UNIVERSITÀ DEGLI STUDI DI PAVIA

Department of Internal Medicine and Therapeutics
Unit of Cellular and Molecular Pharmacology and Toxicology

MPEP, a Metabotropic Glutamate Receptor 5 (mGluR5) negative allosteric modulator, protects from hepatic ischemic injury both *in vitro* and *ex vivo*

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Doctorate in Biomedical Sciences

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LIST OF ABBREVIATIONS

AC: adenylyl cyclase
ACPD: (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid
AD: Alzheimer's disease
ADP: adenosine diphosphate
ago-PAM: agonist-positive allosteric modulator
ANOVA: analysis of variance
ALT: alanine aminotransferase
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANT: adenine nucleotide translocator
APS: ammonium persulphate
AST: aspartate transaminase
ATP: adenosine trisphosphate
BSA: bovine serum albumine
CaM: Calmodulin
CaMK: CaM-dependent kinase
cAMP: cyclic adenosine monophosphate
CaN: Calcineurin
CDPPB: 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide
CHO: Chinese hamster ovary
CHPG: *(R,S)*-2-chloro-5-hydroxyphenylglycine
CNS: central nervous system
CPG: *(S)*-4-carboxyphenylglycine
CPPHA: *N*-[5-chloro-2-[(-1,3-dioxoisoindolin-2-yl)methyl]phenyl]-2-hydroxybenzamide
CRD: cysteine-rich domain
CREB: cAMP-responsive element binding protein
CS: cold storage
CTD: C-terminal domain
CTRL: control
CyCPG: *(R,S)*-α-cyclopropyl-4-CPG
CYP: cytochrome 450
DAG: diacylglycerol
DFB: 3,3′-difluorobenzaldazine
DHPG: *(R,S)*-3,5-dihydroxyphenylglycine
DMSO: dimethyl sulphoxide
ECL: extracellular loop
eF: elongation factor
eF2K: elongation factor 2 kinase
eNOS: endothelial nitric oxide synthase
ENS: enteric nervous system
ER: endoplasmic reticulum
ERK1/2: extracellular signal-regulated kinase 1/2
ETC: electron transport chain
FXS: fragile X syndrome
GABA: gamma aminobutyric acid
GDH: glutamate dehydrogenase
GERD: gastroesophageal reflux disease
GP: glycogen phosphorylase
GPCR: G-protein coupled receptor
GRK: G-protein-coupled receptor kinase
GRM5: glutamate metabotropic receptor 5 gene
GS: glutamine synthetase
GSH: glutathione
HD: Huntington's disease
HIF-1α: Hypoxia-induced factor-1α
ICL: intracellular loop
iGluR: ionotropic glutamate receptor

IL: interleukine
INM: inner nuclear membrane
iNOS: inducible nitric oxide synthase
IP3: inositol 1,4,5-trisphosphate
I/R: ischemia/reperfusion
IRI: ischemia/reperfusion injury
JNK: Jun N-terminal kinase
KRH: Krebs/Henseleit buffer
LDH: lactate dehydrogenase
L-PAG: liver phosphate-activated glutaminase
logP: lipophilicity value
LTD: long-term depression
LTP: long-term potentiation
MAPK: mitogen-activated protein kinase
MCPG: (*S*)-α-methyl-4-carboxyphenylglycine
MDA: malondialdehyde
mGluR: metabotropic glutamate receptor
MPEP: 2-Methyl-6-(phenylethynyl)pyridine
MTEP: 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine
mTOR: mammalian target of rapamycin
NAM: negative allosteric modulator
NCBI: national center for biotechnology information
NMDA: N-methyl-D-aspartate
NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells
NO: nitric oxide
NOS: nitric oxide synthase
PA: partial agonist
PAM: positive allosteric modulator
PBS: phosphate buffered saline
PD: Parkinson's disease
PEP: phosphoenolpyruvate
PI3K: phosphoinositide 3-kinase
PIP: phosphotinositides
PKA: protein kinase A
PKB: protein kinase B (also AKT)
PKC: protein kinase C
PLCβ: phospholipase Cβ
PP1: protein phosphatase 1
PVDF: polyvinylidene difluoride
Quis: quisqualic acid
RCI: respiratory control index
RGS: regulators of g protein signaling
RNS: reactive nitrogen species
ROS: reactive oxygen species
RT-PCR: real time-polymerase chain reaction
SAM: silent allosteric modulator
SAPK: stress-activated protein kinase pathway
SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SHANK3: SH3 and multiple ankyrin repeat domain 3
SOD: superoxide dismutase
TEMED: N,N,N,N'-tetra-methyl-ethylendiamine
TM: transmembrane
TMD: transmembrane domain
TMJD: temporomandibular joint dysfunction
TNF-α: tumor necrosis factor-α
UTR: untranslated region
VFD: venus flytrap domain

AMINO ACID RESIDUES

Ala: alanine
Arg: arginine
Asn: asparagine
Asp: aspartate
Cys: cysteine
Gln: glutamine
Glu: glutamate
Gly: glycine
His: histidine
Ile: isoleucine
Leu: leucine
Lys: lysine
Met: methionine
Phe: phenylalanine
Pro: proline
Ser: serine
Thr: threonine
Trp: tryptophan
Tyr: tyrosine
Val: valine

GLOSSARY

Competitive agonist: a molecule which binds to the orthosteric binding site and initiates a physiological response.

Competitive antagonist: a molecule which binds to the orthosteric binding site and inhibits the physiological action of another.

Inverse agonist: a molecule that binds to the same receptor as an agonist, but induces a pharmacological response opposite to that agonist.

Orthosteric site: binding site of the endogenous agonist.

Allosteric site: binding site topographically distinct from the orthosteric binding site.

Positive allosteric modulator (PAM): a molecule that can strengthen the action of the orthosteric ligand by binding to the allosteric site.

Negative allosteric modulator (NAM): a molecule that can weaken the action of the orthosteric ligand by binding to the allosteric site.

Silent allosteric modulator (SAM): a molecule that binds to the allosteric site without produce any effects and inhibiting both PAM and NAM.

Partial agonist: PAM that occupies the allosteric binding site, but induces only a partial activation of the receptor signaling.

Partial antagonist: NAM that binds to the allosteric binding site, but induces only a partial blockade of the receptor signaling.

Ago-PAM: ago-potentiator: an allosteric ligand that functions as PAM and an allosteric agonist.

Molecular switch: subtle structural changes that alter the pharmacology of a molecule (from PAM to NAM) or the selectivity for a subtype receptor (from mGluR4 to mGluR2)

EC_{50}: the concentration of a drug that gives half-maximal response.

IC_{50}: the concentration of an inhibitor where the response (or binding) is reduced by half.

Potency: the concentration required to produce an effect of given intensity.

Efficacy: the relationship between receptor occupancy and the ability to initiate a response.

Affinity: how well a drug can bind to a receptor.

ABSTRACT

Glutamate, as excitatory amino acid, exerts its role by ionotropic receptors (iGluRs) as well as metabotropic receptors (mGluRs). iGluRs, further subdivided in AMPA, kainate and NMDA receptors, are deputed to fast responses. Metabotropic receptors, instead, are coupled to G proteins and regulate slower responses. They are categorized into three groups according to their sequence homology, G-protein coupling and ligand selectivity. Group I includes mGluR1 and mGluR5 and is coupled to $G_{\alpha q/11}$ protein, which activates phospholipase Cβ, causing phosphoinositides hydrolysis and formation of inositol 1,4,5-trisphosphate and diacylglycerol. This pathway causes calcium mobilization and activation of protein kinase C. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) are mainly coupled to $G_{i/o}$ proteins, inhibiting adenylyl cyclase (Ferraguti & Shigemoto 2006).

Metabotropic glutamate receptor subtype 5 functions as homodimer. Each protomer is composed of a large N-terminal extracellular domain, linked to the seven helices of the transmembrane domain by a cysteine-rich domain, necessary for dimerization and signal transmission. At the intracellular level, the C-terminus tail interacts with a plethora of scaffold proteins, which regulate receptor expression and networks with other receptors. The N-terminal domain represents the orthosteric binding site, in which both agonists and antagonists can trigger or inhibit, respectively, the activation of the receptor. Since orthosteric ligands are poorly selective and generate a "all-or-none" response, allosteric modulators were developed to finely tune the response. Differently from orthosteric molecules, allosteric ones bind to a pocket within the transmembrane domain, from where they can positively or negatively modulate the receptor (Niswender & Conn 2010).

mGluR5 is principally located in the central nervous system, where it regulates several physiological processes, for instance synaptic plasticity. However, mGluR5 has been involved in the onset of some pathological diseases, including fragile X syndrome, Huntington's disease, chronic pain, drug addiction, Parkinson's and Alzheimer's diseases (Nicoletti et al. 2011). Recently, mGluR5 has been detected also in peripheral tissues, such as in liver, pancreas and gastrointestinal tract (Ferrigno et al. 2017).

In particular, mGluR5 plays a crucial role in cerebral ischemia (Bao et al. 2001). In fact, the inadequate blood supply reduces the energy production, leading to an imbalance of ion gradients across membranes. Thus, membrane depolarization activates several voltage-sensitive and receptor-operated ion channels, enhancing release of glutamate. Although glutamate is most exclusively present intracellularly, during ischemic stroke it raises to toxic levels in the extracellular space, triggering a cascade of detrimental events, known as excitotoxicity (Taylor et al. 1995). Like in cerebral ischemia, also in hepatic ischemia glutamate and mGluR5 are fundamental. According to the temperature at which hepatic ischemia has achieved, it can be subdivided into warm or normothermic (37°C) and cold ischemia (4°C) (Baumann et al. 1989). Warm ischemia generally occurs in the setting of transplantation, trauma as well in some types of toxic liver injury, sinusoidal obstruction and sleep apnea, while cold ischemia is applied to organ to reduce hepatic metabolism during preservation before transplantation. Except for fatal events, the ischemic period is generally accompanied by the restoration of oxygen delivery, i.e. the reperfusion, and the global process is called Ischemia/Reperfusion Injury (IRI). During IRI, hepatocyte plasmatic membranes show morphological changes and cells are subjected to dramatic enzyme release, collapse of the ionic and electrochemical gradients, ATP depletion, mitochondrial impairment, cellular acidosis, Kupffer cells activation, ROS and RNS production and inflammatory processes generation. All these mechanisms lead to cell death, either for apoptosis or necrosis (Arias et al. 2001).

In hypoxic conditions, isolated hepatocytes show ameliorated viability after the pharmacological blockade of mGluR5 with the negative allosteric modulator 2-Methyl-6-(phenylethynyl)pyridine (MPEP) (M Storto et al. 2000). Nevertheless, it has been reported that MPEP reduces ATP content in murine BV-2 microglia cells and astrocytes, in a receptor-dependent manner (Chantong et al. 2014; Xu et al. 2014); in both cases the authors hypothesized a mGluR5-mediated effect. It is well known that ATP depletion is the main cause of ischemic stress, so these data look incongruous. However, up to date, it is still unknown whether MPEP is able to deplete ATP in hepatocytes.

The first part of this project was aimed to confirm MPEP protection and investigate whether other negative allosteric modulators (3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP) and Fenobam) had similar properties in anoxic isolated rat hepatocytes. Thus, cell viability was monitored by both trypan blue uptake and lactate dehydrogenase release into the buffer. We confirmed the data published previously from Storto and co-workers, in which

MPEP protected from ischemic injury (M Storto et al. 2000). In our work, MPEP 30 µM treatment exhibited a significant decrease in the mortality rate curve respect to anoxic controls in both trypan blue exclusion and LDH release, considering the intervals 0-75' and 0-90'. Although hepatocytes treated with MTEP showed a trend similar to MPEP-administered cells, the viability resulted significant only in trypan blue assay in the time-lapse 0-60' for MTEP 3 µM. The mortality rate curve of Fenobam 50 µM in the intervals 0-60' and 0-75' in trypan blue assay was significantly different to untreated ischemic sample. We supposed that the differences between MPEP and Fenobam could be due to the lipophilic nature of the molecules: MPEP is more permeable than Fenobam and could exert more easily its function also on intracellular mGluR5 receptors (Jong et al. 2014). In addition, Fenobam is a urea-derivative negative allosteric modulator, so it presents a chemotype distinct from MPEP (Porter et al. 2005). Because of the structural differences, MPEP and Fenobam, even though they bind to the transmembrane domain, interact with different binding residues (A Pagano et al. 2000; Pari Malherbe et al. 2003; Malherbe et al. 2006). The effect due to MPEP administration compared to Fenobam treatment could be also explained according to the rank order of drug potency: MPEP > Fenobam (Porter et al. 2005). Conversely, the administration of the orthosteric antagonist (*S*)-4-carboxyphenylglycine (CPG) did not ameliorate hepatocytes viability, maintaining a mortality rate similar to anoxic controls. This could be attributable to the fact that, probably, 100 µM and 200 µM were too low concentrations of CPG, while its IC_{50} in HEK-293 cells was calculated to be more than 1000 µM (Kingston et al. 1995; Porter et al. 2005), respect to MPEP IC_{50} of 36 nM (Acher n.d.).

Once ascertained that MPEP, MTEP and Fenobam protected from ischemic injury, we explored whether and how ATP could be involved. In their experiments, Chantong and co-workers reported that MPEP administration depleted ATP in microglial cells (Chantong et al. 2014). It is worth noting that ATP depletion is one of the first consequences after electrochemical gradient impairment. Thus, these data appeared to be in contrast with the well-known ability of MPEP to make cells more resistant to ischemic injury (M Storto et al. 2000). We evaluated ATP alterations in isolated rat hepatocytes in anoxic conditions in presence or absence of negative (MPEP, MTEP, Fenobam) or positive (3,3′-difluorobenzaldazine (DFB) along with the agonist *(R,S)*-3,5-dihydroxyphenylglycine (DHPG)) allosteric modulators. ATP concentration was followed in time before and after N_2 insufflation. Hepatocytes treated with MPEP had a significantly lower ATP content respect to controls before anoxia, more evidently for MPEP 30 µM; nonetheless, MPEP-treated cells

showed a slower decline in ATP following N_2 insufflation when compared to anoxic controls or DHPG-treated hepatocytes. After 30 minutes of pharmacological treatment and 30 of exposure to anoxia, ATP levels decreased with all negative allosteric modulators treatment when compared to ischemic controls, although only hepatocytes administered with MPEP 3 µM and 30 µM exhibited ATP levels significantly higher respect to ischemic controls.

Since mitochondria are the production engine of ATP, we investigated the effect of Negative Allosteric Modulators (NAMs) administration on ATP production in isolated mitochondria. Isolated mitochondria were treated 15 minutes with MPEP, MTEP, Fenobam and CPG, all at 0.3 µM, 3 µM and 30 µM. As observed in isolated primary hepatocytes, MPEP and MTEP induced a dose-dependent decrease in ATP level, although more evident and significant with MPEP treatment. ATP concentration in samples treated with Fenobam and CPG, instead, remained almost constant and with values near to the controls. Furthermore, in order to understand whether our molecules could affect mitochondrial function, we assessed respiratory control index, membrane potential, ROS production, I and II mitochondrial complexes function and F_1F_O-ATPase activity. Our experiments demonstrated that MPEP and MTEP altered ATP content without affecting mitochondria functionality. Moreover, NAMs did not uncouple mitochondria, suggesting that ATP depletion occurred in a mitochondria-independent fashion. However, ATP was significantly depleted by MPEP 30 µM. Hence, we assessed an acellular solution containing ATP in presence or absence of MPEP, MTEP, Fenobam and CPG. As detected in isolated hepatocytes and mitochondria, ATP decreased in a dose-dependent way for MPEP and MTEP, while remained unaffected for Fenobam and CPG. Our data suggested that, although the protection provided, only MPEP and MTEP depleted ATP both *in vitro* and in acellular solution, without affecting any functional role of mitochondria or impairing complexes deputed to respiration.

In the second part of this work, we assessed whether the use of a preservation solution containing MPEP could protect the mice livers in two *ex vivo* models of Ischemia/Reperfusion (I/R) injury: cold (4°C) and warm (37°C) I/R injury. Isolated livers from both wild type and knockout mice were preserved at 4°C for 18 hours and successively reperfused at 37°C for one hour for cold I/R injury. The UW preservation solution, as well Krebs-Henseleit buffer used for the reperfusion, contained either MPEP 0.3 µM or DHPG. Perfusate samples were collected at the end of cold preservation (the so-called “wash out”) and during the reperfusion process.

To assess liver condition after 18 hours cold storage, lactate dehydrogenase and transaminase release were evaluated in the wash out and during the reperfusion. LDH release from livers preserved with MPEP 0.3 μM and of mGluR5 knockout organs was significantly lower than in controls, while AST and ALT release was significantly reduced with MPEP-treatment. These data indicated that the pharmacological blockade of mGluR5 or the absence of the receptor protected from ischemic injury progression. Moreover, apoptosis was gauged measuring Bax/Bcl-2 ratio (Produit-Zengaffinen et al. 2009), and no differences were observed between MPEP-treated livers and wild type control sample, suggesting that MPEP could be protective regarding apoptosis.

We evaluated whether the protection mediated by MPEP resulted from a metabolic shift from aerobiosis to anaerobiosis, quantifying protein expression of Hypoxia Inducible Factor-1α (HIF-1α) and Glycogen Phosphorylase (GP). In our experiments, the administration of MPEP did not alter HIF-1α or glycogen phosphorylase respect to anoxic controls, suggesting that other mechanisms were involved in the improvement of liver viability.

When glutamate release induced by pathological stimuli becomes excessive in the extracellular space of central nervous system, intracellular calcium concentration increases and triggers a detrimental downstream cascade, involving upregulation of Tumor Necrosis Factor-α (TNF-α), nitric oxide synthase, mitochondria dysfunction, ROS and RNS production (Kritis et al. 2015). Similarly, in a model of cold ischemia and warm reperfusion, rat hepatocytes viability was decreased by the imbalance of calcium homeostasis (Elimadi & Haddad 2001). Thus, we further investigated mGluR5 ability to control inflammatory mediators in a model of ischemia/reperfusion, evaluating (TNF-α) and inducible isoform of Nitric Oxide Synthase (iNOS) protein expression. The blockade of the receptors by MPEP or its absence in knockout livers significantly reduced both TNF-α and iNOS protein expression, in comparison with controls. In line with published data that reported the presence of TNF Response Element on iNOS gene (Medeiros et al. 2007; Eberhardt et al. 1996), we supposed that the blockade of mGluR5, through TNF-α, selectively reduces iNOS expression. In order to support this hypothesis, protein expression of the endothelial constitutive isoform of NOS (eNOS) was detected: no changes occurred between MPEP-treated livers and controls. A non-specific decrease of all NOS isoforms would make the negative modulators of mGluR5 allosteric strongly contraindicated in various conditions associated with transient ischemia, including multiple organ failure (Ferrigno, Di Pasqua, et al. 2015; Nijveldt et al. 2003; López et al. 2004).

To verify whether ATP levels alteration occurred also in a model of isolation/reperfusion organ, we evaluated ATP content in cold preserved liver. ATP concentration was higher in MPEP-treated samples when compared to controls, while KO and untreated livers presented similar ATP content. These data, in agreement with *in vitro* results, suggest that ATP depletion could occur also in a receptor-independent way. It has recently demonstrated that the triple bond of MPEP reacts with the thiol group of GSH, generating GSH-conjugates (Ferrigno et al. 2017; Zhuo et al. 2015). In addition to alkyne, MPEP and MTEP contain a pyridine, a highly reactive group that could be subjected to alkylation, oxidation, nucleophilic substitutions and so on. Thus, we supposed that MPEP could react in some way with ATP.
Besides cold ischemia, also normoxic (or warm) ischemia was taken into consideration, since it occurred in trauma and elective liver surgery, as well in some types of toxic liver injury, hypovolemic shock, sinusoidal obstruction, Budd-Chiari syndrome and sleep apnea. Thus, we performed ischemia/reperfusion also in normoxic conditions. Also in this case, preservation and reperfusion solution were added with MPEP or DHPG plus DFB. Livers functionality was evaluated by means of lactate dehydrogenase release in the wash out and during the reperfusion and pro-inflammation was assessed by gauging TNF-α released using an ELISA assay. Liver protection has been observed also in normoxic condition. In fact, MPEP-treated and mGluR5 knockout organs showed a decrease in both LDH and TNF-α release, when compared to controls, as observed in cold ischemia.

The administration of the orthosteric agonist DHPG in both *in vitro* and *ex vivo* experiments displayed a trend similar to that of ischemic controls. This could be due to the fact that mGluR5 could be saturated by glutamate, as previously demonstrated (M Storto et al. 2000), or to the rapid desensitization of the receptor by DHPG-induced depolarization (Huang & van den Pol 2007).

Summarizing, by our *in vitro* data, we firstly showed that MPEP and MTEP depleted ATP from hepatocytes and mitochondria without affecting mitochondrial functionality. ATP-depleting ability seems to have no consequences on mGluR5-mediated protection for I/R injury. Then, we confirmed that MPEP reduced the damage caused by anoxia in isolated hepatocytes. Furthermore, we revealed that also MTEP and Fenobam had a positive effect on cell viability. More notably, we demonstrated for the first time that the blockade of mGluR5 with MPEP protected mice livers from cold and warm ischemia/reperfusion injury in *ex vivo* models.

Because of mGluR5 is involved in the onset of several pathologies not only in the central nervous system, it would represents a compelling pharmacological target also for the handling of diseases in peripheral organs, such as ischemia/reperfusion injury of the liver. To date, the molecular mechanisms of mGluR5 NAMs protection in ischemia are not fully understood, even because in diverse cell types the signaling pathways appear different. Thus, further investigation on molecular signaling would be useful to enlarge our knowledge.
MPEP is a very permeable molecule and its administration in the CNS could trigger unexpected pathways. Hence, its use is limited to the research field in order to clarify the mechanisms in which it is involved. For this reason, in preclinical and clinical studies other mGluR5 negative allosteric modulators were employed. However, several clinical trials were discontinued because of the poor efficacy of these negative allosteric modulators (Petrov et al. 2014). Further research in the optimization of these molecules appears necessary. A novel approach is focused on the development of "bitopic" ligands, which are molecules able to bind to both the orthosteric and allosteric sites, with the purpose of combine the "all-or-none" responses of orthosteric ligands with the higher selectivity of the allosteric molecules (Kamal & Jockers 2009).

Our results support the idea that the advancement in the understanding of the molecular pathophysiology of liver injury might lead to the development of novel therapeutic alternatives, whose the mGluR5 negative allosteric modulators represent a feasible and truthful example.

INTRODUCTION

Glutamate (Glu) and glutamine (Gln) are two fundamental amino acids, involved in the synthesis of proteins, amino sugars and nucleotides. Glutamine is crucial for promoting and maintaining cell function, survival and proliferation, whereas glutamate, the most abundant intracellular amino acid, regulates ammonia homeostasis (Newsholme et al. 2003). Glutamate and glutamine are indeed strictly connected, in a glutamine-glutamate cycle (Nissim 1999). Glutamate and ammonia produce glutamine, via Glutamine Synthetase (GS), a cytosolic ATP-dependent enzyme, in the perivenous region. On the other hand, hepatic glutamine is metabolized and converted into glutamate and ammonia (NH_3) by the hepatic isoform of Phosphate-Activated Glutaminase, L-PAG, localized into the mitochondria of periportal cells (Watford 2000). L-PAG works at pH optimum between 7.8 and 8.2 and it is sensitive to small changes in pH, NH_4^+ and glucagon. It has been demonstrated that L-PAG and Glutamate Dehydrogenase (GDH) function is lowered in acidosis (pH 6.8) and increased in alkalosis (pH 7.6) and glutamine synthesis is stimulated in acidosis (Nissim 1999). During acute acidosis, glutamate is converted to alanine via transamination and transported in the extracellular compartment. Glutamate can be also synthetized via glucose conversion of α-ketoglutarate by glutamate dehydrogenase. It works as both substrate and product in many distinct reactions and it is involved in the biosynthesis of some amino acids, for instance L-proline and L-arginine (Wakabayashi et al. 1991; Murphy et al. 1996), and it works also as a neurotransmitter (Watkins & Evans 1981) (Figure 1.1).

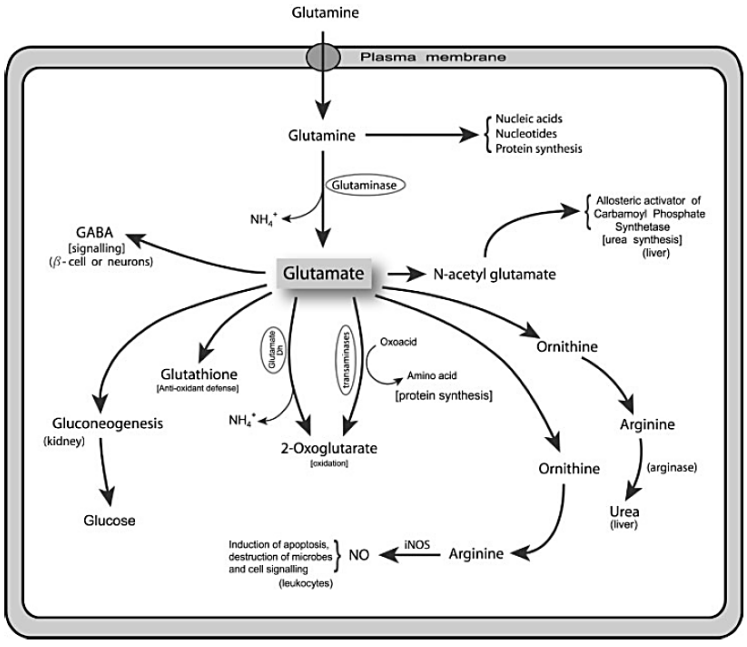


Figure 1.1 Overview of glutamate metabolism. Glutamate is synthetized from glutamine through glutaminase activity. Glutamate, in turn, could be converted into many products (ornithine, glutathione, GABA) involved in different metabolic pathways (Newsholme et al. 2003).

Glutamate, as excitatory amino acid, exerts its function by ionotropic receptors (iGluRs) as well as metabotropic receptors (mGluRs) (Figure 1.2). Ligand-gated ionotropic receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors. As they are glutamate-gated ion channels, they regulate rapid responses once activated. Metabotropic glutamate receptors are G-Protein Coupled Receptors (GPCRs) and they mediate slower responses. They are subdivided into three groups, according to their sequence homology, G-protein coupling and ligand selectivity. Group I includes mGluR1 and mGluR5, group II counts mGluR2 and mGluR3, while group III encompasses mGluR4, mGluR6, mGluR7 and mGluR8 (Ferraguti & Shigemoto 2006).

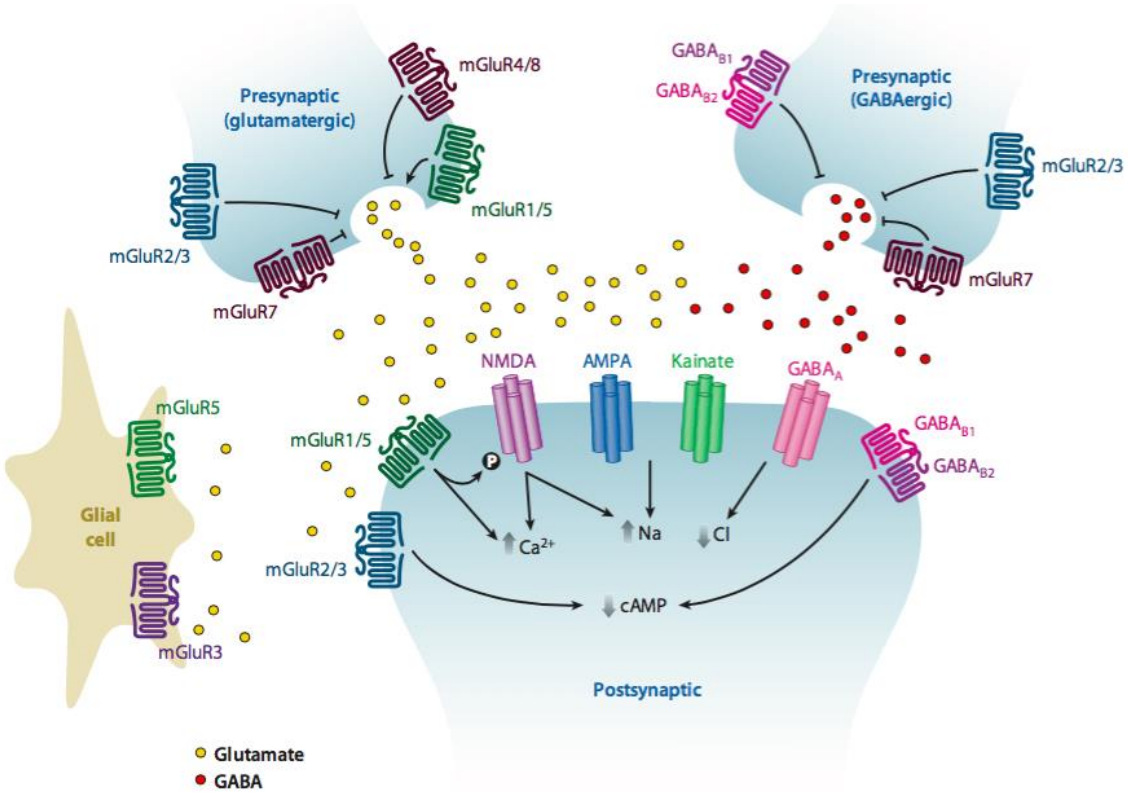


Figure 1.2. Schematic representation of glutamate receptors. Ionotropic glutamate receptors (iGluRs) are localized mainly at postsynaptic level, while metabotropic glutamate receptors (mGluRs) could be present on presynaptic, glial as well postsynaptic cells (Niswender & Conn 2010).

1.1 Genetics

In humans, metabotropic glutamate receptor subtype 5 (hmGluR5) is encoded by the gene *GRM5*, which is a large gene of more that 540,000 base pairs of genomic DNA located on chromosome 11, at position 11q14.2-q14.3 (Corti et al. 2003) (Figure 1.3).

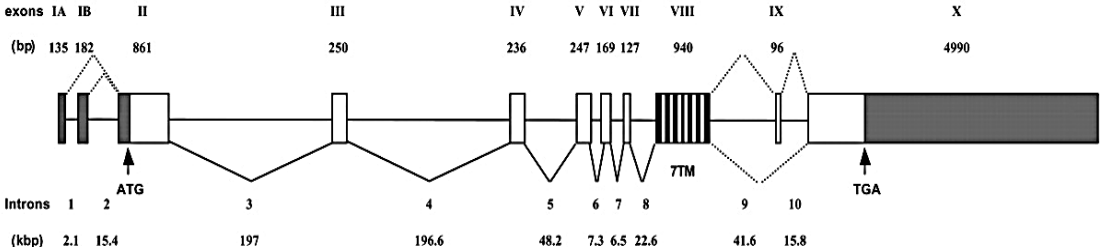


Figure 1.3. Genomic organization of human metabotropic glutamate receptor subtype 5 (*GRM5*). Exons are represented as boxes, and the untranslated regions in exons IA, IB, II, and X are shaded. The seven transmembrane spanning regions (7TM) contained in exon VIII are displayed as vertical stripes. The position of the translational start (ATG) and stop (TGA) codons are marked by arrows. The size of the exons and introns given in base pairs (bp) and kilobase pairs (kbp), respectively, are not drawn to scale (Corti et al. 2003).

As reported on NCBI, *GRM5* gene is conserved among vertebrates, such as chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish and frog (Figure 1.4), and it has orthologue in invertebrates, such as *C. elegans*. mGuR5 (NP_000833 for human, NP_058708 for rat and NP_001074883 for mouse) presents three splice variants: mGluR5a, mGluR5b, that is 32 amino acids longer that isoform a, and mGluR5d, 267 amino acids C-terminal shorter than mGluR5a (Matosin et al. 2017). Although mGluR5a and mGluR5b appear to have the same pharmacological profiles, they differ in the expression and in function. At this purpose, Mion and co-workers reported an opposite effect of mGluR5a and mGluR5b: the first suppresses neuronal maturation, whilst the second promotes neurite growth (Mion et al. 2001). Few studies documents post-transcriptional regulation mechanisms. It has been shown that microRNA miR-128a represses GRM5 3'-Untranslated Region (UTR), suppressing mGluR5 expression (Kocerha et al. 2014). A similar decrease has been obtained with hypermethylation of the promoter (Kordi-Tamandani et al. 2013).

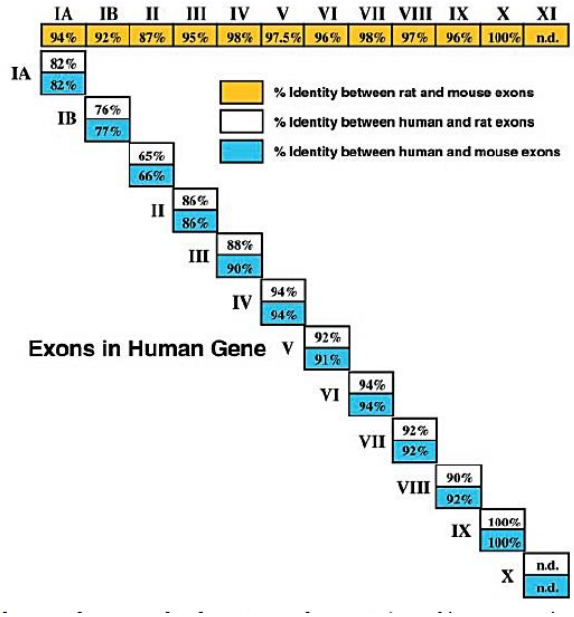


Figure 1.4. Schematic diagram showing the percentage of identity among human, rat and mouse GRM5. The percentage of identity between homologous *GRM5* exons in rat and mouse is represented by yellow boxes, the homology between human and rat exons is displayed by white boxes, while between human and mouse exons blue boxes (Corti et al. 2003).

1.2 Distribution

1.2.1 Distribution in the central nervous system

Since glutamate is a crucial neurotransmitter, it is not surprising that the Central Nervous System (CNS) is rich in ionotropic as well metabotropic receptors and transporters for glutamate. Ionotropic glutamate receptors localize principally in the postsynaptic membranes of glutamatergic synapses, whereas metabotropic glutamate receptors are widely distributed in both neuronal and glial cells in glutamatergic synapses, and also in GABAergic and dopaminergic systems (Ferraguti & Shigemoto 2006).

mGluR1, mGluR3, mGluR5 and mGluR7 are extensively diffused throughout the brain, while mGluR2, mGluR4 and mGluR8 expression is more restricted to specific brain areas. Only mGluR6 is not present in the brain and spinal cord, but it has been detected in retina (Ferraguti & Shigemoto 2006).

In particular, mGluR5 is highly expressed in telencephalic regions, comprising the cerebral cortex, hippocampus, subiculum, olfactory bulbs, anterior olfactory nucleus, olfactory tubercle, striatum, nucleus accumbens and lateral septal nucleus (Abe et al. 1992; Shigemoto

et al. 1993; Romano et al. 1995). In the hippocampus, mGluR5 is abundantly detected in pyramidal and granule cells (Shigemoto et al. 1997). In the brainstem, a notably expression of mGluR5 is found in the shell regions of the inferior colliculus, superficial layers of the superior colliculus and caudal subnucleus of the trigeminal nucleus. In the cerebellar cortex, instead, little expression was observed in Golgi cells, and no expression has been detected in Purkinje and granule cells (Neki et al. 1996; Négyessy et al. 1997). In the spinal cord, mGluR5 is largely found in the superficial dorsal horn (Jia et al. 1999). Moreover, several studies have revealed a differential regulation of mGlu receptors expression during CNS development. In particular, mGluR5 rises perinatally, up to two weeks after birth, and then its expression declines (López-Bendito et al. 2002). Despite the distribution of the two isoforms of metabotropic glutamate receptor 5, mGluR5a and mGluR5b, appears similar, mGluR5a expression is most abundant in the young rat, while mGluR5b is most present in the adult rat (Romano, van den Pol, et al. 1996) (Figure 1.5).

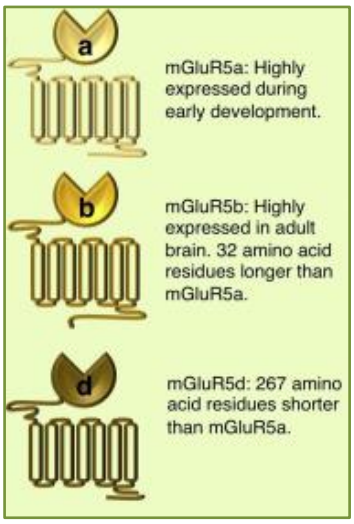


Figure 1.5. Schematic representation of mGluR5 splice variants. mGluR5a is mostly expressed during early development; mGluR5b, instead, is 32-amino acids longer than mGluR5a and is expressed in adult brain, while mGluR5d is the shortest isoform (Matosin et al. 2017).

1.2.2 Distribution in peripheral tissues

L-glutamate is not only an essential excitatory amino acid at brain level, but represents also an important signaling molecule in peripheral tissues. Thus, even if metabotropic glutamate receptors are mainly located in the central nervous system, they have been recently

detected in peripheral tissues too, under both physiological and pathological conditions (Julio-Pieper et al. 2011).

mGluR5 expression has been observed in different districts of the gastrointestinal tract. At mouth level, it is constitutively expressed in the trigeminal ganglion (Lee & Ro 2007) and the masseter muscle (Lee et al. 2006), which could be involved in the Temporomandibular Joint Dysfunction (TMJD). In addition, it has been found in the terminal region of axon innervating human dental pulp, where it mediates the receptor-mediated inflammatory nociception (Kim et al. 2009). mGluR5 has also been identified at the gastroesophageal level, in which it is responsible for the Gastroesophageal Reflux Disease (GERD) (Frisby et al. 2005; Ferrigno et al. 2017). Because of its own nervous system, called Enteric Nervous System (ENS), the intestine is capable to respond to reflex stimuli independently of the CNS. As well at cerebral level, the presence of mGluR5 has been shown in neurons of jejunum, ileum and colon, suggesting its involvement in the regulation of intestinal motility (Hu et al. 1999; Liu & Kirchgessner 2000). Moreover, mGluR5 expression has been detected also in enteric glia in the ileum and colon of rodents and pigs (Nasser et al. 2007). Immunofluorescence studies and western blot analysis have demonstrated that mGluR5 is present in the tongue, where it is associated to inflammation and pain (Liu et al. 2012). Besides the presence of iGluRs, mGluRs in the pancreas have been recently discovered (Brice et al. 2002). Both mRNA and protein of mGluR5 were found in rat and human islet of Langerhans, implying its role in the regulation and homeostasis of glucose.

Since the heart is an innervated organ, it is not surprising that mGluR5 in human is located in the atrial intramural ganglia, atrial and ventricular cardiocytes and bundle of Hiss (Gill et al. 2007). The administration of the agonists induced a significant increase in arterial pressure and heart rate (Tsuchihashi et al. 2000).

mGluR5 has been discovered also in the male reproductive system. It has been reported that mGluR5 mRNA and protein are present in the testis of rat and it is abundantly expressed in human seminiferous tubuli and in the tail of spermatozoa (Storto et al. 2001).

The presence of metabotropic glutamate receptors was documented in the immune system. In fact, RT-PCR and western blot analysis demonstrated that mGluR5 is expressed in whole mouse thymus, isolated thymocytes and a thymic stromal cell line, whereas flow cytometry revealed that mGluR5 expression is induced during thymocyte maturation (Storto *et al.*, 2000). Additionally, mGluR5 was largely expressed in dendritic cells and lymphocytes of the thymic medulla and weakly present in cortical lymphocytes (Rezzani et al. 2003). On the contrary, mGluR5 is constitutively expressed in human peripheral blood lymphocytes, where

activates adenylate cyclase signaling instead of phospholipase C pathway (Pacheco et al. 2004; Pacheco et al. 2007).

mGluR5 has been found also in rat pinealocytes (Yamada et al. 1998), in mouse and rat adrenal gland (Scaccianoce et al. 2003), in mouse osteoclast (Morimoto et al. 2006), in the retina (Dhingra & Vardi 2012), in endothelial cells from human skin (Collard et al. 2002).

Tissue	**Group I**		**Group II**		**Group III**			
	mGluR1	**mGluR5**	mGluR2	mGluR3	mGluR4	mGluR6	mGluR7	mGluR8
Adrenal gland	✓	✓	✓	✓	✓		✓	
Bone cells	✓	✓			✓	✓		✓
Heart	✓	✓	✓	✓				
Colon		✓			✓		✓	✓
Duodenum	✓				✓			✓
Endothelium	✓	✓			✓			
Esophagus	✓	✓			✓			
Ileum		✓	✓	✓				✓
Kidney			✓	✓	✓			
Liver		✓		✓				
Lung				✓	✓			
Pancreas		✓	✓	✓	✓			✓
Skin	✓	✓	✓	✓				
Stem cells		✓			✓			
Stomach	✓	✓	✓	✓	✓	✓	✓	✓
T cells	✓	✓						
Testis	✓	✓	✓	✓	✓	✓		✓
Thymus	✓	✓	✓	✓	✓			

Table 1.1. Localization of metabotropic glutamate receptors in peripheral tissues. Metabotropic glutamate receptors are classified into three groups and they are differentially localized outside the central nervous system. In the second column the peripheral tissues in which mGluR5 has been detected, for instance liver and gastrointestinal tract (Julio-Pieper et al. 2011, modified).

1.2.3 mGluR5 in the liver

The first evidence of the presence of metabotropic glutamate receptors in hepatocytes was proposed by Sureda et al. In that study, they demonstrated that the incubation of primary cultures of rat hepatocytes with two agonists, quisqualate and ACPD, stimulated polyphosphoinositide hydrolysis (Sureda et al. 1997). These data indicated that the activation of a Group I mGlu receptor occurred, although the subtype targeted was unknown because the agonists could bind both the mGluR1 and mGluR5. The presence of mGluR5, and not of mGluR1, in hepatocytes was subsequently confirmed by Storto et al. In fact, they detected mGluR5 mRNA and protein expression in rat liver, isolated hepatocytes and hepatoma cells by RT-PCR and immunoblotting and immunostaining analysis, respectively (M Storto et al. 2000). Beside the presence of mGluR5, Storto and collaborators showed also that the activation of the receptor by means of agonists (ACPD and quisqualate) worsened cell damage in anoxic hepatocytes, whereas the administration of a negative allosteric modulator (MPEP) protected liver cells from necrosis. Lately, the crucial role of glutamate in the activation of mGlu receptors during hypoxic conditions was confirmed using cells from knockout (KO) mice for mGluR5 (Storto et al. 2004). The onset of ischemic damage was delayed and viability was improved in cells from KO mice, as well in mouse hepatocytes treated with MPEP, compared to wild type control hepatocytes. Furthermore, the administration of negative allosteric modulators demonstrated that ROS production, Malondialdehyde (MDA) formation and thiol group oxidation were reduced in isolated hepatocytes treated with tert-butylhydroperoxide. The same phenomena were observed in mice treated with acetaminophen, in which a depletion of GSH was also observed (Storto et al. 2003). In a more recent study, it has been shown that MPEP administration in mouse protected the liver from damage induced by lipopolysaccharide (LPS) (Jesse et al. 2009).

1.2.4 Intracellular distribution

Electron microscopy studies demonstrated that the presence of metabotropic glutamate receptor subtype 5 is not restricted only to postsynaptic membranes or plasmatic membrane of cells outside the CNS, but most of this receptor (50–90%) is intracellular (Jong et al. 2014). mGluR5 has been found on nuclear membranes, where it can induce changes in nuclear

calcium (O'Malley et al. 2003). In fact, Ca^{2+} oscillations were specific because carbachol stimulation of endogenous muscarinic receptors did not prompt this pattern of calcium release. Moreover, Ca^{2+} oscillations were inhibited not only by calcium chelators, but also by MPEP, the negative allosteric modulator selective for mGluR5. Jong and co-workers, by means of immunogold particles, detected mGluR5 on both nuclear membranes, inner and outer, and on Endoplasmic Reticulum (ER) membranes (Figure 1.6). The use of differential permeabilization, in conjunction with antibodies directed to the mGluR5 N- or C- terminals, suggested that the N-terminus is directed towards the lumen of nuclear or ER membranes, while the C-terminus towards the nucleoplasm or the cytoplasm (Jong et al. 2014). With this kind of topology appears obvious that ligands have to be permeable or transported within the lumen. Glutamate, because of its negative charge, can not pass through membranes, so its transport is coupled to exchangers or vesicles. In addition, the EC_{50} for glutamate activation of intracellular mGluR5 is about 61 μM, an irrelevant value compared to the high intracellular glutamate concentration (Jong & O'Malley 2017). Conversely, MPEP, having a lipophilicity value (logP) of 3.3, permeates membranes, while DHPG, quisqualate and glutamate itself have log P values of -2.4, -3.9 and -2.7, respectively (Jong et al. 2014).

