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FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DE COIMBRA

mGlu7 receptor modulation of synaptic transmission and short-term plasticity at the CA1 and dentate gyrus areas of the hippocampus

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Hilde Lavreysen (Janssen Pharmaceutica NV) e do Doutor Ken Veys (Janssen Pharmaceutica NV) e supervisão do Professor Doutor Carlos B. Duarte (Universidade de Coimbra)

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2014

The work presented in this thesis resulted from a partnership between the University of Coimbra and Janssen Pharmaceutica NV. All experimental activities were performed at Janssen Pharmaceutica NV Beerse I, a Johnson & Johnson pharmaceutical research and development facility in Beerse, Belgium.

Beerse, 2014

Acknowledgments

I would like to specify my gratitude to my supervisors: Dr. Hilde Lavreysen, Dr. John Kemp and Dr. Hamdy Shaban for all their input and critics that helped the development of this work.

To Dr. Ken Veys an especial thank you for improving all the possible aspects of this year and creating a strong support for the ones to come.

To Erik, Ilse, Jacobine, Sophie, Sara and Nuno for their useful guidance and patience.

To all the people working in the lab that have made my frequents quests in the search of something much shorter than needed.

To all my colleagues that I had the pleasure to meet and share this experience and have really improve it.

Ao Daniel Rial, Profesor Rodrigo, e restantes membros do grupo das Purinas do CNC.

Ao Professor Carlos Duarte e todos os elementos do seu grupo.

Um especial obrigado a Elvira por toda a paciencia e conselhos e todas as experiencias que nunca foram demais.

To Iryna for the advices and talks putting me right back on track.

Á Rita pela longas horas em que o seu suporte foi vital para tudo.

Aos meus pais pelo incondicional e constante apoio que muitas vezes foi necessário e sempre me levou a seguir em frente.

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Abbreviations

- DHPG,(R,S)-3,5-dihydroxyphenylglycine
- ACPD,(α)-1-aminocyclopentane-trans-1,3-dicarboxylic acid
- 12(S)-HPETE, 12(S)-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid
- MPEP, 2-methyl-6-(phenylethynyl)pyridine hydrochloride
- CNQX,6-cyano-7-nitroquinoxaline-2,3-dione
- ATF2, activating transcription factor 2
- AMPA,α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- AMPAR, AMPA receptors
- AA, arachidonic acid ()
- ATF2, activating transcription factor 2
- BAPTA,1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
- CA1, cornus ammonis 1
- Ca^{2+}, calcium
- CA3, cornus ammonis 3
- CaM, calmodulin;
- CaMKII, Ca 2 /calmodulin-dependent protein kinase II;
- CREB,cAMPresponse element-binding
- CNS, central nervous system
- CO_2, Carbon dioxide
- CV, coefficient of variation
- CREB, cAMP response element-binding;
- CRD, cysteine-rich domain
- AP-5, D-2-amino-5-phosphonopentanoate
- DAG, dyacylglycerol
- DAT, Dopamine transporter
- DG, dentate gyrus
- DHPG, (R,S)-3,5-dihydroxyphenyl-glycine
- eCB, endocannabinoid
- Ecm extracellular matrix
- eEF, eukaryotic translation elongation factor
- eEF2, eukaryotictranslation elongation factor 2
- eEF2K, eukaryotic translation elongation factor 2 kinase
- EF1A, elongation factor 1A
- eIF4, eukaryotic initiation factor 4
- EPSC, excitatory postsynaptic current
- EPSP- Excitatory postsynaptic potential
- ERK, extracellular signal-regulated kinase
- ERK, extracellular signal-regulated kinase
- fEPSP field excitatory Post Synaptic potential

- FMRP, fragile X mental retardation protein
- FXS, fragile X syndrome
- GPCR, G protein-coupled receptors
- GABA, γ-aminobutyric acid
- GABAB, the α-aminobutyric acid receptor B
- GAD, Glutamate decarboxylase
- GDI, guanine nucleotide dissociation inhibitor
- GIRK, G protein gated inwardly rectifiying potassium
- GKAP, guanylate-kinase-associated protein
- GPCR, G protein-coupled receptor
- GRIP, glutamate receptor interacting protein
- HFS, high frequency stimulation
- HEK , human embryonic kidney
- Hz, Hertz
- iGluR, ionotropic glutamate receptor
- IP3, inositol trisphosphate
- IP3R, inositol trisphosphate receptor
- IPSC, inhibitory postsynaptic current
- JNK, Jun N-terminal kinase
- KO, knockout
- L-AP4, l-amino-4-phosphonobutyric acid
- LBR, extracellular ligand-binding region
- LFS, low-frequency stimulation
- LTP,long-term potentiation
- LTD, long term depression
- LY341495, (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid
- LY367385, (S)-()--amino-4-carboxy-2-methylbenzeneacetic acid
- m1-LBR, mGluR1-LBR
- MAP1B, microtubule-associated protein 1B
- MAPK, mitogen activated protein kinase
- MAPK, mitogen activated protein kinase
- MCPG, α-methyl-4-carboxyphenylglycine
- MDD major depressive disorder
- MEA,Micro-electrode arrays
- mGlu receptor, metabotropic glutamate receptor
- MEK, MAP kinase or ERK kinase
- mEPSP Miniature excitatory Post Synaptic potential.
- mGluR, metabotropic glutamate receptor
- Mnk1, MAPK-interacting kinase 1
- mossy fiver – stratum lucidum interneurons (MF-SLIN)

- mTOR, mammalian target of the rapamycin
- mTOR, PI3K-Akt mammalian target of the rapamycin
- Na^{+}- Sodium
- NAc Nucleus accumbens
- NAM negative allosteric modulator
- NAT noradrenaline transporter
- NCS, neuronal Ca2+ sensor
- NF-B, nuclear factor-B
- NMDA N-methyl-D-aspartic acid
- NMDA, N-methyl-D-aspartate
- NMDAR, NMDA receptors
- NP, neuronal pentraxin
- NPR, neuronal pentraxin receptor
- NPR, neuronal pentraxin receptor
- PPD,paired pulse depression
- PPF, Paired pulse facilitation
- PPR,paired pulse ratio
- PAM positive allosteric modulator
- PDZ, post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
- PI3K, phosphoinositide 3-kinase;
- PICK, protein interacting with C kinase
- PIKE, PI 3-kinase enhancer
- PKC, protein kinase C
- PLA2, phospholipase A 2
- PLC, phospholipase C
- PP1, protein phosphatase 1
- PP2A, protein phosphatase 2A
- PP2B, protein phosphatase 2B
- PPG, R-S-phosphonophenylglycine
- PP-LFS, paired-pulse LFS
- Munc-13, Protein unc-13 homolog
- PSD, postsynaptic density
- PTK, protein tyrosine kinase
- PTPs, protein tyrosine phosphatases
- Rab5, ras in the brain protein 5
- Rap1, repressor activator protein 1
- RSK1, ribosomal S6 kinase-1
- S6K, S6 kinase
- SC, Schaffer collaterals
- sEPSP, spontaneous excitatory Post Synaptic potential
- SERT, serotonin transporter
- siRNA – small interfering ribonucleic acid
- STEP, striatal-enriched tyrosine phosphatase
- TACE, tumor necrosis factor--converting enzyme
- TMD, heptahelical transmembrane domain
- VFD, Venus flytrap domain
- MCPG,α-Methyl-4-carboxyphenylglycine

Abbreviations

Abstract and research aims

The metabotropic glutamate subtype receptor 7 (mGlu7 receptor) is the most highly conserved, the predominant of the group III mGlu receptor in the presynaptic active zone and the most widely distributed among mGlu receptors, suggesting a critical role in regulating excitatory and inhibitory synaptic transmission in the central nervous system (CNS). Nonetheless the characterization of the functional role of the mGlu7 receptor in the CNS has been hampered by the lack of selective agents and limited to mGluR7 knockout mouse studies until valuable pharmacological tools for studying its function are being developed.

The main aim of this work was to investigate its role in the control of hippocampal basal synaptic transmission and short-term plasticity. Hippocampal slices from adult and immature Sprague Dawley rats were used to record electrophysiological events using paired-pulse stimulation and a Short-High stimulation paradigm in Schaffer fiber-CA1 synapses and/or dentate gyrus medial perforant path-granule cells synapses.

When tested in the CA1 area L-2- amino-4-phosphonobutyric acid (L-AP4, 3 µM to 1 mM) demonstrated a very different EC_{50} for adult ad immature rats, 1.13 mM and 10.44 µM, respectively.

The diverse allosteric modulators used (AMN802, MMPIP and ADX71743) have demonstrated a promising potential when tested in diverse types of cells expressing the mGlu7 receptor. However when tested in brain slices in CA1 none of them modified in a relevant manner the effect of 600 µM of L-AP4 ((0.74±0.1); MMPIP (10 µM) and L-AP4 (600 µM), 0.56±0.09; ADX71743 (10 µM) and L-AP4 (600 µM), 0.80±0.09; AMN082 (1 µM) and L-AP4 (600 µM), 0.66±0.1). In the other hand, in the dentage gyrus medial perforant path, LY341495 reversed the effect of L-AP4 (0.97±0.05) and, controversially, MMPIP potentiated it (MMPIP plus L-AP4, 0.51±0.06).

In view of the proposed role of mGlu7 as a “low-pass filter”, its role in spontaneous and NMDA evoked spiking was tested with the used of L-AP4. The compound demonstrated a negative effect over spontaneous events (L-AP4 (600 µM), 94.6%± 12) nonetheless when applied before or after elevated doses of NMDA it led to a further increase of the events evoked (NMDA 100 µM and L-AP4 600 µM, 143.4%± 2.85; L-AP4 600 µM and NMDA 100 µM, 1101.4%± 14).

The presence of $GABA_A$ receptor antagonist picrotoxin (PTX) partially reverted the effects of L-AP4 (600 µM) (0.94±0.04 – with PTX vs. 0.74±0.1 –without PTX) and in the presence of 10 µM PTX L-AP4 did not led to an alteration of the PPR.

L-AP4 at a concentration of 600 μM significantly reduces both the values for the peak and area under the curve of NMDAR mediated potentials, indicating that the compound is modulating this receptor response (Peak value: L-AP4 600 μM, 0.53±0.04; Area under the curve: L-AP4 600 μM, 0.55±0.03)

The results suggest that L-AP4 may be acting on different targets than mGlu receptors with a possible agonist effect over GABAa receptors and/or antagonism of NMDAR responses. This still allows the presence of a small window for mGlu7 receptors that need to be further explored with relevant models and more selective compounds tested in those same models.

Keywords: mGlu receptors; synaptic plasticity; synaptic transmission; hippocampus; multi-electrode array; fEPSP

Resumo e Objectivos do Trabalho

O recetor metabotrópico de glutamato do subtipo 7 (recetor mGlu7) é o recetor mais conservado entre espécies, sendo dos elementos do grupo III dos recetores mGlu o que predominantemente se encontra na zona pré-sináptica, este é também o mais amplamente distribuído entre os receptores metabotrópicos de glutamato. Estas características sugerem um papel crítico na regulação da transmissão sináptica excitatória e inibitória no sistema nervoso central (SNC). No entanto, a caracterização funcional deste recetor no SNC continua a ser limitada à existência de modelos knockout para o mGlu7 e pela falta de agentes seletivos até que novos agentes farmacológicos sejam desenvolvidos.

O principal objetivo deste trabalho foi discernir o papel que este recetor tem no controlo da transmissão sináptica basal e na plasticidade de curta duração no hipocampo. Para tal, foram obtidos registos eletrofisiológicos a partir de fatias de hipocampo de ratos Sprague Dawley adultos e jovens. Estes registos foram obtidos após estimulação por pulso pareado tanto nas fibras de Schaffer-CA1 como no caminho perfurante medial – células granulares do giro dentado.

Quando testado na área CA1 o agonista ácido L-2- amino-4-fosfonobutirico (L-AP4, 3 µM até 1 mM) demonstrou um EC50 muito diferente entre ratos adultos e imaturos, 1.13 mM e 10.44 µM respetivamente.

Os diversos moduladores alostéricos usados (AMN802, MMPIP e ADX71743) demonstram um potencial promissor quando testados em diversos tipos de linhas celulares que expressam o recetor mGLU7. Contudo quando testados em fatias de cérebro na área CA1 do hipocampo nenhum dos compostos modificou de maneira significativa os efeitos da aplicação de 600 µM de L-AP4 (0.74±0.1; 10 µM MMPIP e 600 µM L-AP4: 0.56±0.09; 10 µM ADX71743 e 600 µM L-AP4: 0.80±0.09; 1 µM AMN082 e 600 µM L-AP4: 0.66±0.1). Em contraste no giro dentado, o antagonista LY341495, reverteu os efeitos do L-AP4 (0.97±0.05) e contrariamente ao esperado, o MMPIP potenciou os efeitos do agonista (MMPIP e L-AP4: 0.51±0.06).

Tendo em conta o pressuposto papel do recetor como filtro passa-baixo, o seu potencial papel no controlo da atividade neuronal espontânea mediada por NMDA foi testado usando o L-AP4. Este composto diminuiu o número de eventos espontâneos (600 µM L-AP4: 94.6%± 12) no entanto, quando aplicado antes ou depois de doses elevadas de NMDA conduziu a um aumento dos eventos evocados por NMDA (100 uM NMDA e 600 uM L-AP4: 143,4% ± 2,85; 600 uM L-AP4 e 100 uM NMDA: 1101,4% ± 14).

Na presença de picrotoxina (PTX), um antagonista dos recetores GABAA, os efeitos de L-AP4 foram parcialmente revertidos(600 uM L-AP4: com PTX: 0.94±0.04 versus sem PTX:0.74±0.1) e na presença de 10 μM PTX este não induziu a alteração de PPR.

O L-AP4 a uma concentração de 600 uM reduz significativamente tanto os valores de pico como a área sob a curva dos potenciais mediados por NMDAR, indicando que o composto estará a modular a resposta do recetor (valor de pico: L-AP4 600 uM, 0,53 ± 0,04; área sob a curva: L-AP4 600 mM, 0,55 ± 0,03).

Os resultados sugerem que o L-AP4 pode estar a atuar em alvos diferentes dos recetores mGlu com um possível efeito agonista sobre os recetores GABAa e/ou antagonista das respostas do NMDAR. Contudo, os dados parecem também indicar a presença de uma pequena janela de ação para os recetores mGlu7, que precisa de ser mais explorada em modelos relevantes e testada com compostos mais seletivos.

Palavras chave: receptores metabotropicos de glutamato; plasticidade sináptica; transmissao sináptica; hipocampo

1 Glutamatergic Neurotransmission in the hippocampus

1.1 Glutamate and its receptors

Glutamate is considered the main excitatory transmitter in the hippocampus with its actions mediated via two main classes of receptors: ionotropic and metabotropic receptors. The ionotropic glutamate receptors are ligand-gated ion channels in charge of the vast majority of fast excitatory neurotransmission in the CNS. Once a correct concentration of glutamate is released and binds to these receptors it will lead to channel opening, with the resulting predominant Na^+ influx leading to membrane depolarization. Ionotropic glutamate receptors can be classified into three major groups which are named after their selective agonists: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartic acid (NMDA), and kainate receptors and all are heteromeric structures consisting of four subunits which define the properties of the receptor such as its kinetic parameters and/or its ion permeability. AMPA receptors (AMPAR) are composed of a combination of four subunits: GluR1, GluR2, GluR3, and GluR4 (or GluR A–D)[6]. The presence or absence of the GluR2 subunit in the complex will determine several biophysical properties of the receptor. NMDA receptors (NMDAR) are heteromultimers composed of NR1 and NR2A–D subunits, having slower kinetics when compared to AMPAR or kainate receptors (KaR), possibly because of its slow dissociation rate for glutamate. As a result, while glutamate is bound, NMDA channels have shown the capability of undergoing repeated opening.[7] NMDAR activation requires binding of glycine prior to glutamate binding, leading to channel opening and sodium ions (Na^+) and calcium ions (Ca^{2+}) entering the cell. Besides the need for glycine, channel opening can only occur when the magnesium ions (Mg^{2+}) block is removed from NMDAR while the membrane is depolarized (a reference value are currents above -35 millivolts (mV)) , since at resting membrane potentials glutamate binding does not lead to channel opening. Kainate receptors are composed of combinations involving GluR5, 6, 7 and KA1, 2 subunits which alone are non-functional and are retained in the endoplasmic reticulum, but can combine with GluR5–7 to form surface-localized functional receptors. [8, 9]

The metabotropic glutamate receptors (mGlu receptors) are G protein coupled receptors (GPCR) that transduce extracellular signals into G protein activation through biomembranes. They belong to same family as the gamma-aminobutyric acid receptor B ($GABA_B$ receptor), the calcium-sensing receptor, and some taste and pheromone receptors. L-glutamate is their principal agonist. The mGlu receptors are categorized into three groups, which comprise eight subtypes. Each of the groups has a specific regional distribution in the brain and displays a distinct pharmacological profile. All of these characteristics will be further discussed.[10]

1.2 The cornus ammonis 1(CA1) area of the hippocampus

The cornus ammonis 1(CA1) CA1 area of the hippocampus is together with the cornus ammonis 3(CA3) area the principal pyramidal cell fields in the hippocampus. See Figure 1. Being one of the most studied areas of the hippocampus it has revealed playing a role in a diversity of function ranging from autobiographical memory, mental time travel, autonoetic consciousness[11], encoding and retrieval of extinguished fear[12]. It is also preferentially affected in cases of hippocampal atrophy such as in AD. [13]

With the exception of stratum radiatum giant cells, the principal neurons in the hippocampal subfields are a quite homogeneous population of glutamate- releasing pyramid-shaped neurons and GABAergic interneurons. In the CA1 and CA3 areas pyramidal neurons bear numerous spines receiving a single excitatory synapse. Although single-cell-labeling studies indicate that all pyramidal neurons have a local axonal arbor, most excitatory synapses in the hippocampal subfields are of extraneous origin, arriving from a multitude of sources. Although long-term potentiation (LTP) was first described in the dentate gyrus, most studies are done in the CA1 region; this is because of the relative ease of obtaining field potential recordings and intracellular recordings in this region.[5]

1.3 Dentate gyrus of the hippocampus

The dentate gyrus (DG) “filters” incoming neuronal activity from the entorhinal cortex to the hippocampus and in this way, helps to modulate neuronal transfer to the hippocampus proper[14]. Of particular relevance are some features of its perforant pathway synapses such as the distinct features of

medial and lateral perforant pathway inputs, the direct input to CA1 that plays important role in feed-forward inhibition, an input-specific subunit composition of NMDARs and a complementary distribution of mGlu receptors at the medial and lateral perforant pathway. Differential regulation of the medial and lateral perforant path by different mGlu receptors has been established by using selective Group II and Group III agonists and antagonists. Group III metabotropic receptors have mainly a role regulating glutamate release at the lateral perforant pathway, while Group II mGlu receptors can serve as autoreceptors at the medial perforant path. Activation of presynaptic Group II mGlu receptors at the medial perforant pathway is associated with a reduction on synaptic transmission and resulted in a reduction of short-term depression.[15]

A very useful characteristic is that in response to paired pulse stimuli lateral perforant pathway synapses show marked facilitation, while medial perforant path synapses show less facilitation or even depression using a paired-pulse paradigm. If a longer train of stimulus is used the medial perforant path input shows significant depression, while the amplitude of the synaptic events evoked with the stimulation of the lateral pathway shows minimal change. Since the ratio of the excitatory postsynaptic potentials (EPSP) to fiber response is greater in the medial pathway and the observed short-term depression converts to facilitation in lower extracellular calcium concentration ([Ca^{2+}]), it is very likely that the initial release probability is lower at lateral pathway synapses. Discrepancy between the quanta sensed by NMDAR and AMPAR is observed in the lateral, but not in the medial perforant path, indicating that silent synapses are present only at lateral pathway synapses. In the CA1 area, perforant pathway axons display both Group II and Group III mGlu receptors, while mGlu7a receptor and mGlu4 are detected in active zones, mGlu2 can be found in preterminal zones. Segregation of these mGlu receptors to different zones of the presynaptic terminal and their different signaling suggest that

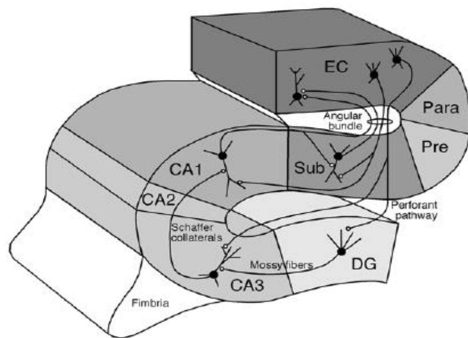

Figure 1 The hippocampal formation. Neurons in layer II of the entorhinal cortex project to the dentate gyrus and the CA3 field of the hippocampus proper via the perforant pathway. Neurons in layer III of the entorhinal cortex project to the CA1 field of the hippocampus and the subiculum via the perforant and alvear pathways. The granule cells of the dentate gyrus project to the CA3 field of the hippocampus via mossy fiber projections. Pyramidal neurons in the CA3 field of the hippocampus project to CA1 via Schaffer collaterals. Pyramidal cells in CA1 project to the subiculum. Both CA1 and the subiculum project back to the deep layers of the entorhinal cortex.[5]

they could be involved in distinct regulatory roles. [16, 17]

On the other hand, activation of mGlu receptors is involved in the induction of a large amplitude or long-lasting late phase LTP of AMPAR-mediated transmission induced by strong or repeated stimulation protocols since it can be blocked by the use of, for example, both antagonists 2-[(1S,2S)-2-carboxycyclopropyl]- 3-(9H-xanthen-9-yl)-D-alanine (LY341495) and 2-Methyl-6-(phenylethynyl) pyridine (MPEP). The involvement of group I mGlu receptors in LTP induced by intense stimulation is thereby consistent with spillover of synaptically released glutamate to the perisynaptic location of group I mGlu receptors.[18]

The enhancement of nicotinic acetylcholine receptor dependent LTP is also done by mGlu receptors. mGlu receptors have also been shown to contribute in metaplastic changes in LTP, being involved in the facilitation of LTP by prior stimulation (priming), modulating LTP of AMPAR-mediated transmission at the mossy fibre synapse to CA3 principle neurons and influencing LTP induction by the setting of a molecular switch which after activation stays on for hours. The switch can, however, be actively reset using low frequency stimulation.[6,19]

2 The Structure and classification of mGlu receptors

The mGlu receptors are members of the class C subclass of receptors within the GPCR superfamily. There are four main subclasses of Class C receptors and mGlu receptors are included in the subclass II. All members of this family possess a large extracellular ligand binding domain, also referred to as the Venus Flytrap Domain (VFD), a prototypical heptahelical transmembrane domain (TMD), and an intracellular carboxy terminus. Class C receptors also have a short cysteine-rich domain (CRD) situated between the VFD and the TMD regions, except $GABA_BR$.[13, 14]

To date, eight subtypes of mammalian mGlu receptors are known, which are categorized into three groups according to sequence similarity, location in the nervous system, and pharmacologic properties (see Table 1).*In vitro* studies have shown that group I receptors are coupled to G_q and the G_q-like family of G proteins and the stimulation of phospholipase C and release of intracellular calcium, whereas the group II and III receptors couple to the G_i and G_o family of G proteins and the inhibition of adenylyl cyclase.[20] On the other hand *in vivo,* mGlu receptors are coupled to multiple signal transduction pathways that lead to the modulation of, for example, voltage-gated calcium and /or potassium channels.[21, 22]

The Structure and classification of mGlu receptors

Evidence indicates that mGlu receptors, like other neurotransmitter receptors, are oligomeric protein complexes mainly homodimers in which protomers within the dimeric complexes are linked at numerous points. Nonetheless some groups have reported the presence of heterodimers composed of one mGlu subunit partnered with others subunits of different class C GPCR or for example mGlu2 and mGlu4 subunits (but not mGlu2 and mGlu1) can heteromerize. [23, 24] The existence of higher-order structures *in vivo*, such as tetramers formed from dimers of dimers, has not been yet ruled out.

Table 1 Classification of the mGluRs

Family receptor	Coupling	Key localization and actions	Group/subtype-selective pharmacological agents
Group I			
$mGlu_1$	Excitatory G_q-coupled	Most often postsynaptic at glutamatergic synapses. Indicated in synaptic plasticity, including long-term. potentiation/depression (LTP/LTD). Cerebellar localization in granular cell and parallel fibre layers.	Agonists: DHPG, 1*S*,3*R*-ACPD, quisqualate Antagonist: LY393675 Inverse agonist (or allosteric antagonist): LY367385
$mGlu_5$	Excitatory G_q-coupled	Most often postsynaptic at glutamatergic synapses, also found in glial cells. High expression in several forebrain regions including hippocampus and amygdala. Indicated in synaptic plasticity, especially some forms of cortical and hippocampal LTD.	Agonists: DHPG, 1*S*,3*R*-ACPD, quisqualate, CHPG Inverse agonist (or allosteric antagonist): MPEP
Group II			
$mGlu_2$	Inhibitory G_i/G_o-coupled	Localization largely presynaptic on glutamatergic and other neurotransmitter synapses. High expression in forebrain regions including hippocampus and amygdala; also in certain layers with the cortex and cerebellum. Linked to hippocampal LTD and regulation on medial perforant path.	Agonists: DCG-IV, 2*R*,4*R*-APDC, 1*S*,3*R*-ACPD, LY354740, LY379268 Antagonist: LY341495 Potentiator: 4-MPPTS (LY487379), 4-APPES, CBiPES
$mGlu_3$	Inhibitory G_i/G_o-coupled	Widely expressed in glial cells but also discrete localization both pre- and postsynaptic on glutamatergic and other neurotransmitter synapses. Expression within forebrain regions including hippocampus and thalamus. Linked to neurotropin release from glial cells.	Agonists: DCG-IV, 2*R*,4*R*-APDC, 1*S*,3*R*-ACPD, LY354740, LY379268 Antagonist: LY341495
Group III			
$mGlu_4$	Inhibitory G_i/G_o-coupled	Localization both pre- and postsynaptic on glutamatergic and other neurotransmitter synapses. Presynaptic in cerebellar parallel fibres and linked to cerebellar plasticity and motor learning.	Agonists: L-SOP, ACPT-1, L-AP4 Antagonist: MSOP, MAP4, CPPG
$mGlu_6$	Inhibitory G_i/G_o-coupled	Expression confirmed only in retinal bipolar ON cells. Knockout animals reported to have visual acute deficits.	Agonists: L-SOP, L-AP4 Glutamate-site antagonist: MSOP, MAP4
$mGlu_7$	Inhibitory G_i/G_o-coupled	Localization both pre- and postsynaptic on glutamatergic and other neurotranmitter synapses in limbic and cortical regions. Has lower affinity for glutamate than other mGlu subtypes and only presynaptic inhibitory mGlu localized to active zone of synapses. Thought to serve a classical autoreceptor function.	Agonists: L-SOP, L-AP4 Antagonist: MSOP, MAP4, LY341495 (100-fold lower affinity than group II)
$mGlu_8$	Inhibitory G_i/G_o-coupled	Localization largely presynaptic on glutamatergic and other neurotranmitter synapses. High expression in forebrain regions including hippocampus and amygdala. Linked to regulation of lateral perforant path.	Agonists: L-SOP, L-AP4, 3,4-DCPG Antagonist: MSOP, MAP4

3 mGlu receptors characteristic structural domains and elements

3.1.1 The Venus Flytrap Domain

The VFD of the mGlu receptors is a large bilobed structure of nearly 600 amino acids. Its crystal structures, show that the two VFDs within both protomers face away from each other. Lobe 1 is situated on top of lobe 2, which in turn sits on top of the CRD.

