functional $GABA_B$ receptors. These changes in receptor functionality are equally obtained with the selective deletion of the $GABA_{B1}$ 7-TM domain. Altogether, these data confirm the fact that the signal induced by the agonist proceeds from the $GABA_{B1}$ VFT domain to the $GABA_{B2}$ 7-TM domain (Monnier et al., 2011). However, the cooperativity between the domains of the $GABA_{B1}$ and $GABA_{B2}$ receptors subunits is necessary in order to have a functional and responsive $GABA_B$ receptor. Indeed, it has been demonstrated that $GABA_{B1}$ 7-TM domain increases the efficiency of $GABA_{B2}$ to couple with the G protein (Galvez et al., 2001) while the $GABA_{B2}$ VFT domain improves the agonist affinity mediated by the VFT domain of $GABA_{B1}$ (Kaupmann et al., 1998).

Like other metabotropic receptors, also $GABA_B$ receptors have different isoforms and splice variants. It is worth to remember two different isoforms of the $GABA_{B1}$ receptors: the $GABA_{B1a}$ and $GABA_{B1b}$ subunits. The $GABA_{B1a}$ isoform was found in the

presynaptic terminal while the $GABA_{B1b}$ isoform was detected in the postsynaptic component (Kaupmann et al., 1997). These isoforms differ because of the presence of two protein sequences, or sushi domains, in the N-terminus sequence of the $GABA_{B1}$ receptor, although both the receptor proteins undergo comparable activation mechanisms and express identical VFT conformation (Kaupmann et al., 1997; Frangaj et al., 2017).

As far as the pharmacology of the $GABA_B$ receptor is concerned, one of the best described agonist of $GABA_B$ receptor is Baclofen, an analogue of GABA (Bowery et al., 2002). Baclofen is a drug approved for muscle spasticity but also finds other applications in drug addiction and muscle spasms. Beyond this well-known compound, a relative high number of compounds are available acting as orthosteric agonists, antagonists and allosteric modulators, which interact with $GABA_{B1}$ or $GABA_{B2}$ receptor subunits in specific regions (Kniazeff et al., 2011; Frangaj et al., 2017). Interestingly, activation by allosteric modulators of the $GABA_{B2}$ receptor subunit is sufficient to trigger some intracellular events like the activation of the ERK/CREB pathway, underlining a possible therapeutic role for this class of compounds (Tu et al., 2007; Frangaj et al., 2017).

Signal transduction, receptor-mediated effects and clinical implications

The $GABA_B$ protein shows a lifecycle similar to that of other Class C GPCRs proteins, starting from the post-transcriptional modifications of the proteins to the insertion in the cell surface. The regulation of all these events alters the number of functional the $GABA_B$ receptors in the plasma membrane (Benke et al., 2012). A crucial step of the $GABA_B$ protein trafficking is the early heterodimerization of the $GABA_{B1}$ and $GABA_{B2}$ proteins in the ER of the cells. Specifically, $GABA_{B1}$ possesses an arginine-based ER retention/retrieval signal (RXR) in the C-terminal domain which prevents the trafficking

of the receptor subunits. This block is mediated by the coat protein complex I (COP I) which binds the RXR sequence (Calver et al., 2001; Brock et al., 2005). The heterodimerization with the $GABA_{B2}$ subunit masks this retention site and allows the $GABA_{B1}$ receptor subunits to leave the ER. In contrast, $GABA_{B2}$ receptor subunits do not show this type of control (Benke et al., 2012).

Heterodimerization of the receptor subunits is also implicated in the endocytosis and degradation of the protein. In fact, $GABA_B$ receptors internalize as heterodimers and $GABA_{B2}$ receptor subunit seems to control this event (Grampp et al., 2008). Little is known about the degradation processes but what is crucial is the fine and fast control of the balance between degradation and recycling of the $GABA_B$ receptors (Benke et al., 2012) In conclusion, the data so far available suggest that $GABA_B$ receptors constitutively participate to events of trafficking and internalization/transport to the cell surface at a high rate, mediated by different factors like the activation of the classical clathrin-dependent pathway. The physiological consequences of these movements are the presence of a pool of intracellular receptors which can be rapidly recruited and transported to the cell surface by shifting the balance from degradation/recycling to the expression in plasma membrane (Benke et al., 2012).

The desensitation events participate to control and regulate the membrane $GABA_B$ receptor availability. There are three main different mechanisms that were suggested to be involved in the desensitation of the $GABA_B$ receptors (Kanaide et al., 2007; Benke et al., 2012). In the cerebellum, the main mechanism of desensitation is mediated by the G protein coupled receptors kinase (GRKs) 4 and 5 which promote agonist-induced desensitation of $GABA_B$ receptors by direct association between GRK4/5 with $GABA_{B2}$ proteins. For this reason, this process is indicated as phosphorylation-independent desensitation event (Kainade et al., 2007). In the cortex and hippocampus, the predominant desensitation mechanism is a phosphorylation-dependent process. This

kind of desensitation is mediated by protein kinase C (PKC) and NEM-sensitive fusion protein (NSF). Specifically, PKC promotes the phosphorylation of specific sites in the $GABA_B$ receptors to induce the desensitation. The NSF sequence masks this site and avoids the desensitation. The characteristics of this process are not completely clarified, but the entire event seems to be influenced by activation of intracellular pathway involving Ca^{2+} signal and PKC induction (Pontier et al., 2006). Finally, the third main mechanism of desensitization depends on the binding of the $GABA_B$ receptors to the potassium channel tetramerization domain-containing protein (KCTD). This interaction creates a sort of proteasome which can modify many pharmacological parameters of the $GABA_B$ receptor, like agonist affinity, and promotes its desensitation. The precise mechanism is not completely understood, and it seems to acquire unique characteristics among regions in the CNS (Schwenk et al., 2010). Opposite to these mechanisms of desensitation, it is worth to remember that some phosphorylative events are implicated in the stabilization of the $GABA_B$ receptor in the plasma membrane. One example is the PKA-AMPK phosphorylation of the serine 783 in the C terminus domain of the $GABA_{B1}$ and the $GABA_{B2}$ receptor subunits, which stabilize the activity of the orthosteric agonist Baclofen (Kuramoto et al., 2007).

The $GABA_B$ receptors are generally coupled to the Gαi and Gαs proteins and they can regulate three different signal pathways: G protein activated inwardly-rectifying K^+ channels (GIRK), inhibition of the voltage-gated Ca^{2+} channels (Ca_v) and inhibition of the PKA-adenylyl cyclase pathway (Bettler et al., 2004). In general, the $GABA_B$ receptor activation leads to the inhibition of cAMP production mediated by the Gα subunit of the G protein. This event carries out a repression of the activation of intracellular pathway and a block of the mobilization of neurotransmitter vesicles. In opposite, $GABA_B$ receptors coupled to the Gαs protein can induce the production of the

cAMP and this dualistic activity seems to be important for the synaptic plasticity (Terunuma, 2014 and 2018).

The Gβγ subunit is linked to the modulation of GIRK channels and Ca_v channels (Fernández-Alacid et al., 2009). The $GABA_B$ receptor can strongly induce the activation of GIRK proteins which in turn favour the inhibition of neurotransmitters release by causing hyperpolarization (Ladera et al., 2008). This event is preferentially a postsynaptic event while the block of Ca_v channels is mainly a presynaptic process. The Ca_V channels, particularly the P/Q ad N-type Ca^{2+} channels, are robustly blocked by the action of $GABA_B$ agonists. This process leads to the repression of Calcium influx and neurotransmitters release beyond the inhibition of calcium-dependent processes. Consequently, these events explain why Baclofen is used to block muscle spasticity and spasms (Benke et al., 2012; Terunuma, 2018). However, it is worth reminding that these processes are not generally strictly correlated to their pre/post synaptic localization (Benke et al., 2012).

These events and the different mechanisms of the activation of the $GABA_B$ receptors have a strong influence in the effects carried out by these receptors in physiological and pathological condition. The $GABA_B$ receptor mediates both the slow and the prolonged inhibition which is important for different mechanisms of neuronal plasticity and memory formation (Terunuma, 2018). The $GABA_B$ receptors are altered in different neurological disorder like epilepsy, depression, drug addiction and pain. These findings confirm why, beside its use to treat muscle spasticity and spasms, Baclofen is considered for other therapeutic applications like the control of pain. Drug addiction as well as the inhibition of cognitive process still represent an important field of application of $GABA_B$ receptors, due to the positive effects obtained with Baclofen (Bowery, 2016).

Localization, synaptic distribution and dimerization

The $GABA_B$ receptor is one of the most widespread inhibitory receptor in the CNS. It is widely expressed in the cortex, cerebellum and hippocampus, especially in the interneurons, where it can alter the excitability of neuronal circuits. The $GABA_B$ receptors are presynaptic receptors but they can be found also at the postsynaptic site. This different synaptic localization reflects structural and functional characteristics. In fact, presynaptic receptors possess the $GABA_{B1a}$ isoform while the postsynaptic receptors possess the $GABA_{B1b}$ isoform. In addition, postsynaptic $GABA_B$ receptors is strictly bind to the GIRK protein family which in turn promotes hyperpolarization and synaptic plasticity (Terunuma et al., 2018).

Until its discovery, the $GABA_B$ receptor draw attention due to its heterodimeric nature. Indeed, the $GABA_B$ receptor is an obligatory heterodimer. It has been already discussed the implication of heterodimerization of $GABA_{B1}$ and $GABA_{B2}$ receptors subunits in all the receptor processes, from the mechanism of modification from the ER to the internalization / trafficking events. The trend of the $GABA_B$ receptors to heterodimerize is also reflected by its capability to find different interactions with other receptors, even if they do not belong to the Class C GPCR.

First, the $GABA_B$ receptor can strictly interact with $GABA_A$ channels and it can carry out a positive or negative interaction in inhibiting neurotransmission (Tao et al., 2013). In addition, it has been observed that $GABA_B$ receptor can interact with different glutamatergic receptors. The NMDA and AMPA ionotropic receptors can modulate the activity of $GABA_B$ receptors as well as the mGlu1 receptor activity can strictly influence the $GABA_B$ receptor activation and life cycle. In the same manner, the $GABA_B$ receptor can modulate mGlu1 receptors through a direct interaction or by alterating the intracellular pathways (Kantamneni et al., 2013; Vergassola et al., 2018). Finally, it has been demonstrated that the $GABA_B$ receptors can heterodimerize with

several other receptors like tyrosine kinase receptor (RTKs) and insulin like growth factor-1 receptor (IGF-1R) (Terunuma, 2016).

AIM OF THE RESEARCH

During my PhD student course, my research aimed at characterizing from a pharmacological and functional point of view the metabotropic glutamate receptors in the presynaptic nerve terminals (here referred as synaptosomes) of different area of rodents CNS. I focused on two main research projects:

- The first one was dedicated to the pharmacological characterization of mGlu2/3 autoreceptors in cortical and spinal cord nerve endings. Starting from previous work (Di Prisco et al., 2016), the novelty of the study I was involved in relied on the use of new ligands able to differentiate between the mGlu2 and the mGlu3 receptor proteins and of a new experimental approach which allowed the "immuno-pharmacological characterization" of the two receptors under investigation.

  Based on the results from these experiments and considering previous data it was concluded that homomeric mGlu3-preferring autoreceptors dimers preferentially account for the modulation of glutamate release from spinal cord synaptosomes while mGlu2-preferring, mGlu3-containing heteromeric assembly are present in cortical synaptosomes where they control glutamate overflow.

  During my PhD training, this study was also extended to investigate whether the presynaptic mGlu2/3 autoreceptors in spinal cord nerve endings colocalize and functional cross-talk with the presynaptic 5-HT_{2A} heteroreceptors. The results suggested the existence of the physical association between these two receptors that cross-talk in an antagonist-like fashion. This interaction could have important therapeutic implications for the treatment of spinal cord diseases typified by excess of glutamate.

  Finally, I started to characterize the mGlu2/3 receptors in the mouse hippocampus. In collaboration with Professor Emanuele Sher at Ely Lilly

laboratories, I approached the pharmacological characterization of these receptors by an electrophysiological approach. Specifically, I recorded the excitatory post synaptic potential from CA1 and DG hippocampal slices in order to evaluate the impact on the glutamatergic transmission of Group II mGlu receptors in this area.

- The second project aimed at confirming the presence and at demonstrating the functional role of presynaptic mGlu1 heteroreceptors controlling GABA release in cortical nerve endings. The study unveiled the colocalization and the functional cross-talk linking presynaptic mGlu1 heteroreceptors and $GABA_B$ autoreceptors in GABAergic nerve endings. Differently from the mGlu2/3-5-HT_{2A} receptor complex, mGlu1 and $GABA_B$ receptors do not physically interact, but functionally cross-talk to control glutamate and GABA release in cortical nerve endings. We proposed that this interaction is mediated by functional processes which control the $GABA_B$ expression and trafficking in-out the cell surface, affecting the efficacy of the orthosteric agonist Baclofen at the $GABA_B$ receptors. The use of mice lacking mGlu1 receptor confirmed our studies. These overall results suggest a complex mechanism of interaction linking mGlu1 and $GABA_B$ receptors in the cortical presynaptic nerve terminal which could have important physio-pathological consequences.

RESULTS AND DISCUSSION

The mGlu2/3 receptors structural and functional characterization

The mGlu receptors are strictly involved in the development of several neurological diseases and neuropsychiatric disorders (Nicoletti et al., 2011), which in most of the cases are typified by an excess of glutamate or by the hyperactivity of neurotransmitters exocytosis. In this context, the role of mGlu receptors belonging to the second group is particularly attractive because of the negative feedback mechanism of control they can exert at presynaptic terminals where the release of transmitters occurs. Here, they can be activated by external glutamate, when its concentration increases pathologically in the synaptic cleft, leading to the reduction of exocytosis.

The pharmacological characterization of mGlu2/3 receptors has been slowed down by the lack of ligands able to discriminate between the two receptor proteins. The mGlu2 and the mGlu3 receptor proteins share about the 70% of sequence homology and most of the ligands that bind these receptors interact with both the receptor proteins. The lack of selective ligands for the two receptor proteins also limited so far to evaluate the homo and heterodimerization of these receptors.

In recent years, new ligands were synthesized able to recognize the mGlu2 and the mGlu3 receptors. Furthermore, anti-mGlu2 and anti-mGlu3 receptors antibodies became available to be used for deciphering the protein composition of the native receptors in selected neuronal populations (Gupta et al., 2008). Taking advantage from these new tools, I investigated the pharmacological profile of presynaptic release-regulating mGlu2/3 autoreceptors and their possible homo/heterodimerization in mouse cortex and spinal cord. In particular, the research aimed at understanding the expression

and the pharmacological profile of these receptors in different CNS regions as well as their homodimerization or heterodimerization with other receptors.

To achieve this aim, I used the technique of the superfusion of a monolayer of synaptosomes in order to characterize the contribution of the mGlu2 and the mGlu3 receptors in the release-regulating activity of presynaptic Group II mGlu receptors. Synaptosomes were also used in biochemical/immunocitochemical studies to confirm the presence of the two receptor proteins in this compartment of chemical synapses. Please refers to the section "Methods" for technical details.

On the existence of mGlu2 and mGlu3 receptor proteins in cortical synaptosomes

Experiments were first performed to confirm the expression of the mGlu2/3 receptor proteins in cortical nerve endings and possibly to evidentiate different associations of the two receptor proteins (Cartmell and Schoepp, 2000, Niswender and Conn, 2010).

The results from Western Blot analysis unveiled the presence of mGlu2/3 receptor proteins in cortical synaptosomes (Figure 1A). As a first approach I used an antibody able to recognize both the mGlu2 and the mGlu3 receptor proteins. This anti-mGlu2/3 antibody recognized proteins having a mass consistent with the presence of both the monomeric and the dimeric form of the mGlu2/3 receptors. The analysis was then carried out with selective anti-mGlu2 and anti-mGlu3 antibodies. The specificity of the two antibodies was confirmed thank to a collaboration with Ferdinando Nicoletti and Giuseppe Battaglia (Neuromed, see Olivero et al., 2017, Figure 4, also for the negative controls), by using knocked out mice lacking respectively the mGlu2 or the mGlu3 receptor proteins. The selective anti-mGlu2 antibody unveiled a mGlu2-immunoreactivity compatible with the monomeric (about 100 kDa) and the dimeric (about 200 kDa) form of the receptor protein (Figure 1B) in cortical lysates.

Concomitantly, the selective anti-mGlu3 antibody highlighted a clear immunopositivity corresponding to the dimeric form (about 200 kDa) of the protein and a slight signal for the monomeric form (about 100 kDa) of mGlu3 receptor protein (Figure 1C).

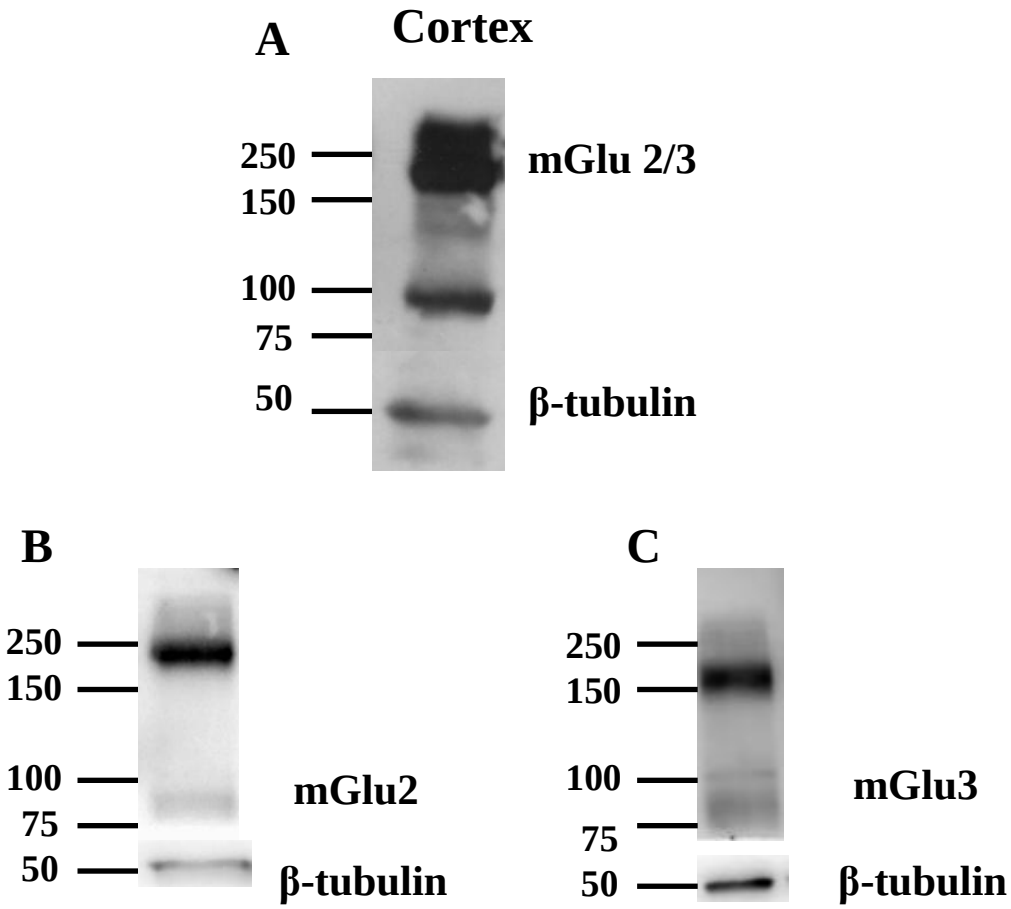


Figure 1: Western Blot analysis with selective anti Group II mGlu receptor antibodies in cortical synaptosomes. Western blot analysis of cortical synaptosomal lysates by using the selective anti-mGlu2/3 antibody (A), anti-mGlu2 antibody (B) and anti-mGlu3 antibody (C). β-butulin was used as neuronal marker and loading control. The figure shows a representative image of five analysis carried out in different days. **A)** The anti-mGlu2/3 antibody recognizes a band size compatible with the monomeric (about 100 kDA) and dimeric (about 200 kDa) form of the mGlu2/3 receptor proteins in cortical synaptosomes. **B)** The selective anti-mGlu2 antibody recognizes a band size compatible with the monomeric (about 100 kDA) form and more pronounced band size compatible with the dimeric (about 200 kDa) form of the mGlu2 receptor proteins in cortical synaptosomes. **C)** The selective anti-mGlu3 antibody recognizes a band size compatible with the monomeric (about 100 kDA) form and more

pronounced band size compatible with the dimeric (about 200 kDa) form of the mGlu3 receptor proteins in cortical synaptosomes.

Confocal analysis was then carried out to confirm the presence of the mGlu2/3 receptor proteins in cortical glutamatergic synaptosomes (see also Di Prisco et al., 2016). Experiments were aimed at highlighting the colocalization of the mGlu2/3 immunopositivity with the syntaxin-1α (Stx-1α) immunofluorescence (Figure 2, line 1), here used as a marker of presynaptic particles, as well as with the immunopositivity for the vesicular glutamate transporter 1 (VGLUT1), here used as a selective marker of glutamatergic synaptosomes (Figure 2, line 2). The quantification of the protein colocalization revealed that 71 ± 2% of VGLUT1-positive synaptosomes also expressed mGlu2/3-immunoreactivity (Figure 2, line 1 panel "c"), and that the 64±4% of the sintaxin1α-positive synaptosomes were mGlu2/3-immunoreactive (Figure 2, line 2 panel "f").

Then, the distribution of the mGlu2 and the mGlu3 receptor proteins was analyzed by quantifying the colocalization of the mGlu2-/mGlu3-immunoreactivities with the VGLUT1 immunofluorescence by using the selective antibodies (Figure 2, line 3 and 4). The quantification of the protein colocalization revealed that 91±4% of VGLUT1-positive synaptosomes also expressed mGlu2-immunoreactivity (Figure 2, line 3, panel "i"), while the 74±4% of the VGLUT1-positive synaptosomes were also mGlu3-immunoreactive (Figure 2, line 4, panel "l").

Finally, I analysed the colocalization of mGlu2 and mGlu3 receptor proteins in cortical glutamatergic synaptosomes (Figure 2, line 5). The quantification indicated that 83±4% of the mGlu2-positive particles were also positive for mGlu3-immunoreactivity, while the 71±3% of the mGlu3-positive synaptosomes were also mGlu2-immunoreactive (Fgure 2, line 5, panel "o").