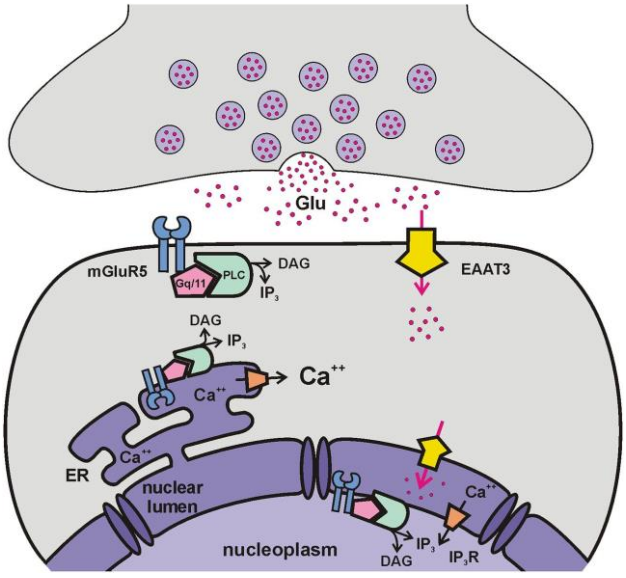


Figure 1.6. Suggested model of mGluR5 localization at cell surface and at intracellular level. Besides the presence of mGluR5 at cell surface, mGluR5 has been found also intracellularly, on nuclear and endoplasmic reticulum membranes. According to this model, the topology of intracellular receptors indicates that the Venus flytrap domain is towards the lumen, while the C-terminal domain is addressed to cytoplasm (Jong et al. 2014).

1.3 Structure

Metabotropic glutamate receptors, as well as most Class C GPCRs, act as dimer, both homodimer and heterodimer (Doumazane et al. 2011). Each protomer consists of a large extracellular N-terminal domain linked to the intracellular C-terminal domain by the seven helices of the transmembrane domain (Figure 1.7).

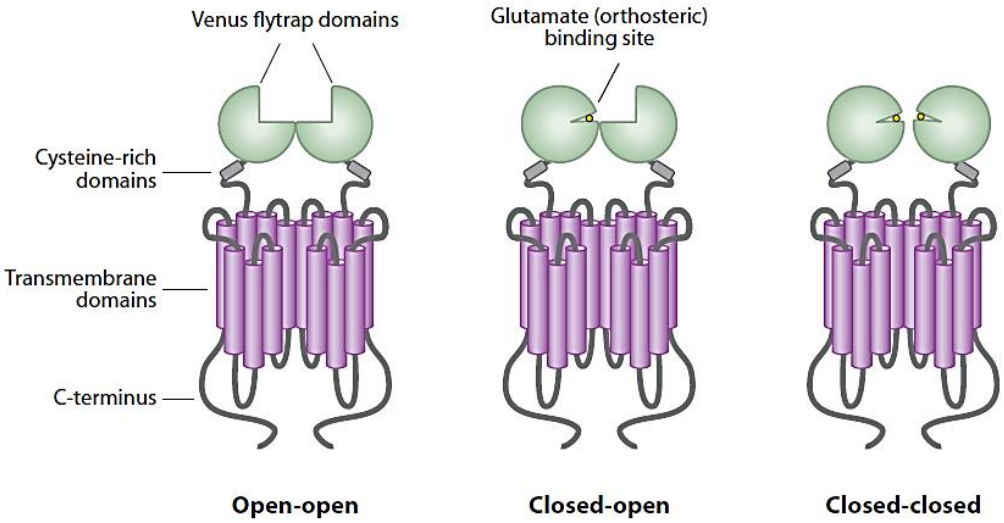


Figure 1.7. Diagram of mGluR5 structure and distinct activity states. mGluR5 contains a large extracellular N-terminal domain composed of the Venus Flytrap Domain (VFD), that binds glutamate and the other orthosteric ligands, and the Cysteine-Rich Domain (CRD), which transmits the signal to the seven helices of the Transmembrane Domain (TMD), that represents the binding site of allosteric modulators. The C-Terminal Domain (CTD), the intracellular portion of the receptors, is linked to several proteins and it is subjected to modifications, such as phosphorylation, which regulate the receptor activity. mGluR5 acts as homodimer: in the open-open state (the two VFDs are unbound) the receptor is inactive; in the closed-open (one orthosteric binding site occupied and one free) and in the closed-closed state (both VFDs bind to glutamate or orthosteric ligand) the receptor is active (Niswender & Conn 2010).

1.3.1 The Venus Flytrap Domain

Metabotropic glutamate receptors contain a large extracellular N-terminal domain, uncommon for Class C GPCRs. It is called Venus Flytrap Domain (VFD) and holds the glutamate-binding site. The VFD structure was first proposed by O'Hara in 1993 (O'Hara et al. 1993) and the crystal structure of the mGluR1-VFD was solved in 2000 by Morikawa and collaborators (Morikawa et al. 2000). Subsequently, several structures of mGluR1-VFD alone (Tsuchiya et al. 2002) and co-crystallized with different ligands were elucidated (Dobrovetsky et al. 2009). Different studies about the crystal structures of N-terminal mGluR5

(Dobrovetsky et al. 2010a), mGluR3 (Muto et al. 2007; Wernimont et al. 2007) and mGluR7 (Muto et al. 2007; Dobrovetsky et al. 2010b,) confirmed the high similarity of the overall structures of the Venus flytrap domains across the three groups. Recent evidence reveals that two VFDs dimerize together, back to back, and important conformational changes are induced when agonists bind to one or both Venus flytrap domains (Jingami et al. 2003) (Figure 1.8).
Although it has well documented that Class C GPCRs heterodimerize, it does not occur for mGluR5. mGluR1 and mGluR5 share 60% of their amino acid sequence, but they do not heterodimerize (Romano, Yang, et al. 1996). More recently, Fuxe and colleagues revealed that mGluR5 does not generate true heterodimer, but it is co-localized and functionally linked to other receptors, such as adenosine ones (Fuxe et al. 2014).

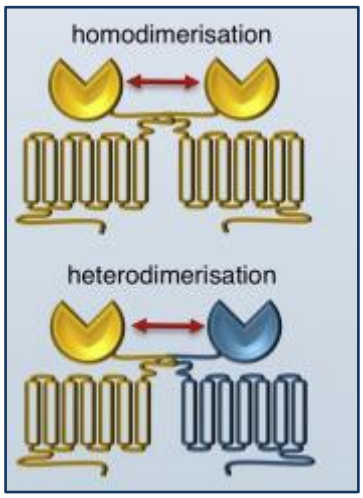


Figure 1.8. Metabotropic glutamate receptor dimerization. Metabotropic glutamate receptors act as dimers, both homodimers (at the top) or heterodimers (at the bottom). mGluR5 forms only homodimers with others mGuR5 and could co-localize with other receptors, such as adenosine receptors (Matosin et al. 2017).

The dimeric bilobal rearrangement can exist into three main states: open-open, open-closed, and closed-closed. The open-open conformation is stabilized by antagonists and makes the receptor inactive; the open-closed and closed-closed conformations, instead, are induced by the binding of ligand to one or two protomers.
The mutation of residues that prevent the closure of the VFD can switch the pharmacology of antagonists to agonists (Bessis et al. 2002), suggesting that the relative orientation of these domains is essential for receptor activation. For glutamate binding, several conserved residues span the two lobes and make critical contacts with the glutamate molecule (Pin et al. 2003; Kuang et al. 2003; Acher & Bertrand 2005). Moreover, the Venus flytrap domain also bind

divalent cations, such as magnesium and calcium, which can potentiate or activate the receptor (Kunishima et al. 2000; Kubo et al. 1998; Francesconi & Duvoisin 2004).

1.3.2 The Cysteine-Rich Domain

The Cysteine-Rich Domain (CRD) is linked to the Venus flytrap domain towards the following transmembrane domain. The CRD is approximately 80 amino acids long and consists of nine crucial cysteine residues, eight of them are connected by disulfide bridges (Muto et al. 2007). The CRD plays a central role, since it transmits the conformational changes induced by orthosteric agonist binding from the Venus flytrap domain to the transmembrane and C-terminal domains. In fact, crystallization and mutagenesis studies have demonstrated that the signal induced by ligand binding is carried from the VFD through the CRD, because the ninth CRD cysteine form a disulfide bridge with a cysteine in lobe 2 of the Venus flytrap domain (Muto et al. 2007; Rondard et al. 2006). Moreover, when the VFD cysteine involved in the disulfide bridge with the CRD is mutated, as reported by for mGluR2 and mGluR5, the receptor can dimerize and bind orthosteric ligands appropriately, but it is not able to propagate intracellular signaling; however when an allosteric ligand binds to the transmembrane domain, it can induce a normal signal transduction (Rondard et al. 2006; Niswender & Conn 2010). In addition, it has been shown that the truncation of the mGluR5 extracellular domain did not affect the constitutive activity of the receptor, suggesting the involvement of transmembrane and C-terminal domains in the receptor basal activity (Goudet et al. 2004).

1.3.3 The Transmembrane Domain

The Transmembrane Domain (TMD) of metabotropic glutamate receptors, as all others GPCRs, holds seven spanning membrane α-helices (TM1-7), in which the allosteric binding site resides. Although mGluRs share low homology with family A GPCRs, for long time structures were proposed on the basis of crystallization of rhodopsin-like receptors. It has been demonstrated that the central portion of the second Intracellular Loop (ICL2) regulates

G-protein-coupling specificity, since it recognizes the extreme C-terminal end of the α-subunit (Havlickova et al. 2003). ICL2 (about 30 residues) is the most variable loop and is regulated by kinases, among them G-protein-coupled Receptor Kinase 2 (GRK2), involved in the desensitization of the receptor (Gomeza et al. 1996; Pin et al. 1994; Dhami et al. 2004). The third intracellular loop (ICL3) is the most conserved one, it activates G-proteins and, together with ICL1 and C-terminus of the receptor, controls the effective interaction with G-protein. Moreover, ICL3 represents the binding site of allosteric modulators, both positive and negative (De Blasi et al. 2001; Jeffrey Conn et al. 2009). The crystal structure of the transmembrane domain has been solved really recently (Doré et al. 2014) and the number of the residues are based on the Ballesteros-Weinstein scheme, modified subsequently by Pin and co-workers. The Ballesteros-Weinstein numbering nomenclature was thought for GPCRs and is composed of two numbers: the first one refers to the helix (1-7), the second one is attributed starting from to the most conserved residue (50) in each helix (Ballesteros & Weinstein 1995). Later, Pin and colleagues modified this notation, using as reference position other residues: the central glycine for TM1, the central phenylalanine for TM2, the lysine close to the end of TM3, the tryptophan at the end of TM4, the central leucine in TM5, and the central tryptophan of TM6. The only index position that has been maintained is the proline in the NPxxY motif of TM7 (Pin et al. 2003). The residues of the transmembrane domain do a plethora of interactions among themselves, important to allosteric modulators binding, to stabilize active or inactive conformation, to transduce the signal from the VFD to the intracellular domain. Cys $644^{3.29}$ at the N-terminus end of TM3 stabilizes a conserved disulfide bridge with Cys 733 in the second extracellular loop (ECL2), forming a critical bond in anchoring ECL2. In turn, ECL2 interacts with ECL1, with one residue in the N-terminal of TM1 (Tyr 730 of ELC2 to Asp 577 of TM1), with two residues in the C-terminal of TM2 (Val 729 to Ala $637^{2.58}$ and Leu 731 to Leu $635^{2.56}$), and with one residue in TM3 (Leu 731 to Gln $647^{3.32}$). Moreover, the position of ECL2, which spans to the top of the receptor, is critical since it hinders the entrance to the allosteric site (Figure 1.9).

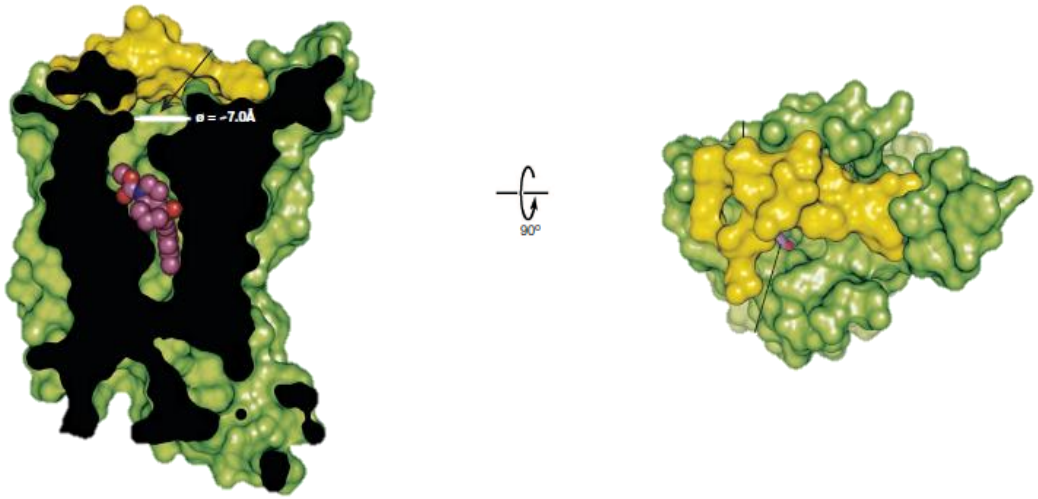


Figure 1.9. Allosteric binding pocket entrance. The configuration of the helical bundle and the position of Extracellular Loop 2 (ECL2, in yellow) combine to restrict the entrance to the allosteric pocket. Surface representation of the mGluR5 transmembrane domain view in parallel to the membrane plane, on the left, and view from the extracellular space on the right (Doré et al. 2014).

An important feature that characterizes the inactive state is the so-called "ionic lock", i.e. interhelical interactions (Figure 1.10). In mGluR5, as in the other GPCRs, the ionic lock is made by a salt bridge of 2.7 Å of distance between Lys $665^{3.50}$ in TM3 and Glu $770^{6.35}$ in TM6. Additionally, Lys $665^{3.50}$ interacts to Ser 613 in ICL1 (2.4 Å of distance), reinforcing the block. A second lock connects TM6 to TM3 by means of ICL1, because Arg $668^{3.53}$ binds to the Ser 614 in ICL1 (3.1 Å of distance). Conversely, to enhance constitutive activity, it is sufficient to substitute Ser 613, Glu $770^{6.35}$ or Lys $665^{3.50}$ with an alanine. As well, changing Ser 613 with a lysine results in charge repulsion with Lys $665^{3.50}$, lead to a constitutive activation of the receptor. Instead, substituting Ser 614 with an aspartic acid, the hydrogen bond is substituted by a strong ionic interaction with Arg $668^{3.53}$, increasing the second lock. The crystal structure of TMD reveals also that two residues in the seventh helix (Pro $820^{7.50}$ and Lys $821^{7.51}$) are highly conserved across family C of GPCRs (Doré et al. 2014).

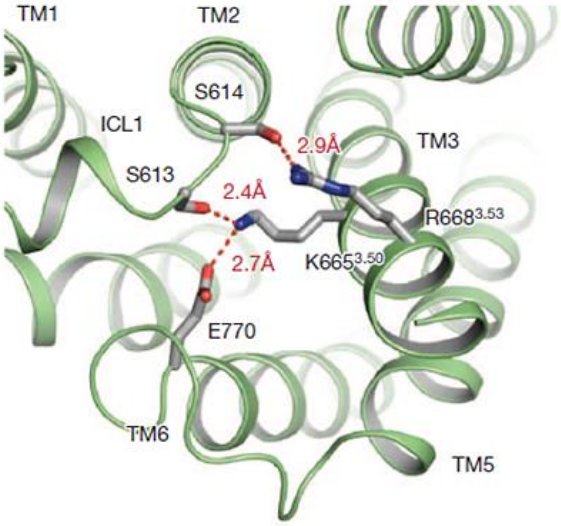


Figure 1.10. The ionic lock motif of mGluR5. The ionic lock represents the interhelical interactions that occur in the inactive state if the receptor to hinder the allosteric binding site. The salt bridge is made by. Lys $665^{3.50}$ in TM3 and Glu $770^{6.35}$ in TM6. Additionally, the block I reinforced by the interaction between Lys $665^{3.50}$ and Ser 613 in ICL1. Carbon is shown in grey, nitrogen in blue and oxygen in red (Doré et al. 2014).

1.3.4 The C-terminal Domain

The C-Terminal Domain (CTD) of metabotropic glutamate receptors faces to the inner part of the cell. CTD represents an important region for modulating G-protein coupling and is the site of many phosphorylations and protein-protein interactions (Niswender & Conn 2010). Uematsu and colleagues have recently demonstrated that protein kinase A (PKA) directly phosphorylates Ser 870, a residue located within the Calmodulin (CaM) binding site and close to protein kinase C (PKC) site (Ser 839) and to the $G_{\alpha q/11}$-protein-coupling region in the C-terminus (Lys 827-Lys 850) (Uematsu et al. 2015; Mao et al. 2008). Ser 870 is phosphorylated under physiological conditions in neostriatal neurons, but when intracellular cAMP levels increase, hyperphosphorylation occurs. Moreover, administration of DHPG 100 μM in HEK293T cells increased ERK1/2 phosphorylation and consequently induced calcium oscillations, whereas in cells in which Ser 870 is substituted with an alanine, phosphorylation can not occur and ERK signaling is compromised (Uematsu et al. 2015).

Besides PKA, PKC is involved in the phosphorylation of several residues. The major PKC phosphorylation site on C-terminal domain is Ser 901, close to calmodulin binding site, with whom it competes for the binding site. In fact, it is known that if CaM binds to C-terminal region, PKC can not phosphorylate Ser 901; reciprocally, if PKC is bound, CaM binding is inhibited (Minakami et al. 1997; Lee et al. 2008) (Figure 1.11). Furthermore, Ser 901

phosphorylation by PKC decreased mGluR5 surface expression, suggesting the involvement of PKC in mGluR5 trafficking, while calmodulin overexpression increased mGluR5 surface expression (Lee et al. 2008).

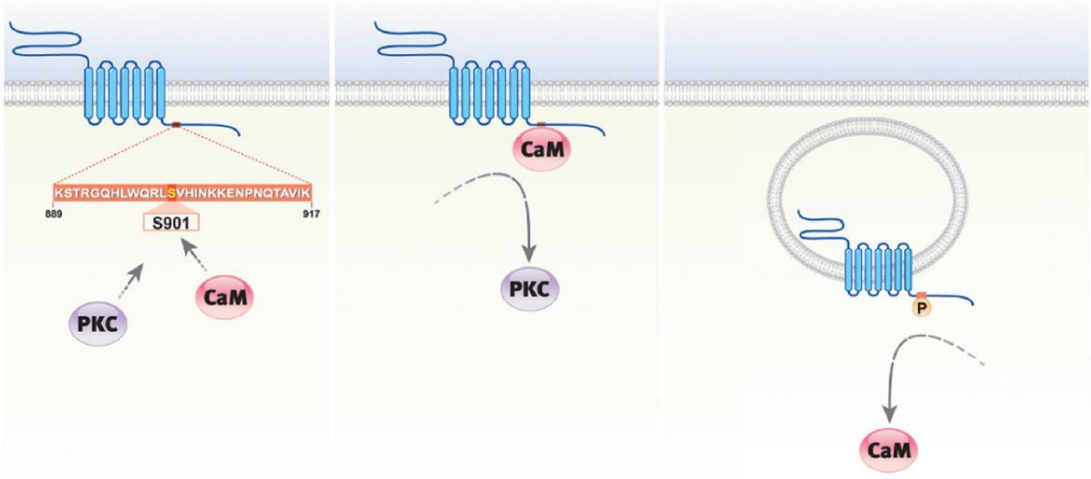


Figure 1.11. Model of PKC–CaM regulation of mGluR5 surface expression. Protein Kinase C (PKC) and Calmodulin (CaM) compete for the same residue, Ser 901, determining mGluR5 trafficking (on the left). The binding of CaM to Ser 901 stabilizes receptor surface expression (in the middle), while PKC phosphorylation of Ser 901, inhibiting CaM binding, decreases receptor surface expression (on the right) (Lee et al. 2008).

PKC phosphorylates also Ser 839, inducing calcium oscillations (Kawabata et al. 1996), Thr 681, implicated in G-protein coupling in ICL2 (Francesconi & Duvoisin 2000), Thr 606, Ser 613, Thr 665, Ser 881 and Ser 890 linked to receptor desensitization (Gereau & Heinemann 1998).

The first part of C-terminal tail contains a conserved motif of 5 amino acids (KSVSW or KSVTW), that is recognized by the catalytic isoform of protein phosphatase 1 (PP1) (Croci et al. 2003), while the last residues of the C-terminus bind to the scaffolding protein Tamalin (Kitano et al. 2002). In addition, C-terminal portion interacts also with several scaffolding proteins, which physically tether together different proteins, generating macromolecular signal complexes (Figure 1.12). One of the earliest mGluR5 interactor identified was Homer-1a (Brakeman et al. 1997). It belongs to the family of Homer proteins, subdivided in long Homers (Homers 1b, 1c, 2, 3) and short Homer (Homer 1a), which share the binding consensus sequence PPxxFR. Long Homer proteins have a C-terminal coiled coil domain and two leucine zipper motifs necessary for the dimerization and the formation of multiprotein complexes (Tadokoro et al. 1999; Sun et al. 1998); short Homers, instead, lack the coiled coil

domain and act as dominant negative proteins (Xiao et al. 2000; Fagni et al. 2002). Homer isoforms can also interact with other proteins, such as SH3, multiple ankyrin repeat domains (Shank) and Preso1 (Piers et al. 2012), affecting molecular signaling of mGluR5. In addition, Homer proteins are also involved in the localization and trafficking of the receptor (Thomas 2002).

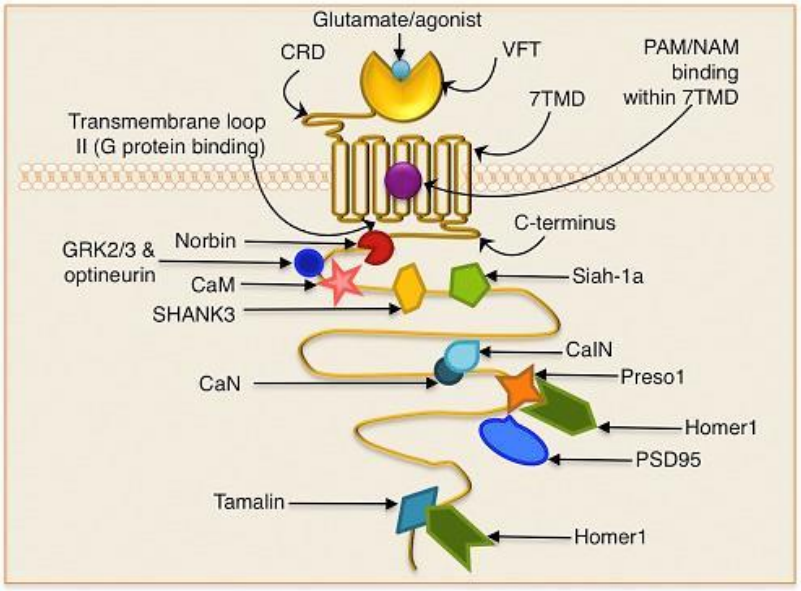


Figure 1.12. Schematic representation of mGluR5 proteins that interact directly with C-terminal domain. The intracellular CTD of mGluR5 is the site of many protein–protein interactions, including binding of Norbin, G-protein Regulating Kinases 2/3 (GRK2/3), Calmodulin (CaM), SH3 and multiple ankyrin repeat domain 3 (SHANK3), Calcineurin (CaN), Preso1, Homer1, and Tamalin (Matosin et al. 2017).

Scaffolding proteins are involved also in the physical association between mGluR5 and NMDA Receptors (NMDARs) (Tu et al. 1999) (Figure 1.13). Furthermore, mGluR5 are biochemically coupled to NMDAR via PKC (Niswender & Conn 2010). As a consequence of these molecular and biochemical interactions, mGluR5 activation by the agonist DHPG enhances NMDAR functionality (Gregory et al. 2011; Won et al. 2012), while the administration of negative allosteric modulators, such as MPEP, decreased NMDA function (O'Leary et al. 2000).

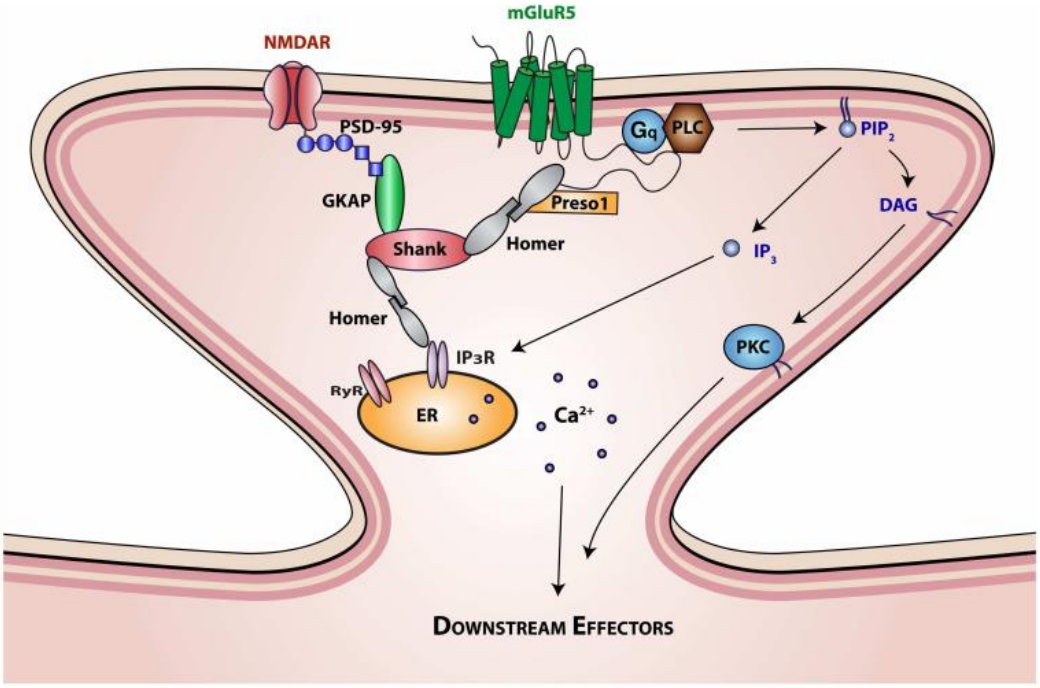


Figure 1.13. Interaction of mGluR5 and NMDAR. Metabotropic Glutamate Receptor 5 and N-Methyl-D-Aspartate Receptor are biochemically and physically linked together. The scaffolding proteins interacting with mGluR5 CTD interact also with NMDAR. It follows that the activation of mGluR5 causes the activation of NMDAR, likewise mGluR5 inhibition leads to NMDAR inactivation (Piers et al. 2012).

1.4 Signaling

Group II and III metabotropic glutamate receptors are coupled mainly to $G_{i/o}$ proteins, inhibiting Adenylyl Cyclase (AC), while group I mGluRs couple to $G_{\alpha q}/G_{11}$ and activate phospholipase Cβ (PLCβ), leading to the hydrolysis of phosphoinositides (PIPs) and formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). This pathway causes calcium mobilization and activation of protein kinase C (PKC) (Yin & Niswender 2014) (Figure 1.14).

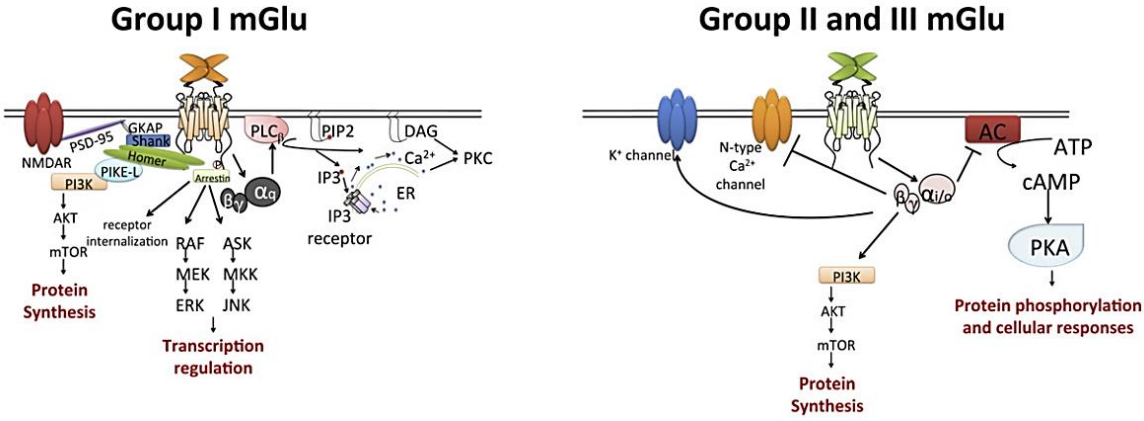


Figure 1.14. Schematic signaling of metabotropic glutamate receptors. Metabotropic glutamate receptors are G-protein coupled receptors. Group I receptors (mGluR1 and mGluR5) are coupled to $G_{\alpha q}/G_{11}$ and, through phosphoinositides hydrolysis (PIPs), formation of Inositol 1,4,5-trisphosphate (IP3) and Diacylglycerol (DAG), activate protein kinase C (PKC) and the kinases pathway (on the left). Group II and III receptors, instead, are coupled to $G_{i/o}$ proteins, inhibiting Adenylyl Cyclase (AC) (on the right) (Yin & Niswender 2014, modified).

mGluR5-generated calcium responses can be oscillatory or transient, according to the cell type in which the receptor is activated: in heterologous cells, hippocampal and spinal cord neurons the activation of mGluR5 leads to oscillatory responses, while in striatal neurons a fast transient peak occurs, followed by a sustained increase (Flint et al. 1999; Romano et al. 2001; Kettunen et al. 2002; Jong et al. 2005). mGluR5-mediated calcium increase activates several downstream signaling pathways, among them calmodulin (CaM), which in turn regulates CaM-dependent kinases (CaMKs), such as CaMKII and CaMKIV, altering gene transcription and translation (Wang & Zhuo 2012). CaMK activation upregulates cAMP-Responsive Element Binding protein (CREB), serum response factor and histone deacetylase (Swulius & Waxham 2008). Moreover, it has been shown that also elongation Factor 2 Kinase (eF2K) is upregulated, it phosphorylates eF2, inhibiting protein synthesis and, at the same time, upregulating translation of specific synaptic products (Park et al. 2008). Besides CaMK effectors, mGluR5 induces Mitogen-Activated Protein Kinase (MAPK) pathways, i.e. the Extracellular signal-Regulated Kinase (ERK1/2) pathway, the p38 MAPK pathway and the c-Jun N-terminal Kinase/Stress-Activated Protein Kinase pathway (JNK/SAPK) (Wang et al. 2007). In addition, transcription and translation can be modulated also by MAPK signaling, since MAPK activates ETS-domain transcription factor (Elk-1), CREB, activator protein 1, activating transcription factor 2, c-Jun, c-Rel and Nuclear Factor κ-light-chain-enhancer of activated B cells (NF-κB) (Yang & Sharrocks 2006; Wang et al. 2007; Gladding et al. 2009), and ERK1/2 pathway helps the formation of the eukaryotic translation initiation factor 4E

(eIF4E) complex, necessary for translation (Banko et al. 2006). mGluR5 is able to activate also the enzyme phosphoinositide 3-kinase (PI3K), which stimulate downstream kinases, such as protein kinase B (PKB, also known as AKT) and phosphoinositide-dependent kinase 1 (PDK 1) (Rong et al. 2003; Ronesi & Huber 2008; Franke et al. 1997; Chan et al. 1999; Vanhaesebroeck & Alessi 2000). These kinases activate in turn the mammalian target of rapamycin (mTOR), that leads to protein translation initiation (Hou & Klann 2004) (Figure 1.15).

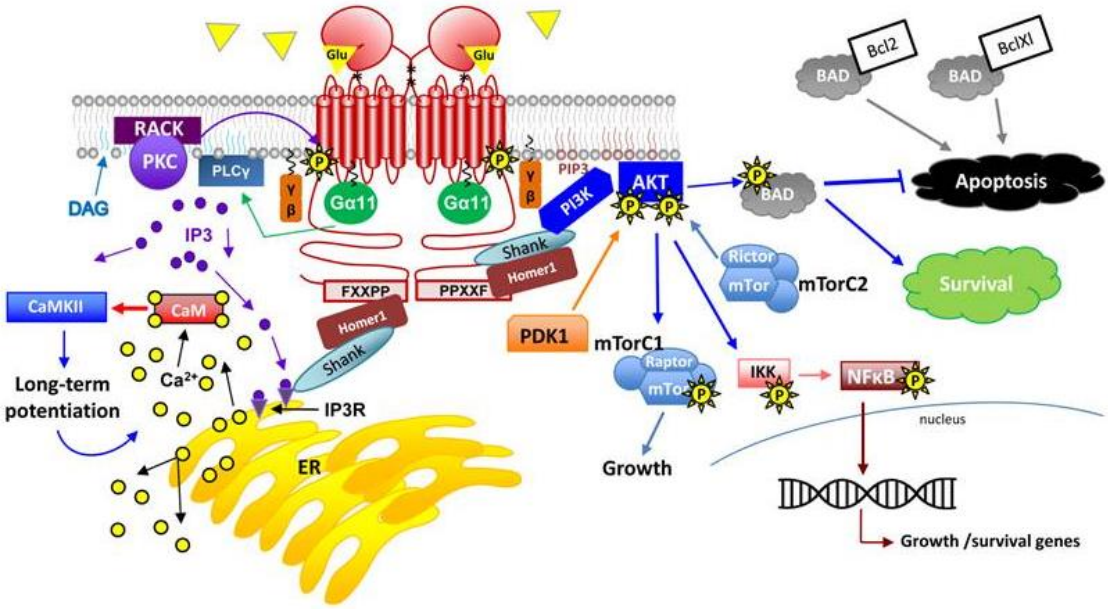


Figure 1.15. mGluR5 signaling pathway. When glutamate binds to the Venus flytrap domain, the receptor is in its active state. The downstream cascade begins with G-protein dissociation, then the second messenger systems are activated: PKB, PLCγ, PI3K/AKT/mTorC1 and 2, IP3/DAG, NF-κB and CaM (Willard & Koochekpour 2013).

1.5 Desensitization, Endocytosis & Trafficking

Metabotropic glutamate receptor 5 responsiveness is affected at G-proteins level as well as at receptor level. Concerning G-protein, receptor activity is attenuated by a family of proteins called Regulators of G protein Signaling (RGS), able to distinguish specific members of G-protein α subunits. For instance, RGS4 interacts with $G_{\alpha q/11}$ proteins, accelerating GTPase activity of α subunits of heterotrimeric G-proteins and preventing the subsequent activation of PLC (Schwendt & McGinty 2007; Dhami & Ferguson 2006). However, shortly after exposure to an agonist, mGluR5 desensitizes. The typical model for desensitization

involves GPCR Kinase (GRK) 2-mediated phosphorylation, which induces β-arrestins binding, separating receptors and heterotrimeric G proteins (Ferguson 2001; Sorensen & Conn 2003). In addition, also PKC is implicated in receptor desensitization: PKC directly phosphorylates serine and threonine residues, antagonizing Calmodulin binding and inhibiting signal cascade (Dhami & Ferguson 2006; Minakami et al. 1997).

The receptor internalization (or endocytosis) that ensues agonist desensitization, called also homologous desensitization, can occur by both clathrin-dependent or independent pathways (Dhami & Ferguson 2006) (Figure 1.16). However, metabotropic glutamate receptor 5, like many others GPCRs, undergoes also constitutive endocytosis, that means that it is internalized also in absence of ligands or inverse antagonists, such as MPEP, although this process is enhanced in presence of ligands. It has revealed that, at 37°C, mGluR5 time course of endocytosis had a time constant of 8.5 min, with a endocytic rate of 11.7%/min (Fourgeaud et al. 2003). Besides it has been established that mGluR5 endocytosis is a clathrin-independent process (Fourgeaud et al. 2003), the most plausible internalization route resulted from caveolar/lipid raft signaling (Francesconi et al. 2009). Lipid rafts and caveolae, in association with cholesterol and glycosphingolipids, are specialized membrane microdomains (Simons & Ikonen 1997). According to Francesconi and colleagues model, mGluR5 associate with lipid rafts and its constitutive internalization is partially facilitated by caveolar/raft-dependent endocytosis (Francesconi et al. 2009).

Ensuing constitutive endocytosis, mGluR5 is localized in the recycling compartments, before reused again to the cell surface in about 3.5 hours. Analysis revealed that, most of times, mGluR5 is not present in lysosomes and the receptor is recycled and not synthetized *de novo* (Trivedi & Bhattacharyya 2012).

mGluR5 ability to traffic towards the plasmatic membrane varies radically for truncated forms of the receptor. Truncation of the large C-terminal tail did not impede plasmatic membrane expression, whereas it has been shown that the transmembrane domain was fundamental. In fact, mGluR5 truncated after the first, second or seventh transmembrane helices trafficked efficiently to the cell surface in heterologous cells. Conversely, mGluR5 truncation of TM5 or TM6 fully caused the failure of the receptor to traffic to the plasmatic membrane both in heterologous cells and neurons (Chang & Roche 2017).

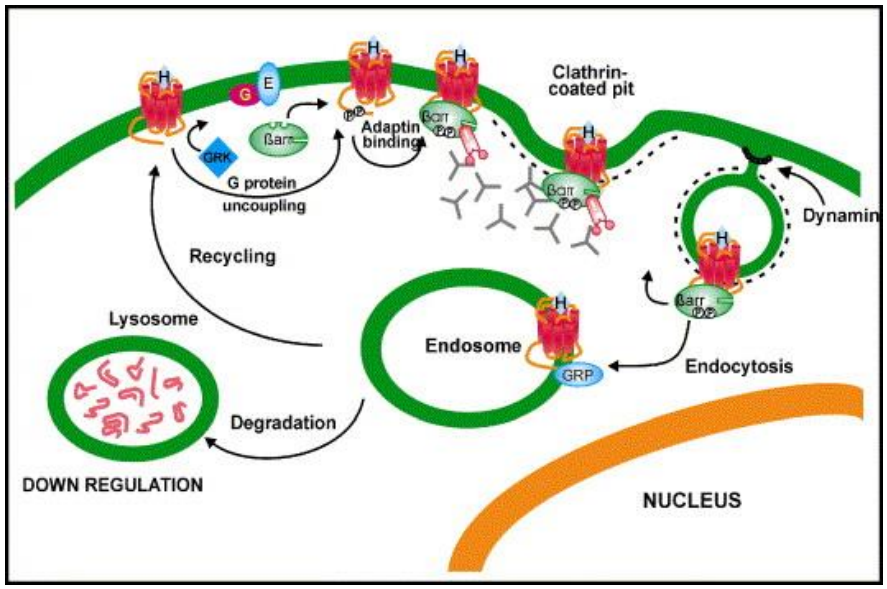


Figure 1.16. Diagram of GPCR desensitization and resensitization following agonist activation. Shortly after exposure to an agonist, mGluR5 desensitizes, by both clathrin-dependent or independent pathways. Moreover, metabotropic glutamate receptor 5 undergoes also constitutive endocytosis, that means that it is internalized also in absence of ligands or inverse antagonists. After internalization, the receptor is usually recycled, or it is degraded by lysosomes (Dhami & Ferguson 2006).

For intracellular mGluR5, instead, little is known. Although there might be some sequences that address the receptor in the appropriate localization, these nuclear localization signal motifs sequences are recently discovered only for mGluR5 directed to Inner Nuclear Membrane (INM). This signal motif, 25 amino acids long found at C-terminal tail, represents a new motif sequence for INM trafficking. In addition, once at the INM, mGluR5 is stably retained in this region via interactions with chromatin (Sergin et al. 2017).

1.6 Ligands

Metabotropic glutamate receptor 5 possesses two main binding pockets: the orthosteric site, located in the Venus flytrap domain, and the allosteric pocket, placed within the seven helices of the transmembrane domain. In both pockets molecules that can activate (agonist) or inactivate (antagonist) the receptor bind to it, triggering or inhibiting, respectively, the downstream molecular signaling (Spooren et al. 2001) (Figure 1.17).

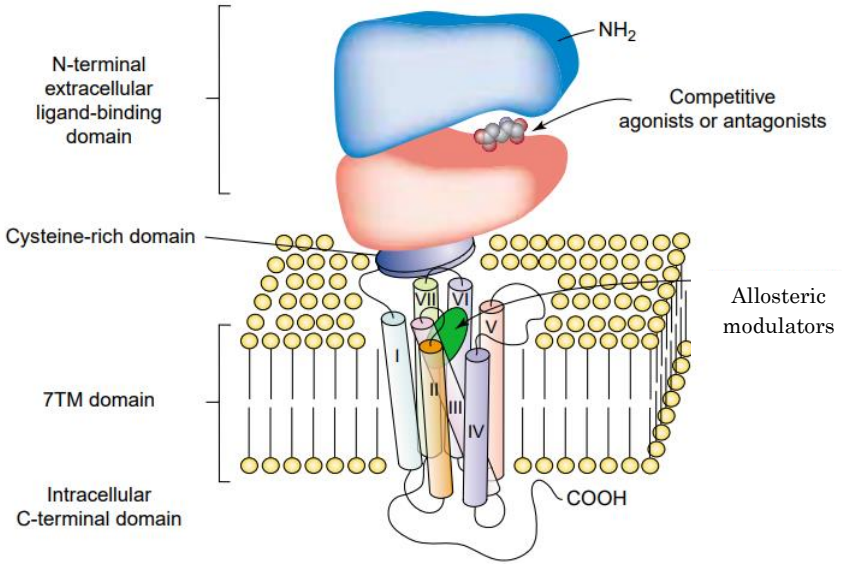


Figure 1.17. Representation of mGluR5 binding sites. The orthosteric binding site is placed within the two lobes of the Venus flytrap domain of the extracellular N-terminal domain. Conversely, the allosteric binding site is located within the transmembrane domain (Spooren et al. 2001, modified).

1.6.1 Orthosteric binding pocket

The identification of orthosteric ligands for mGluR5 has presented and presents still now some hindrances. It has been documented that glutamate can target ionotropic as well metabotropic receptors (Ferraguti & Shigemoto 2006). It is known that glutamate interacts with eight different metabotropic receptors. The crucial residues in the orthosteric binding site of group I metabotropic glutamate receptors share 100% of homology (Table 1.2), highlighting the difficulty to identify a selective agonist for a specific subtype. In addition, the superimposition of the crystal complex formed by L-Glutamate and mGluR1 to the L-Glutamate and mGluR5 complex revealed that the orthosteric sites were identical (Mølck et al. 2014) (Figure 1.18).

Subtype	64	68	100	150	151	152	173	174	175	223	305	306	310	328	394	396
mGluR1	Y	R	W	G	S	S	S	A	T	Y	D	G	R	K	D	K
mGluR5	Y	R	W	G	S	S	S	A	T	Y	D	G	R	K	D	K

Table 1.2. Sequence alignment of the orthosteric site of group I metabotropic glutamate receptors. The orthosteric binding site of mGluR1 and mGluR5 share 100% homology in the amino acid sequence (Mølck et al. 2014, modified).

About mGluR5 orthosteric binding site, the side chains of L-Glutamate makes polar interactions with Ser 152, Thr 175 and Asp 305; also the backbone atoms are involved in polar interactions with Ser 152, Ser 173 and Thr 175; the acidic moiety interacts with both conserved (Arg 68 and Lys 396) and non-conserved polar residues (Tyr 64 and Ser 173).

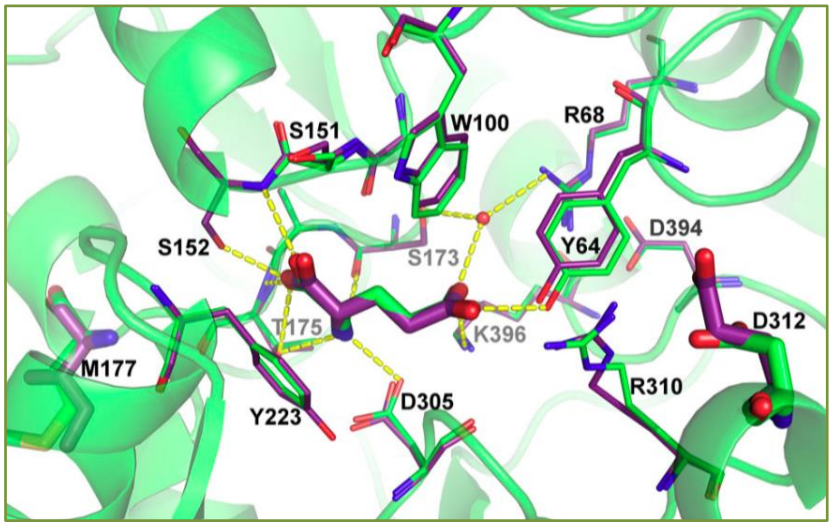


Figure 1.18. Superimposition of mGluR1 and mGluR5 structures interacting with glutamate. The overlapped crystal structures of mGluR1 (in purple) and mGluR5 (in green) showed the residues involved in the interaction between the glutamate and the receptor. Only two residues differ between mGluR1 and mGluR5 (in bold stick). Polar interactions are indicated in yellow dotted lines (Mølck et al. 2014).