Glutamate binds within a cleft molded between lobes 1 and 2 inducing a large 31-degree conformational shift leading to the closure of both lobes closer by more than 20 Å.[25]

Based on the crystal structures of mGlu1 with bound glutamate or the antagonist α-methyl-4-carboxyphenylglycine (MCPG), two primary configurations are recognized: an open/open form and a closed/open form. The open/open form is seen in the presence of antagonists while the closed/open form was observed when glutamate was added (the conformation that is acquired is also depending at least in part on the presence of stabilizing di- or trivalent cations). Both conformations are observed when no ligands are present creating the hypothesis that the VFD has a dynamic equilibrium such that in the absence of ligand, the so-called open-open/R (resting) conformation is favored.[26] In all mGlu receptors, the conformational change initiated in the VFD is then transmitted to the TMD (via the CRD)and will result in the activation or inhibition of an effector protein coupled to the receptor (either directly or through other proteins).

In homodimeric mGlu receptors, a single molecule of glutamate bound to only one of the two protomers can lead to activation of the receptor complex, nonetheless it has been suggested that for the full activation of the receptor an agonist molecules may need to be bound to both protomers[26], it has been also postulated that agonist bound to both protomers may induce an “insulated state” leading to the desensibilization of the receptor.[10, 27, 28]

3.1.2 The VFD Dimer Interface

One interesting observation about the dimer interface between the two VFDs is that it likely participates in initiating the receptor activation process in a hydrophobic patch of residues. Co-crystallizations of mGlu1 VFD with gadolinium and glutamate, together with mutagenesis experiments, have shown that the ion is bound to a site within the lobe 2–lobe 2 interface.[29, 30]

3.1.3 The Glutamate-Binding Pocket

The glutamate binding pocket in the mGlu receptors occupies a relatively small cavity within the VFD compared to other class C receptors and is highly selective for glutamate over other amino acids being constituted by approximately 12 residues in the binding pocket that establish bonds with glutamate (additional bonding interactions may happen with more complex agonists and antagonists). See Figure 2. Several residues at the pocket are highly conserved between mGlu receptors; being those who interact with the α-carboxy and α-amino groups of the glutamate ligand, with special focus in four residues (Thr-188, Asp-208, Tyr-236, and Asp-318 in mGlu1) that are essential for ligand binding and are a signature feature of all mGlu receptors and most, if not all, class C amino acid–binding receptors.[4, 31]

If we compare the amino acids that interact with the α-carboxy and α-amino groups of the glutamate ligand with the residues that establish bonds with the side chain of the glutamate ligand the last ones are not so conserved and are the principal determinants of receptor subtype selectivity for orthosteric agonists and.[4, 31]

Orthosteric mGlu agonists and antagonists can be divided into three groups:

- Nonselective compounds with little selectivity among the eight subtypes,
- Compounds that display selectivity towards group I, group II, or group III receptors,
- Drugs that show substantial selectivity toward a single receptor subtype. [29]

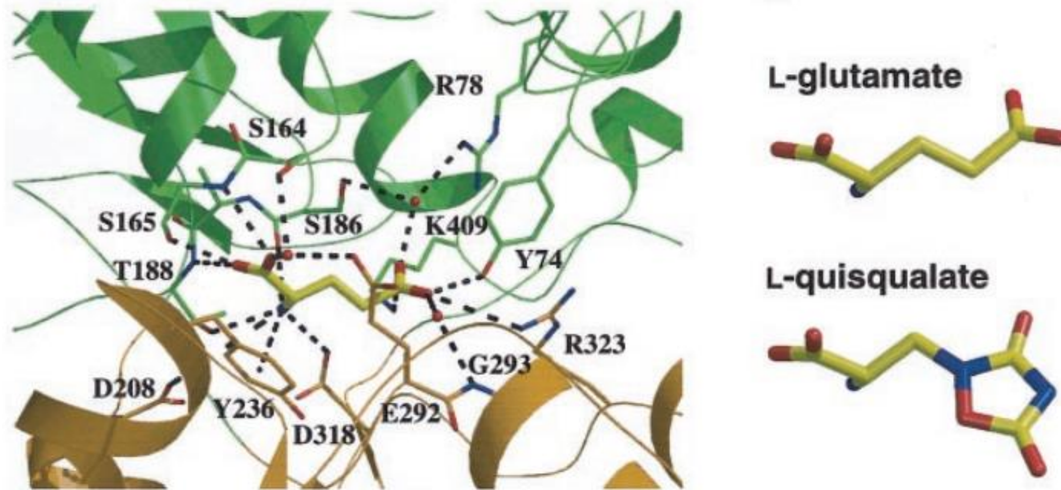

Figure 2 A close-up view of the ligand binding site of mGlu1. The ligand binding site residues of m1-LBR contacting L-glutamate (yellow) are shown as a ribbon diagram. L-Glutamate contacts the LB1 interface (green) and the LB2 interface (brown) of m1-LBR in the crystal structure. Red balls are water molecules. Broken lines indicate polar interactions. Also presented are the chemical structures of L-glutamate and L-quisqualate. These compounds are shown in the best orientation toward the binding site of m1-LBR (mGlu1- extracellular ligand-binding region (m1-LBR)).[4]

Most mGlu ligands belong to the group-selective class, and the molecular basis for this selectivity has been determined. For example the highly potent group I selective agonist quisqualate binds in the glutamate pocket in an orientation that allows additional interactions not possible with the glutamate molecule. See Figure 2. A prominent feature of group III receptors is their selective activation by phosphonate- or phosphate-containing compounds such as L-2-amino-4-phosphonobutyric acid (L-AP4), being this selectivity mediated by a cluster of amino acids in the glutamate pocket, primarily composed of the side chains of lysines and arginines. Agonist selectivity across mGlu groups may be mediated by a very small number of residues in the binding pocket. For example, mutating lysine 74 together with lysine 317 or lysine 74 and glutamate 287 to the equivalent amino acids in mGlu1 result in an incomplete switch of its pharmacologic profile and if a the double mutation is made the receptor displays an affinity for quisqualate that are similar to the wild type mGlu1.[4, 29]

3.1.4 The Cysteine-Rich Domain

The CRD, which links the VFD to the TMD, is composed of approximately 60–70 amino acids, where 9 are cysteines, which are highly conserved throughout all mGlu receptors and is important for the signaling from the VFD to the TMD. Truncated forms of mGlu3, mGlu4 and mGlu8, which included all of the VFD plus part or all of the CRD, were retained in intracellular compartments when transfected into mammalian cells. However, the constructs encompassing the VFD but completely devoid of the CRD were secreted and retained their ligand-binding capabilities, suggesting that the VFD and the CRD fold independent of one another and that the CRD is not essential for ligand binding.[29, 32, 33]

3.1.5 The Heptahelical Transmembrane

It has always been assumed, based on hydropathy plots, that mGlu receptors have a prototypical heptahelical TMD topology and experiments have already confirmed this hypothesis demonstrating an alternating pattern of cytoplasmic to extracellular tags beginning with intracellular loop 1 through to extracellular loop 3. [34] See Figure 3.

It should also be focused that unlike other GPCR family members, mGlu receptors couple to heterotrimeric G proteins via their second intracellular loop domain instead of the third intracellular loop domain utilized by Class 1 and 2 GPCRs.[26, 35]

The Heptahelical domain of mGlu5 like rhodopsin-like receptors can be activated directly by positive allosteric modulators such as in the case of $GABA_{B2}$.[36-40]

3.1.6 The Role of Intracellular Loops 2 and 3 in Receptor Activation

Insertion of fluorophores into the third intracellular loop of the receptor mGlu1α prevented its membrane trafficking, suggesting that this loop has a role in the formation of a functional receptor. Insertion into the first and second intracellular loops caused a decrease and an increase signals in FRET study, respectively, on glutamate binding. A model has been proposed in which agonist binding leads to a shift in the intracellular domains leading to the second intracellular loops of each monomer to move closer (explaining the increase in the FRET signal).A concentration–response effect is seen with increasing concentrations of extracellular glutamate which are correlated with both increased FRET in the second intracellular loop dimer and increased release of intracellular calcium. Several intracellular proteins have been shown to modulate G protein coupling and receptor activation. GPCR kinases

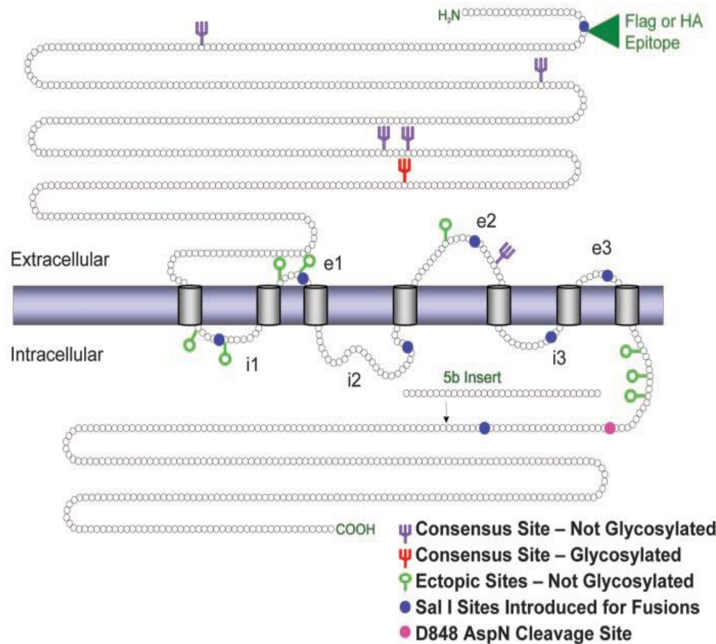

Figure 3 Topological profile of mGluR5.

The confirmed seven transmembrane domain topology of mGluR5 is shown along with the location of all of the manipulations made in the study.[2, 3] The site of introduction of N-terminal FLAG and HA tags is shown by the green triangle (amino acid 22). Sites of inserted Sal I restriction sites are indicated by the blue circles. These correspond to aminoacid 608 in i1, amino acid 641 in e1, aminoacid 688 in i2, amino acid 721 in e2, aminoacid 769 in i3, amino acid 799 in e3, and amino acid 869 in the C-terminal tail. Unused native or ectopically introduced glycosylation sites are indicated by the purple and green symbols, respectively, and the glycosylated native site at Asn-444 is indicated by the red symbol. The identified Asp-N cleavage site at Asp-848 is indicated in pink.

(GRKs) phosphorylate rhodopsin-like GPCRs and promote the binding of arrestins, resulting in the desensitization and internalization of the targeted receptor. GRK-mediated inhibition of mGlu1, however, is phosphorylation and α-arrestin independent. GRK2 inhibits mGlu1 by binding to a region in the second intracellular loop, presumably preventing the binding of the G protein. Together these findings confirm that second intracellular loop in the mGlu receptors are an important determinant of G protein selectivity and is essential for activation of the receptor. [2,41, 42]

4 mGlu Receptors distribution on the adult rat brain

The distribution of mGlu1, mGlu3, mGlu5 and mGlu7 receptor is widespread throughout the brain, while mGlu2, mGlu4 and mGlu8 are more restricted to specific brain regions. In humans expression of mGlu6 has been found mainly in the retina, and only very low level can be found outside of this structure. In the adult rat, mGlu receptors are mainly expressed in neuronal cells with the exception of mGlu3 that is also extensively expressed in glial cells throughout the brain. Furthermore, the expression of mGlu3 and of mGlu5 has been found to be up-regulated in reactive astrocytes.[43] See Figure 4 for reference of the following parts.

4.1 Distribution of group I mGlu receptors

Immunoreactivity for this group has been extensively found in the CNS, being most intense in Purkinje cells of the cerebellar cortex and mitral/tufted cells of the olfactory bulb with a strong expression also being detected in neurons of the lateral septum, globus pallidus, entopeduncular nucleus, ventral pallidum, magnocellular preoptic nucleus, most of the thalamic nuclei but not in the reticular nucleus, substantia nigra and dorsal cochlear nucleus (for mGlu1). Intense expression has been seen mainly in telencephalic regions, including the cerebral cortex, hippocampus, subiculum, main and accessory olfactory bulbs, anterior olfactory nucleus, olfactory tubercle, striatum, nucleus accumbens, lateral septal nucleus, shell regions of the inferior colliculus, superficial layers of the superior colliculus and caudal subnucleus of the spinal trigeminal nucleus (for mGlu5).[43]

4.2 Distribution of group II mGlu receptors

The most intense expression of mGlu2 is present in Golgi cells in the cerebellar cortex, mitral cells of the accessory olfactory bulb, external part of the anterior olfactory nucleus and some neurons in the entorhinal and parasubicular cortices (at an electron-microscopic level, immunoreactivity for mGlu2/3 has frequently been detected in small unmyelinated axons, especially in pre-terminal portions of axons, rather than in axons terminals). mGlu3 is expressed extensively throughout the CNS.[43]

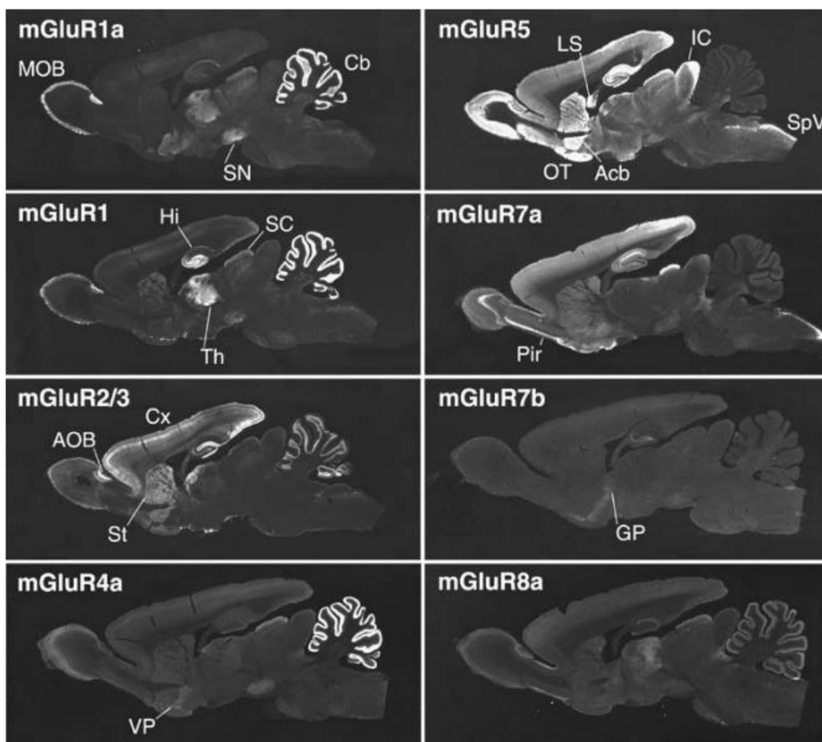

Figure 4 Distribution of mGluRs in the adult rat brain. Immunoreactivities for mGluR1α (mGluR1a), mGluR1, mGlu2/3, mGlu4a, mGluR5, mGlu7a, mGlu7b and mGlu8 in parasagittal sections (AOB accessory olfactory bulb, Acb accumbens nucleus, Cb cerebellum, Cx neocortex.

4.3 Distribution of group III mGlu receptors

Amongst group III mGlu receptors, the distribution of mGlu7 receptor is the most extensive being detected in the main olfactory bulb, olfactory tubercle, neocortex, piriform cortex, hippocampus including CA1-CA3 and dentate gyrus, septum, striatum, accumbens nucleus, claustrum, amygdaloid complex, preoptic region, hypothalamus, thalamus, superior colliculus, locus coeruleus, dorsal cochlear nucleus and dorsal horn of the spinal cord. The distribution of mGlu7b is more limited than that of mGlu7a receptor where most regions showing mGlu7b receptor immunoreactivity also display mGlu7a receptor immunoreactivity the inverse is not true. In the hippocampus, for example, mGlu7a receptor is seen throughout all dendritic layers being strongest in the strata oriens and radiatum, followed by the stratum lacunosum moleculare, whereas mGlu7b

receptor has been observed only in the terminal zone of the mossy fibers.The labeling of mGlu7a receptor in the pyramidal cell layer is very weak but strong in CA3 dendritic fields such as the stratum lacunosum moleculare, especially in the inner layeradjacent to the dentate molecular layer. Both isoforms appearin presynaptic zones of asymmetrical and symmetrical synapses with no clear differentiation.[44]

The medial perforant path terminal zone in the dentate gyrus, namely the middle third of the molecular layer, was also prominently immunopositive for mGlu7a receptor as in contrast to the lateral perforant path which has a more prominent immunolabeling for mGlu8.[44]These differential locations of the receptors seem to have a functional relevance on the pharmacological manipulation that can affect this two signaling paths.[14, 16] This aspect is further discussed with some of the results obtained when modulating the medial perforant path. In CA3 stratum lucidum, the somatodendritic decoration observed for mGlu7b receptor was stronger than for mGlu7a receptor. The strong terminal labeling for mGlu7a receptor on the somatodendritic profiles of interneurons was distributed most densely in the stratum oriens/alveus border zone in the CA1 area. As in mGlu7a receptor labeling, mGlu7b receptor-labeled puncta were mostly found on mGlu1a-labeled interneurons. Hippocampal thin sections used in mGlu7 receptor mRNA in situ hybridization revealed no significant marking in the CA3 st. radiatum, suggesting that the mRNA for mGlu7 receptor was absent in CA3 stratum radiatum interneurons. In contrast, CA3 pyramidal neurons, dentate granule cells, and some hilar neurons were intensely labeled. In contrast, mossy fiber pyramidal cells synapses displayed reversible depression by L-AP4 (200 μM), indicating that persistent L-AP4 inhibition is peculiar to mossy fiber pyramidal cells synapses and does not result from an inability to wash the agonist from slices.[45]

mGlu6 is mainly restricted to the retina and the expression of mGlu4 is most intense in cerebellar granule cells. Prominent expression of mGlu4 is also observed in the periglomerular cells and granule cells of the main olfactory bulb, olfactory tubercle, entorhinal cortex, CA1-3 and hilus of the hippocampus, lateral septum, septofimbrial nucleus, striatum, rostral part of the intercalated amygdaloid nucleus, thalamic nuclei, lateral mammillary nucleus, pontine nuclei and dorsal horn.

The expression pattern of mGlu8 is more restricted than that of mGlu7 receptor being expressed in the main and accessory olfactory bulbs, anterior olfactory nucleus, piriform cortex, entorhinal cortex, pontine nuclei, and lateral reticular nucleus of the medulla oblongata.[43]

5 Synaptic distribution of mGlu receptors

While fast excitatory neurotransmission is universally mediated by ionotropic glutamate receptors that require their localization in the postsynaptic membrane specialization of glutamatergic synapses; mGlu receptors are found more broadly distributed throughout various membrane compartments of both neuronal and glial cells. mGlu mediated regulation of transmitter release is not limited to glutamatergic synapses (in which the activation of these receptors often requires high-frequency or repetitive stimulation so extrasynaptic mGlu receptors on dendrites and axons can be activated) but also present in GABAergic synapses and on the dopaminergic system.[46, 47] See Figure 5.

Group I receptors are generally expressed perisynaptically at postsynaptic sites, where they usually form an annulus around a central core of ionotropic glutamate receptors. The mGluR2 receptor is

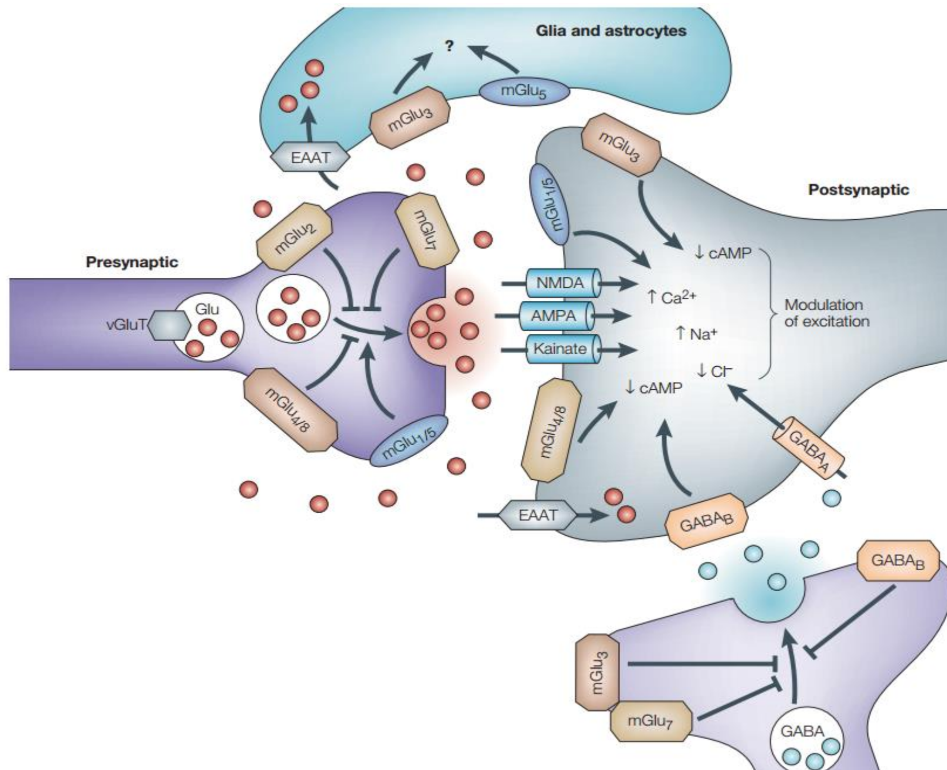

Figure 5 Hypothetical synapse illustrating the general synaptic localization and function of glutamatergic receptors and transporters.The ionotropic glutamate receptors NMDA, kainate and AMPA subtypes, largely function to mediate fast receptor transmission, but also mediate the use dependent changes required for neuronal plasticity. The vesicular transporters (vGluT 1 and vGluT 2) load glutamate into vesicles presynaptically. The glial, astrocyte and postsynaptic glutamate transporters (excitatory amino-acid transporters, EAAT1–5) are thought to mediate the uptake of glutamate and therefore termination of synaptic transmission. The mGlu receptors have a diverse synaptic localization and function pre- and postsynaptically to modulate neurotransmitter release and postsynaptic excitability, respectively. mGlu1 and mGlu5 (group I) are mostly localized perisynaptically both in the presynaptic as postsynaptic components, mGlu4, mGlu7 receptor and mGlu8 (group III) at the presynapse, while mGlu2 and mGlu3 (group II) do not show any preference.

located pre- and post-synaptically but is thought to be predominantly located at the pre-terminal portion of axons where it functions as an autoreceptor.[44, 48, 49]

Group III mGlu receptors are primarily found at the presynaptic active zone where they probably function as glutamate autoreceptors.mGlu7 receptor are located in pyramidal cells of the hippocampus and in bipolar cells of the retina, in different densities at the same synaptic terminal, depending on the nature of the postsynaptic neuron. Based on the specific presynaptic localization and its relative low glutamate affinity, mGlu7 receptor appears to function as a low-pass filter[50], inhibiting synapses firing above a certain frequency. Supporting this idea are genetic studies that have discovered that mice with ablation of mGlu7 receptor show behavioral deficits (further discussed ahead).[46, 47, 51, 52] See Figure 5.

Interesting Shigemoto et al[44]demonstrated that the density of mGlu7 receptor labeling in the active zone of the presynaptic membrane was much higher in those terminals that made synapses with mGlu1α-immunopositive dendritic shafts.[50] See Figure 6.The apparently complete segregation of mGlu7 receptor between two synapses within single boutons suggests that coupling of the receptor with its effector is likely to be spatially restricted and probably membrane delimited. As well Scanziani et al[53]established that the pharmacological and physiological properties of Schaffer collateral terminals from the same axon are different, and seem to depend on the target cells that they innervate.This was demonstrated by the fact thatL-AP4 reduced transmitter release from Schaffer collateral terminals that made synapses with interneurons, but not those terminals sharing a synapse with CA1 pyramidal cells. These observations led to the hypothesis that the presynaptic properties of receptors modulating transmitter release are influenced by the target cell and not exclusively by the presynaptic neuron. [54]

As autoreceptors located in glutamatergic neurons, mGlu7 receptors regulate glutamate release, while as heteroreceptors located in GABAergic interneurons, they participate in the regulation of GABA activity.[52, 55, 56]

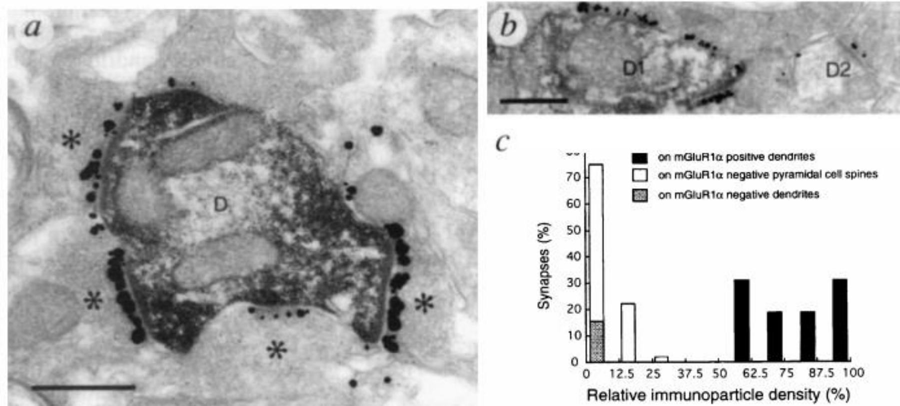

Figure 6 Localization study fragment that has shown that mGlu7 receptor is more restricted to the presynaptic grid at the site of vesicle fusion on mGlu1 receptors immunopositive neurons.[71]

6 Differential mGlu7 receptor expression/density during brain development

Manahan-Vaughan and Reymann[57] have revealed that L-AP4 inhibits LTP in vivo with a variation in sensitivity to the drug occurring between CA1 and DG and the age of the animal (8 and 12 weeks). One important point is that the concentrations that they used were low when compared to the ones needed for the activation of mGlu7. Nonetheless they report that at 12 weeks of age in CA1 concentration higher than 100 µM reduced the amplitude of LTP induced by tetanization compared to controls. In 8 weeks old animals a dose-dependent effect was observed over fEPSPs baseline, with a more pronounced in the DG (all effects were blocked as well by α-methyl-4-carboxyphenylglycine (MCPG)).