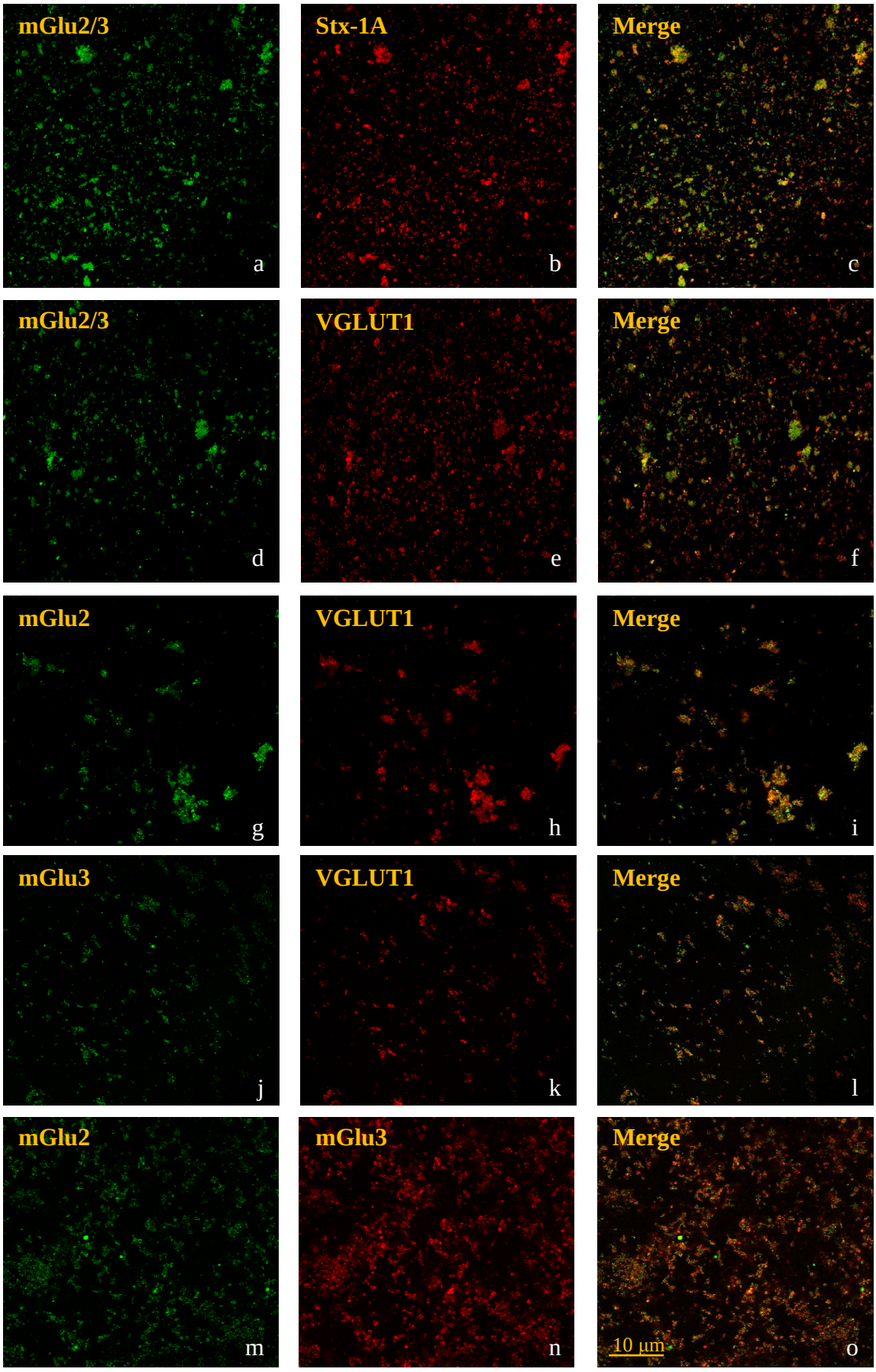
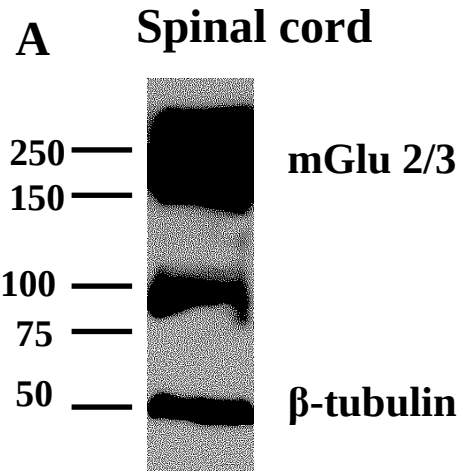

mGlu2/3
a
Stx-1A
b
Merge
c
mGlu2/3
d
VGLUT1
e
Merge
f
mGlu2
g
VGLUT1
h
Merge
i
mGlu3
j
VGLUT1
k
Merge
l
mGlu2
m
mGlu3
n
Merge
10 µm
o

Figure 2: Confocal analysis of Group II mGlu receptor proteins in cortical synaptosomes. Confocal analysis of the colocalization of mGlu2/3 receptor proteins with syntaxin-1A (line 1), with VGLUT1 (line 2) and of the mGlu2 and of the mGlu3 receptor proteins immunoreactivities with VGLUT1 (line 3 and 4) in cortical nerve endings. The image also report the colocalization of mGlu2-positive particles with mGlu3-immunoreactivity in cortical nerve endings (line 5). The figures show representative images of five experiments carried out on different days.

On the existence of mGlu2 and mGlu3 receptor proteins in spinal cord synaptosomes

I then performed a comparable analysis of the Group II mGlu receptors in mouse spinal cord synaptosomes.

Again, Western Blot analysis was carried out to confirm the expression of mGlu2/3 receptor proteins in spinal cord synaptosomal lysates (see also Di Prisco et al., 2016). Results unveiled the presence of mGlu2/3 receptor proteins in spinal cord synaptosomes (Figure 3A). In the synaptosomal lysates the anti-mGlu2/3 antibody recognized proteins having a mass consistent with the presence of both the monomeric and the dimeric form of the mGlu2/3 receptor proteins. The analysis was then carried out with the selective anti-mGlu2 and anti-mGlu3 antibodies (see also Olivero et al., 2017). The anti-mGlu2 antibody confirmed the presence of mGlu2-immunoreactivity compatible with the monomeric (about 100 kDa) and dimeric (about 200 kDa) form of the receptor protein (Figure 3B). Similarly, the selective anti mGlu3 antibody unveiled a clear immunopositivity corresponding to the dimeric form (about 200 kDa) of the protein but a slight immunopositivity for the monomeric form (about 100 kDa) of the receptor protein (Figure 3C).



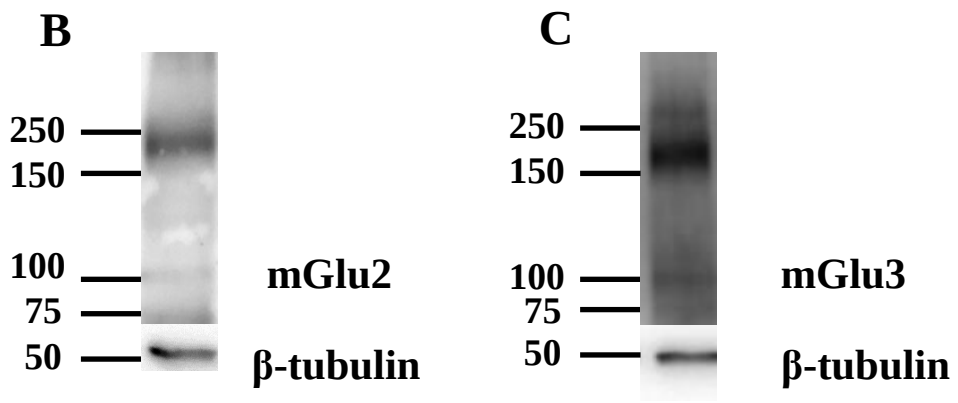
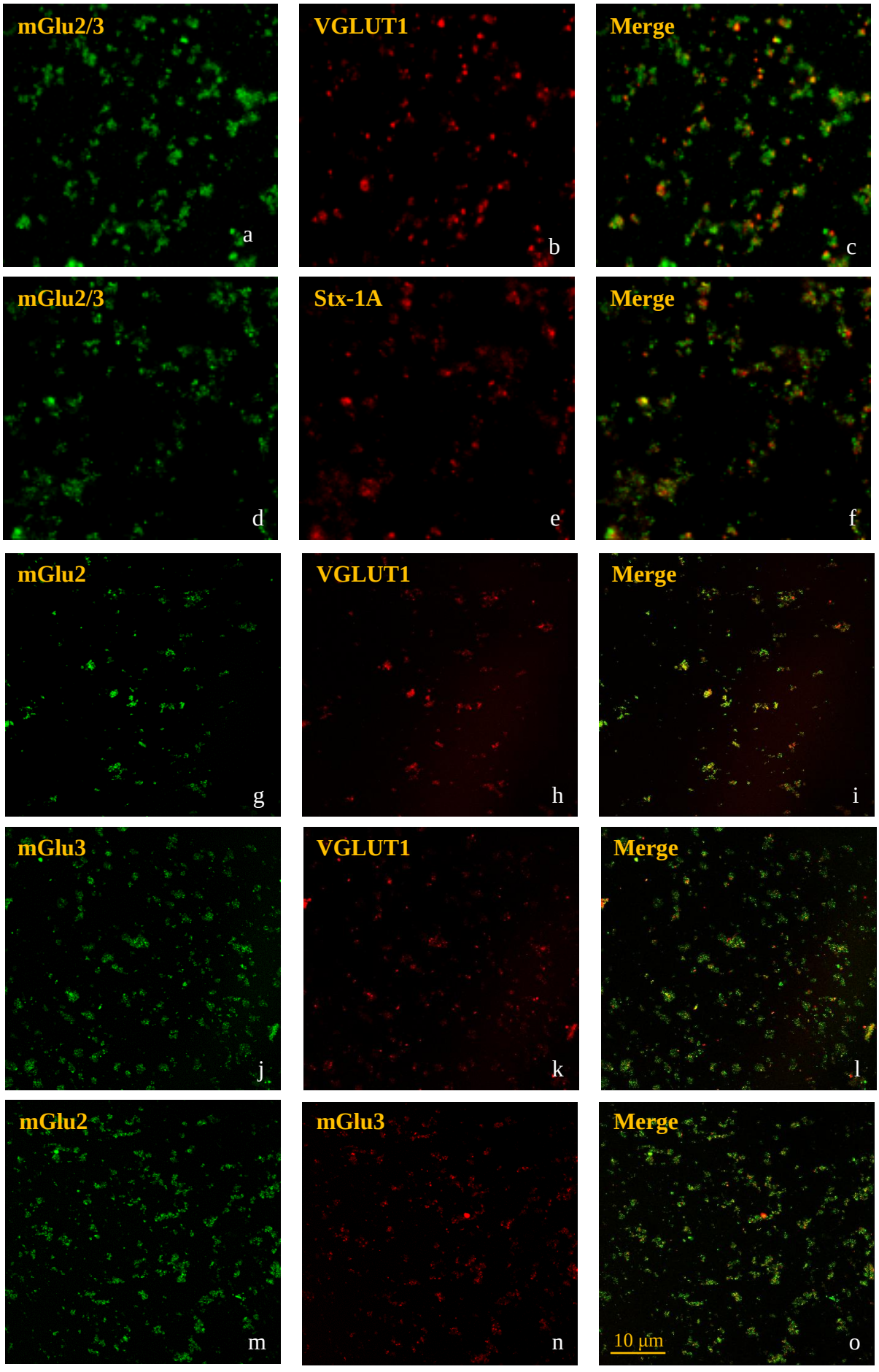


Figure 3: Western Blot analysis with selective anti Group II mGlu receptor antibodies in spinal cord synaptosomes. Western blot analysis in spinal cord synaptosomal lysates with the anti-mGlu2/3 antibody (A), the anti-mGlu2 antibody (B) and the anti-mGlu3 antibody (C). β-butulin was used as a neuronal marker and loading control. The figure shows a representative image of five analysis carried out in different days. **A)** The anti-mGlu2/3 antibody recognizes a band size compatible with the monomeric (about 100 kDA) and dimeric (about 200 kDa) form of the mGlu2/3 receptor proteins in spinal cord synaptosomes. **B)** The selective anti-mGlu2 antibody slightly recognizes a band size compatible with the monomeric (about 100 kDA) form and a more pronounced band compatible with the dimeric (about 200 kDa) form of the mGlu2 receptor proteins in spinal cord synaptosomes. **C)** The selective anti-mGlu3 antibody recognizes a band compatible with the monomeric (about 100 kDA) form but a more pronounced band compatible with the dimeric (about 200 kDa) form of the mGlu3 receptor proteins in spinal cord synaptosomes.

Confocal analysis was then performed to confirm the presence of the mGlu2/3 receptor proteins in spinal cord glutamatergic synaptosomes (see also Di Prisco et al., 2016). To highlight the presence of the mGlu2/3-immunoreactivity in spinal cord glutamatergic particles I carried out experiments to unveil the colocalization of the mGlu2/3-positive particles with the syntaxin-1α (Stx-1α) immunofluorescence (Figure 4, line 1) and the colocalization of mGlu2/3-positive particles with VGLUT1 immunofluorescence (Figure 4, line 2). The quantification of the signalling revealed that 80±3% of VGLUT1-positive synaptosomes also expressed mGlu2/3-immunoreactivity (Figure 4, line 1, panel "c"), while the 68±4% of the sintaxin1α-positive synaptosomes were also mGlu2/3-immunoreactive (Figure 4, line 2, panel "f").

Then, the presence of the mGlu2 and the mGlu3 receptor proteins was confirmed in the spinal cord glutamatergic presynaptic terminals by analysing the colocalization of the mGlu2-positive and the mGlu3-positve particles with the VGLUT1 fluorescence (Figure 4, line 3 and 4). The quantification of the protein colocalization revealed that 56±3% of VGLUT1-positive synaptosomes also expressed mGlu2-immunoreactivity (Figure 4, line 3, panel "i"), while the 47±7% of the VGLUT1-positive synaptosomes were also mGlu3-immunoreactive (Figure 4, line 4, panel "l").

Finally, I analysed the colocalization of mGlu2 and mGlu3 receptor proteins in spinal cord synaptosomal particles (Figure 4, line 5). The quantification indicated that 33±6% of the mGlu2-positive particles also expressed mGlu3-immunoreactivity, while the 52±9% of the mGlu3-positive synaptosomes were also mGlu2-immunoreactive (Figure 2, line 5, panel "o").


mGlu2/3
a
VGLUT1
b
Merge
c
mGlu2/3
d
Stx-1A
e
Merge
f
mGlu2
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h
Merge
i
mGlu3
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VGLUT1
k
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l
mGlu2
m
mGlu3
n
Merge
10 µm
o

Figure 4: Confocal analysis of Group II mGlu receptors in spinal cord synaptosomes Confocal analysis of the colocalization of mGlu2/3 receptor proteins with syntaxin-1A (line 1), with VGLUT1 (line 2) and of the mGlu2 and the mGlu3 receptor protein immunoreactivities with VGLUT1 (line 3 and 4) in spinal cord nerve endings. The figure also reports colocalization of mGlu3-positive with mGlu2-immunoreactivities in spinal cord nerve endings (line 5). The figure shows representative images of five experiments carried out on different days.

To briefly summarize, the confocal microscopy of the mGlu2 and the mGlu3 receptor proteins unveiled that these proteins are robustly expressed in cortical glutamatergic synaptosomes, with an apparent slight predominance of the mGlu2 receptor proteins. This could suggest the existence of a heterodimeric association of mGlu2 and mGlu3 proteins in the same particles. This conclusion is well in line with the results from Western blot analysis with the mGlu2/3 antibody and the selective anti-mGlu2 or the anti-mGlu3 antibodies, although the possibility that mGlu2 and mGlu3 homodimers also colocalize in the same particles deserves confirmation. Comparable conclusions could be proposed based on the results from Western blot analysis and confocal microscopy in spinal cord synaptosomes, although the percentage of colocalization of the two receptor proteins in these regions differs from that observed in the cortex.

The pharmacological characterization of the mGlu2/3 receptors in cortical and spinal cord synaptosomes

To definitively assess whether the Group II mGlu autoreceptors in cortical and spinal cord synaptosomes differ in term of protein composition and pharmacological profile, I approached the pharmacological characterization of the two receptors by a functional point of view, by using "the up-down superfusion of a thin layer of synaptosomes" to monitor the activity of selected agonists/antagonists on the release of glutamate.

The "Up and down perfusion of a thin layer of synaptosomes" technique is a suitable approach to study the modulation of neurotransmitters release mediated by presynaptic receptors. The technique was first described by Maurizio Raiteri and colleagues (Raiteri et al., 1974) and it is nowadays used, with minor modification, to characterize by a functional point of view the subunit composition and the role of presynaptic release-regulating receptors. Briefly, synaptosomes are layered as a monolayer on microporous filters and up-down superfused with physiological solutions. Then, the superfusate

fractions are collected to monitor the release of preloaded tritiated or endogenous transmitters. The continuous up-down superfusion of these particles assures the rapid removal of any endogenous compounds actively released by synaptosomes, before they can interact and activate structures such as receptors or carriers present on the nerve endings, thereby excluding any indirect effects. The activation of receptors on the synaptosomal membranes only can be achieved by adding selective receptor ligands to the perfusion medium. Under these experimental conditions, any ligand-induced changes to the transmitter release can be attributed exclusively to the interaction of the ligands with the respective receptors on nerve endings. In the experiments described below, the release of glutamate was triggered by exposing synaptosomes transiently (90 sec) to a mild depolarizing stimulus (12 mM or 15 mM KCl solution). The efficiency of the presynaptic mGlu2/3 autoreceptors in controlling glutamate exocytosis was measured by quantifying the changes in the depolarization evoked transmitter exocytosis elicited by the concomitant presence of mGlu2/3 receptor ligands and the KCl solution (Fig 5, see also the section “Methods”).

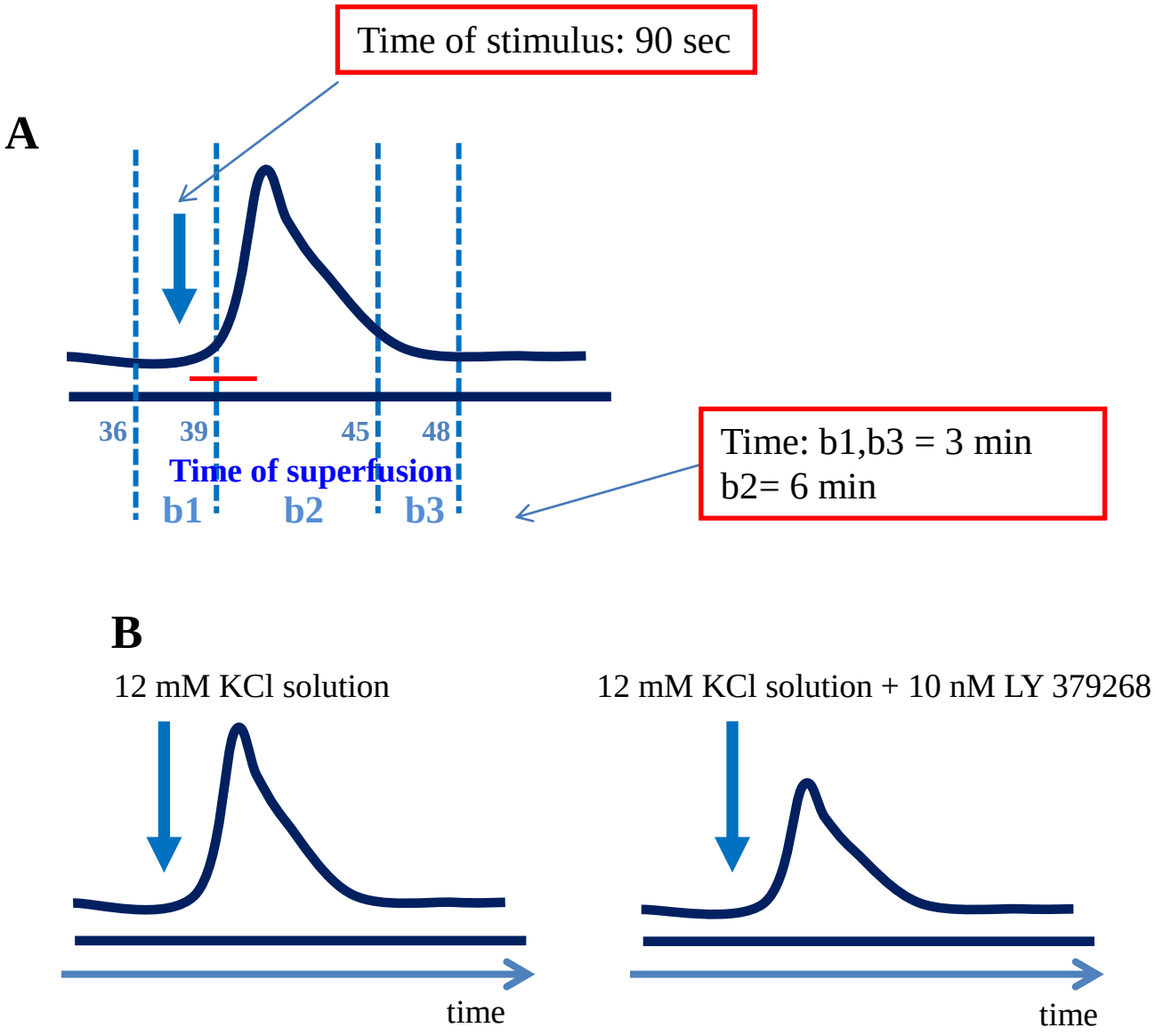


Figure 5: Time-schedule of the superfusion experiments. Schematic representation of the time course in an "up down superfusion" experiments. **A)** After labelling with radioactive tracer (i.e. [^{3}H]D-ASP to monitor glutamate exocytosis), synaptosomes are layered at the bottom of thermostated superfusion chambers (Raiteri et al., 1974, see also the section "Methods"). Synaptosomes are then exposed transiently (90 sec) to KCl enriched solution in the presence (**B** bottom, right) or in the absence (**B** bottom, left) of receptor agonist(s). Fractions are collected as follows: b1, a 3 min fraction before (t = 36-39 min) and b3, a 3 min fraction after (t = 45-48 min) a six min fraction b2 (t = 39-45 min) containing the KCl-evoked transmitter overflow. **B)** Schematic representation of the time course of the transmitter release elicited by the depolarizing stimulus alone (left) or in the presence of the mGlu2/3 agonist LY379268 (right).

Ligands	Properties
LY 379268	mGlu2/3 agonist
LY 341495	mGlu2/3 antagonist
NAAG	mGlu3 agonist
LY 541850	mGlu2 agonist mGlu3 antagonist
LY 566332	mGlu2 PAM
LY 2389575	mGlu3 NAM
BINA	mGlu2 PAM
ML337	mGlu3 NAM
Anti mGlu2 antibody	Anti NH_2-mGlu2 antibody
Anti mGlu3 antibody	Anti NH_2-mGlu3 antibody

Table 1: The list of the mGlu2/3 ligands used for the pharmacologically characterization of the native Group II mGlu receptors. The table reports the compounds used to perform the functional superfusion studies, including orthosteric, allosteric molecules and selective antibodies for the mGlu2/3 receptor proteins. For each ligand is reported the predicted membrane target.

Effects of mGlu2/3 orthosteric ligands on the mGlu2/3 autoreceptors in cortical and spinal cord nerve endings

Previous studies (see Di Prisco et al., 2016) unveiled the activity of different orthosteric ligands at the mGlu2/3 autoreceptors in cortical (Table 2) and spinal cord (Table 3) nerve terminals.

The first main finding of the study was that LY 379268, a broad spectrum orthosteric agonist acting at both mGlu2 and mGlu3 receptors (Schoepp et al., 1999), dramatically decreases the glutamate exocytosis in a region-dependent, concentration-sensitive fashion. LY 379268 inhibited in a concentration-dependent manner the [^{3}H]D-ASP overflow evoked by 12 mM KCl solution from cortical nerve endings with an apparent EC_{50} of 1.50±1.15 nM and reaching the maximal inhibitory activity when added up to 10 nM (Table 2). Similarly, LY 379268 also inhibited in a concentration-dependent manner the [^{3}H]D-ASP overflow evoked by 15 mM KCl solution from spinal cord nerve endings. In this case the apparent EC_{50} amounted to 0.15±0.37 pM and a maximal inhibitory effect exerted by the agonist was when added up to 0.1 nM (Table 3). In both cortical and spinal cord synaptosomes, the broad spectrum orthosteric antagonist LY 341495 efficiently prevented the inhibitory effect elicited by LY 379268 (Table 2 and 3).