1.6.1.1 Orthosteric agonists

Quisqualic acid (Quis) was the first identified agonist for metabotropic glutamate receptors (Figure 1.19). At low concentrations, it demonstrated higher potency and selectivity for group I mGluRs (potency or EC_{50}=0.03-3 μM for mGluR1 and EC_{50}=0.02-0.3 μM for mGluR5) (Schoepp et al. 1999a) when compared with the other two groups, while, at higher concentrations, it activated mGluR3 (EC_{50}=40-220 μM) (Palmer et al. 1989; Sacaan et al. 1998). In addition, quisqualate interacted also with ionotropic glutamate receptors, showing potent agonism for AMPA receptors (Watkins et al. 1990).

A second agonist able to stimulate phosphoinositide hydrolysis mediated by mGluR1 and mGluR5 was *(1S,3R)*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) (Palmer et al. 1989; Manzoni et al. 1990; Darryle D. Schoepp et al. 1991; Schoepp & Hillman 1990) (Figure 1.19). Among the five *cis* conformations and the four *trans* conformations (Tückmantel et al. 1997), the only isomer that owned agonist activity was *(1S,3R)*-ACPD (Irving et al. 1990; D

D Schoepp et al. 1991; Cartmell et al. 1993; Schoepp et al 1992). Although ACPD activated all mGluRs, it was the first agonist capable to discriminate from ionotropic receptors, at concentrations of up to 100 μM (Schoepp et al. 1999b).

(R,S)-3,5-dihydroxyphenylglycine (DHPG) is the most popular group I selective agonist (Figure 1.19). It was synthetized starting from 3,5-dihydrobenzaldehyde (Christensen et al. 1983). Between the two enantiomers, it was determined that *(S)*-3,5-DHPG was ten-fold more potent than *(R)*-3,5-DHPG in stimulating phosphoinositide hydrolysis (Richard Baker et al. 1995). The potency of *(S)*-3,5-DHPG for the different metabotropic glutamate receptors subtypes has been characterized: mGluR1a=6.6 μM, mGluR5a=2 μM, mGluR2,4,7,8>1000 μM, mGluR3=106 μM (Conn & Pin 1997).

The docking studies of DHPG-mGluR5 complex have identified the interacting amino acid residues in the binding pocket of the receptor. The phenyl ring of DHPG makes π-π interactions with Trp 100, one hydroxyl group of DHPG interacts with Arg 310, whereas the other one makes hydrogen bonds with Tyr 64, Lys 396 and with the water molecule situated near Arg 64, the same residues involved in the binding between the acidic moiety of glutamate and the receptor (Mølck et al. 2014).

Recent studies reported that, under certain conditions, *(S)*-3,5-DHPG could interact with NMDA receptors. However, these data appear controversial. In fact, from one hand, *(R,S)*-3,5-DHPG (300 μM) did not potentiate NMDA response, and the current triggered by NMDA receptors was reversibly reduced by the administration of 100 μM *(S)*-3,5-DHPG (Yu et al. 1997; Contractor et al. 1998). From the other one, at concentrations ranging from 1-10 μM, *(S)*-3,5-DHPG strengthened NMDA-induced depolarization in murine CA1 region of the hippocampus (Fitzjohn et al., 1996; Attucci et al., 2001). Furthermore, isolated cord neurons treated with 50-100 μM *(S)*-3,5-DHPG showed potentiated NMDA-induced calcium responses (Krieger et al. 2000). In addition, *(S)*-3,5-DHPG appeared to act as a co-agonist at the glycine site of NMDA receptor, potentiating NMDA responses (Contractor et al. 1998).

Successively, another phenylglycine derivative was developed, starting from 2-chloro-5-benzoyloxybenzaldehyde: *(R,S)*-2-chloro-5-hydroxyphenylglycine (CHPG) (Figure 1.19). Differently from DHPG, Doherty and colleagues demonstrated that CHPG activates mGluR5a, but not mGluR1a, showing selectivity within the group I. However, like DHPG, CHPG potentiates NMDA-induced depolarization in hippocampal CA1 neurons (DOHERTY et al. 1997). Nevertheless, recently, Kammermeier showed that CHPG was able to activate mGluR1a and mGluR1b with similar potency and efficacy (Kammermeier 2012).

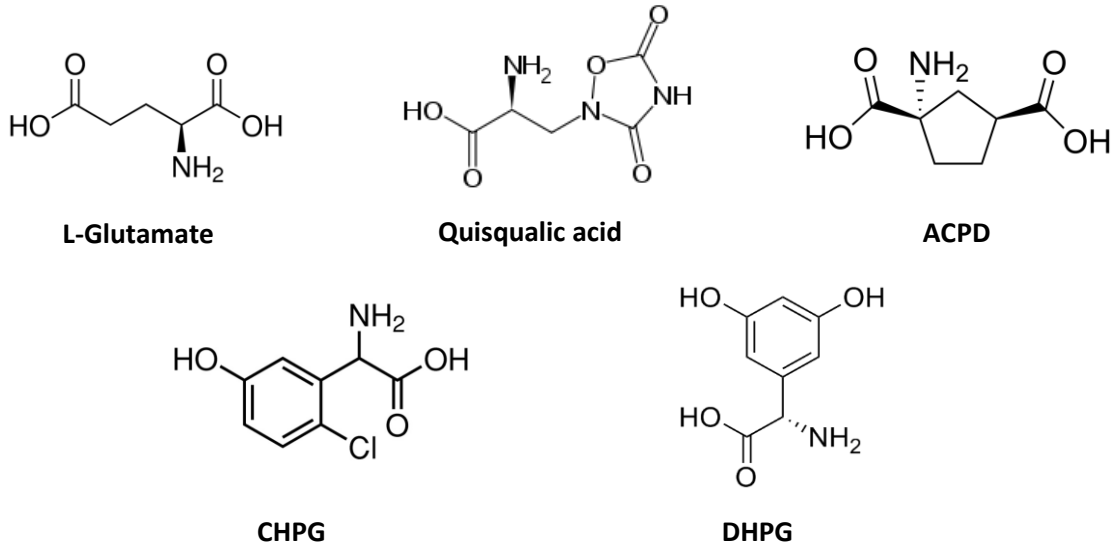


Figure 1.19. Orthosteric agonist of mGluR5. Chemical structures of several mGluR5 orthosteric agonists. Starting from the top: L-glutamate (Glu), quisqualic acid (Quis), 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), 2-chloro-5-hydroxyphenylglycine (CHPG) and dihydroxyphenylglycine (DHPG).

1.6.1.2 Orthosteric antagonists

The orthosteric antagonists, as previously outlined, are those molecules that bind to the receptor in the same site in which the endogenous ligand binds to, inhibiting the downstream signaling cascade.

For group I metabotropic glutamate receptors, the first selective competitive antagonists described were phenylglycine derivatives. *(S)*-4-carboxyphenylglycine (*(S)*-4-CPG) showed a more potent antagonism activity for mGluR1 (IC_{50}=20–80 mM), compared with mGluR5 (IC_{50}>500 mM). Also α-methyl-4-carboxyphenylglycine ((*S*)-4-MCPG) demonstrated to be a more potent antagonist for mGluR1 (IC_{50}=20–500 μM) and a less potent one for mGluR5 (IC_{50}=200–1000 mM). However, both *(S)*-CPG and *(S)*-MCPG antagonized all groups of mGluRs (Schoepp et al. 1999b; Bräuner-Osborne et al. 2000). *(S)*-4-carboxy-3-hydroxyphenylglycine (*(S)*-4-C3HPG) inhibits mGluR1, but it is a partial agonist for mGluR5 (Brabet et al. 1995) and an agonist for mGluR2 (Bräuner-Osborne et al. 2000). It was observed that only the *S* isoform owned the mGluRs antagonist activity, whereas the *R* forms antagonized NMDA receptors (Birse et al. 1993; Eaton et al. 1993).

Besides, it has been observed that the ability of phenylglycine derivatives to antagonize mGluR5 is dependent on the agonist administered to elicit the functional response, a

phenomenon called agonist-dependent antagonism. For example, *(S)*-4-CPG showed a greater antagonist activity on *(S)*-3,5-DHPG-triggered calcium release in Chinese Hamster Ovary (CHO) cells than of L-glutamate-mediated release. Vice versa, α-cyclopropyl-4-CPG (*(R,S)*-CyCPG) was found to have a higher ability to antagonize L-glutamate-evoked Ca^{2+} release than that mediated by *(S)*-3,5-DHPG (Doherty et al. 1999).

Subsequently, to enhance the affinity and potency of the compounds, a second generation of group I mGluR antagonists was developed. Substituting the α-methyl group of MCPG with a α-thioxanthylmethyl, Eli Lilly Company synthetized LY 367366. LY 367366, with an IC_{50} ranging between 3-6 μM, competes for both mGluR1 and mGluR5, and with a lower extent for the other mGluRs (IC_{50}>10 mM). Other compounds and mixtures were produced, such as LY 393675 (Schoepp et al. 1999b), LY 393053 (Chen et al. 2000), LY 367385 and LY 339840 (Kingston et al. 2002), but no one showed a strong selectivity for mGluR5.

Although finding an orthosteric agonist selective only for mGluR5 has been emerged so difficult, thanks to new technologies and the continuous discoveries, it looked to be more possible to detect a selective orthosteric antagonist. In fact, because of the antagonists prevent the closure of the VTD and additional non-conserved residues, such as Tyr 311 and Asp 312, appear available in the open-conformation and not in the close one (Topiol et al. 2011). However, to date, any selective competitive antagonist for mGluR5 has been developed yet.

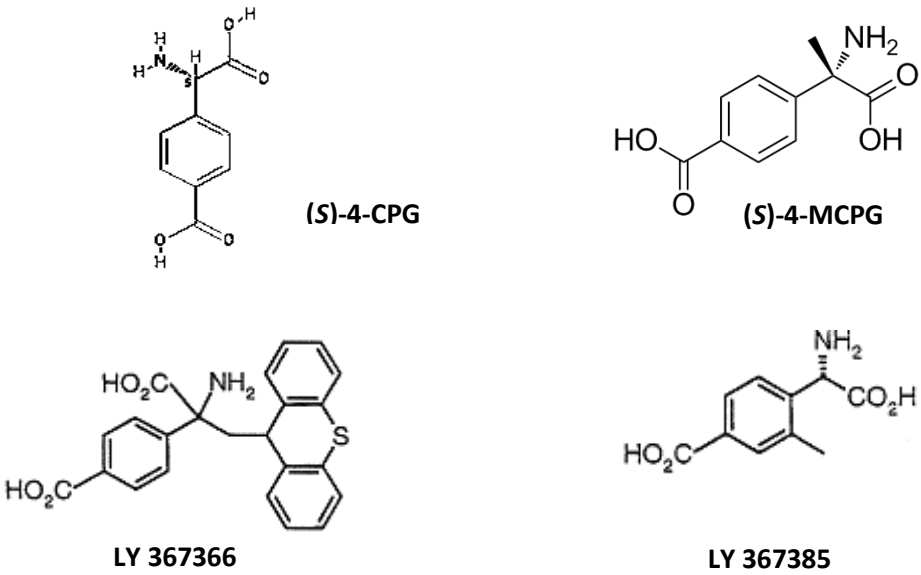


Figure 1.20. Orthosteric antagonist of mGluR5. Chemical structures of multiple mGluR5 orthosteric antagonists. Starting from the top: *(S)*-4-carboxyphenylglycine (*(S)*-4-CPG), *(S)*-α-methyl-4-carboxyphenylglycine (*(S)*-4-MCPG), LY 367366 and LY 367385.

1.6.2 Allosteric binding pocket

Because of the high degree of sequence homology of the orthosteric binding pocket and the subsequent low selectivity of orthosteric ligand, most effort was addressed to target the transmembrane domain, in which allosteric binding site resides (Mølck et al. 2014), because the use of allosteric modulators offers several advantages (Wood et al. 2011a). First of all, allosteric binding sites are less conserved than orthosteric ones, thus high subtype selectivity is more easily accomplished. Secondly, the effects of allosteric modulators are saturable and once allosteric pockets are occupied, no further effects are detected. Thirdly, allosteric modulators are effective only in presence of the endogenous or an orthosteric ligand, resulting in temporal and spatial activity of the endogenous ligand. Fourthly, chemical tractability is improved.

The first mGluR5 allosteric ligands to be discovered were Negative Allosteric Modulators or NAMs (Gasparini et al. 1999; Varney et al. 1999a). Besides NAMs, also Positive and Silent Allosteric Modulators (PAMs and SAMs, respectively) were designed.
Negative allosteric modulators act inhibiting receptor response; on the contrary, PAMs potentiate the ability of orthosteric ligand to activate the receptor. SAMs, instead, do not affect receptor functionality, but compete for the binding of other allosteric modulators, preventing NAM-induced receptor inhibition or PAM-induced receptor potentiation. Further research lead to the development of the so called “ago-PAMs”, that are positive allosteric modulators with intrinsic allosteric agonist activity (Noetzel et al. 2012).

To discriminate whether an allosteric modulator binds to mGluR5 or mGluR1, mutational studies were performed. Mutations in transmembrane helices 3, 5, 6 and 7 revealed overlap of the allosteric binding pockets of the two subtypes (A Pagano et al. 2000). In fact, NAM-mediated receptor inhibition can occur when one of eight conserved residues in mGluR5 or one of 4 conserved residues in mGluR1 were mutated, such as Phe 788 in mGluR5 and Phe 801 in mGluR1 or Tyr 792 in mGluR5 and Tyr 801 in mGluR1. In mGluR5 the mutation of Trp 785 TM6 to Ala affects mGluR5 NAMs, although mGluR1 NAM binding was not altered by the same mutations. This suggests that there might be significant differences in how NAMs bind to the subtype receptor, mainly due to the non-conserved residues. For example, mGluR5 NAMs interact deeper in the binding pocket with Trp 785 and

Thr 781, while mGluR1 NAMs bind higher in the allosteric pocket, to Thr 815 and Ala 818 (Mølck et al. 2014).

Mülhemman and colleagues described for the first time 6 key residues located in the TMD region of mGluR5 which are involved in its allosteric regulation by DFB, a positive allosteric modulator: Ser $657^{3.39}$, Leu $743^{5.47}$, Thr $780^{6.44}$, Trp $784^{6.48}$, Phe $787^{6.51}$ and Met $801^{7.39}$ were crucial in DFB binding (Goudet et al. 2004; Julie A O'Brien et al. 2003; Mühlemann et al. 2006) (Figure 1.21). In fact, mutations of these residues affected importantly DFB binding and effect. The mutation of Leu $743^{5.47}$ in Val or Ala decreased significantly both potency and efficacy of DFB. As well, Thr $780^{6.44}$ to Ala conversion totally blocked DFB effect, whereas mutagenesis of Trp $784^{6.48}$ to Ala or Phe caused an increase of the DFB-mediated potentiation of the receptor. Conversion of Phe $787^{6.51}$ to Ala, instead, transformed DFB into a weak NAM. All these residues were shown to interact also with NAMs, except for Met $801^{7.39}$, suggesting that a partial overlap of DFB and MPEP binding sites occurred.

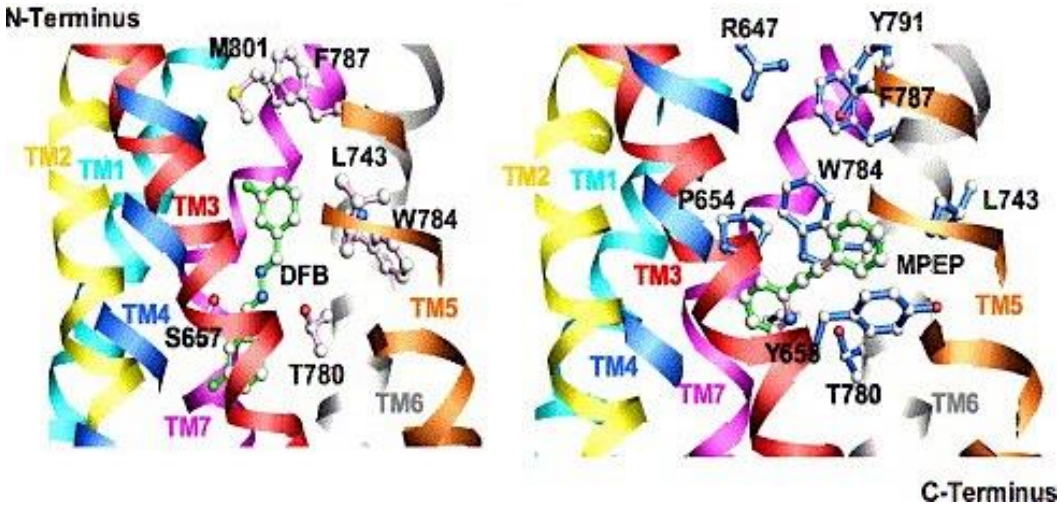


Figure 1.21. mGluR5 allosteric pocket complexed with DFB (A) and MPEP (B). The molecular modelling of the mGluR5 allowed to determine the residues involved in the binding of a positive allosteric modulator, DFB (on the left, in green). However, these residues are the same involved in the interaction between the receptor and the negative allosteric modulator MPEP, except for Met 801 in TM7 (on the right, in green). TM1 (cyan), TM2 (yellow), TM3 (red), TM4 (blue), TM5 (orange), TM6 (grey) and TM7 (magenta) (Mühlemann et al. 2006).

Pagano and colleagues first, and Malherbe and co-workers then, proposed possible interactions between negative allosteric modulators and mGluR5 allosteric pocket (Adriana Pagano et al. 2000; P. Malherbe et al. 2003; Malherbe et al. 2006). In their site-directed

mutagenesis coupled with three-dimensional receptor-based pharmacophore modelling, they compared MPEP and Fenobam, two NAMs with distinct chemotypes. They observed that some residues were common to both NAMs: Pro 654$^{3.36}$, Tyr 658$^{3.40}$, Thr 780$^{6.44}$, Trp 784$^{6.48}$, Phe 787$^{6.51}$, Tyr 791$^{6.55}$ and Ala 809$^{7.47}$. However, Arg 647$^{3.29}$ and Ser 657$^{3.39}$ interacted only with Fenobam, while Leu 743$^{5.47}$ bound exclusively to MPEP (Figure 1.22). The aliphatic hydroxyl side chain of Ser 657$^{3.39}$ could form a hydrogen bond with the 4-oxo group of the imidazole of Fenobam (Figure 1.22).

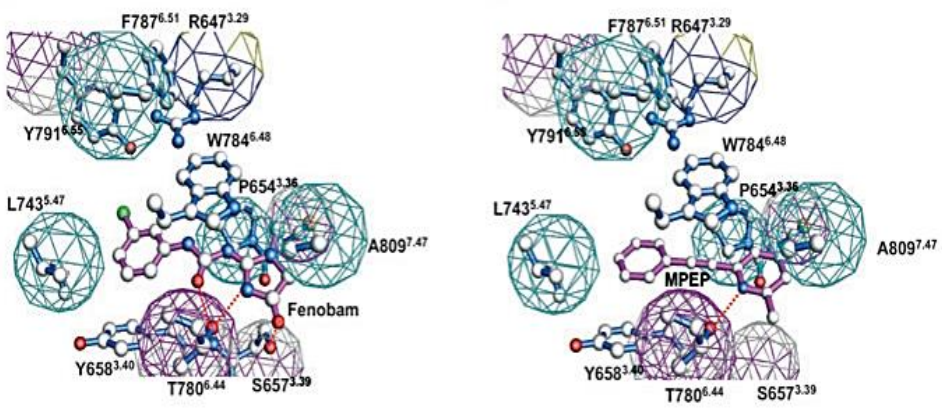


Figure 1.22. Essential residues for Fenobam and MPEP. The two negative allosteric modulators possess two different chemotypes, and interact differently within the allosteric binding pocket. For instance Thr 780$^{6.44}$ binds to both Fenobam (on the left, in magenta) and MPEP (on the right, in magenta), while Ser 657$^{3.39}$ interacts only with Fenobam and Leu 743$^{5.47}$ binds exclusively to MPEP. Hydrogen bonds are visualized by dotted lines (Malherbe et al. 2006).

The mutation of some of these fundamental residues (Arg 647$^{3.29}$ Ala, Pro 654$^{3.36}$ Ser, Ser 657$^{3.39}$ Cys, Tyr 658$^{3.40}$ Val, Thr 780$^{6.44}$ Ala, Trp 784$^{6.48}$ Ala, Phe 787$^{6.51}$ Ala and Ala 809$^{7.47}$ Val) (Figure 1.23) led to the loss of Fenobam binding and consequent failed inhibition of quisqualate-induced intracellular calcium mobilization. The conversion of Pro 654$^{3.36}$ Ser resulted in a reduction in binding affinity also for MPEP by 40-fold and potency by 15-fold. The conversion of Tyr 658 to a Phe increased the affinity and potency of both Fenobam and MPEP, although the mutation had much stronger effect on Fenobam than MPEP, because the chlorine added in Fenobam prompted the stacking interactions. These results and the models suggested that MPEP is surrounded by highly hydrophobic residues, making more p-stacking than H-bonds. Differently, the forces involved in Fenobam binding are equally both H-bonds and p-stacking. This could explain the higher affinity of MPEP for mGluR5 than that of Fenobam (KD 3.1 and 55.5 nM, respectively). The mutation of Trp 784$^{6.48}$ in alanine was

observed to completely inhibit the inverse agonist effect of MPEP, whereas the conversion of Phe 787$^{6.51}$ and Tyr 791$^{6.55}$ to Ala produced an important decrease in the MPEP inverse agonist activity.

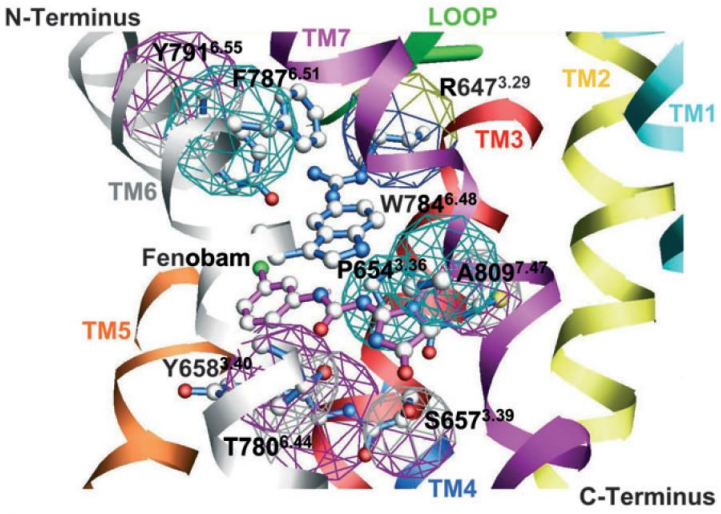


Figure 1.23. Three-dimensional modelling of mGluR5 allosteric pocket complexed with the negative allosteric modulator Fenobam. Overview of Fenobam (in magenta) interactions with the residues in the transmembrane domain. TM1 (cyan), TM2 (yellow), TM3 (red), TM4 (blue), TM5 (orange), TM6 (grey) and TM7 (magenta) (Malherbe et al. 2006).

In 2014, when finally human mGluR5 transmembrane structure was crystallized, residues involved in NAM binding were revealed using mavoglurant (Doré et al. 2014) (Figure 1.24). The 3-methylphenyl ring of mavoglurant occupied a pocket between Ala 810$^{7.40}$ and Pro 655$^{3.40}$, surrounded by Ile 625$^{2.46}$, Gly 628$^{2.49}$, Ser 654$^{3.39}$, Ser 658$^{3.43}$ and Tyr 659$^{3.44}$. The 3-methyl substituent of mavoglurant formed hydrogen bonds with the side chains of Tyr 659$^{3.44}$, Thr 781$^{6.46}$, the main-chain carbonyl of Ser 809$^{7.39}$ and a water molecule at the bottom of the allosteric pocket. The alkyne linker passed through a narrow groove between Tyr 659$^{3.44}$, Ser 809$^{7.39}$, Val 806$^{7.36}$ and Pro 655$^{3.40}$. These interactions partially clarify the prevalence of an alkyne linker in mGluR5 negative allosteric modulators. The saturated bicyclic rings resided within a mainly hydrophobic pocket restricted by Val 806$^{7.36}$, Met 802$^{7.32}$, Phe 788$^{6.53}$, Trp 785$^{6.50}$, Leu 744$^{5.44}$, Ile 651$^{3.36}$, Pro 655$^{3.40}$ and Asn 747$^{5.47}$.

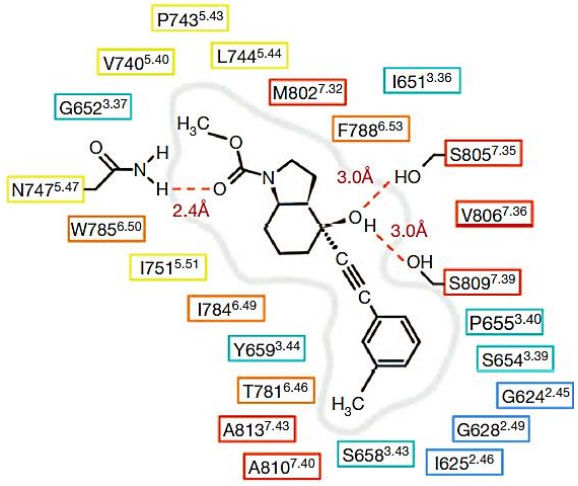


Figure 1.24. The mGluR5 allosteric pocket complexed with Mavoglurant. Interactions between the allosteric binding pocket and the negative allosteric modulator Mavoglurant. Hydrogen bonds are depicted as dashed red lines, the distances between heavy atoms are measured in angstroms (Å) (Doré et al 2014).

1.6.2.1 Positive allosteric modulators

It is well demonstrated that mGluR5 and NDMARs are physically and biochemically linked together, so that Positive Allosteric Modulators (PAMs) of metabotropic glutamate receptor subtype 5 were originally synthetized with the aim of indirectly modulate NMDA receptor function in pathological conditions (Cleva & Olive 2011).

The first mGluR5 PAM to be developed was 3,3′-difluorobenzaldazine (DFB) in 2003 by researchers at Merck, characterized by low potency and solubility in aqueous solutions, and was brain barrier impermeant. DFB did not show intrinsic agonist activity, but it strengthened threshold responses to glutamate, quisqualate, and DHPG in fluorometric Ca^{2+} assays up to 6-fold, with EC_{50} values between 2 and 5 μM. In addition, at 10 to 100 μM, it shifted mGluR5 agonist concentration-response curves approximately two times towards the left (J. A. O'Brien et al. 2003).

Successively, *N*-[5-chloro-2-[(-1,3-dioxoisoindolin-2-yl)methyl]phenyl]-2-hydroxybenzamide (CPPHA) (O'Brien et al. 2004) and 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDPPB) (Lindsley et al. 2004) were disclosed. It has been reported that CDPPB had a greater efficacy in both human and rat (EC_{50} 27 nM and 98 nM, respectively) compared to CPPHA (EC_{50} in human=150-180 nM) (Stauffer 2011). However, CPPHA bound to a different binding site respect to MPEP-like ligands (Chen et al. 2007). Interestingly, DFB and CPPHA have been shown to have differential modulatory effects on the activation and phosphorylation of ERK1/2 and calcium mobilization, suggesting possible differential PAM signaling. This disclosure supports the concept that PAMs which occupy

distinct allosteric binding sites and likely involve unique modulator-receptor conformations can enable different downstream signaling responses (Zhang et al. 2005).

Besides these molecules, second-generation compounds were synthetized. Addex Therapeutics developed a structurally different class of piperidinyl 1,2,4-oxadiazoles headed by (*S*)-(4-fluorophenyl)[3-[3-(4-fluorophenyl)-1,2,4-oxadiazol-5-yl]piperidin-1-yl]methanone (ADX 47273) (Liu et al. 2008).

Another class of mGluR5 PAMs has been represented by acetylene moiety, such as N-methyl-5-(phenylethynyl)pyrimidin-2-amine (MPPA) (Sharma et al. 2009), (4-hydroxy-piperidin-1-yl)(4-phenylethynyl)phenyl)methanone (VU0092273) (Rodriguez et al. 2010) and N-cyclobutyl-6-((3-fluorophenyl)ethynyl)nicotinamide hydrochloride (VU0360172) (Rodriguez et al. 2010), the last two projected by Vanderbilt University.

In addition, a distinct chemotype containing N-aryl piperazine and not acetylene was developed by AstraZeneca, with its CPPZ as lead compound (SLASSI et al. 2007).

Furthermore, to avoid potential metabolic and toxicological drawbacks of triple bond molecules, the acetylene typical of MPEP-derived PAM such as MPPA was replaced. Thus, exploiting the chemical scaffold of CPPHA, a new class of mGluR5 PAMs, containing a lipophilic ether tail linked to the benzamide group, was synthetized. These molecules interacted within a site different from MPEP pocket and displayed high potency in activating mGluR5 (EC_{50} concentrations extending from 33 nM to 1.3 μM). These ligands include *N*-(5-chloropyridin-2-yl)-4-propoxybenzamide (VU0001850), 4-butoxy-*N*-(2-fluorophenyl)-benzamide (VU00402237) and 4-butoxy-*N*-(2,4-difluorophenyl)benzamide (VU0357121) (Hammond et al. 2010).

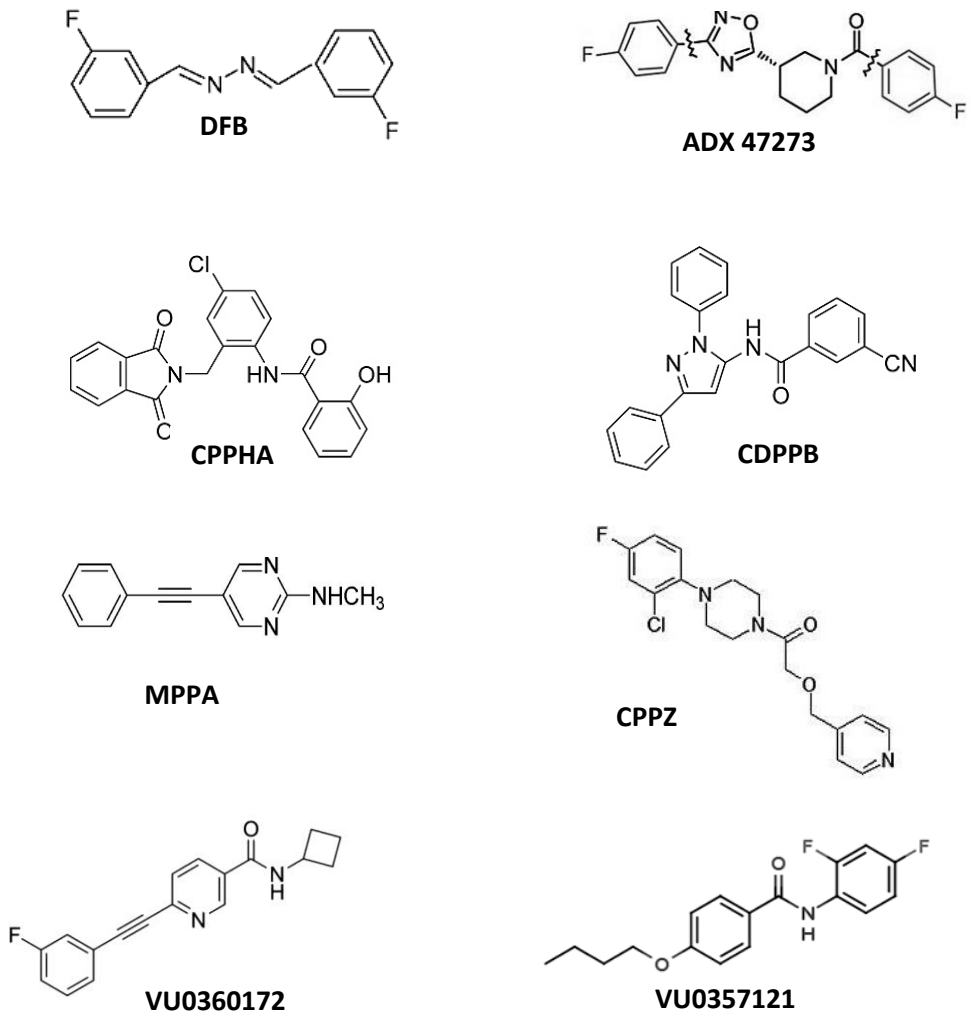


Figure 1.25. Positive allosteric modulators of mGluR5. Chemical structures of several mGluR5 Positive Allosteric Modulators (PAMs). Starting from the top: DFB, ADX 47273, CPPHA, CDPPB, MPPA, CPPZ, VU0360172 and VU0357121.

1.6.2.2 Negative allosteric modulators

SIBIA Neuroscience first discovered selective mGluR5 NAMs, such as 6-methyl-2-(phenylazo)-pyridin-3-ol (SIB-1757) and (*E*)-2-methyl-6-styryl-pyridine (SIB-1893) (Figure 1.26). In the phosphoinositide hydrolysis assay carried on cell lines expressing the human metabotropic glutamate receptor subtype 5a (hmGluR5a), it has been demonstrated that SIB-1757 and SIB-1893 have IC_{50} values of 3.7 μM and 3.5 μM, respectively. Moreover, in rat neonatal brain slices, SIB-1757 and SIB-1893 inhibited agonist DHPG-evoked inositol phosphate accumulation in both hippocampus and striatum by 60% to 80% (Varney et al. 1999b).

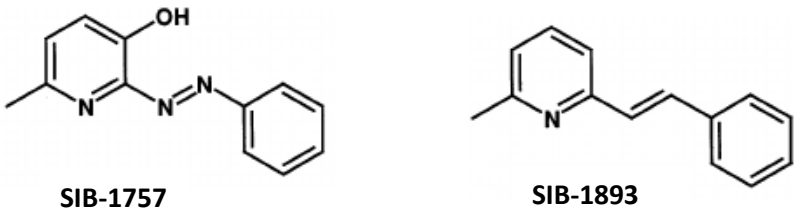


Figure 1.26. Negative allosteric modulators of mGluR5. Chemical structures of the first Negative Allosteric Modulators (NAMs) SIB-1757 and SIB-1893.

Modifications of SIB-1757 and SIB-1893 structures led to the development of 2-Methyl-6-(phenylethynyl)pyridine (MPEP), a negative allosteric modulator that fully inhibits quisqualate-stimulated PI hydrolysis (IC_{50}=36 nM) in human recombinant cells (Gasparini et al. 1999). Similarly, in the rat hippocampus, 10 mg/kg MPEP given intraperitoneally blocked PI hydrolysis induced by the agonist CHPG (Anderson et al. 2002), stabilizing the inactive conformation of mGluR5. In addition, because of its highly membrane permeability (lipophilic value (LogP) of 3.3), MPEP inhibits also agonist-mediated calcium variations within isolated striatal nuclei (Jong et al. 2005). MPEP receptor occupancy varies among species. For example, receptor occupancy in rat brain can last up 2 hour for 75%, while in mouse brain 75% receptor occupancy can be maintained for 15 minutes (Anderson et al. 2003). However, MPEP is not particularly selective for mGluR5. In fact, it has been reported that MPEP can also interact with mGluR4, acting as a positive and not negative allosteric modulator (Mathiesen et al. 2003), and it can have electrophysiological effects also on human NMDA and kainate receptors (Lea & Faden 2006).

3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pridine (MTEP), a thiazol derivative of MPEP with improved aqueous solubility, was lately developed at Merck Laboratories (Cosford et al. 2003). In cultured rat cortical neuronal cells, MTEP 0.2 μM fully blocked CHPG-induced PI hydrolysis, and at 0.02 μM lower but important reduction in the agonist-induced phosphoinositide hydrolysis occurred, suggesting that MTEP acted as a PLC-coupled mGluR5 antagonist (Lea et al. 2005). Similar to MPEP, MTEP characterization has shown that receptor occupancy *in vivo* exhibited significant species variability. In rodent brain, MTEP also lasts 75% receptor occupancy for 2 hours, whereas, in murine brain 75% occupancy is sustained for about 15-30 minutes (Anderson et al. 2003).

Differently from its precursor, MTEP demonstrated its highly selectivity for mGluR5 and poor effects on other mGluR and iGluR subtypes (Anderson et al. 2002; Cosford et al. 2003).

In vivo and *in vitro* studies in rats report that MTEP is mainly metabolized by cytochrome P450 (CYP) isoforms CYP1A1/2, CYP2C6 and CYP2C11 (Green et al. 2006) and the following metabolites are produced: hydroxymethyl, pyridine N-oxide, a novel ring opened acetamide/aldehyde, thiazole oxide and carbon dioxide (Yang & Chen 2005).

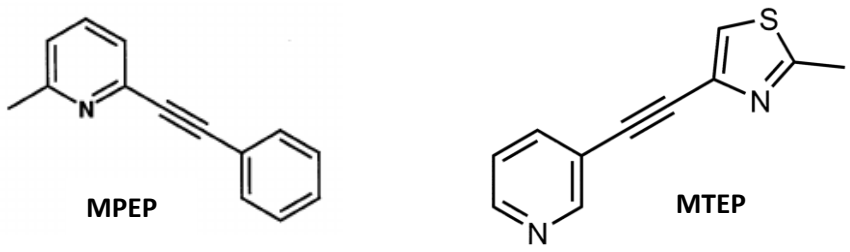


Figure 1.27. Negative allosteric modulators of mGluR5. Chemical structures of MPEP and MTEP.

Dipraglurant (ADX48621) from Addex (Dipraglurant structure disclosed in International nonproprietary names for pharmaceutical substances (INN). WHO Drug Information 2009;23:328), mavoglurant (AFQ056, methyl-(3a*R*,4*S*,7a*R*)-4-hydroxy-4-[(3-methylphenyl)ethynyl]octahydro-1*H*-indole-1-carboxylate) from Novartis (Vranesic et al. 2014) and basimglurant (RG7090, RO4917523, 2-chloro-4-[1-(4-fluoro-phenyl)-2-methyl-1H-imidazol-4-ylethynyl]-pyridine) from Roche (Jaeschke et al. 2015) are all negative allosteric modulators sharing MPEP chemotype. Since alkyne-containing molecules have long been correlated with risk for metabolic activation and ensuing hepatotoxicity, pharmaceutical industries undertook to improve their formulations in order to modify the release and the pharmacokinetics of the drugs (Emmitte 2017).

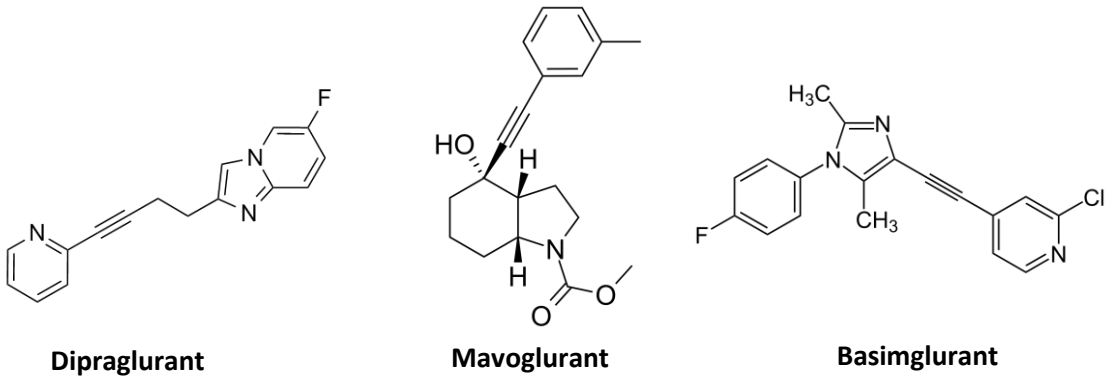
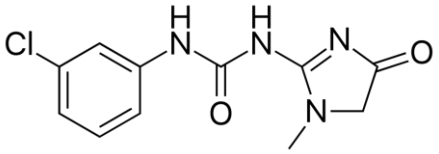


Figure 1.28. Negative allosteric modulators of mGluR5. Chemical structures of Dipraglurant, Mavoglurant and Basimglurant.

During the years, compounds with a chemotype distinct from the prototypical MPEP alkyne were employed to develop new compounds. N-(3-chlorophenyl)-N'-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl)urea, also known as Fenobam was synthetized at McNeil Laboratories as potential anxiolytic, although its molecular target was unknown (Hunkeler & Kyburz 1980). Only recently it has been revealed that Fenobam selectivity inhibited mGluR5 activity, not interacting with rat mGlu1a, 2, 4, 7 and 8 (Ceccarelli et al. 2007). Fenobam presents high affinity for both human and rat mGluR5 (K_d of 31.1 and 53.6 nM, respectively), with an IC_{50} of 58 nM and its binding is temperature-dependent, improving considerably at 25°C (Porter et al. 2005). Fenobam has been reported to be extensively metabolized by rats *in vivo*, while *in vitro* Fenobam remains unmetabolized for 55% (Wu et al. 1995).



Fenobam

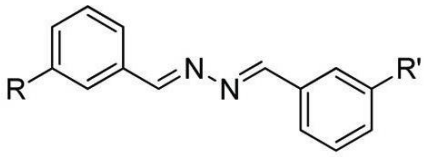
Figure 1.29. Negative allosteric modulators of mGluR5. Chemical structures of Fenobam characterized by a different chemotype than MPEP.

1.6.3. Molecular switches

Slightly modifications of allosteric modulators structure can potentially cause huge changes in receptor pharmacology-response as well in subtype selectivity (Wood et al. 2011b). This emerging phenomenon is termed "molecular switch".

About the pharmacology mode, alterations can transform a weak molecule into a potent one or can modify a NAM into a PAM, a SAM, a PA or an ago-PAM, or vice versa.

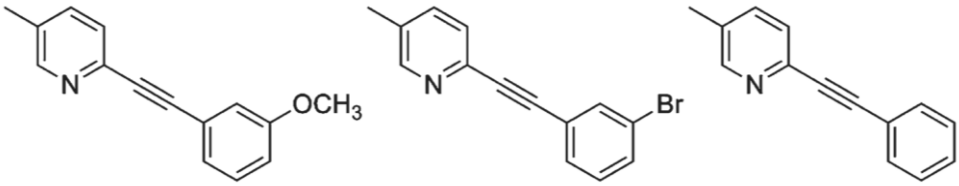
Subtle modifications of DFB scaffold produce molecules with different pharmacological properties. In fact, fluorine replacement with methoxy group produces a negative allosteric modulator (3,3'-dimethoxybenzaldazine or DMeOB), while fluorine substitution into chlorine leads to a silent allosteric modulator (3,3'-dichlorobenzaldazine or DCF) (J. A. O'Brien et al. 2003) (Figure 1.30).



1. DFB	**R,R'=F**	**PAM**
2. DOMeB	**R,R'=OMe**	**NAM**
3. DCB	**R,R'=Cl**	**SAM**

Figure 1.30. Molecular switches of DFB. Slightly modifications in DFB structure change the nature of the molecule: from PAM, DFB could be transformed in a NAM or even in a silent allosteric modulator (Stauffer 2011).

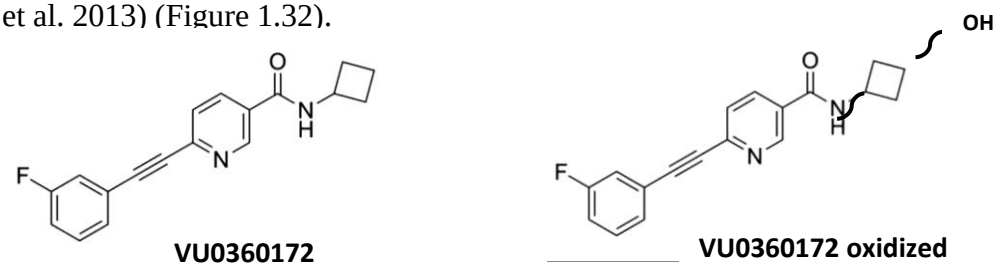
About 2-methyl-6-(2-phenylethynyl)pyridine, modifications of MPEP scaffold lead to both partial antagonists, such as M-5MPEP and Br-5MPEP, and SAM such as 5MPEP (Rodriguez et al. 2005) (Figure 1.31).