When L-AP4 is applied in younger animal (neonatal) a reduction in synaptic transmission is observed with concentrations one to two orders of magnitude lower than the ones necessary for the same degree of effect on adult animals. L-AP4 activates mGlu4 and 8 at much lower concentrations than those required to activate mGlu7 receptor, suggesting that the group III mGlu subtype modulating transmission is a high affinity receptor in neonates and a low affinity receptor in adults (effects of L-AP4 could be blocked by LY341495 in both neonates and adults).[1, 58]

Through the use of L-AP4, (S)-3,4-dicarboxyphenylglycine (DCPG) and N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCC) Ayala[1] et al. got more insight into the

potential contribution of mGLu4, 7 and 8. They report that the selective mGlu8 agonist (DCPG) has a significant effect in slices from neonatal rats but does not reduce synaptic transmission in adult slices, and the mGlu4 selective positive allosteric modulator, PHCCC, was unable to potentiate the L-AP4-induced effects at either age tested. Based on these results they hypothesized that group III mGlu receptors role in the regulation of transmission at the SC-CA1 synapse is developmentally regulated.

In support of their possibility that mGlu7 receptor is the main group III mGlu subtype involved in regulating transmission at SC-CA1 synapses in adults, immunohistochemical analysis of Group III mGlus in the adult rat hippocampus revealed that mGlu4a is primarily localized to the inner third of the molecular layer and mGlu8 is found in the lateral perforant path terminal zone. In the other hand the mGlu7a receptor is distributed in all dendritic layers throughout the hippocampus (also see “Receptor distribution in the brain and hippocampus”).

Antibodies to mGlu7a receptor rat hippocampus of animals with 7 days exposed that this receptor is not highly expressed in the stratum radiatum of the CA1 region as it is in the adult.[59] Leading to the hypothesis that mGlu7 receptor expression and receptor reserve are not higher in neonates than adults. Therefore it is possible that another mGlu subtype or isoform mediate higher potency of L-AP4 in neonates and cannot be “detected” with the methods applied so far.

The hypothesized developmental switch in expression of a receptor with high affinity for glutamate to one with low affinity suggests that during the neonatal development there is a requirement for tighter control of glutamate release. This can be supported by the fact that the formation of inhibitory synapses occurs mostly postnatally in the developing rat brain, particularly in the CA1 subfield of the rat hippocampus, the development of GABAergic inhibitory control is ongoing in animals up to postnatal day 10 and some studies suggest that inhibitory responses cannot be evoked until postnatal days 14. [60, 61]

This lack of inhibitory control in the neonatal animals could increase the vulnerability to excessive excitatory activity and expression of a receptor with high sensitivity to glutamate concentrations during these early stages of postnatal development provides a protective mechanism.

7 mGlu7 receptor knock-out and knock-down models

Animal models of mGlu7 receptor KO have shown reduction in fear-mediated freezing - immediate and delayed - responses during electric foot shocks; impairment in the ability to associate unconditional averse stimuli, conditional stimuli in both fear and conditioned taste aversion responses, and increased seizure susceptibility. This diverse phenotype creates a possible link between mGlu7 receptor and the maintenance of homeostatic neuronal excitability. [62]

They also present an impaired working memory, dysregulated stress responses, spatial working memory deficit, reduced anxiety and depression. Slices prepared from mGlu7 receptor-/- mice were slightly more excitable under control conditions and a slower recovery from frequency facilitation in cortical slices (suggesting that the receptor as a role as a frequency-dependent regulator in presynaptic terminals).[3, 14, 55, 63-73]

Based on these findings, mGlu7 receptor is considered a promising target for the development of wide-spectrum therapeutic drugs. Some fine examples include the fact that adult siRNA-induced knockdown of mGlu7 receptors reduces anxiety in the mouse and the mice present an antidepressant-like phenotype[74-76]

Systemic mGlu7 receptor activation reduced alcohol consumption and preference, as well as, locomotor stimulating and rewarding properties of ethanol. The blockage of mGlu7 receptor expression in the NAc, using shRNA-expressing lentiviral vectors blocked increased ethanol consumption and preference in a two-bottle choice drinking. The great majority of NAc neurons are GABAergic medium spiny neurons and lentiviral vectors are not selective for any neuronal subtype, leading the authors to the hypothesis that the majority of the mGlu7 receptor-shRNAs were expressed in GABAergic neurons. [77]

On the other hand the KO animals also demonstrate deficits in amygdala-dependent fear learning, have a dysregulated hypothalamice pituitaryeadrenal axis, with increases in hippocampal BDNF levels (which are also observed following chronic treatment with anxiolytics or antidepressants).[51]

Recent studies show that the Wistar Kyoto rat, an animal model of increased anxiety, has increased levels of mGlu7 receptor in subregions of the hippocampus; although it is worth noticing that mGlu7 receptor is not altered in other models of anxiety and depression.[78, 79]

mGlu7 receptor knock-out and knock-down models

At CA1 synapses the two mGlus expressed at highest levels are mGlu5 and mGlu7. With a deletion of mGlu5 the induction of LTP of NMDAR is prevented without any kind of effect over LTP of AMPAR. On the other hand the deletion of mGlu7 receptor allows LTP to be induced and that once the potentiation reaches a steady level there is no apparent difference between mGlu7 KO mice and their littermate controls. However, the initial decremental phase of LTP, known as short-term potentiation is significantly attenuated in the mGlu7 KO mouse. In these animals less frequency facilitation during, and less post-tetanic potentiation following, a high frequency train is present. These results show that the absence of mGlu7 receptors results in alterations in short-term synaptic plasticity in the hippocampus[80].

The use of short interfering RNA targeting mGlu7 receptor in the adult mouse brain had no effect on forced swim test behavior or locomotor activity test suggesting that antidepressant-like effects observed in KO animals may reflect an adaptive phenotype, it also should be considered that the behaviour of different inbred strains of mice varies greatly and the influence of the background strain used in knockout studies may influence the results seen in behavioral tests.[73, 74, 81, 82]

A relevant observation done with the KO and heterozygous mice for mGlu7 receptor is that a GABAergic dysfunction is present (decreased levels of glutamic acid decarboxylase 65 and 67 kDa and increased reelin proteins in the hippocampus - both in mGlu7−/+ and mGlu7 KO mice) suggesting that mGlu7 receptor is involved in the regulation of GABAergic system activity at the level of GABA synthesized enzymes, specific proteins expressed by GABAergic neurons and metabotropic receptor for GABA. If GABAB autoreceptors on GABAergic nerve terminals are downregulated in mGlu7 receptor KO mice, the release of GABA, which is regulated by these receptors, should be increased, resulting in the anxiolytic phenotype of these animals since drugs that increase GABA activity are the most effective antianxiety agents. However this effect is contradicted by the decreased levels of both GADs.[83] The observed increased level of reelin expression in both mGlu7−/+ and mGlu7 KO mice, suggested improvements in circuitry plasticity and increased numbers of synapses in animals lacking mGlu7 receptor, but this has not been confirmed.[84]

Another very interesting model is a knock-in mice lacking the PDZ-Ligand motif of mGlu7a receptor that has revealed an impaired PKC-dependent autoinhibition of glutamate release, spatial

working memory deficits (although their exploratory behavior is normal), and increased susceptibility to pentylenetetrazol [85]a phenotype somehow similar to that of the mGlu7 receptor KO.

It had been previously demonstrated that PICK1 binding to the C-terminal region of mGlu7a receptor is crucial for agonist-triggered presynaptic signaling in vivo as well as for presynaptic mGlu7a receptor clustering in vitro[86] but apparently, the PDZ-ligand motif of mGlu7a receptor is not essential for synaptic localization in vivo. L-AP4 at a concentration of 400μM induced a significant decrease in the frequency but not amplitude of sEPSCs, and this effect was strongly antagonized by the PKC inhibitor GF109203X (10 μM) in cells prepared from both wild type and mGlu7a receptor AAA/AAA mice.[85] The authors commented that in hippocampal neurons, L-AP4 induced effects were seen at considerably low concentrations (10 μM) because of the presence of high-affinity group-III mGlu receptors that mask the effects of the mGlu7a receptor mutation.[85]

8 Signaling pathways commonly associated with the mGlu7 receptor activation

Two main function have been attributed to mGlu7. One is its involvement in the frequency-dependent regulation of glutamate and the other it that is serves as a low-pass filter[50] for neuroprotection in cases of high concentrations of glutamate being released.[61, 72, 76, 84, 87-90]

Simultaneously a role in disinhibition has been demonstrated in various brain regions either by direct or indirect mechanisms. [91]

mGlu7 is typically associated with $G_{i/o}$ proteins and negatively coupled to adenylate cyclase and Ca^{2+} channels (of which the type and density does seems to affect the receptor signaling).[92-94]The receptor has also been associated to a suppression of voltage- gated Ca^{2+} channels (both N- and P/Q-type) [93, 95]and inhibits synaptic transmission via a presynaptic mechanism depending on calmodulin, PKC and the scaffolding protein PICK1.[86, 96]On the other hand some studies show that mGlu7 receptor inhibits glutamate release through a PKC-independent decrease in activity of P/Q-type Ca2+channels and by diminishing cAMP in hippocampal nerve terminals[94]

Its activation is usually linked to an inhibition of glutamate release from presynaptic terminals where it acts as an autoreceptor and inhibition of GABA release from interneurons where it acts as a

heteroreceptor. They also are negatively coupled to AC-dependent intraterminal pathway, and co-localize, but do not functionally cross-talk, with $GABA_B$ autoreceptors.[97]

Even though mGlu7 is “classically located” at the presynaptic component its action can affect postsynaptic elements and the results of some studies point towards postsynaptic located mGlu7-mediated effects. The receptor has been shown to act on postsynaptic targets such as suppressing $GABA_A$ receptor-mediated postsynaptic response.[98] See Figure 7

Gu et al[47] revealed a mechanism in which mGlu7 activation increases the actin-depolymerizing activity of cofilin via an ERK-dependent mechanism. This mGlu7-induced actin depolimerization results in less NR1 binding to actin assembly, which may contribute to the dissociation of NMDARs from the scaffold protein complex. See Figure 7.

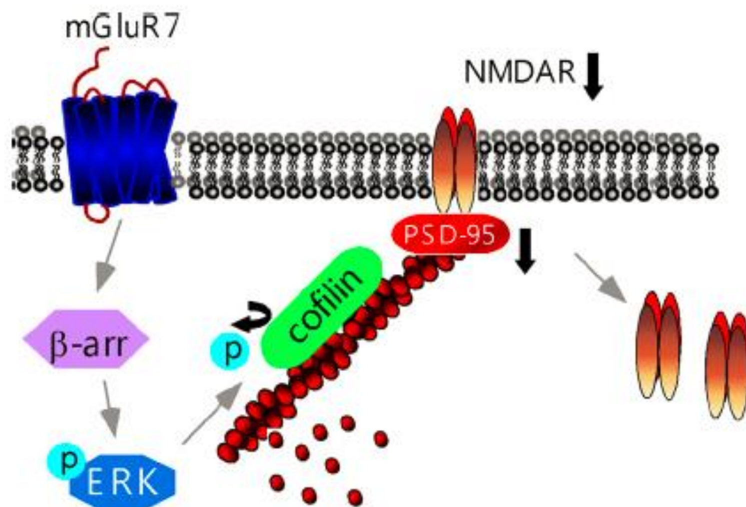

Figure 7 Schematic model for mGluR7 regulation of NMDARs. Activation of mGluR7, via the ERK pathway, triggers the increase of cofilin activity by decreasing its phosphorylation, leading to the increased actin depolimerization. Consequently, the NR1/PSD-95/ actin interactions are interfered, causing the internalization of NMDARs and the reduction of NMDA responses.[99]

9 Metabotropic glutamate receptors role in short term synaptic plasticity

Action potentials generated near the cell body propagates down the axon where they open voltage-gated Ca^{2+} channels leading to an increase in $[Ca^{2+}]_i$ that will trigger the rapid release of vesicles containing neurotransmitter that will act on receptors on the postsynaptic cell and/or autoreceptors. Virtually all types of synapses studied up to date are regulated by a variety of short- and long-lasting processes, which lead to a decrease or increase in synaptic strength. Repeated activation at some synapses leads to synaptic enhancement and facilitatory processes dominate (facilitation); at others the result is a decrease in synaptic strength and depression prevails. In most cases, it is apparent that multiple processes are present, and the result can be a combination of facilitation and depression in which synaptic strength is highly dependent on the details of the timing and strength of synaptic activation as well as the characteristics of the synapses and neurons involved. Leading to the definition of short term synaptic plasticity as the whole mechanisms of use-dependent plasticity in the time scale of tens of milliseconds to several minutes.[100]

In terms of enhancement most of the mechanisms involved are, as demonstrated by quantal size analysis, presynaptic in origin, with an effect on number of release sites, active zones containing clusters of vesicles, the number of vesicles released, the probability of release of these vesicles and highly related to Ca^{2+} concentration in the presynapse . Of these mechanism involved some specific sites of regulation should be focused such as the action potential waveform, activation of diverse Ca^{2+}channel with different kinetic and pharmacological properties, facilitation trigger and the readily releasable pool modification, residual $[Ca^{2+}]_i$, reserve pool size, metabotropic and ionotropic autoreceptors modulation and activity, Ca^{2+}-ATPase, regulation of residual $[Ca_{2C}]_i$ in augmentation, and mitochondrial regulation of residual $[Ca^{2+}]_i$ in PTP.[100]

The search for molecular mediators of short-term synaptic plasticity has revealed importance for elements such as some postsynaptic mechanisms, presynaptic ionotropic receptors, and metabotropic receptors; however it has been more difficult to identify the molecules responsible for facilitation, PTP, and depression. Some of the primary difficulties in this identification are that manipulations affecting the baseline level of transmission indirectly influence the magnitude of short-term plasticity. An assumed relationship that has become a standard means of evaluating the possibility of a

neuromodulator having a presynaptic or postsynaptic site of action is evaluating if an increase in the initial probability of transmitter release decreases the magnitude of synaptic enhancement, and, conversely, a decrease in the probability of release results in larger synaptic enhancement or reduced synaptic depression. Nevertheless an analysis of quantal content should be performed. Often neurons are connected by a large number of synaptic contacts and determining the quantal content of baseline recordings is not straightforward. In that case, measures of PSP or PSC amplitude should be made, along with tests for postsynaptic effects such as measuring miniature PSP or PSC amplitudes.

At synapses where NMDA receptors are present, it is relevant the use-dependent block of NMDA receptors to detect changes in the probability of release. Using styryl dyes such as FM1-43, allow the assessment of vesicle turnover rates to low-frequency stimulation since it is often experimentally difficult to measure the baseline level of synaptic transmission. [101]

Another common complication occurs when synapses are manipulated for a prolonged time before the effects on plasticity are measured as in the case of genetic mutations where a variety of homeostatic mechanisms allow neuronal systems to respond to the initial effect of a manipulation with secondary changes.[100]

Even they only compose a minor part postsynaptic mechanisms also mediate short-term plasticity, adding a complexity level to presynaptic mechanisms. One way they participate is when saturation of postsynaptic receptors can limit responses, especially when the probability of release is high. As a consequence, synaptic currents can underestimate the modulation of the presynaptic component at some synapses. A practical example is that AMPA receptor saturation influences the time course of recovery from depression.[102] This underestimation can also occur when postsynaptic receptors desensitize, making them unavailable for subsequent activation, and leading to short-term decreases in synaptic responses and no distinction between a decrease in available vesicles or a reduction in release probability can be make.[103]

As already stated extracellular calcium is of the factor that influence synaptic transmission efficiency both at presynaptic and postsynaptic compartments[28] and interestedly as found by Thanawal and Regehr "calcium dependence of p [probability of release] is insufficient to account for the calcium dependence of the EPSC size, and that the effective RRP [ready releasable pool] size is calcium dependent. Our experiments indicate that neuromodulators that regulate presynaptic voltage-gated calcium channels (VGCCs) regulate both p and effective RRP size"[104] reinforcing the concept of mGlu receptors having a role in controlling short term plasticity.

10 Pharmacological tools for mGlu7 receptor modulation

10.1 The broad mGlu Group III agonist L-AP4

This compound is a Group III selective agonist. It is associated with a reduction of fEPSPs at SC-CA1 synapses mediated by mGlu7 receptor. Its $EC_{50}$for mGlu7 is around 160 – 500 µM and 0.4 – 1.2 µM for other members of group III (mGlu4, 6, 8).[47]

This compound has demonstrated other effects such as neuroprotective profile at high concentration (mM range) against NMDAR-induced but not kainate-induced insult[105], it reduces the short-term depression evoked by high frequency stimulation.[106] In the PFC L-AP4 (200 µM) causes a strong reversible reduction of the amplitude of NMDAR-EPSC and AMPAR-EPSC, without altering the paired pulse ratio of NMDAR-EPSC but significantly increasing the paired pulse ratio of AMPAR-EPSC. This suggests that L-AP4regulates NMDAR-EPSC mainly via a postsynaptic mechanism, whereas regulating AMPAR-EPSC through a presynapticaction.[47] Group III mGluRs are primarily coupled to $G_{i/o}$ proteins and their activation by L-AP4 lead to inhibition of cAMP formation and PKA activation.[99, 107]Other than the PKA signaling pathway previous studies have shown that L-AP4 can activate PI3K, MAPK (ERK1/2) pathways in cultured cerebellar granule cells.[107]

L-AP4 also produces an inhibitory effect on GABA release in the hippocampus, striatum, substantia nigra, and cerebral cortex[98], while inhibiting non-vesicular glutamate release in the NAc[108] and inhibits glutamate-mediated EPSPs in the hippocampus[109]

10.2 The mGlu7 selective agonist/positive allosteric modulatorAMN082

AMN082 is a recent allosteric agonist with its binding site in the transmembrane region of the receptor. The compound is orally active, penetrates the brain-blood barrier, and elevates plasma stress hormones in an mGlu7-dependent manner[51]. Its EC_{50} for mGlu7 is 64-290 nM (transfected mammalian cells expressing mGlu7,cAMP accumulation and GTPγS binding studies)[51]and no significant activity at related G protein-coupled receptors (GPCR) or ion channels in concentration lower than3 mM/l is detected.[74]

AMN082 does not reduce synaptic transmission at the SC-CA1 synapse as it would be expected[1] and it also induces robust internalization of mGlu7 receptor and produces a rapid loss of surface mGlu7 receptor, being this observation one of the potential causes for the contradictory effects of AMN082[110] as, for example, its anxiolytic effects.[110]

AMN082 is pro-nociceptive when injected in the amygdala. Its effects include increased frequency, but not amplitude, of spontaneous EPSCs but had no effect on miniature EPSCs, decreased evoked IPSCs and frequency, but not amplitude, of spontaneous and miniature IPSCs.[111] AMN082 as well blocked the acquisition of fear learning, facilitated fear extinction, and impaired long-term potentiation in the amygdala[112] and *in vivo* inhibits the acquisition of Pavlovian fear conditioning, susceptibility to seizures, anxiety and depression and both cocaine self-administration, reinstatement of drug seeking behavior[90]; reduces time spent in REM sleep, decreasing the number of REM sleep episodes and their mean duration leading simultaneously to an increase in total time spent awake and longer episode duration of wakefulness[113]; and produces antidepressant-like events trough the modulation of glutamatergic signaling.[114]

AMN082 concentration dependently lowered Nucleus accumbens (NAc) extracellular GABA and increased extracellular glutamate but had no effect on extracellular dopamine levels contrary to what happens with L-AP4.[90, 115] In hippocampal synaptosomal preparations AMN082 acting through mGlu7 as a heteroreceptor also inhibiting K^+ evoked GABA release being this effect addictive to the inhibition caused by baclofen (a specific antagonist of $GABA_B$ receptor).[97]

Met-1 (the main metabolite of AMN082) itself was found to possess an antidepressant effect, as would be expected from itsin vitro profile of a mixed serotonin transporter (SERT), noradrenaline transporter (NAT) and dopamine transporter (DAT) inhibitor. This should be taken in account when data generated from systemic administration of AMN082 needs to be interpreted with caution due to the potential of AMN082's effects being mediated through non-mGlu7 receptor elements.[87]

Furthermore, it was recently reported that AMN082 does not activate mGlu7 receptor in CHO cells as assessed by measuring mGlu7 receptor-mediated calcium responses in cells expressing the promiscuous G protein, $G_{\alpha 15}$.[116]This suggests that the pharmacology of AMN082 may be complex, and that this compound may not serve as a general agonist of mGlu7 receptor across multiple responses and cell populations..[117]

10.3 The negative allosteric modulator MMPIP

MMPIP is considered the first allosteric mGlu7 receptor-selective negative allosteric modulator (NAM) (IC_{50}= 26 nM ininhibitingL-AP4-induced Ca^{2+}mobilizationin CHO cells expressing mGlu7 linked to $G_{\alpha 15}$).[118]

Electrophysiological studies reveal that, in contrast to the orthosteric antagonist LY341495, MMPIP is unable to block agonist-mediated responses at the Schaffer collateral-CA1 synapse, a location at which neurotransmission has been shown to be modulated by mGlu7 receptor activity.[89] This is an “unexpected contradiction” since the compound showed promising results in other studies. Niswender et al[89] reported, when testing a small library of MMPIP-derived compounds, which the pharmacology of these ligands seems to be context-dependent, and MMPIP exhibits differences in negative cooperativity in certain cellular backgrounds. This leads to a warning that when results with the effects of MMPIP are analyzed the system in which they were obtained should be carefully taken in account.

Suzuki et al[116] reported that MMPIP was able to antagonize L-AP4 and AMN082-induced inhibition of cAMP accumulation in CHO cells coexpressing rat mGluR7 with $G_{\alpha 15}$. However in the absence of agonists, MMPIP caused a further increase in forskolin-stimulated cAMP levels, contrary to LY341495 (competitive antagonist). This leads to the conclusion that MMPIP may act as an inverse agonistic or may act as an allosteric antagonists blocking specific signaling pathways while permitting other intracellular signaling. An idea recently proposed as “permissive antagonism” by Kenakin.[119]

Behavioral studies demonstrated that MMPIP administration *in vivo* impaired non-spatial and spatial cognitive performances in the object recognition test and the object location test, increased time tocomplete the task in the 8-arm radial maze test without increasing error, and decreased social interaction. MMPIP had no effects on locomotor activity, rotarod performance, prepulse inhibition, and maternal separation-induced ultrasonic vocalization in rat pups, stress-induced hyperthermia in mice, or the tail suspension test. Curiously MMPIP did not alter the threshold for induction of seizures by electrical shock or PTZ.[118, 120, 121]

Overall, the combination of in vitro pharmacology and electrophysiology results suggest that MMPIP exhibits differential degrees of negative cooperativity in different cellular contexts and is not as effective at antagonizing mGlu7 receptor activity at a central glutamatergic synapse as first hypothesized.

10.4 The negative allosteric modulator ADX71743

ADX71743 is a new compound considered to be an mGlu7-especific NAM bioavailable following subcutaneous administration and brain-penetrant (IC_{50}=63 nM in inhibiting the calcium influx caused by L-AP4 in HEK293 cell line stably coexpressing mGlu7 with a chimeric $G_{i\alpha}$ protein).[87]

In hippocampal slices ADX71743 lead to attenuation of L-AP4-induced synaptic depression confirming its activity at the native receptor. *In vivo*, ADX71743 caused no impairment of locomotor activity or activity on rotarod in mice, having an anxiolytic-like profile in the marble burying and elevated plus maze tests. It also leads to a small reduction in amphetamine-induced hyperactivity in mice. The compound was inactive in the mouse forced swim test.[87]

10.5 Potent orthosteric antagonist LY341495

LY341495 is a potent and selective orthosteric antagonist for the group II mGlu receptors, being a non-selective antagonist for group III however is considered one of the most potent. The compound exhibits 10-fold selectivity for mGlu2 and three over mGlu8 receptors. Furthermore, LY341495 is near six-fold selective for mGlu8 receptors when compared with mGlu7 or mGlu6 receptors.[122]

The IC_{50} value for human mGlu7a is 990nM (receptors expressed in non-neuronal cells)[123]nevertheless 100 μM of the compound does not affect either STP or LTP at SC-CA1 synapses when tested in brain slices.[124]

LY-341495 had no effects in the elevated plus maze and stress-induced hyperthermia tests in mice, as well as on punished drinking (Geller–Seifter's test) and differential reinforcement of low rates of responding in rats.[125]

It is concluded that behavioral profile of mGlu2/3receptor antagonists as represented by LY-341495 is different from that of conventional anxiolytic and antidepressant drugs. [65]

11 mGl7 receptor internalization

mGlu7 receptor expressed in neurons undergoes both constitutive and activity-driven endocytosis[126], being its constitutive endocytosis is clathrin-independent[127]

L-AP4 and AMN082 treatment increased both mGlu7a and mGlu7b receptor internalization at mossy fiver – stratum lucidum interneurons (MF-SLIN) transmission and SC-CA1 synapses, respectively. In the case of the L-AP4 outcome it can be antagonized by MSOP and cannot be re-produced with the non-mGlu7 receptor group III mGlu agonists ACPT-1 and DCPG.[110, 128]

This phenomenon has revealed that repeated agonist exposure discloses a facilitatory role of mGlu7 receptors on glutamate release, which is mediated by stimulation of inositol phospholipid hydrolysis and membrane translocation of Protein unc-13 homolog (Munc-13).[92-94, 129]

It should be noted that agonist induced internalization of the receptor does not happen in all situation as, for example, mGlu7 receptor does not undergo agonist-induced endocytosis in human embryonic kidney (HEK) cells[130] and this influence the way data obtained in this system of expression should be analyzed and interpreted.

12 mGlu receptors as drug targets

12.1 Alzheimer's disease

Some works have shown that altered synaptic structure, function, and plasticity precede the characteristic neurodegeneration and contribute to the early learning and memory deficits of AD.[131] Mutations in amyloid precursor protein (APP) that lead to its abnormal proteolysis and cleavage and generates amyloid β protein (Aβ) that eventually aggregate into senile plaques which for long time have been considered one of the markers for AD. But even though Aβ aggregates are observed in AD patients, the typical cognitive and memory impairments are now known to precede the occurrence of amyloid plaques shifting the research from the plaques to the soluble Aβ peptides. The presence of various forms of Aβ oligomers in various models both *in vitro* and *in vivo* lead to a depression of synaptic transmission through an LTD-like mechanism that also inhibit LTP-like phenomena. This effect is established through the inhibition of glutamate transporters that will create an accumulation of extracellular glutamate levels and activation of mGlu receptors and NMDARs to levels that result in AMPAR endocytosis and ultimately to loss of structural and functional synapses or spines. At physiological levels it is important to know that APP mRNA interacts with FMRP and is rapidly translated in response to mGlu activation but not much more is known. APP levels are elevated in

synaptoneurosomes of Fmr1KO mice and may contribute to the altered mGlu-LTD and cognitive deficits in fragile X syndrome [132-134]

12.2 *Fragile X Syndrome Mental Retardation*

Fragile X syndrome (FXS) is commonly associate with a loss-of-function mutations in Fmr1, which encodes an RNA-binding protein, fragile X mental retardation protein (FMRP) that associates itself with dendritic mRNAs, RNA granules, translating polyribosomes and is hypothesized to function as a translational regulator of dendritic mRNAs. FMRP is a major regulator of mGlu dependent protein synthesis and plasticity since Fmr1 mRNA is translated in response to this receptors' activation as do others mRNAS including Psd-95, amyloid precursor protein (APP), elongation factor 1a (Ef1a), Map1b, and Arc which also interact with FMRP. In FXS mouse models hippocampal and cerebellar mGlu-LTD are altered and following recent reports (where FMRP switches from a translational suppressor to an activator in response to mGlu receptors through either dephosphorylation or ubiquination of FMRP or disassociation of FMRP from cytoplasmic FMRP interacting protein (CYFIP1), a recently identified eIF4E binding protein (4EBP)) in the absence of FMRP, as in fragile X syndrome, loss of steady-state translational suppression will create an increased protein levels of FMRP target mRNAs as well as a deficit in mGlu-induced translation of these mRNAs. Genetic reduction of mGlu5 reverses deficits in experience-dependent plasticity in the visual cortex, dendritic spine density, and hippocampal-dependent learning in FXS models supporting the role of these receptors in this pathology. [135, 136,137] See Figure 8.