Although the LY 379268 robustly activates mGlu2/3 receptors in both cortical and spinal cord synaptosomes, the impressive difference in the potency of the agonist in the two synaptosomal preparations seems best interpreted by assuming the involvement of two different mGu2/3 receptor subtypes. Specifically, it was proposed the presence of a high affinity LY 379268-sensitive binding site in spinal cord terminals and of a low affinity LY 379268-sensitive binding site in cortical terminals.

Di Prisco and colleagues (2016) also tested the impact of two other orthosteric ligands: the N-acetylaspartylglutamate (NAAG) and the (1S,2S,4R,5R,6S)-2-amino-4-

methylbicyclo[3.1.0]hexane-2,6-dicarboxylic acid (we refer to as LY 541850). Both in vivo and in vitro studies suggested that NAAG is a selective agonist at the mGlu3 receptors (Neale et al., 2011). Differently, LY 541850 is typified by a double-faced profile since it acts as an agonist at the mGlu2 receptors and as an antagonist at the mGlu3 receptors (Sanger et al., 2013; Hanna et al., 2013).

Differently from the effect of LY 379268, the ligand NAAG was unable to inhibit the 12 mM KCl evoked [3H]D-ASP overflow from cortical synaptosomes, even when added at higher concentration (100 nM) (Table 2). In opposite, NAAG efficiently inhibited glutamate release from spinal cord synaptosomes with an apparent EC_{50} amounting to 0.16±0.28 pM, comparable to EC_{50} of LY 379268 in the same synaptosomal preparation (Table 3). To note, the NAAG-induced inhibition of the KCl-evoked release of tritium in spinal cord synaptosomes was prevented by the concomitantly exposure to LY 341495 (Table 2 and 3).

The effect of LY 541850 was also tested both in cortical and spinal cord presynaptic terminals. As far as double-faced compounds are concerned, the technique of the "up-down superfusion of synaptosomes" represents a method of choice to evaluate the efficacy of compound with a double activity (agonist/antagonist). In superfused synaptosomes, the functional effects elicited by receptors can be only induced by agonists exogenously added to the superfusion medium, because antagonists are expected to be inactive on their own (Raiteri et al., 1974; Marchi et al., 2015), providing evidence that the receptors they bind do not adopt a constitutive active state (Musante et al., 2008). Accordingly, the results observed when testing LY 541850 are well in line with the previous observations. LY 541850 mimicked LY 379268 in controlling glutamate exocytosis from cortical synaptosomes, compatible with an agonist-like activity at mGlu2 autoreceptors in these terminals. In contrast, LY 541850 failed to modulate glutamate exocytosis from spinal cord synaptosomes but it efficiently

prevented the effect of the mGlu2/3 agonist LY 379268, compatible with an antagonist-like activity on the mGlu3 autoreceptors in these terminals (Table 2 and 3).

The previous results so far described allowed the authors to propose the presence of Group II mGlu receptors with a different pharmacological profile in the two CNS regions. The functional results seemed best interpreted by assuming the existence of presynaptic release regulating mGlu2-preffering autoreceptors in cortical nerve endings and of a presynaptic mGlu3-preferring autoreceptors in spinal cord glutamatergic nerve endings. Despite the difficulty to predict the composition and the relative contribution of the mGlu2 and the mGlu3 receptors in both synaptosomal preparations, the results from Confocal microscopy and from Western blot analysis in synaptosomal lysates seemed predictive of the presence of mGlu3 homodimers in spinal cord nerve endings and of mGlu2/3 heterodimeric association in cortical synaptosomes (Di Prisco et al., 2016).

Stimulus	[3H]D-ASP overflow	% of change
12 mM KCl	1.13 ± 0.09	/
0.1 nM LY 379268	0.63 ± 0.12	-44.25 (*)
0.1 nM LY 379268 100 nM LY 341495	1.27 ± 0.10	+12.38
100 nM LY 541850	0.58 ± 0.07	-48.68 (*)
100 nM NAAG	1.18 ± 0.02	+4.42
100 nM LY 541850 100 nM LY 341495	1.22 ± 0.03	+7.96

Table 2: Effect of orthosteric ligands of mGlu2/3 receptors on the 12 mM KCl-evoked [^{3}H]D-ASP release from cortical synaptosomes. The table reports the effect of orthosteric agonists of mGlu2/3 LY 379268, NAAG and LY 541850 and the competitive antagonist LY 341495 added concomitantly to the 12 mM KCl-evoked release from cortical nerve endings. Based on previous results, LY 541850 was used as an agonist in cortical synaptosomes.* significant difference (at least $P < 0.05$) versus 12 mM KCl evoked release (see also Di Prisco et al., 2016).

Stimulus	[^{3}H]D-ASP overflow	% of change
15 mM KCl	1.92 ± 0.13	/
0.1 nM LY 379268	0.76 ± 0.13	-60.42 (*)
0.1 nM LY 379268 100 nM LY 341495	1.89 ± 0.08	-1.57
100 nM LY 541850	1.85 ± 0.08	-3.65
0.01 nM NAAG	0.82 ± 0.1	-57.3 (*)
100 nM LY 541850 0.1 nM LY 379268	1.82 ± 0.23	-5.2
0.01 nM NAAG 100 nM LY 341495	1.55 ± 0.14	-19.27

Table 3: Effect of orthosteric ligands of mGlu2/3 receptors on the 15 mM KCl-evoked [^{3}H]D-ASP release from spinal cord synaptosomes. The table reports the effect of orthosteric agonists of mGlu2/3 LY 379268 and NAAG and competitive antagonists LY 341495 and LY 541850 when added up with the 15 mM KCl-evoked release from spinal cord nerve endings. Based on previous results, LY 541850 was used as an antagonist in spinal cord synaptosomes. *significant difference (at least $P < 0.05$) versus 15 mM KCl evoked release (see also Di Prisco et al., 2016).

Effects of mGlu2/3 allosteric modulators on the mGlu2/3 autoreceptors in cortical and spinal cord nerve endings

Based on the results obtained with the study of mGlu2/3 orthosteric ligands, I carried out superfusion experiments aimed at further implement the pharmacological characterization of the presynaptic release regulating mGlu2/3 receptors in both cortical and spinal cord nerve endings. To achieve this aim I used a double approach: one is the use of ligands that could discriminate the participation of the two receptor proteins to the receptor assemblies and the other one is the innovative immuno-pharmacological approach by using selective antibodies. Selective positive (PAM) and negative (NAM) allosteric modulators, namely the mGlu2 PAMs LY 566332 and BINA, and the mGlu3 NAMs LY 2389575 and ML337, were used to this aim.

Before showing results obtained, however, some aspects deserve discussion. It is recognized that positive allosteric modulators or PAMs increase the receptor mediated activation elicited by submaximal concentration of orthosteric agonists while negative allosteric modulators prevent the activation mediated by the orthosteric agonists regardless the amount of agonist applied. On the other hand, in order to select a suitable concentration of ligands used in our study we considered the concentration-effect relationship of LY 379268 in the cortex and in the spinal cord nerve terminals (Di Prisco et al., 2016). Specifically, I used selected concentrations of the orthosteric agonist leading to a partial inhibition (for the experiments to test the efficacy of PAMs) and to an almost total inhibition (for the experiments to test the efficacy of NAMs).

The first ligands tested are from the LY series synthesized by Jim Moon and colleagues in the Eli Lilly laboratories. These ligands are the compounds LY 566332 and LY 2389575 which behave as almost pure mGlu2 receptor PAM and mGlu3 receptor NAM respectively.

The results of functional experiments showed that both LY 566332 and LY 2389575 were not able to modify on their own the KCl evoked glutamate exocytosis both in cortical and spinal cord synaptosomes. In the same experiments, 1 μM LY 566332 amplified the 3 nM LY 379268-induced inhibition at cortical presynaptic terminals but at the same concentration it did not modify the 30 pM LY 379268-induced inhibition in spinal cord presynaptic terminals, even when added concomitantly to a lower concentration of the orthosteric agonist. On the other side, 1 μM LY 2389575 recovered the inhibition of the glutamate release mediated by 30 pM of LY 379268 from spinal cord synaptosomes. Interestingly, the same concentration of LY 2389575 partially blocked the 10 nM LY 379268 in cortical synaptosomes.

Comparable results were also obtained with the two others allosteric modulators: the mGlu2 receptor PAM BINA and the mGlu3 receptor NAM ML-337 (not shown, see Olivero et al., 2017).

Altogether, these results appear well in line with previous findings with the mGlu2/3 orthosteric agonists. They confirmed the preferential role of mGlu3 in controlling the glutamate exocytosis in spinal cord presynaptic nerve endings and the role of the mGlu2 receptors in the cortical nerve endings. However, the capacity of the mGlu3 NAMs to decrease the LY 379268-induced inhibition in presynaptic cortical nerve endings also suggested that mGlu3 participates to the control of the Group II mGlu receptors activity in cortical nerve endings.

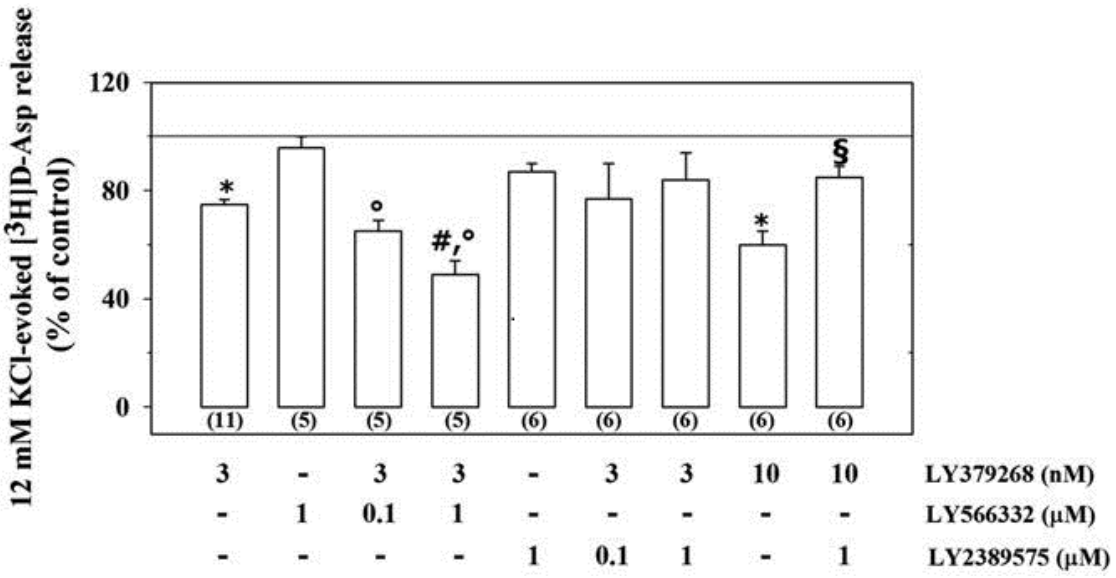


Figure 6: Effect of mGlu2/3 allosteric modulators in cortical synaptosomes. Effect of the allosteric modulators LY 566332, PAM mGlu2, and LY 2389575, NAM mGlu3, on the inhibition of 12mM KCl-evoked [^{3}H]D-ASP overflow elicited by the mGlu2/3 agonist LY 379268 in cortical nerve endings. The 12 mM KCl evoked release of tritium over the basal release amounted to 1.39±0.36 nCi (% of control). Data are the ± SEM of at least four experiments run in triplicate. *P < 0.05 versus the 12 mM KCl-evoked tritium overflow; °P < 0.05 versus the 12 mM KCl/1μM LY 566332 evoked tritium overflow; #P < 0.05 versus the 12 mM KCl/3 nM LY 379268 evoked tritium overflow; §P < 0.05 versus the 12 mM KCl/10 nM LY 379268 evoked tritium overflow (see also Olivero et al., 2018, figure 1).

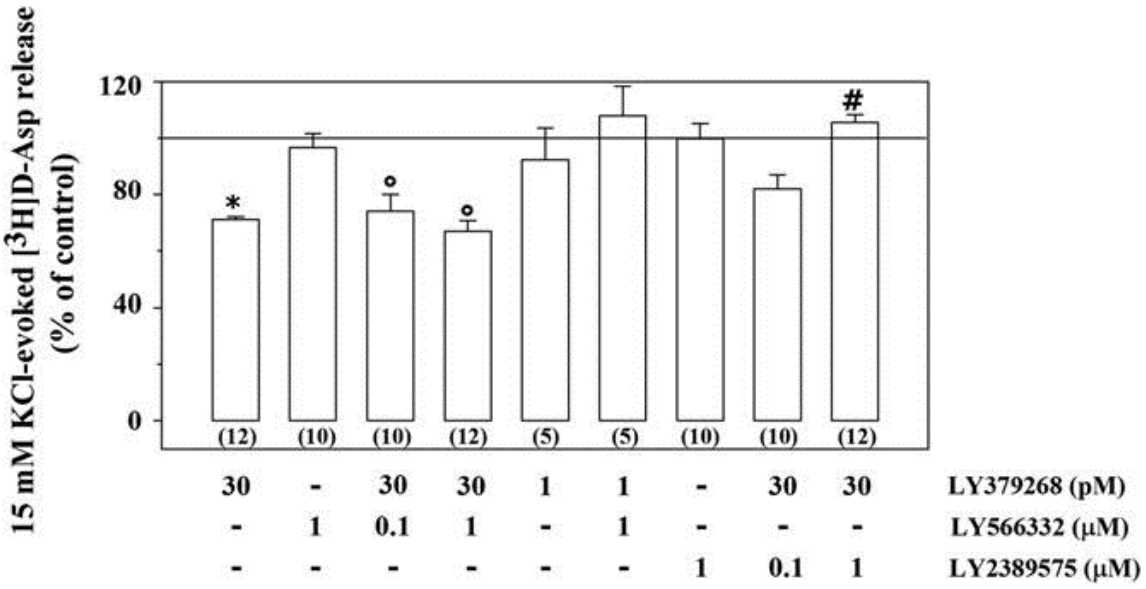


Figure 7: Effect of mGlu2/3 allosteric modulators in spinal cord . Effect of allosteric modulators LY 566332, PAM mGlu2, and LY 2389575, NAM mGlu3, on the inhibition of 15 mM KCl-evoked [^{3}H]D-ASP overflow elicited by the mGlu2/3 agonist LY 379268 in mouse spinal cord synaptosomes. The 15 mM KCl-evoked release of tritium over the basal release amounted to 4.23±0.79 nCi. Results are expressed as a percentage of the 15 mM KCl-evoked tritium overflow (% of control). Data are the mean ± SEM of at least four experiments run in triplicate. *P < 0.05 versus the 15 mM KCl-evoked tritium overflow; °P < 0.05 versus the 15 mM KCl/1μM LY 566332-evoked tritium overflow; #P < 0.05 versus the 15 mM KCl/30 pM LY 379268-evoked tritium overflow (see also Olivero et al., 2018, figure 2).

Effects of selective anti-mGlu2 and anti-mGlu3 antibodies on the mGlu2/3 autoreceptors in cortical and spinal cord nerve endings

Beside the classic pharmacological approach used to characterize the mGlu2/3 autoreceptors profile in the spinal cord and the cortical mouse synaptosomes, the innovative "immuno-pharmacological approach" was used to implement the analysis. This approach relies on the use of selective antibodies to define the pharmacological profile of GPCRs proteins (Gupta et al., 2008; Musante et al., 2008; Di Prisco et al., 2016). Specifically, the binding of a selective antibody to a ligand binding pocket in the extracellular domain can cause the activation or the block of the receptor activity, consequently mimicking the effect of an agonist or antagonist at the receptor involved (Musante et al., 2008; Olivero et al., 2017). These considerations are well in line with the observation that circulating autoantibodies in patients suffering from different autoimmune diseases are associated to a downstream of the action of selective GPCRs in CNS (Makita et al., 2007; Lancaster et al., 2010).

In our superfusion experiments, the synaptosomal particles are incubated with selective anti-mGlu2 antibody (1:1000) and anti-mGlu3 antibody (1:1000) before adding the radioactive tracer (for further details see section "Methods"). Then, the impact of the incubation with antibodies was evaluated on the inhibitory effect exerted by the orthosteric agonist LY 379268 on the KCl evoked release.

The first result observed was that anti-mGlu2 and anti-mGlu3 antibodies did not modify on their own the 12 mM or 15 mM KCl evoked release from both cortical and spinal cord nerve terminals respectively (Figure 8 and 9). It was found that anti-mGlu2 antibody abolished the inhibitory effect of LY 379268 in cortical synaptosomes but, in the same experimental condition, it was ineffective in spinal cord synaptosomes. In contrast, the anti-mGlu3 antibody blocked the LY 379268-induced inhibition of glutamate release in spinal cord synaptosomes, but it less, although significantly,

blocked the LY 379268-induced inhibition of glutamate release in cortical synaptosomes (Figure 8 and 9, see also Olivero et al., 2017, figure 8 and 9). Altogether, these results suggest that selective antibodies preferentially act as antagonists in these experiments.

To conclude, the selective anti-mGlu2 and anti-mGlu3 confirmed the previous results obtained by using orthosteric and allosteric modulators of the mGlu2/3 receptors. In fact, they suggest that the mGlu3 receptors are crucial in the control of the LY 379268-induced inhibition in spinal cord nerve endings while the mGlu2 receptors play a main role in the LY 379268-induced inhibition in cortical synaptosomes, where also the mGlu3 receptors participate.

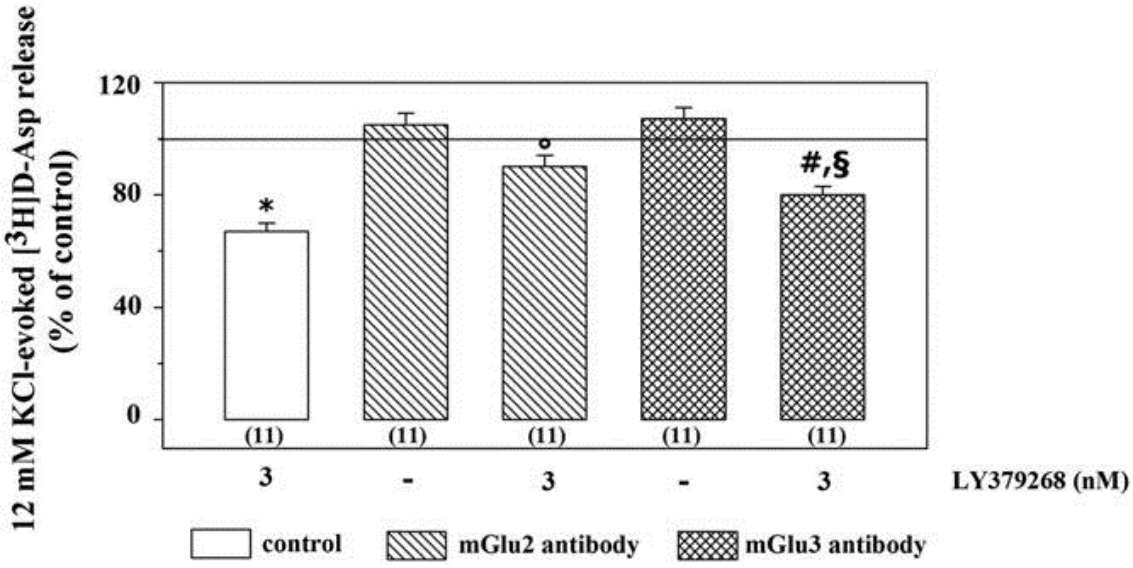


Figure 8: Effect of the selective anti-mGlu2 and anti-mGlu3 antibodies in cortical synaptosomes. Effects of the incubation of cortical synaptosomes with the anti-mGlu2 or the anti-mGlu3 antibodies on the 12 mM KCl/LY379268-evoked release of [^{3}H]D-ASP. The 12 mM KCl-evoked release of tritium amounted to the following: control synaptosomes, 1.48±0.43 nCi; anti-mGlu2 antibody incubated synaptosomes, 1.63±0.27 nCi; anti-mGlu3antibody incubated synaptosomes 1.48±0.29 nCi. Results are expressed as percentage of the 12 mM KCl-evoked [^{3}H]D-ASP release (% of control). Data are the mean ± SEM of at least four experiments run in triplicate. *P < 0.05 versus the 12 mM KCl-evoked tritium overflow from control cortical synaptosomes; °P < 0.05 versus the 12 mM KCl/3 nM LY 379268-evoked tritium overflow from control cortical synaptosomes; #P < 0.05 versus the 12 mM KCl-evoked tritium

overflow from cortical synaptosomes incubated with the anti-mGlu3receptor antibody; §P < 0.05 versus the 12 mM KCl/3 nM LY379268-evoked tritium overflow from control cortical synaptosomes (see also Olivero et al., 2018, figure 8).

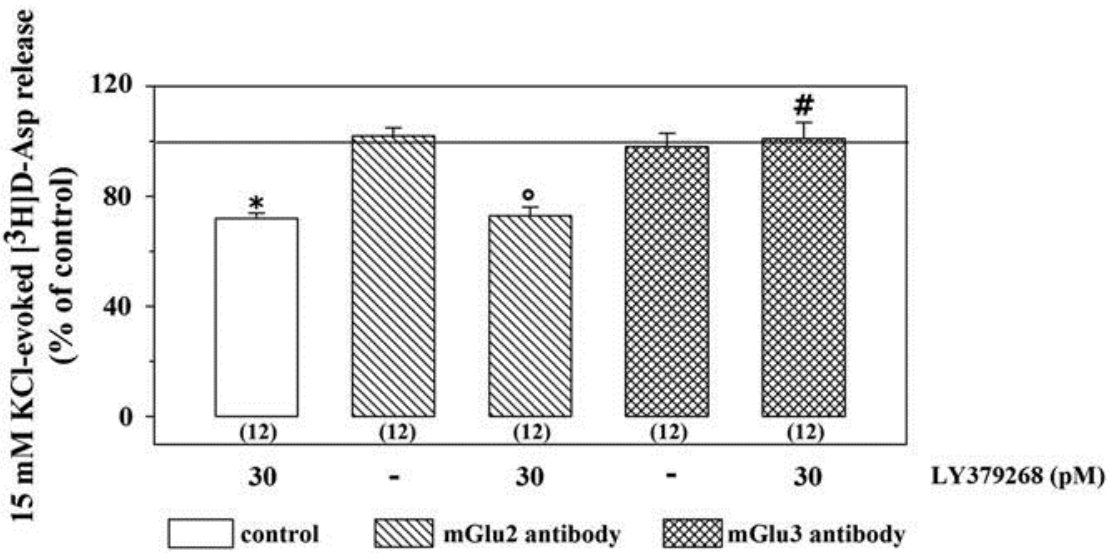


Figure 9: Effect of the selective anti-mGlu2 and anti-mGlu3 antibodies in spinal cord synaptosomes. Effects of the incubation of spinal cord synaptosomes with the anti-mGlu2 or the anti-mGlu3 antibodies on the 15 mM KCl/LY 379268-evoked release of [^{3}H]D-ASP. The 15 mM KCl-evoked release of tritium amounted to the following: control synaptosomes 4.76±0.69 nCi; anti mGlu2 antibody incubated synaptosomes, 3.21±0.55 nCi; anti mGlu3antibody incubated synaptosomes 3.98±0.42 nCi. Results are expressed as percentage of the 15 mM KCl-evoked [^{3}H]D-ASP release. Data are the mean ± SEM of at least four experiments run in triplicate. *P < 0.05 versus the 15 mM KCl-evoked tritium overflow from control spinal cord synaptosomes; °P < 0.05 versus the 15 mM KCl-evoked tritium overflow from spinal cord synaptosomes incubated with the anti-mGlu2receptor antibody; #P < 0.05 versus the 15 mM KCl/30 pM LY 379268 evoked tritium overflow from control spinal cord synaptosomes (see also Olivero et al., 2018, figure 9).