M-5MPEP **Br-5MPEPy** **5MPEP**
~50% PA **~50% PA** **SAM**

Figure 1.31. Molecular switches of MPEP. Modifications of MPEP scaffold lead to synthesis of both partial agonists (M-5MPEP and Br-5MPEPy) and silent allosteric modulators (5MPEP) (Wood et al. 2011).

Molecular switches have been observed also *in vivo*, as documented for VU0360172, a pure mGluR5 PAM, which is subjected to oxidation by cytochrome P450 (CYP) lead to a potent ago-PAM, that is a positive allosteric modulators having intrinsic agonist activity (Rook et al. 2013) (Figure 1.32).



VU0360172 **VU0360172 oxidized**

Figure 1.32. *In vivo* molecular switch. The mGluR5 pure positive allosteric modulator VU0360172 could be directly modified by the activity of cytochrome P450 *in vivo*, leading to a PAM with intrinsic agonist activity (ago-PAM) (Rook et al. 2013).

Besides the modes of pharmacology, molecular switches can affect receptor subtype selectivity. In this case, modifications of allosteric modulators scaffold make a molecule selective for another receptor than the "original". These kinds of molecular switches are limited to mGluR4 PAMs and no reports about mGluR5 have been found yet (Wood et al. 2011b).

1.7 Role

1.7.1 Physiological roles

Metabotropic glutamate receptor 5 is highly expressed in the central nervous system, thus it plays important roles in development, synaptic function, learning and memory (Nicoletti et al. 2011). For instance, mGluR5 activation prompted NMDAR-dependent Long-Term Potentiation (LTP) and caused significant changes in postsynaptic calcium concentration, which in turn modifies synaptic efficacy at neuronal synapse. Likewise, in cerebellum, mGluR5 activation induces also Long-Term Depression (LTD), in which calcium is released from intracellular stores, resulting from the endocytosis of AMPA receptors. Another mechanism proposed to mGluR5-dependent LDT is calcium-independent and involves interactions with the scaffolding protein Homer (Baudry et al. 2012). Furthermore, mGluR5 modulates central reward pathways and could affect also feeding. It has been shown that the administration of MTEP to diet-induced obese rats mediates feeding suppression (Bradbury et al. 2004).

1.7.2 Pathological roles

Although mGluR5 is involved in a plethora of physiological processes, evidences reported that mGluR5 hyperactivation as well as hypoactivation can affect the canonical molecular signaling, leading to important consequences. It has been established that glutamatergic hyperactivation in cortex, thalamus, amigdala and hippocampus, correlates with anxiety and fragile X syndrome, whereas hypofunctionality is associated to schizophrenia (Ritzen et al. 2005).

Nowadays, allosteric modulators appear really fascinating, because they overcome orthosteric ligand drawbacks linked to the "all-or-none" response. For this reason, mGluR5 allosteric modulators, both positive and negative, are being taken into consideration for the treatment of several pathologies at central nervous system level, including Huntington's, Parkinson's and Alzheimer's diseases, chronic pain, drug addiction, as well at peripheral level, such as gastroesophageal reflux and tumors.

1.7.2.1 Schizophrenia

Schizophrenia (MIM 181500) is, under several points of view, a complex neuropsychiatric syndrome. In fact, it is characterized by different kinds of symptoms, divided in positive, negative and cognitive. Among positive symptoms there is persistent psychosis, represented by the typical loss of contact with reality, consisting of hallucinations and disorganized speech and behavior. The social withdrawal, emotional blunting, reduced energy and incapacity to feel pleasure are considered negative symptoms. Also cognitive dysfunctions appear in schizophrenia, such as impairment in memory (Kahn et al. 2015). Thus, all these symptoms affect the everyday life of people suffering from that syndrome, up to loss of independence (Harvey 2014).

Moreover, schizophrenia is complex because of the inheritance and risk factors. Several studies have shown that relatives of a proband with schizophrenia have higher chance to be affected by this disorder than relatives of control (Kendler & Diehl, 1995), and the probability is much greater within homozygous twins (Sponsoring initiatives in the molecular genetics of mental disorders. In: Genetics and Mental Disorders: Report of the NIMH Genetics Workgroup. Bethesda, Md.: NIH 98-4268 1998). It has been reported that people born in late winter are most prone to develop schizophrenia, because of the fetal brain could be exposed to maternal respiratory infections and malnutrition, such as folic acid and vitamin D deficiency (Kahn et al. 2015). Also the paternal age appears to be important: in fact children whose fathers were rather old are more subjected to schizophrenia (Malaspina et al. 2001).

Furthermore, schizophrenia is a complicated disease since the molecular mechanisms are not completely understood yet. Most studies focused on the hyperactivation of dopaminergic system (Gray & Roth 2007; Reynolds 2004). Thus, dopamine D_2 receptor antagonists have been developed as antipsychotic drugs. Not only their beneficial effects are limited to the

treatment of positive symptoms, but also cause several adverse effects, mainly at extrapyramidal motor neurons (Gray & Roth 2007). Recent analysis revealed that glutamatergic system impairment is also associated to schizophrenia. It is well established that NMDA receptors are involved in learning and memory, and a hypofunction of these receptors causes cognitive defects as in schizophrenia (Coyle 2012; Weickert et al. 2013). Furthermore, NMDA receptors (NMDARs) play a crucial role since a defect in its transmission produces an abnormal increase of dopamine (Snyder & Gao 2013). Drugs targeted to NMDAR are problematic because their activation triggers a rise of calcium within the cells, leading to excitotoxicity (Anon n.d.). To overcome that drawback, a new approach has been considered, i.e. targeting metabotropic glutamate receptors. Since mGluR5 and NMDARs are strictly associated through scaffolding proteins linked to C tails of mGluR5, such as Homer, the activation of mGluR5 could lead to NMDARs activation too (Tu et al. 1999).

1.7.2.2 Fragile X syndrome

Martin-Bell syndrome, an X-linked intellectual retardation, was described for the first time in 1943 (Martin & Bell 1943). Later, the existence of a break, subsequently termed "fragile site", on the X chromosome was discovered by Lubs (Lubs 1969). Only in 1991 the gene responsible for this syndrome was identified on the X chromosome at position q27.3, and named Fragile X Mental Retardation 1 gene (*FMR1*), and hence the term Fragile X Syndrome (FXS) (Verkerk et al. 1991).

FXS (MIM 300624) is the leading factor of autism and one of the most frequent causes of inherited intellectual disability worldwide (Crawford et al. 2001; Garber et al. 2008). This syndrome affects approximately 1 in 2500-4000 males and 1 in 7000-8000 females (Jewell et al. 2016), which display milder symptoms than males (Hersh et al. 2011). Patients with FXS present symptoms that include moderate to severe mental retardation, attention deficits, anxiety, hyperactivity, developmental delays, self-injury, aggressiveness, seizures, increased responses to sensory stimuli (Bailey et al. 2008).

Fragile X syndrome is induced by mutations of the X-linked *FMR1* (MIM 309550) gene, which encodes for the Fragile X Mental Retardation Protein, FMRP (Pieretti et al. 1991; Fu et al. 1991; Verkerk et al. 1991). The most recurrent mutation of *FMR1* is the expansion of a

CGG trinucleotide repeat in the 5′ untranslated region (UTR) of the gene. This syndrome is more severe as the increase of the number repeats. In fact, CGG triplet is repeated up to 45 times in healthy people, expansion between 45 and 54 repeats characterizes "intermediate" phenotype, expansion between 55 and 199 trinucleotide repeats is classified as premutation, while more than 200 CGG repeats represent a full mutation (Kronquist et al. 2008). In the full mutation case, the expanded CGG trinucleotide is recognized as a CpG island; consequently *FMR1* is hypermethylated and the chromatin condensed. Although the coding region of the gene remains intact, both changes, one structural (the triplet expansion) and one epigenetic (hypermethylation) lead to transcriptional silencing (Sutcliffe et al. 1992; Eberhart & Warren 1996; Coffee et al. 1999; Coffee et al. 2002; Godler et al. 2010).

The loss of FMRP results in an increased Long-Term Depression (LTD) at the Schaffer Collateral-CA1 (SC)-CA1 pyramidal cell synapses in the hippocampus, due to an excessive activation of group I metabotropic glutamate receptors, specifically mGluR5 (Huber et al. 2002). Carrying on these studies, Bear and colleagues proposed the so-called "mGluR theory", according to four events observed at the synapse level (Bear et al. 2004). First of all, fragile X mental retardation protein acts as a translational repressor, since it is an RNA-binding protein (Brown et al. 2001; Zalfa et al. 2003); secondly, protein synthesis at the synapse is potentially triggered by the activation of metabotropic group I receptors (Weiler et al. 1997); thirdly, the absence of FMRP induces an upregulation of mGluR5-mediated signaling pathways (Huber et al. 2002); lastly, many of the results of mGluR activation depend on the synaptic translation of mRNA (Karachot et al. 2001; Zho et al. 2002; Huber et al. 2000; Raymond et al. 2000). Among the molecular pathways triggered by mGluR5 activation, are included the association of the receptor to Homer proteins, the activation of upstream regulators of dendritic mRNA translation, such as ERK/MAPK-interacting kinase (Mnk1)/eukaryotic Initiation Factor 4E (eIF4E) pathway, and the PtdIns-3-k/mammalian target of rapamycin (mTOR)/p70S6K pathway (Nicoletti et al. 2011). Hence, the research has been focused on the negative allosteric modulators of mGluR5 as potential treatments of FXS. Yan and colleagues have treated mice with a negative allosteric modulator of mGluR5, MPEP, recovering anxiety and seizures, the major symptoms of FXS (Yan et al. 2005). Successively, it has been shown that the crossbreeding of FXS mice with mGluR5 knock-out mice corrected several fragile X phenotypes, due to a reduction of the expression of 50% of mGlu5 receptors (Dölen et al. 2007).

While the administration of DHPG, an orthosteric agonist of group I mGluR, showed an increase in LTD at the SC-CA1 synapse in hippocampal rat (Palmer et al. 1997), acute

therapy of fragile X mice with the negative allosteric modulator CTEP rescued long term-depression, corrected protein synthesis, prevented epileptic events and reduced macroorchidism. Besides, chronic treatment with CTEP restored learning and memory deficits, elevated locomotor activity and increased spine density (Michalon et al. 2012). Another selective NAM, GRN-529, was found to reduce repetitive behaviors and aberrant social interactions in autistic mice (Silverman et al. 2012).

On human adults, Fenobam, already investigated as an anxiolytic in a number of phase II studies in the early 1980s (Friedmann et al. 1980; Pecknold et al. 1982; Itil et al. 1978) showed improved clinical behaviors and no adverse effects (Berry-Kravis et al. 2009). Although AFQ056, also known as mavoglurant, was in phase II clinical trials for adults with FXS, Novartis announced that the company discontinued AFQ056 development program in fragile X syndrome, following the negative results obtained in a large international clinical trial in adults as well in adolescents. In fact, in both placebo and controlled trials, patients administered with mavoglurant did not show improvement over placebo in any outcome measures (Letter from Novartis CEO, 2014). Unfortunately, metabotropic glutamate receptor 5 NAMs present negative side effects. For example, MPEP worsens psychosis induced by phencyclidine (PCP) in rats, causing cognitive impairments and psychotomimetic effects (Campbell et al. 2004), as well Fenobam in early clinical trials, albeit with an unclear mechanism yet (Friedmann et al. 1980; Pecknold et al. 1982; Itil et al. 1978).

1.7.2.3 Anxiety disorders

Anxiety disorders are mental illness characterized by important feelings of anxiety and fear. These emotional states may provoke also physical symptoms, such as a fast heart rate and tremor. Anxiety disorders include several pathologies: Generalized Anxiety Disorder (GAD), specific phobia, social anxiety disorder, separation anxiety disorder, agoraphobia, panic disorder, obesity and selective mutism. Often, people suffer from more than one anxiety disorder (Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association (5th ed.). Arlington: American Psychiatric Publishing. 2013), and, sometimes, they can occur with other mental diseases, such as major depressive disorder and personality disorder. The causes are imputable to both genetic and environmental factors (https://www.nimh.nih.gov/health/topics/anxiety-disorders/index.shtml#pub2), such as the presence of mental disorders in the family, child abuse and poverty (Craske & Stein 2016).

The diagnosis of mental disorders is long and complex because of the complexity of symptoms, which can be shared to other diseases, for instance drug abuse and heart disease (Craske & Stein 2016; Testa et al. 2013). Although anxiety disorders are highly treatable nowadays, only a small percentage of the 40 million adults affected in US receives cures (https://adaa.org/about-adaa/press-room/facts-statistics). The typical treatment consists in the administration of antidepressant, barbiturates or benzodiazepines, accompanied by psychological support (Craske & Stein 2016). The molecular mechanisms that cause anxiety disorders are not fully understood. Anxiety disorders are believed to result from disruption in the balance of activity in the emotional centers or in the higher cognitive centers of the brain (Martin et al. 2009). Moreover, it has been demonstrated that abnormalities in serotonergic and adrenergic functions are implicated in the pathogenesis of depression and anxiety (Baldwin & Rudge 1995). In addition, a hyperactivation of metabotropic glutamate receptor 5 has been observed in the amygdala of patients affected by anxiety disorders (Romano et al. 1995; Valenti et al. 2002). The inhibition of mGluR5 by means of negative allosteric modulators showed exiting results. For instance, MPEP was found to exert anxiolytic-like effects in several anxiety-like behavior tests, including elevated-plus maze, social exploration, fear-potentiated startle, Vogel-conflict and light-dark box test (Hovelsø et al. 2012). Furthermore, MTEP had anxiolytic-like properties in contextual fear conditioning following acute or sub-chronic treatment, indicating that tolerance did not develop to the anxiolytic effect of MTEP (Gravius et al. 2008). Both MPEP and MTEP exhibited antidepressant-like effects in an animal model of depression, the olfactory bulbectomy model (Pałucha et al. 2005). Fenobam anxiolytic properties were also evaluated in clinical studies (Pecknold et al. 1982). It has been documented that mGluR5 knockout mice displayed an antidepressant-like behavioral phenotype (Li et al. 2006). Inhibition of mGluR5 may have therapeutic potential in the treatment of depression and anxiety disorders. Additionally, the antidepressant efficacy of tricyclic antidepressants and selective serotonin reuptake inhibitors might be improved by concomitant treatment with mGluR5 negative allosteric modulators.

1.7.2.4 Chronic pain

Pain is an unpleasant sensory and emotional experience, which begins with a peripheral stimulus and undergoes a physiological process, resulting in the sensation of pain.

Acute pain is defined as a temporal normal sensation, triggered by the nervous system. Conversely, it is considered chronic pain the pain that persists for at least 12 weeks (https://www.ninds.nih.gov/Disorders/All-Disorders/Chronic-Pain-Information-Page; https://medlineplus.gov/magazine/issues/spring11/ articles/spring11pg5-6.html). According to origin, clinical features and mechanisms, pain could be also classified in nociceptive, inflammatory, dysfunctional or neuropathic pain (Costigan et al. 2009).

A study conducted by the American Institute of Medicine of The National Academies reported that more than 100 million of Americans and 1.5 billion of people worldwide suffer from chronic pain, manifestation of several pathologies, such as fibromyalgia, osteoarthritis, migraine, temporomandibular joint disorders, dental pain, neuropathic pain, visceral pain hypersensitivity disorders and postsurgical pain (Woolf 2011). Indeed, chronic pain is the main cause of long-term disability in the United States. Moreover, the aforementioned disease represents an important problem also for the entire society, which spends out at least $560-$635 billion per year (http://www.prweb.com/pdfdownload/8052240.pdf).

Several mechanisms are involved in the generation and maintenance of chronic pain, among them those induced by glutamate receptors. In fact, after injury, glutamate-induced plasticity is crucial in the raised synaptic efficacy, taking place in the dorsal horn of the spinal cord at the synapse between primary afferent terminals and second order neurons. This mechanism, termed activity-dependent central sensitization, induces post-injury pain hypersensitivity (Woolf 1983), and it shares characteristics common to the hippocampal Long-Term Potentiation (LTP) (Ji et al. 2003), fundamental for memory and learning (Nicoll 2003). Although both iGlu and mGlu receptors are involved in chronic pain induction and maintenance, ionotropic receptors could not be considered good targets for pain treatment because of their adverse effects, such as psychotomimetic effects and amnesia (Chappell et al. 2014). Conversely, since metabotropic receptors are able to modulate the responses, they represent targets of greater interest. However, activation of mGlu receptors can enhance or reduce cell excitability, depending on the localization of the specific subtype activated. In fact, except subtype 6 expressed exclusively in the retina (Vardi et al. 2000), all the other mGluRs are expressed in the so-called pain neuraxis, encompassing peripheral nerve endings, dorsal root ganglia, dorsal horns of the spinal cord, and supraspinal sites (Varney & Gereau 2002). Furthermore, numerous mGlu receptors are also present in glial cells, comprising astrocytes, oligodendrocytes, and microglia (Saab et al. 2006; D'Antoni et al. 2008; Byrnes et al. 2009). In rodent peripheral sensory afferents, activation of mGluR1 and mGluR5 by agonist administration (DHPG and CHPG) induces pronociceptive effects (Bhave et al. 2001;

K. Walker et al. 2001), enhancing Transient Receptor Potential Vanilloid 1 (TRPV1) function. TRPV1 is the major endogenous transducer of noxious heat and also responds to inflammatory molecules and capsaicin (Bhave 2001; Hu 2002). Even though mGlu1 and mGlu5 receptors are both essential for chronic pain, studies utilizing orthosteric or allosteric antagonists have revealed that mGlu5 receptors represent a better therapeutic target for the management of chronic pain. DHPG administration in spinal cord dorsal horn mice neurons induces pain sensitivity through the activation of the Extracellular Regulated Kinase (ERK) signaling, while treatment with MPEP reduces inflammation-induced ERK activation and inflammatory pain plasticity (Karim et al. 2001). It has been shown that activation of mGluR5 induces nociception in the amigdala (Li & Neugebauer 2003; Tappe-Theodor et al. 2011), and produces antinociceptive effects in the Periaqueductal Gray matter (PAG) (Maione et al. 2000). Analgesia has been enhanced in several models of persistent pain using different negative allosteric modulators, as described for MPEP (Kolber et al. 2010; Fisher & Coderre 1996), Fenobam (Montana et al. 2009), SIB-1757 and SIB-1893 (Varney et al. 1999a; Dogrul et al. 2000) and ADX10059 (Marin & Goadsby 2010), which initially demonstrated better efficacy for migraine management than placebo. Successively the clinical trial in phase IIa was discontinued for long term treatments because it caused transaminases increase (http://www.addextherapeutics.com/investors/press-releases/news-details/?tx_ttnews%5Btt_news%5D%20%20=%2088&cHash=d075a6fa63acce925582f8f481fb8dc3).

1.7.2.5 Drug addiction

Addiction is a complicated chronically relapsing illness defined by excessive drug intake, repeated failing attempts and stopping or reducing drug use, drug-seeking and self-administration, tolerance and withdrawal, and continued drug intake in spite of negative consequences (American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. Vol. 4th Edition. Washington DC: American Psychiatric Press; 1994).
Human aspects of drug addiction can not be easily studied, thus animal models of addiction have been extensively studied and have yielded a great amount of information about drug reward and reinforcement, drug-seeking and relapse behaviors. Among all receptors and mechanisms, several lines of evidence suggest that glutamate neurotransmission plays a key

role in the processes of drug addiction and relapse (Olive 2009). Chiamulera and co-workers reported that hyperlocomotion in response to acute administration of different doses of cocaine was not affected in mice carrying a deletion of the GRM5 and intravenous self-administration of cocaine is avoided (Chiamulera et al. 2001). Furthermore, they confirmed that the administration of mGluR5 negative allosteric modulator MPEP reduced cocaine-self administration, without causing non-specific motor effects. Therefore, genetic or pharmacological inhibition of mGluR5 function have been shown to decreases cocaine self-administration as well as cocaine-induced hyperlocomotion, suggesting that inhibition of mGluR5 may be advantageous in treating cocaine addiction in humans.

Others studies revealed that, besides MPEP, also MTEP, another mGluR5 negative allosteric modulator, attenuated intravenous self-administration of cocaine, nicotine, heroin, morphine and diminished voluntary ethanol consumption in rodent models, without altering food intake (Pałucha et al. n.d.; Olive 2009). However, the mechanisms supporting these evidences are controversial. On one hand, MPEP has been found to raise thresholds for intracranial electrical self-stimulation (Harrison et al. 2002), demonstrating that mGluR5 inhibition negatively influences brain reward function, which may manage the ability of mGluR5 antagonists to suppress active drug self-administration. On the other hand, MPEP did not alter drug-induced lowering of brain reward stimulation levels, but it is proposed to alter the discriminative stimulus properties of distinct drugs, as revealed for cocaine, nicotine and ethanol (Besheer et al. 2006).

In spite of several researches on animals, to date there are no clinical studies about the efficacy of mGluR5 negative allosteric modulators in handling addictive disorders. This lack of clinical data makes it difficult to speculate whether and in what extend the inhibition of mGluR5 could be beneficial in the treatment of drug addiction and alcoholism in humans.

1.7.2.6 Parkinson’s disease

Parkinson's Disease (PD) is a progressive and chronic disorder of the central nervous system, that influence movement (http://www.pdf.org/about_pd). In the US, nearly one million people are affected with Parkinson's disease (http://www.pdf.org/parkinson_statistics) and about 7 to 10 million people worldwide. The incidence of the disease increases with age and men are more prone to PD than women (https://parkinsonsnewstoday.com/parkinsons-disease-statistics/). Parkinson's syndrome affects neurons in the substantia nigra. These dying

neurons produce dopamine, a chemical neurotransmitter that sends messages to the part of the brain that controls movement. As PD progresses, the amount of dopamine produced decreases and the patient is unable to control movements (http://www.pdf.org/about_pd). The principal symptoms include tremor of muscles, rigidity and stiffness, slow movement and impaired balance (https://www.merriam-webster.com/dictionary/Parkinson%27s%20disease). Despite a lot of researches, actually, there are no effective drugs, but current therapy is focused on symptoms reduction. To date, L-DOPA, a precursor of dopamine, represents the gold-standard therapy for Parkinson's disease. Unfortunately, L-DOPA causes motor fluctuations and dyskinesia in 80% of the patients, leading to the so-called L-DOPA-Induced Dyskinesia (LID). Metabotropic glutamate receptor subtype 5 has been found to be highly expressed in substantia nigra and to be hyperactivated by L-DOPA administration (Fabbrini et al. 2007). Hence, mGluR5 has been investigated as a target for PD treatment (Rylander et al. 2010). The first evidence of the potential to modulate excessive glutamatergic neurotransmission inhibiting mGluR5 has been published by Spooren and colleagues (Spooren et al. 2000). They reported that MPEP could attenuate unilateral rotating behavior in the rodent 6-hydroxydopamine (6-OHDA) lesion model. The same effects were found using MTEP. However, MTEP has been demonstrated to have not translational potential, thus other molecules were examined (Mela et al. 2007). The effects of Fenobam, an mGluR5 negative allosteric modulator already tested in humans for anxiety disorders, were evaluated in rodent and nonhuman primate models of PD. In rats and monkeys, both acute and chronic treatment with Fenobam attenuated the L-DOPA-induced dyskinesia (Rylander et al. 2010). In addition, mavoglurant, or AFQ056, has reached phase II of different clinical trials, but it was discontinued because of the lack of efficacy demonstrated in humans (Petrov et al. 2014; Trenkwalder et al. 2016).

1.7.2.7 Huntington’s disease

Huntington’s disease (HD; MIM 143100) was described for the first time in 1872 by George Huntington as a hereditary form of chorea. HD is an autosomal dominant neurodegenerative disorder, associated to the loss of neurons in the striatum and the cortex (Allbutt 1918). Huntington’s disease is the most common inherited neurodegenerative disease (Finkbeiner 2011). Male and female are affected equally, and HD is most frequent in Western

population (3-7 individuals in 100.000), then in Asiatic and African populations (https://ghr.nlm.nih.gov/condition/huntington-disease#statistics). HD is characterized by motor deterioration, cognitive decay, behavioral disturbance and psychiatric disease (Ross & Tabrizi 2011). The symptoms generally appear at the third or fourth decade of individual life, and, since HD is a progressive disease, the clinical manifestations worsen after its onset. Involuntary movements (hence the name "chorea") are soon followed by gait dysfunctions, postural instability, lack of balance and incoordination. Moreover, eye movement is also affected, and dysarthria and dysphagia subsequently appear. Furthermore, psychiatric and cognitive disorders usually come out during disease progression, including personality alterations, obsession, compulsion, depression and dementia (Piira et al. 2013).

Although Huntington's disease was discovered at the end of the 18th century, the gene has been only recently identified. *HUNTINGTIN* (HTT) gene (MIM 613004) is mapped in 4p16.3, and an abnormal glutamine CAG trinucleotide expansion in exon 1 is the leading cause of HD (MacDonald et al. 1993). In fact, wildtype HTT contains from 15 to 35 CAG repeats, while in the pathologic form of HTT the trinucleotide expansion is increased up to 180 repeats, resulting in a mutant protein. The normal function of the htt protein has already unclear, but it might play a role in internal cell signaling, maintenance of cyclic adenosine monophosphate response element binding protein and preventing neuronal toxicity (Nucifora et al. 2001).

Despite the recognized cause of HD, actually there is no effective drug, since the therapeutic strategies are addressed to the management of symptoms, rather than targeting the cause. These approaches include the use of neuroleptics or benzodiazepines to cure the chorea, antiparkinsonian molecules to enhance hypokinesia, antidepressants and antipsychotics for the handling of depression and personality changes. Another recent approach is the use of metabotropic glutamate receptor 5 modulators, considering that, as many other neurodegenerative pathologies, HD is associated with excitotoxicity and dysregulation of glutamate neurotransmission (Fabiola M Ribeiro et al. 2014). Moreover, Anborgh and colleagues have demonstrated that mGluR5 interacts with the Htt mutant-binding protein optineurin, which substitutes GRK2 in the desensitization of the receptor (Anborgh et al. 2005). mGluR5 activation is coupled to $G_{\alpha q/11}$ proteins, which lastly leads to calcium release and the activation of protein kinase C, resulting in neurotoxicity. On the other hand, mGluR5 activates cell signaling pathways important for cell survival and proliferation, such as ERK and AKT, leading to neuroprotection (Rong et al. 2003; Hou & Klann 2004; Mao et al. 2005). Thus, the activation or the inhibition of mGluR5 by agonists or antagonists is still

controversial. Acute blockage of mGluR5 is neuroprotective, while chronic MPEP treatment inhibits protective pathways and cell survival, leading to basal neuronal death. Nevertheless, HD mutant mice treated with MTEP exhibit increased locomotor activity (Ribeiro et al. 2014). However, agonist administration could be harmful in the same way: DHPG treatment overstimulates calcium release, causing neuronal death (Tang et al. 2005; Doria et al. 2013). Interestingly, it has been reported that PAM administration may enhance the activation of pro-survival signaling pathways, avoiding calcium release and consequent excitotoxicity. A series of PAMs exhibit neuroprotective role on HD mice, activating AKT signaling: DFB, VU1545 and CDPPB, the last improves also recognition memory (Doria et al. 2013).

1.7.2.8 Pathological role in peripheral tissues

Metabotropic glutamate receptor 5, as in the brain, has been observed to be located and involved in both physiological and pathological functions in peripheral tissues. For instance, in the gastrointestinal tract, a hyperactivation of the receptor correlates to Gastroesophageal Reflux Disease (GERD). The administration of the mGluR5 negative allosteric modulator MPEP has led to a decrease in reflux events through inhibition of Transient Lower Sphincter Relaxation (TLESR) (Ferrigno et al. 2017). Another negative allosteric modulator, ADX10059 from Addex Therapeutics, was associated to an improvement in clinical symptoms characterized by reduced acid reflux (Keywood et al. 2009). Unfortunately, ADX10059 showed poor tolerability (dizziness and nausea) and poor efficacy in the refractory gastroesophageal reflux disease (Zerbib et al. 2010). Recently, it has been reported that mavoglurant decreased the incidence of postprandial reflux episodes in both animals and humans with moderate or severe GERD (Rouzade-Dominguez et al. 2017). Pharmacological or genetic blockade of mGluR5 in the pancreas has been observed to decrease insulin secretion, regulating glucose homeostasis (Storto et al. 2006). Since glutamate has been established to regulate proliferation, migration and survival of neuronal progenitor cells and immature neurons during brain development, it is proposed to play a crucial role as a potential growth factor also in tumor development (Stepulak et al. 2014). mGluR5 has been reported to be involved in osteosarcoma, prostate cancer, glioma, medulloblastoma and Squamous Cell Carcinoma (SCC). DHPG-induced expression of

mGluR5 in SCC has been found to correlate with the advancement of the pathology, while the inhibition with MPEP abolished cell migration and invasion (Teh & Chen 2012).

1.8 Glutamate in ischemia

Ischemia is caused by a reduction of blood flow and oxygen, due to a transient or permanent vessel occlusion (Nishizawa 2001). The inadequate blood supply reduces the energy production, leading to an imbalance of ion gradients across membranes (Hansen et al. 1982; Jiang et al. 1992). Thus, membrane depolarization occurs and several voltage-sensitive and receptor-operated ion channels are activated (Fujiwara et al. 1987; Silver & Erecińska 1990). The opening of both sodium and calcium channels as well chloride ones is coupled to an enhanced release of glutamate. Although glutamate is a fundamental neurotransmitter, most exclusively present intracellularly (99.99%) (Danbolt 2001), during ischemic stroke it raises to toxic levels in the extracellular space, triggering a cascade of detrimental events (Taylor et al. 1995; Barbour et al. 1988; Phillis et al. 1997). In fact, the excessive glutamate release induced by pathological stimuli increases, in turn, intracellular calcium concentration, destroying calcium homeostasis. The consequent downstream cascade involve upregulation of nitric oxide synthase, mitochondria dysfunction, oxidative phosphorylation imbalance, ROS and RNS production, ER stress and lysosomal enzyme release. Also cystine uptake can be inhibited by glutamate accumulation. The reversal of the action of the cystine/glutamate antiporter exacerbates the aforementioned events, since cystine is employed for GSH synthesis (Bridges et al. 2012). GSH depletion impacts the ability of the cells to scavenge free radicals, leading to ROS accumulation and alteration in calcium homeostatic mechanisms causing cell death, being a vicious cycle (Fukui et al. 2009).

To avoid or reduce all these phenomena, several therapies were introduced. Since rapid glutamate transmission is mediated by ionotropic receptors, it has been proposed to administer iGlu receptor antagonists to abort upstream the excitotoxic cascade, such as MK-801 or dizocilpine, selective for NMDA receptors (Pohorecki et al. 1990). Alternatively, because of ischemic cascade consists of various passages, it has been suggested the use of calcium channel blockers, for instance nimodipine, to block the downstream effects of this detrimental pathway (Gelmers 1985). Further researches lead to the discovery of allosteric modulators for metabotropic glutamate receptors, enabling the finely tuning of receptors

responses. Makarewicz and co-workers occluded carotid artery, inducing ischemia in adult gerbil. They demonstrated that the administration of MTEP, a negative allosteric modulator for mGluR5, reduced cerebral damage from 90% to 60% (Makarewicz et al. 2006). In neurons, MPEP and SIB-1893 have been shown to prevent phosphoinositol hydrolysis induced by the selective mGluR5 agonist (*R*,*S*)-2-chloro-5-hydroxyphenylglycine (CHPG), improving cell viability. The authors proposed that mGluR5 non-competitive antagonists exerted their neuroprotective effects also by modulating NMDA receptors (O'Leary et al. 2000). Similar results were obtained in tissues other than brain. For example, in their experiment, Storto and colleagues induced anoxia on isolated rat hepatocytes. Glutamate release was evaluated in anoxic as well in normoxic hepatocytes, showing higher levels in anoxic condition when compared to oxygenated hepatocytes. Assessed the involvement of glutamate in hepatic ischemia, they speculated the possible role of metabotropic glutamate receptor subtype 5. The administration of quisqualate, a competitive agonist of mGluR5, decreased cell viability after 45 minutes of ischemia, reaching the maximum level after 90 minutes. On the contrary, the treatment with MPEP, a negative allosteric modulator for mGluR5, improved hepatocytes viability and induced recovery after ischemic injury (M Storto et al. 2000).

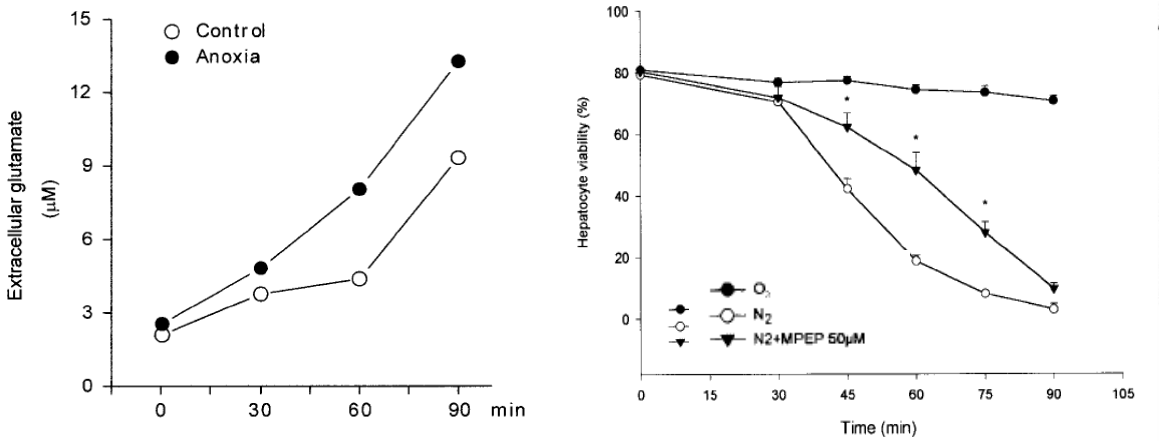


Figure 1.33. Hepatocytes in normoxic and anoxic conditions. Glutamate concentration increases in anoxic conditions compared to normoxic conditions (on the left). The administration of mGluR5 negative allosteric modulator MPEP increased hepatocyte viability respect to anoxic controls (M Storto et al. 2000).

1.9 Hepatic ischemia/reperfusion injury

Since the liver is involved in several biological processes, it needs elevated levels of oxygen. To fulfill the high demand of oxygen, the liver is provided by a well-branched vascularization and the two main delivering blood vessels are the hepatic artery and the portal vein. Although the dual vascularization, ischemic events could occur. According to the temperature at which is achieved, hepatic ischemia/reperfusion injury can be subdivided into warm and cold ischemia: the first one takes place at body temperature (37°C), while the latter at 4°C (Baumann et al. 1989). Warm ischemia occurs in the setting of transplantation, trauma and elective liver surgery, as well in some types of toxic liver injury, hypovolemic shock, sinusoidal obstruction, Budd-Chiari syndrome and sleep apnea, in which the blood flow is temporarily suspended. Cold ischemia, instead, is applied to reduce hepatic metabolism during organ preservation before transplantation (Tanaka & Wanless 1998; Papadopoulos et al. 2013). Except for fatal events, the ischemic period is generally accompanied by the restoration of oxygen delivery, i.e. the reperfusion, and this process is called Ischemia/Reperfusion Injury (IRI) (Elias-Mirò, Jimnez-Castro and Peralta, 2012).

In the ischemic phase, hypoxia presents several consequences. First of all, hepatocytes show morphological changes at plasmatic membrane level, consisting mainly in blebs generation. Blebs contain cytosol and endoplasmic reticulum, while mitochondria and lysosomes are excluded. Cells volume increases about 30%-50%, and when blebs burst, the release of enzymes and intracellular catabolites occurs, together with the collapse of the ionic and electrochemical gradients. In addition, hypoxia elicits mitochondrial impairment, cellular acidosis and Kupffer cells activation (Lemasters 1999). The second phase, reperfusion, consists into rewarming the organ at 37°C, and hepatic circulation is restored. During this latter phase, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) arise, triggering a further Kupffer cells activation within two hours. After 48 hours post transplantation, the inflammatory process begins, causing neutrophils infiltration, cytokines releasing and proteases activation (Carden & Granger 2000)

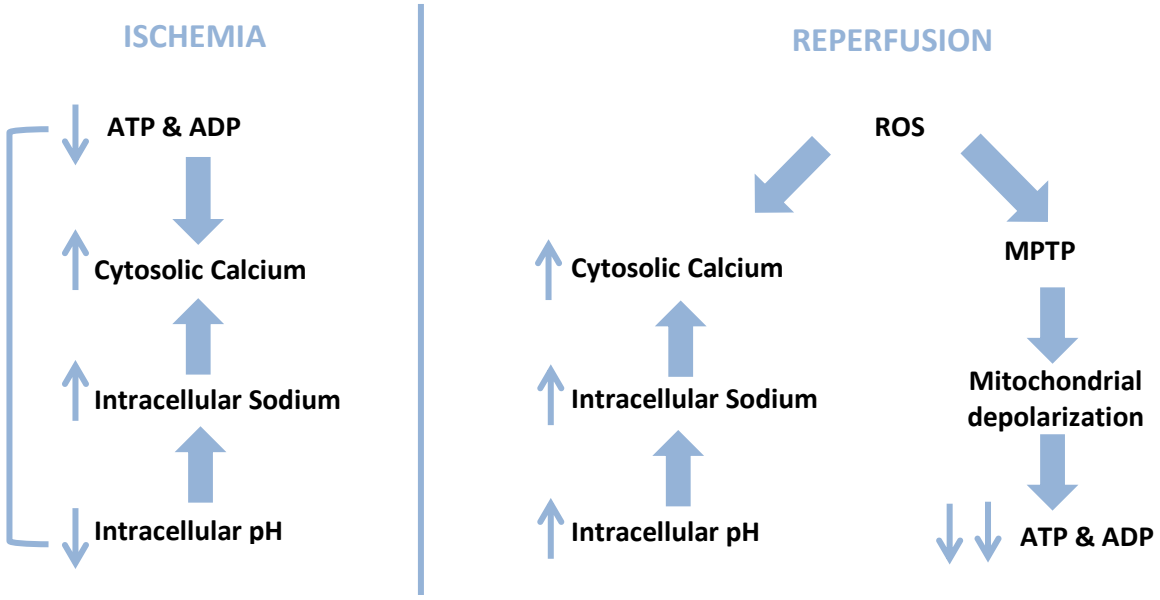


Figure 1.34. Schematic representation of ischemia/reperfusion injury signaling.

1.9.1 ATP depletion

The major event of ischemic damage is the reduction of Adenosine Triphosphate (ATP) synthesis, accomplished by mitochondria. ATP is an essential energy substrate: its hydrolysis, in fact, provides energy for several metabolic and biochemical reactions involved in development, adaptation and cell survival. ATP production in an aerobic cell is particularly effective when the degradation of glucose and fatty acids is coupled to the complexes located into the inner membrane of mitochondria to drive oxidative phosphorylation. Oxidative phosphorylation is mediated by an electron transport chain and establishes a transmembrane electrochemical gradient by supporting the accumulation of protons in the intermembrane space of the mitochondria. This gradient is used as an energy source by ATP synthase during the synthesis of an ATP molecule from a molecule of Adenosine Diphosphate (ADP) and an inorganic phosphate.

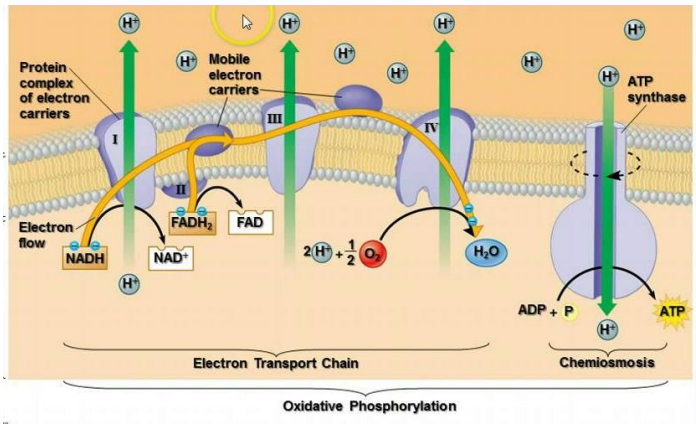


Figure 1.35. Electron transport chain. The coupling of electron transfer through mitochondrial complexes and proton transfer across membrane creates the proton gradient necessary to ATP formation in F_1F_O-ATP synthase.

Without oxygen, oxidative phosphorylation halts: the proton gradient between the intermembrane space and the inner mitochondria is abolished, thus ATP synthesis is interrupted. This rapid fall in intracellular ATP induces a cascade of events leading to reversible cell damage, which, over time, gradually becomes irreversible, causing cell death and destruction of the parenchymal tissue. Thus, to survive, the cell shifts its metabolism from aerobiosis to anaerobiosis, during which glycolytic substrates are able to rescue sinusoidal endothelial cells from lethal cell injury (Nishimura et al. 1998). Unfortunately, glucose does not protect hepatocytes against anoxic injury since hepatocytes lack hexokinase IV, which converts glucose in glucose 6-phosphate in the glycolytic process. However, in certain conditions, hepatocytes employ glycogen to produce energy (Bradford et al., 1986). In fact, once glycogen is transformed in glucose 1-phosphate by glycogen phosphorylase, and subsequently in glucose 6-phosphate by phosphoglucomutase, it can enter the glycolytic cycle. When glucose and glycogen are sold out, hepatocytes exploit fructose or, in addition, they can enhance gluconeogenesis (Nieminen et al. 1990).

Another consequence of depletion of ATP is the conversion of adenosine in hypoxanthine, substrate of xanthine dehydrogenase. In ischemic conditions, xanthine dehydrogenase is converted in xanthine oxidase, which converts in turn hypoxanthine in xanthine and urate, with the release of free radicals (Reddy et al. 2004). Furthermore, ATP depletion causes the destruction of plasmatic membranes, because Na^+/K^+ ATPases can not control the electrochemical gradient anymore, inducing cellular oedema.

1.9.2 Intracellular acidosis

Another process occurring during I/R injury is intracellular acidosis. The increased production of protons due to metabolic modifications quickly saturates the buffering capacity of the cell. Intracellular acidosis alters the physiological functioning of the cell by accumulating intracellular Na^+ through the activation of Na^+/H^+ exchangers, ensued by Ca^{2+} raised due to activation of Na^+/Ca^{2+} exchangers, increasing the production of free radicals, changing the affinity of several proteins and their tertiary structures, inhibiting enzymes, and disrupting the function of sarcoplasmic pumps and carriers (Martin et al. 2006). The main source of protons during ischemia comes from the production of lactate from pyruvate by Lactate Dehydrogenase (LDH). The accumulation of extracellular lactate greatly reduces the effectiveness of the lactate/proton co-transporter, preventing the removal of protons. Additionally, the residual metabolic activity also contributes to acidosis, as the hydrolysis of an ATP molecule releases a proton (Gourdin & Dubois 2013). However, the naturally occurring acidosis in ischemic conditions has been shown to be highly protective, because of the suppression of degradative enzymes, including proteases, phospholipases and endonucleases, and inhibition of permeability transition pore. Nevertheless, during reperfusion oxygen and physiological pH are restored, mitochondrial permeability transition pore opens and cell death rapidly occurs (Kim et al. 2003).

1.9.3 Calcium overload

In physiological conditions, intracellular calcium concentration is maintained constant (10^{-7} M) by specific transporters localized both within plasmatic and organelles membranes, such as endoplasmic reticulum and mitochondria (Berridge 1993). Intracellular calcium plays a pivotal role in the activation of many enzymes involved in several functions, including muscle contraction and cell signalling. During ischemia, instead, since transporters have an altered behaviour, a detrimental increase of calcium intracellular concentration occurs (Sakon et al. 2002). The elevated amount of Ca^{2+} triggers the activation of numerous enzymes: proteases, responsible for cytoskeleton damages and vesicles formation; phospholipases, causing deformation of plasmatic membrane; endonucleases, which determine chromatin condensation (Schanne et al. 1979). In addition, I/R induced-calcium levels activate the calpains, proteases able to degrade membrane, cytoplasmic and nuclear substrates, leading to

the breakdown of cellular architecture and finally apoptosis (Momeni 2011; Croall & Ersfeld 2007). Intracellular Ca^{2+} accumulation is also associated to calcium pyrophosphate complexes and uric acid generation, which bind to inflammasomes. Inflammasomes enhance production of cytokines, such as IL-1β and TNF-α, exacerbating I/R injury (Kalogeris et al. 2012). At mitochondria level, the ever-greater calcium concentration stimulates ATP synthase, pyruvate dehydrogenase, isocitrate dehydrogenase, that attempt to bend-aid ATP depletion (Schanne et al. 1979).