12.3 *Parkinson's disease*

Decreasing levels of dopamine due to midbrain neurons degeneration is one of the hallmarks of PD. Affection of the DA neurons of the substantia nigra compact leads to characteristic motor phenotypes where reduction of spontaneous movements, rigidity, bradykinesia, and tremor are present. Of the various mouse models for PD one of the few characteristic that may be found in almost all them is the absence of indirect pathway mGlu-LTD that cause a balance shift toward LTP within the striatum, and the activity in the indirect pathway may actually be enhanced. Supporting this idea are rescue

experiments with a partial restoral of indirect pathway mGlu-LTD by inhibition of the degradation of endocannabinoids in combination with D2 receptor activation that have enhanced locomotor activity in dopamine-depleted mice. Besides the involvement of dopamine in LTD, it can hyperpolarize MSNs through D2 receptors and GIRK channel opening, and modulate direct-pathway activity. Further investigation is needed in this promising work.[138]

12.4 Anxiety and stress disorders

Anxiety disorders represent a group of conditions that range from generalized anxiety, panic attacks, post-traumatic stress disorder, and obsessive-compulsive syndrome to social phobias.

The group II mGlu agonists seem to be important in reversing anxiety states by maintaining homeostasis in select brain regions related to anxiety as proved by the compound LY354740 (mGlu2/3 agonist) that has progressed into Phase II clinical trials for anxiety disorders. The use of this compound produced a lasting and concentration-dependent blockade of the fear potentiated startle response in rats, with a near complete blockade in fear-enhanced startle with no effects on basal startle nor producing ataxia, sedative/hypnotic effects or suppressing motor coordination, effects that are typically associated with benzodiazepine treatment. Group II mGlu agonists probably produce their pharmacological effects by inhibiting glutamate release from excitatory inputs to crucial brain structures, such as the amygdala and hypothalamus.[139]

12.5 Epilepsy

Various authors have already discovered polymorphisms of the mGlu7 (GRM7) gene association with schizophrenia.[140-144]

In a rat model that developed spontaneous recurrent seizures after pilocarpine-induced status epilepticus, L-AP4 was significantly less effective in inhibiting field excitatory postsynaptic potentials (EPSPs) in hippocampal slices. In rats that did not develop pilocarpine-induced status epilepticus, field EPSPs were highly sensitive to L-AP4. These results point towards a down-regulate group III mGlu receptors in cases of recurrent seizures and association with deteriorated autoregulation of glutamate release. [145]

In the dentate gyrus of patients suffering from temporal lobe epilepsy, group III mGlu-mediated inhibition of synaptic transmission was almost lost in the subgroup with Amon's horn sclerosis.[146]

12.6 Schizophrenia

Although dopaminergic dysfunction has been a major hypothesis for the schizophrenia pathology, glutamatergic dysfunction has been suggested as an alternative hypothesis for schizophrenia pathology based on the observations of psychosis induced by PCP, which is a noncompetitive antagonist of NMDA1, the NMDA-type ionotropic glutamate receptor. [147]Similar to epilepsy, polymorphisms for mGlu7, mGlu4 and mGlu8 have potential influence in schizophrenic profiles.[148-150]

Wierońska et all also report that when using AMN082, it had no effect on amphetamine induced hyperactivity but induced an enhancement of MK-801-induced hyperactivity and DOI-induced head twitches in mice and that this effects were not observe din KO animal and these were less sensitive for DOI-induced effect than their wild type littermates.[151]

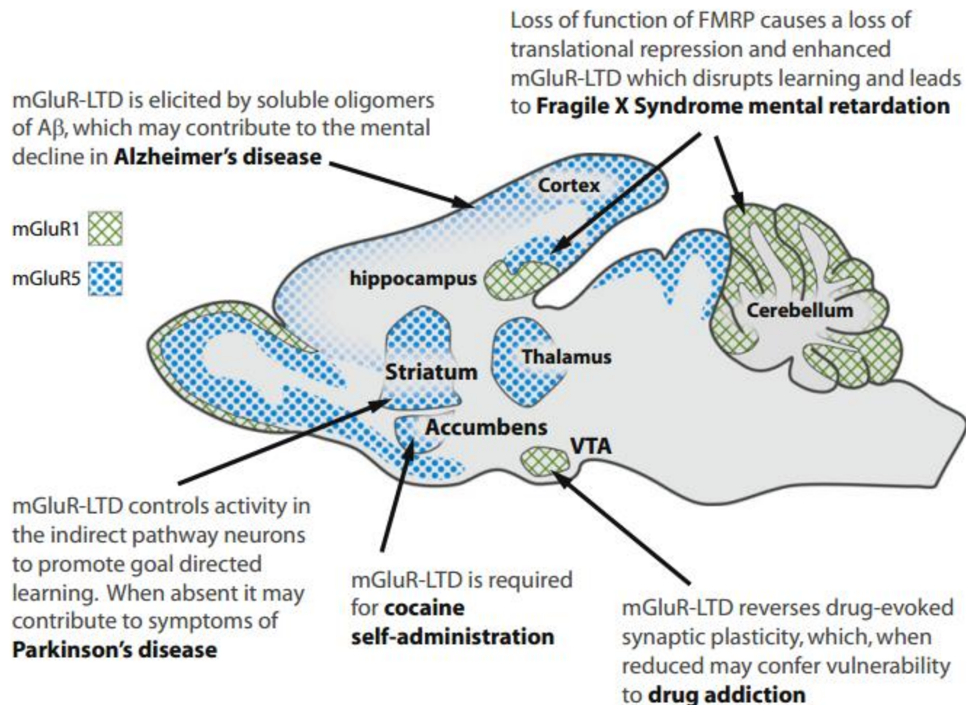

Figure 8 mGlu-LTD in Health and Disease

Experimental evidence suggests the involvement of mGlu-LTD in goal-directed learning and cerebellar circuit adjustment during motor learning. Excessive mGlu-LTD has been linked to Alzheimer's disease and fragile X syndrome, while a loss of mGlu-LTD in the striatum may contribute to Parkinson's symptoms. Finally, reduced

mGlu-LTD in the midbrain has been suggested to confer a vulnerability of drug addiction. The group 1 mGlu receptors, mGlu1 and mGlu5, are differentially expressed in most brain regions and are coexpressed in some, based on Ferraguti and Shigemoto43. mGlu1 expression is indicated by the green cross-hatching, and mGlu5 expression pattern is indicated by the blue cross-hatching. Lighter cross-hatching indicates a lower level of expression.

13 Methods and materials

13.1 Ethical note

All procedures were performed in compliance with Belgian law (Royal Decree on the protection of laboratory animals, dd. April 6, 2010). The study protocols were approved by the animal experimental ethical committee of Janssen Research and Development (Beerse, Belgium). The facility was AAALAC-accredited.

13.2 Animals

All experiments were carried out on horizontal slices from the hippocampus of male Sprague Dawley rats (Charles River, Germany) of 3 to 5 weeks old, weighing 150 to 300g, or immature rats of 10 to 15 days, weighing 15 to 40g (Charles River, Germany). The slices were 300 µm thick for the adult animals and 400 µm for the immature. The rats were housed in individually ventilated cages (IVC) with sawdust bedding at 22 ^{0}C on a 12/12h light/dark cycle. Adult animals were housed in groups of 5 rats per cage (44 x 34 x 20 cm) with *ad libitum* access to food and water while immature male rats stayed with all their littermates and mother under the same conditions as previously stated.

13.3 Brain slice preparation

Adult rats were anesthetized with 3 to4% isoflurane, weighted, and decapitated with a guillotine while immature rats were decapitated with large scissors.

Their heads were submersed in a recipient with ice-cold preparation solution composed of (in mM): $MgSO_4.7H_2O$ 8.3; NaCl 124; $NaH_2PO_4.H_2O$ 1.25; KCl 2.7; $NaHCO_3$ 26; $CaCl_2$ 2; D-glucose.H_2O 18; Ascorbic acid 2; pH 7.25-7.35; osmolarity 300 to 320 mOsm. For the older rats - >200g - the preparation and cutting solution was composed (in mM): Sucrose 234; KCl 2.5; NaH_2PO_4 1.25; $NaHCO_3$ 25; Glucose 11; $CaCl_2$ 0.5, $MgCl_2$ 7. Using high concentrations of Mg^{2+} blocked NMDAR mediated signaling which is commonly associated to neurotoxicity.[152-154] The skin was removed with large operating scissors (Medicon 03.05.14). With delicate scissors (Medicon 02.21.91) a cut was made through the foramen magnum through the sagittal suture up to caudal level of the eye orbits as well as a cut through both lambdoid sutures. With the help of a curved forceps the bone pieces were removed and the brain was cleaned with a few drops of cooled preparation solution. The brain was

then rapidly removed from the skull with a curved spatula (KLSMartin – 35-683-16) and placed in fresh ice-cold preparation buffer. All solutions were previously bubbled with 95% O_2 and 5% CO_2.

The brain was then transferred to a Petridish filled with ice-cold preparation solution and containing a filter paper (Whatman 1PS phase separator filter papers) for visual aid. The cerebellum and prefrontal cortex were removed using two scalpels. The brain was glued horizontally on the base plate of the vibratome using cyanoacrylate glue and supported by a small square agar block (3%). The brain was positioned so that the posterior part was facing the blade of the vibratome (Leica VT 1200S). The tray of the vibratome was filled with ice-cold cutting solution (same composition as the preparation solution) bubbled with 95% O_2 and 5% CO_2 and cooled to reduce neuron activity (Julabo f12 Cooling system – temperature is maintained around 4°C in the bath).

Horizontal brain slices were cut with the vibratome at a velocity of 0.09-0.1 mm/s. Hippocampi were dissected out of the slices with the help of two scalpels and incubated for 15-20 minutes at 35^{0}C on nylon meshes immersed in a glass vessel filled with ACSF (in mM $MgSO_4.7H_2O$ 1 -the concentration was decreased so that NMDAR-mediated response could be taken into account-; NaCl 124; $NaH_2PO_4.H_2O$ 1.25; KCl 2.7; $NaHCO_3$ 26; $CaCl_2$ 2; D-glucose.H_2O 18; Ascorbic acid 2; pH 7.25-7.35; osmolarity 300±10 mOSM)[155] bubbled with 95% O_2 and 5% CO_2. The slices were left to recover for at least 45 minutes in oxygenated ACSF at room temperature (22ºC ±1). Slices were placed on a nylon mesh in the recording chamber and immobilized with an U-shaped platinum frame and continuously perfused with ACSF at 30^{0}C ± 2 at a rate of 1.7–2.2 ml/min.

13.4 Electrophysiological recordings

13.4.1 fEPSP acquisition

The system was composed of 2 PatchStar Micromanipulators (Scientifica) for control of the stimulating and recording electrode. The perfusion rate was controlled with a peristaltic pump (PPS2, Multichannel System) and the temperature of the ACSF was controlled by a heated perfusion tube bubbled with 95% O2 and 5% CO2).

The slices were visualized via differential interference contrast microscopy (DIC) using infrared light on an upright fixed-stage microscope (Olympus BX51W1) with a 5X Zeiss A-Plus and a10X

ocular (Olympus, Belgium). The images were captured by a CCD camera, Retiga Exi (QImaging, BC, Canada) onto a computer display running QCapture Software 2.98 with the QCam Driver DLL 2.08 (QImaging).

The stimulation signals were generated by a stimulus isolator (ISO-Stim 01D) which was controlled by the patch clamp amplifier via TTL pulses. Field potentials were acquired with a MultiClamp 700B amplifier controlled by the pCLAMP 10.4 software. The data was digitized with a Digidata 1440 (Molecular Devices). The data was low-pass filtered at 5 kHz, sampled at 10 kHz, and analyzed offline.

The recording electrodes were made of thick-walled borosilicate glass capillaries with an outer diameter of 1.5 mm and an inner diameter of 0.86mm (Harvard apparatus) and were pulled with a P97 or P87 Flaming/Brown Micropipette Puller (Sutter Instruments). The electrodes had a tip diameter of 1µm, corresponding to a resistance of 1-3 MΩ. The stimulating electrode was a monopolar Tungsten Electrode with a tip impedance of 1 MΩ. Fine precision movement of the pipette was accomplished by PatchStar, a stepper motor with anti-backlash in combination with LinLab software control (Scientifica Ltd., UK).

13.4.1.1 In the CA1 are of the hippocampus

fEPSPof the hippocampal CA1 area were recorded by positioning the recording pipet near the stratum radiatum and the stimulation electrode in the Shaffer collaterals (as shown in Figure 9). Such recordings are usually accompanied by a pronounced presynaptic fiber volley, an indicator of presynaptic intraneuronal response.[5, 156]

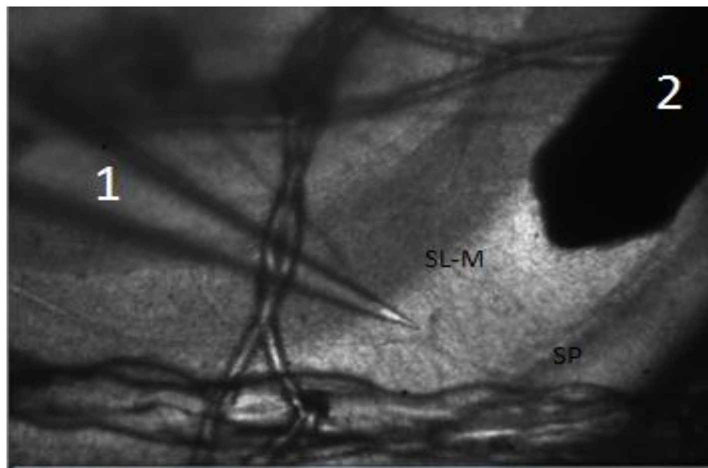

Figure 9Example of the positioning of the recording (1) and stimulating (2) for recording fEPSP in the hippocampal CA1 area in an adult rat brain tissue slice.

13.4.1.2 In the DG MPP of the hippocampus

Responses to selective stimulation of the MPP were evoked by positioning the recording and stimulating electrodes in the middle one-third of the molecular layer of the DG (as shown in Figure 10). The correct placement of the electrodes was verified by measuring pairs of stimuli with an interstimulus interval of 100 ms. Stimulation of the MPP shows a smaller second stimulus compared to the first, known as pair pulse depression[14] as this pathway has a low probability of release. In this area the use of paired pulses validates the electrodes position since it was already demonstrated that such stimulation of the MPP and LPP evokes responses displaying paired-pulse depression and facilitation, respectively.[157, 158]

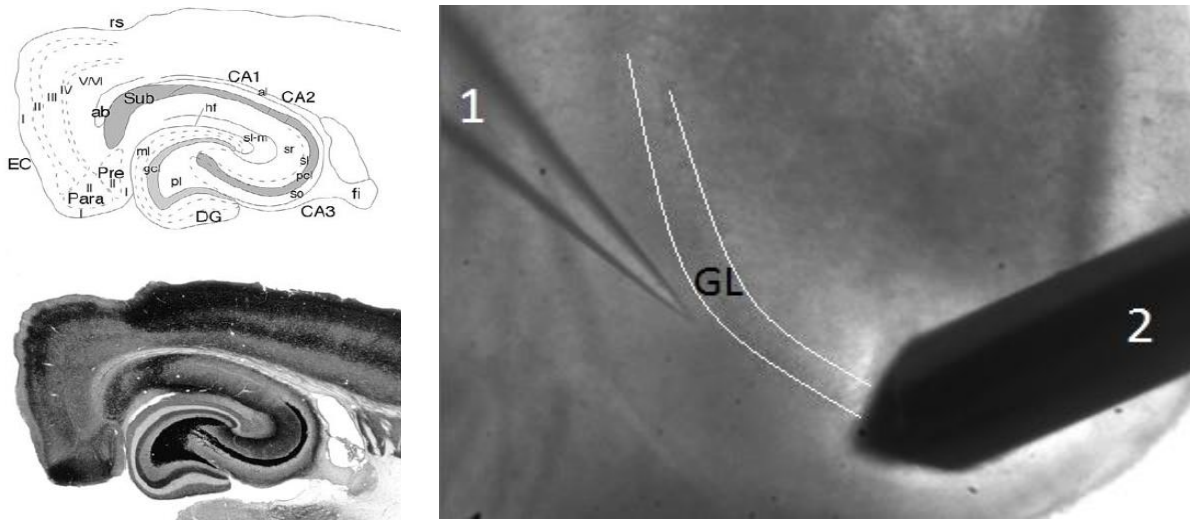

Figure 10Right side - Example of the positioning of the recording (1) and stimulating (2) for recording fEPSP in the hippocampal DG MPP in an adult rat brain tissue slice. Left side -The outer band corresponds to the terminal zone of the lateral perforant pathway; the middle unstained region corresponds to the terminal zone of the medial perforant pathway; the inner band corresponds to the zone of termination of the associational and commissural pathways of the dentate gyrus. ab, angular bundle; al, alveus; CA1, CA1 field of the hippocampus; CA2, CA2 field of the hippocampus; CA3, CA3 field of the hippocampus; DG, dentate gyrus; EC, entorhinal cortex; fi, fimbria; gcl, granule cell layer of the dentate gyrus; hf, hippocampal fissure; ml, molecular layer of the dentate gyrus; Para, parasubiculum; pcl, pyramidal cell layer of the hippocampus; pl, polymorphic layer of the dentate gyrus; Pre, presubiculum; sl, stratum lucidum of CA3; sr, stratum radiatum of the hippocampus; sl-m, stratum

13.4.2 MEA

Multi-electrode array (MEA -Ayanda Biosystems SA, Switzerland) extracellular recordings were also performed in acute brain slices to study synaptic transmission of the Schaffer collateral to the CA1 area. The MEAs consisted of 60 platinum electrodes (30 µM diameter; 100 µM interspacing) that protruded 25–35 µM into the tissue slice.

Individual tissue slices were placed with a drop of standard ACSF on top of the electrodes and secured with a platinum grid, providing a steady and sufficient contact between the electrodes and the tissue. The hippocampal network in the tissue slice was correctly orientated onto the electrodes using an inverted microscope which was connected to a digital CMOS camera (TCA-3.0C, Tucsen Image Technology Inc., NY, USA). The images of the tissue slices were imported on a PC. This made it possible to designate a stimulation site with ease and to store the configuration for analysis.

The tissue was perfused at a rate of 1 to 2.5 ml/min with ACSF at 32 ^{0}C (TC02, MultiChannel Systems).

A clear signal was obtained in CA1 by stimulating the Schafer collaterals near the CA3 area with two different electrodes generating paired pulse bipolar current. The stimulation was designed in the MC_Stimulus II software (Multi Channel Systems) and consisted of a positive pulse followed by a negative pulse, each lasting 100µs .This stimulation protocol was applied at 1/60 Hz.

The MEA chip were carefully rinsed after each experiment by different steps.

First, they were rinsed with pure water (milliQ Water), followed by70% ethanol to remove compounds and avoid bacterial contamination. Residual ethanol was removed by rinsing again with pure water. During all the cleaning procedures care was taken not to damage the electrodes in the chip by not applying pressurize air onto the electrodes. The connection between the MEA chips and the pre-amplifier were also cleaned with a tissue slightly soaked in 70% ethanol and were allowed to dry before placing the chip.[159]

13.4.2.1 Spiking activity

Spontaneous spiking activity[3] was measured using the same procedure as described for the fEPSP recordings on the MEAs except that no electrical stimulation was delivered. Data was filtered with a Butterworth 2 pole filter in with a high pass cutoff frequency at 250 Hz. Threshold criterion for spike

detection was defined as a minimum of 20 pA. A similar recording system was used as described above except the microscope was a Zeisss Axiovert 135. [160]

13.4.3 Stimulation protocols and recordings

The correct stimulation strength was determined by means of input-output curves, which were constructed by incrementally increasing the stimulus intensity and recording the evoked response.

Baseline stimulation strength was set to evoke a response that corresponded to about 70% of the maximum attainable fEPSP that showed no clear contamination by population spikes. Recordings were started after obtaining a stable baseline during a minimum of 20 minutes. Compounds were delivered after 30 minutes of stable baseline recording.

For recording fEPSPs paired pulses with a 30ms interpulse interval at 1/60 Hz were used.

The compounds were washed out for a period of 30 min to verify if the fEPSPs could return to baseline levels or if the effect of the compound was persisted.

To validate the glutamatergic origin of fEPSP an inhibitory cocktail was added. The cocktail was composed of kynurenic acid (10mM) and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 50µM) dissolved in ACSF. All recording in which the obvious effect of the cocktail in reduction of the fEPSP was not observed were discarded.

13.5 Chemicals and compounds

All chemical were purchased from Sigma-Aldrich and compounds were either purchased from Tocris Bioscience or synthetized *in situ* at Janssen Pharmaceutica.

13.6 Data analysis

Data files from fEPSPs recording using borosilicate pipettes were opened in Clampfit 10.2.The baseline potential was recorded in an interval of 20 to 50ms before the first stimulus artifact. The peak amplitude (mV) of both fEPSPs were measured by using a peak finding algorithm functioning within a user set interval that was defined as the period starting after the presynaptic volley and the end of

the signal. Data was imported to Microsoft Excel for the analysis. Peak amplitude was normalized to the mean the last 5 minutes of the baseline recording. Values for each pharmacologically modulated condition were considered as the averaged of the last 5 minutes in which the compound(s) where present in the recording chamber.

Data files from MEA recordings were opened using MC_Rack software (Multi Channel Systems). Data was filtered with a Butterworth 2nd order filter with a Low Pass Cutoff Frequency at 200 Hz for all channels.The criteria for peak value determination were the same as previously described. Per slice recordings from five electrodes were normalized to the baseline and averaged. These electrodes were near the area of interest (CA1) and showed a stable baseline during a minimum of 30 minutes and showed a clear decrease in amplitude after adding kynurenic acid and CNQX.

The concentration-response curves were fit to a Hill equation: $B = Bmax/(1 + (IC_{50}/[agonist])^{nH})$, where B, B_{max}, IC_{50}, [agonist], and n_H are percentage of inhibition, maximum inhibition, half inhibition concentration, agonist concentration, and Hill coefficient, respectively.[161]

Values are represented as mean ± Standard Error of the Mean (SEM) from n slices from a minimum of 3 animals. Statistical significance was assessed by a one-tailed Student's t test and repeated measures with ANOVA for the baseline versus pharmacologically modulated conditions. A p value of 0.05 or less was considered to account for significant differences in the different conditions.

14 Results

To evaluate the role of mGlu7 in basal synaptic transmission and short-term synaptic plasticity fEPSP were recorded in acute brain slices from both adult and immature SD rats using multiple combinations of compounds.

Paired-pulse phenomena are short-term forms of plasticity operating in the millisecond to second range. Two stimuli are delivered to the afferent fiber and the resulting post synaptic potentials are either depressed or enhanced somehow revealing the probability of release of those fibers and how they are modulated.[162] The first and second response to the pair of pulses delivered are designated as fEPSP1 and fEPSP2, respectively. The paired pulse ratio (PPR) is defined as the ratio of fEPSP2 over fEPSP1. A PPR bigger than one is called paired-pulse facilitation and shows the second EPSP bigger than the first. In paired-pulse depression the ratio is less than one. See Figure 11 for reference.

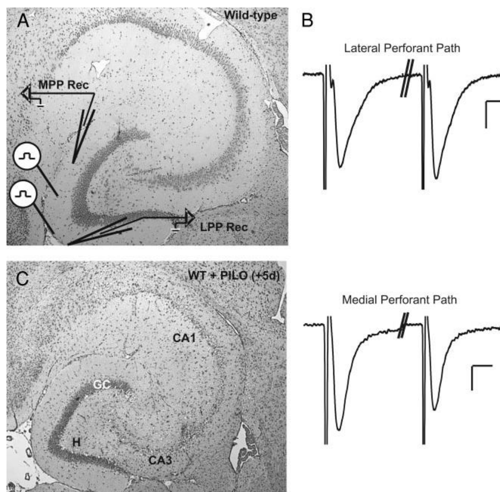

Figure 11 A: representative transverse hippocampal slice taken from a saline- injected wild-type (WT) control animal fixed and stained with hematoxylin after recording. Also shown are the characteristic placement of stimulating and recording electrodes in the medial perforant path (MPP) and lateral perforant path (LPP). MPP and LPP responses were evoked by placing the stimulating and recording electrodes into the middle or outer molecular layers, respectively. B: electrodes were correctly positioned if paired pulse stimulation (150-ms interpulse interval) produced depression in the MPP, and facilitation in the LPP. Calibration bars 5 ms, 0.3 mV. Concentration response of mGlu7 agonist (L-AP4) in the CA1 area of adobe

One of the main problems when trying to discern the different contributions of Group III mGlu receptors over the control of synaptic transmission and short term plasticity resides in the absence of selective compounds to modulate their activity. However, when working with compounds that have a similar structure and affinity as glutamate, one can rely on the low affinity that the mGlu7 receptor has for glutamate (mM range). Because higher concentrations of the compounds are needed for mGLu7 compared to mGlu4 and mGlu8 it is possible to distinguish the contribution of the different receptors.

However, there is a tradeoff: high concentrations of compounds may lead to off-target effects that could negatively influence the results obtained, as will be shown in this chapter.

Ayala et al[1] have demonstrated that synaptic transmission at the SC-CA1 synapse is developmentally regulated by changes in the ratios of the different Group III mGlu receptors subtypes. Their conclusion was derived from the fact that the agonist L-AP4 only had an effect in neonatal rats at low concentrations and higher concentrations had an effect in both neonatal and mature rats.

These observations led to the first objective for this thesis, namely to define the optimal working concentration of L-AP4 for the activation of mGluR7 in brain slices in adult and immature rats. Similarly, it was relevant to define the optimal concentration of the potent non-selective agonist LY341495 in both age groups.

Therefore fEPSP were recorded of SC-CA1 synapses from immature rats (10 to 15 days) and adult rats (7 to 9 weeks) with increasing concentrations of the compounds.

14.1 L-AP4 concentration-response curve in animals with 7 to 9 weeks

In the adult animals the agonist only started having a significative effect with concentrations above 300 µM, with the highest concentration 1 mM having the greatest effect decreasing the peak value to 55 ± 9% ($p<0.01$)of baseline values. See Figure 12.