The mGlu2/3 and 5-HT_{2A} heterodimerization in spinal cord nerve terminals

The term heterodimerization refers to the association of two receptor proteins, independently if the two receptors involved belong to the same group (mGlu2/3 receptor heterodimer) or to receptors that are activated by different transmitters, as in the case of the mGlu2/3 and 5-HT_{2A} receptor-receptor interaction.

The 5-hydroxytryptamine2A receptors (5-HT_{2A}) is a serotonergic metabotropic receptor of the 5-HT receptor family. Beyond its involvement in a number of a physiological processes like the modulation of learning and memory and the control of excitatory events, this receptor attracted the interest of scientists because of its role as specific target of therapeutics against antipsychotic disorders. Contrasting results are present in literature concerning the role of the 5-HT_{2A} receptor as modulator of chemical transmission. In particular, evidence have been provided suggesting both excitatory and inhibitory activity of this receptor. Actually, electrophysiological studies unveiled that the activation of this receptor can induce excitatory postsynaptic currents in layer V pyramidal cells (Aghahanian and Marek, 1999). In addition, it was demonstrated that this receptor is expressed in glutamatergic synapse with a presynaptic distribution were this receptor was associated to an inhibitory activity on glutamatergic transmission (Marcoli et al., 2001; Wang et al., 2006).

In recent years it was demonstrated that Group II mGlu receptors and 5-HT_{2A} receptors can heterodimerize in the cortex of mammals (Delille et al., 2012). Surprisingly and in way unexpectedly, it was proposed that the two receptors interact in antagonist-like manner (Delille et al., 2013), as suggested by the finding that activation of mGlu2/3 receptors reduced the 5-HT_{2A} receptor-induced EPSCs, while mGlu2/3 antagonist/negative allosteric modulators reinforces the 5-HT_{2A} mediated excitations of

L5P cells (Marek et al., 2000). Starting from the data supporting the existence of the mGlu2/3 receptors in spinal cord glutamatergic nerve endings, I investigated whether these terminals also possess 5-HT_{2A} receptors and if mGlu2/3 and 5-HT_{2A} receptors can be functionally coupled to control glutamate release in spinal cord synaptosomes.

The results demonstrated that 5-HT_{2A} receptors are expressed in the spinal cord glutamatergic terminals where they colocalize with mGlu2/3 receptors. This conclusion relies on functional studies supported by immunochemical evidence. In fact, superfusion experiments unveiled that the inhibitory effects on the 15 mM KCl-evoked release of [^{3}H]D-ASP elicited by either LY 379268 (0.1 nM), used in a concentration causing a submaximal inhibition, or by (±)DOI (100 nM), used in a concentration eliciting the maximal inhibition, was almost abolished when the two agonists were concomitantly added (not shown, see Olivero et al., 2018). The loss of function in controlling glutamate release of both agonists when concomitantly added was best interpreted by assuming the colocalization and the functional cross-talk of the two receptors and also supported that the two receptors interact in an antagonist-like fashion. Indeed, it is known that the activation of two colocalized receptors controlling the exocytosis of the same transmitter should lead to an inhibitory effect amounting to the sum of the two effects elicited by the agonists alone (Musante et al., 2008; Pittaluga, 2016; Olivero et al., 2018). However, the concomitant activation of the two receptors blocked the presynaptic release-regulating activity of both the two agonists consistent with a reciprocal antagonism of the releasing function. The close localization of the two receptors was further supported by results from Confocal microscopy showing a large colocalization of mGlu2/3 and 5-HT_{2A} receptor proteins in spinal cord synaptosomes and from Western blot analysis (not shown, see Olivero et al., 2018).

In order to confirm the possible heterodimerization of the mGlu2/3 and the 5-HT_{2A} receptors, immunoprecipitation studies were performed in spinal cord terminals to

demonstrate the physical interaction between these receptors. The results showed a strong physical linking anti mGlu2/3 antibody immunoprecipitate from spinal cord synaptosomal lysates were also positive for 5-HT_{2A} receptors (not shown, see Olivero et al., 2018, figure 5). In addition, when 5-HT_{2A} antagonist were concomitantly added to the stimulus in the presence of a low, ineffective, concentration of LY 379268 (1 pM), the broad spectrum mGlu2/3 agonist became efficacious and was able to induce a marked inhibition of glutamate release in these terminals. In other words, these experiments demonstrated that blocking the 5-HT_{2A} receptors increases the response of the mGlu2/3 receptors agonist LY 379268 in spinal cord nerve terminals (not shown, see Olivero et al., 2018).

The oligomerization between mGlu2/3 and 5-HT_{2A} receptors is an example of antagonist like cross-talk linking two receptors belonging to different group which can tune the release of glutamate and involve the activation of mGlu2/3 receptors. This may be particularly important in those diseases that are typified by an hyperglutamatergicity in spinal cord. Hypothesis could be also proposed for the mechanism of receptor heterodimerization and for the control of glutamate facilitation mediated by the Group II mGlu receptors/5-HT_{2A} receptor complex. The main hypothesis is that 5-HT_{2A} can modulate the trafficking and the insertion of mGlu2/3 receptors in the spinal cord synaptosomal plasma membranes. According to this view, the exposure of synaptosomes to 5-HT_{2A} antagonists causes a significant increase in the amount of mGlu2/3 receptor proteins in the plasma membranes of synaptosomes (Olivero et al., 2018). The increased expression of the mGlu2/3 receptor protein could be consistent with a rapid changes in the number and, possibly, in the composition of presynaptic mGlu2/3 autoreceptors. This observation is particularly intriguing since it indirectly implies some important consequences. First, there is the possibility that a ready-releasable pool of mGlu2/3 receptor proteins exists in spinal cord glutamatergic nerve

endings. Second, mGlu2/3 receptor proteins could rapidly traffic in-out terminal plasma membranes. Third, antagonist activity of non-glutamatergic receptors linking to mGlu2/3 receptors can modulate these rapid in-out movements, controlling the insertion of these receptors in plasma membranes. However, this kind of effect could be more complex in vivo (De La Fuente Revenga et al., 2019).

In conclusion, the fact that the mGlu2/3 receptors can oligomerize with 5-HT_{2A} receptor opens the possibility to have a new way to indirect control the glutamatergic transmission in spinal cord. However, the exact nature of this interaction and which receptor between mGlu2 and mGlu3 forms the physical link with 5-HT_{2A} need further investigation.

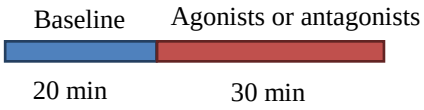
Electrophysiological characterization of mGlu2/3 receptors in mouse hippocampus slices

Building from the characterization of mGlu2/3 in cortical and spinal cord synaptosomes (Di Prisco et al., 2016; Olivero et al., 2017) but also from data in the literature (Gu et al., 2008) I designed, with the help of my supervisors Francesca Pasqui and Emanuele Sher, a study to characterize the effects of mGlu2/3 receptors modulators in mouse hippocampal slices, utilizing electrophysiological readouts. This is the gold standard technique to study synaptic transmission. In the hippocampus, mGlu2/3 receptors were already reported to have an important role in the control of glutamate release and to participate to different events like long-term synaptic plasticity (Upreti et al., 2013).

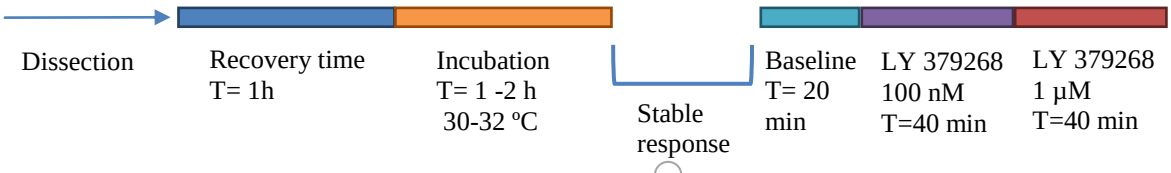
In this section I am going to report the results obtained during my foreign experience at Eli Lilly laboratories (Erl Wood Manor, Sunninghill Road, Windlesham , GU20 6PH, UK), where my work is followed and led by Dr. Emanuele Sher. It was focused on the recording of excitatory postsynaptic potential (fEPSP) from the Cornu Ammonis-1 (CA1) and the Dentate Gyrus (DG) of hippocampal slice of adult mice, in order to characterize the mGlu2/3 receptors expressed in these areas and their contribution to the modulation of synaptic transmission. The results were obtained utilizing two different protocols, which are shown below. Based on these preliminary observations, we suggested that both mGlu2 and mGlu3 participate to the modulation of synaptic transmission of these regions.

- ***Protocol A:*** In these experiments, fEPSPs were recorded in the DG or in the CA1 areas of mouse hippocampal slices. After stable responses were obtained for at least 20 minutes, I applied different compounds for a suitable time. We call this “Protocol A”. Minor modification were made in some experiments to

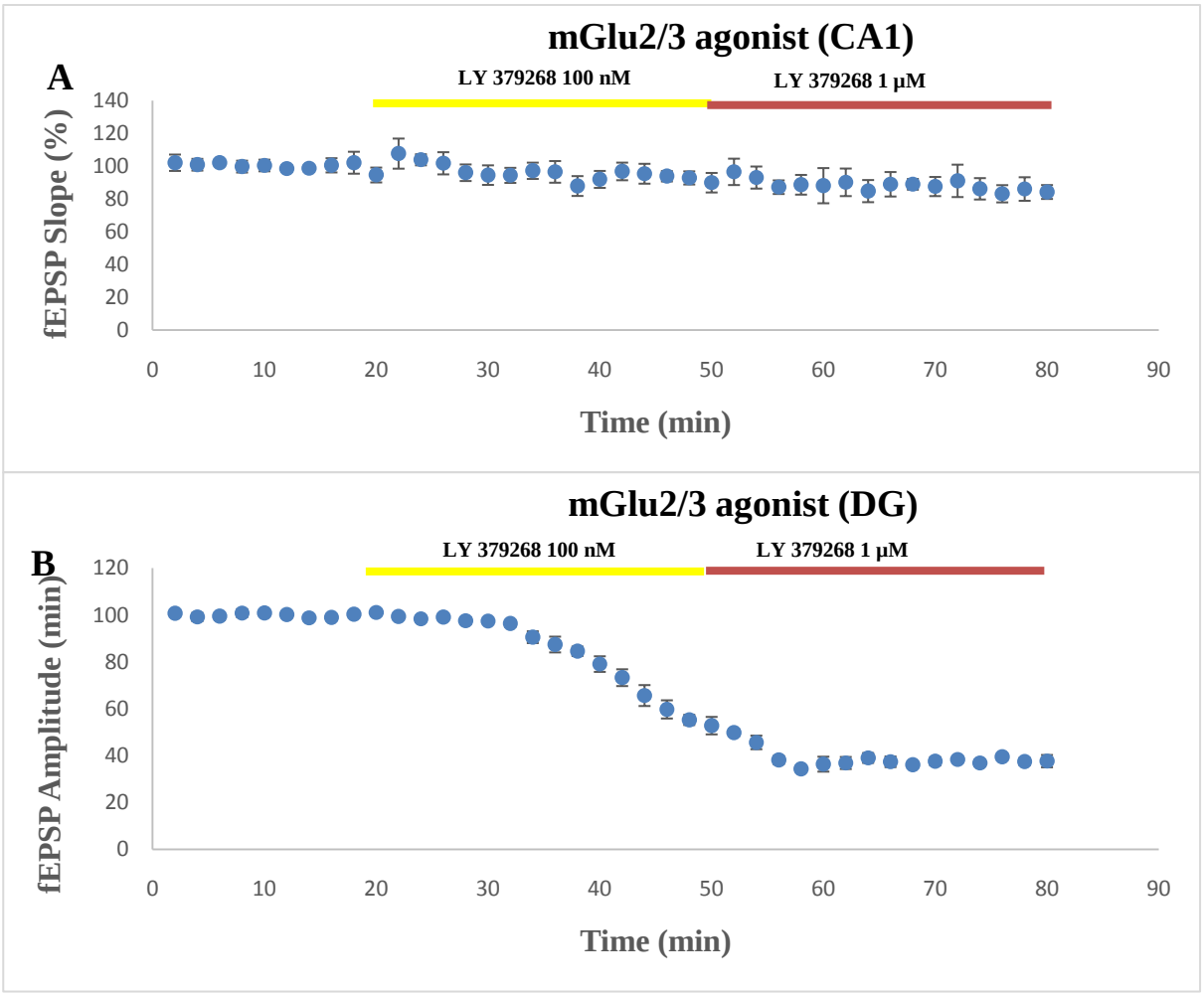
the time of drug application, in order to obtain a stable response. A general example of the protocol is reported below

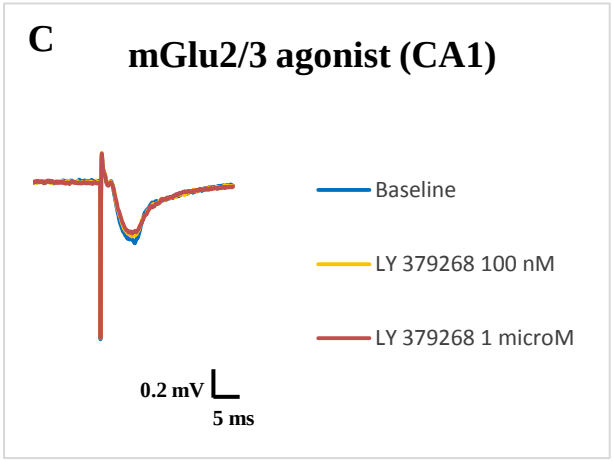


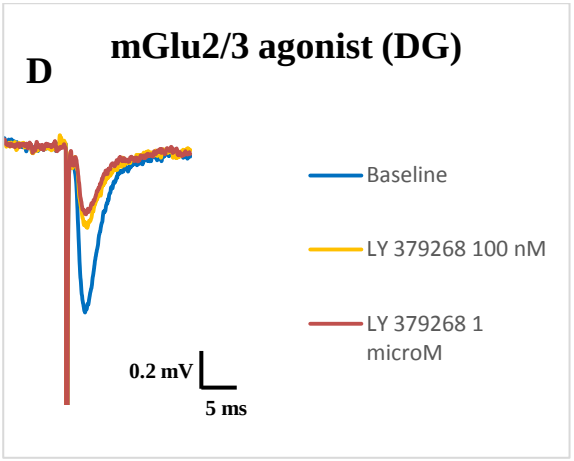
- ***Protocol B:*** In these experiments, fEPSPs were recorded only in the DG of mouse hippocampal slices. Briefly, the following protocol was used to perform most of the experiments shown in this report: after the dissection and after at least 1 h with the slices recovering in normal aCSF (recovery time), between 2 and 4 slices were incubated with ACSF and antibodies at defined concentrations for 1-2 h. Then, the experiments were carried out starting from a stable response (for at least 20 minutes) followed by addition of the compounds (40 minutes per compound). The protocol is reported below in details. In this "Protocol B" two types of compounds were used: Abcam mGlu2 antibodies 15672 and anti Alomone anti mGlu3 antibodies AGC-017 (the same antibodies used for the characterization of mGlu2/3 in cortical and spinal cord synaptosomes, see also Di Prisco et al., 2016 and Olivero et al., 2018). For the first antibody two different concentrations were used: 6.5 mg/ml and 13 mg/ml. For the second only one concentration was used: 13 mg/ml. In addition, we performed an incubation in the same manner with only ACSF as vehicle control. The time and temperature of incubation were the same for both antibodies and vehicle. As mentioned above, in order to reduce the amounts of antibodies used, and to improve the incubation, we carefully put between 2 and 4 slices in a small well of a 24 multi wells filled with ACSF constantly bubbled O_2 95% / CO_2 5%.



The results will be reported below, following the same protocols just described. For each compound, antibodies and peptides experiments the fEPSPs time course and traces will be shown. I will finally summarise all the data (treatments and controls) in bar histograms graphs.


A
mGlu2/3 agonist (CA1)
LY 379268 100 nM
LY 379268 1 µM
fEPSP Slope (%)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Time (min)
B
mGlu2/3 agonist (DG)
LY 379268 100 nM
LY 379268 1 µM
fEPSP Amplitude (min)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Time (min)


C
mGlu2/3 agonist (CA1)
Baseline
LY 379268 100 nM
LY 379268 1 microM
0.2 mV
5 ms


D
mGlu2/3 agonist (DG)
Baseline
LY 379268 100 nM
LY 379268 1 microM
0.2 mV
5 ms

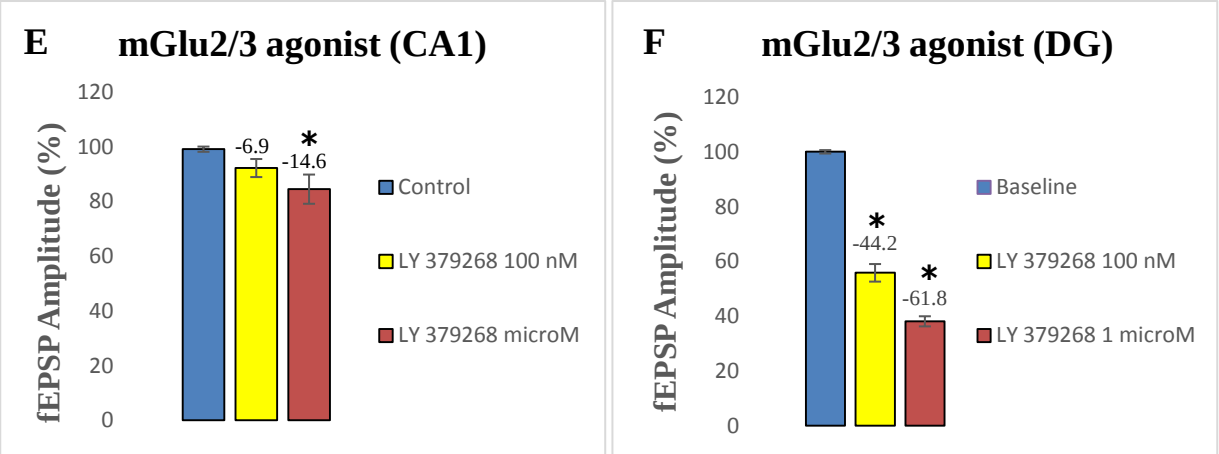


Figure 10: Different effect of the mGlu2/3 agonist LY 379268 in the CA1 and DG of hippocampus slice. The effect of the mGlu2/3 agonist LY 379268 on the synaptic transmission in the CA1 and DG of hippocampus slice.

A: Pooled data (n=4) for the fEPSP slope time course, recording from *stratum radiatum*. Each point represents the average of 4 sweeps and the mean ±SEM of 4 indipendent slices. Compounds were applied for the time indicated by the bars: at 20 min the LY 379268 100 nM in yellow; at 50 min the LY 379268 1 µM in red.

B: Pooled data (n=3) for the fEPSP Amplitude time course, recording from the molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 3 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 100 nM LY 379268 in yellow; at 50 min the1 µM LY 379268 in red.

C: Example traces from an individual experiment recording from *stratum radiatum* of CA1. Each trace represents the last point for each condition in the experiment: baseline in blue, 100 nM LY 379268 in yellow and 1 µM LY 379268 in red.

D: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, 100 nM LY 379268 in yellow and 1 µM LY 379268 in red.

E: Graph summarising the results for the agonist applied in CA1. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

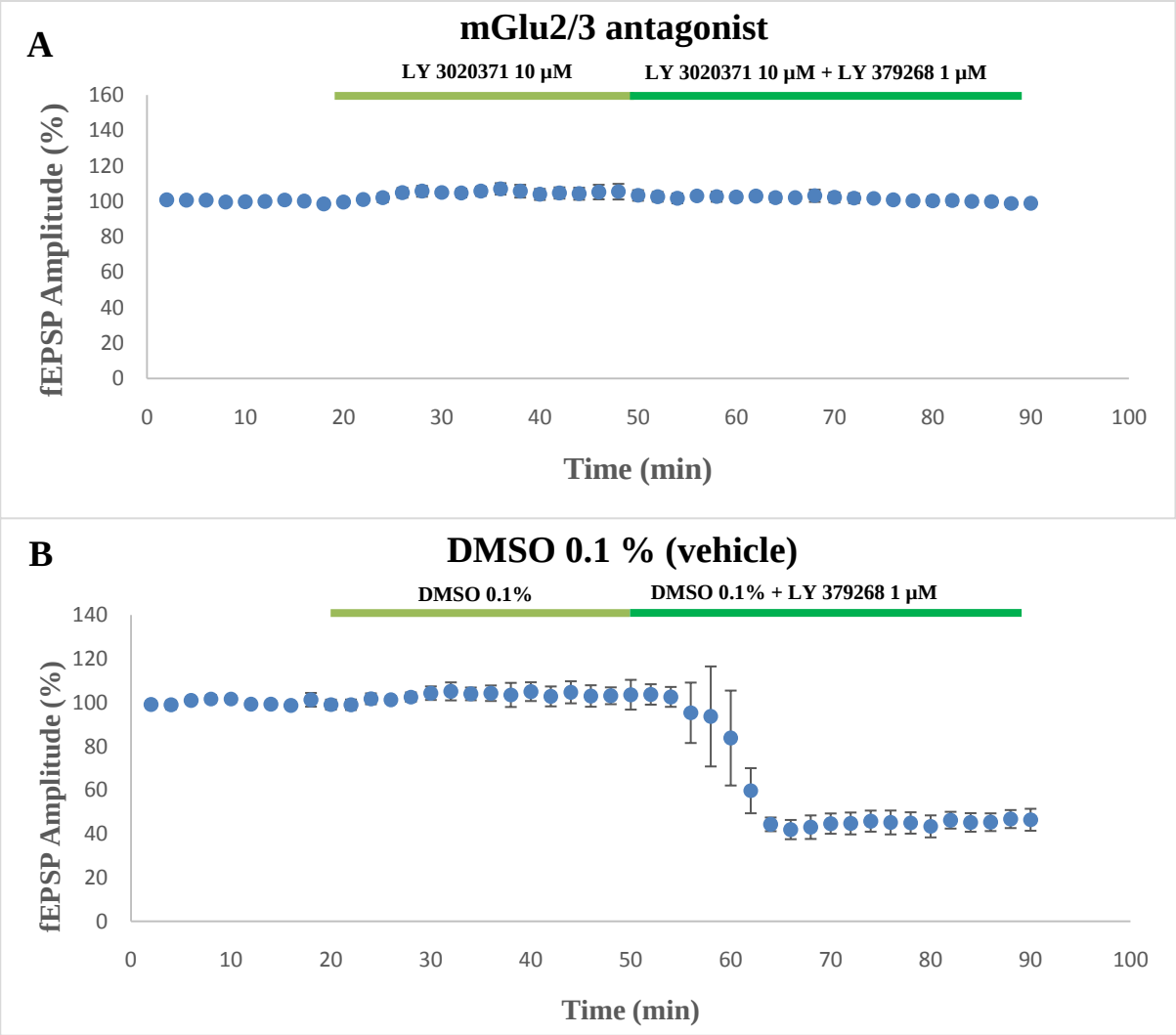
-100 nM LY 379268 slightly decrease the synaptic transmission by 6.9% (93.1% of baseline recorded).