1.9.4 Kupffer cells activation

Kupffer cells are specialized macrophages within the liver, localized between sinusoids wall and blood vessels. Their task is the defense of the liver from bacteria, viruses and other exogenous compounds. During ischemia/reperfusion process, these cells are produced in an excessive extent, and their products, such as cytokines, are consequently overexpressed, exacerbating the injury. Among cytokines are included: interleukine-1 (IL-1), interleukine-6 (IL-6) and tumor necrosis factor (TNF)-α (Wanner et al. 1996). IL-1 appears within minutes during I/R damage and induces ROS formation, whereas IL-6 release is delayed and its administration in rats protects from warm ischemia/reperfusion injury (Camargo et al. 1997). TNF-α, instead, plays a dual role in the liver: at certain conditions it promotes hepatocyte proliferation and liver regeneration, while during liver damage it mediates cell death (Schwabe & Brenner 2006). In fact, in IRI, TNF-α induces Kupffer cells to produce more TNF-α (Shirasugi et al. 1997). In turn, TNF-α release enhances oxidative stress and the delivery of chemokines, leading hepatocytes to apoptosis (Imanishi et al. 1997). Furthermore, the reperfusion worsens the scenery because TNF-α spreads throughout the organ and the body (Nastos et al. 2014), recalling other chemokines and neutrophils (Colletti et al. 1990).

In a rat model of ischemia/reperfusion, the isolated Kupffer cells release TNF-α almost five times more when compared with sham-operated controls (Wanner et al. 1996).

Moreover, the presence of IL-12 has been shown to activate both IL-1 and TNF-α (Lentsch et al. 1999).

1.9.5 ROS

During both phases of I/R process, Reactive Oxygen Species (ROS) are overproduced: superoxide anion ($O_2•^-$), hydroxyl radical (OH•), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2). They are generally synthesized within mitochondria by the electron transport chain, in peroxisomes by soluble enzymes, such as xanthine oxidase and aldehydeoxidase, in the endoplasmic reticulum by P450 system or in nuclear membrane. In physiologic conditions, cells have specific endogenous systems, able to reduce ROS effect. Superoxide Dismutase (SOD), reduced Glutathione (GSH), vitamins A, E, C, some elements (selenium, zinc and magnesium) and other molecules, such as melatonin and bilirubin, are the most important antioxidant, involved in the counterbalancing ROS production and inactivation (Sauer et al. 2002). Nevertheless, at certain conditions, this equilibrium can shift in favour of radicals: oxidative stress condition is so established, and cellular environment passes from reducing to oxidant.

Oxidative stress impairs cells through a variety mechanisms, including lipid peroxidation, DNA oxidation, and enzyme denaturation.

During ischemia, intracellular hypoxia stimulates ROS production through complexes I and III of the mitochondrial Electron Transport Chain (ETC) (Guzy & Schumacker 2006). Moreover, cytochromes are in the reduced state, and electrons pass directly to O_2. Therefore, a big number of superoxide anions are easily produced (Becker 2004). When O_2 emerges during reperfusion, xanthine oxidase produces $O_2•^-$ and H_2O_2, which are converted to OH• through Fe^{2+} oxidation. Besides xanthine oxidase, mitochondria and other ROS-producing systems contribute to the $O_2•^-$ and H_2O_2 formation.

ROS also upregulate nuclear transcription factors such as NF-κB and the subsequent release of TNF-α and IL-1 (Cursio et al. 1999).

1.9.6 Nitric oxide

Nitric Oxide (NO) is an important cellular signalling molecule: it modulates vascular tone and it is involved in angiogenesis. Besides the reduction of nitrites or nitrates, the constitutive endothelial calcium-dependent Nitric Oxide Synthase (eNOS) provides the basal release of NO in physiological conditions. However, during inflammation, NO is produced by the induced Nitric Oxide Synthase (iNOS) isoform (Golwala et al. 2009). Recently, a novel

isoform of NOS has been found: the mitochondrial NOS (mtNOS). Actually mtNOS has not completely understood: some works reported that it could be a sort of eNOS, on the contrary, other publications revealed similarities with iNOS (Lacza et al. 2009).

During liver I/R injury, NO reduction is associated with the worsening of the hepatic damage. In this particular condition, the NO steady state production decreases, due to a reduced eNOS activity. The fall of available nitric oxide induces oxidative stress, apoptosis, leukocyte adhesion and microcirculatory tone (Lemasters 1999). In addition, the high amount of both NO and ROS allow reactions between them, yielding Reactive Nitrogen Species (RNS), such as peroxynitrites ($ONOO^{\bullet -}$). These toxic molecules, like ROS, modify primary structure of proteins and their functionality (L. M. Walker et al. 2001).

On the contrary, restoration of NO to more physiological levels diminishes the liver ischemic injury, enhancing hepatic oxygenation and sinusoidal microcirculation. In fact, being a vasodilator, nitric oxide improves hepatic oxygenation and sinusoidal microcirculation (Siriussawakul et al. 2010).

There is a still open controversy about the detrimental or beneficial effects of NO in ischemia/reperfusion injury. In a rat model of hepatic I/R, Wang and co-workers observed an increase in iNOS mRNA and protein expression on the first day following hepatic reperfusion. Upregulation of iNOS correlated with increased hepatic injury, as shown by elevated serum levels of Alanine aminotransferase (ALT) and Aspartate Transaminase (AST), two markers for liver damage. Administration of a non-selective nitric oxide synthase inhibitor significantly increased AST and ALT, whereas administration of a selective iNOS inhibitor significantly decreased transaminases levels. These data suggest that the deleterious effects of the non-selective NOS inhibitor were due to inhibition of eNOS, while the protective effects of the selective inhibitor were caused by inhibition of iNOS (Wang et al. 1998).

1.9.7 Cell death

Ischemic stress prepares cells for damage and will eventually induces cell death, but cell injury is generally displayed during the reperfusion of the organ (Caldwell-Kenkel et al. 1989). For many years, I/R-induced cell death was supposed to occur by necrosis or oncosis. However, it is now documented that cells can also be programmed to die by cellular signaling

mechanisms through apoptosis (Jaeschke 1991). Moreover, another process has been described as "necrapoptosis", in which a common death signal or toxic stress appears and culminates in either necrosis or apoptosis (Lemasters 1999).

Necrosis is an unplanned event that involves groups of cells, depleted of any ATP. The most relevant feature of necrosis is the swelling of cells and their constituent organelles, ensued by plasma membrane rupture and the release of hepatic enzymes, such as lactate dehydrogenase and transaminases (Jaeschke 1991). Cell lysis interests hepatocytes in warm ischemia, while during cold ischemia sinusoidal endothelial cells are more prone to necrosis (Jaeschke & Lemasters 2003). In addition, necrosis is characterized by important mitochondrial dysfunction, succeeded by rapid permeability transition (Arias et al. 2001).

A detailed morphological research established cell shrinkage, chromatin condensation, changes in plasma membrane lipids, DNA degradation and apoptotic bodies formation are the hallmarks for apoptosis (Arias et al. 2001). Since it is a programmed mechanism, apoptosis requires energy, in form of ATP, to be accomplished. It is triggered by specific signals, such as TNF-α and Fas ligand, which activate a cascade of caspases and lead to death individual cells. Apoptosis is a complicated process, in which an extrinsic and intrinsic pathways crosstalk between themselves, and in which several proteins are recruited, for example those belonging to Bcl-2 family. Bcl-2 family is composed of many proteins, among them Bcl-2 and Bax. Bcl-2 has an antiapoptotic function, whereas Bax is proapoptotic (Tsujimoto & Shimizu 2000). Therefore, in a murine model of I/R, it has been demonstrated that Bcl-2 overexpression protects livers after reperfusion (Selzner et al. 2002). Similarly, Bax ablation attenuated the apoptotic injury induced by I/R in liver through the inhibition of a caspase (Ben-Ari et al. 2007).

Although differences in morphology and signals characterize each death modalities, they can share common pathways, for instance the Mitochondrial Permeability Transition Pore (MPTP) (Kim et al. 2003). The Mitochondrial Permeability Transition (MPT) is the massive swelling and depolarization of mitochondria, which occur in oxidative stress or in calcium overload conditions. In these circumstances a pore into the inner mitochondrial membrane opens, molecules of less than 1500 Da can freely pass across the membranes, leading to oxidative phosphorylation uncoupling and ATP is not produced anymore (Baines 2009). Mitochondrial permeability transition initiates both in necrosis and in apoptosis after reperfusion. When the onset of MPT is widespread and implicates the greatest amount of mitochondria in a cell and ATP is strongly depleted, the cell undergoes to necrotic cell death. On the contrary, when mitochondrial permeability transition develops slowly and not involves

all the mitochondria within a cell, ATP content may recover. In this case, partial ATP recovery prevents necrotic cell death, addressing the cell to apoptosis. However, if ATP levels fall again, necrosis substitutes apoptosis. Thus, interconversion between apoptotic and necrotic cell killing is controlled by the availability of ATP (Kim et al. 2003).

AIM OF THE STUDY

Metabotropic glutamate receptor subtype 5 (mGluR5) belongs to Group I mGluRs and it is coupled to $G_{\alpha q/11}$ protein. Through phospholipase Cβ activation, this pathway causes calcium mobilization and protein kinase C stimulation (Ferraguti & Shigemoto 2006). mGluR5 is composed of a large N-terminal extracellular domain, linked to the seven helices of the transmembrane domain by a cysteine-rich domain, necessary for dimerization and signal transmission. At the intracellular level, the C-terminus tail interacts with a plethora of scaffold proteins, which regulate receptor expression and networks with other receptors. The N-terminal domain represents the orthosteric binding site, in which both agonists and antagonists can activate or inhibit, respectively, the activation of the receptor. Since orthosteric ligands are poorly selective and generate a "all-or-none" response, allosteric modulators were developed to finely tune the response. Differently from orthosteric molecules, allosteric ones bind to a pocket within the transmembrane domain, from where they can positively or negatively modulate the receptor (Niswender & Conn 2010). mGluR5 is mainly located in the central nervous system, where it regulates both physiological and pathological processes (Nicoletti et al. 2011). Recently, mGluR5 has been detected also in peripheral tissues, such as in liver, pancreas and gastrointestinal tract (Ferrigno et al. 2017).

From the literature, it is known that the metabotropic glutamate receptor subtype 5 is involved in ischemia, both at cerebral and hepatic level (M Storto et al. 2000). The ischemic period is generally accompanied by the restoration of oxygen delivery, i.e. the reperfusion. The global process is called Ischemia/Reperfusion Injury (IRI) and causes detrimental effects within the cells. Starting from morphological changes, the cells are subjected to biochemical and electrochemical changes: ATP depletion is accompanied by mitochondrial impairment, Kupffer cells activation, generation of reactive species of oxygen and nitrogen, inflammation and cell death (Arias et al. 2001).

In hypoxic conditions, isolated hepatocytes showed ameliorated viability after the pharmacological blockade of mGluR5 with the negative allosteric modulator 2-Methyl-6-(phenylethynyl)pyridine (MPEP) (M Storto et al. 2000). Nevertheless, it has been reported that MPEP reduces ATP content in murine BV-2 microglia cells and astrocytes, in a receptor-dependent manner (Chantong et al. 2014; Xu et al. 2014); in both cases the authors hypothesized a mGluR5-mediated effect. It is well known that ATP depletion is the main cause of ischemic stress, so these data look incongruous. However, up to date, it is still unknown whether MPEP is able to deplete ATP in hepatocytes.

The first part of this study had multiple aims: first of all to confirm MPEP protection and investigate whether other mGluR5 allosteric and orthosteric antagonists (MTEP, Fenobam, CPG) have similar properties; then, to clarify the mechanisms involved in the protection mediated by MPEP; and, finally, to investigate the mechanism of MPEP-mediated ATP depletion.

In the second part of the project, we wanted to assess whether MPEP protection was maintained in *ex vivo* models. In fact, we know that hepatocytes treated with MPEP exhibited improved viability after ischemic injury, but no one reported any protection occurring in the whole organ. For this reason, we evaluated whether the administration of MPEP could improve liver conditions in two models of ischemia/reperfusion injury: cold (4°C) and warm (or normothermic (37°C)) ischemia/reperfusion.

MATERIALS & METHODS

3.1 Materials

Collagenase was provided by Worthington Biochemical Corporation, Lakewood, New Jersey, United States. Belzer UW® Cold Storage Solution was purchased from Bridge To Life, Columbia, United States. 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes Inc., Oregon, United States.
All other chemicals used for experiments were of analytical grade and were purchased from Sigma Aldrich (Milano, Italy).

3.1.1 Animals

Original studies in animals have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Italian Ministry of Health and the Ethical Committee of the University of Pavia. Balb c mice and mGlu5 receptor knockout mice, provided by Charles Rivers Laboratories, weighting 20-30 g, were allowed free access to water and food until the beginning of all the experiments.

3.1.2 Antibodies

The mouse antibody anti-Tubulin was purchased from Sigma Aldrich. Santa Cruz Biotechnology, INC. provided the rabbit polyclonal antibodies anti-Bax, anti-eNOS, anti-Actin, the goat polyclonal anti-TNF-α and the mouse monoclonal anti-Bcl-2. The mouse monoclonal anti-HIF-1α was purchased from Abcam, while the rabbit polyclonal anti-GP was from Agrisera. Cayman Chemical provided the rabbit polyclonal antibody anti-iNOS.
Specific peroxidase-conjugated anti-IgG antibodies were from Santa Cruz Biotechnology.

3.1.3 Drugs

6-Methyl-2-(phenylethynyl)pyridine (MPEP), 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) and Fenobam were purchased from Sigma Aldrich (Milano, Italy). 3,5-Dihydroxyphenylglycine (DHPG), 3,3'-Difluorobenzaldazine (DFB) and (*S*)-4-Carboxyphenylglycine (4-S-CPG) were provided by Tocris Cookson Ltd (Bristol, UK).

3.2 *In vitro* experiments

3.2.1 Hepatocyte isolation

Hepatocytes were isolated from male Wistar rats (200-250 g, fasted for 18 hours) by collagenase perfusion of the liver, according to Croce et al. (Croce et al. 2004).

3.2.1.1 Apparatus

For hepatocytes isolation a perfusion system is required. The perfusion system was composed of a peristaltic pump, heat exchanger, water bath, bubble trap, reservoir, oxygenator (O_2CO_2, 95% and 5% respectively) and autoclavable tubing (Figure 3.1). The perfusion buffer passes from the reservoir through a bubble trap and a Y tube, connected to a manometer and to the portal vein cannulated. The perfusate outflowing through the thoracic caval vein was collected in the reservoir again and recirculated.

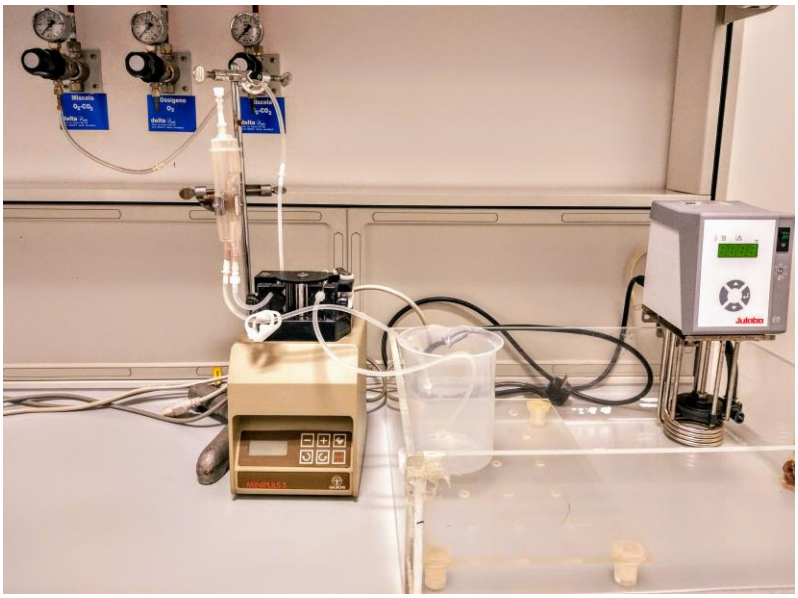


Figure 3.1. Perfusion system for hepatocytes isolation. The perfusion apparatus is composed of a peristaltic pump, heat exchanger, water bath, bubble trap, reservoir, oxygenator and autoclavable tubing.

3.2.1.2 Solutions

HANK 10X in 500 ml ultrapure water as final volume: NaCl 40 g
KCl 2 g
Na_2HPO_4 0.2 g

Krebs 10X: **Solution A:** $NaHCO_3$ 2.1 g in 40 ml of ultrapure water saturated with O_2CO_2
Solution B: NaCl 6.95 g
KCl 0.355 g
KH_2PO_4 0.163 g
MgSO4 * 7 H_2O 0.295 g in 40 ml of ultrapure water.
Then, B was mixed with A solution to a final volume of 100 ml.

KRH 10X in a final volume of 500 ml ultrapure water: NaCl 33.60 g
KCl 1.86 g
KH_2PO_4 0.68 g
MgSO4 * 7 H_2O 1.48 g
HEPES 29.79 g

It was necessary to dilute 10-fold and add other reagents to these solutions daily, just before the experiment. The washing solution, the collagenase solution and Krebs solution were heated at 37°C, pH 7.4 and saturated with O_2CO_2, while KRH solution was heated at 37°C at a pH of 7.4.

Washing solution in a final volume of 200 ml of ultrapure water: Hank 10X 1:10
EDTA 53.3 mg
BSA 2.66 g

Collagenase solution in a final volume of 150 ml of ultrapure water: Hank 10X 1:10
Collagenase 75 mg
$CaCl_2$ 60 mg

Krebs solution in a final volume of 200 ml of ultrapure water: Krebs 10X 1:10
HEPES 600 mg
BSA 2 g
$CaCl_2$ 125 mM 1:100

KRH solution in a final volume of 200 ml of ultrapure water: KRH 10X 1:10
BSA 0.08 g
$CaCl_2$ 100 mM 1:50

Percoll 36% in a final volume of 200 ml of ultrapure water and Hank 10X 1:10

3.2.1.3 Isolation procedure

Male Wistar rats (200-250 g), fasted for 18 hours, were anesthetized intraperitoneally by means of 40 μg/kg sodium pentobarbital in saline and depth of anesthesia was monitored by tail pinch and sniffing reflex. After a midline incision of the abdomen, the animals were heparinized with 2000/5000 U/kg of body weight, to avoid blood clotting. Then, the viscera were placed sideways and the hepatic portal vein was cannulated. Firstly, livers were perfused

with the washing solution saturated with O_2CO_2 for 5 minutes at 37°C, then livers were reperfused with the collagenase solution saturated with O_2CO_2, for 7 minutes, at 37°C, or until the pins left a sign into the liver. So, when collagen was degraded, Krebs solution was used to wash the organ from the previous solution. Hepatic cells were layered on top of a Percoll suspension and centrifuged at 500 x g for 3 minutes. After removing the supernatant and the Percoll layer, hepatocytes were suspended in KRH buffer and incubated at 37°C (final cell density: $1x10^6$/mL).

3.2.1.4 Pharmacological treatment

Hepatocytes, at a final cell density of $1x10^6$/mL, were incubated 30 minutes at 37°C in rotavapor (Figure 3.2), with the following molecules:

- MPEP: 0.3 μM, 3 μM, 30 μM
- MTEP: 0.3 μM, 3 μM, 30 μM
- Fenobam: 1 μM, 10 μM, 50 μM
- (*S*)-4-CPG: 100 μM, 200 μM
- DHPG 100 μM + DFB 10 μM
- Vehicle (KRH + DMSO)

Stock solutions were prepared in DMSO for MPEP, Fenobam and DFB, MTEP and DHPG were solubilized in ultrapure water, (*S*)-4-CPG in water plus NaOH 10 M, at high temperature.

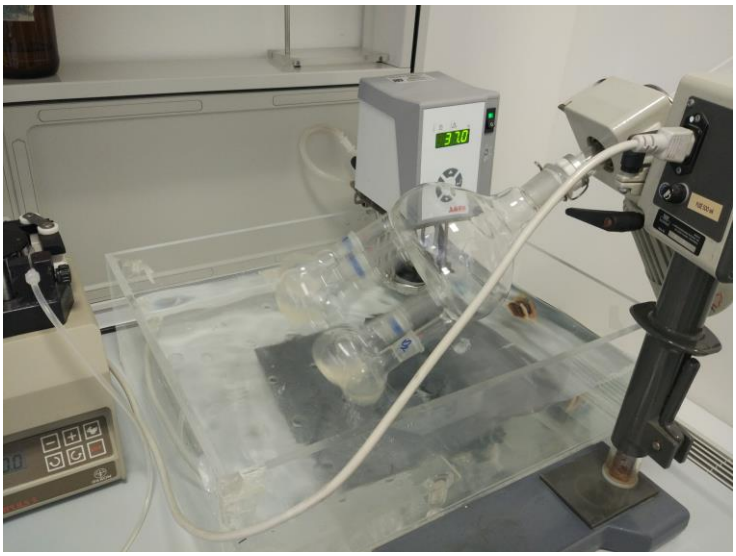


Figure 3.2. Pharmacological treatment of hepatocytes in rotavapor, at 37°C.

3.2.1.5 Warm ischemia experiments on rat hepatocytes

Anoxia was induced by blowing nitrogen for 2 minutes into hermetically sealed vials containing the cell suspension, while control vials were exposed to an oxygen-containing atmosphere (Figure 3.3). The sealed vials were maintained at 37°C in a water bath for 90 minutes and cell samples were collected at 0', 30', 45', 60', 75' and 90'.

Figure 3.3. N_2 insufflation into sealed vials to make cells anoxic.

3.2.1.6 *In vitro* assays on hepatocytes

3.2.1.6.1 Measurement of Cell Viability: trypan blue exclusion and LDH release

Cell viability was monitored by both Trypan blue exclusion and release of lactate dehydrogenase into the medium, as described respectively by Piccinini et al. and by Wieme and Demeulenaere.

Trypan blue exclusion assay is based on the principle that intact plasma membranes in living cells exclude this dye, while dead cells do not (Piccinini et al. 2017). Briefly, trypan blue was diluted 10-fold in PBS and 1:10 aliquot of hepatocytes was added to this solution. Hepatocytes were loaded on Burker chamber and blue cells, those in which trypan blue entered, were counted, obtaining the percentage of dead cells.

The cytosolic enzyme lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. When oxygen is absent or in short supply, LDH converts pyruvate, the final product of glycolysis, to lactate.

$$\text{PYRUVATE + NADH} \xleftrightarrow{\text{LDH}} \text{LACTATE + NAD}^+$$

LDH is considered a marker for the evaluation of cellular damage: in fact, it is released when cells burst out.
Hepatocytes viability was assessed through release of lactate dehydrogenase into the perfusate (Wieme & Demeulenaere 1970) by means of a spectrophotometer at a wavelength of 340 nm, every 6 seconds in one minute. To evaluate LDH concentration, it was necessary to measure its activity adding to supernatant saturating concentration of NADH and pyruvate. In this way, the enzyme consumes the substrates at a rate proportional to its concentration. Applying Lambert-Beer's law to the slope of the obtained curve and knowing the molar extinction of NADH, activity of LDH released from damaged cells was calculated and expressed in mU/min/g.

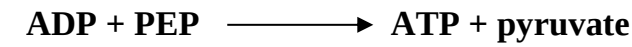
3.2.1.6.2 Measurement of ATP

Adenosine triphosphate (ATP) is a marker for cell viability because it is present in all metabolically active cells and its concentration declines very rapidly when cells undergo necrosis or apoptosis. ATP represents also a marker of the energetic status of the cell, as it rapidly falls in detrimental conditions.

Hepatocyte ATP is measured with the ATPlite monitoring system, based on firefly (*Photinus pyralis*) luciferase. The production of light is due to the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration and was measured by using a Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader.

Briefly, at stabilized times, isolated hepatocytes were precipitated in TCA 30% and centrifuged at 10,000 x g for 5 minutes at 4°C. The supernatant was collected and diluted 40 times in 100 mM phosphate buffer pH 7.75 and assayed. ATP variation in presence of mGluR5 modulators was measured by the luminescence method using the ATPlite luciferine/luciferase kit (Perkin Elmer Inc., Waltham, Massachusetts), according to manufacturer's instructions with minor changes. Lysis buffer and luciferine/luciferase buffer (50 µl each) were added to 100 µl of cells ($1x10^6$/ml in KRH buffer) in a white 96-well plate, then the luminescence was measured in a Victor^2 Perkin Elmer luminometer.

Afterwards, Adenosine Diphosphate (ADP) evaluation was performed, treating the same samples with Phosphoenolpyruvate (PEP) and Pyruvate Kinase enzyme (PK). PK is able to catalyze the pyruvate production, starting from ADP and PEP.



By this step, all the ADP present in the sample was converted to ATP and, in this manner, it was possible to obtain a total ATP value, given adding ATP and ADP. The values of ATP and ADP were calculated by subtraction (Gorman et al. 2003).

3.2.2 Mitochondrial isolation

Whole livers (9 g) were washed with ice-cold saline and processed immediately for mitochondria isolation by standard techniques using differential centrifugation.

3.2.2.1 Solutions

Homogenation buffer, pH 7.2: Sucrose 0.25 M
HEPES 5 mM
EDTA 1 mM

Washing buffer, pH 7.2: Sucrose 0.25 M
HEPES 5 mM

Respiration buffer, pH 7.1: Sucrose 110 mM
EGTA 0.5 mM
$MgCl_2$ *6 H_2O 3 mM
K-gluconate 60 mM
KH_2PO_4 10 mM
HEPES 20 mM

Rh-123 buffer, pH 7.2: Sucrose 250 mM
HEPES 10 mM
$MgCl_2$ 2 mM
KH_2PO_4 4 mM
Succinate 6 mM
Rotenone 1 μM
Rhodamine (Rh)-123 0.3 μM
Oligomycin 1 μg/ml

3.2.2.2 Procedure

Livers were homogenized in ice-cold homogenation buffer using a teflon/glass Potter homogenizer (B. Braun, Melsungen, Germany). The homogenate was filtered and then centrifuged at 660 x g for 10 minutes at 4°C. The supernatant was taken up and centrifuged again for 10 minutes at 10,000 x g at 4°C. The resulting pellet was resuspended in the washing buffer and centrifuged again 10 minutes at 10,000 x g. The supernatant was discarded, while the pellet kept on ice. Single mitochondrial preparations were obtained for each individual animal (n = 4–6) and mitochondrial protein concentration was determined using the Lowry method (Vairetti et al. 2012; Ferrigno, Vairetti, et al. 2015).

3.2.2.3 Pharmacological treatment

Isolated mitochondria were incubated 15 minutes with the following mGluR5 negative allosteric modulator and orthosteric agonist, respectively:

- MPEP: 0.3 μM, 3 μM, 30 μM
- DHPG: 1 μM, 10 μM, 100 μM
- Vehicle (DMSO)

Stock solutions were prepared in DMSO for MPEP and in ultrapure water for DHPG.

3.2.2.4 *In vitro* assays on mitochondria

3.2.2.4.1 Mitochondrial membrane potential (ΔΨ)

Mitochondrial membrane potential (ΔΨ) was assessed by measuring the uptake of the fluorescent dye rhodamine (Rh)-123, as previously described (Vairetti et al. 2012).
Rhodamine 123 is a cationic fluorescent dye, employed to specifically mark respiring mitochondria. At low concentrations, the probe is not toxic and diffuses according to the negative membrane potential across the mitochondrial inner membrane (Emaus et al. 1986; Chazotte 2011). Variations of ΔΨ are engendered, directly or indirectly, by the proton

movements taking place across the mitochondrial inner membrane during oxidative phosphorylation: under physiological conditions, protons are actively extruded by respiration and passively taken up through ATP synthase during ATP synthesis. Similarly, membrane polarization by administration of respiratory substrates to isolated mitochondria induces Rh-123 quenching, whereas depolarization by ADP addition stimulates fluorescence retrieval (Baracca et al. 2003). Thus, impairment of membrane potential causes loss of the dye and, consequently, the fluorescence intensity (Chazotte 2011).

In practice, the basal fluorescence of rhodamine 123 was measured ((rhodamine 123) in) using a Perkin Elmer LS 50B fluorescence spectrometer at 503 (excitation wavelength) and 527 nm (emission wavelength). After 15 minutes treatment with mGluR5 modulators, mitochondria, at a final concentration of 0.6 μg/μl, were incubated at dark in Rh-123 buffer for 15 minutes. Then, the suspension was centrifuged at 15,000 x g for 10 minutes at room temperature. The supernatant was collected and the fluorescence of Rh-123 was measured again ((rhodamine 123) out). ΔΨ (mV) was calculated according to the following relationship:

$$\Delta\Psi = -59 \log \text{(rhodamine 123) in / (rhodamine 123) out}$$

Each measurement was performed in triplicate immediately after sacrifice on fresh mitochondria preparations.

3.2.2.4.2 Respiratory Control Index (RCI)

Since reduction of oxygen is a critical step in the process of mitochondrial electron transport and ATP synthesis, measurement of mitochondrial oxygen consumption provides a convenient way to assess mitochondrial function. Respiratory Control Index (RCI) expresses the tightness of the coupling between respiration and phosphorylation. To evaluate the respiration process, state 3 and state 4 rates have to be gauged, because RCI is the ratio between the rates of state 3 and state 4 (Silva & Oliveira 2012). State 3 is considered the actively respiring state, whereas the slower rate after all the ADP has been phosphorylated to form ATP is referred to as state 4. It is possible to determine the Phosphate/Oxygen ratio (P:O ratio between ATP formed and oxygen consumption) by assessing the decline in oxygen

concentration during the fast burst of state 3 respiration after adding a known amount of ADP (Figure 3.4).

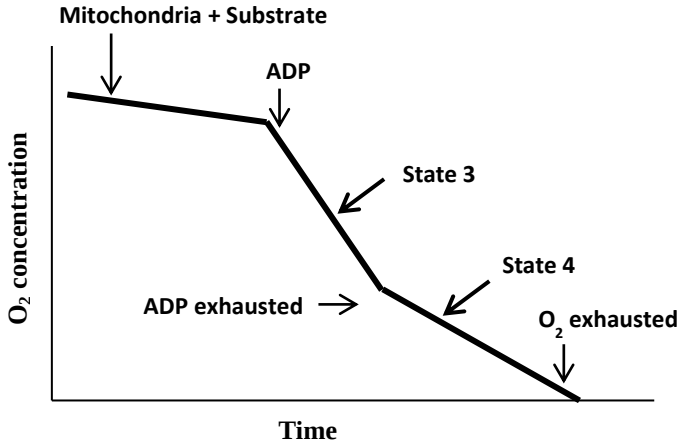


Figure 3.4. Respiratory control index curve. Respiratory control index is the ratio between state 3 and state 4. In a mitochondria solution containing substrates, the addition of ADP causes the rapid decrease of oxygen up to ADP is exhausted (state 3), then the reaction proceeds until oxygen concentration is zero (state 4).

In our experiments, respiratory control index was measured by means of a Clark-type oxygen electrode in a sealed mitochondrial chamber at 25°C (Figure 3.5). After the calibration of the electrode to set the correct atmospheric pressure, voltage and temperature, mitochondria (1 mg/ml) were added to 2 ml of the respiration buffer and the system was allowed to equilibrate in few seconds. Mitochondrial respiration was initiated by addition of 10 mM succinate plus 1 µM rotenone, while oxidative phosphorylation was initiated by addition of 5 mM ADP. In the case of negative allosteric modulator effect evaluation, MPEP 30 µM was added to mitochondrial solution and incubated for 4 minutes before the addition of succinate and rotenone. Each measure was performed in triplicate.

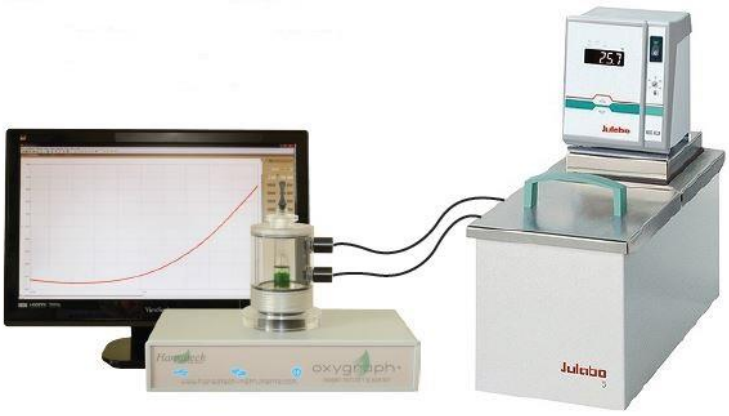


Figure 3.5. Clark-type electrode apparatus.

3.2.2.4.3 Radical oxygen species formation

Reactive Oxygen Species (ROS) include both the so-called "free radicals" (superoxide radical, hydroxyl radical) and molecules containing oxygen. Generally, ROS production takes place during normal metabolic processes involving oxygen. However, in certain conditions an excessive release of ROS occurs, causing several damages to proteins, lipids and DNA, molecules highly sensitive to oxidative stress.

The mitochondrial determination of ROS was obtained using the conversion of the 2',7'-Dichlorofluorescein Diacetate (H_2DCFDA) to a fluorescent molecule, the 2',7'-Dichlorofluorescein (DCF). The H_2DCFDA works as a probe: it enters the organelle where its two acetyl groups are removed by intracellular esterases and, in the presence of ROS, it is oxidized to DCF.

Mitochondria ($1x10^6$) were resuspended in respiration buffer and H_2DCFDA 5 μM (Molecular Probes, Inc.) was added. After 30 minutes of incubation at 25°C, the suspension was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was lysed by 300 μl of deionized water. The vortexed solution was transferred to a black 96-well plate for fluorescence measurements by a Perkin Elmer Victor^2 microplate reader (excitation 485 nm, emission 530 nm) (Storto et al. 2003).

3.2.2.4.4 Respiratory chain complexes I & II function through ATP production

Synthesis of ATP occurs through oxidative phosphorylation (OXPHOS) along the respiratory or Electron Transport Chain (ETC). It includes four enzymatic complexes (complexes I–IV), embedded in the inner mitochondrial membrane, and F_1F_O-ATP synthase/hydrolase (also called Complex V). The ETC catalyzes the transfer of reducing equivalents from high-energy compounds produced by the reactions of the Krebs cycle, such as NADH and $FADH_2$, to oxygen. Electrons from NADH pass through complex I to complex III and complex IV via ubiquinone, producing H^+ protons in the intermembrane space. This proton gradient creates the mitochondrial membrane potential that is coupled with ATP synthesis by complex V from ADP and P*i*. ATP is released from the mitochondria in exchange for cytosolic ADP using a carrier, Adenine Nucleotide Translocator (ANT). $FADH_2$ electrons, instead, pass through complex II to complex III and IV, but only these latter two complexes can pump protons into the intermembrane space.

To trigger the electron transport chain in isolated mitochondria, pyruvate and malate are introduced as substrate to produce NADH, which will transfer its electrons starting from complex I, while succinate is added to initiate ETC by complex II, together with rotenone, to inhibit complex I and consequent ETC in the revers sense.

In our experiments, fresh isolated mitochondria 20 µl (about 1 µg) were added to 1980 µl of the respiration buffer, at 25°C, in water bath. After a 15 minutes treatment with mGluR5 modulators, an aliquot of the mitochondrial suspension was loaded on a white 96-well plate and basal ATP was measured at Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader. To evaluate Complex I function, a mixture of malate 2 mM and pyruvate 5 mM was introduced into mitochondrial suspension and an aliquot was transferred to the plate and read again. Then, ADP 2.5 mM was added and another aliquot was read at two consecutive times. To assess Complex II function, a mixture of succinate 10 mM and rotenone 0.5 µM was added to mitochondrial suspension after ATP basal lecture and an aliquot was transferred to the plate and read again. Then, ADP 2.5 mM was added and another aliquot was read at two consecutive times.

Stock solutions were prepared in ultrapure water for succinate, malate and pyruvate, while rotenone was solubilized in DMSO.

3.2.2.4.5 The F_1F_O ATPase activity

The mitochondrial F_1F_O-ATP synthase/hydrolase, commonly referred also as Complex V, is a multimeric protein located in the inner membrane. F_1 domain, exploiting energy in the form of a proton gradient generated by the Electron Transport Chain (ETC), in the presence of P_i phosphorylates ADP to form ATP. This mechanism, known as oxidative phosphorylation, is responsible for about 90% of ATP generation in mammalian cells. However, when cells are deprived from oxygen, such as in ischemia, this enzyme can perform the reverse reaction, hydrolyzing ATP in ADP, accompanied by transportation of H^+ back to intermembrane space (Wang et al. 2017).

The F_1F_O ATPase activity was assayed using the Complex V MitoCheck from Cayman (Cayman Chemical, Ann Arbor, USA), according to the manufacturer's instructions. In this assay, ATP was dephosphorylated by Complex V to generate an ADP molecule. The ADP produced was then utilized by Pyruvate Kinase (PK) to convert Phosphoenolpyruvate (PEP) into pyruvate with the concomitant generation of ATP. Pyruvate, in the presence of NADH and lactate dehydrogenase, is then reduced to lactate and NAD^+ (Figure 3.6). The rate of NADH oxidation is monitored at 340 nm.

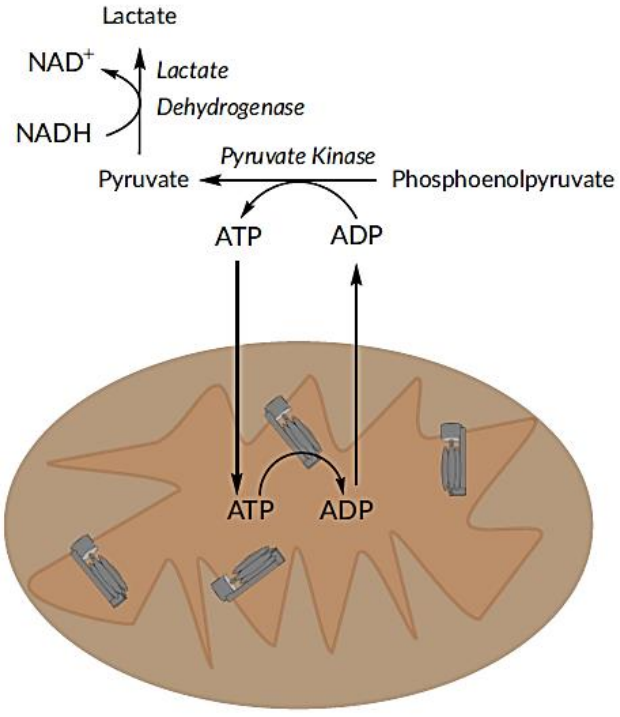


Figure 3.6. Schematic reactions of Complex V activity assay.

3.3 Experiments in acellular solutions

The direct effect of both allosteric and orthosteric antagonists on ATP was assayed in tubes containing neither hepatocytes nor mitochondria, but only a buffered solution. Each vial contained one of the following concentrations of ATP: 10-5-2.5-1.25 μM and one of the ensuing molecules: MPEP, MTEP, Fenobam or CPG at one of these concentrations: 0.3-3-30 μM. ATP content was measured at Victor^2 Multilabel Counter Wallac, Perkin Elmer microplate reader.

3.3 *Ex vivo* experiments

3.3.1 Ischemia/reperfusion model

3.3.1.1 Apparatus

For cold ischemia a polyethylene tube, ice and preservation solution are needed, as illustrated in Figure 3.7.

Figure 3.7. Static cold preservation. The organ is usually conserved in a bag filled of preservation solution and kept to 4°C. In our experiments we used a plastic tube.

For warm ischemia and reperfusion a more elaborated system is required. A typical perfusion apparatus includes several components: peristaltic pump, heat exchanger, bubble trap, reservoir, jacketed perfusion chamber, oxygenator and tubing (Figure 3.8). The perfusion buffer passes through a bubble trap and a Y tube, connected to a manometer and to the portal vein. The perfusate outflowing through the thoracic caval vein was collected in the perfusion chamber, before flowing back to the reservoir by the peristaltic pump.

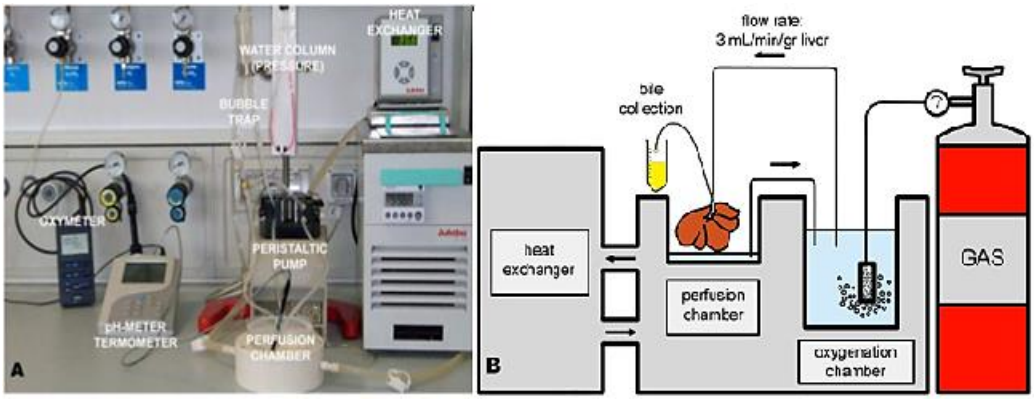


Figure 3.8. Perfusion apparatus. A. The system used into experiments. **B.** The schematic representation of perfusion system. Generally, the apparatus is composed by heat exchanger, perfusion chamber, reservoir, oxygenator, bubble trap and peristaltic pump.

3.3.1.2 Solutions

Krebs-Henseleit solution: $NaHCO_3$ 25 mM
NaCl 118 mM
KCl 4.7 mM
KH_2PO_4 1.25 mM
$MgSO_4$ * 7 H_2O 1.2 mM
$CaCl_2$ 1.25 mM
HEPES 10 mM

UW solution, pH 7.4: Pentafraction 50 g/L
Lactobionic Acid 35.83 g/L
KH_2PO_4 3.4 g/L
$MgSO_4$ * 7 H_2O 1.23 g/L

Raffinone pentahydrate 17.83 g/L

Adenosine 1.34 g/L

Allopurinol 0.136 g/L

Total glutathione 0.922 g/L

KOH 5.61 g/L

3.3.1.3 Procedure

Livers were isolated from Balb c mice and mGlu5 receptor knockout mice, anesthetized with 40 mg/kg of sodium pentobarbital in saline, administered intraperitoneally. After median laparotomy followed by bilateral subcostal incision, the animal received 200 units of heparin per 100 g of body weight via the abdominal inferior caval vein (5000 U/mL, Marvecs Services, Agrate Brianza - MI) to prevent blood clotting and the consequent blood pressure decrease (Cheung et al. 1996). The needle was inserted in a portion of the vessel partially covered of fat, enabling the protection of the hole left by needle extraction. Then the bile duct was cannulated with a 0.61 mm polyethylene tubing (Intramed, Becton-Dickinson, Loveton Circle, MD - USA) to collect bile. The cannulation was made easier by closing the bile duct with a 4/0 silk suture ligation, placed in distal position. In this way the bile duct swelled upstream, becoming more discernible (Figure 3.9A). Then a cut was opened in the bile duct with the spring scissors, and slant-shaped polyethylene tubing was inserted (Figure 3.9B). When the tubing was completely filled with bile, it was fixed with 4/0 silk suture ligation (Figure 3.9C) (Ferrigno et al. 2013).

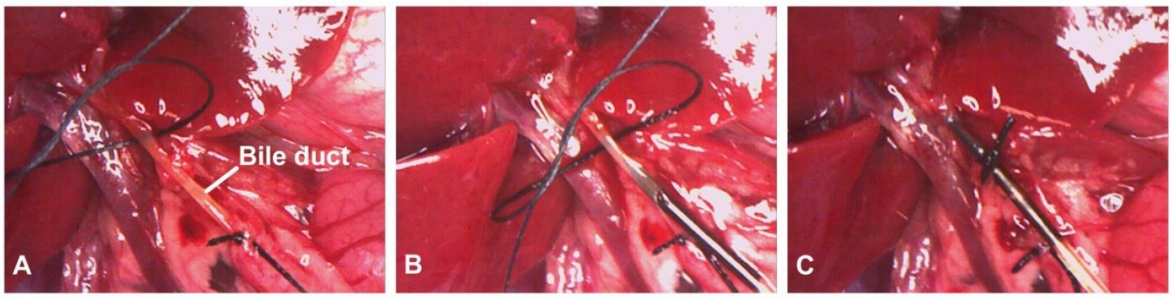


Figure 3.9. Bile duct cannulation. A. The bile duct is ligated with a 4/0 silk suture. **B.** A cut is opened in median position and slant-shaped polyethylene tubing is inserted. **C.** When the tubing completely fills with bile, it is fixed with a 4/0 silk suture ligation (Ferrigno et al. 2013).