The PPR was significantly altered with lower concentrations, starting at 10 µM, and maintaining a similar level of reduction with the increasing concentrations. This may indicate a postsynaptic local of action, contrary to the expected presynaptic effect due to activation of mGlu7. See Figure 13.

The EC_{50} calculated (1.13 mM) was extrapolated from the fit to the Hill equation since the range of concentrations used was not wide enough to cover the “real” EC_{50}. As stated the concentrations used were not higher than 1 mM due to solubility problems, possible neurotoxic and off-target effects. Nonetheless the result is similar to the ones found in the literature when tested in animal animals brain slices.[1] See Figure 14.

A clear washout of the compound was present during the experiments even tough not to baseline levels but it should be kept in mind the high concentrations of the compound used and as well the total time of incubation of the slice which also influences the final viability of the slice.

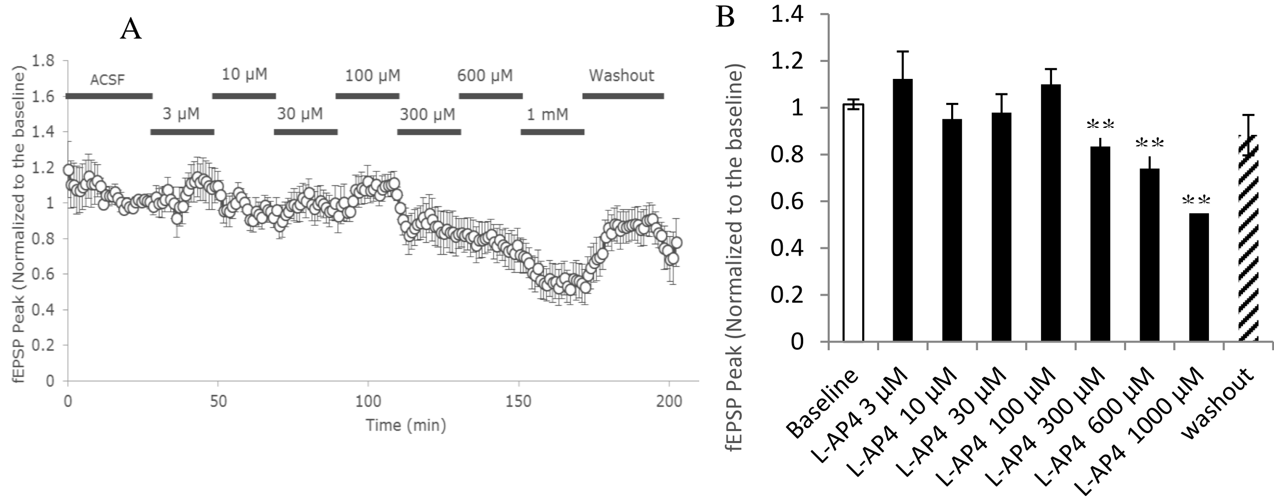

Figure 12 **Concentration response of L-AP4 over fEPSP recorded in the hippocampal CA1 area of adult animals.** A. L-AP4 significantly reduces synaptic transmission at the SC-CA1 synapse in the adult hippocampus at concentrations higher than 100μM with a reduction of near 50% at 1 mM as also shown in Ayala et al[1]. fEPSPs were elicited by stimulating the SC.B. Bar graph shows the averaged effect of each concentration of the fEPSPs.L-AP4 (300 μM), 0.83±0.1, p<0.01; L-AP4 (600 μM), 0.74±0.1, p<0.01;L-AP4 (1000 μM), 0.55±0.09, p<0.01. A total of 7 slices from 5 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

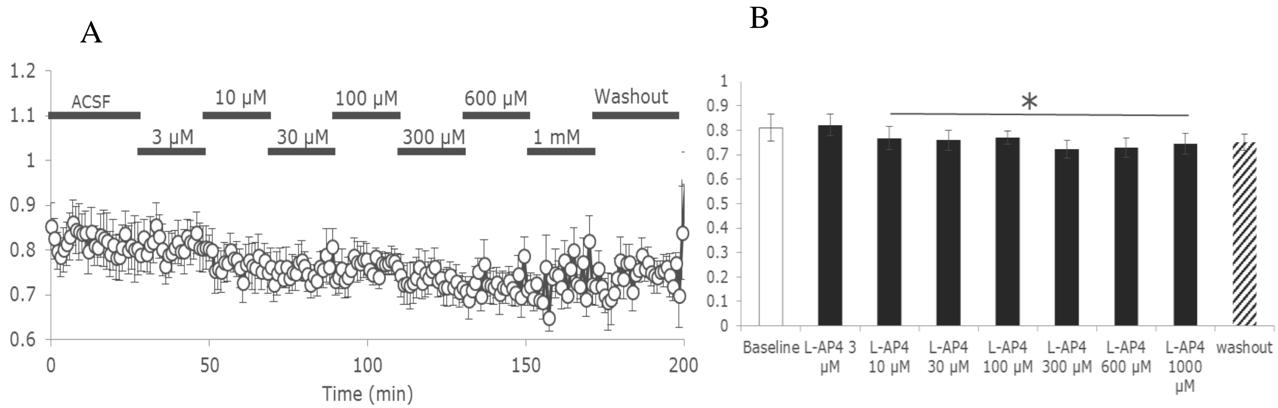

Figure 13 **Concentration response of L-AP4 over PPR of paired pulses fEPSP recorded in the hippocampal CA1 area of adult animals.** A. L-AP4 leads to a reduction of the ratio with concentrations higher than 10 µM suggesting that the compound is acting on receptors presynaptically localized. The reduction on PPR was expected since Group III mGlu are mainly located presynaptically. Bar graph shows the averaged effect of each concentration of the PPR. Baseline, 0.81±0.06; L-AP4 (300 µM), 0.72±0.04, p<0.01; L-AP4 (600 µM), 0.73±0.04, p<0.01;L-AP4 (1000 µM), 0.74±0.09, p<0.05. A total of 7 slices from 5 animals were averaged. Data (Mean ± S.E.M.).*p<0.05, **p<0.01, two-tailed Student's t test.

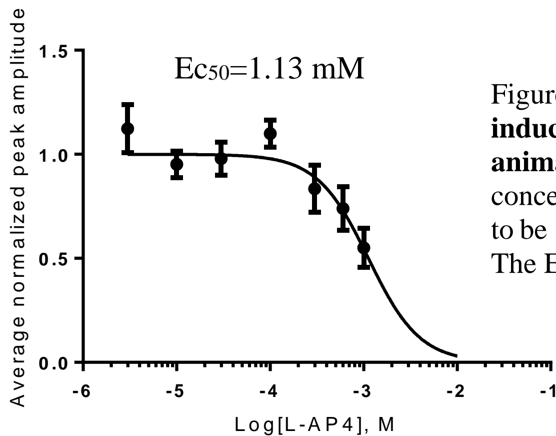

Figure 14 **Depression of fEPSP peak (mean ±S.E.M.) induced by increasing concentrations of L-AP4 in adult animals SC-CA1 synapses of the hippocampus**. The concentration producing half-maximal depression is predicted to be 1.13 mM. A total of 7 slices from 5 animals were averaged. The EC_{50} was extrapolated from the fit to the Hill equation.

14.2 L-AP4 concentration-response curve in animals with 10 to 15 days

In the immature animals the agonist started having a significative effect already with the first concentration used (L-AP4 3 μM, 56±2% of baseline). This indicates that at this age high affinity receptors are responsible for the effect of the compound. It would have been relevant to use lower concentrations to have a more appropriate curve of effect. See Figure 15.

The PPR was significantly altered with all concentrations used, and similarly to adult animals, maintained at a similar level of reduction with the increasing concentrations (around 70% of baseline values) except with the last concentration used (1 mM) in which a small increase was observed indicating that possible off-targets were activated at this concentration. See Figure 16.

The EC_{50} calculated (10.44 μM) could be more precise if lower concentrations were used as the shape of the concentration-response curve is almost linear. See Figure 17.

When compare to results found in the literature of brain slices incubation of young animals (around 10 days old) the value obtained (10.44 μM) is quite lower.[1]

A washout of the compound was present during the experiments even though not as evident as in the adult animals. Similarly the high concentrations of the compound, the total time of incubation of the slice and the higher sensibility of young tissue to damage may account for this diminished washout.

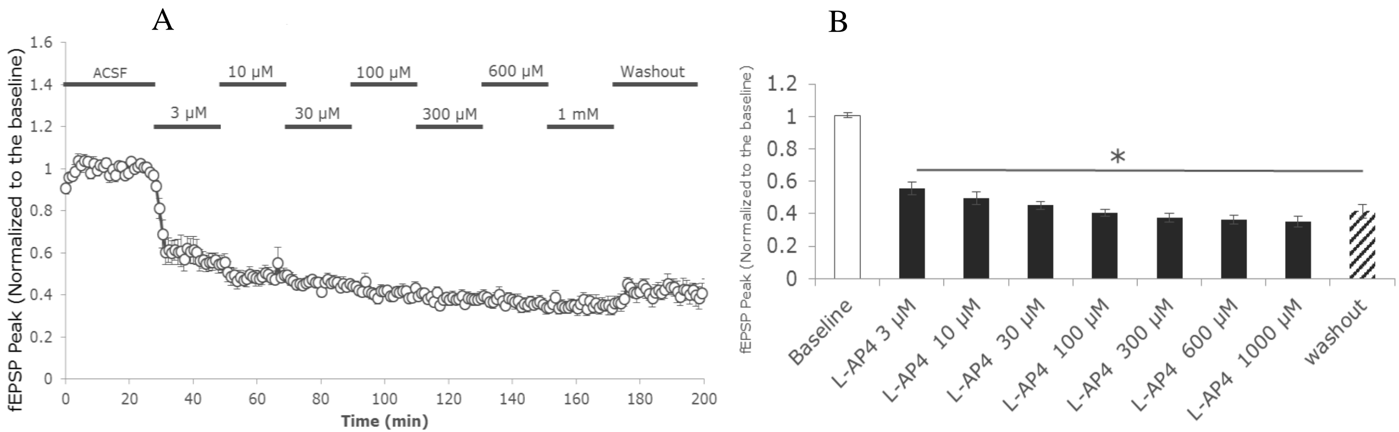

Figure 15 [1]Concentration response of L-AP4 over fEPSP recorded in the hippocampal CA1 area of immature rats. A. L-AP4 significantly reduces synaptic transmission at the SC-CA1 synapse at concentrations as low as 3 µM. fEPSPs were elicited by stimulating the SC. B. Bar graph shows the averaged effect of each concentration of the fEPSPs. L-AP4 (3 µM), 0.56±0.02, $p<0.01$; L-AP4 (1000 µM), 0.35±0.03, $p<0.01$. A total of 7 slices from 5 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*$p<0.05$, **$p<0.01$, two-tailed Student’s t test.

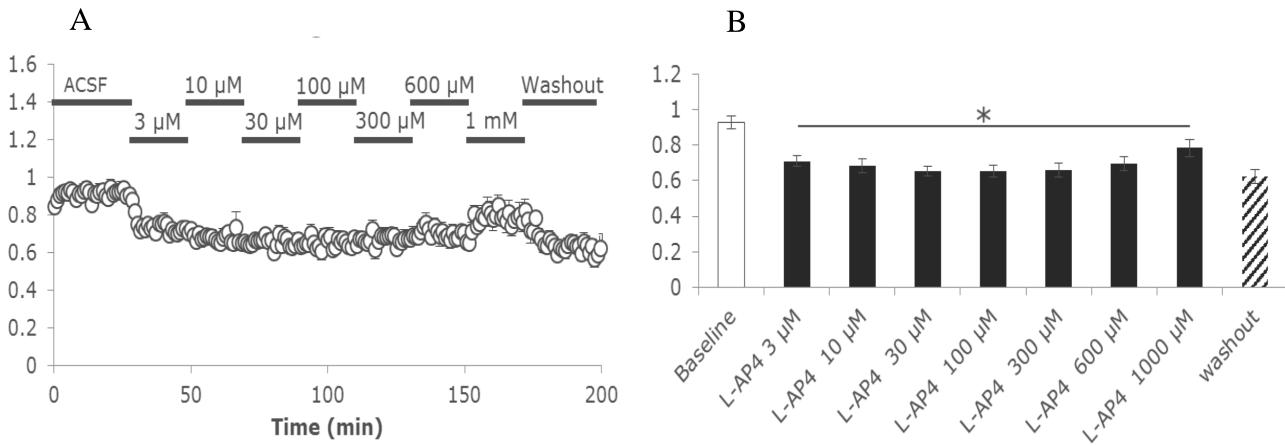

Figure 16Concentration response of L-AP4 over PPR of paired pulses fEPSP recorded in the hippocampal CA1 area of adult animal. A.L-AP4 leads to a reduction of the ratio with concentrations higher than 10 µM suggesting that the compound is acting on receptors presynaptically localized. The reduction on PPR was expected since Group III mGlu are mainly located presynaptically. B. Bar graph shows the averaged effect of each concentration of the PPR. Baseline, 0.92±0.04; L-AP4 (3 µM), 0.70±0.03, $p<0.05$; L-AP4 (1000 µM), 0.78±0.04, $p<0.05$. A total of 7 slices from 4 animals were averaged. Data (Mean ± S.E.M.).*$p<0.05$, **$p<0.01$, two-tailed Student’s t test.

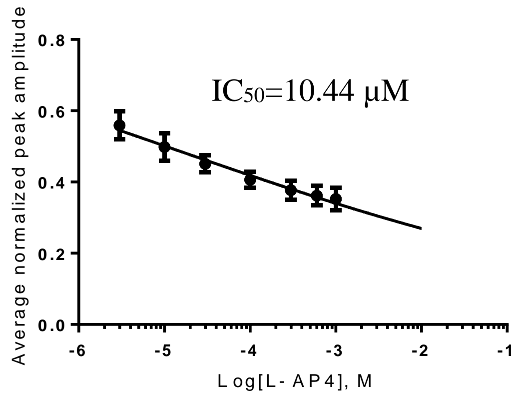

Figure 17 Depression of fEPSP peak (mean ±S.E.M.) induced by increasing concentrations of L-AP4 in immature rats SC-CA1 synapses of the hippocampus. The concentration producing half-maximal depression is predicted to be 10.44 µM. A total of 7 slices from 4 animals were averaged. The EC50 was extrapolated from the fit to the Hill equation.

14.3 Effects of mGlu7 agonist (L-AP4 600µM) on basal synaptic transmission and short-term synaptic plasticity of DG MPP-granule cells synapses

Zhai et al[157] and Nisnbaum et al[163] demonstrated that of all mGlu receptors, mGlu8 has the most control on basal synaptic transmission in the DG LPP However, in the MPP, the Group II elements seem to have a role in the control of synaptic transmission. However all these studies used low concentrations of L-AP4 compared to the values that should act on mGlu7, and state that the small effects observed on MPP using these low concentrations could be due to allow affinity receptor as mGlu7.

Bough et al[14] demonstrated a reduction of mGlu7 signaling after pilocarpine treatment which has been shown to induce status epilepticus.[164] Attributing to the receptor an important role in the generation of hyper-excitability in the dentate and the receptor could therefore contribute to epileptogenesis. It should be noted that only animals lacking mGlu7 have increased susceptibility to seizures while mGlu1, 2, 4, 5 and 8 do not.

Therefore the effects of L-AP4 were testes over basal fEPSP recorded with a paired pulse stimulus in the DG MPP of adult animals. The objective was discern the possible effects of activating mGlu7 in one of the main “input” pathways of the hippocampus during basal synaptic transmission and short-term plasticity events.

Data collected from 3 adult animals showed that the application of L-AP4 significantly reduced the peak value of fESPS at the MPP-granule cells synapses (68±3% of baseline values, $p<0.01$) with a clear washout being present. See Figure 18. The compound as well led to an increase of the PPR, indicating a possible presynaptic local of action of the agonist in this area, validating a possible action of mGlu7. See Figure 19. This demonstrated that both the CA1 and DG MPP area of adult animals are sensible to alterations of their fEPSP by the use of the agonist and that the mGlu7 receptor may have a role in the control of the MPP in contrast to the control of the LPP by mGlu8. [157]

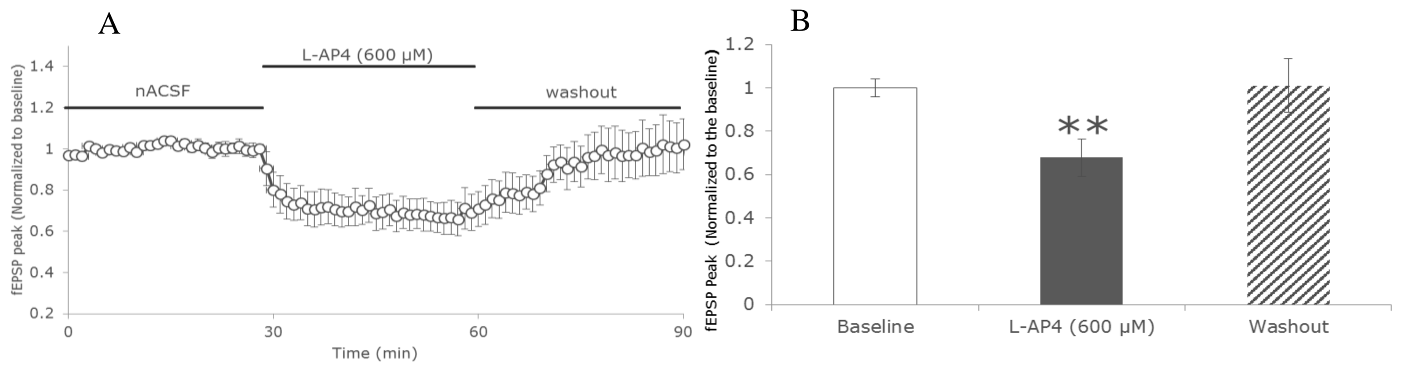

Figure 18 **Effect of L-AP4 over fEPSP recorded in the hippocampal DG MPP in adult animals.** A. L-AP4 significantly reduces synaptic transmission at the MPP-granule cells synapses at 600 µM. fEPSP were elicited by stimulating near the granular layer of the DG. B. Bar graph shows the averaged effect of L-AP4 over the fEPSPs. L-AP4 (600 µM), 0.68±0.03, p<0.01. A total of 7 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

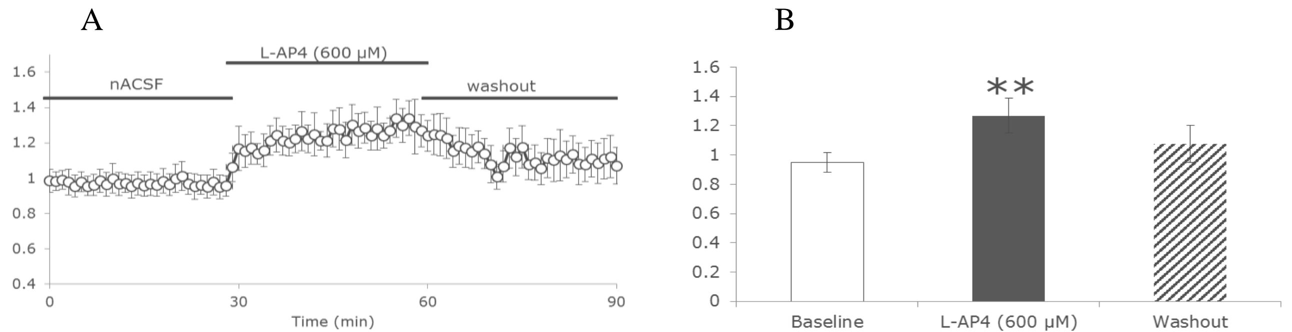

Figure 19 **Effect of L-AP4 over PPR of paired pulses fEPSP recorded in DG MPP of adult animals.** A. L-AP4 significantly increases the ratio at the MPP-granule cells synapses at 600 µM. fEPSP were elicited by stimulating near the granular layer of the DG. This suggesting that the compound is acting on receptors presynaptically localized adding validation of the response being mediated by mGlu7. B. Bar graph shows the averaged effect of L-AP4 over the fEPSPs ratio. Baseline, 0.94±0.07; L-AP4 (600 µM), 1.27±0.1, p<0.01.A total of 7 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

14.4 LY341495 (potent non-selective antagonist) concentration-response curve in adult rat SC-CA1 synapses

Due to the lack of potent and selective antagonists for mGlu7 the next best (commercially available) solution is the potent and non-selective compound LY341495. With the hypothesis that the role of mGlu7 signaling is different in adult and immature animals concentration-response curves were elaborated. These curves were expected to help choose a working concentration for the compound and as well to discern differences in the response in the different ages of the animals. Simultaneously it was tested if the higher concentration used could prevent the effects of the working concentration of L-AP4.

Possible biphasic LY341495 concentration response in adult animals

When tested in CA1 are of adult animals the antagonist significantly reduced both the fEPSP peak value (see Figure 20) and PPR (see Figure 21) in the CA1 area at all concentrations tested and did not seem to reverse the depressing effects of L-AP4.

The effect over the peak amplitude seems to have a biphasic response with and accentuated decrease at 0.3 µM to around 80% of baseline values and later with 10 and 30 µM there is another deflection that disappears when the concentration is elevated to 100 µM.

The reduced PPR maintained the same degree of decrease with all concentrations used. However in the presence of both compounds the PPR was lower than the initial baseline or the decrease caused by the increasing concentrations of the antagonist.

A washout of the effects is present even tough not very accentuate and it could have benefit from a longer time of exposure to aCSF.

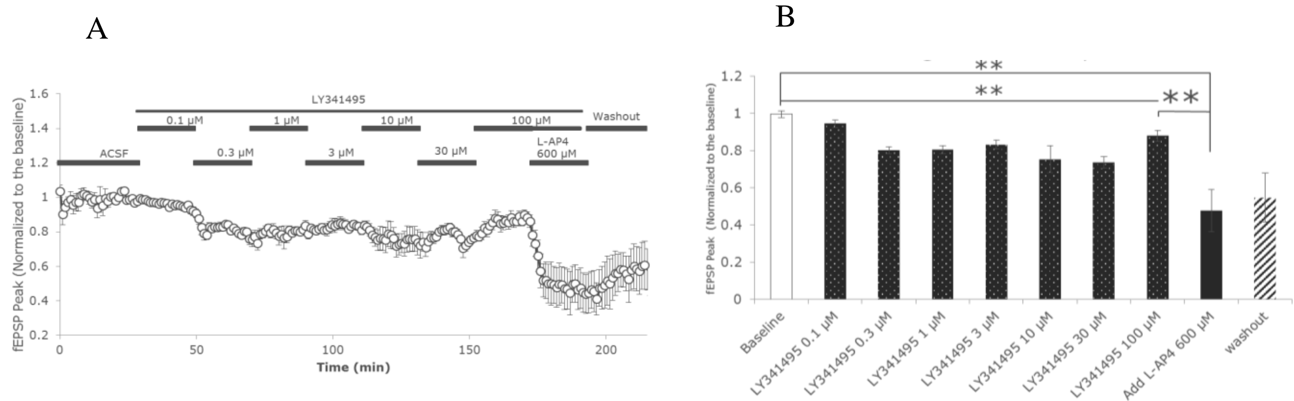

Figure 20 Effect of the antagonist LY341495 over fEPSP recorded in the hippocampal SC-CA1 synapses in adult animals. A. The compound significantly reduced synaptic transmission at all concentrations tested and did not seem to reverse the depressing effects of L-AP4 at the concentration used. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC . B. Bar graph shows the averaged effect of increasing concentrations of LY341495, and the addiction of 600 µM to the last concentration of the antagonist used, over the fEPSPs. LY341495 (0.3 µM), 0.80±0.01, p<0.01; LY341495 (30 µM), 0.73±0.03, p<0.01; LY341495 (100 µM), 0.74±0.03, p<0.01; LY341495 (100 µM) and L-AP4 (600 µM), 0.47±0.1, p<0.01 to baseline and LY341495 at 100 µM. A total of 6 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

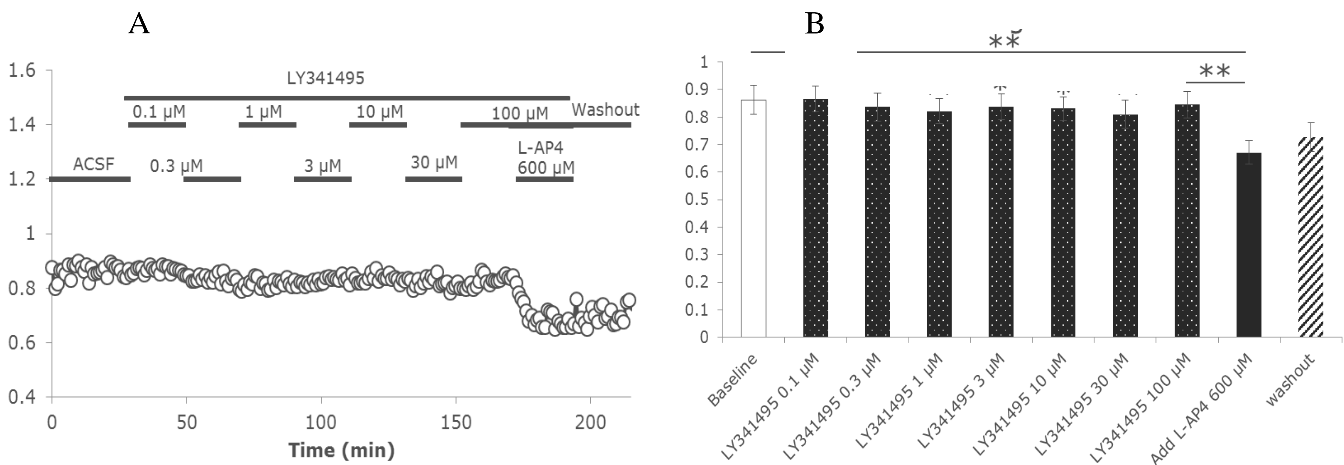

Figure 21 **Effect of the antagonist LY341495 over PPR of paired pulses fEPSP recorded in the hippocampal SC-CA1 synapses in adult animals.** A. The agonist at concentrations of 1,3,10 and 30 µM decreased PPR with significance. At 100µM the compound did not alter the PPR and did not interfere with the reduction caused by L-AP4, which reached similar levels in the absence of this antagonist. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. This suggesting that the compounds are acting on receptors presynaptically located. B. Bar graph shows the averaged effect of L-AP4 over the fEPSPs. Baseline, 0.95±0.06; LY341495 (0.3 µM), 0.84±0.05, p<0.01; LY341495 (30 µM), 0.84±0.05, p<0.01; LY341495 (100 µM), 0.84±0.05, p<0.01; LY341495 (100 µM) and L-AP4 (600 µM), 0.67±0.04, p<0.01 to baseline and LY341495 at 100 µM. A total of 6 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

14.5 LY341495 (potent non-selective antagonist) concentration-response curve in immature rats SC-CA1 synapses

In the immature animals a similar situation, compared with the older animals, was present, only with a more defined decrease of fEPSP peak that started already at the first concentration tested (see Figure 22). They also present an almost constant level of depression that was not accentuated concentration dependently. In this animals the antagonist was, as well, not capable of preventing the effects of L-AP4, and the presence of both compounds led to an accentuated decrease of the PPR that was otherwise similarly depressed by the other concentrations of LY341495. See Figure 23.