-1 µM LY 379268 slightly although significantly depressed the synaptic transmission by 14.6% (85.4% of baseline recorded).

F: Graph summarising the results for the agonist applied in DG. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

-100 nM LY 379268 significatly decrease the synaptic transmission by 44.2% (55.8% of baseline recorded).

- 1 μM LY 379268 significatly depressed the synaptic transmission by 61.8% (38.2% of baseline recorded).


A
mGlu2/3 antagonist
LY 3020371 10 µM
LY 3020371 10 µM + LY 379268 1 µM
fEPSP Amplitude (%)
Time (min)
B
DMSO 0.1 % (vehicle)
DMSO 0.1%
DMSO 0.1% + LY 379268 1 µM
fEPSP Amplitude (%)
Time (min)

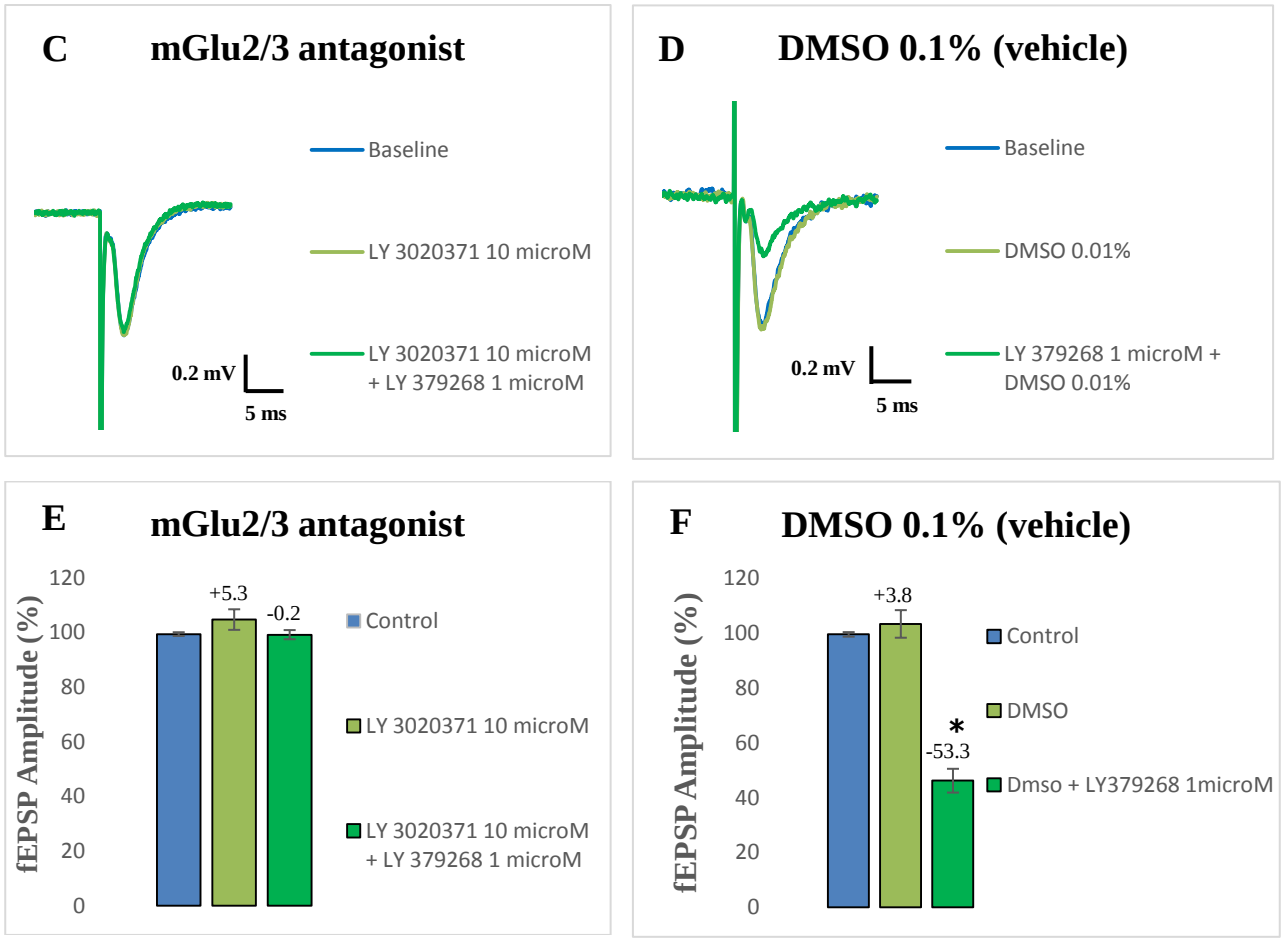


Figure 11: Effect of the mGlu2/3 antagonist LY 3020371 in DG. The effect of the mGlu2/3 antagonist LY 3020371 against the agonist LY379268 at the higher concentration in the DG of hippocampus slice. The vehicle DMSO 0.1% was evaluated for possible interaction with the synaptic transmission.

A: Pooled data (n=4) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 10 µM LY 3020371 in light green and at 50 min the 1 µM LY 379268 + 10 µM LY 3020371 in green.

B: Pooled data (n=4) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the DMSO 0.1% in light green and at 50 min the DMSO 0.1% + 1 µM LY 379268 in green.

C: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, 10 µM LY 3020371 in light green and 1 µM LY 379268 + 10 µM LY 3020371 in green.

D: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, DMSO 0.1% in light green and DMSO 0.1% + 1 µM LY 379268 in green.

E: Graph summarising the results for the antagonist. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- 10 µM LY 3020371 did not affect the synaptic transmission: +5.3% (105.3% of baseline recorded).
- 10 µM LY 3020371 + 1 µM LY 379268 did not depress the synaptic transmission: -0.2% (99.8% of baseline recorded).

F: Graph summarising the results for the vehicle DMSO 0.1%. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- DMSO 0.1% did not affect the synaptic transmission: +3.8% (103.8% of baseline recorded).
- DMSO 0.1% + 1 µM LY 379268 depressed the synaptic transmission by -53.3% (46.7% of baseline recorded).

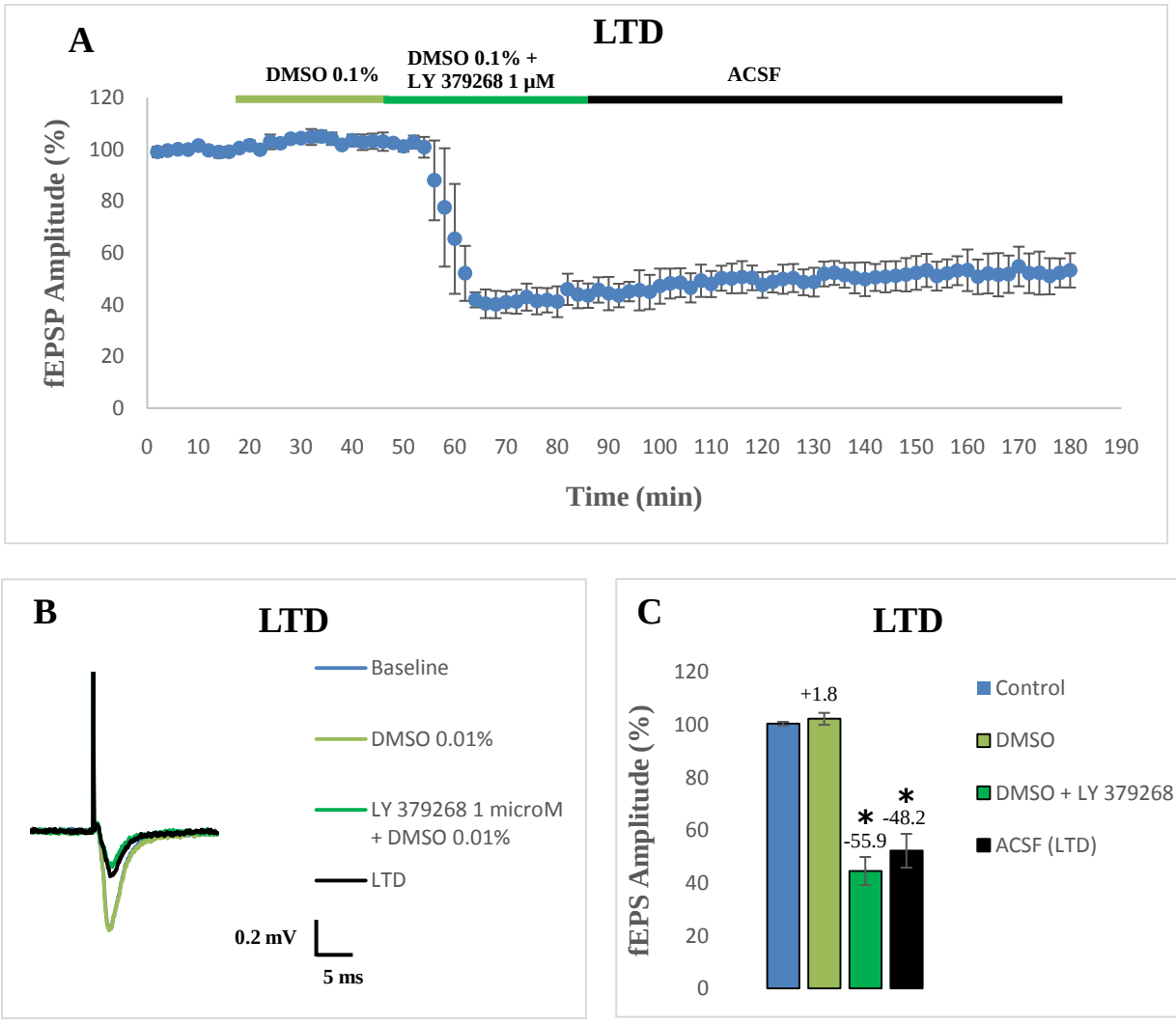


Figure 12: Pharmacological LTD mediated by mGlu2/3 agonist in DG. The graphs show the effect of the agonist LY 379268 at the higher concentration after the wash out of the compound in the DG of mouse hippocampal slices. The vehicle DMSO 0.1% was evaluated to avoid possible interaction with the synaptic transmission and compounds

A: Pooled data (n=4) for the fEPSP Amplitude time course, recording from the molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the DMSO 0.1% in light green, at 50 min the DMSO 0.1% + 1 µM LY 379268 in green and at 90 min the ACSF in black.

B: Example traces from an individual experiment recording from the molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, DMSO 0.1% in light green, DMSO 0.1% + 1 µM LY 379268 in green and ACSF in black.

C: Graph summarising the results for the vehicle DMSO 0.1% and the following Pharmacological LTD induced byt the agonist LY 379268. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- DMSO 0.1% did not affect the synaptic transmission: +1.8% (101.8% of baseline recorded).

- DMSO 0.1% + 1 μM LY 379268 depressed the synpatic transmission: -55.9% (44.1% of baseline recorded).

- After ACSF applied, the synaptic transmission was not able to recover for the rest of the experiment -48.2 % (51.8 % of baseline recorded).

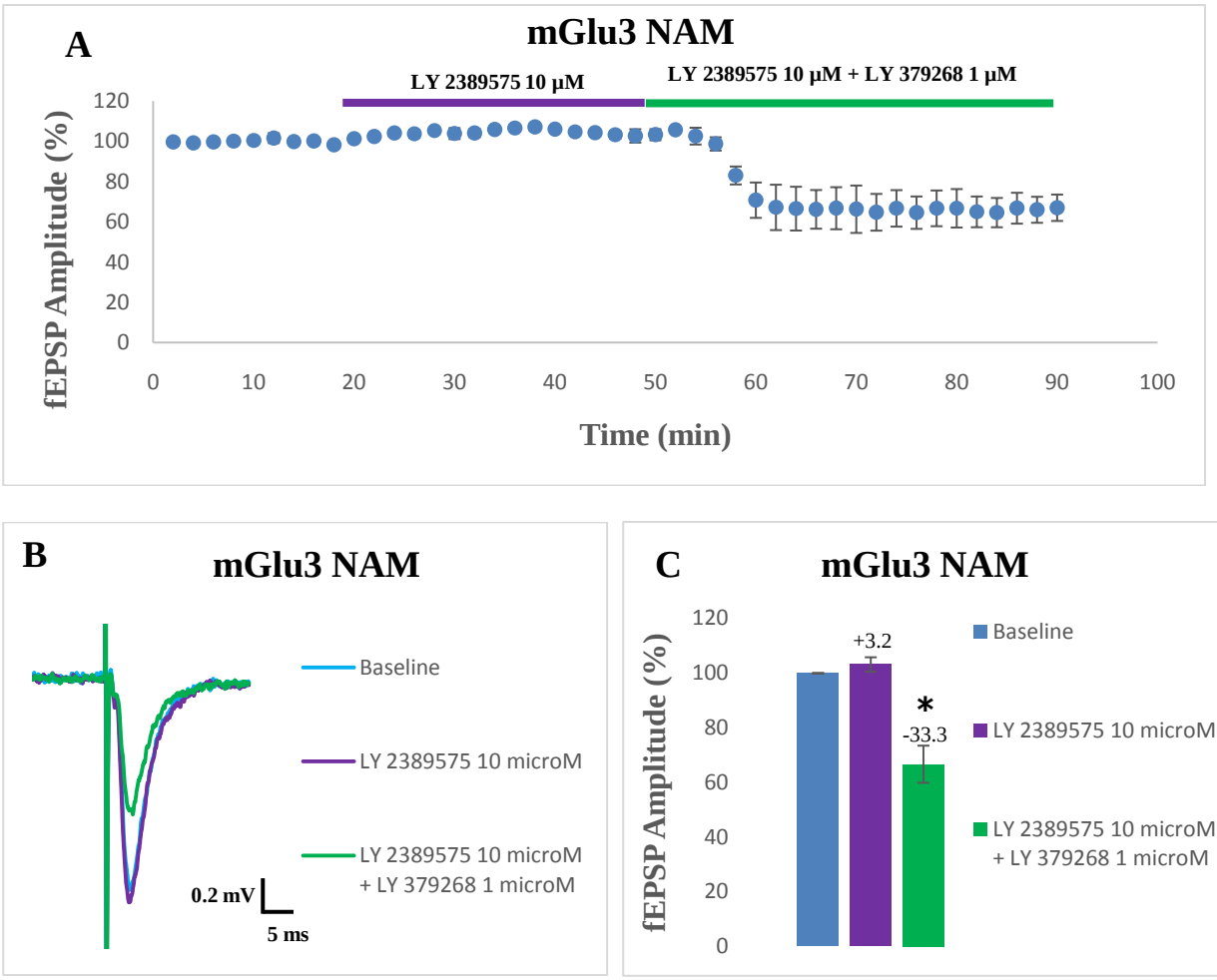


Figure 13: Effect of the mGlu3 NAM LY 2389575 in DG. The effect of the mGlu3 NAM LY 2389575 versus the agonist LY 379268 at the higher concentration in the DG of hippocampus slice.

A: Pooled data (n=5) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 5 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 10 µM LY 2389575 in purple and at 50 min the 10 µM LY 2389575 + 1 µM LY 379268 in green.

B: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, 10 µM LY 2389575 in purple and 10 µM LY 2389575 + LY 379268 1 µM LY 379268 in green.

C: Graph summarising the results for the NAM mGlu 3. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 2389575 10 µM did not affect the synaptic transmission: +3.2% (103.2% of baseline recorded).

- LY 2389575 10 µM + LY 379268 1 µM decrease the synaptic transmission: -33.3% (66.7% of baseline recorded).

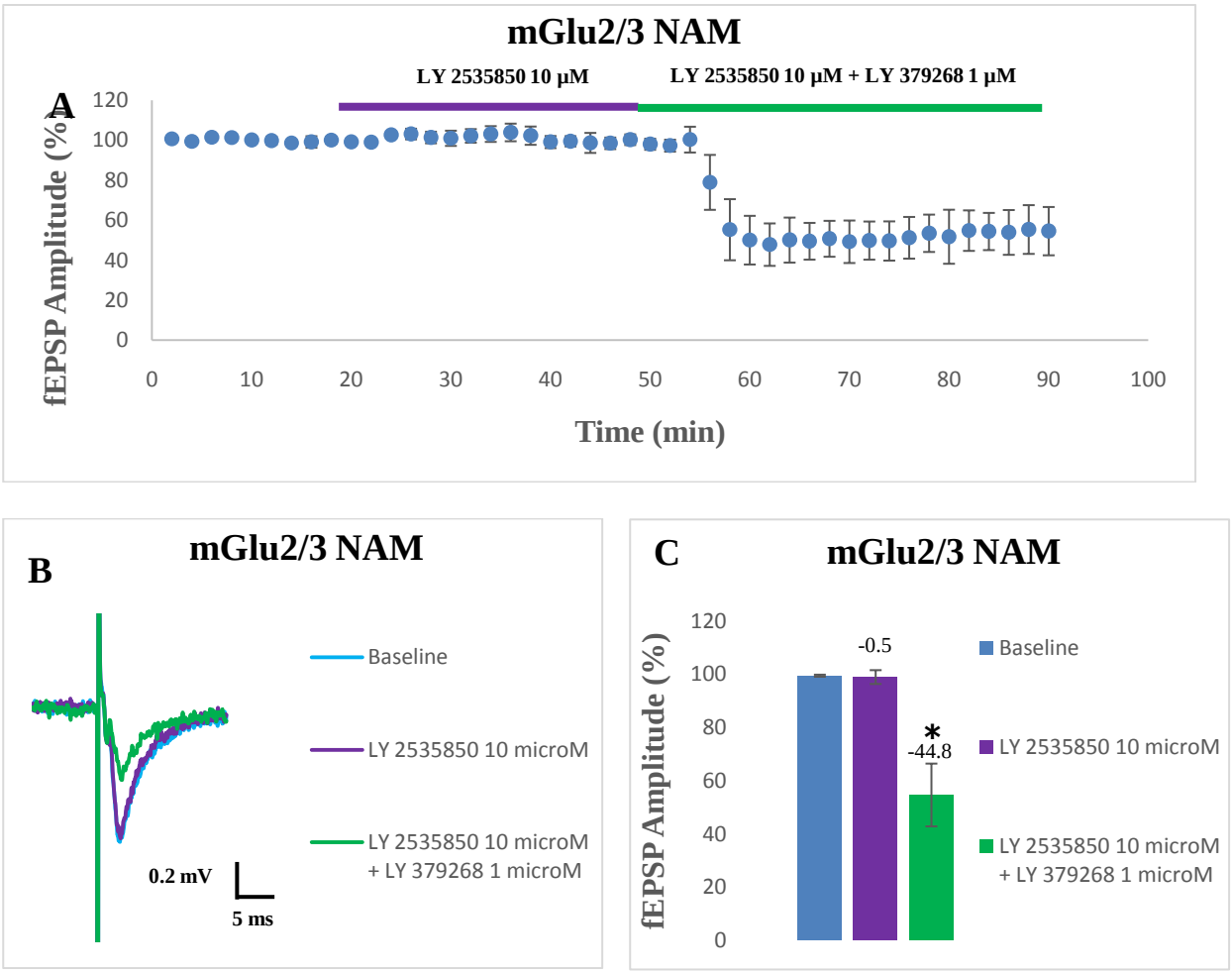


Figure 14: Effect of mGlu2/3 NAM LY 2535850 in the DG. These experiments show the effect of the mGlu2/3 NAM LY 2535850 versus the agonist LY 379268 at the higher concentration in the DG of hippocampus slice.

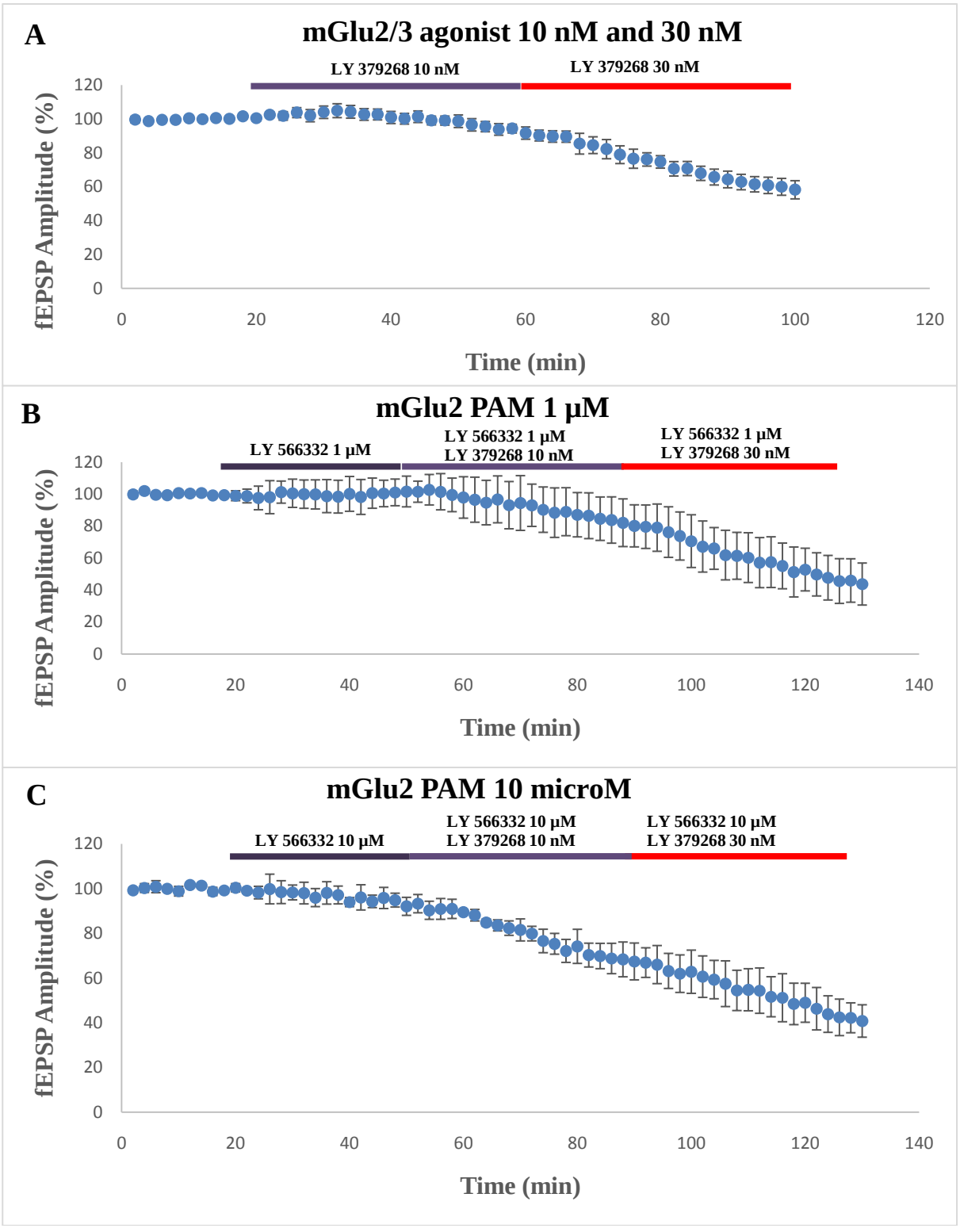
A: Pooled data (n=5) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 5 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 10 µM LY 2535850 in purple and at 50 min the 10 µM LY 2535850 + 1 µM LY 379268 in green.

B: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: 20 min baseline in blue, 10 µM LY 2389575 in purple and 10 µM LY 2389575 + 1 µM LY 379268 in green.

C: Graph summarising the results for the NAM mGlu2/3 LY 2535850. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 2535850 10 µM did not affect the synaptic transmission: -0.5% (99.5% of baseline recorded).