Two 4/0 silk sutures were inserted under the portal vein in a proximal position, and two loose double knots were prepared. To avoid leakage through the duodenal vein during perfusion, it was important to place at least one of the sutures between the duodenal branch of the portal vein and the liver (Figure 3.10A). The fat tissue surrounding the portal vein was carefully scrabbled with two cotton-buds. Then, the portal vein was cannulated with a 22 gauge catheter, stabilized by 6/0 silk suture (Johnson & Johnson, Arlington, UK) (Figures 3.10B and 3.10C) to allow the perfusion (Ferrigno et al. 2013).

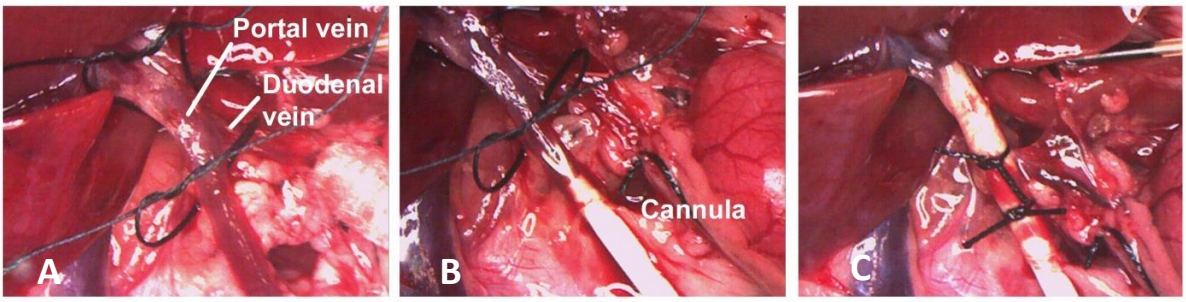


Figure 3.10. Portal vein cannulation. A. Two 4/0 silk sutures are inserted under the portal vein in proximal position. **B.** Portal vein is cannulated with a 22G catheter. **C.** The catheter is ligated with a 6/0 silk suture (Ferrigno et al. 2013).

Afterwards, abdominal inferior caval vein was cut open. Thus, to eliminate all the remaining blood, the liver was washed out in situ with 50 mL of modified Krebs/Henseleit Buffer (KHB) via the portal vein cannula.

3.3.1.4 Cold ischemia/reperfusion model

Then, the liver, free from ligaments, after a washing with the preservation solution Belzer-University of Wisconsin at 4°C (UW, ViaSpanTM, DuPont Pharmaceuticals, Wilmington, DE, USA), was placed into polyethylene tube containing 20 ml UW solution and preserved for 18 hours at 0-4°C.

Concluded the static cold preservation time, the liver was put at room temperature for 10 minutes and successively reperfused with oxygenated (O_2CO_2, 95% and 5%, respectively) KHR buffer at pH 7.4, for 1 hour at 37°C. Reperfusion flux was maintained constant during reperfusion. Samples of the reperfusion solution were collected at 0', 30' and 60'.

At the end of reperfusion, liver samples were snap frozen in liquid nitrogen for further analysis.

3.3.1.5 Warm ischemia/reperfusion model

After isolation, liver was washed out with Krebs-Henseleit buffer and ischemia was performed perfusing for 30 minutes deoxygenated KRH, bubbling the solution with N_2CO_2 (95% and 5%, respectively), at 37°C. Afterward, liver was reperfused with oxygenated KRH solution at 37°C for 2 hours. Samples of the reperfusion solution were collected at 0', 30', 60' 90' and 120'. At the end of reperfusion, liver samples were snap frozen in liquid nitrogen for further analysis.

3.3.1.6 Pharmacological treatment

Isolated livers were treated with MPEP 0.3 μM or vehicle DMSO, added to the preservation solution during cold storage (18 hours, for cold ischemia) or the deoxygenated Krebs-Henseleit buffer (30 minutes, for warm ischemia) and during oxygenated reperfusion.

3.3.2 *Ex vivo* assays

3.3.2.1 Perfusate analysis

Liver parenchyma viability was assessed through release of lactate dehydrogenase into the effluent perfusate, as previously described, and through release of aspartate transaminase and alanine aminotransaminase.

Aspartate Transaminase (AST) is an enzyme belonging to the class of transferases, detectable at very high concentration in the bloodstream as a result of liver and muscle damage. Alanine Aminotransferase (ALT), differently from AST, is a generic cell necrosis index, present at elevated plasma concentration as result of liver and muscle injury of different nature. Therefore it is assessed in combination with more specific markers. For this

reason, the AST/ALT ratio is commonly used as an index of liver damage and can be quantified in serum or in perfusate.

Transaminases were assessed by an automated Hitachi 747 analyser (Roche/Hitachi, Indianapolis, IN, USA), with the contribution of Doctor Vittoria Rizzo, Department of Molecular Medicine, IRCCS Policlinico San Matteo, Pavia.

3.3.2.2 Lowry Protein Assay

The Lowry assay (Folin-Ciocalteau) is applied to quantify the protein content in a specific sample. The Folin reactive (a mixture of tungstate, molybdate and sodium phosphate) and a copper sulphate solution are added to the sample, determining a color change directly related to the protein amount. The absorbance of the sample is measured spectrophotometrically at 550 nm and the high sensitivity of this method allows quantifying up to 5 μg proteins in a 0.2 ml volume.

Two reagents, A and B (Table 3.1), were mixed in a ratio of 50 parts of A and 1 part of B, making a final solution C (A+B= C, Table 3.1). The samples were analyzed in duplicate.

Copper ions (Cu^{2+}) in C solution react in a basic environment with $CO\text{-}NH_2$ protein groups, producing the violet color of the mixture. This reaction is specific for polypeptides because almost two $CO\text{-}NH_2$ groups are required.

Afterwards, Folin reactive was added (D solution, Table 3.1). The copper protein complex reduced tungstic acid and molybdic acid to tungstate blue and molybdate blue. Folin reactive was added only at the end, because in absence of Cu^{2+} ions, it would bind exclusively to aromatic residues, leaving proteins undetected.

To quantify the sample protein content, it was necessary to build a calibration curve, by means of Bovine Serum Albumin (BSA) standard at the concentration of 2 mg/ml. Seven points of the curve were prepared, making serial dilution in deionized water and adjusting the final volume to 200 μl. As the samples, also the curve was analyzed in duplicate. To fix the zero point of the curve, a blank sample was prepared and treated like other samples. Both standards and samples were supplemented with 1 ml of C solution (Table 3.1), stirred and incubated for 10 minutes at room temperature. Then, 100 μl of Folin reactive (D solution) were added to all tubes. After 30 minutes of incubation, the absorbance was detected spectrophotometrically at 550 nm.

The absorbance value was used to determine the protein concentration according to Lambert-Beer law:

$$\mathbf{A = \varepsilon_{\lambda} \times C \times l}$$

(**A**=absorbance; $\boldsymbol{\varepsilon_{\lambda}}$=molar extinction coefficient; **C**=sample concentration; **l**=optical path in cm).

Solution A	NaK 268 mg/l, Na_2CO_3 23.4 g/l, NaOH 4 g/l
Solution B	$CuSO_4$ at 1.56 % in distilled water
Solution C	Solution A + Solution B with a ratio of 50:1
Solution D	Folin + distilled water with a ratio of 1:1

Table 3.1. Summary of Lowry solutions.

3.3.2.3 SDS-PAGE

3.3.2.3.1 Liver tissue extracts preparation

Liver tissue samples (about 50 mg) were homogenized in ice-cold CelLytic Buffer (500 µl) supplemented with Protease Inhibitor Cocktail (10 µl/mL) and centrifuged at 15,000 x g for 10 minutes, as previously described (Di Pasqua et al. 2016). The collected supernatant was divided into new tubes and 4 µl of it were used to quantify the protein content by means of the Lowry method. After that, the supernatant was divided into aliquots containing the same amount of proteins and it was reduced with SDS 2XR, 2% β-mercaptoethanol.

3.3.2.3.2 Polyacrylamide Gel Electrophoresis (SDS PAGE)

Electrophoresis is a technique consisting in differentiated migration of charged molecules in electric field. Molecules are separated according to their charge, size and the applied electric field.

Sodium Dodecyl Sulphate (SDS) is an anionic detergent that stably binds to proteins, denaturating them. In case of excess of SDS, the proteins acquire a constant amount of negative charge *per* unit mass: all the protein-SDS complexes will move toward the anode and, according to the gel sorting properties, their mobility (and therefore the distance travelled after a certain period) is inversely proportional to $\log_{10}$ of their molecular weight. Using standards with known molecular weight together with samples, it is possible to establish the molecular weight of the proteins in the sample.

Protein sorting in SDS-PAGE was carried out by means of a chamber for electrophoresis MINI-PROTEAN III, Bio-Rad. The sorting gel dimensions were 88x55 mm and a thickness of 1.5 mm.

Proteins package themselves in the stacking gel, which had a polyacrylamide concentration of 3%, whereas they separated each other in the linear running gel, in which the polyacrylamide concentration was 7.5% or 10%.

3.3.2.3.3 Acrylamide gels preparation

Two glass plates, outer and inner, were joined together vertically, using a special support.

The prepared running gel solution (Table 3.2) was poured between the two glass plates by the use of a syringe. Once the gel is polymerized, it formed a web capable of separating proteins, according to their molecular weight.

Afterwards, the stacking gel was dispensed (Table 3.2) over the running gel and it allows to a special teflon comb to form the sample loading wells.

Solutions	Running Gel 7.5%	Running Gel 10%	Stacking Gel 3%
Acrylamide 30%	2250 µl	3000 µl	250 µl
Tris HCl 1.5 M pH 8.8	2250 µl	2250 µl	-
Tris HCl 1.25 M pH 6.8	-	-	250 ml
SDS 10%	90 µl	90 µl	25 ml
Water	3870 µl	3120 µl	1700 µl
TEMED 1%	450 µl	450 µl	250 µl
APS 10%	90 µl	90 µl	25 µl
Total Volume	**9000 µl**	**9000 µl**	**2500 µl**

Table 3.2. Polyacrylamide gel solutions.

TEMED (N,N,N,N'-Tetra-Methyl-Ethylendiamine) and APS (Ammonium Persulphate), which are the two cross-linking and polymerizing agents in the reaction, were added just before pouring the solutions between the glass plates.
The samples run simultaneously with a standard mixture of known molecular weight protein. The electrophoresis was carried out for about 2 hours at a constant voltage of 100 Volts, at room temperature. The used buffer for the run was composed of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.

3.3.2.4 Western Blotting

This technique consists of moving proteins, previously separated through SDS-PAGE, from within the gel onto a membrane made of Polyvinylidene Difluoride (PVDF). A MINI TRANS-BLOT Bio-Rad chamber was used and the transfer occurred through the application of an electric field with constant amperage of 200 mA, at 4°C for 2 hours. The procedure consisted in preparing a "sandwich" putting in contact the polyacrylamide gel and the PVDF membrane, the latter previously activated by soaking it first in methanol for few seconds and then in water.

Both the gel and the PVDF were gently pressed together by suitable supports. Therefore, to ensure a more closely contact, two layers of sponges and blotting paper were used, soaked in blotting buffer (20 mM Tris, 150 mM glycine, 20% methanol). During the transfer, the gel was turned towards the negative pole, while the membrane was close to the positive one. This is necessary because, since proteins have a negative charge, will tend to migrate toward the positive pole and, therefore, towards the PVDF membrane.

3.3.2.5 Immunoblotting

The PVDF membrane was incubated for 2 hours at 4°C, under mild stirring, with 5% Blotting-Grade Blocker or BSA in TBS (20 mM Tris/HCl, 500 mM NaCl, pH 7.5) to block any non-specific sites. Afterwards, the membrane was washed twice in TBS 1X 0.1% Tween 20 and incubated overnight, under mild stirring, with specific primary antibodies depending on the protein of interest:

- **anti-HIF-1α**, **anti-GP**, **anti-TNF-α**, dilution 1:2,000;
- **anti-α-Tubulin, anti-Bax, anti-Bcl-2, anti-eNOS, anti-iNOS,** dilution 1:1,000;
- **anti-Actin**, dilution 1:10,000.

After that, the PVDF was washed every 5 minutes for 30 minutes, under stirring and at room temperature with washing buffer PBS 1X (Na_2HPO_4 8 mM, NaH_2PO_4-H_2O 2 mM, NaCl 140 mM, pH 7.4, 0.1% Tween 20), in order to remove the excess of antibody. Then, the membrane was incubated for 1 hour at 4°C with appropriate peroxidase-conjugated secondary antibody diluted 1:2,000 for all antibodies, except for HIF-1α, GP e TNF-α, diluted 1:3,000.
Subsequently, the membranes were washed again with PBS 1X. In the presence of the substrate, the peroxidase conjugated secondary antibody, developed a chemiluminescent reaction that identified the protein of interest, exposing the membrane to the analyzer of digital images ChemiDoc XRS+, Bio-Rad.
For the chemiluminescence reaction the Bio-Rad kit, which included two reagents to be mixed in the ratio 1:1, was used. Reagents must be added on the membrane in order to allow the development of the light reaction. At the end, the PVDF was exposed for different times

(from a minimum of 30 seconds to a maximum of 10 minutes) to the analyzer of digital images ChemiDoc XRS+, Bio-Rad.

The intensity of the bands was quantified with the software Image Lab, Bio-Rad.

In order to cover again the same PVDF membrane with new antibody, without any interference in the new marking due to the previous one, the secondary antibody conjugated with peroxidase could be inhibited.

In practice, at the end of the immunoblotting development, the PVDF membrane was rehydrated using the washing buffer for few minutes. Subsequently, it was placed in a 1% solution of NaN_3 in PBS 1X and left under stirring at room temperature for about 2 hours. At this point, the excess NaN_3 was removed with several washes with PBS 1X, and the membranes were re-probed with another antibody, without the need of performing again the non-specific sites blocking procedure.

3.4 Statistical analysis

Statistical analysis was performed by means of R Statistical software (v. 3.3.0) and the graphical interface R Studio (v. 1.0.143). Normality and homogeneity of variances were verified by means of Shapiro's Test and Levene's Test (car package), respectively. In the majority of cases, data had normal distribution and where analyzed with ANOVA, followed by Tukey's HSD Test for multiple comparisons. The non-parametric Dunn's Test was used for data not following the normal distribution (dunn.test package). In case of repeated measures against time, significance was analyzed by fitting data in a Linear Mixed-Effects model (NLME package).

RESULTS

4.1 *In vitro* experiments

Isolated hepatocytes were treated 30 minutes with various negative allosteric modulators of metabotropic glutamate receptor 5: MPEP, MTEP and Fenobam, and the orthosteric antagonist CPG, at different concentrations. Anoxia was induced insufflating N_2 for 2 minutes.

4.1.1 Measurement of cell viability: trypan blue exclusion and LDH release

Hepatocytes viability was monitored by both trypan blue uptake and Lactate Dehydrogenase (LDH) release into the buffer. The times considered for the measures were: 0 (after N_2 insufflation), 30, 45, 60, 75 and 90 minutes.

The administration of MPEP 30 µM improved significantly the viability of anoxic hepatocytes respect to anoxic controls in both trypan blue exclusion assay (Fig.4.1 A) and LDH release (Fig.4.1 B). In particular, comparing the curves of mortality rate by using Linear Mixed Effects (LME) model emerged that, in the 0-90' time lapse, the mortality rate for MPEP 30 µM-treated hepatocytes was lower than in anoxic control (p=0.003 in trypan blue assay and p=0.004 in LDH release). The p value for this differences in the interval of 0-75' in trypan blue and LDH release were, respectively, p=0.00009 and p=0.009.

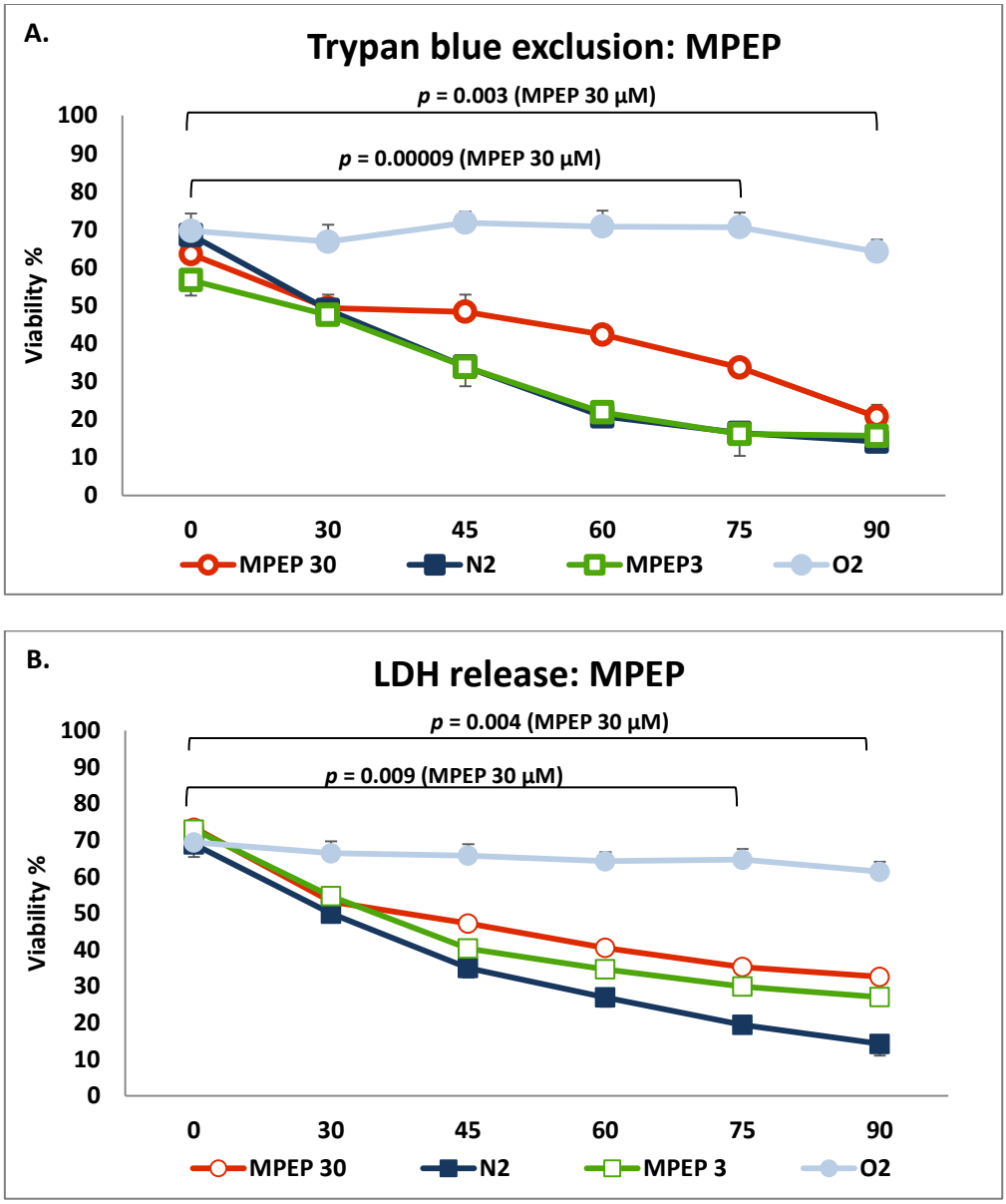


Figure 4.1. Cell viability after MPEP treatment. The curves of mortality rate were significantly lower in MPEP 30 µM-administered hepatocytes, compared to anoxic controls, at 0-90' and 0-75' intervals, as observed in both trypan blue exclusion (**A**) and LDH release (**B**) assays. The significant *p* values are indicated in the graph.

Linear mixed effects model analysis revealed that the slope of mortality rate was significantly improved only for hepatocytes treated with MTEP 3 µM, in the time lapse 0-60', compared to ischemic controls (p=0.047) (Fig.4.2 A).

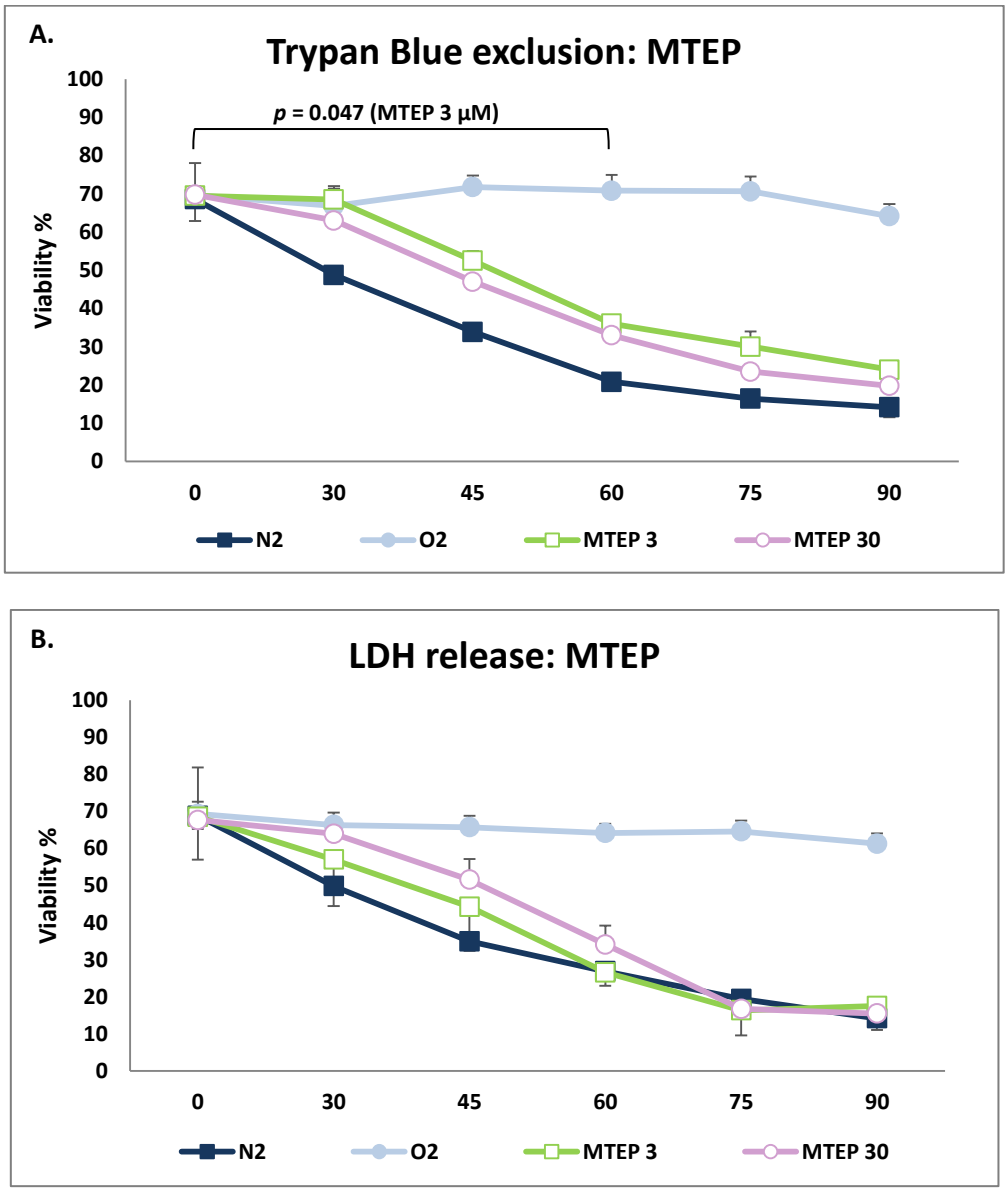


Figure 4.2. Cell viability after MTEP treatment. MTEP 3 µM enhanced significantly the viability of anoxic hepatocytes when compared to anoxic controls in trypan blue exclusion assay, in the interval 0-60' (p=0.047).

According to LME model, in trypan blue exclusion assay the mortality rate curves for Fenobam 50 µM were significantly different compared to untreated anoxic hepatocytes curves at the intervals 0-60' and 0-75' (p=0.03 and p=0.05, respectively). Consistent with ANOVA statistics plus Tukey's Honest Significant Difference (HSD) test, after 45 minutes of ischemia, cells administered with both Fenobam 1 µM and 10 µM showed a significant rise in cell viability respect to anoxic controls (p=0.05 and p=0.003, respectively) (Fig.4.3 A). Although not significant, Fenobam treatment reduced LDH release compared to ischemic controls (Fig.4.3 B).

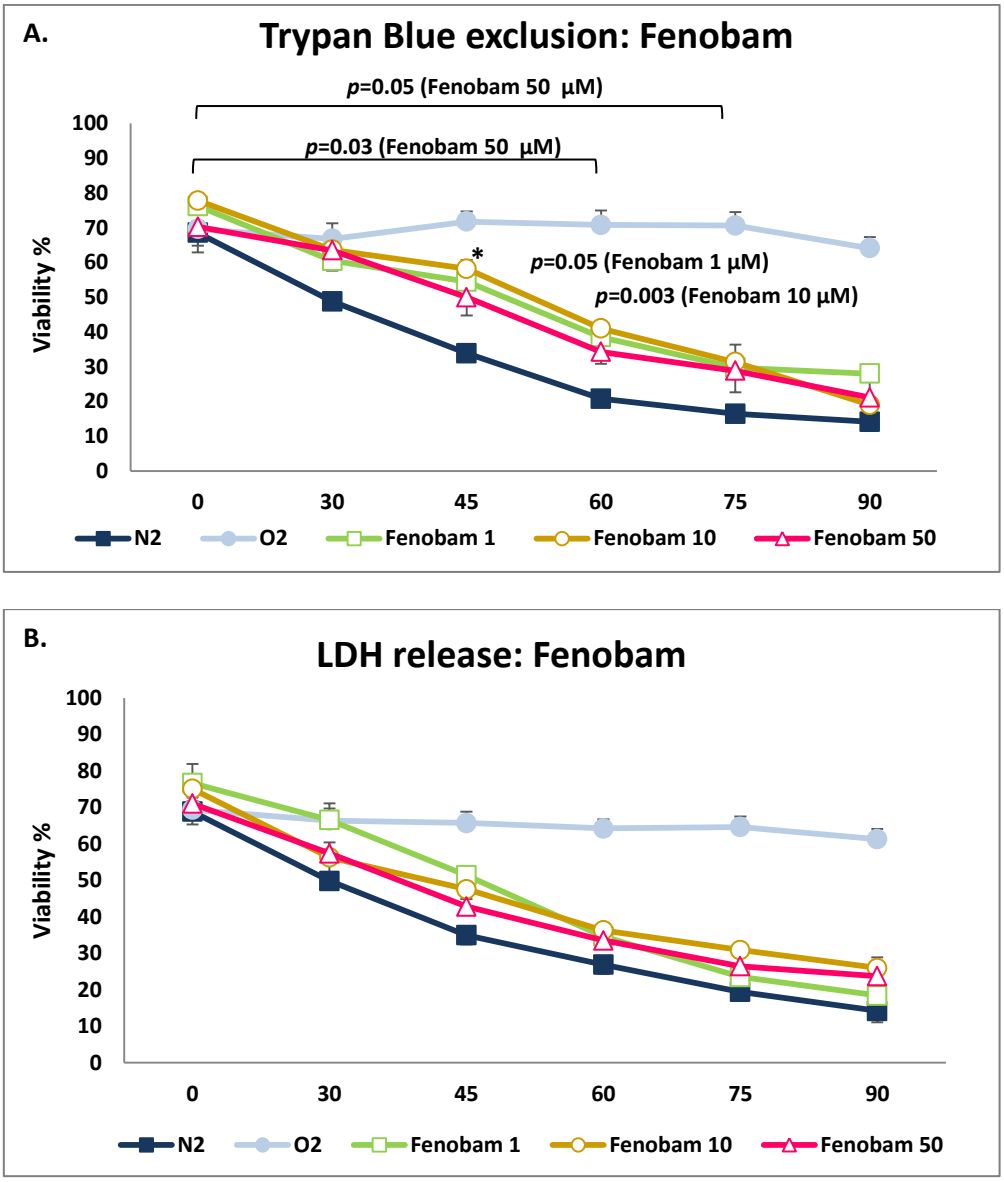


Figure 4.3. Cell viability after Fenobam treatment. Fenobam at different concentrations, 1 µM, 10 µM and 50 µM, enhanced significantly the viability of anoxic hepatocytes respect to anoxic controls, as showed with trypan blue exclusion assay (**A**). Although less evident and not significantly, Fenobam administration reduced LDH release (**B**). The significant p values are indicated in the graph.

Anoxic hepatocytes administered with the orthosteric antagonist CPG at both 100 µM and 200 µM showed a decline in cell viability comparable with anoxic controls, as demonstrated by Trypan blue assay (Fig.4.4 A) and LDH release (Fig.4.4 B).

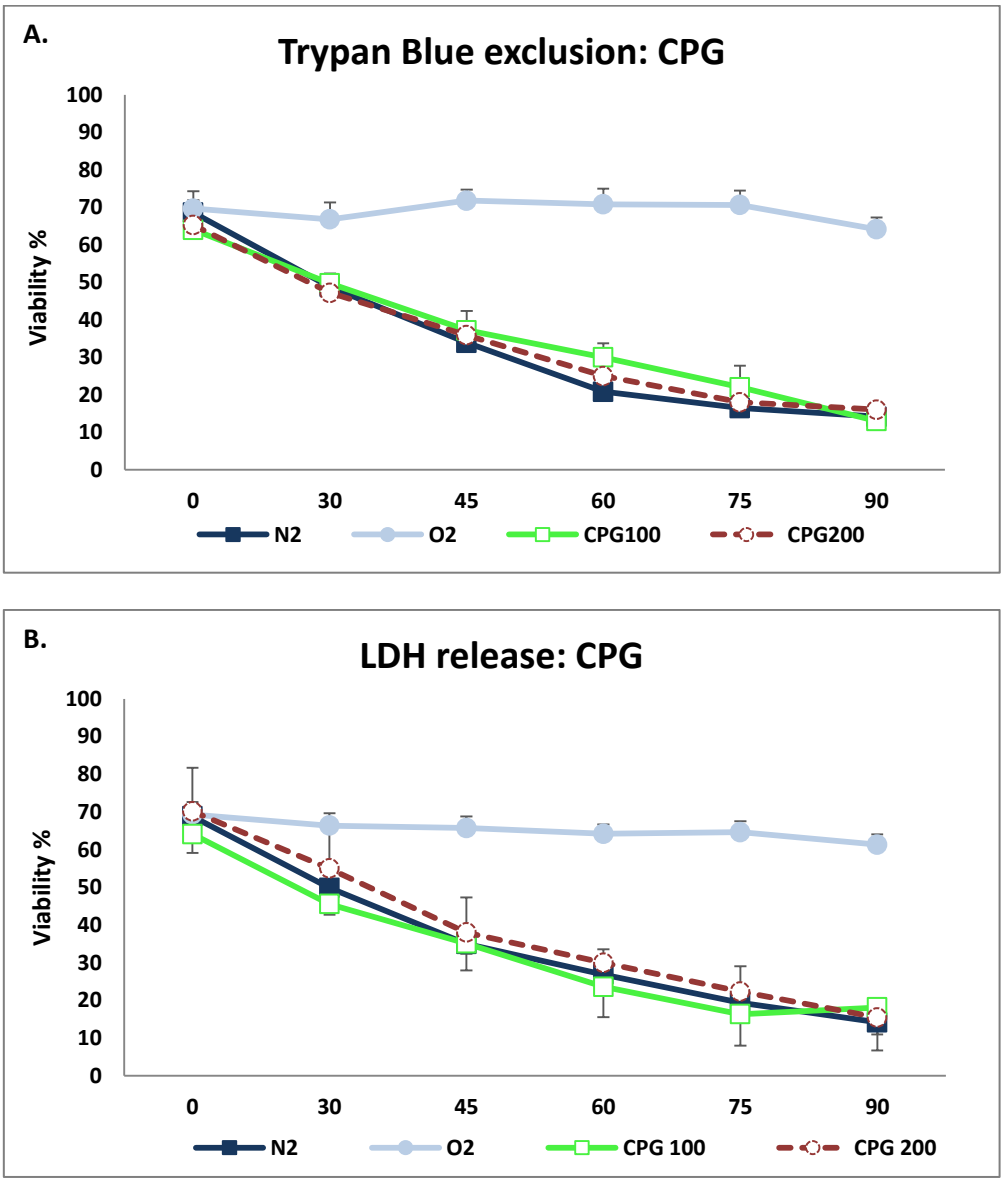


Figure 4.4. Cell viability after CPG treatment. CPG administration did not improve the viability of anoxic hepatocytes, which had a trend comparable to anoxic controls, in both trypan blue exclusion assay (**A**) and LDH release assay (**B**).

4.1.2 Measurement of ATP on hepatocytes during ischemia

ATP concentration was followed in time before and after N_2 insufflation. Samples treated with MPEP had a significantly lower ATP content respect to controls before anoxia; nonetheless, MPEP-treated cells showed a slower and significant decline in ATP following N_2 insufflation after 30 and 45 minutes for both MPEP 3 µM (p=0.002 and p=0.04, respectively) and MPEP 30 µM (p=0.014 and p=0.045, respectively), when compared to anoxic controls (Figure 4.5). Statistical analyses were calculated with ANOVA plus Tukey's HSD test.

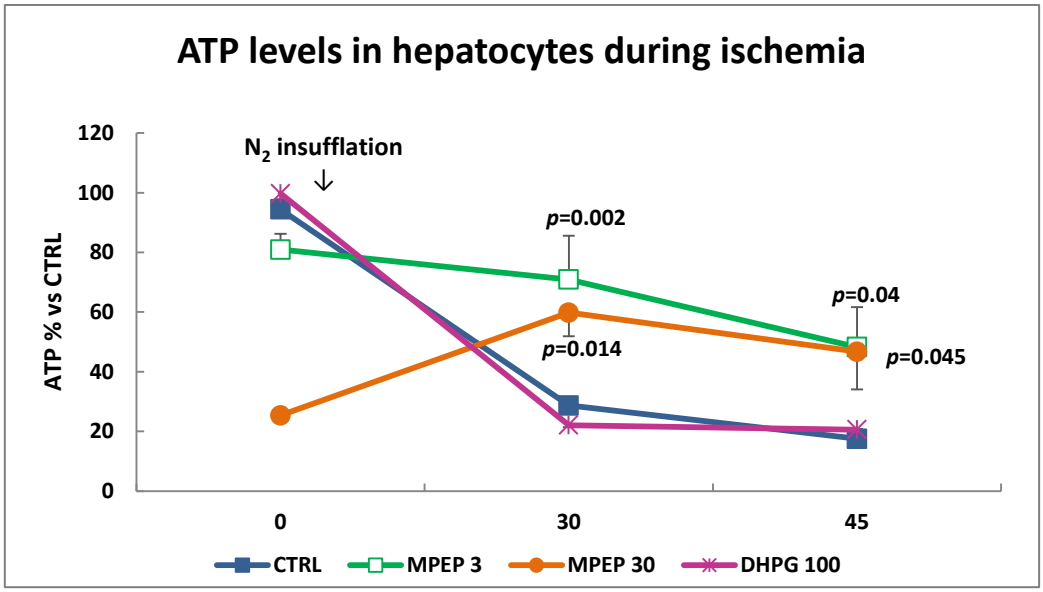


Figure 4.5. ATP content in isolated hepatocytes, before and after ischemia. Hepatocytes treated with MPEP 3 µM and 30 µM exhibited a significant higher content of ATP after 30 minutes and 45 minutes of anoxic exposure, when compared to DHPG-administered and anoxic untreated cells. The significant p values are indicated in the graph.

After 30 minutes of pharmacological treatment and 30 minutes of anoxia, ATP levels in primary hepatocytes treated with MPEP 3 µM and 30 µM were significantly higher respect to anoxic controls (p=0.002 and p=0.014, respectively), while no statistical differences were observed with the other treatments (Figure 4.6). Data were statistically analyzed with ANOVA plus Tukey's HSD test.

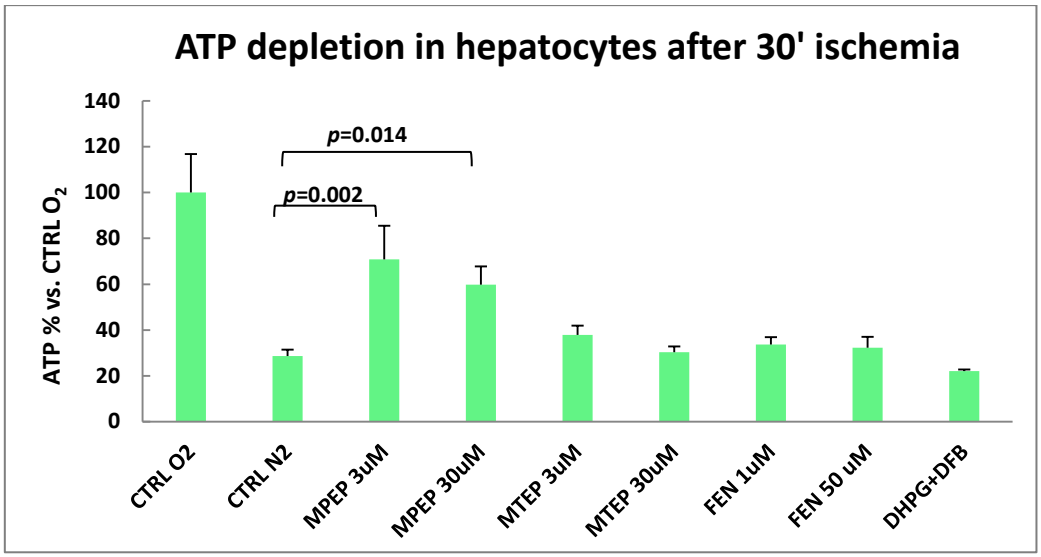


Figure 4.6. ATP depletion in isolated hepatocytes after 30 minutes of ischemia. Hepatocytes treated with MPEP 3 µM and 30 µM exhibited a significant ATP content after 30 minutes of ischemia, respect to anoxic controls. The administration of other negative allosteric modulators or orthosteric agonist did not alter ATP levels in anoxic cells. The significant *p* values are indicated in the graph.

4.1.3 Measurement of ATP on hepatocytes during oxygenated conditions

After 15 minutes of pharmacological treatment, MPEP administration reduced ATP levels in isolated oxygenated hepatocytes in a dose-dependent manner. ATP decrease was significant for MPEP 3 µM and 30 µM, when compared to controls (p=0.064 and p=0.003, respectively) (Figure 4.7). Statistics were realized with ANOVA plus Tukey's HSD test.

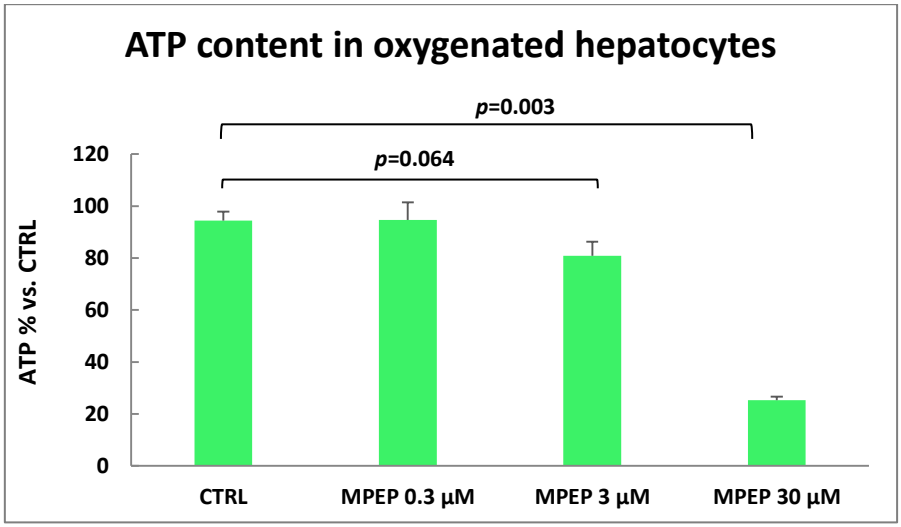


Figure 4.7. ATP levels in oxygenated primary hepatocytes. MPEP 3 µM and 30 µM significantly reduced ATP levels respect to controls in isolated oxygenated hepatocytes. The significant *p* values are indicated in the graph.

4.2.1 Mitochondrial ATP content

ATP levels were measured in isolated mitochondria after 15 minutes of pharmacological treatment. MPEP administration at 3 µM and especially at 30 µM significantly decreased ATP content, when compared to controls ($p<1x10^{-7}$ and $p<1x10^{-5}$, respectively). MTEP, although at lesser extent, significantly reduced ATP levels only at 30 µM with regard to controls ($p<7x10^{-7}$). Fenobam and CPG, instead, did not alter ATP concentration respect to controls (Fig. 4.8). Statistical analyses were performed with ANOVA plus Tukey HSD.

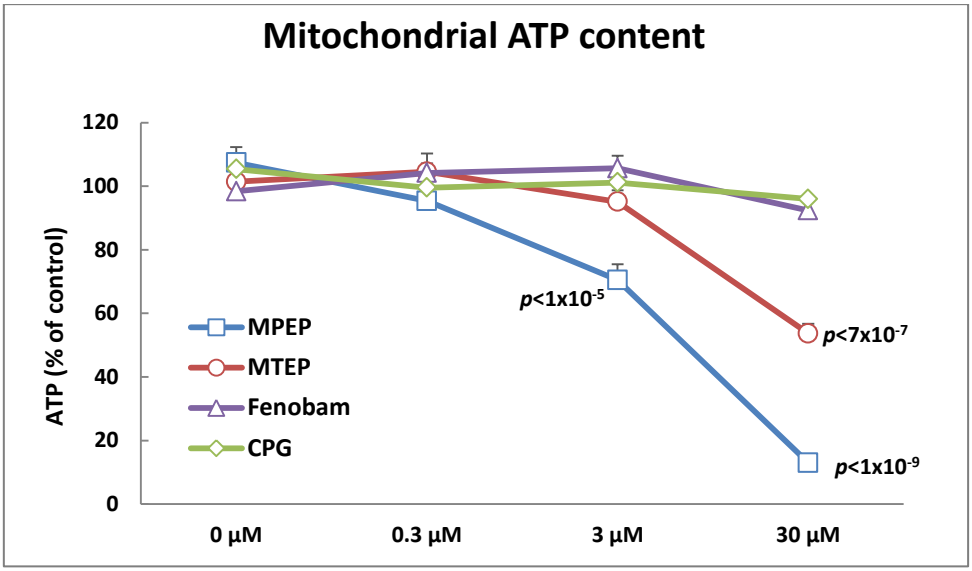


Figure 4.8. Mitochondrial ATP content. Treatment with MPEP 3 µM and 30 µM and MTEP 30 µM significantly reduced ATP levels, respect to controls, in isolated mitochondria. Fenobam and CPG did not modify ATP content, when compared to controls. The significant *p* values are indicated in the graph.

4.2.2 Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨ) was evaluated by measuring the uptake of the fluorescent dye rhodamine (Rh)-123, after 15 minutes pharmacological treatment with mGluR5 allosteric and orthosteric antagonists.

No differences were observed in mitochondrial membrane potential using MPEP, MTEP, Fenobam and CPG, all at 30 µM, respect to controls. All the treatments were significantly different from uncoupled mitochondria, obtained by freezing-thawing cycles (Figure 4.9).

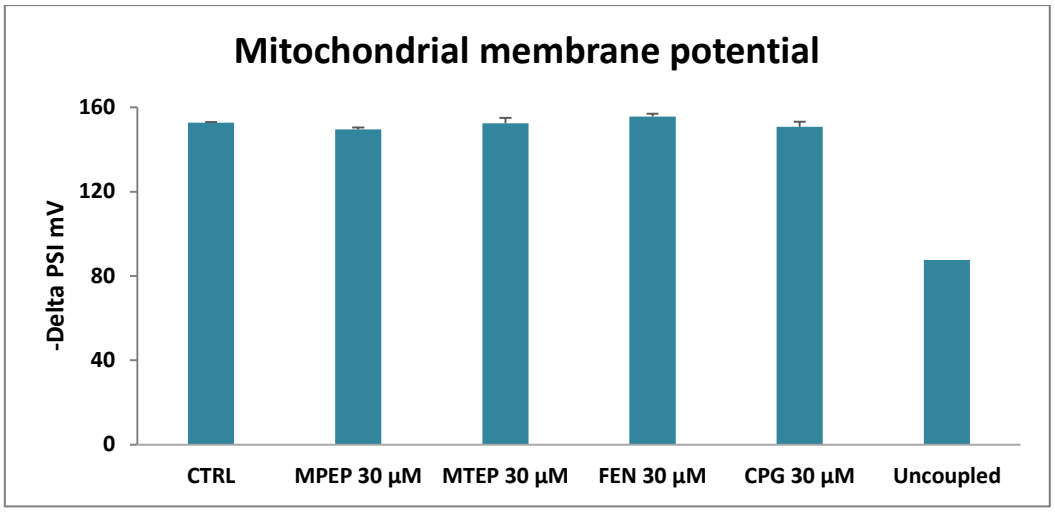


Figure 4.9. Mitochondrial membrane potential. Pharmacological treatment with MPEP, MTEP, Fenobam and CPG at 30 µM did not change mitochondrial membrane potential, when compared to controls.