A washout of the effects is present even tough not very accentuate and it could have benefit from a longer time of exposure to aCSF as in the adult animals. See 0

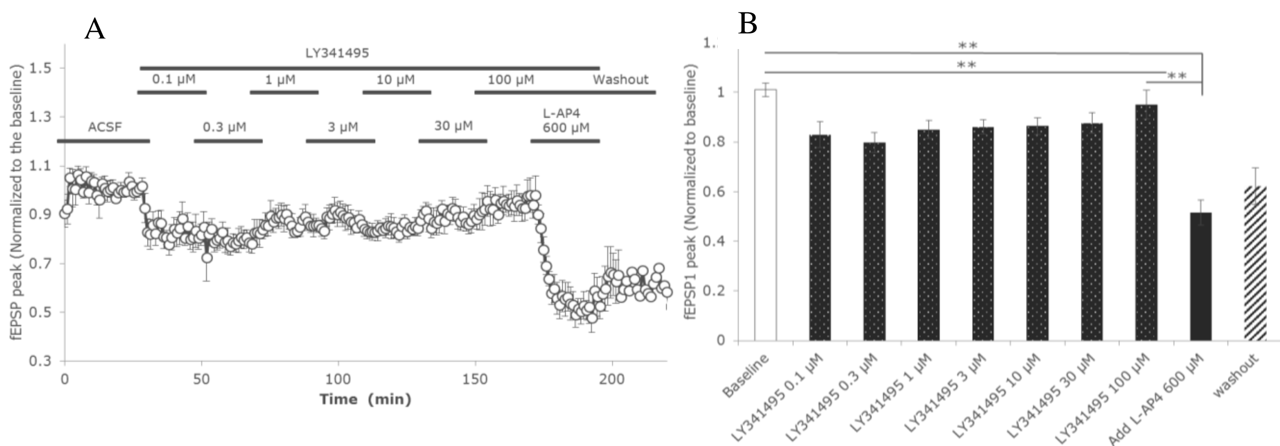

Figure **22 Effect of the antagonist LY341495 over fEPSP recorded in the hippocampal SC-CA1 synapses in adult animals**. A. The compound significantly reduced synaptic transmission at all concentrations tested and did not seem to reverse the depressing effects of L-AP4 at the concentration used. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of increasing concentrations of LY341495, and the addiction of 600 µM to the last concentration of the antagonist used, over the fEPSPs.LY341495 (0.3 µM), 0.80±0.04, p<0.01; LY341495 (100 µM), 0.95±0.05, p<0.01; LY341495 (100 µM) and L-AP4 (600 µM), 0.52±0.06, p<0.01 to baseline and LY341495 at 100 µM. A total of 6 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student’s t test.

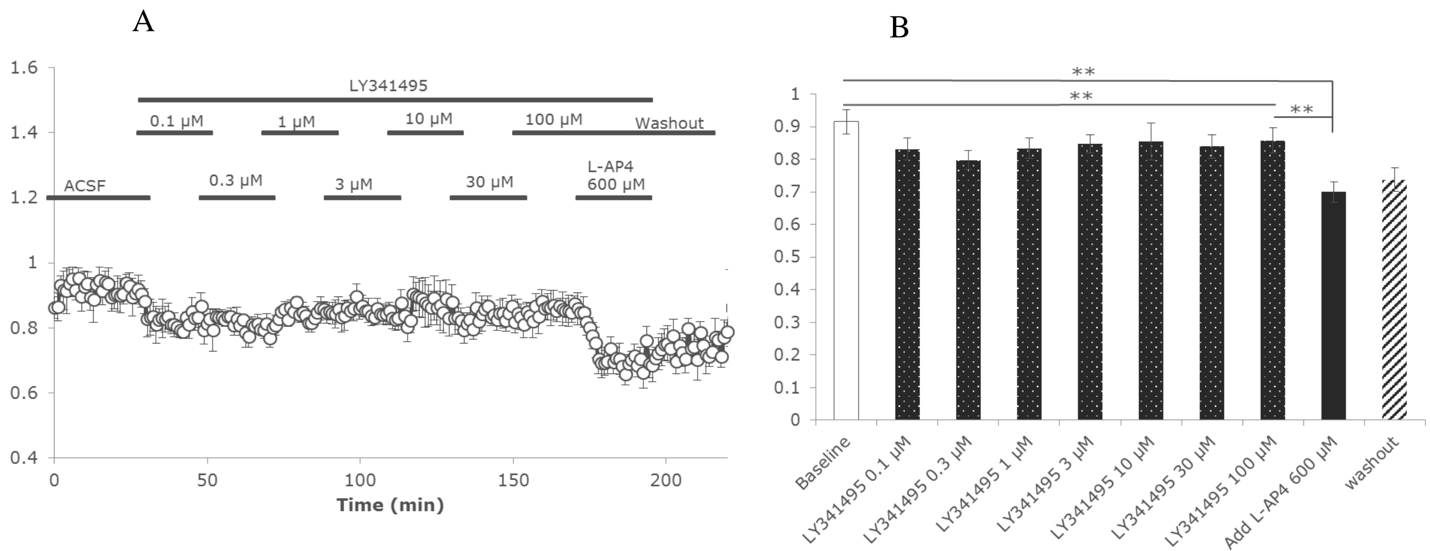

Figure 23 **Effect of the antagonist LY341495 over PPRof paired pulses fEPSP recorded in the hippocampal SC-CA1 synapses of immature rats.** A. The agonist significantly decreased PPR at all concentrations used and could revert the reduction predicted to happen with L-AP4. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. This suggesting that the compounds are acting on receptors presynaptically located. B. Bar graph shows the averaged effect of L-AP4 over the fEPSPs. Baseline, 0.91±0.04; LY341495 (0.3 µM), 0.80±0.03, p<0.01; LY341495 (100 µM), 0.86±0.04, p<0.01; LY341495 (100 µM) and L-AP4 (600 µM), 0.70±0.03, p<0.01 to baseline and LY341495 at 100 µM.A total of 6 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

14.6 Effects of NAMs (MMPIP and ADX71743), antagonist (LY341495) and agonist/PAM (AMN082) preincubation over the influence of L-AP4 in adult animals CA1 area and DGMPP basal synaptic transmission and PPR

As already stated, the lack of selective pharmacological compounds has limited the study of mGlu7 function in the CNS. MMPIP, ADX71743 and AMN082 are recent compounds stated as selectively allosteric modulators when tested in diverse models.[18, 110, 120, 165-167]

It is therefore of interest to test them against the effects of L-AP4 in the CA1 and DG MPP of adult rats. It was predicted that both NAMs would attenuate the depressing effect of L-AP4, while for AMN082, it could either potentiate the effect of L-AP4 acting as a PAM or due to its induction of receptor internalization and desensitization act as a NAM. Nevertheless it should be focused that the compounds present a rich and yet uncharacterized pharmacology with various potential unknown target and the possibility that their metabolites

14.6.1 In SC-CA1 synapses of hippocampus of adult animals

14.6.1.1 NAM – MMPIP

This compound did not had an effect by itself over basal synaptic transmission has demonstrated by the lack of change in the peak amplitude (see Figure 24) and PPR (see Figure 25). Surprisingly this compound considered to be a NAM led an apparent increase of the effects of the agonist L-AP4 (see Figure 24).

When both compounds were present the variation on the PPR made it unfeasible for analysis and no conclusion can or should be drawn from it. Repeating the experience could be helpful to evaluate any change possible present.

A non-complete washout of the compounds was present.

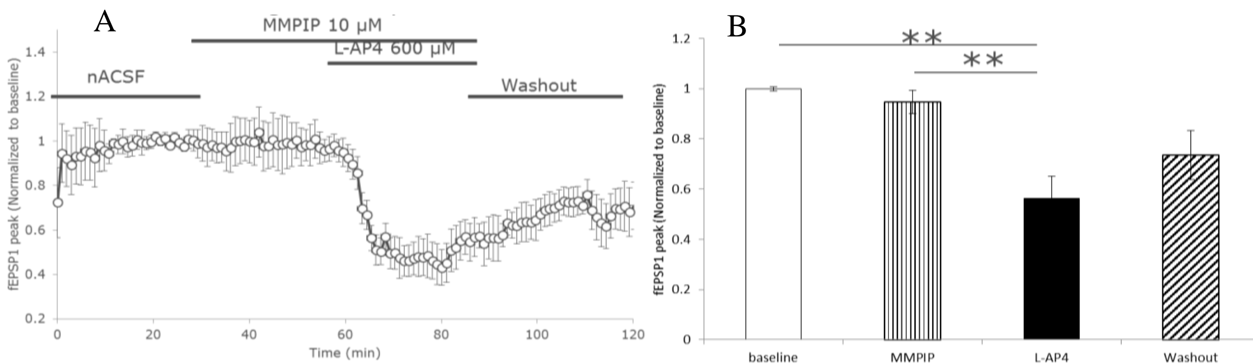

Figure 24 **Effect of preincubation with the NAM MMPIP over the expected depression of fEPSP recorded in the hippocampal SC-CA1 synapses in adult animals.** A. The compound did not significantly modify basal synaptic transmission by itself. This concentration of the compound did not as well reverse the depressing effects of L-AP4.fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of 10 µM of MMPIP and the addiction of 600 µM of L-AP4. MMPIP (10 µM), 0.95±0.05, p>0.05; MMPIP (10 µM) and L-AP4 (600 µM), 0.56±0.09, p<0.01.A total of 7 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student’s t test.

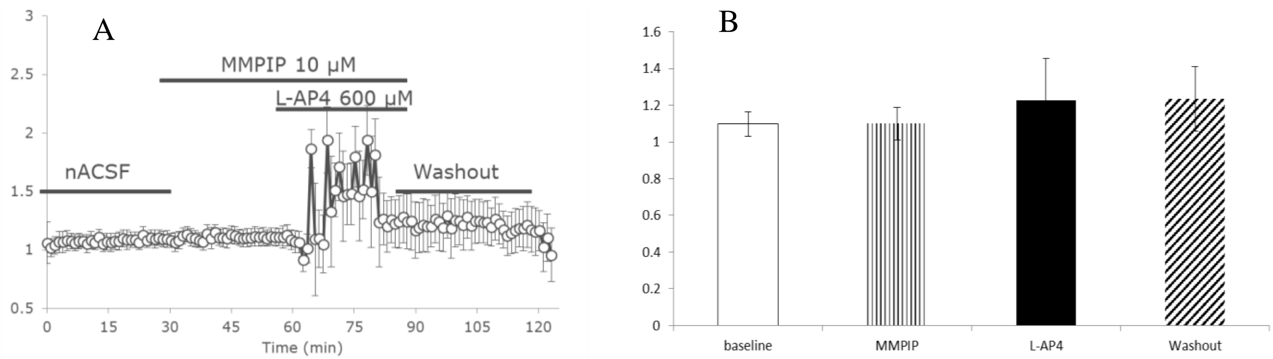

Figure 25 **Effect of preincubation with the NAM MMPIP over the expected alteration of PPR induced by L-AP4 recorded in the hippocampal SC-CA1 synapses in adult animals.** A. The compound did not significantly modify PPR by itself but unusual modifications of the PPR during the incubation with L-AP4 were presented and led to a non significative alteration when compared to the baseline. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of 10 µM of MMPIP and the addiction of 600 µM of L-AP4. Baseline, 1.1±0.07; MMPIP (10 µM), 1.1±0.09, p>0.05; MMPIP (10 µM) and L-AP4 (600 µM), 0.22±0.09, p>0.05.A total of 7 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

14.6.1.2 NAM – ADX71743

This compound did not had an effect by itself over basal synaptic transmission has demonstrated by the lack of change in the peak amplitude (see Figure 26) and PPR (see Figure 27).

ADX71743 was able to reduce the effect of L-AP4 to levels at which the decrease ceases to be significative (see Figure 26) and there is no alteration on the PPR (see Figure 27). Nonetheless it should be focused that the p values are close to a minimum to significance.

A washout of the small effect that L-AP4 had was not present, it may be due to an interaction of the compounds or a conditioning by their presence, but it should be noticed that there is no significant chance of both peal amplitude and PPR during all the experiment.

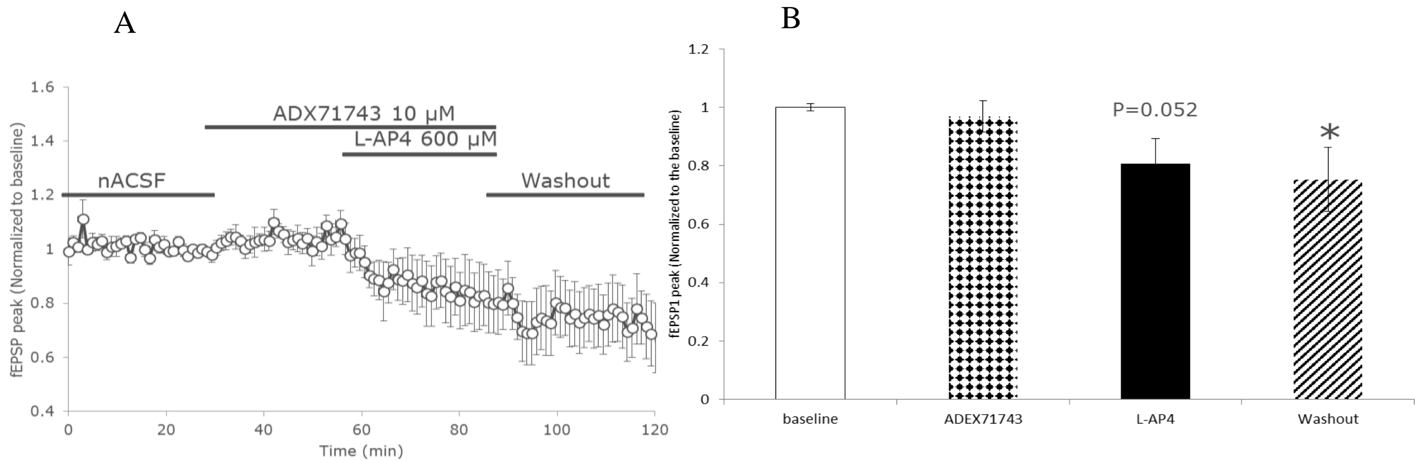

Figure 26 **Effect of preincubation with the NAM ADX71743 over the expected depression of fEPSP recorded in the hippocampal SC-CA1 synapses in adult animals.** A. The compound did not significantly modify basal synaptic transmission by itself. However the excepted effect of L-AP4 was not as pronounced leading to a non-significant change. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of 10 µM of ADX71743 and the addiction of 600 µM of L-AP4. ADX71743 (10 µM), 0.96±0.05, p>0.05; ADX71743 (10 µM) and L-AP4 (600 µM), 0.80±0.09, p>0.05. A total of 9 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

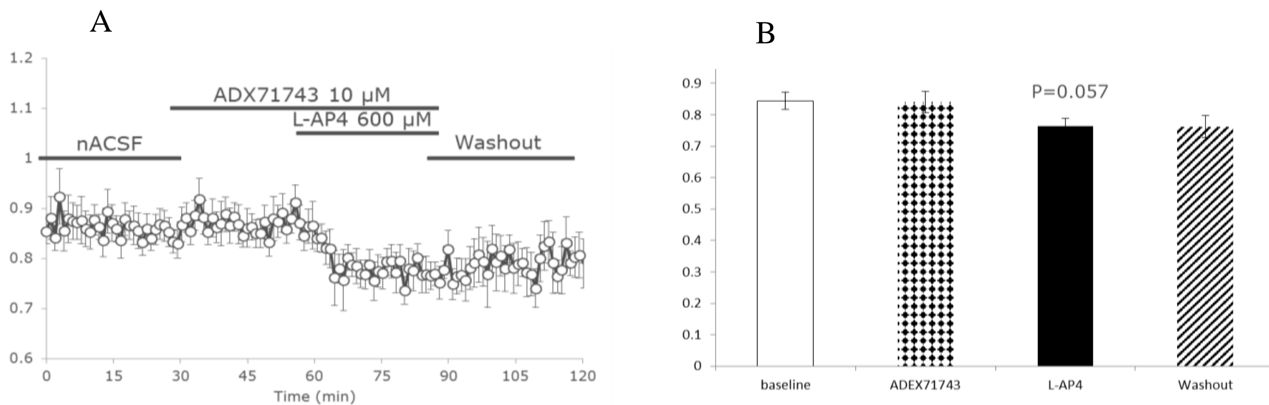

Figure 27 **Effect of preincubation with the NAM ADX71743over the expected alteration of PPR induced by L-AP4 recorded in the hippocampal SC-CA1 synapses in adult animals**. A. The compound did not significantly modify PPR by itself but unusual modifications of the PPR during the incubation with L-AP4 were presented and led to a non significative alteration when compared to the baseline. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of 10 µM of ADX71743 and the addiction of 600 µM of L-AP4. Baseline, 0.85±0.02; ADX71743 (10 µM), 0.84±0.03, p>0.05; ADX71743 (10 µM) and L-AP4 (600 µM), 0.76±0.03, p>0.05.A total of 7 slices from 4 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student's t test.

14.6.1.3 Selective agonist/PAM - AMN082

This compound did not had an effect by itself over basal synaptic transmission has demonstrated by the lack of change in the peak amplitude (see Figure 28) and PPR (see Figure 29).

The compound did not change the predicted effects of L-AP4 regarding the alteration of peak amplitude (see Figure 28) but it did alter the PPR change induced by the non-selective agonist. This slight alteration of PPR (see Figure 29) may indicate a preconditioning of the receptor due to the presence of the NAM. Other fact may explain this small difference is that AMN082 may modify some of the off-targets of L-AP4 and therefore somewhat alter the final response induced by the last one.

A non-complete washout of the compounds was present however there was considerable variability of the results.

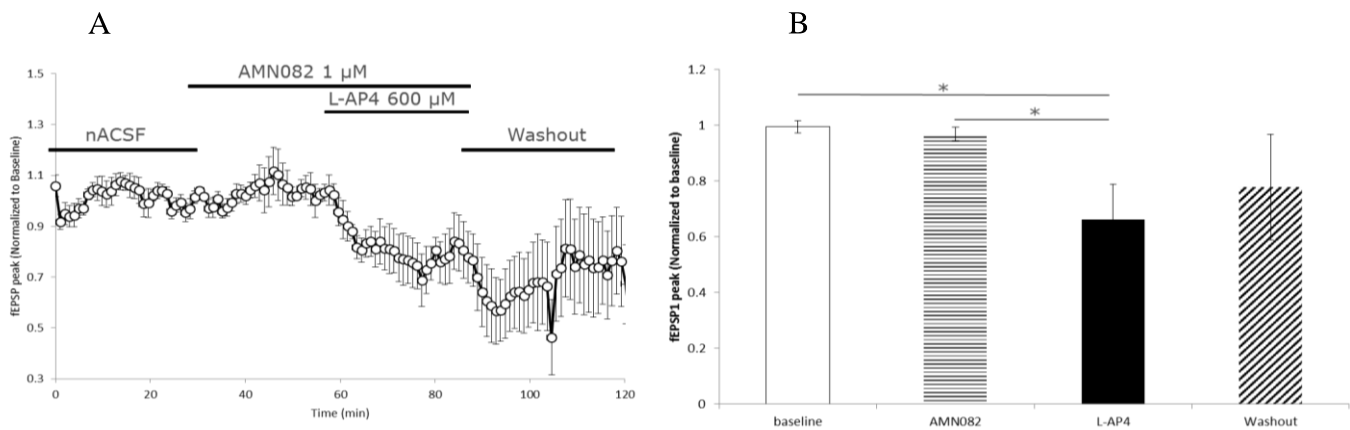

Figure 28 **Effect of preincubation with the PAM AMN082 over the expected depression of fEPSP recorded in the hippocampal SC-CA1 synapses in adult animals.** A. The compound did not significantly modify basal synaptic transmission by itself neither alter the effect of L-AP4 since this one is similar to a situation with no preincubation of the NAM. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of 1 μM of AMN082 and the addiction of 600 μM of L-AP4. AMN082 (1 μM), 0.97±0.02, $p>0.05$; AMN082 (1 μM) and L-AP4 (600 μM), 0.66±0.1, $p<0.05$.A total of 7 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*$p<0.05$, **$p<0.01$, two-tailed Student's t test.

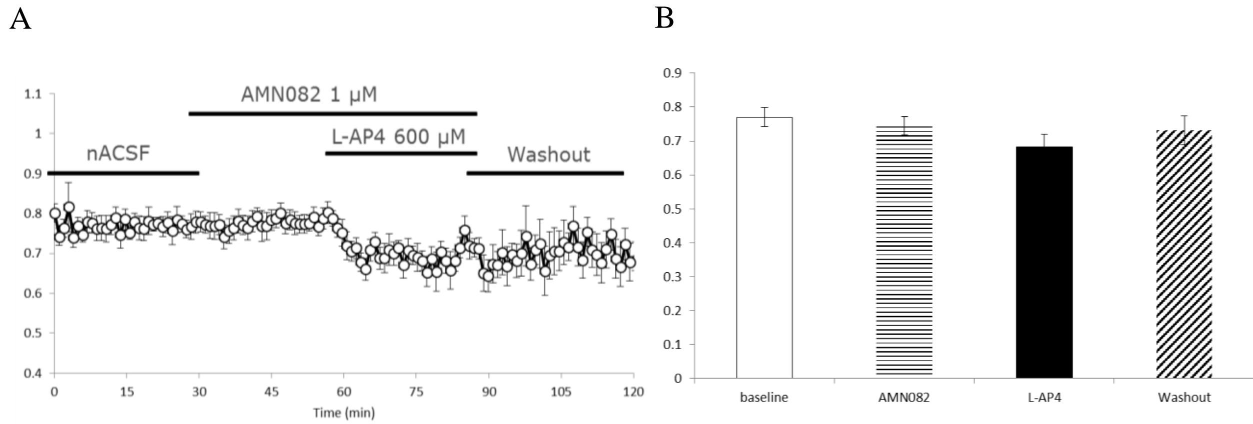

Figure 29 **Effect of preincubation with the PAM AMN082 over the expected alteration of PPR induced by L-AP4 recorded in the hippocampal SC-CA1 synapses in adult animals.** A. The compound did not significantly modify PPR by itself. In the presence of both compound no significative change in PPR was detected only a tendency to decreased. fEPSP were elicited by stimulating near the CA3 area for stimulation of the SC. B. Bar graph shows the averaged effect of 10 µM of MMPIP and the addiction of 600 µM of L-AP4. Baseline, 0.77; AMN082 (1 µM), 0.74±0.02, p>0.05; AMN082 (1 µM) and L-AP4 (600 µM), 0.68±0.04, p>0.05.A total of 7 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01, two-tailed Student’s t test.

14.6.2 In DG MPP-granule cells synapses of the hippocampus of adult animals

As in CA1 the effects of the compound were tested in the DG MPP. Plotted simultaneously with the condition in which a pre-incubation with a compound is done before adding L-AP4 at 600 µM to the solution is an experiment in which no compound is added but L-AP4 to the circulating solution. The first situation is represented by the filled circles with the designated compound and its concentration indicated. The second is represented with open circles and the period of time correspondent to the pre-incubation period is blanked out for facilitating the observation of effect by the first compound itself. For an easier visualization of the effects the results are plotted in the end of this section.

In 14.6.2.1 the compound did not had an effect by itself over basal synaptic transmission has demonstrated by the lack of change in the peak amplitude and PPR (see Figure 30). The antagonist did prevented the effects of L-AP4 in this synapses by maintaining both peak amplitude and PPR with the same levels than baseline. See Figure 30

14.6.2.1 Potent and orthosteric antagonist- LY341495

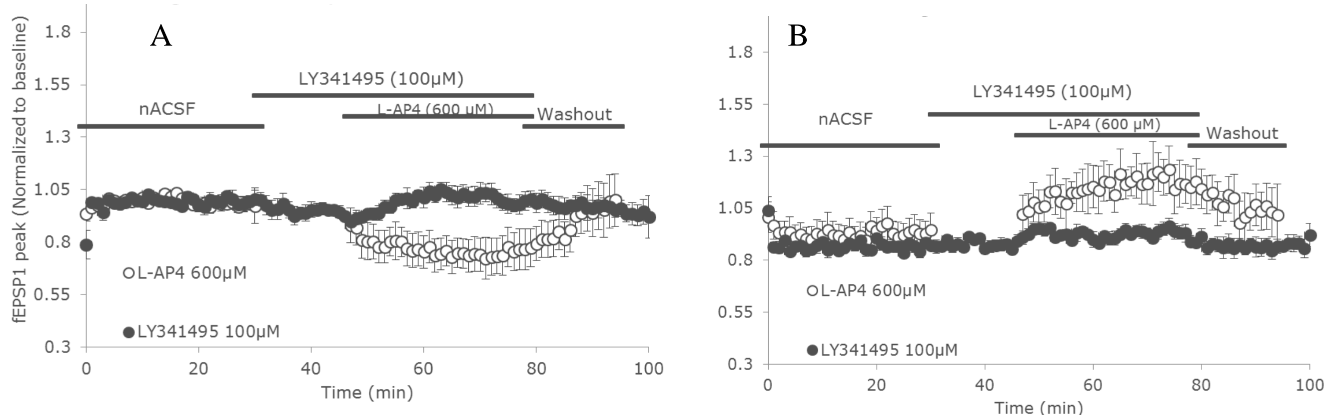

Figure 30 **Effect of preincubation with the antagonist LY341495 over the expected depression of fEPSP caused by L-AP4 recorded in the hippocampal DG MPP-granule cells synapses in adult animals.** A. The compound by itself did significantly alter basal synaptic plasticity but blocked the expected effect of L-AP4 maintain the signal at baseline level. B. The same occurred with PPR with no significant change detected even in the presence of L-AP4. fEPSP were elicited by stimulating near the granular layer of the DG. A total of 7 slices from 3 animals were averaged.

14.6.2.2 mGlu7 selective PAM - AMN082

The compound did not had an effect by itself over basal synaptic transmission nor has it changed the effects of L-AP4 on this synapses has demonstrated by the lack of change in the peak amplitude and PPR (see Figure 31).