- LY 2535850 10 µM + LY 379268 1 µM depressed the synaptic transmission: -44.8% (55.2% of baseline recorded).


A
mGlu2/3 agonist 10 nM and 30 nM
LY 379268 10 nM
LY 379268 30 nM
fEPSP Amplitude (%)
Time (min)
B
mGlu2 PAM 1 µM
LY 566332 1 µM
LY 566332 1 µM
LY 379268 10 nM
LY 566332 1 µM
LY 379268 30 nM
fEPSP Amplitude (%)
Time (min)
C
mGlu2 PAM 10 microM
LY 566332 10 µM
LY 566332 10 µM
LY 379268 10 nM
LY 566332 10 µM
LY 379268 30 nM
fEPSP Amplitude (%)
Time (min)

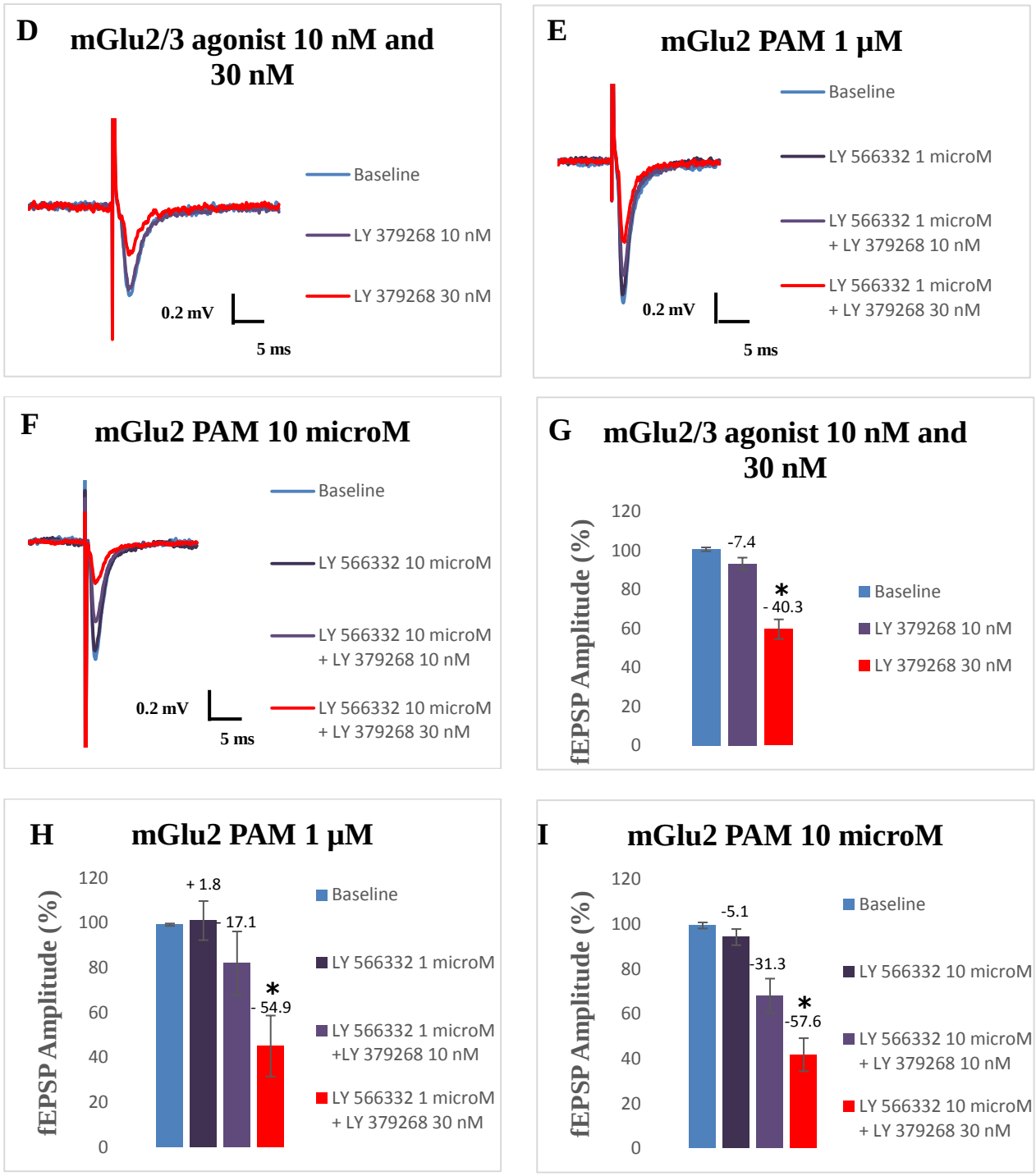


Figure 15: Effect of mGlu2/3 PAM LY 566332 in DG. The effect of the mGlu 2 PAM LY 566332 1 µM and 10 µM followed by the agonist LY 379268 at the lower concentration (10 nM and 30 nM) in the DG of hippocampus slice.

A: Pooled data (n=4) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 10 nM LY 379268 in purple and at 50 min the 30 nM LY 379268 in red.

B: Pooled data (n=3) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 3 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 1 µM LY 566332 in dark purple, at 50 min the 1 µM LY 566332 + 10 nM LY 379268 in purple and at 90 min the 1 µM LY 566332 + 30 nM LY 379268 in red.

C: Pooled data (n=4) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 10 µM LY 566332 in dark purple, at 50 min the 10 µM LY 566332 + 10 nM LY 379268 in purple and at 90 min the 10 µM LY 566332 + 30 nM LY 379268 in red.

D: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, 10 nM LY 379268 in purple and 30 nM LY 379268 in red.

E: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, 1 µM LY 566332 in dark purple, 1 µM LY 566332 + 10 nM LY 379268 in purple and 1 µM LY 566332 + 30 nM LY 379268 in red.

F: Example traces from an individual experiment recording from molecular layer of DG. Each trace represents the last point for each condition in the experiment: baseline in blue, 10 µM LY 566332 in dark purple, 10 µM LY 566332 +10 nM LY 379268 in purple and 10 µM LY 566332 + 30 nM LY 379268 in red.

G: Graph summarising the results for the agonist LY 379268 at 10 nM and 30 nM. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 379268 10 nM slightly reduced the synaptic transmission: -7.4% (92.6% of baseline recorded).
- LY 379268 30 nM depressed the synaptic transmission: -40.3% (59.7% of baseline recorded).

H: Graph summarising the results for the PAM mGlu2 LY 566332 at 1 µM. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 566332 1 µM did not affect the synaptic transmission: +1.8% (101.8% of baseline recorded).

- LY 566332 1 µM + LY 379268 10 nM slightly depressed the synaptic transmission: -17.1% (82.9% of baseline recorded).

- LY 566332 1 µM + LY 379268 30 nM significantly depressed the synaptic transmission: -54.9% (45.1% of baseline recorded).

I: Graph summarising the results for the mGlu2 PAM LY 566332 at 10 µM. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 566332 10 µM did not affect the synaptic transmission: -5.1% (94.9% of baseline recorded).

- LY 566332 10 µM + LY 379268 10 nM depressed the synaptic transmission: -31.3% (68.7% of baseline recorded).

- LY 566332 10 µM + LY 379268 30 nM significantly depressed the synaptic transmission: -57.6% (42.4% of baseline recorded).

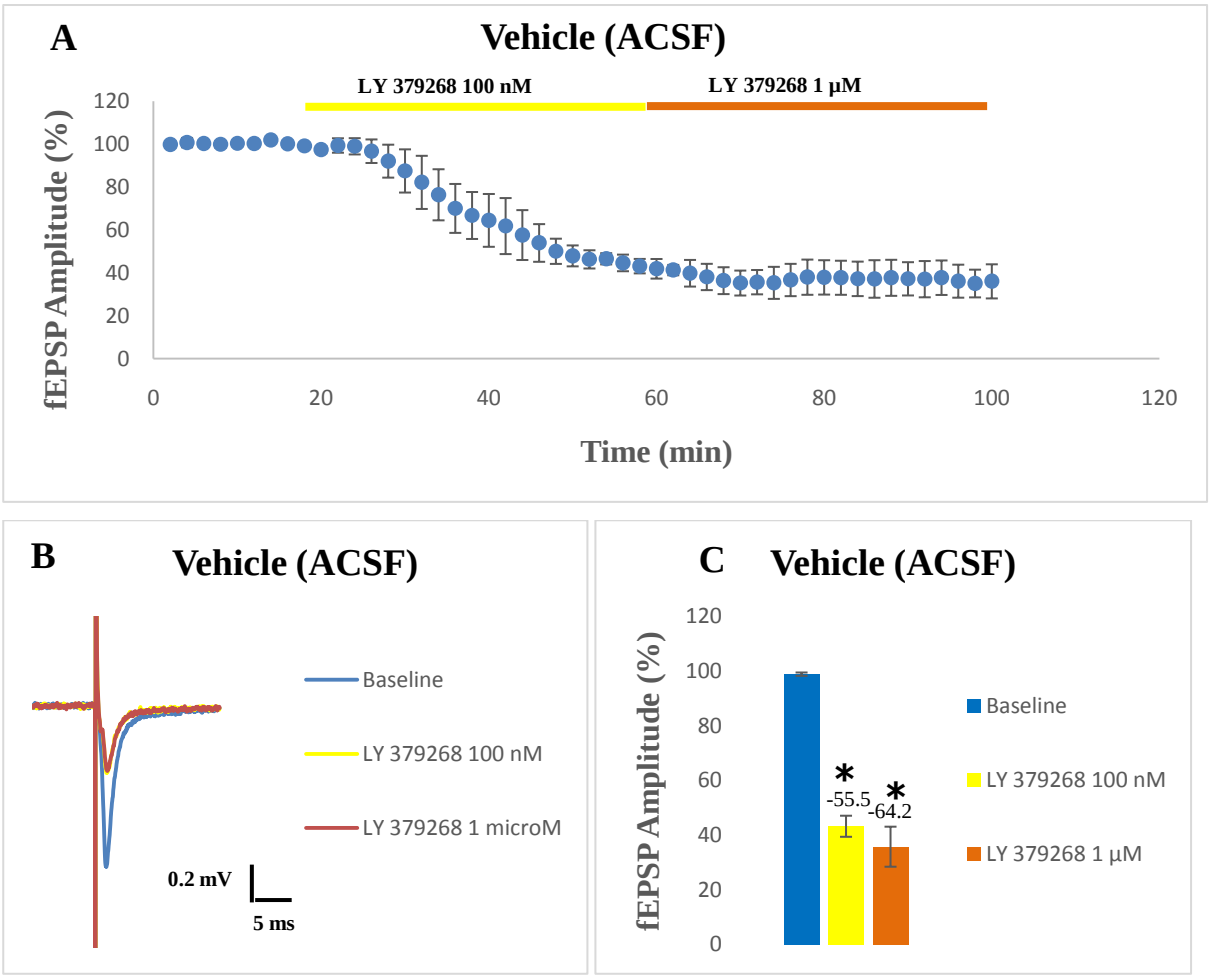


Figure 16: Effect of two concentrations of mGlu 2/3 agonist LY 379268, 100 nM and 1 µM, on vehicle treated slices. These experiments represent the Control for antibodies experiments.

A: Pooled data (n=5) showing the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 5 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 100 nM LY 379268 in yellow and at 50 min in the 1 µM LY 379268 in orange.

B: Example trace from an individual experiment recording from molecular layer of DG. Each sweep represents the last point for each condition in the experiment: baseline in blue, 100 nM LY 379268 in yellow and 1 µM LY 379268 in orange.

C: Graph summarising the results for the synaptic blocking effects of the mGlu2/3 agonist LY 379268. Each column represent the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 379268 100 nM significatly depressed the synaptic transmission: -55.5% (44.4 of baseline recorded).

- LY 379268 1 µM significatly depressed the synaptic transmission: -64.2% (35.8 of baseline recorded).

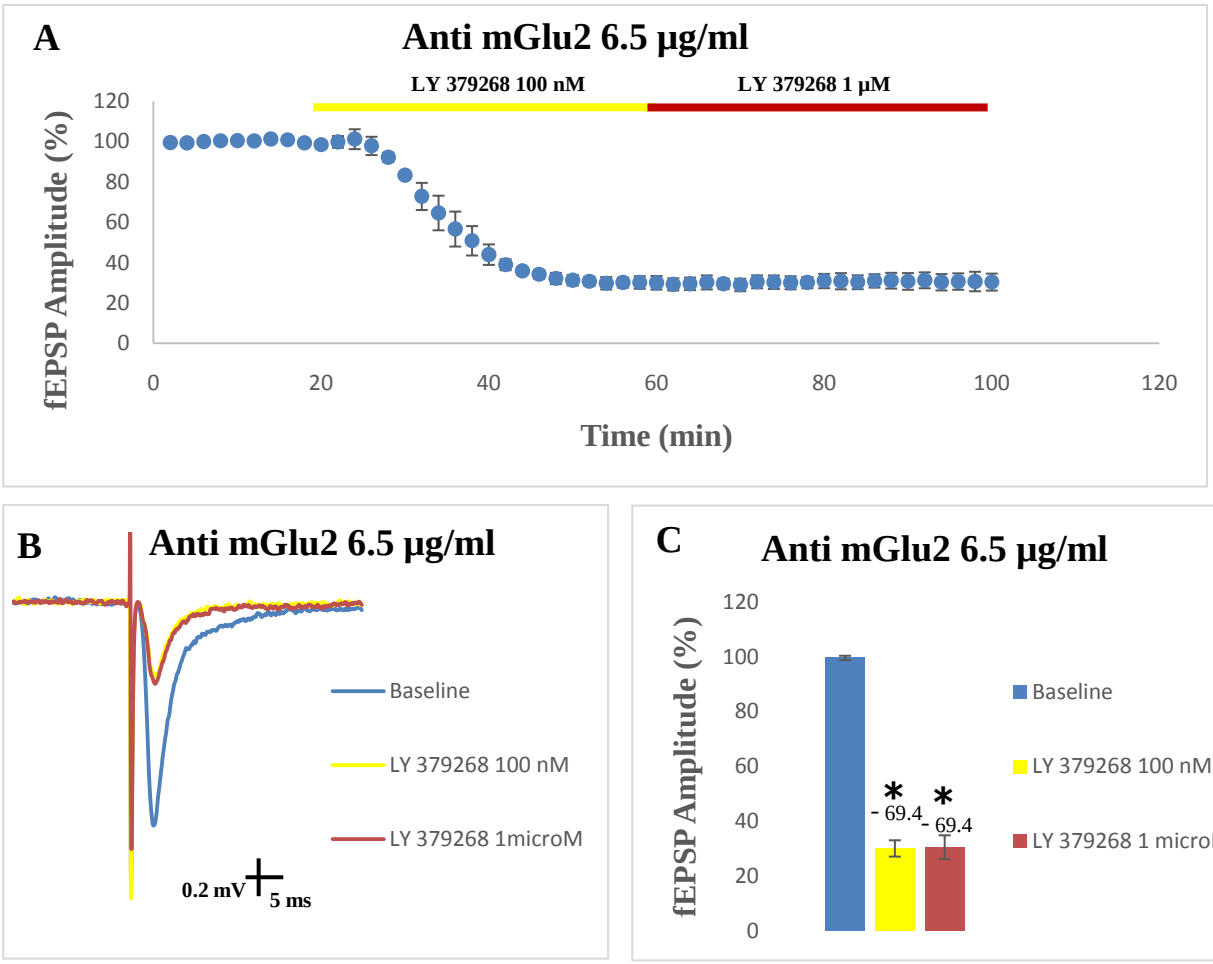


Figure 17: Effects of pre-incubation with the anti mGlu2 antibody (6.5 µg/ml) on LY 379268 (100 nM and 1 µM) responses. There is no prevention of the inhibition mediated by the agonist LY 379268.

A: Pooled data (n=3) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 3 independent slices. Compounds were applied for the time indicated by the bars: at 20 min the 100 nM LY 379268 in yellow and at 60 min the 1 µM LY 379268 in red.

B: Example traces from an individual experiment recording from molecular layer of DG. Each sweep represents the last point for each condition in the experiment: baseline in blue, 100 nM LY 379268 in yellow and 1 µM LY 379268 in red**.**

C: Graph summarising the results with the mGlu2 antibody. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 379268 100 nM signifincatly depressed the synaptic transmission: -69.4% (30.6 of baseline recorded).

- LY 379268 1 µM signifincatly depressed the synaptic transmission: -69.4% (30.6 of baseline recorded).

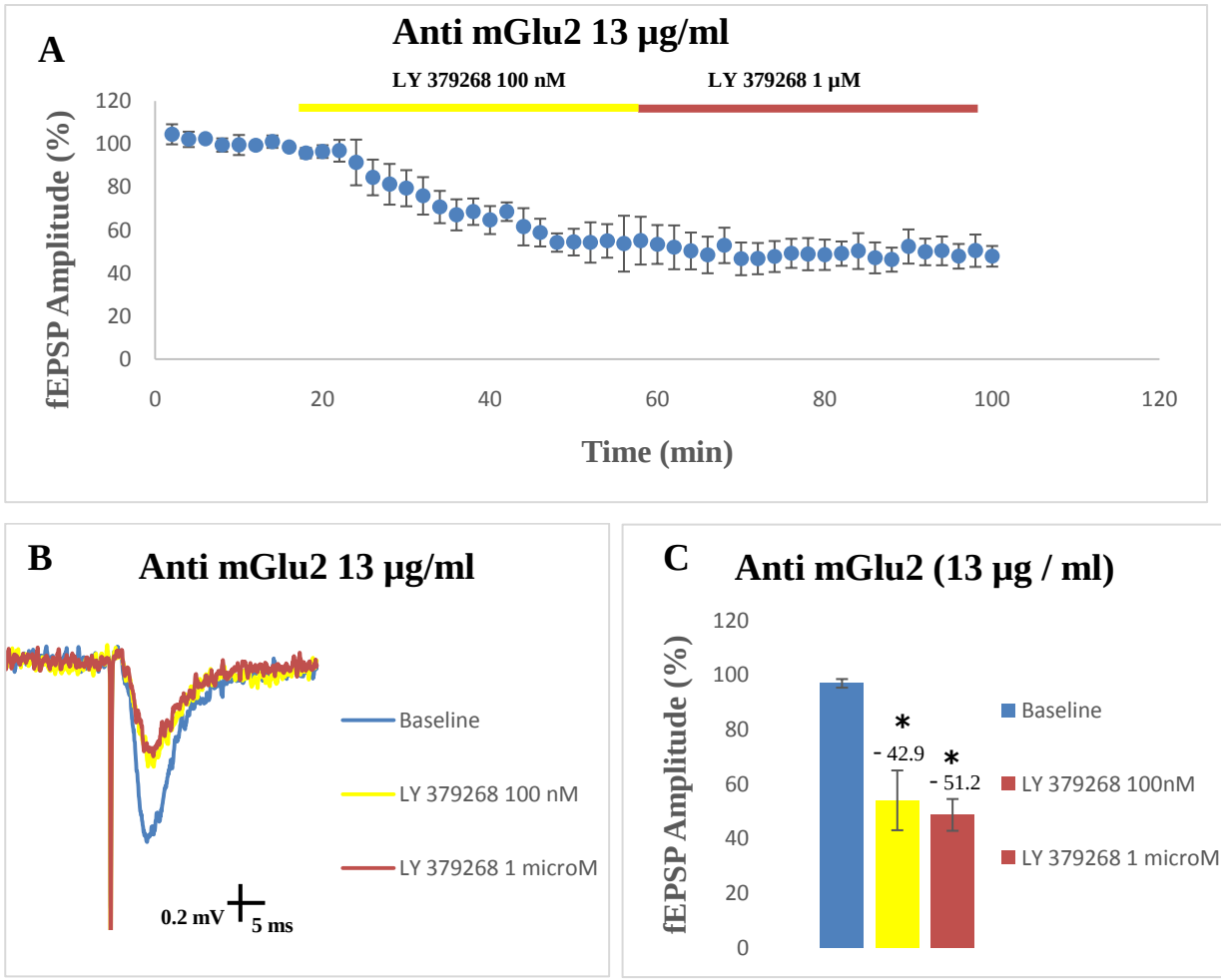


Figure 18: Effects of pre-incubation with the anti mGlu2 receptor antibody (13 µg/ml) on LY 379268 (100 nM and 1 µM) responses. This concentration of antibody partially prevents the agonist-induced inhibition of synaptic transmission.

A: Pooled data (n=4) for the fEPSP Amplitude time course, recording from molecular layer of DG. In this and subsequent figures, each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Drugs were applied for the time indicated by the bars: at 20 min the 100 nM LY 379268 in yellow and at 60 min the 1 µM LY 379268 in red.

B: Example traces from an individual experiment recording from molecular layer of DG. Each sweep represents the last point for each condition in the experiment: baseline in blue, 100 nM LY 379268 in yellow and 1 µM LY 379268 in orange.

C: Graph summarising the results with the mGlu2 antibody. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 379268 100 nM depressed the synaptic transmission: -42.9% (57.1 of baseline recorded).

- LY 379268 1 µM depressed the synaptic transmission: -51.2% (48.8 of baseline recorded).

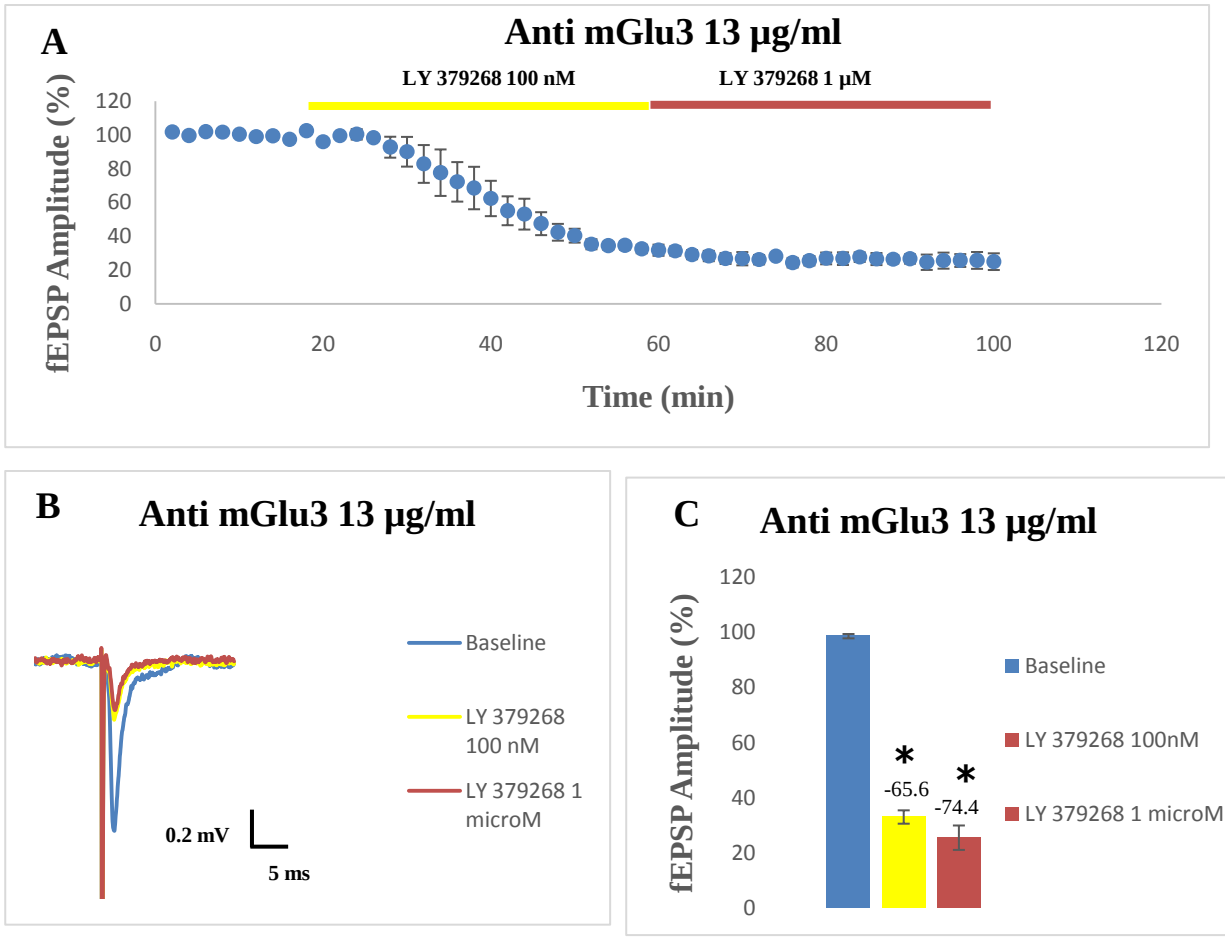


Figure 19: Effects of pre-incubation with the anti mGlu 3 receptor antibody (13 µg/ml) on LY 379268 (100 nM and 1 µM) responses. This antibody did not prevent the agonist inhibitory effects on synaptic transmission.