4.2.3 Mitochondrial respiratory control index

Respiratory Control Index (RCI) expresses the tightness of the coupling between respiration and phosphorylation. RCI was assessed by means of a Clark-type oxygen electrode in a sealed mitochondrial chamber at 25°C. Mitochondria treated with MPEP 30 µM showed similar values of mitochondrial respiratory control index of control mitochondria. The difference between treated and uncoupled mitochondria was significant (Fig.4.10).

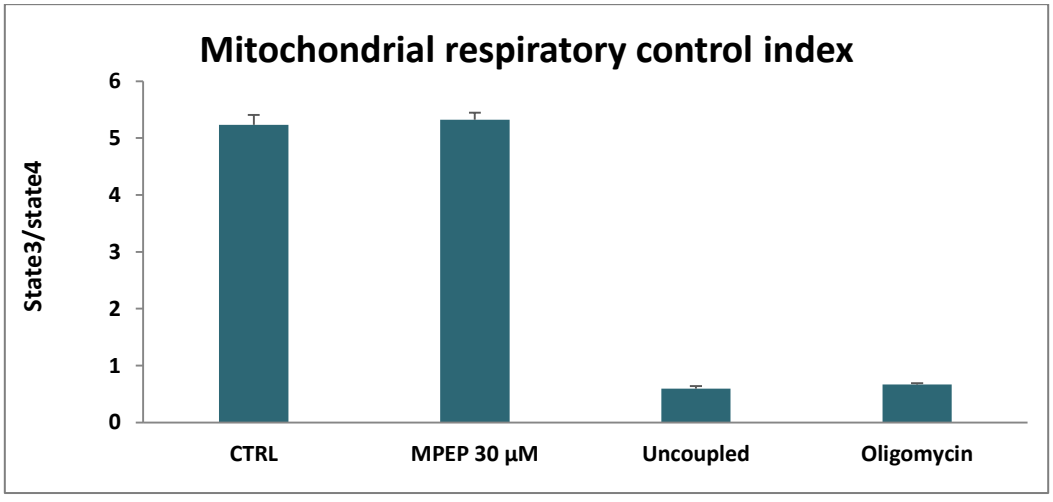


Figure 4.10. Mitochondrial respiratory control index. The administration of MPEP 30 µM did not change the mitochondrial respiratory control index respect to untreated mitochondria.

4.2.4 ROS formation

Supraphysiological levels of Radical Oxygen Species (ROS) can damage macromolecules, such as DNA, proteins and lipids, leading to oxidative stress. ROS formation in isolated mitochondria after pharmacological treatment was evaluated by Dichlorodihydrofluorescein Diacetate (H_2DCF-DA) assay.

The administration of MPEP slightly decreased ROS production in a dose-dependent fashion, although not significantly, respect to controls. DHPG, instead, did not alter ROS production when compared to controls (Fig. 4.11).

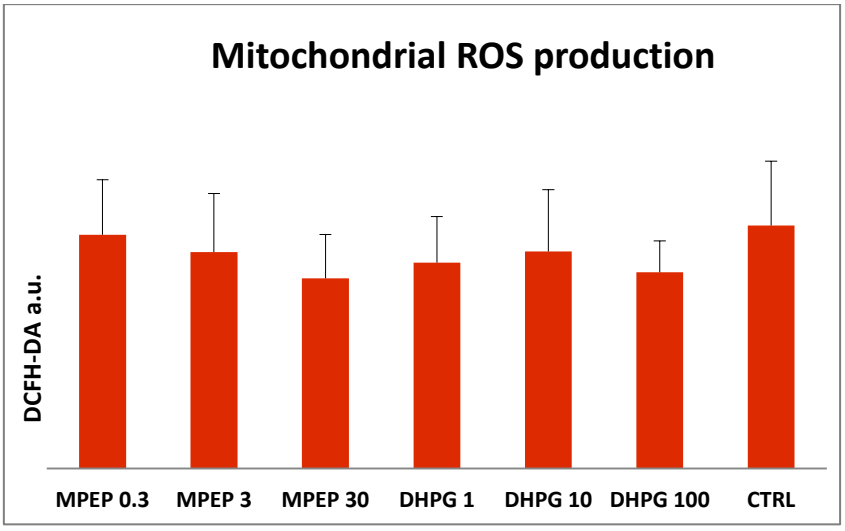


Figure 4.11. Mitochondrial ROS production. The differential treatments of mitochondria did not alter ROS production when compared to untreated mitochondria.

4.2.5 Mitochondrial complex I and complex II

Complexes I and II are part of the electron transport chain in the oxidative phosphorylation process. To evaluate the functionality of the two complexes, specific substrates (malate and pyruvate for complex I and succinate and rotenone for complex II) were added to mitochondrial solutions. In both complexes, MPEP 30 µM treatment reduced significantly ATP content compared to untreated mitochondria (p=0.00000005 and p=0.00005, respectively) (Fig.4.12). The administration of MPEP 3 µM decreased significantly ATP levels in complex I (p=0.004) (Fig. 4.12 A).

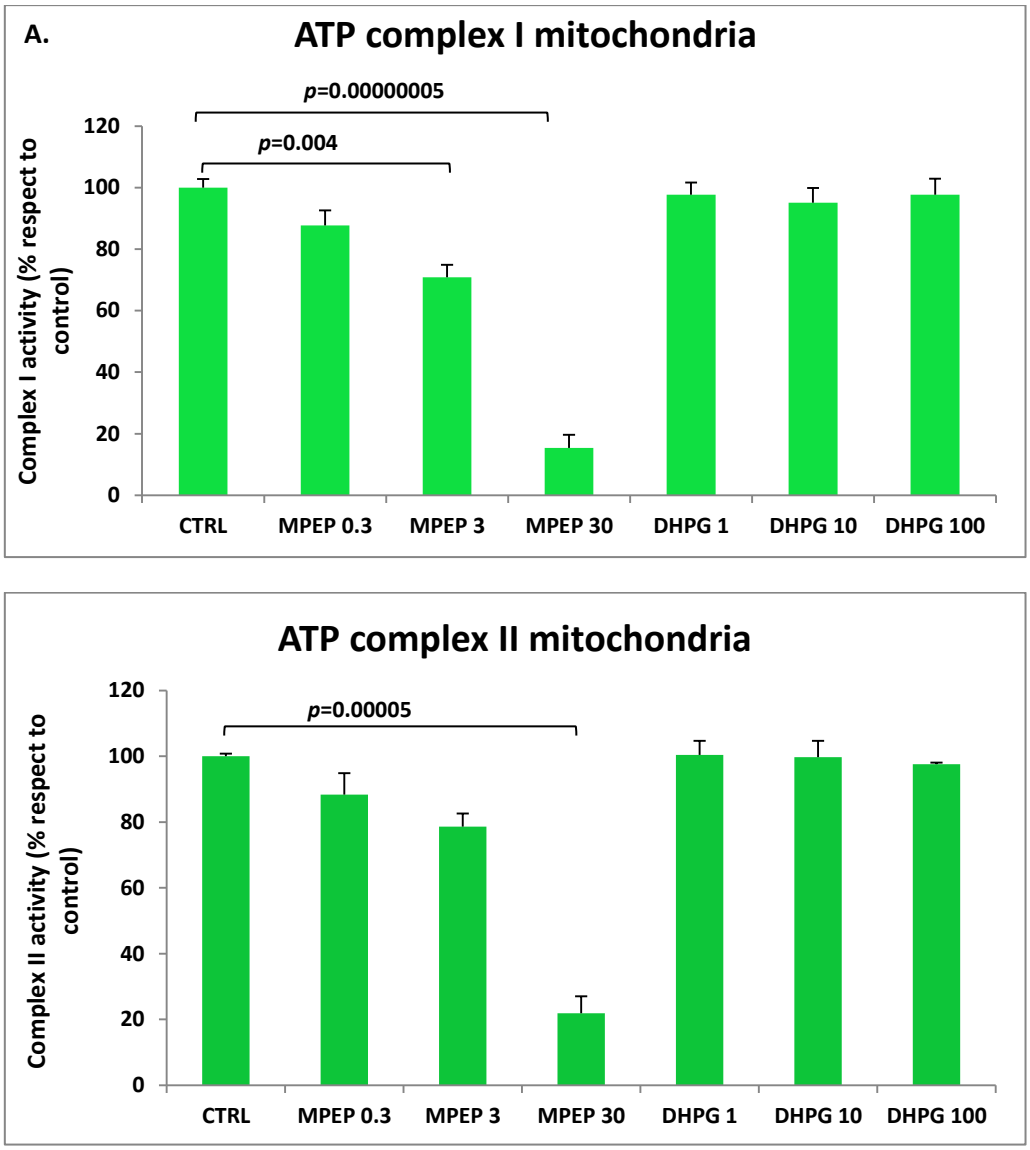


Figure 4.12. Mitochondrial complex I and II. ATP content of mitochondria treated with MPEP 30 µM reduced significantly when compared to controls in both complex I and complex II (p=0.00000005 and p=0.00005, respectively). Also MPEP 3 µM-administered mitochondria exhibited a significant decrease of ATP levels respect to untreated samples.

4.2.6 The F_1F_O ATPase activity

F_1F_O ATPase, or Complex V, is the last step in the oxidative phosphorylation, in which ATP molecules are generated from ADP and P*i*. Complex V activity was measured in isolated mitochondria treated with MPEP 30 µM, MTEP 30 µM, Fenobam 30 µM and CPG 30 µM. No significant differences were found between the treated samples and controls. Oligomycin, an ATP synthase inhibitor, was used as negative or uncoupler controls (Fig. 4.13).

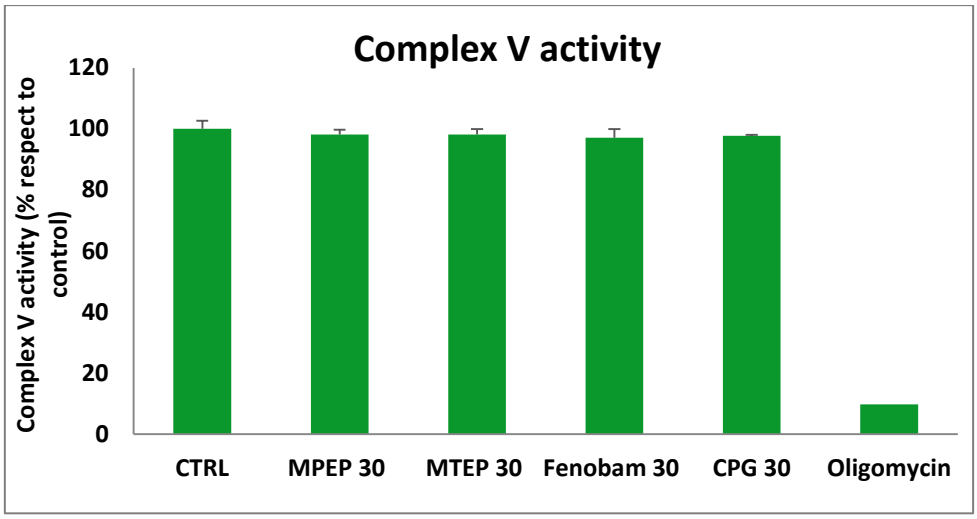


Figure 4.13. Mitochondrial complex V activity. F_1F_O ATPase activity was measured following pharmacological treatment, with MPEP, MTEP, Fenobam and CPG at 30 µM. No significant differences were observed between the treated mitochondria and the controls, while significant differences were found comparing treated samples to uncoupled mitochondria administered with oligomycin.

4.3 ATP in acellular solutions

ATP content was assessed not only in isolated hepatocytes and mitochondria, but also in acellular solutions. MPEP and MTEP at 30 µM significantly reduced the ATP concentration in ATP acellular solutions (p=0.048 and p=0.002, respectively), with marked depletion ability for MPEP. Fenobam and CPG, instead, did not alter the ATP level in vitro (Fig. 4.14). Statistical analyses were performed with ANOVA plus Tukey's HSD test.

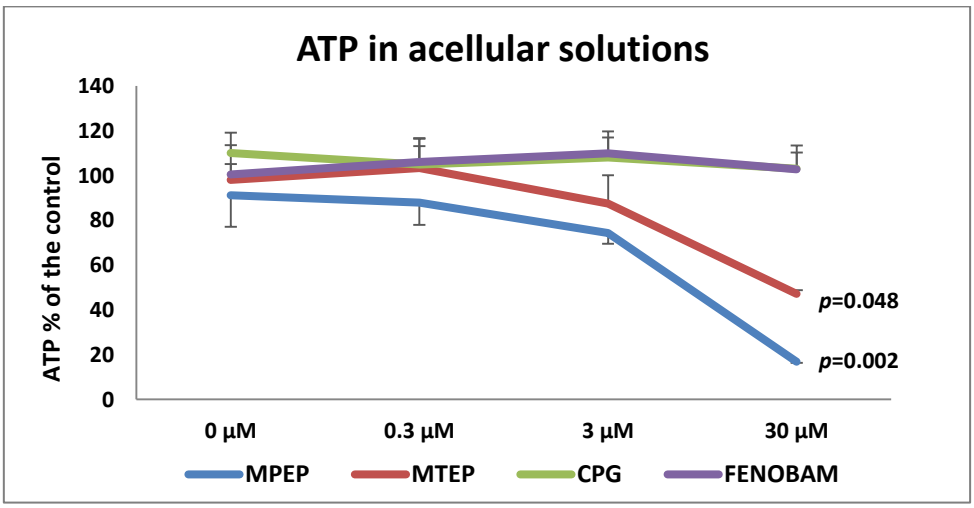


Figure 4.14. ATP content in acellular solutions. ATP level in acellular solutions added with MPEP and MTEP at 30 µM significantly decreased the ATP concentration (p=0.048 and p=0.002, respectively), with remarkable depletion ability for MPEP. Fenobam and CPG, instead, did not alter the ATP concentration in vitro.

4.4.1 *Ex vivo* experiments: cold ischemia/reperfusion

4.4.1.1 Perfusate analysis in cold ischemia/reperfusion

Livers exposed to 18 hours of static cold preservation in UW solution with and without MPEP at 0.3 µM, as well mGluR5 knockout livers, were subjected to 2 minutes of washing with KRH buffer. Then the livers were reperfused for 60 minutes with oxygenated KRH solution, at 37°C, to mimic organ reimplant.

Liver parenchyma viability was assessed through release of lactate dehydrogenase and aspartate transaminase and alanine aminotransferase into the effluent perfusate.

4.4.1.1.1. LDH release

Lactate dehydrogenase is released when cell lysis occurs and LDH values were assessed spectrophotometrically.

During the wash out, LDH release of livers preserved with MPEP was significantly lower than that released by control livers (p=0.04). Also mGluR5 knockout livers showed a significant decrease in LDH release when compared to controls (p=0.05) (Figure 4.15). Statistical analyses were performed with ANOVA plus Tukey's HSD test.

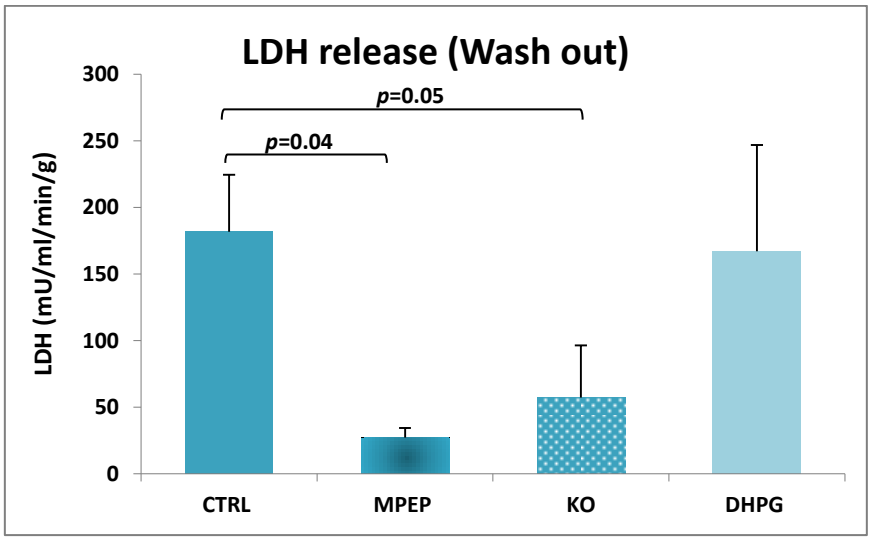


Figure 4.15. LDH release in wash out. Livers preserved with MPEP 0.3 µM released significantly less LDH when compared to controls (p=0.04) in the wash out solution. Also mGluR5 knockout livers showed a significant reduction in LDH release compared to controls (p=0.05).

LDH release was evaluated also during the reperfusion, every 15 minutes. According to linear mixed effects model, the mortality rate curves of MPEP-treated and mGluR5 knockout livers were significant when compared to anoxic controls (p=0.022 and p=0.014, respectively). Moreover, LDH release from KO livers is comparable to that from MPEP-treated organs.

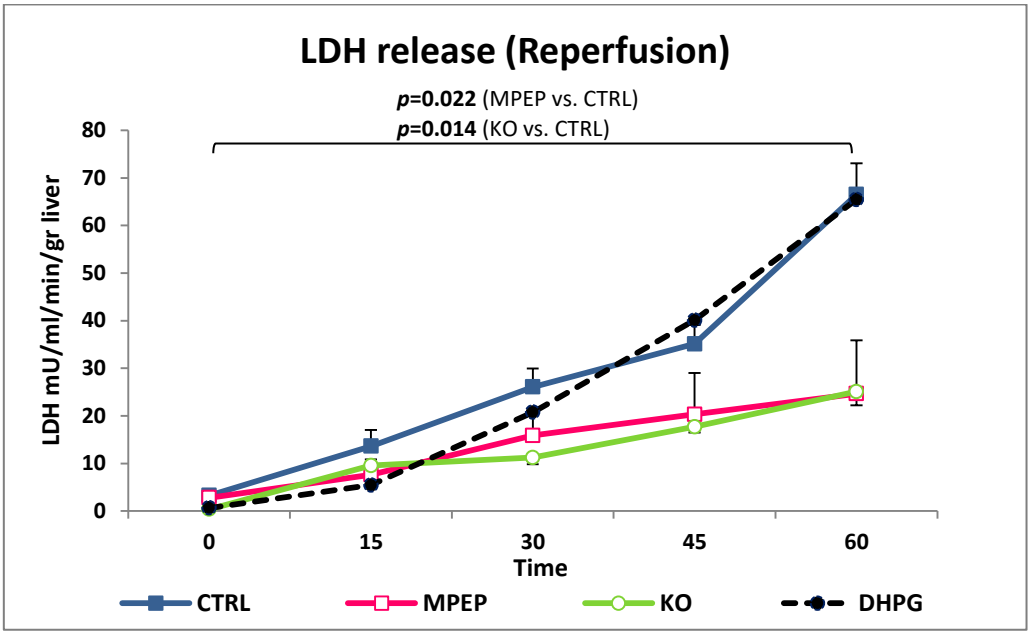


Figure 4.15. LDH release during reperfusion. During reperfusion, lactate dehydrogenase release was significantly lower in livers preserved with MPEP 0.3 µM or in mGluR5 knockout organs respect to controls. The significant *p* values are indicated in the graph.

4.4.1.1.2. Transaminases release

Aspartate Transaminase (AST) and Alanine Aminotransferase (ALT), considered markers for liver damage, were evaluated in both wash out and in the perfusate. Transaminases were assessed by an automated Hitachi 747 analyser.

AST levels decreased in wash out in livers treated with MPEP 0.3 µM and the reduction became significant after 30 minutes and 60 minutes of reperfusion respect to controls (p<0.05 and p=0.02, respectively). No differences were detected in mGluR5 knockout livers when compared to controls.

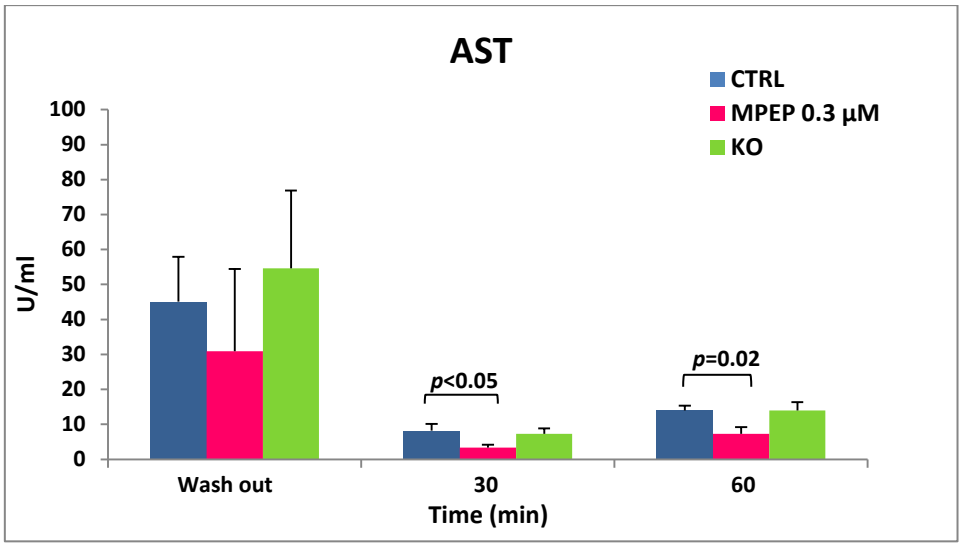


Figure 4.16. AST release. Aspartate transaminase release lowered in wash out in livers preserved with MPEP 0.3 µM, when compared to controls and the decrease achieved the significance after 30 minutes and 60 minutes of reperfusion (p<0.05 and p=0.02, respectively). AST release was not altered in mGluR5 knockout livers respect to controls.

ALT concentration significantly diminished in livers treated with MPEP 0.3 µM in wash out (p=0.020) and after 30 minutes (p=0.054) and 60 minutes (p=0.055) of reperfusion respect to controls. In mGluR5 knockout livers no differences were observed when compared to controls.

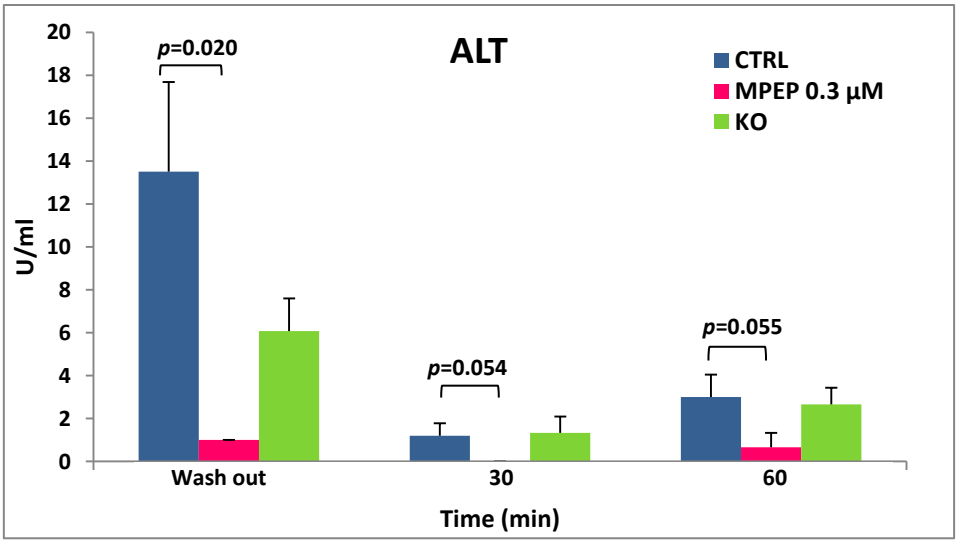


Figure 4.17. ALT release. Alanine aminotransferase release significantly decreased in livers treated with MPEP 0.3 µM in wash out (p=0.020) and during the reperfusion, at both 30 and 60 minutes (p<0.054 and p=0.055, respectively) respect to controls. No differences were observed in ALT release in mGluR5 knockout livers when compared to controls.

4.4.1.2 ATP content

ATP levels were evaluated on homogenized tissue samples by means of luciferine/luciferase assay. Although not significant, ATP content was higher in MPEP-treated and mGluR5 knockout organs when compared to anoxic controls (Figure 4.18).

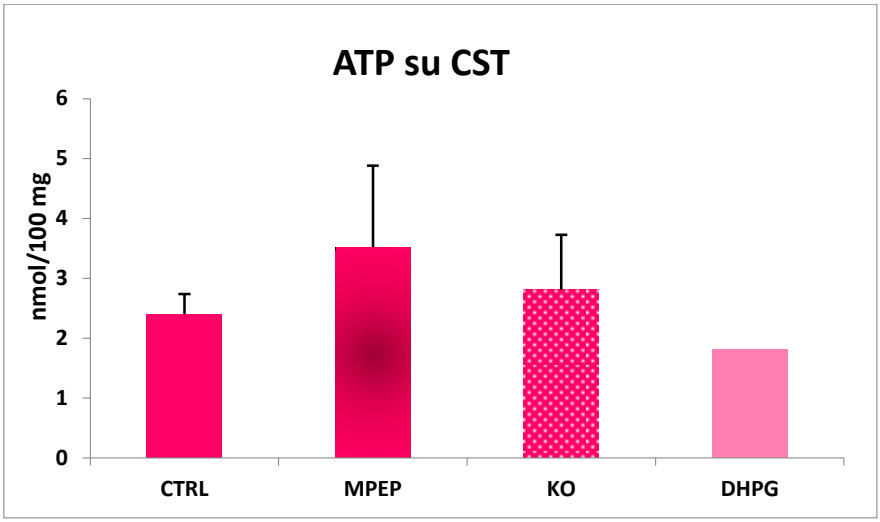


Figure 4.18. ATP levels. Albeit not significant, ATP content in livers treated with MPEP and in mGluR5 knockout organs was higher than in ischemic controls.

4.4.1.2 Western blot analysis in cold ischemia/reperfusion

4.4.1.2.1 Bax and Bcl-2 protein expression

Bax and Bcl-2 belong to Bcl-2 family of proteins which regulate apoptosis. Bax is a pro-apoptotic protein, while Bcl-2 is an anti-apoptotic one. Bax and Bcl-2 protein expression were assessed by means of western blot analysis. No differences were found between MPEP-treated and wild type livers in both Bax (Fig.4.19A) and Bcl-2 protein expression (Figure 4.19B).

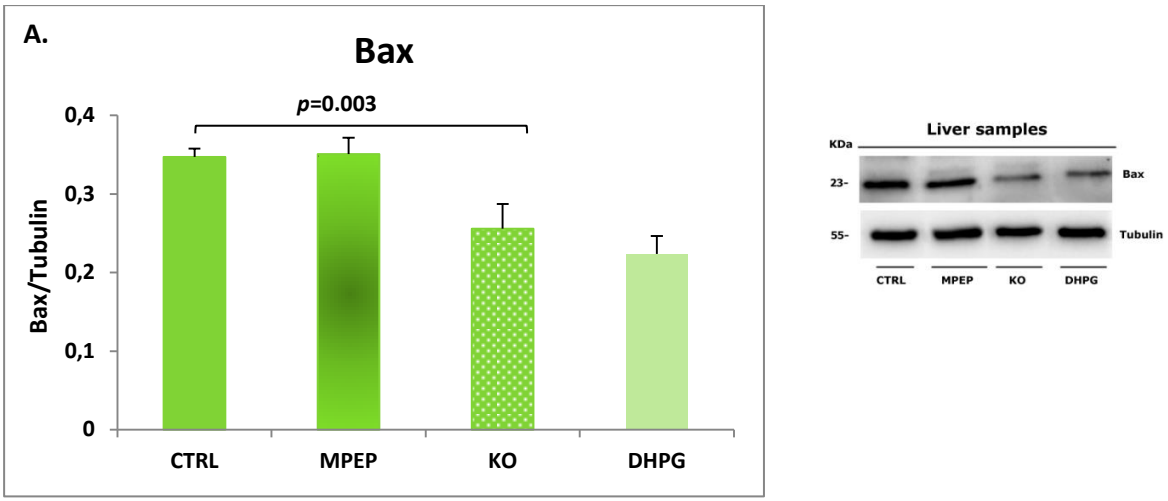


Figure 4.19A. Bax protein expression. Administration of MPEP did not alter Bax protein expression when compared to controls. A significant decrease, instead, has been revealed in mGluR5 knockout livers (p=0.003).

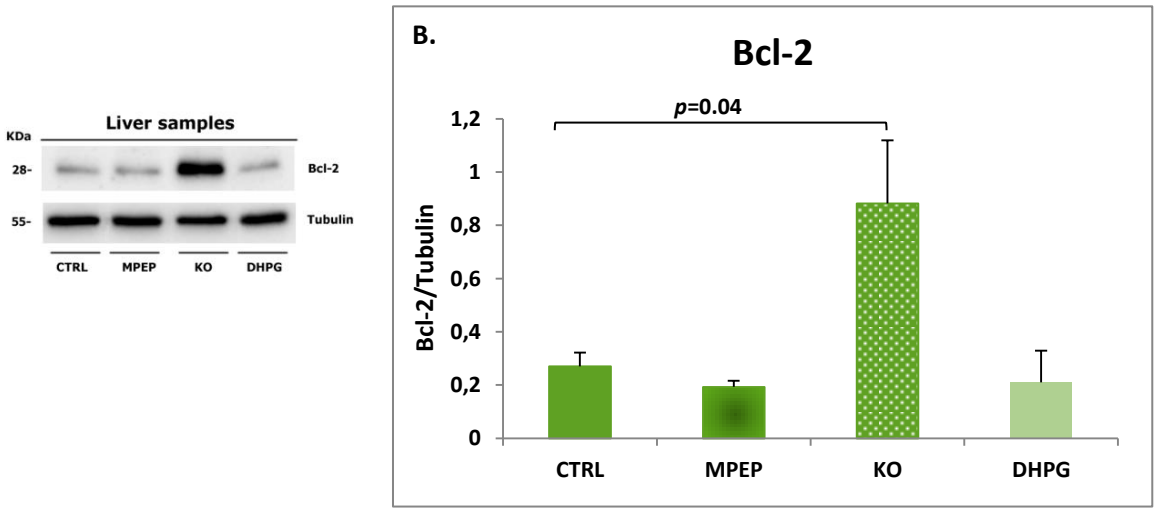


Figure 4.19B. Bcl-2 protein expression. Administration of MPEP did not alter Bcl-2 protein expression respect to controls, while a significant increase has been revealed in mGluR5 knockout livers (p=0.04).

The ratio of Bax/Bcl-2 is considered a marker for the susceptibility of a cell to apoptosis and it was also evaluated. A significant decrease occurred between MPEP-treated livers and control group (p=0.06) and also in KO organs when compared to controls (p=0.001). Livers administered with DHPG showed a Bax/Bcl-2 ratio similar to controls (Figure 4.20). Statistical analysis were performed with Kruskal–Wallis test plus Dunn's test.

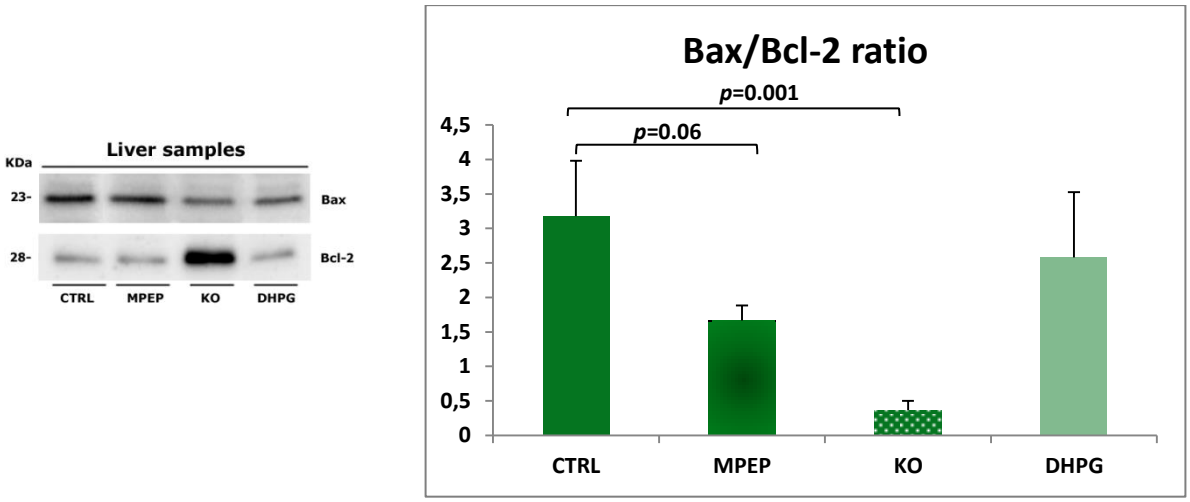


Figure 4.20. Bax/Bcl-2 ratio. Bax/Bcl-2 ratio significantly decreased in MPEP-treated and in KO livers compared to the control group (p=0.06 and p=0.001), while Bax/Bcl-2 ratio in DHPG-administered livers showed similar trend to that of controls.

4.4.1.2.2 HIF-1α protein expression

Hypoxia-Inducible Factor-1α (HIF-1α) is one of the main transcription factor expressed in hypoxic conditions. HIF-1α activation induces a metabolic shift from aerobiosis to anaerobiosis, favoring glycolytic process and blocking Krebs's cycle (Semenza 2000). HIF-1α protein expression was quantified by means of western blot analysis. No differences were detected in HIF-1α protein expression in livers treated with MPEP 0.3 µM or DHPG 100 µM, as well mGluR5 knockout livers, when compared to controls (Figure 4.21).

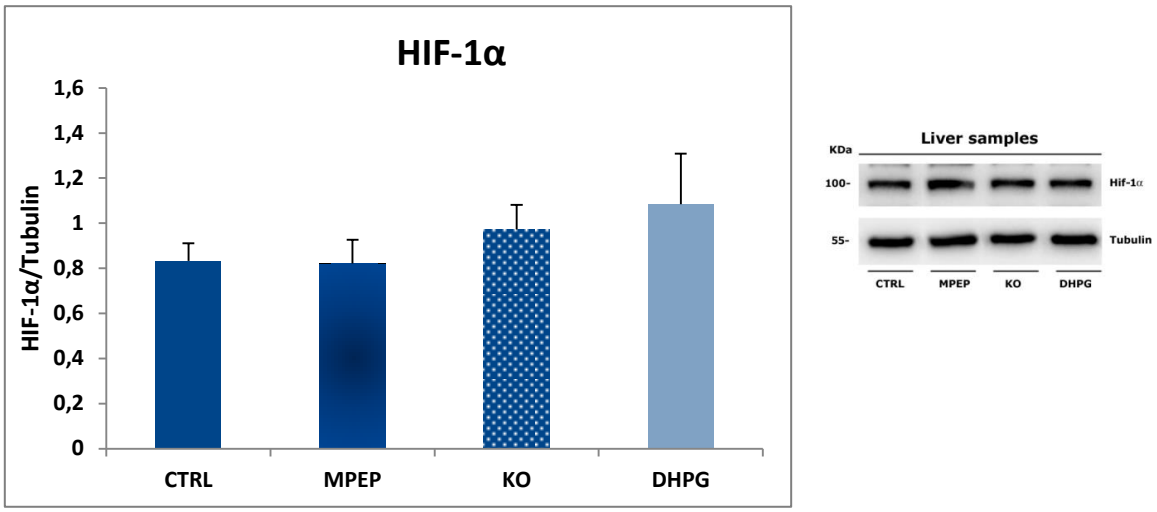


Figure 4.21. HIF-1α protein expression. No differences in HIF-1α protein expression were found among the four groups at the end of reperfusion.

4.4.1.2.3 Glycogen phosphorylase protein expression

Glycogen Phosphorylase (GP) is a fundamental enzyme during glycogenolysis, in which breaks up glycogen to form glucose molecules. It works in hypoglycemic conditions, eliciting available glucose for glycolysis.

Liver samples were collected at the end of reperfusion and analyzed for glycogen phosphorylase protein expression. No differences were observed in GP protein levels in treated, untreated and knockout organs (Figure 4.22).

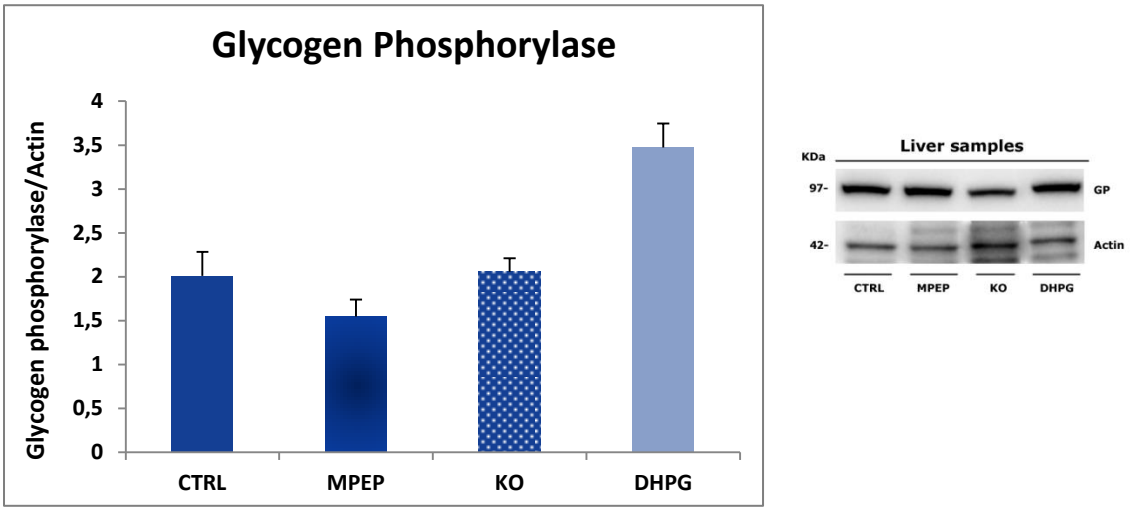


Figure 4.22. Glycogen phosphorylase protein expression. At the end of reperfusion, livers preserved with MPEP 0.3 µM, DHPG 100 µM and mGluR5 knockout organs showed no changes in glycogen phosphorylase protein expression when compared to controls.

4.4.1.2.4 TNF-α protein expression

Tumor Necrosis Factor-α (TNF-α) is one of the first protein released during organ reperfusion, after preservation at 4°C, and it is considered as a marker for liver damage (Tilg et al. 2006). To assess the inflammation extent, TNF-α protein expression was quantified by using western blot analysis. MPEP-treated livers showed a significant decrease in TNF-α protein expression, compared to control group (p=0.036). Also knockout group displayed a significant decline in TNF-α protein expression, respect to the control group (p=0.020). DHPG treatment induced a release of TNF-α comparable to controls (Figure 4.23). Statistical analyses were calculated using ANOVA plus Tukey's HSD test.

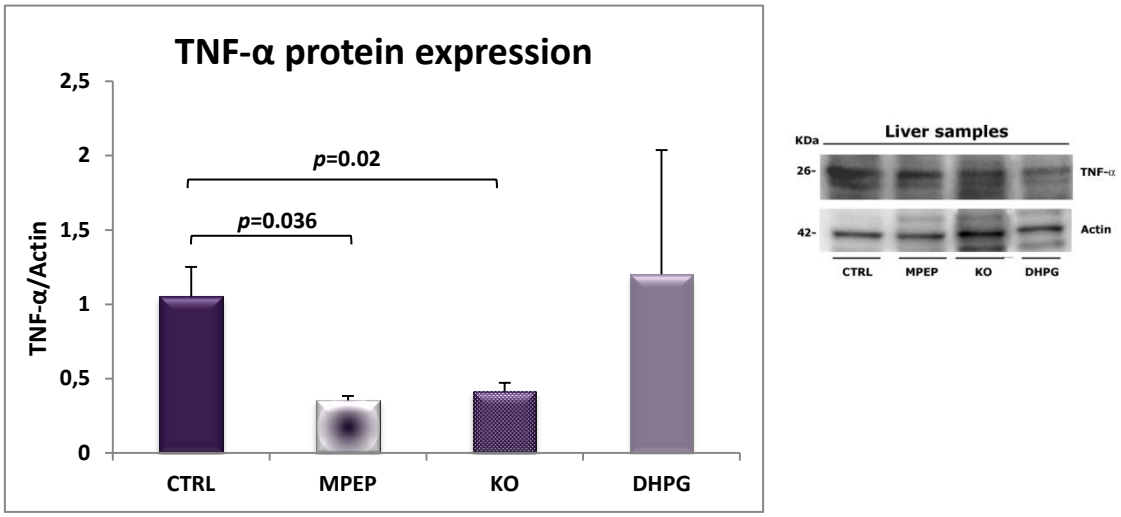


Figure 4.23. TNF-α protein expression. Livers preserved with MPEP 0.3 µM and mGluR5 knockout livers showed a significant lowering in TNF-α protein expression respect to controls at the end of reperfusion (p=0.0036 and p=0.020). DHPG-treated organs, instead, displayed TNF-α protein amount comparable to control livers.

4.4.1.2.5 iNOS protein expression

The inducible isoform of Nitric Oxide Synthase (iNOS) is expressed as a consequence of inflammatory processes. After stimulation, iNOS synthetizes nitric oxide in excess, leading to ROS formation (Casillas-Ramírez et al. 2006). Western blot analysis of liver samples collected at the end of reperfusion revealed that in both MPEP-treated and knockout livers a significantly reduced iNOS protein expression occurred respect to controls (p=0.0031 and p=0.0074, respectively, according to ANOVA plus Tukey's HSD test) (Figure 4.24).

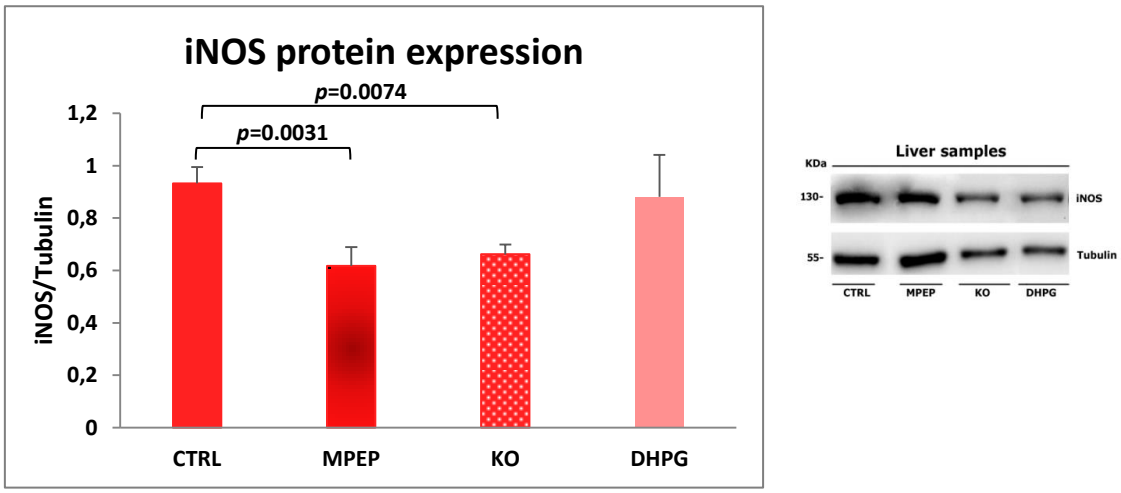


Figure 4.24. iNOS protein expression. Livers preserved with MPEP 0.3 µM and mGluR5 knockout livers showed a significant lowering in iNOS protein expression respect to controls at the end of reperfusion (p=0.0031 and p=0.0074). DHPG-treated organs, instead, displayed iNOS protein amount comparable to control livers.

4.4.1.2.6 eNOS protein expression

Differently from iNOS, the endothelial isoform of Nitric Oxide Synthase (eNOS) is constitutively expressed at vascular level. eNOS is an homeostatic regulator that controls vascular tone, cellular proliferation, leucocytes adhesion and platelets aggregation, hence favoring the correct cardio-circulatory system functioning (Förstermann & Sessa 2012).
eNOS protein expression of samples collected after 60 minutes of reperfusion was analyzed by means of western blot technique. eNOS protein levels were not altered with administration of MPEP 0.3 µM and DHPG 100 µM respect to controls. Although not significant, mGluR5 knockout livers showed an increase in eNOS protein expression (Figure 4.25).