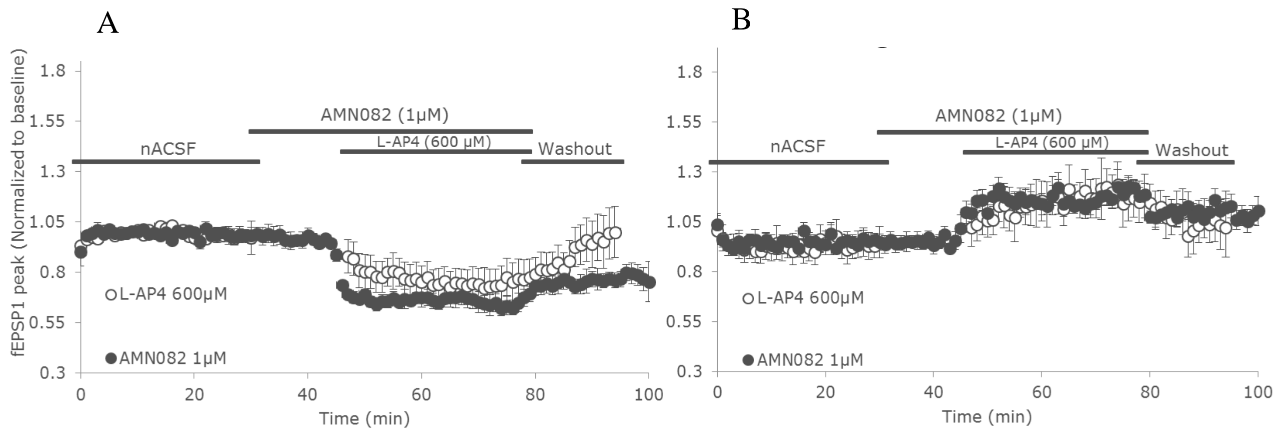

Figure 31 **Effect of preincubation with the mGlu7 selective PAM AMN082 over the expected depression of fEPSP caused by L-AP4 recorded in the hippocampal DG MPP-granule cells synapses in adult animals.** A.The compound by itself did significantly alter basal synaptic plasticity neither blocked the expected effect of L-AP4. B. The same occurred with PPR with no significant change detected with AMN082 and an increase of the ratio after the addiction of L-AP4. fEPSP were elicited by stimulating near the granular layer of the DG. A total of 7 slices from 3 animals were averaged.

14.6.2.3 mGlu7 selective NAM - ADX71743

In 14.6.2.3 the compound did not had an effect by itself over basal synaptic transmission nor has it changed the effects of L-AP4 on this synapses has demonstrated by the lack of change in the peak amplitude and PPR (see Figure 32).

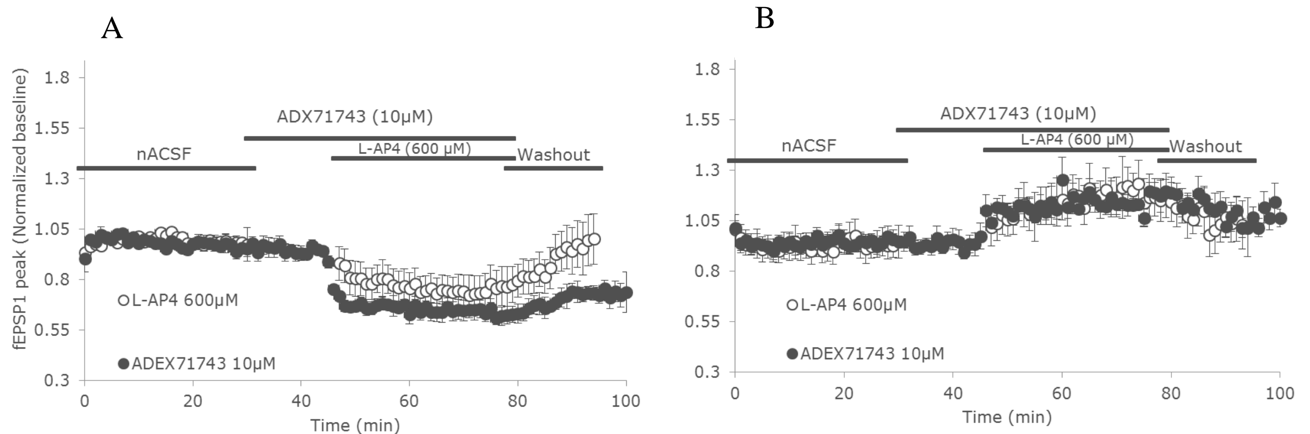

Figure 32 **Effect of preincubation with the mGlu7 selective NAM ADX71743 over the expected depression of fEPSP caused by L-AP4 recorded in the hippocampal DG MPP-granule cells synapses in adult animals.** A. The compound by itself did significantly alter basal synaptic plasticity neither blocked the expected effect of L-AP4. B. The same occurred with PPR with no significant change detected with ADX71743 and an increase of the ratio after the addiction of L-AP4. fEPSP were elicited by stimulating near the granular layer of the DG. A total of 7 slices from 3 animals were averaged.

14.6.2.4 mGlu7 selective NAM – MMPIP

In 14.6.2.4 the compound did not had an effect by itself over basal synaptic transmission has demonstrated by the lack of change in the peak amplitude and PPR (see Figure 33). It did, as in the SC-CA1 synapses (see Figure 24), and contrary to expected, lead to an enhancement of the effect of L-AP4 nonetheless this time with a clear PPR that was similar to the one with L-AP4 alone (see Figure 33 non filled circles) but contrasting to the previous experiments (see Figure 19). The washout was not evident indicating a long term modification of the transmission on the fibers.

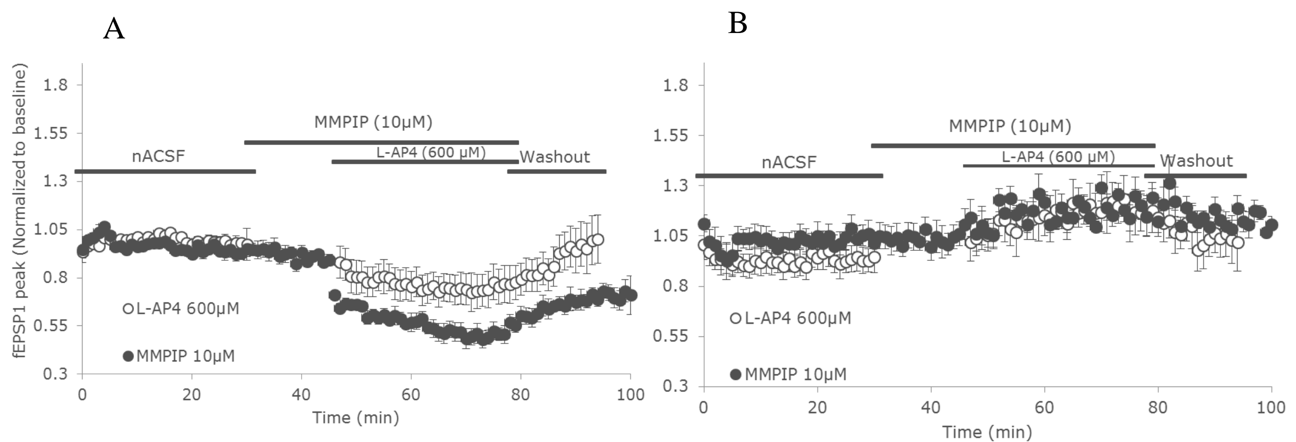

Figure 33 **Effect of preincubation with the mGlu7 selective NAM MMPIP over the expected depression of fEPSP caused by L-AP4 recorded in the hippocampal DG MPP-granule cells synapses in adult animals**. A. The compound by itself did significantly alter basal synaptic plasticity. Contrary to expected the compound lead to a further decrease in the signal depression caused by L-AP4. B. A similar PPR was present but it did not reach significance. fEPSPs were elicited by stimulating near the granular layer of the DG. A total of 7 slices from 3 animals were averaged.

PAMs and NAMs ?

Although mGluR agonists can differentially activate receptors between groups, most compounds do not discriminate for specific receptor subtypes within groups. To study the mGlu7 receptor subtype in rats hippocampal slices, the actions of the selective group III agonist, L-AP4 (600 µM) were measured and without and with the preincubation and presence of antagonists, PAMs and NAMs, stated as selective in the literature, of the receptor.

In the CA1 area of adult animals none of the compound tested significantly altered the effect of L-AP4. It should be mentioned that the PPR measurement in the situation of preincubation and presence MMPIP is far from what would be expected and may indicated lack of viability in some slices and should not be taken in account. Also noticeable is the lack of a clear washout in the experiments in which ADX71743 is used, that may be due to chemical characteristics of the compound interacting with the components of the setup. Other possible explanation is that the combination of both ADX71743 and L-AP4 could induce a strong modification of the slices that was not revertible or could even be toxic and led to cell death.

See Figure 34

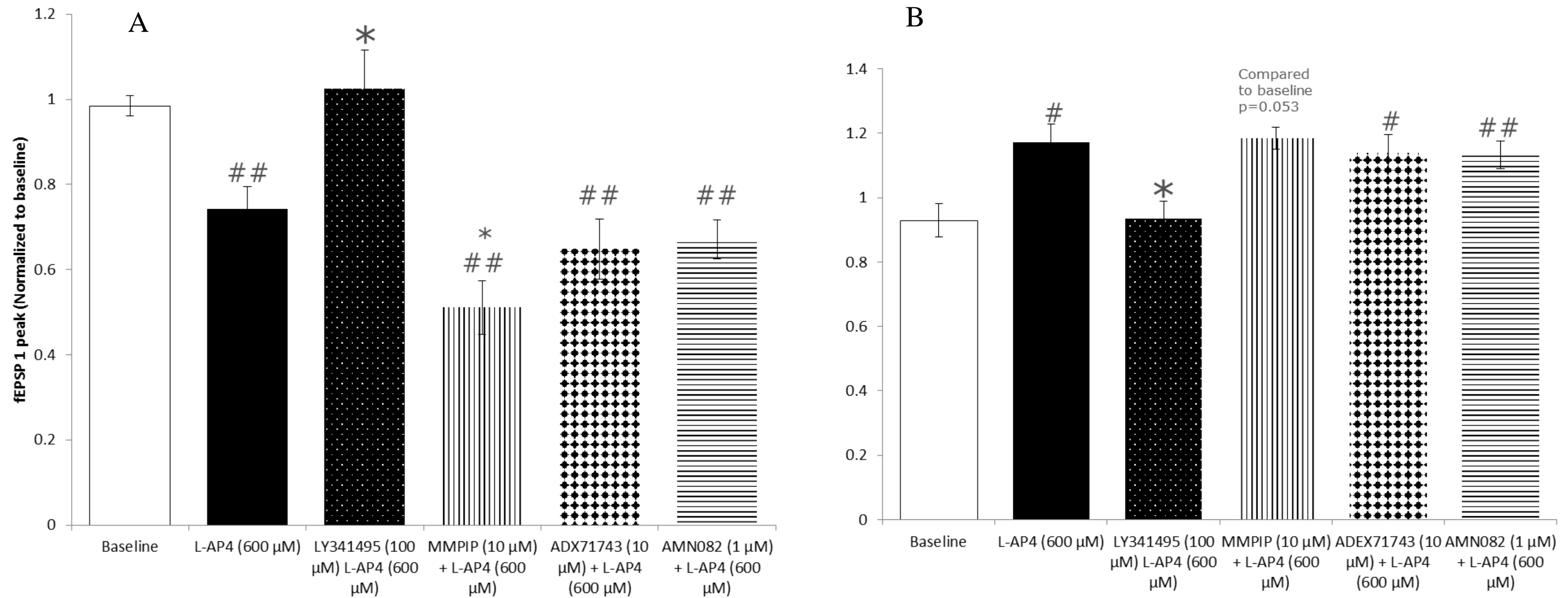

Figure 34 A. Bar graphs showing the averaged effect over fEPSP1 peak (normalized to baseline) and B. PPR of the different compounds preincubation over L-AP4 effects. Recordings in the hippocampal DG MPP-granule cells synapses of adult animals. The depressing effect of L-AP4 is reversed by preincubating the slices with 100 μM of LY341495 accompanied by a non-alteration of the PPR. When a preincubation with 10 μM of the mGlu7 NAM is done the effects of L-AP4 over synaptic transmission seem augmented with a higher decrease of the peak value but is accompanied of a non significative alteration of the PPR when compared to baseline value (however a tendency to increase is observed). Data (Mean ± S.E.M.) was normalized to the baseline.*$p<0.05$, **$p<0.01$ compared to depressed level of L-AP4 (600 μM); # $p<0.05$, ##$p<0.01$ compared to baseline, two-tailed Student's t test.

Values present in A: L-AP4, 0.68±0.08; LY341495 plus L-AP4, 0.97±0.05; MMPIP plus L-AP4, 0.51±0.06; ADX71743 plus L-AP4, 0.64±0.07; AMN082 plus L-AP4, 0.67±0.05.

Values present in B: L-AP4, 1.17±0.06; LY341495 plus L-AP4, 0.93±0.06; MMPIP plus L-AP4, 1.14.±0.05; ADX71743 plus L-AP4, 1.2±0.03; AMN082 plus L-AP4, 0.95±0.03.

14.7 Spontaneous and NMDA induced spiking activity modulation by mGlu7 receptor

mGlu7 is considered, due to its characteristics, a low pass filter[50, 168] and so could serve as part of a gating mechanism that would not allow extensive release of glutamate. The laminar organization of the hippocampus allows information transmission between its areas in easy to understand manner that can be quantified.

L-AP4 at a concentration of 400 µM induces a significant decrease in the frequency but not amplitude of sEPSCs. Simultaneously a role of the receptor involvement in the frequency-dependent regulation of glutamate release has been proposed.[169]

So measurement of spontaneous spiking and chemically evoked spiking could allow an evaluation of the potential influence of mGlu7 on spatiotemporally coordinated activity in the hippocampal neural network. It should be kept in mind that the receptor does as well regulate the release of GABA and the inhibitory network of adult animals should not be forgotten.[97]

In the absence of electrical stimulation spiking activity of hippocampal brain slices from adult animals was measure using MEA. Spiking activity of the CA1 area and DG of the hippocampus were measured indiscriminately.

The line graphs show the average total spike occurred in each minute in each condition while the graph bar in B show the average of total spikes in each condition. The graph bar in C show the normalized to baseline average of event in each condition. One point to remember is the variability between slices of even the same animals of the number of total spontaneous events.

14.7.1 Effect of the agonist L-AP4 (600 µM) on spiking activity on adult rat hippocampus

In 0 L-AP4 has a negative effect over the number of spontaneous events with a 94.6%± 12 of baseline levels. See Figure 35.

Washout of the effect was not present and it could be an indication that either the compound induces a long term silencing of spontaneous activity or that the quality of the slices was compromised and therefore spontaneous activity was lost over time.

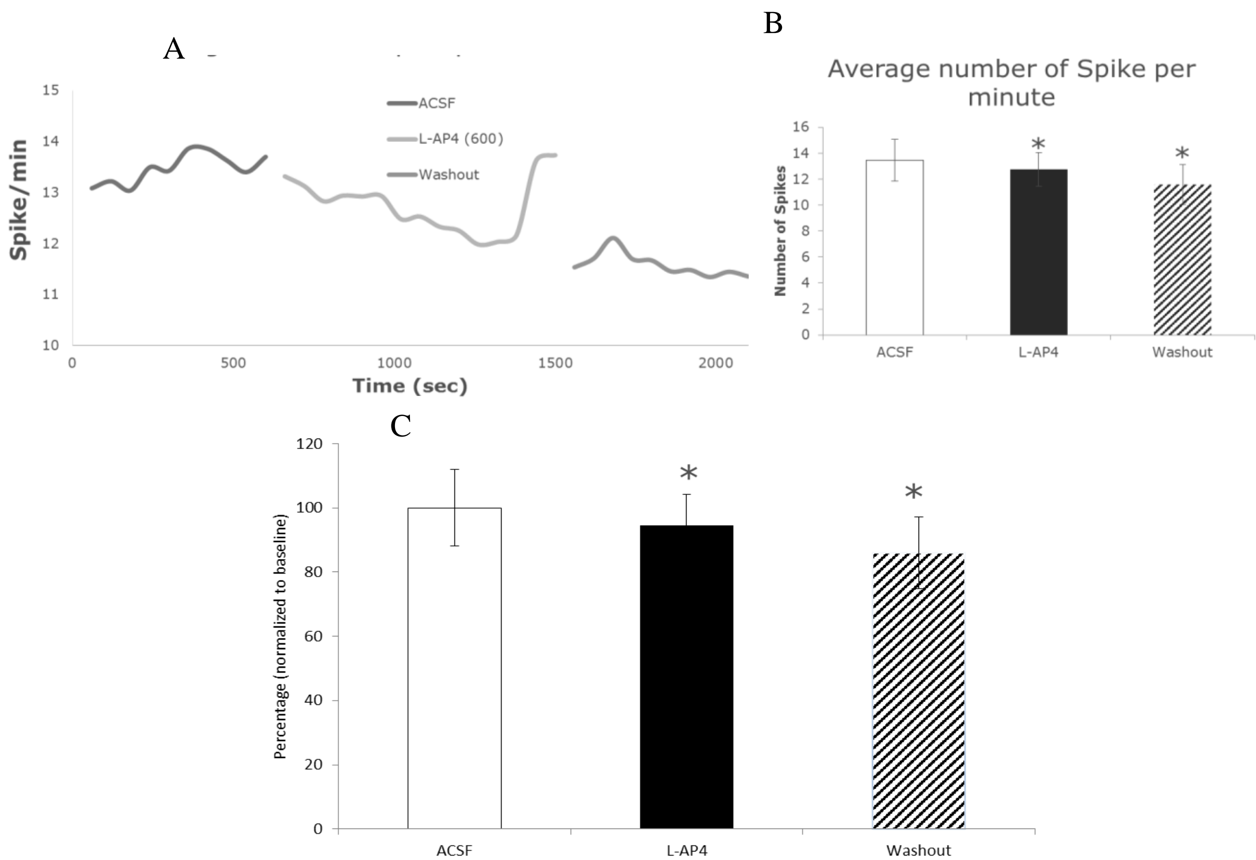

Figure 35 **The agonist L-AP4 at 600 µM lead to a significant reduction in the number of spikes registered by minute.** A. Representation of average number of spikes per minute. B. Bar graph showing the values of average number of spikes per minute in each situation. C. Percentage normalized to baseline of the average number of spikes per minute. A total of 8 slices from 4 animals were averaged. L-AP4, 94.6%± 12. Data (Mean ± S.E.M.) was normalized to the baseline or as a percentage of that normalization.*p<0.05, **p<0.01, two-tailed Student’s t test.

14.7.1.1 Effect of the agonist L-AP4 (600 µM) over 50 µM NMDA induced Spiking

At 50 µM NMDA induced an evident and consistent increase in activity in adult brain slices and this was greatly increased by the addition of L-AP4 at working concentration (see Figure 36) demonstrating an inverse effect than the one presented when L-AP4 is added without the presence of NMDA (see Figure 35). This may indicate that the targets of L-AP4 either differ in the two situations or that depending on the activity being spontaneous or induced (application of NMDA) the receptor(s) affected may have opposite effects. A clear washout of the effects of both NMDA and L-A4 was present even though it did not reached baseline level.

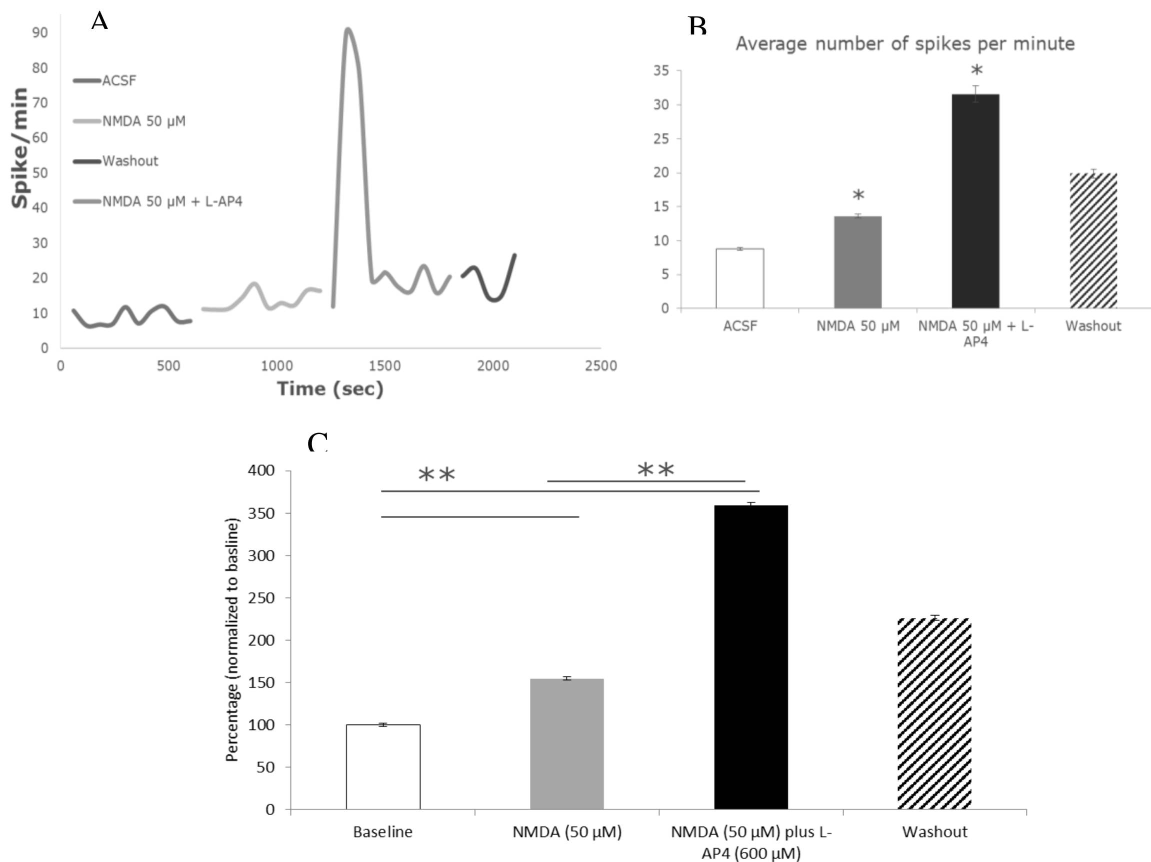

Figure 36 **NMDA (50 µM) led to an increase of the average number of spikes per minute in adult rat hippocampus.** A. Representation of average number of spikes per minute. B. Bar graph showing the values of average number of spikes per minute in each situation. C. Percentage normalized to baseline of the average number of spikes per minute.L-AP4 at 600 µM led to a significant increase in the number of spikes by minute induced by this concentration of NMDA. NMDA 50 µM, 154.8%± 2; NMDA 50 µM and L-AP4 600 µM, 358.9%± 3.8. A total of 15 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline or as a percentage of that normalization.*p<0.05, **p<0.01, two-tailed Student's t test.

14.7.1.2 Influence of the antagonist LY341495 (100 µM) on the effect of agonist L-AP4 (600 µM) over 50 µM NMDA induced Spiking

Single application of 600 µM of L-AP4 led to a reduction of spontaneous spiking activity with no evident washout of this effect. The short time application of the agonist (300 sec) should be enough for the activation of mGlu7 and not inducing a strong internalization/ desensitization of the receptor (internalization studies as an antibody feeding assay should have been realized to confirm this assumption). The hypothesized activation of the receptor should have led to a reduction of glutamate release that created the decrease in spontaneous spiking. See Figure 35.

Application of L-AP4 after or before high concentration of NMDA (1 mM) led to an increase in spiking activity (not shown). Even with lower concentrations (50 µM of NMDA) L-AP4 was able to increase spiking activity induced by NMDA in an accentuated way. See Figure 36. This effect was not prevented by the preincubation and presence of the antagonist LY341495 at working concentration. In this last experiment the antagonist itself led to an increase of the spiking activity that when ompared to the effects of L-AP4 is small but should not be overviewed. See Figure 37.

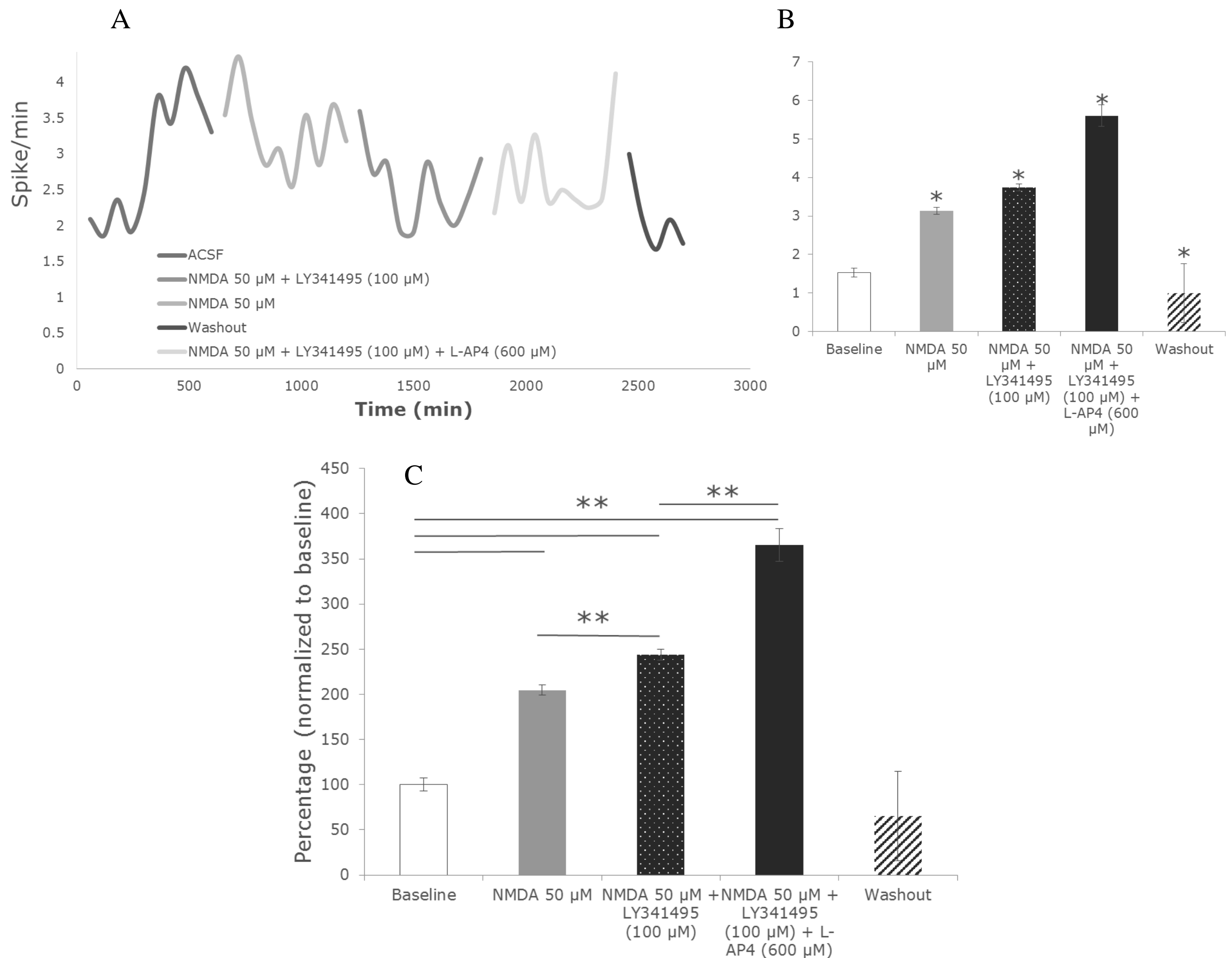

Figure 37 **NMDA (50 µM) led to an increase of the average number of spikes per minute in adult rat hippocampus.** A. Representation of average number of spikes per minute. B. Bar graph showing the values of average number of spikes per minute in each situation. C. Percentage normalized to baseline of the average number of spikes per minute. The antagonist LY341495 L-AP4 at 600 µM led to a significant increase in the number of spikes by minute induced by this concentration of NMDA.NMDA 50 µM, 204.5%± 8; NMDA 50 µM and LY341495 100 µM, 243.8%± 8;NMDA 50 µM and LY341495 100 µM and L-AP4 600 µM, 365.3%± 20 A total of 15 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline or as a percentage of that normalization.*p<0.05, **p<0.01, two-tailed Student's t test.