A: Pooled data (n=4) for the fEPSP Amplitude time course, recording from molecular layer of DG. Each point represents the average of 4 sweeps and the mean ±SEM of 4 independent slices. Drugs were applied for the time indicated by the bars: at 20 min the 100 nM LY 379268 in yellow and at 60 min the 1 µM LY 379268 in red.

B: Example traces from an individual experiment recording from molecular layer of DG. Each sweep represents the last point for each condition in the experiment: baseline in blue, 100 nM LY 379268 in yellow and 1 µM LY 379268 in red.

C: Graph summarising the results with the mGlu3 antibody. Each column represents the average of the average of the last 3 points (6 minutes) for each concentration application for each individual experiment.

- LY 379268 100 nM depressed the synaptic transmission: -65.6% (34.4 of baseline recorded).

- LY 379268 1 µM drammaticly depressed the synaptic transmission: -74.4% (25.6 of baseline recorded).

Our goal was to use a gold standard technique to investigate the modulation of synaptic transmission by mGlu2/3 receptors, using a pool of different compounds and antibodies. At the beginning, the experiments were performed, separately, in two different mouse hippocampal area, CA1 and Dentate Gyrus. The fEPSPs were measured in both regions and fEPSP Slopes were analysed for CA1 while fEPSP Amplitudes were analysed for DG. We started by using the well known agonist LY 379268 in both regions and we obtained different results, as shown in Figure 10. We observed a major impact of this compound in the DG with minimal effects in CA1. The strong effects of this mixed 2/3 agonist in the perforant pathway to the Dentate Gyrus brought us to focus all subsequent experiments on this area.

Figure 11 shows the second step of our study: evaluating the effects of the antagonist LY 3020371. This novel compound has already been shown to display a potent and selective antagonist action on mGlu2/3 receptor (Witkin J.M. et al., 2015). Here we found that the antagonist completely blocked the effect of the agonist (used at the higher concentration of 1 μM) in the DG. Two important things have to be noted to fully appreciate the selective effects of the antagonist. The first was that this compound had no effect by itself on synaptic transmission, as shown in the 30 minutes of perfusion after the baseline. The second was that DMSO 0.1%, here used as vehicle to solubilize the antagonist, had no effects either, nor on the baseline nor on the inhibition mediated by the agonist.

The inhibition of synaptic transmission mediated by the agonist LY 379268 is a complex event and one of the consequence is the promotion of a so-called pharmacological Long Term Depression (LTD). This was demonstrated by the experiments reported in Figure 12. After the agonist was applied for a short time, the slice was washed for 90 min with normal aCSF and we showed that the inhibition was still present long after removal of the agonist.

Once established the effects of mGlu2/3 agonists and mGlu2/3 antagonists on acute and long term synaptic depression, we tried to better understand the relative contribution of mGlu2 versus mGlu3 receptors.

As mentioned before, these receptors subtypes have a high sequence homology which, in part, explain the low number of selective compounds available (Niswender and Conn, 2010; Nicoletti et al., 2011). In order to further characterize the individual contribution of these two receptor subtypes, we evaluated a pool of novel compounds. In particular, we focused on the negative allosteric modulators (NAMs) LY 2389575 and LY 2535850, and the positive allosteric modulator (PAM) LY 566332.

The mGlu3 NAM LY 2389575 (Figure 13) and mGlu2/3 NAM LY 2535850 (Figure 14) showed a similar and limited ability to prevent the effect of the agonist LY 379268 utilised at 1 µM, even if LY 2389575 was slightly more potent. Figure 20 summarises the results of the comparison between NAMs and the agonist. The robust inhibition induced by LY 379268, was still observed in the presence of the NAMs, in contrast to our previous results with the orthosteric antagonist.

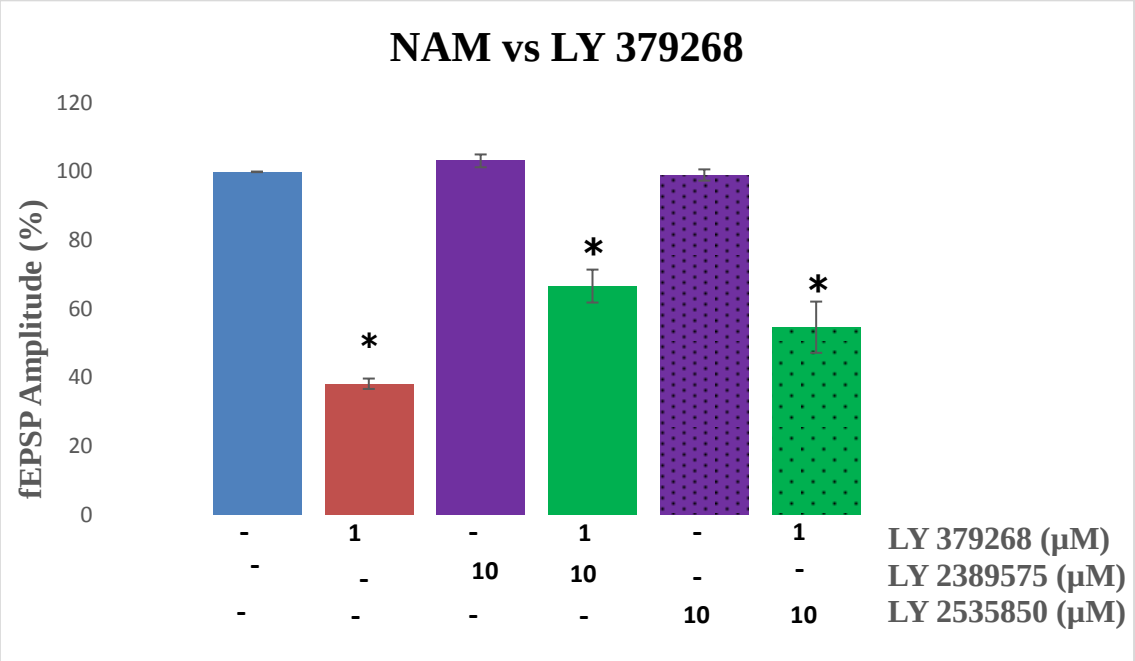


Figure 20: Effects of mGlu NAM in DG The graph shows a summary of the effects of the NAMs LY 2389575 (10 µM) and LY 2535850 (10 µM) versus the agonist LY 379268 (1 µM). Data are the mean ±SEM of 4 experiments. ***** significantly different from the baseline ($p<0.05$).

Only the mGlu3 NAM had a statistically significant effect in preventing the agonist effects, but the effects were small and unlikely to suggest a preferential role of mGlu3. The error bars are pretty large and we only tested one concentration of each NAM. Therefore, any conclusion would be premature, at this stage.

On the other hand, Figure 15 showed the interesting effects of the mGlu 2 PAM LY 566332 which showed a nice enhancement of the inhibition mediated by low concentrations of the agonist LY 379268. The figure shows in order the effect of LY 379268 at 10 nM and 30 nM, then two different concentrations of the PAM 1 µM and 10 µM alone and in the presence of the agonist. As before, PAM has no effect by itsef but it increased the inhibition at both concentrations of agonist.

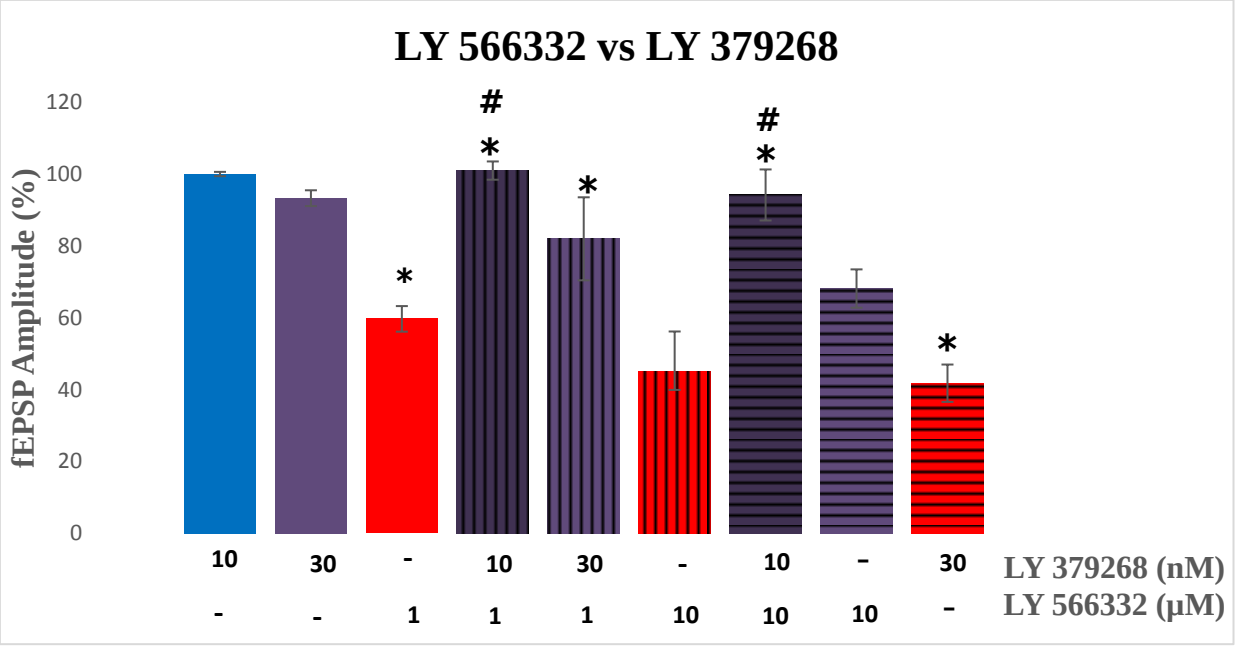


Figure 21: Effects of mGlu 2 PAM in DG. The graph shows a summary of the effect of the mGlu2 PAM (10 µM) versus the agonist LY 379268 (1 µM). Data are the mean ±SEM of 4 experiments.

*****significantly different from baseline ($p<0.05$); #significantly different from LY 379268 10 nM ($p<0.05$).

The bar graph above (Figure 21) shows a summary of the effects of the mGlu2 PAM. The PAM was effective at 1 µM, but the higher concentration of 10 µM showed a slightly more pronounced effect and less variable results. After these experiments with various pharmacological agents, in order to further evaluate the individual contribution of mGlu2 and mGlu3 receptors in the modulation of glutamatergic synaptic transmission in the mouse hippocampal DG, we used selective antibodies directed against the extracellular domain of the receptors. The antibodies used are the same antibodies utilised for the "immuno-pharmacological" characterization of the mGlu2/3 receptors in cortical and spinal cord synaptosomes (see also Olivero et al., 2017). As described above, the pre incubation with selective antibodies was used to try to induce a selective block of one of these receptor subtypes, and therefore a prevention of the inhibitory effects of the dual mGlu2/3 agonist LY 379268.

As a control, as shown in Figure 16, we performed experiments to show that similar pre-incubations with the vehicle alone was not affecting the ability of LY379268 to inhibit synaptic transmission.

Figure 17 shows that pre-incubation with a lower concentration of the anti mGlu 2 receptor antibody (6.5 µg/ml) did not prevent the inhibition mediated by the agonist LY 379268 at either 100 nM or 1 µM.

More interesting, Figure 18 and 19 show the effects of anti mGlu 2 and 3 receptor antibodies, respectively, after pre-incubations with higher antibody concentrations (13 µg/ml).

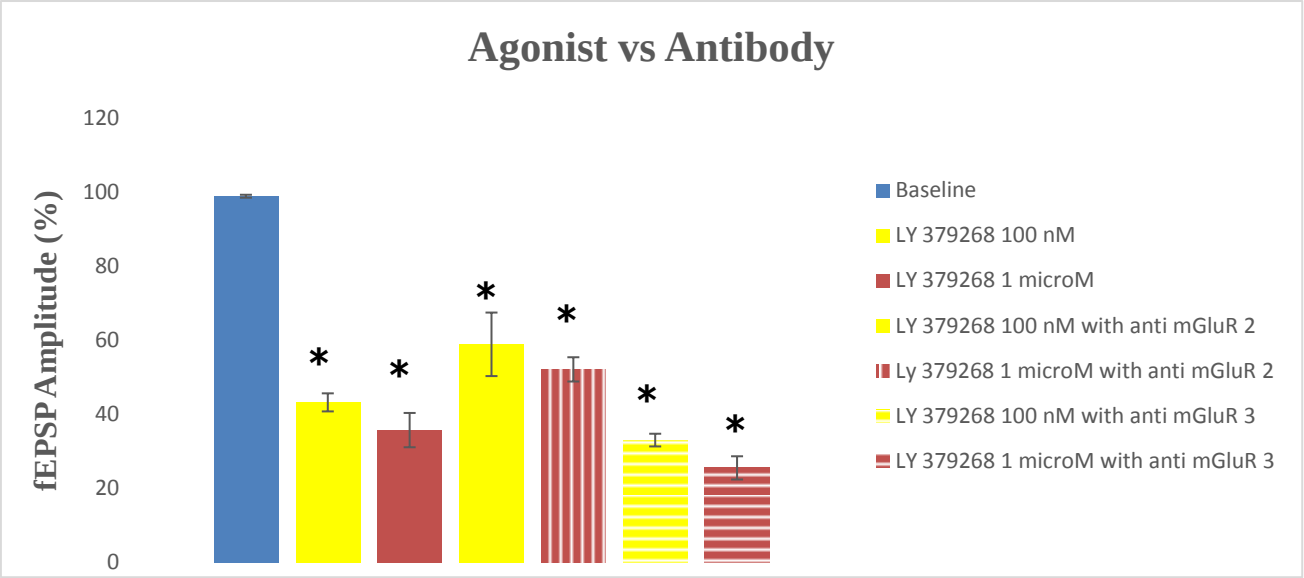


Figure 22: **Effects of anti mGlu2/3 effects in DG.** The graph shows a summary of the effects of mGlu 2 and 3 receptor antibodies (13 µg/ml) against the effects of the agonist LY 379268 at 100 nM and 1 µM. Data are the mean ±SEM of at least 3 experiments. * Significantly different from Baseline ($p<0.05$).

As shown in the summary graph (Figure 22), all the LY 379268 effects, with or without antibody pre-incubation, are significantly different from baseline. We can conclude that the two antibodies did not prevent synaptic inhibition mediated by this agonist. However, in contrast with the lower antibody concentration, after pre-incubation with a higher concentration of the anti mGlu 2 receptor antibody, LY379268 was causing slightly less inhibition. More experiments and with different antibody concentrations and/or pre-incubation times would be needed to substantiate this trend. This small inhibitory effect was not seen with the mGlu3 receptor antibody (if anything, a trend towards an enhanced agonist effect was seen).

Altogether these results suggest some points for discussion:

- There is a stronger control of synaptic transmission by mGlu2/3 receptors in the DG rather than in CA1 of the mouse hippocampus. However, the identification of the individual contribution of mGlu2 and mGlu3, carried out with NAMs, PAMs and antibodies was less clear cut.

- The antibodies experiments may suggest a differential impact of the two antibodies on the modulation of synaptic transmission by mGlu receptors in the DG area. The trend in the data suggests that blocking mGlu2 receptors, but not mGlu3 receptors, with selective antibodies could partially prevent LY 379268 effects. The effects were small but consistent with the partial potentiation of LY379268 effects which we found using the selective mGlu2 receptor PAM LY566332.

mGlu1 and $GABA_B$ in cortical synaptosomes: a receptor-receptor functional cross talk.

The second part of my work aimed at investigating the presence and the role of mGlu1 receptors in the GABAergic terminals and to assess whether these receptors can functionally interact with $GABA_B$ autoreceptors in these terminals.

The $GABA_B$ and mGlu1 receptors have a widespread distribution in the CNS where they control glutamate and GABA transmission. Both receptors are expressed at the presynaptic level (Ladera et al., 2008; Pittaluga, 2016, Vergassola et al., 2018). Specifically, the $GABA_B$ receptor exists as autoreceptor in GABAergic nerve terminals and as heteroreceptor in non-GABAergic terminals (Bowery et al., 2002). Differently, the mGlu1 receptors exist as autoreceptor in cortical glutamatergic presynaptic terminals (Pittaluga, 2016), but, despite the clear localization of these receptors in cortical GABAergic interneurons, the possibility that mGlu1 receptors control GABA exocytosis still is matter of debate.

As a first approach, Western blot analysis was carried out to confirm the presence of the mGlu1 and of $GABA_B$ receptor proteins in the cortical synaptosomal lysates, as described in figure 23.

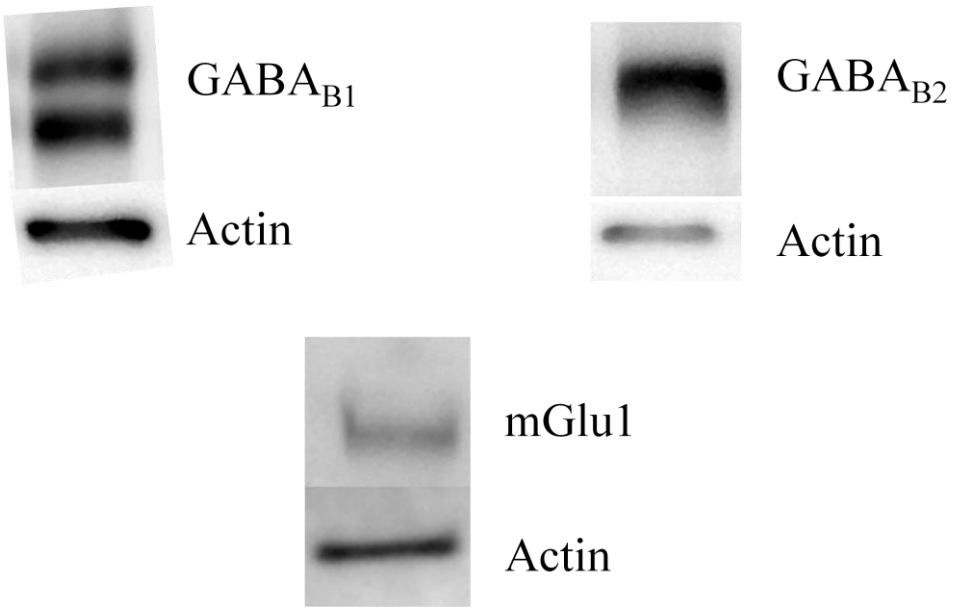


Figure 23: Western Blot analysis of the GABA$_{B1}$, GABA$_{B2}$ and mGlu1 receptor proteins in cortical synaptosomes. The picture shows the Western Blot analysis of cortical synaptosomal lysates with the selective anti-GABA$_{B1}$, anti-GABA$_{B2}$ and anti-mGlu1 antibodies. Actina was used as neuronal marker loading control. The figure shows a representative images of five analysis carried out in different days.

Then we analysed with Confocal microscopy whether the two receptors colocalize in vesicular GABA transporter (VGAT)-positive GABAergic particles (not shown, see Vergassola et al., 2018, figures 1 and 8). The results were largely confirmatory of the presence of both receptors in presynaptic GABAergi and glutamatergic particles.

These findings were predictive of the existence of both receptors in cortical nerve endings. In order to confirm their existence at this level but also owing to evidentiate their functional interaction, we moved to the functional studies performed with the "up and down superfusion" system. It was investigated whether mGlu1 ligands can modify on their own the GABA exocytosis from cortical synaptosomes (Musante et al., 2010, Zucchini et al., 2013). Figure 24 shows that neither 3,5-DHPG nor LY 367385 caused significant changes in the 12 mM KCl evoked [^{3}H]GABA release from cortical GABAergic terminals. However, when concomitantly added to (±)baclofen the two ligands significantly influenced the effect of the orthosteric agonist $GABA_B$ agonist. In particular, the mGlu1 agonist 3,5-DHPG (30 μM) significantly reduced the inhibition induced by the $GABA_B$ agonist baclofen (3 μM) while the mGlu1 antagonist LY 367385 (0.03, 0.1 and 1 μM) significantly reinforced it (Figure 24). These observations led to propose the existence of presynaptic mGlu1 receptors on cortical GABAergic nerve terminals and their functional cross talk with the $GABA_B$ autoreceptors (Vergassola et al., 2018).

This conclusion was further supported by data obtained with cortical synaptosomes isolated from the *Grm1*$^{crv4/crv4}$ mice (Bossi et al., 2016; Vergassola et al., 2018). This is a mouse mutant model bearing a genetic inactivation of the mGlu1 receptor coding gene resulting in the lack of the mGlu1 receptor proteins. We found that in mouse cortical synaptosomes from the inhibitory effect elicited by (±)baclofen on the 12 mM KCl [^{3}H]GABA release was more pronounced when compared to wild type animals (not shown, see Vergassola et al., 2018, figure 5). In addition, Western Blot analysis

indicated a significant increase of the $GABA_{B2}$ receptor proteins expression in the synaptosomal lysates, that would account for the increased activity of the $GABA_B$ autoreceptors in controlling GABA exocytosis (Moller et al., 2017). Even if further studies are required to completely elucidate the mechanism in these mutant mice, our data are well line with the antagonist-like cross-talk linking the mGlu1 and the $GABA_B$ receptors in GABAergic cortical nerve endings.

Evidence in literature suggested that $GABA_B$ receptors and mGlu1 receptors can physical interact at least in some regions of the CNS like hippocampus (Tabata et al., 2004) and that this interaction could depend on Ca^{2+} ions. In release experiments, the physical interaction between the $GABA_B$ and the mGlu1 receptors was so far excluded by immunoprecipitation studies. Moreover, we could not investigate the role of external calcium because this ion is essential to transmitter exocytosis.

Nonetheless, the results above described claims for a receptor cross-talk linking mGlu1 and the $GABA_B$ receptors in an antagonist-manner. The impact of this functional cross-talk deserves further study to be clarified.