14.8 Effect of noncompetitive antagonist of $GABA_A$ receptor Picrotoxin (PTX (10 μM)) over L-AP4 (600 μM) induced alterations in adult CA1 area of the hippocampus

mGlu7 receptor is known to act on glutamatergic and GABAergic signaling modulating synaptic transmission. Simultaneously a role in disinhibition has been demonstrated in various brain regions either by direct or indirect mechanisms. mGlu7 heteroreceptors activation have been shown to inhibit GABA exocytosis being negatively coupled to adenyl cyclase and do not cross-talk with pre-synaptic $GABA_B$ autoreceptors.[97] mGlu7 immunoreactivity is mainly restricted to GABAergic inputs of interneurons that receive innervation from mGlu7-enriched glutamatergic terminals.[98] The receptor has been shown to act on postsynaptic targets such as suppressing $GABA_A$ receptor-mediated postsynaptic response. Therefore the activation of the receptor in a situation where $GABA_A$ is inhibited would allow an evaluation of direct effect on the glutamatergic and/or GABAergic systems. See Figure 38.

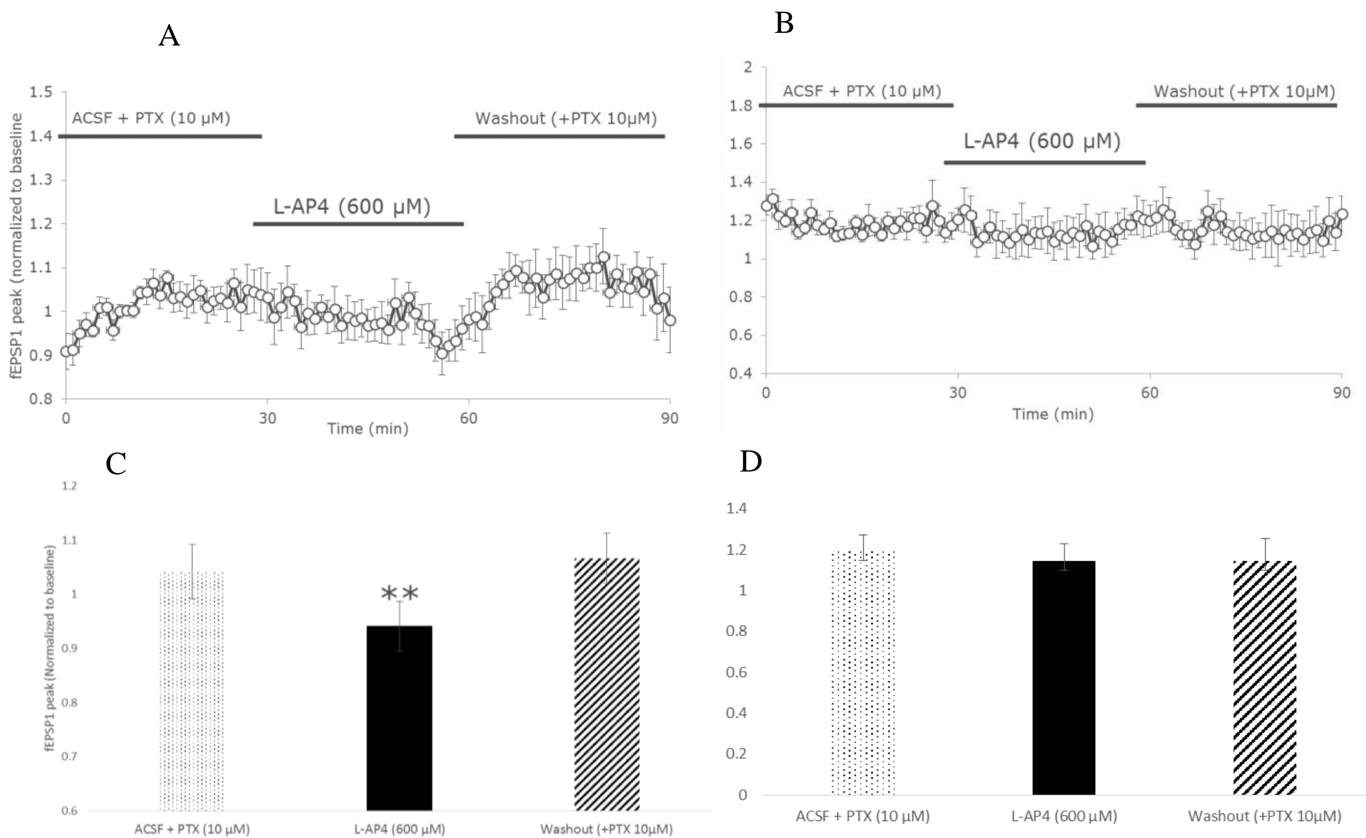

Figure 38 **Effect of L-AP4 over fEPSP and PPR of paired pulses fEPSP recorded in SC-CA1 synapses of the hippocampus of adult animals.** A.L-AP4 significantly decreased fEPSP1 but not in the same degree as in the absence of picrotoxin (0.94±0.04 – with PTX vs 0.74±0.1 –without PTX of baseline value, $p<0.01$).B. In the presence of 10 μM PTX L-AP4 did not led to an alteration of the PPR. C and D. Bar graph shows the averaged effect of L-AP4 over the fEPSP and PPR.A total of 7 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*$p<0.05$, **$p<0.01$, two-tailed Student's t test.

The presence of PTX diminishes the effects of L-AP4 revealing a potential target of the compound

The initial objective of this experiment was to isolate the different components possibly involved in the depression of fEPSP by L-AP4. As the hypothetical target of the working concentration of L-AP4, mGlu7 can act at both glutamatergic and GABAergic terminals.

Surprisingly when the $GABA_A$ receptor was inhibited using PTX the effect L-AP4, even though still present, was not as prominent as when the agonist is applied alone. See Figure 38. This may indicated that either L-AP4 may be acting directly as a GABAergic agonist or the prolonged presence of L-AP4 could have led to an internalization of mGlu7 leading to an increase/disinhibition of GABA release. It should be focused that in the presence of PTX there was no change in the PPR when L-AP4 was added. This could indicate a possible postsynaptic action of L-AP4 when alone which may indicate a different target than mGlu7 since this receptor is mainly presynaptic.

14.9 NMDAR mediated synaptic potential in adult CA1 area modulation by increasing concentrations of the agonist L-AP4

Using a similar procedure as Weitlauf et al[170] and concepts used by Coan and Collingridge[171] in which NMDAR-mediated synaptic responses can be isolated as a CNQX-resistant component of evoked potential in the absence of extracellular Mg^{2+}[172], the effect of activating mGlu7 were tested.

NMDAR mediated responses were isolated in an Mg^{2+}free medium in the presence of picrotoxin (25 µM) and CNQX (50 µM) to block $GABA_A$R-mediated and AMPAR-mediated signaling, respectively. Glycine (10 µM), a NMDAR coagonist/allosteric potentiator, was likewise added to facilitate the activation of NMDARs since it was demonstrated to markedly potentiated the effect of NMDA on these receptors.[173]

The signal obtained after the removal of magnesium and the pharmacological blockade of GABAR and AMPAR demonstrated secondary population spikes appearing after a primary population spike, as described by Abe et al.[172] Even though this first population spike is usually insensitive to APV, suggesting it is not directly mediated by an NMDAR component[171] using the described protocol, it has demonstrated that this peak is sensitivity to PCP (30 µM), an activity dependent antagonist. See Figure 39 and Figure 40.

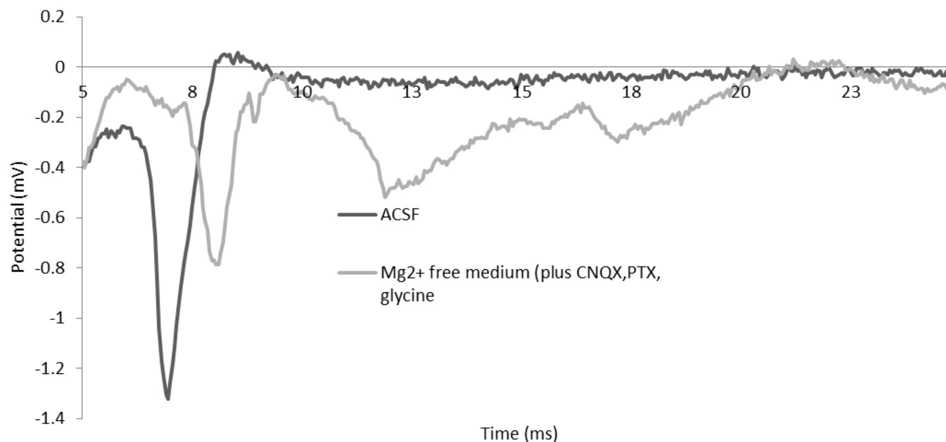

Figure 39 **Representative somatic population spike from adult CA1 area traces from one slice from one animal**. The traces demonstrating differences between a typical AMPAR mediated response in ACSF and the response when the medium is changed so a NMDAR response is isolated. The most evident differences when compared to the typical population spike include a decrease in the signal amplitude and an increase of the area under the curve. The signal is also accompanied by an increase number of population spikes that have an irregular frequency.

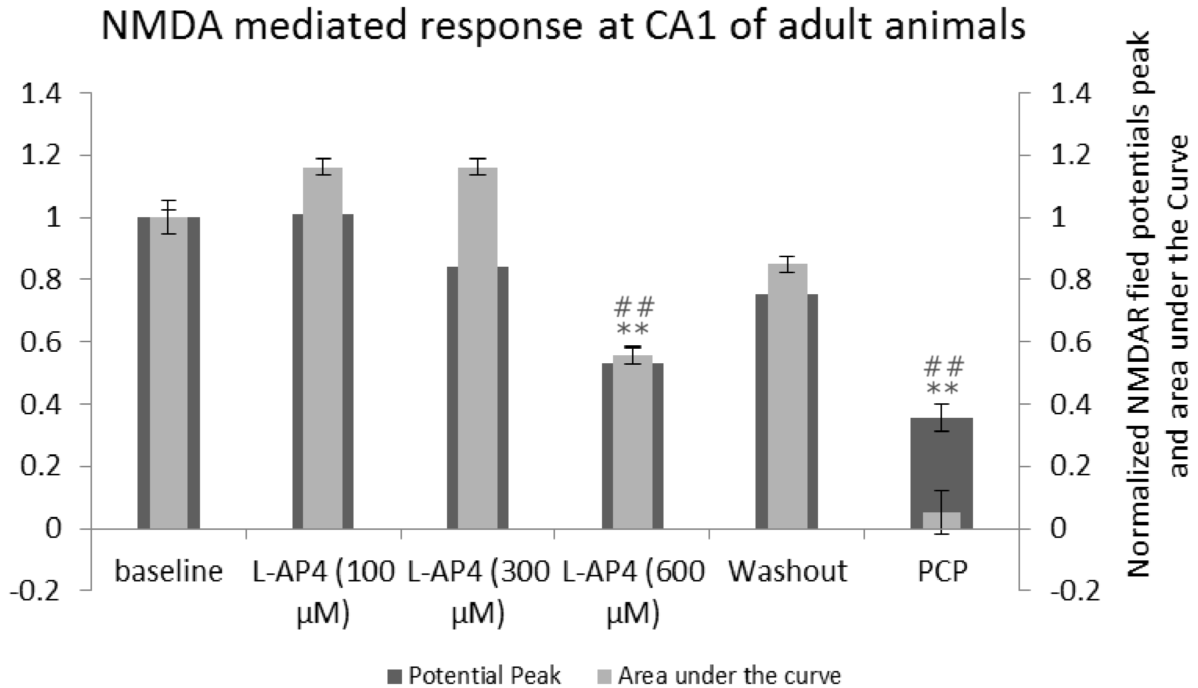

Figure 40 **Increasing concentrations of L-AP4 over NMDAR mediated potential in recorded in the hippocampal CA1 area of adult animals.** L-AP4 at a concentration of 600 µM significantly reduces both the values for the peak and area under the curve indicating that the compound is modulating a NMDAR-dependent response. The antagonist PCP reduced with significance as well values for the peak and area under the curve, demonstrating the possible NMDAR origin of the potential and characteristics measured. Bar graph shows the averaged effect of the compound over the peak of the NMDA mediated potential and area under the curve from the signal. Peak value: L-AP4 600 µM, 0.53±0.04; PCP30 µM, 0.35±0.05.Area under the curve: L-AP4 600 µM, 0.55±0.03; PCP30 µM, 0.05±0.07. A total of 6 slices from 3 animals were averaged. Data (Mean ± S.E.M.) was normalized to the baseline.*p<0.05, **p<0.01 (for peak values); # p<0.05, ## p<0.01 (for values of the area under the curve), two-tailed Student's t test.

L-AP4 application led to a decrease of NMDAR-mediated fEPSP

With the same rationale as the previous experiments (14.8) this set of experiences was aimed at discerning if L-AP4 leads to a reduction of fEPSP peak amplitude by mediating either excitatory or inhibitory neurotransmission. This added to the fact that the mGlu7 receptor has been involved in the control of NMDAR surface expression[174] led to the question of whether L-AP4 has an effect over NMDAR mediated fEPSPs. The results show that at the working concentration L-AP4 has a possible effect over the strength of NMDAR mediated fields (peak amplitude) and over general firing of the

receptor (area under the curve as an indicative of the non-coordinated firing of various NMDAR). See Figure 40. The results indicate that the possible activation of mGlu7 leads to a reduction of glutamate and this affects the activation of NMDAR by this neurotransmissor. However further studies should be done to isolated, for example, AMPAR mediated fEPSP and also verify the effects of L-AP4 over the responses of this receptor. Using patch-clamping in brain slices and isolated NMDAR-, AMPAR-mediated currents could also be helpful.

15 Discussion

The main aim of this project was to discern if the mGlu7 receptor has any role in the modulation of basal synaptic transmission and short-term plasticity in both the CA1 area and the DG MPP. As already discussed, one of the main complications of discerning the role of this receptor on synaptic activity and plasticty is the lack of specific modulators. The ones available can affect all Group III receptors or members of Group I or II, and the concentrations needed to activate mGlu7 may even affect other receptors and/or channels.

These facts create the necessity to carefelly choose the right concentration for each compound used. L-AP4 is the most commonly used agonist for Group III receptor subtypes. Eventhough it is not selective for mGlu7, with an EC_{50} around 1 mM (two orders of magnitude over mGlu4 and mGlu8), using it at high enough concentrations should allow the activation of the receptor. At this high concentration one should however not rule out the potential of activating other receptors or channels.

fEPSP concentration-response curves in hippocampal slices (CA1) from either adult or immature animals allowed the calculation of the EC_{50}. In adult it was close to the one calculated by Ayala et al (1 mM vs. 1.12 mM), nonetheless the EC_{50} in immature rats was considerably lower (25 µM vs. 10.44 µM). The differences between the two age groups indicate that lower affinity receptors have a more relevant contribution at older ages, suggesting a developmental change in the receptor ratio in the hippocampus CA1 area. What is hypothesized is that on immature animals the lack of an mature inhibitory network needs to be compensated with the higher affinity receptors that will have a more strict control over the total quantity of glutamate present in the synapse so it does not reach neurotoxic levels.[1]

These results allowed to choose 600 µM as a working concentration for adult animals for L-AP4, which significantly decreased fEPSP amplitude in both the CA1 and DG MPP (see 0 and 14.2).This concentration is similar to the one used in literature.[96, 175]

The fit of the concentration response curve would have benefited greatly from more data at higher concentrations. Nonetheless no concentrations higher than 1 mM were used due to off-target interactions that have already been identified at lower concentration[122] as well as potential neurotoxic effects and limits of the compound solubility in the bath solution.

LY341495 concentration-responses in adult rat SC-CA1 synapses seem to present a biphasic profile (see 14.4 and 0) with the first deflection occurring at low concentrations up to 1 µM and the second

after 3 µM. If we take in account the IC_{50} values for this compound we could hypothesize that the first component of the biphasic response was mediated by mGlu2/3 or mGlu8 but due to the very low density of these receptors in CA1 other elements could also be responsible. The second component of the response could have involved mGlu7 and the elements of Group I, especially mGlu5 that has a high density in this area of the hippocampus.[44] At higher concentrations (100 µM) off-targets should also be considered. Since even though the peak value was significantly reduced compared to baseline it was still also significantly higher than the value for 30 µM. It has already been demonstrated that LY341495 at 100 µM had little or no effect on NMDAR, AMPAR or KaR-mediated responses on motoneurones[122], this leads to the hypothesis of another mGlu receptor being involved.

Concentrations between 1µM and 30µM led to a reduction of the PPR while 100 µM returned the ratio close to baseline levels.

When L-AP4 (600 µM) was added in the presence of LY341495 (100 µM) it led to a significant increase in the response that was observed with L-AP4 alone (see 14.4).This effect was unexpected since the concentration of the antagonist used should block the response from both Group II and III and therefore should have blocked the effect of L-AP4.

As stated by Howson and Jane[122] this could possibly be explained by another receptor being activated at higher concentrations of L-AP4 or by is a saturable removal mechanism for the agonist. The changes in PPR also point towards a possible postsynaptic receptor being involved, thus excluding mGlu7 which is mainly localized presynaptically.

The long exposure, with increasing concentrations, of either the agonist or antagonist could also have led to desensitization and/or internalization of receptors.[110] The usage of imaging techniques, either live or *post-hoc*, could help evaluate the receptor internalization and explain some of the results obtained.

These effects were not observed in the DG MPP where L-AP4 at 600 µM (see 14.3) produced a decrease of peak amplitude (0.68±0.08, $p<0.01$) with a corresponding increase in PPR (baseline, 0.95±0.06; 600 µM L-AP4, 1.23±0.1, $p<0.01$). In this same area the preincubation and presence of LY341495 (100 µM) during the application of L-AP4 (600 µM) did not led to a depression of the peak amplitude (1.02±0.09, $p>0.05$) neither an increase of the PPR (baseline, 0.95±0.06; 100 µM LY341495 and 600 µM L-AP4, 0.93±0.06, $p>0.05$).

In immature rats 0.1 µM of LY341495 decreased the peak amplitude (0.83±0.03, $p<0.01$) and PPR (baseline, 0.91±0.04; 0.1 µM LY341495, 0.83±0.03; $p<0.01$) and the higher concentrations had a similar effect. Exceptions were peak amplitude depression for concentrations of 30 µM and 100 µM (peak amplitude with 30 µM, 0.87±0.04; $p<0.05$ and peak amplitude with 100 µM, 0.95±0.06, $p<0.01$) and the PPR with 100 µM (PPR with 100 µM, 0.86±0.04, $p<0.01$).

When L-AP4 (600 µM) was added in the presence of LY341495 (100 µM) it led to a reduction of the fEPSP peak amplitude and PPR. The reduction of peak amplitude was not as marked as the one observed during the concentration response with L-AP4 alone at the same concentration (L-AP4 (600 µM), 0.36±0.03; LY341495 (100 µM) plus L-AP4 (600 µM), 0.51±0.05, $p<0.05$). The reduction on PPR was similar in both situations (L-AP4 (600 µM), 0.7±0.04; LY341495 (100 µM) plus L-AP4 (600 µM), 0.7±0.03, $p>0.05$, baseline values are being taken in account for the comparison).

As commented by Gee et al[176] the discovery and development of compounds specifically targeting mGlu7 is lagging behind the successful discovery of compounds for the other groups of mGlu receptors.

The first mGlu7-selective allosteric agonist AMN082 has contradictory effects in various behavioral test batteries when compared to KO and siRNA-mediated knockdown. Also, AMN082 has been shown to induce a rapid and long-lasting internalization of mGlu7 that translates into functional antagonism of the receptor.[110] Additionally, its primary metabolite, N-benzhydrylethane-1, 2-diamine, inhibits serotonin and norepinephrine reuptake transporters.[166]

This compound was expected to alter the response of L-AP4, either by increasing its effect by acting as a PAM or by working as an allosteric agonist. The latter would lead to the internalization of the receptor (as previously stated), lowering the number of receptors that are available for activation. This leads to the effect that the decrease of basal synaptic transmission is less pronounced.

In a similar way, both MMPIP and ADX71743 have produced incongruent results in behavioral paradigms despite displaying promising pharmacological properties in vitro. ADX71743 was shown to have robust anxiolytic-like effects in the elevated plus-maze and the marble burying test.[87] MMPIP showed no effects in a battery of anxiety-like and depression related tests but was active in spatial learning tasks.[116, 118]

All three compounds are likely to act via allosteric sites in more lipophilic domains rather than at the VFTD. This increased lipophilicity may be also associated with undesirable off-target effects.

Furthermore, single and/or different allosteric ligands do not necessarily similarly affect all downstream signaling pathways of a given receptor.

When tested in the CA1 and in the DG MPP areas of the hippocampus none of allosteric modulators led to changes in basal synaptic transmission at the concentrations used. These concentrations were based on previous results from the System Biology Group - Electrophysiology Section at Janssen Pharmaceutica (data not published) and a search in relevant literature.[87, 90, 114, 118]In the CA1 area the preincubation and presence of all three modulators with the agonist led to changes in peak amplitude and PPR that are not significantly different from those when L-AP4 is applied alone. In the DG MPP MMPIP augmented the depression on peak amplitude caused by L-AP4 accompanied by a trend to increase the PPR value (p=0.053). However some remarks had to be taken into consideration.

In the case of peak amplitude modifications, the presence of ADX71743 affected the depression caused by L-AP4 leading it to not reach significance when compared to its baseline.

In the case of PPR modifications the simultaneous presence of MMPIP and L-AP4 resulted in high variability that biased possible valid comparisons.

In the experiment where ADX71743 (in both CA1 and DG MPP) was used the PPR present at baseline was smaller than other experiments and the effect of the compounds was not reverse during washout which could be due to the lipophilicity of the compound.[176]

Using the MEAs capacity to evaluate the electrophysiological properties of multiple simultaneous electrodes in diverse areas of brain slices the hypothesis that mGlu7 may be involved in the modulation of spiking activity was accessed. The expected effects over spontaneous spiking were originally that the agonist would either decrease this event due to the reduction of glutamate release or increase it due to reduction of GABA release.

First it was verified that L-AP4 (600 µM) had a negative effect on the average number of spontaneous spiking events. The effect of the increasing concentrations of NMDA (not showed) has allowed the extrapolation of a concentration (50 µM) that would induce a significant increase in spiking activity and so create a window for pharmacological modulation of this property.

The results even though not conclusive, do indicate an effect of L-AP4 on NMDA induced spiking and on spontaneous spiking. This effects could possibly be mediated by mGlu7, especially on high concentrations of NMDA, as working as an autoreceptor targeting GABAergic interneurons. Nonetheless other receptor and mechanism could also be involved.

When mGlu7 is activated has an heteroreceptor it is linked to inhibition of of GABA exocytosis (both AMN802 and L-AP4 inhibited K^+ evoked release of GABA from hippocampal synaptosomes)[97] and as an autoreceptor the postsynaptic targets are commonly GABAergicinterneurons further decreassing the net ouput of GABA release. AMN082 a stipulated mGlu7 especific NAM/agonist was also shown to decrease GABA release from non vesicular origins.[98]

The effects stated in literature elicited the question of whether the reduction of peak amplitude caused by L-AP4 in the CA1 could be related to GABAergic neurotransmission or solely glutamatergic. Picrotoxin (PTX, 10 µM), a noncompetitive antagonist at $GABA_A$ receptors, was used to block postsynaptic responses to the release of GABA. In the presence of PTX, L-AP4 (600 µM) reduced peak amplitude (10 µM PTX and 600 µM L-AP4, 0.94±0.05, $p<0.01$) with no change in the PPR (baseline, 1.20±0.08; 10 µM PTX and 600 µM L-AP4, 1.15±0.08, $p>0.05$). In spite of a reduction of peak aplitude being present it was considerably lower compared to L-AP4 alone (600 µM L-AP4, 0.74±0.1; 10 µM PTX and 600 µM L-AP4, 0.94±0.05, $p<0.01$).

Taking all these results into consideration it was suggested that L-AP4, which was considered and used as a mGlu7 agonist may have acted on different target at the concentrations used to activate the receptor.

Further experiments could elaboratethe potential off-targets of L-AP4.A relevant experimentwould be to do a concentration response curve in both areas with the same concentration in KO animals for mGlu7. It would be preferred to use conditional KO so that developmental compensation could not influence the results obtained.

One of the hypothesized targets, from the results of PTX diminishing the effects over basal synaptic transmission of L-AP4, is $GABA_AR$. Accessing if L-AP4 at 600 µM (the concentration used through most of this work in adult animals) is behaving as an agonist of $GABA_AR$, perforated-patch-clamp recordings, which do not alter $[Cl^-]_I$[177], measuring chloride reverse potential using GABA, L-AP4 and GABA receptors antagonist could use to validate the hypothesis.

A more complex hypothesis could be based on the agonist induced receptor internalization. Long presence of AMN082 or L-AP4 has been shown to promote the receptor internalization[110] leading to a relief of the receptor inhibitory effect over the release of GABA and glutamate. The net effect of the

receptor internalization could lead to a net decrease of glutamate release due to increase GABA release thus decreasing fEPSPs peak amplitude.[94]

To further discern the mechanism by which L-AP4 may lead to a reduction in peak amplitude and based on the results by Gu et Al[47] demonstrating a functional relation between these two, NMDAR mediated population spikes were pharmacologically isolated and the effect of L-AP4 tested. The agonist at a concentration of 600 μM demonstrated an effect on NMDAR mediated potentials leading to a decrease of both the peak amplitude of the potentials and of the area under the curve of the signal. The parameters measured evidenced a similar behavior during and sensibility to PCP (an activity dependent NMDAR antagonist) especially the area under the curve which was reduced close to zero in the presence of 30 μM of PCP.

16 Conclusions

Throughout this work a great deal of controversial data and possible hypotheses has emerged. This further confirms the complex role of this receptor in the brain as well as the need for truly selective compounds that allow a careful evaluation of its function.

It is also hypothesized that L-AP4, the most commonly used agonist for this receptor, may act on different off-targets at a concentration thought to activate mGlu7, potentially masking the true relevance and action of activating this receptor. It is difficult to confirm whether the agonist is really acting on mGlu7 since the receptor is both an autoreceptor and heteroreceptor and the final effect of its activation will depend on the net effect as a result of its expression in different cell types.

Further experiments involving more sophisticated and sensible techniques would be needed to advance discoveries in this field, especially due to the complex signaling, anchoring and internalization of this receptor. The choice of the models used is also an important factor since the paradoxal findings in literature could be related to the different models they use, especially non-neuronal cells and/or models that do not involve the entire complexity and physiology of a complete neuronal network.

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