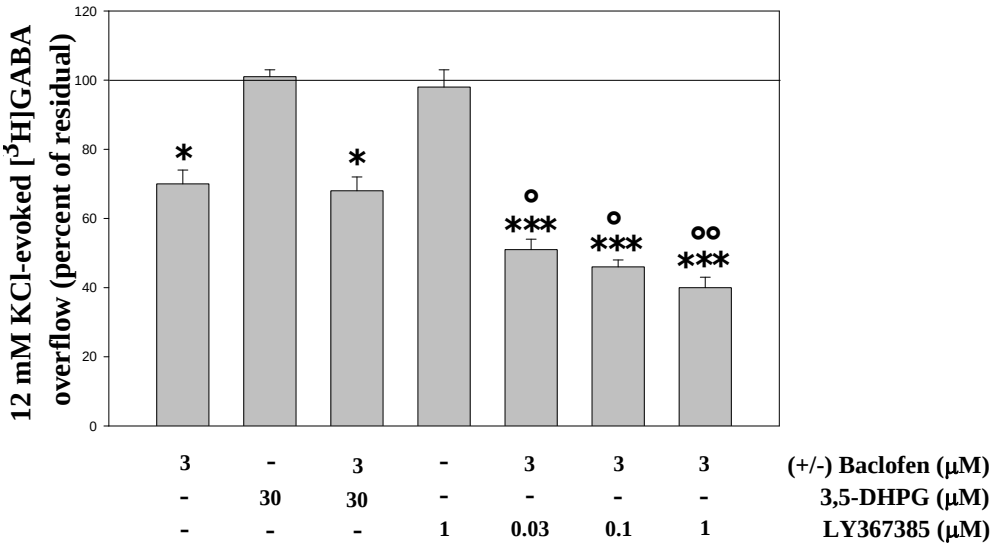


Figure 24: Effect of mGlu1 receptor ligands on the GABAergic transmission. Effects of 3 mM (±) baclofen, 30 mM 3,5-DHPG and LY 367385 (0.03–1 mM) alone or concomitantly added on the 12 mM KCl-induced [^{3}H]GABA overflow from mouse cortical nerve terminals. Results are expressed as percentage of the 12 mM KCl-induced [^{3}H]GABA overflow (percent of residual). Data are the means SEM of five experiments run in triplicate.*$p < 0.05$ vs 12 mM KCl overflow; ***$p < 0.001$ vs 12 mM KCl overflow; °$p < 0.05$ vs 12 mM KCl/3 mM (±)baclofen overflow;°°°$p < 0.001$ vs the 12 mM KCl/3 mM (±) baclofen overflow.

CONCLUSION

The aim of my PhD course and of this work was to approach the study of the neurotransmission in the CNS and, in particular, the mechanisms of control of transmitter release from selected populations of nerve endings.

I worked at different projects dedicated either to the pharmacological characterization of presynaptic release-regulating mGlu receptors and to analyse their interactions with other receptors. The main model used to perform these studies was the model of isolate nerve endings or synaptosomes. I focused on the pharmacological profiles of the mGlu2/3 receptors in selected regions of the CNS as well as on their ability to hetero/oligodimerization with other receptors.

The results debated in this thesis brings new insights about the mechanisms of the receptor oligomerization as well as on the receptor-receptor cross-talk in the CNS, in particular at the presynaptic terminals. The overall data unveil new possibilities to control glutamatergic and GABAergic transmission, in particular in the cortex and the spinal cord of adult rodents. Beyond the limits of our studies, my results lead to the following main conclusions:

- ❖ The integration of signals derived from glutamatergic and GABAergic neurotransmission can finely modulate one each other through the activation of oligomeric receptors assemblies that carry out complex “non conventional” responses.
- ❖ The dimerization processes can produce signals which can deeply modify the intraterminal pathways in cells and should be taken into considerations either in physiological and pathological conditions.
- ❖ The “up-down superfusion of a thin monolayer of synaptosomes“ represents, with biochemical support studies, a suitable approach to investigate receptor dimerization and receptor-receptor cross talk in the CNS.

"The scientist is not a person who gives the right answers, he's one who asks the right questions." (Claude Levi-Strauss)

METHODS

Animals

Adult mice (male and female, 3-6 months, strain C57BL/6J) and adult rats (female and male, 3-6 months, strain Sprague Dawley) were obtained from Charles River (Calco, Italy) and were housed in the animal facility of DIFAR, Section of Pharmacology and Toxicology. The experimental procedures were in accordance with the European legislation (European Communities Council Directive of 2010/63/EU) and the ARRIVE guidelines, and they were approved by the Italian Ministry of Health. All animals were kept under environmental controlled conditions (ambient temperature= 22°C, humidity= 40%) on a 12 h light/dark cycle with food and water available ad libitum. Animals were killed by cervical dislocation followed by decapitation and tissue were rapidly removed. In line with the 3Rs rules (replacement, refinement and reduction) any effort was made to reduce the number of animals to obtain statistically reliable results.

The mGlu2 receptor knockout ($mGlu2^{-/-}$) mice, the mGlu3 receptor knockout ($mGlu3^{-/-}$) mice, the double mGlu2/3 receptor knockout ($mGlu2/3^{-/-}$) mice on a CD1 genetic background and their CD1 wild-type (WT) counterparts were kindly provided by Eli Lilly & Company (Indianapolis, IN, USA) (Linden et al., 2005) and housed at IRCSS Neuromed. These mice were individually genotyped for the mGlu2 and the mGlu3 receptors gene by PCR analysis. The experimental procedures were in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number no.50/2011-B). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health. Animal studies were reported in compliance with the ARRIVE guidelines (see British Journal of Pharmacology, McGrath and Lilley, 2015).

The Grm1^{crv4} mice bearing the spontaneous recessive crv4 mutation were also used. The crv4 mutation occurred in the BALB/c/Pas inbred strain and consisted of an intronic insertion of a retrotransposon LTR (Long Terminal Repeat) fragment that disrupted the Grm1 gene splicing, causing the absence of mGlu1 receptor protein. The Grm1$^{crv4/crv4}$ homozygous mice presented mainly motor coordination deficits and bone defects (Conti et al., 2006; Musante et al., 2017). The affected (Grm1$^{crv4/crv4}$) and the control [Grm1$^{+/+}$, wild type (WT)] mice were maintained on the same genetic background by intercrossing Grm1$^{crv4/+}$ mice. The procedures for breeding and genotyping of Grm1$^{crv4/crv4}$ mice were reviewed and approved by the Animal welfare ethical committee of the IRCCS-AOU San Martino-IST National Cancer Research Institute (Genoa, Italy), and the definitive approval obtained by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number 371). To obtain the genotype of the mouse progeny, DNA was extracted from ear clippings according to the manufacturer's protocol (KAPA Mouse Genotyping Kits). Crv4 mutation was detected by DNA polymerase chain reaction (PCR) amplification using specific primers as previously described (Musante et al., 2010; Rossi et al., 2013).

Preparation of synaptosomes

Mouse or rat purified synaptosomes were prepared with a standard percoll gradient protocol from a selective nervous tissue (Dunkely et al., 2008, Summa et al., 2013). The selected CNS tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane (TRIS, final concentration 0.01 M) with a glass/Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (1,000 x g for 5 min) to remove nuclei and debris, and the supernatant was gently layered on a discontinuous percoll gradient (6%, 10%, and 20% v/v in Tris-buffered sucrose). After centrifugation at 33,500 x g for 5 min, the layer between 10% and 20%

percoll (synaptosomal fraction) was collected and washed by centrifugation (20,000 x g for 16 min). Synaptosomes were resuspended in a physiological medium having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.4.

Synaptosomes still represent one of the most suitable preparation to study neurotransmitter exocytosis from presynaptic compartment and, consequently, the modulation of release by presynaptic receptors. The formation of synaptosomes is due to a sequence of precise steps based on centrifugation and percoll separation and based on the properties of the plasma membrane. In fact, isolate nerve endings can be separated from the axon of neurons and, then, they can independently generate small circle bodies. Synaptosomes have a diameters about 1-1.5 μm and they preserve all the structures of the synaptic bottom, keeping alive for few hours in appropriate condition. Therefore, this model can be used to perform neurochemical studies like evaluation of neurotransmitter release or biochemical analysis like Confocal microscopy and Western Blot analysis (Pittaluga, 2016, 2019).

Preparation of mouse hippocampal slices.

Adult male wild type mice (6-10 weeks) were anaesthetized by isoflurane and killed by decapitation. The brain was rapidly removed and placed into ice-cold sucrose cutting solution comprising in (mM): NaCl (87), D-glucose (25), sucrose (75), $NaHCO_3$ (25), KCl (2.5), NaH_2PO_4 (1.25), ascorbic acid (3), $CaCl_2$ (0.5) and $MgCl_2$ (7), saturated with 95% O_2 and 5% CO_2.The hippocampi were removed from the brain, paying attention to the preservation of the whole structure, and transverse slices (350 μm thick) were prepared from the hippocampi isolated from each cerebral hemisphere using a Leica VT1200 S vibrating microtome according to the manufacturer's instructions. Then, slices were transferred to normal artificial cerebrospinal fluid (aCSF) at room temperature, comprising of in (mM): NaCl (124), D-

glucose (10), $NaHCO_3$ (26), KCl (3), NaH_2PO_4 (1.25), ascorbic acid (3), $CaCl_2$ (2) and $MgSO_4$ (1), continuously bubbled with 95% O_2 / 5% CO_2.

Release experiments

After the separation and the isolation from a selected tissue of CNS, synaptosomes were incubated for 15 min at 37°C in a rotary water bath in the presence of [^{3}H]D-Aspartate, an analogous of glutamate (indicated as [^{3}H]D-ASP,f.c.: 50 nM), or [^{3}H]GABA (f.c: 20 nM). In the experiment dedicate to the studies of the impact of selective anti-mGlu antibodies on the modulation of glutamate exocytosis by mGlu receptors, the synaptosomes were incubated for 30 min in the presence of the following antibodies: polyclonal rabbit anti-mGlu3 (1:1000) or monoclonal mouse anti-mGlu2 (1:1000). In these experiments, the radioactive tracer was added at t=15 min of incubation.

Identical portions of the synaptosomal suspension were layered on microporous filters at the bottom of parallel thermostated chambers in a Superfusion System (Raiteri et al., 1974, Pittaluga, 2016; Ugo Basile, Comerio, Varese, Italy). Synaptosomes were transiently (90 s) exposed, at t=39 min, to high KCl-containing medium (12 mM KCl for cortical synaptosomes or 15 mM KCl for spinal cord synaptosomes, NaCl substituting for an equimolar concentration of KCl; Zucchini et al., 2013) in the absence or in the presence of the agonists and/or antagonists. Allosteric or enzymatic modulators were always added at t=39 min concomitantly with agonists or alone. Fractions were collected as follows: two 3 min fractions (basal release), one before (t=36–39 min) and one after (t=45–48 min) and a 6 min fraction (t=39–45 min; evoked release). The collected fractions and the superfused synaptosomes were measured for radioactivity. The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total radioactivity. The KCl-evoked overflow was estimated by subtracting the neurotransmitter content in the first and the third fractions collected

(basal release, b1 and b3) from that in the 6 min fraction collected during and after the depolarization pulse (evoked release, b2). The effect of agonists/antagonists is expressed as percentage of the KCl evoked overflow of tritium in the absence of receptor agonists and antagonists (% of control). Data are always presented as the mean ± SEM of n (numbers indicate in the legend of the figure) independent determinations obtained in separate experiments run in triplicate (three superfusion chambers for each experimental conditions) on different days.

Electrophysiological recordings

Slices were allowed to recover for at least 1h at room temperature prior to commencing the recordings (recovery time). Meanwhile, the multi slice rig was prepared. This setting allows to record simultaneously from two slices in separate chambers and condition. Slice were transferred to submerged recording chambers continuously perfused at 3.5 mL/min with aCSF and maintained at 30-32 °C by means of an inline heater. Slices were secured in place by adherence to a poly-d-lysine coated coverslip and visualized using four digital microscopes (Dino-Lite), one for each chamber. A glass recording electrode [1.5 or 1.2 mm OD, 0.86 mm ID glass, with filament, resistance 1-4 MΩ, filled with aCSF and a bipolar stimulating electrode [made from Ni:Cr (80:20) formvar-coated wire] were put in place. In different experiments, I recorded separately from two different hippocampal areas, the Cornu Ammonis (CA1) and the Dentate Gyrus (DG), respectively. For CA1 recordings, both electrodes were placed in the *stratum radiatum* while stimulating the shaffer collateral pathway; for DG recordings, the electrodes were placed in the molecular layer of the Dentate Gyrus while stimulating the perforant path. Responses were evoked by monophasic constant current stimulation of 0.1 ms duration. Electrodes were carefully advanced into the slice so as to achieve the maximal response. The maximal amplitude (for the DG recordings) and slope (for the CA1 recordings) of

the field excitatory postsynaptic potential (fEPSP) were used as measure of synaptic strength. Stimulus intensity was set to a level eliciting a response about 50% in size of that where a population spike was first observed. In the DG recordings, correct positioning of the electrodes was verified by application of a paired-pulse protocol: application of paired pulses at an inter-stimulus interval of 50-100 ms induces paired-pulse depression in the Medial Perforant Pathway (MPP) but paired-pulse facilitation in the Lateral Perforant Pathway (LPP). Data were filtered at 3 kHz and amplified 500x by the amplifier and digitized at 10 kHz. fEPSP were evoked every 30s (0.03 Hz) and averages of 4 successive trails were analyzed on- and off- line with WinLTP software (Anderson and Collingridge, 2007).

Biotinylation (mGlu2/3 receptor studies)

The amount of mGlu2/3 receptors proteins in synaptosomal plasmamembranes was evaluated by performing surface biotinylation and subsequent immunoblot analysis (Salamone et al., 2014). Briefly, purified synaptosomes were divided into 2 aliquots: the first aliquot was incubated for 20 min with 100 nM MDL11,939 at 37 °C under mild shaking (T), while the other one was kept as control (C). Synaptosomes (T and C) were then treated with sulfo-NHS-SS-biotin (2 mg/ml) in PBS/Ca-Mg of the following composition (mM): 138 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4, 1.5 $MgCl_2$, 0.2 $CaCl_2$, pH 7.4 for 1 h at 4 °C and then incubated in PBS/Ca-Mg with 100 mM glycine for 15 min at 4 °C to quench the reaction. Biotinylated synaptosomes were then lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors). Samples (100 mg) were incubated with NeutrAvidin agarose beads for 1 h at room temperature under shaking. Beads were added to the biotinylated synaptosomes to pull down the biotinylated proteins, as well as to non-biotinylated synaptosomes, to evaluate the specificity of neutravidin pull-down (B).

After extensive washes, samples were boiled for 5 min at 95 °C in SDS-PAGE loading buffer to separate biotinylated proteins from the beads. Eluted fractions were analysed through immunoblot assay (see section "Western blot analysis"). The immunoreactivity of the mGlu2/3 receptors was monitored in the total lysate (L), in the control and in the antagonist-pretreated biotinylated synaptosomes (respectively C and T) and in the streptavidin pulldown of the non-biotinylated synaptosomal lysate (B). β-actin (Sigma Milan, Italy), a cytosolic protein, was used as control to evaluate the specificity of biotinylation reaction.

Immunoprecipitation

Purified synaptosomes were lysed in ice-cold lysis buffer having the following composition: 140 mM NaCl, 20 mMTris, 0.5% Triton X-100, protease inhibitors, pH 7.4. Protein A Dynabeads were incubated with anti-mGlu2/3 1:500, anti-$GABA_{B1}$ 1:500 and anti-$GABA_{B2}$ 1:500 antibodies diluted in PBS containing 0.02% Tween 20 (t-PBS) for 10 min at room temperature. Synaptosomal lysate (200 mg) was added to antibody-bound Protein A Dynabeads (I.P.), as well as to beads without antibody (negative control, B). After an incubation for 25 min at room temperature under shaking, beads were washed three times in t-PBS and then resuspended in SDS-PAGE loading buffer. Samples were boiled at 95 °C for 5 min in order to elute proteins from beads and subjected to Western Blot analysis (see below section"Western blot analysis").

Western blot analysis

The purified synaptosomes were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, protease inhibitors, pH 8.0) and quantified for the protein content with Bradford protocol. Samples were boiled for 5 min at 95°C in SDS-PAGE loading buffer. Proteins were then separated by SDS 7.5% or 10% PAGE and

transferred onto PVDF membranes. Membranes were incubated for 1 h at room temperature in Tris-buffered saline-Twee (t-TBS: 0.02 M Tris, 0.150 mM NaCl and 0.05% Tween 20), containing 5% (w.v-1) non-fat dried milk and then probed with different primary antibodies (see below for entire list) overnight at 4°C. After extensive washes in t-TBS, the membranes were incubated for about 1 h at room temperature with appropriate horseradish peroxidise-linked secondary antibodies (see below for entire list).

In the experiments carried out to verify the specificity of the anti-mGlu2 and anti-mGlu3 antibodies, mouse cerebral cortices (from WT and knockout mice) were dissected out and homogenized at 4°C in Tris–HCl pH 7.5, 10 mM; NaCl, 150 mM; SDS 0.1%, EDTA, 5 mM and complete TM protease cocktail tablets. Proteins (20 μg) from supernatants were separated by 8% SDS-PAGE and transferred on immuno-blot PVDF membranes. Membranes were incubated with mouse anti-mGlu2 (1 h at room temperature, in t-TBS), rabbit anti-mGlu3 receptor (1 h at room temperature, in t-TBS) and mouse anti β-tubulin (overnight at 4°C) antibodies and then for 1 h with the appropriate peroxidise coupled secondary antibodies. Immunoblots were visualized with an enhanced chemiluminescence plus Western blotting detection system. Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec, Cambridge, UK).

Primary antibodies: mouse monoclonal anti mGlu 1 receptor antibody 1:250 (BD Biosciences (San Jose, CA, United States), mouse monoclonal anti-$GABA_{B1}$ receptor antibody 1:500 (Santa Cruz Biotechnology, Dallas, TX, United States), mouse monoclonal anti-$GABA_{B2}$ receptor antibody 1:500 (Santa Cruz Biotechnology, Dallas, TX, United States), mouse monoclonal anti-mGlu2 receptor antibody 1:1000 for cortical synaptosomes and 1:500 for spinal cord synaptosomes (Abcam Cambridge, UK), rabbit monoclonal anti-mGlu3 receptor antibody 1:1000 (Alomone Labs

Jerusalem, Israel), rabbit monoclonal anti-mGlu2/3 receptor antibody 1:2000 (Novus Biologicals Littleton CO, USA), rabbit monoclonal anti-5-HT_{2A} receptor antibody 1:500 (Immunostar Hudson,WI, USA), mouse polyclonal anti-β-tubulin antibody 1:800 (Sigma Milan, Italy), mouse monoclonal anti-β-actin antibody 1:5000 (Sigma Milan, Italy), mouse monoclonal anti-Gapdh antibody 1:10000 (Sigma Milan, Italy).

All horseradish peroxidase-coupled anti-mouse and anti-rabbit secondary antibodies were from Sigma (Milan, Italy).

Confocal analysis

Purified synaptosomes were fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.05% Triton X-100 PBS for 5 min and incubated with primary antibodies (see below for entire list): After extensive washes, synaptosomes were incubated for 1 h at room temperature with the antibodies reported below. Synaptosomes were then applied to coverslips (Musante et al., 2008). Fluorescence images (512×512×8 bit) were then visualized by use of a sixchannel Leica TCS SP5 laser-scanning confocal microscope, equipped with 458, 476, 488, 514, 543 and 633 nm excitation lines, through a plan-apochromatic oil immersion objective 63X/1.4NA. Light collection configuration was optimized according to the combination of chosen fluorochromes. Sequential channel acquisition was performed to avoid crosstalk. A Leica 'LAS AF' software package was used for image acquisition, storage and visualization. The quantitative estimation of co-localized proteins was performed as described previously (Musante et al., 2008; Summa et al., 2013), by calculating the 'co-localization coefficients' (Manders et al., 1993). They express the fraction of colocalizing molecular species in each component of a dual colour image and are based on the Pearson's correlation coefficient, a standard procedure for matching one image with another in pattern recognition. If two molecular species are colocalized, the overlay of their spatial distributions has a correlation value

higher than what would be expected by chance alone. Costes et al. (2004) developed an automated procedure to evaluate the correlation between the green and red channels with a significance level >95%. The same procedure automatically determines an intensity threshold for each colour channel based on a linear least-square fit of the green and red intensities in the image's 2D correlation cytofluorogramme. Costes's approach was carried out by macro routines integrated as plugins (WCIF Colocalization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.51p software (Wayne Rasband, NIH, USA).

For mGlu2/3 studies: I used the following primary antibodies: mouse anti-mGlu2 receptor 1:1000 (Abcam Cambridge, UK), rabbit anti-mGlu3 receptor 1:1000 (Alomone Labs Jerusalem, Israel) and guinea pig anti vesicular glutamate transporters type 1 or VGLUT1 1:500 (Millipore Corporation, Billerica, MA, USA). I used the following conjugated antibodies: donkey anti-rabbit AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying co-localization of mGlu3 receptor and VGLUT1 proteins), with donkey anti mouse AlexaFluor-488 and goat anti guinea pig AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying co-localization ofmGlu2 receptor and VGLUT1 proteins), with donkey anti-rabbit AlexaFluor-488 with goat anti-mouse AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying co-localization of mGlu2 and mGlu3 receptor proteins). All AlexaFluor antibodies are purchased from Sigma, Milan, Italy

For mGlu2/3 and 5-HT_{2a} studies: I used the following primary antibodies: rabbit anti-mGlu2/3 receptor antibody 1:1000 (Novus Biologicals Littleton CO, USA), mouse anti-5-$HT2_A$ receptor antibody 1:200 (Immunostar Hudson,WI, USA), and guinea pig anti-vesicular glutamate transporters type 1 or VGLUT1 1:500 (Millipore Corporation, Billerica, MA, USA). I used the following conjugated antibodies: donkey anti-rabbit AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, confocal

analysis aimed at identifying colocalization of mGlu2/3 receptor and VGLUT1 proteins), with donkey anti-mouse AlexaFluor-488 and goat anti-guinea pig AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying colocalization of 5-$HT2_a$ receptor and VGLUT1 proteins), with donkey anti-rabbit AlexaFluor-488 with goat anti-mouse AlexaFluor-633 (1:1000 both, confocal analysis aimed at identifying colocalization of mGlu2/3 and 5-HT2A receptor proteins). All AlexaFluor antibodies are purchased from Sigma, Milan, Italy.

For $GABA_B$ and mGlu1 studies: I used the following primary antibodies: rabbit anti-mGlu1 receptor antibody 1:500 (BD Biosciences, San Jose, CA, United States), mouse anti-$GABA_{B1}$ receptor antibody 1:500 (Santa Cruz Biotechnology, Dallas, TX, United States), mouse anti-$GABA_{B2}$ receptor antibody 1:500 (Santa Cruz Biotechnology, Dallas, TX, United States), guinea pig anti-vesicular GABA transporter or VGAT 1:300 (Alomone Labs Jerusalem, Israel), and guinea pig anti-vesicular glutamate transporter type 1 or VGLUT1 1:500 (Millipore Corporation, Billerica, MA, USA). I used the following conjugated antibodies: donkey anti-mouse AlexaFluor-647, goat anti-guinea pig AlexaFluor-488, goat anti-rabbit AlexaFluor-555 as appropriate, purchased by Life Technologies Corporation Carlsbad, CA, United States (for colocalization see "Results and Discussion" sections).

Calculations and statistical analysis

Sigma plot 10 data analysis and graphing software package were used for data handling/statistics and for graph drawing. ANOVA was performed followed by Dunnett's test or Newman–Keulsmultiple comparisons test, as appropriate; direct comparisons were performed by Student's t-test. Post hoc tests were done only if F value was significant. Data were considered significant if $P < 0.05$ and reported as *P <

0.05, **P < 0.01 and *** P < 0.001 (*could be change with other symbols where necessary).

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