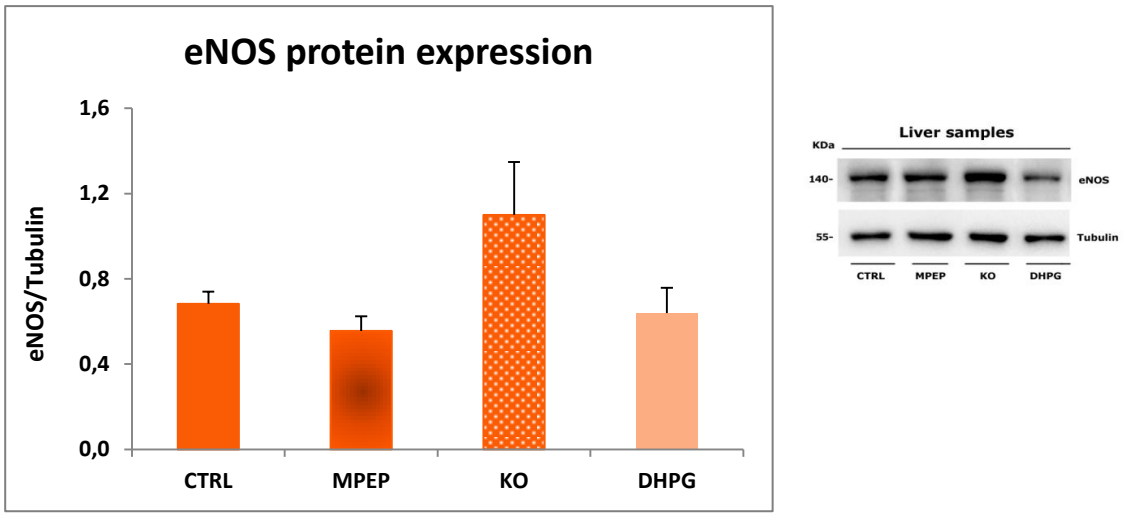


Figure 4.25. eNOS protein expression. At the end of reperfusion, livers preserved with MPEP 0.3 µM exhibited a slightly lowering in eNOS protein expression respect to controls, while knockout livers showed an increase in eNOS protein expression. DHPG-treated samples expressed approximately the same amounts of eNOS of controls.

4.4.2 *Ex vivo* experiments: warm ischemia/reperfusion

4.4.2.1 Perfusate analysis in normothermic ischemia/reperfusion

Livers exposed to 1 hour of static normothermic preservation in KRH solution with and without MPEP at 0.3 µM, DHPG+DFB, as well mGluR5 knockout livers, were subjected to 2 minutes of washing with KRH buffer. Then the livers were reperfused for 120 minutes with oxygenated KRH solution, at 37°C, to mimic organ reimplant.

Liver parenchyma viability was assessed through release of lactate dehydrogenase and aspartate transaminase and alanine aminotransferase into the effluent perfusate.

4.4.2.1.1. LDH release

Lactate dehydrogenase is released when cell lysis occurs and LDH values were assessed spectrophotometrically during the two hours of reperfusion.

According to LME model, the mortality rate curves of MPEP-administered and mGluR5 knockout livers were significant compared to anoxic controls (p=0.05 and p=0.005, respectively) (Figure 4.25).

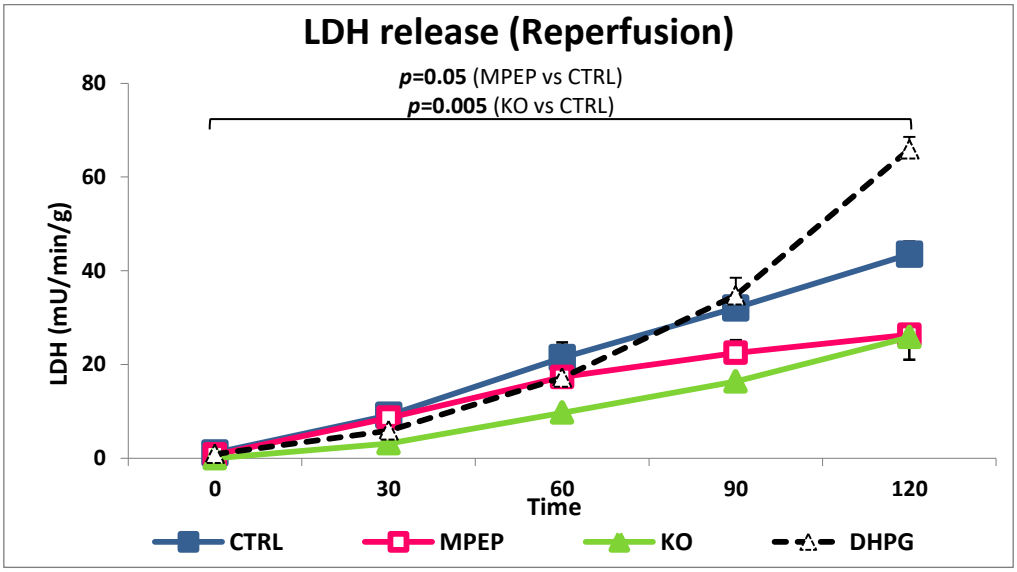


Figure 4.25. LDH release. LDH released was gauged during the reperfusion. During the process, livers preserved with MPEP 0.3 µM and mGluR5 knockout organs released significant less LDH respect to controls. The significant p values are indicated in the graph.

4.4.2.1.2 TNF-α release

Tumor necrosis factor-α was evaluated by means of ELISA test. Ischemic organs administered with MPEP 0.3 µM and mGluR5 knockout livers exhibited a significant reduction in TNF-α release respect to wild type livers (p=0.03 and p=0.04, respectively) (Figure 4.26).

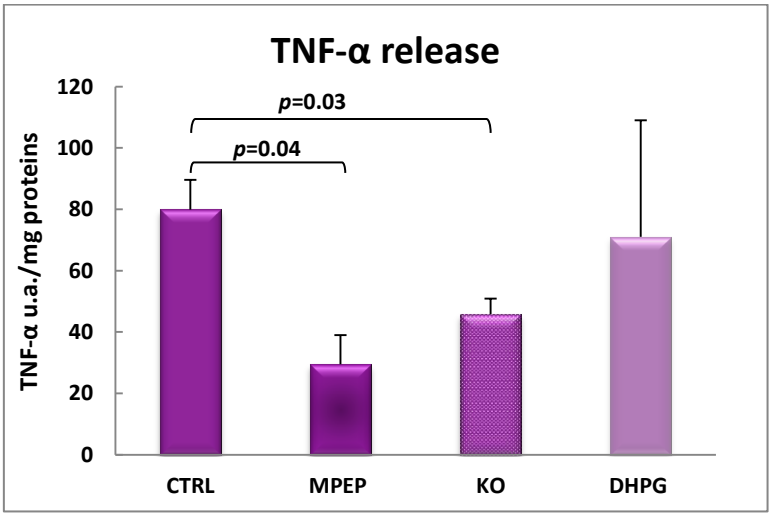


Figure 4.26. TNF-α release. Livers preserved with MPEP 0.3 µM and mGluR5 knockout organs released significant less TNF-α respect to untreated livers (p=0.03 and p=0.04, respectively).

DISCUSSION

In this work we demonstrated for the first time that negative allosteric modulator MPEP protects whole mice livers from cold ischemia/reperfusion injury and warm ischemia/reperfusion injury.

We showed that MPEP and MTEP depleted ATP from hepatocytes and mitochondria without affecting mitochondrial functionality. Furthermore, MPEP- and MTEP-mediated ATP depletion in cells is reasonably not a receptor-mediated process. In fact, ATP depletion occurs also in acellular buffers and it is probably due to the formation of MPEP-ATP or MTEP-ATP adducts, a process similar to the formation of MPEP-GSH adducts reported in literature. Finally, we showed that ATP depletion after MPEP or MTEP administration does not affect the protection from hepatic ischemia/reperfusion injury, to the point that the administration of these molecules induces even an increase of ATP respect to anoxic untreated controls.

5.1 *In vitro* experiments

The first part of this project was aimed to confirm MPEP protection and investigate whether other negative allosteric modulators had similar properties in anoxic isolated rat hepatocytes.

Cell viability was monitored by both trypan blue uptake and lactate dehydrogenase release into the buffer. We confirmed the data published previously from Storto and co-workers, in which MPEP protected from ischemic injury (M Storto et al. 2000). In our work, MPEP 30 µM treatment exhibited a significant decrease in the mortality rate curve respect to anoxic controls in both trypan blue exclusion and LDH release, considering the intervals 0-75’ and 0-90’. Although hepatocytes treated with MTEP showed a trend similar to MPEP-administered cells, the viability resulted significant only in trypan blue assay in the time-lapse 0-60’ for MTEP 3 µM. Lea and colleagues have demonstrated that MPEP was able to inhibit also NMDA receptors even at low concentrations, while its analog MTEP could not block NMDARs until a concentration more than 200 µM. These data suggested that MTEP was more selective for mGluR5, but the protection accomplished was major with MPEP treatment (Lea et al. 2005). Trypan blue assay for Fenobam, instead, displayed significant improvement in hepatocytes viability respect to anoxic controls at 1 µM, 10 µM and 50 µM at 45 minutes, with ANOVA, while fitting linear mixed model showed that the mortality rate curve of Fenobam 50 µM in the intervals 0-60’ and 0-75’ was significantly different to untreated ischemic sample. We supposed that the differences between MPEP and Fenobam could be

due to the lipophilic nature of the molecules: MPEP is more permeable (logP=3.3) than Fenobam (logP=1), and could exert more easily its function also on intracellular mGluR5 receptors (Jong et al. 2014). In addition, Fenobam is a urea-derivative negative allosteric modulator, so it presents a chemotype distinct from MPEP (Porter et al. 2005). Because of the structural differences, MPEP and Fenobam, even though they bind to the transmembrane domain, interact with different binding residues (A Pagano et al. 2000; Pari Malherbe et al. 2003; Malherbe et al. 2006). The effect due to MPEP administration compared to Fenobam treatment could be also explained according to the rank order of drug potency: MPEP > Fenobam (Porter et al. 2005). Interestingly, data reported by Zhang and colleagues provide evidence that different allosteric agonists can differentially modulate coupling of a single receptor to different signaling pathways (Zhang et al. 2005). In their study, CPPHA enhanced ERK1/2 phosphorylation in presence of low amount of agonist, or even in its absence; conversely, at high concentration of the agonist, CPPHA attenuated ERK1/2 phosphorylation. DFB activity, instead, do not depend on the concentration of agonist. Moreover, the activation of mGluR5 with CPPHA could have downstream physiological responses mediated by ERK1/2 phosphorylation and not from calcium mobilization, as occurs, instead, with DFB treatment. Thus, we did not exclude that similar behavior could occur also for MPEP and Fenobam.

The administration of the orthosteric antagonist CPG did not ameliorate hepatocytes viability, maintaining a mortality rate similar to anoxic controls. This could be imputable to the fact that, probably, 100 µM and 200 µM were too low concentrations of CPG, while its IC_{50} in HEK-293 cells was calculated to be more than 1000 µM (Kingston et al. 1995; Porter et al. 2005), respect to MPEP IC_{50} of 36 nM (Acher n.d.).

Once ascertained that not only MPEP, but also MTEP and Fenobam protected hepatocytes from ischemic injury, we explored whether and how ATP could be involved. In their experiments, Chantong and co-workers reported that MPEP administration depleted ATP in microglial cells (Chantong et al. 2014). It is worth noting that ATP depletion is one of the first consequences after electrochemical gradient impairment. Thus, these data appeared to be in contrast with the well-known ability of MPEP to make cells more resistant to ischemic injury (M Storto et al. 2000). We evaluated ATP alterations in isolated rat hepatocytes in anoxic conditions in presence or absence of MPEP, MTEP, Fenobam and DHPG plus DFB. ATP concentration was followed in time before and after N_2 insufflation. Hepatocytes treated with MPEP had a significantly lower ATP content respect to controls before anoxia, more

evidently for MPEP 30 µM; nonetheless, MPEP-treated cells showed a slower decline in ATP following N_2 insufflation when compared to anoxic controls or DHPG-treated hepatocytes. After 30 minutes of pharmacological treatment and 30 minutes of exposure to anoxia, ATP levels decreased with all negative allosteric modulators treatment when compared to ischemic controls, although only hepatocytes administered with MPEP 3 µM and 30 µM exhibited ATP levels significantly higher respect to ischemic controls.

Since mitochondria are the production engine of ATP, we investigated the effect of Negative Allosteric Modulators (NAMs) administration on ATP production in isolated mitochondria. Isolated mitochondria were treated 15 minutes with MPEP, MTEP, Fenobam and CPG, all at 0.3 µM, 3 µM and 30 µM. As observed in isolated primary hepatocytes, MPEP and MTEP induced a dose-dependent decrease in ATP level, although more evident and significant with MPEP treatment. ATP concentration in samples treated with Fenobam and CPG, instead, remained almost constant and with values near to the controls. Furthermore, in order to understand whether our molecules could affect mitochondrial function, we assessed respiratory control index, membrane potential, ROS production, complex I and II function and F_1F_O-ATPase activity. Our experiments demonstrated that MPEP and MTEP altered ATP content without affecting mitochondria functionality. Moreover, NAMs did not uncouple mitochondria, suggesting that ATP depletion occurred in a mitochondria-independent fashion. However, ATP was significantly depleted by MPEP 30 µM. Hence, we assessed an acellular solution containing ATP in presence or absence of MPEP, MTEP, Fenobam and CPG. As detected in isolated hepatocytes and mitochondria, ATP decreased in a dose-dependent way for MPEP and MTEP, while remained unaffected for Fenobam and CPG. Our data suggested that, although the protection provided, only MPEP and MTEP depleted ATP both *in vitro* and in acellular solution, without affecting any functional role of mitochondria or impairing complexes deputed to respiration.

5.2 *Ex vivo* experiments

In the second part of this work, we assessed whether the use of a preservation solution containing MPEP could reduce the susceptibility of liver grafts to cold preservation injury. To mimic what happens *in vivo* during transplantation, *ex vivo* experiments were performed. Isolated livers from both wild type and knockout mice were preserved at 4°C for 18 hours and

successively reperfused at 37°C for one hour. The UW preservation solution, as well Krebs-Henseleit buffer used for the reperfusion, contained either MPEP 0.3 µM or DHPG. Perfusate samples were collected at the end of cold preservation (the so-called "wash out") and during the reperfusion process.

To assess liver condition after 18 hours cold storage, lactate dehydrogenase released in the wash out was evaluated. MPEP-treated and knockout organs showed a significant decrease when compared to controls. LDH release of livers preserved with MPEP 0.3 µM and of mGluR5 knockout organs was significantly lower than in controls. Moreover, LDH released by KO livers is comparable to that from MPEP-treated organs up to 45 minutes of reperfusion. Transaminases release was also gauged. AST levels decreased in livers treated with MPEP 0.3 µM in wash out and the reduction became significant after 30 minutes and 60 minutes of reperfusion respect to controls. ALT concentration significantly diminished in livers treated with MPEP 0.3 µM in wash out, after 30 and 60 minutes of reperfusion respect to controls. No differences were detected in mGluR5 knockout livers when compared to controls for both AST and ALT. Taken together, these data indicated that the pharmacological blockade of mGluR5 or the absence of the receptor protected from ischemic injury progression. Moreover, apoptosis was gauged measuring Bax/Bcl-2 ratio (Produit-Zengaffinen et al. 2009), and no differences were observed between MPEP-treated livers and wild type control samples, suggesting that MPEP could be protective regarding apoptosis.

To evaluate whether the protection mediated by MPEP resulted from a metabolic shift from aerobiosis to anaerobiosis, we quantified protein expression of Hypoxia Inducible Factor-1α (HIF-1α) and Glycogen Phosphorylase (GP). HIF-1α, one of the principal transcription factors induced by oxygen deprivation, promotes glycolysis, blockade of Krebs cycle and glycogen accumulation (Pescador et al. 2010). Glycogen phosphorylase is active in hypoglycemic conditions and intervenes in glycogen breaking to yield glucose molecules. Although it is expressed lately in hypoxic conditions, glycogen phosphorylase is HIF-1α-independent (Favaro et al. 2012). In our experiments, MPEP treatment did not alter HIF-1α or glycogen phosphorylase respect to anoxic controls, suggesting that other mechanisms were involved in the improvement of liver functionality.

When glutamate release induced by pathological stimuli becomes excessive in the extracellular space of central nervous system, intracellular calcium concentration increases and triggers a detrimental downstream cascade, involving upregulation of Tumor Necrosis

Factor-α (TNF-α), nitric oxide synthase, mitochondria dysfunction, ROS and RNS production (Kritis et al. 2015) (Figure 5.1). Similarly, in a model of cold ischemia and warm reperfusion, rat hepatocytes viability was decreased by the imbalance of calcium homeostasis (Elimadi & Haddad 2001).

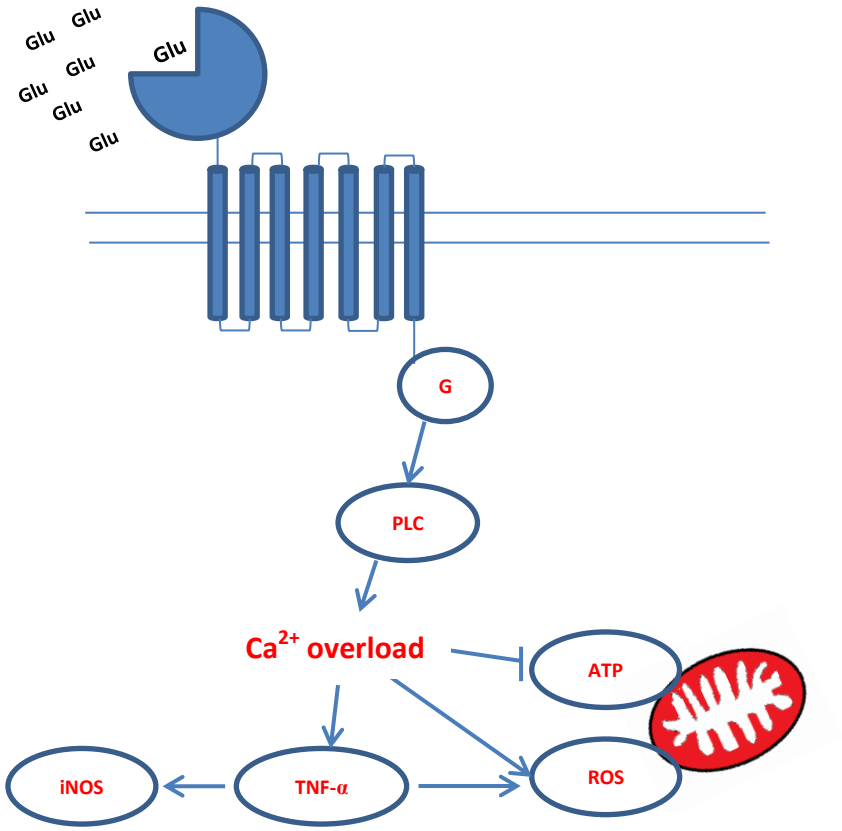


Figure 5.1. Excitotoxic cascade. mGluR5 activation by excessive glutamate causes an increase in calcium concentration that, in turn, leads to inflammatory markers generation.

Although in microglia cells the activation of mGluR5 provoked a decrease in TNF-α expression (Chantong et al. 2014), data about mice livers and isolated hepatocytes reported that MPEP administration led to a decrease of drug-induced ROS and iNOS generation (Storto et al. 2003). Thus, we further investigated mGluR5 ability to control inflammatory mediators in a model of ischemia/reperfusion. To accomplish this purpose, TNF-α and inducible isoform of Nitric Oxide Synthase (iNOS) protein expression were evaluated. The blockade of the receptors by MPEP or its absence in knockout livers significantly reduced TNF-α protein, in comparison with controls. Western blot analysis revealed that in both MPEP-treated and knockout organs a significantly reduction in iNOS protein expression

occurred, respect to controls. It is known that in different cell types and organs, from bowel to macrophages and liver, TNF-α positively correlates with iNOS expression through NF-κB: an increment in TNF-α produces an increase in iNOS and the inhibition of TNF-α leads to the decrease of iNOS (Nandi et al. 2010; Fonseca et al. 2003; Muntané et al. 2000). In line with published data that reported the presence of TNF Response Element on iNOS gene (Medeiros et al. 2007; Eberhardt et al. 1996), we supposed that the blockade of mGluR5, through TNF-α, selectively reduces iNOS expression. In order to support this hypothesis, protein expression of the endothelial constitutive isoform of NOS (eNOS) was detected: no changes occurred between MPEP-treated livers and controls. Thus, it is plausible that, in *in vivo* systems, eNOS, keeping nitric oxide production at physiological levels, could promote the proper functioning of the cardio-circulatory system in critical conditions, such as reperfusion injury following an ischemic event. Moreover, a non-specific decrease of all NOS isoforms would make the negative modulators of mGluR5 allosteric strongly contraindicated in various conditions associated with transient ischemia, including multiple organ failure (Ferrigno, Di Pasqua, et al. 2015; Nijveldt et al. 2003; López et al. 2004).

As outlined previously, in isolated anoxic hepatocytes treated with NAMs we observed a slower decrease in ATP when compared to anoxic controls and, after 30 minutes of anoxia, ATP content was significantly higher in rat hepatocytes administered with MPEP 3 µM and 30 µM respect to anoxic controls. To verify whether a similar trend occurred also in a model of isolation/reperfusion organ, we evaluated ATP content in cold preserved liver. ATP concentration was higher in MPEP-treated samples when compared to controls. Conversely, mGluR5 knockout organs displayed ATP levels similar to controls. It has been reported that MPEP administration reduced ATP content in microglia cells and astrocytes, although in astrocytes mGluR5 is present only in the early development and the authors hypothesized that ATP decrease was due to a kainate receptors.

These results, in agreement with *in vitro* data, suggest that ATP depletion could occur also in a receptor-independent way. It has recently demonstrated that the triple bond of MPEP reacts with the thiol group of GSH, generating GSH-conjugates (Ferrigno et al. 2017; Zhuo et al. 2015). In addition to alkyne, MPEP and MTEP contain a pyridine, a highly reactive group that could be subjected to alkylation, oxidation, nucleophilic substitutions and so on. Thus, we supposed that MPEP could react in some way with ATP.

Besides cold ischemia, also normoxic (or warm) ischemia was taken into consideration, since it occurred in trauma and elective liver surgery, as well in some types of toxic liver injury,

hypovolemic shock, sinusoidal obstruction, Budd-Chiari syndrome and sleep apnea. Thus, beside cold ischemia, we performed ischemia/reperfusion also in normoxic conditions. Also in this case, preservation and reperfusion solution were added with MPEP or DHPG plus DFB. Livers functionality was evaluated by means of lactate dehydrogenase release in the wash out and during the reperfusion and pro-inflammation was assessed by gauging TNF-α released using an ELISA assay. Liver protection has been observed also in normoxic condition. In fact, MPEP-treated and mGluR5 knockout organs showed a decrease in LDH and TNF-α release, when compared to controls or DHPG-administered livers, as previously observed in cold ischemia.

Despite experiments in central nervous system has revealed that the role mediated by DHPG is controversial (Baskys et al. 2005; Masuoka et al. 2016), we obtained that the administration of this orthosteric agonist in both *in vitro* and *ex vivo* experiments displayed a trend similar to that of ischemic controls. This could be due to the fact that mGluR5 could be saturated by glutamate, as previously demonstrated (M Storto et al. 2000), or to the rapid desensitization of the receptor by DHPG-induced depolarization (Huang & van den Pol 2007).

CONCLUSIONS

Metabotropic glutamate receptor 5 (mGluR5) has been extensively studied in the Central Nervous System (CNS). Besides its physiological role in synaptic plasticity, memory and learning, it has been shown that mGluR5 dysregulation could lead to several pathologies, such as fragile X syndrome, schizophrenia and Parkinson's disease (Nicoletti et al. 2011).
Furthermore, mGluR5 plays a crucial role in ischemic events. Its activation causes an excessive release of calcium at intracellular level, leading to an increase of inflammatory markers and reactive oxygen species that could provoke cell death (Taylor et al. 1995; Barbour et al. 1988; Phillis et al. 1997). Conversely, the inhibition of the receptor is correlated to a reduction of cerebral or hepatic damage (Makarewicz et al. 2006; M Storto et al. 2000).
In the first part of this project we focused our attention on *in vitro* experiments. Our purposes were multiple: to confirm MPEP protection and investigate whether other mGluR5 allosteric and orthosteric antagonists (MTEP, Fenobam, CPG) had similar properties; then, to elucidate the mechanisms involved in the protection mediated by MPEP; and, finally, to explore the mechanism of MPEP-mediated ATP depletion. In the second part of the study, we aimed to assess whether MPEP protection was maintained in two *ex vivo* models of ischemia/reperfusion injury: cold (4°C) and warm (37°C) ischemia/reperfusion.
By our *in vitro* data, we firstly showed that MPEP and MTEP depleted ATP from hepatocytes and mitochondria without affecting mitochondrial functionality. ATP-depleting ability seems to have no consequences on mGluR5-mediated protection for I/R injury. Then, we confirmed that MPEP reduced the damage caused by anoxia in isolated hepatocytes. Furthermore, we revealed that also MTEP and Fenobam had a positive effect on cell viability. More notably, we demonstrated for the first time that the blockade of mGluR5 with MPEP protected mice livers from cold and warm ischemia/reperfusion injury in *ex vivo* models.

Because of mGluR5 is involved in the onset of several pathologies not only in the central nervous system, it would represents a compelling pharmacological target also for the handling of diseases in peripheral organs, such as ischemia/reperfusion injury of the liver. To date, the molecular mechanisms of mGluR5 NAMs protection in ischemia are not fully understood, even because in diverse cell types the signaling pathways appear different. Thus, further investigation on molecular signaling would be useful to enlarge our knowledge.
MPEP is a very permeable molecule and its administration in the CNS could trigger unexpected pathways. Hence, its use is limited to the research field in order to clarify the mechanisms in which it is involved. For this reason, in preclinical and clinical studies other mGluR5 negative allosteric modulators were employed, such as Fenobam, Mavoglurant and

Basimglurant. However, several clinical trials were discontinued because of the poor efficacy of these negative allosteric modulators (Petrov et al. 2014). Further research in the optimization of these molecules appears to be necessary. A novel approach is focused on the development of "bitopic" ligands, which are molecules able to bind to both the orthosteric and allosteric sites, with the purpose of combine the "all-or-none" responses of orthosteric ligands with the higher selectivity of the allosteric molecules (Kamal & Jockers 2009).

Our results support the idea that the advancement in the understanding of the molecular pathophysiology of liver injury might lead to the development of novel therapeutic alternatives, whose the mGluR5 negative allosteric modulators represent a feasible and truthful example.

REFERENCES

Abe, T. et al., 1992. Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca2+ signal transduction. *The Journal of biological chemistry*, 267(19), pp.13361–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1320017 [Accessed September 11, 2017].

Acher, F.C., Tocris Bioscience Scientific Review Series. Available at: https://www.funakoshi.co.jp/download/pdf/metabotropic_glutamate_receptors_review.pdf [Accessed October 6, 2017].

Acher, F.C. & Bertrand, H.-O., 2005. Amino acid recognition by Venus flytrap domains is encoded in an 8-residue motif. *Biopolymers*, 80(2–3), pp.357–366. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15810013 [Accessed October 11, 2017].

Allbutt, C., 1918. NOTES FROM A CLINICAL LECTURE ON A CASE OF HUNTINGTON'S CHOREA: Delivered at Addenbrooke's Hospital, Cambridge. *British medical journal*, 1(2988), pp.389–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20768988 [Accessed July 14, 2017].

Anborgh, P.H. et al., 2005. Inhibition of metabotropic glutamate receptor signaling by the huntingtin-binding protein optineurin. *The Journal of biological chemistry*, 280(41), pp.34840–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16091361 [Accessed July 18, 2017].

Anderson, J.J. et al., 2002. [3H]Methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine Binding to Metabotropic Glutamate Receptor Subtype 5 in Rodent Brain: In Vitro and in Vivo Characterization. *Journal of Pharmacology and Experimental Therapeutics*, 303(3), pp.1044–1051. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12438526 [Accessed October 6, 2017].

Anderson, J.J. et al., 2003. In vivo receptor occupancy of mGlu5 receptor antagonists using the novel radioligand [3H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine). *European journal of pharmacology*, 473(1), pp.35–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12877935 [Accessed October 6, 2017].

Anon, 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell*, 72(6), pp.971–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8458085 [Accessed July 17, 2017].

Anon, Editorial SchizophrEnia. Available at: https://www.future-science.com/doi/pdf/10.4155/fmc.13.137 [Accessed August 31, 2017].

Attucci, S. et al., 2001. Activation of type 5 metabotropic glutamate receptors enhances NMDA responses in mice cortical wedges. *British Journal of Pharmacology*, 132(4), pp.799–806. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11181420 [Accessed August 30, 2017].

Bailey, D.B. et al., 2008. Co-occurring conditions associated with FMR1 gene variations: findings from a national parent survey. *American journal of medical genetics. Part A*, 146A(16), pp.2060–9. Available at: http://doi.wiley.com/10.1002/ajmg.a.32439 [Accessed July 11, 2017].

Baines, C.P., 2009. The mitochondrial permeability transition pore and ischemia-reperfusion injury. *Basic research in cardiology*, 104(2), p.181. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2671061/ [Accessed October 17, 2017].

Baldwin, D. & Rudge, S., 1995. The role of serotonin in depression and anxiety. *International clinical psychopharmacology*, 9 Suppl 4, pp.41–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7622823 [Accessed October 16, 2017].

Ballesteros, J.A. & Weinstein, H., 1995. [19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. In pp. 366–428. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1043947105800497 [Accessed July 25, 2017].

Banko, J.L. et al., 2006. Regulation of eukaryotic initiation factor 4E by converging signaling pathways during metabotropic glutamate receptor-dependent long-term depression. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(8), pp.2167–73. Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.5196-05.2006 [Accessed July 24, 2017].

Bao, W.L. et al., 2001. Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia. *Brain research*, 922(2), pp.173–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11743947 [Accessed October 26, 2017].

Baracca, A. et al., 2003. Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F(0) during ATP synthesis. *Biochimica et biophysica acta*, 1606(1–3), pp.137–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14507434 [Accessed October 18, 2017].

Barbour, B., Brew, H. & Attwell, D., 1988. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. *Nature*, 335(6189), pp.433–435. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2901670 [Accessed August 2, 2017].

Baskys, A. et al., 2005. Group I metabotropic glutamate receptors reduce excitotoxic injury and may facilitate neurogenesis. *Neuropharmacology*, 49, pp.146–156. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16023152 [Accessed October 19, 2017].

Baudry, M. et al., 2012. Roles of group I metabotropic glutamate receptors under physiological conditions and in neurodegeneration. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling*, 1(4), pp.523–532. Available at: http://doi.wiley.com/10.1002/wmts.51 [Accessed October 11, 2017].

Baumann, M. et al., 1989. Effects of warm and cold ischemia on mitochondrial functions in brain, liver and kidney. *Molecular and Cellular Biochemistry*, 87, pp.137–145. Available at: https://link.springer.com/content/pdf/10.1007%2FBF00219256.pdf [Accessed September 6, 2017].

Bear, M.F., Huber, K.M. & Warren, S.T., 2004. The mGluR theory of fragile X mental retardation. *Trends in Neurosciences*, 27(7), pp.370–377. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0166223604001328 [Accessed July 11, 2017].

Becker, L.B., 2004. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovascular research*, 61(3), pp.461–70. Available at: https://academic.oup.com/cardiovascres/article-lookup/doi/10.1016/j.cardiores.2003.10.025 [Accessed September 7, 2017].

Ben-Ari, Z. et al., 2007. Bax ablation protects against hepatic ischemia/reperfusion injury in transgenic mice. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society*, 13(8), pp.1181–8. Available at: http://doi.wiley.com/10.1002/lt.21221 [Accessed September 8, 2017].

Berridge, M.J., 1993. Inositol trisphosphate and calcium signalling. *Nature*, 361(6410), pp.315–325. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8381210 [Accessed September 7, 2017].

Berry-Kravis, E. et al., 2009. A pilot open label, single dose trial of fenobam in adults with fragile X syndrome. *Journal of medical genetics*, 46(4), pp.266–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19126569 [Accessed July 12, 2017].

Besheer, J., Stevenson, R.A. & Hodge, C.W., 2006. mGlu5 receptors are involved in the discriminative stimulus effects of self-administered ethanol in rats. *European journal of pharmacology*, 551(1–3), pp.71–5. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0014299906009563 [Accessed October 16, 2017].

Bessis, A.-S. et al., 2002. Closure of the Venus flytrap module of mGlu8 receptor and the activation process: Insights from mutations converting antagonists into agonists. *Proceedings of the National Academy of Sciences of the United States of America*, 99(17), pp.11097–102. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.162138699 [Accessed July 19, 2017].

Bhave, G. et al., 2001. Peripheral group I metabotropic glutamate receptors modulate nociception in mice. *Nature neuroscience*, 4(4), pp.417–23. Available at: http://www.nature.com/doifinder/10.1038/86075 [Accessed July 13, 2017].

Birse, E.F. et al., 1993. Phenylglycine derivatives as new pharmacological tools for investigating the role of metabotropic glutamate receptors in the central nervous system. *Neuroscience*, 52(3), pp.481–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7680790 [Accessed August 29, 2017].

De Blasi, A. et al., 2001. Molecular determinants of metabotropic glutamate receptor signaling. *Trends in pharmacological sciences*, 22(3), pp.114–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11239574 [Accessed July 25, 2017].

Brabet, I. et al., 1995. Phenylglycine derivatives discriminate between mGluR1- and mGluR5-mediated responses. *Neuropharmacology*, 34(8), pp.895–903. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8532171 [Accessed August 29, 2017].

Bradbury, M.J. et al., 2004. Metabotropic Glutamate Receptor mGlu5 Is a Mediator of Appetite and Energy Balance in Rats and Mice. *Journal of Pharmacology and Experimental Therapeutics*, 313(1), pp.395–402. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15590770 [Accessed October 11, 2017].

Brakeman, P.R. et al., 1997. Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature*, 386(6622), pp.284–288. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9069287 [Accessed July 31, 2017].

Bräuner-Osborne, H. et al., 2000. Ligands for glutamate receptors: design and therapeutic prospects. *Journal of medicinal chemistry*, 43(14), pp.2609–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10893301 [Accessed August 29, 2017].

Brice, N.L. et al., 2002. Metabotropic glutamate and GABA(B) receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic beta cells. *Diabetologia*, 45(2), pp.242–52. Available at: http://link.springer.com/10.1007/s00125-001-0750-0 [Accessed August 30, 2017].

Bridges, R.J., Natale, N.R. & Patel, S.A., 2012. System xc^- cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. *British journal of pharmacology*, 165(1), pp.20–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21564084 [Accessed September 19, 2017].

Brown, V. et al., 2001. Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell*, 107(4), pp.477–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11719188 [Accessed July 11, 2017].

Byrnes, K.R. et al., 2009. Metabotropic glutamate receptor 5 activation inhibits microglial associated inflammation and neurotoxicity. *Glia*, 57(5), pp.550–60. Available at: http://doi.wiley.com/10.1002/glia.20783 [Accessed July 13, 2017].

Caldwell-Kenkel, J.C. et al., 1989. Reperfusion injury to endothelial cells following cold ischemic storage of rat livers. *Hepatology (Baltimore, Md.)*, 10(3), pp.292–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2668147 [Accessed September 11, 2017].

Camargo, C.A. et al., Interleukin-6 Protects Liver Against Warm Ischemia/Reperfusion Injury and Promotes Hepatocyte Proliferation in the Rodent. Available at: http://onlinelibrary.wiley.com/store/10.1002/hep.510260619/asset/510260619_ftp.pdf?v=1&t=j7a7z2z6&s=32e44531c8c772d9f2b66a80f08dd9457baf99b5 [Accessed September 7, 2017].

Campbell, U.C. et al., 2004. The mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) potentiates PCP-induced cognitive deficits in rats. *Psychopharmacology*, 175(3), pp.310–8. Available at: http://link.springer.com/10.1007/s00213-004-1827-5 [Accessed July 12, 2017].

Carden, D.L. & Granger, D.N., 2000. Pathophysiology of ischaemia-reperfusion injury. *The Journal of Pathology*, 190(3), pp.255–266. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10685060 [Accessed September 6, 2017].

Cartmell, J. et al., 1993. Subtypes of metabotropic excitatory amino acid receptor distinguished by stereoisomers of the rigid glutamate analogue, 1-aminocyclopentane-1,3-dicarboxylate. *Neuroscience letters*, 153(1), pp.107–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8390029 [Accessed August 30, 2017].

Casillas-Ramírez, A. et al., 2006. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life sciences*, 79(20), pp.1881–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16828807 [Accessed October 2, 2017].

Ceccarelli, S.M. et al., 2007. Rational design, synthesis, and structure–activity relationship of benzoxazolones: New potent mglu5 receptor antagonists based on the fenobam structure. *Bioorganic & Medicinal Chemistry Letters*, 17(5), pp.1302–1306. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0960894X06013989 [Accessed October 6, 2017].

Chan, T.O., Rittenhouse, S.E. & Tsichlis, P.N., 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annual review of biochemistry*, 68(1), pp.965–1014. Available at: http://www.annualreviews.org/doi/10.1146/annurev.biochem.68.1.965 [Accessed July 24, 2017].

Chang, K. & Roche, K.W., 2017. Structural and molecular determinants regulating mGluR5 surface expression. *Neuropharmacology*, 115, pp.10–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27211252 [Accessed October 10, 2017].

Chantong, B. et al., 2014. Inhibition of metabotropic glutamate receptor 5 induces cellular stress through pertussis toxin-sensitive Gi-proteins in murine BV-2 microglia cells. *Journal of Neuroinflammation*, 11(1), p.190. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25407356 [Accessed October 12, 2017].

Chappell, A.S. et al., 2014. Results from clinical trials of a selective ionotropic glutamate receptor 5 (iGluR5) antagonist, LY5454694 tosylate, in 2 chronic pain conditions. *Pain*, 155(6), pp.1140–1149. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24631589 [Accessed July 13, 2017].

Chazotte, B., 2011. Labeling Mitochondria with Rhodamine 123. *Cold Spring Harbor Protocols*, 2011(7), p.pdb.prot5640-prot5640. Available at: http://www.cshprotocols.org/cgi/doi/10.1101/pdb.prot5640 [Accessed October 18, 2017].

Chen, Y. et al., 2000. Evaluation of the activity of a novel metabotropic glutamate receptor antagonist (+/-)-2-amino-2-(3-cis and trans-carboxycyclobutyl-3-(9-thioxanthyl)propionic acid) in the in vitro neonatal spinal cord and in an in vivo pain model. *Neuroscience*, 95(3), pp.787–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10670446 [Accessed August 29, 2017].

Chen, Y. et al., 2007. N- -2-hydroxybenzamide (CPPHA) Acts through a Novel Site as a Positive Allosteric Modulator of Group 1 Metabotropic Glutamate Receptors. *Molecular Pharmacology*, 73(3), pp.909–918. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18056795 [Accessed October 4, 2017].

Cheung, K. et al., 1996. An Optimized Model for Rat Liver Perfusion Studies. *Journal of Surgical Research*, 66(1), pp.81–89. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8954836 [Accessed October 18, 2017].

Chiamulera, C. et al., 2001. Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice. *Nature neuroscience*, 4(9), pp.873–4. Available at: http://www.nature.com/doifinder/10.1038/nn0901-873 [Accessed October 16, 2017].

Christensen, H.N. et al., 1983. Synthesis and transport applications of 3-aminobicyclo[3,2,1]octane-3-carboxylic acids. *Journal of Medicinal Chemistry*, 26(10), pp.1374–1378. Available at: http://pubs.acs.org/doi/abs/10.1021/jm00364a008 [Accessed August 30, 2017].

Cleva, R.M. & Olive, M.F., 2011. Positive allosteric modulators of type 5 metabotropic glutamate receptors (mGluR5) and their therapeutic potential for the treatment of CNS disorders. *Molecules (Basel, Switzerland)*, 16(3), pp.2097–106. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21368721 [Accessed October 4, 2017].

Coffee, B. et al., 1999. Acetylated histones are associated with FMR1 in normal but not fragile X-syndrome cells. *Nature genetics*, 22(1), pp.98–101. Available at: http://www.nature.com/doifinder/10.1038/8807 [Accessed July 11, 2017].

Coffee, B. et al., 2002. Histone modifications depict an aberrantly heterochromatinized FMR1 gene in fragile x syndrome. *American journal of human genetics*, 71(4), pp.923–32. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0002929707603759 [Accessed July 11, 2017].

Collard, C.D. et al., 2002. Neutrophil-derived glutamate regulates vascular endothelial barrier function. *The Journal of biological chemistry*, 277(17), pp.14801–11. Available at: http://www.jbc.org/lookup/doi/10.1074/jbc.M110557200 [Accessed September 11, 2017].

Colletti, L.M. et al., 1990. Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. *Journal of Clinical Investigation*, 85(6), pp.1936–1943. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2161433 [Accessed September 7, 2017].

Conn, P.J. & Pin, J.-P., 1997. PHARMACOLOGY AND FUNCTIONS OF METABOTROPIC GLUTAMATE RECEPTORS. *Annual Review of Pharmacology and Toxicology*, 37(1), pp.205–237. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9131252 [Accessed August 30, 2017].

Contractor, A. et al., 1998. Direct effects of metabotropic glutamate receptor compounds on native and recombinant N-methyl-D-aspartate receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 95(15), pp.8969–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9671788 [Accessed August 30, 2017].

Corti, C. et al., 2003. Gene structure of the human metabotropic glutamate receptor 5 and functional analysis of its multiple promoters in neuroblastoma and astroglioma cells. *The Journal of biological chemistry*, 278(35), pp.33105–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12783878 [Accessed October 6, 2017].

Cosford, N.D.P. et al., 2003. [3H]-methoxymethyl-MTEP and [3H]-methoxy-PEPy: potent and selective radioligands for the metabotropic glutamate subtype 5 (mGlu5) receptor. *Bioorganic & medicinal chemistry letters*, 13(3), pp.351–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12565928 [Accessed October 6, 2017].

Costigan, M., Scholz, J. & Woolf, C.J., 2009. Neuropathic Pain: A Maladaptive Response of the Nervous System to Damage. *Annual Review of Neuroscience*, 32(1), pp.1–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19400724 [Accessed July 12, 2017].

Coyle, J.T., 2012. NMDA receptor and schizophrenia: a brief history. *Schizophrenia bulletin*, 38(5), pp.920–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22987850 [Accessed August 31, 2017].

Craske, M.G. & Stein, M.B., 2016. Anxiety. *The Lancet*, 388(10063), pp.3048–3059. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27349358 [Accessed October 16, 2017].

Crawford, D.C., Acuña, J.M. & Sherman, S.L., FMR1 and the fragile X syndrome: human genome epidemiology review. *Genetics in medicine : official journal of the American College of Medical Genetics*, 3(5), pp.359–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11545690 [Accessed July 11, 2017].

Croall, D.E. & Ersfeld, K., 2007. The calpains: modular designs and functional diversity. *Genome biology*, 8(6), p.218. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17608959 [Accessed September 7, 2017].

Croce, A.C. et al., 2004. Autofluorescence properties of isolated rat hepatocytes under different metabolic conditions. *Photochemical & Photobiological Sciences*, 3(10), p.920. Available at: http://xlink.rsc.org/?DOI=b407358d [Accessed October 18, 2017].

Croci, C. et al., 2003. Group I Metabotropic Glutamate Receptors Bind to Protein Phosphatase 1C. *Journal of Biological Chemistry*, 278(50), pp.50682–50690. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14519764 [Accessed July 31, 2017].

Cursio, R. et al., 1999. A caspase inhibitor fully protects rats against lethal normothermic liver ischemia by inhibition of liver apoptosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 13(2), pp.253–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9973313 [Accessed September 7, 2017].

D'Antoni, S. et al., 2008. Metabotropic Glutamate Receptors in Glial Cells. *Neurochemical Research*, 33(12), pp.2436–2443. Available at: http://link.springer.com/10.1007/s11064-008-9694-9 [Accessed July 13, 2017].

Danbolt, N.C., 2001. Glutamate uptake. *Progress in neurobiology*, 65(1), pp.1–105. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11369436 [Accessed August 2, 2017].

Dhami, G.K. et al., 2004. G Protein-coupled receptor kinase 2 regulator of G protein signaling homology domain binds to both metabotropic glutamate receptor 1a and Galphaq to attenuate signaling. *The Journal of biological chemistry*, 279(16), pp.16614–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14764583 [Accessed July 25, 2017].

Dhami, G.K. & Ferguson, S.S.G., 2006. Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis. *Pharmacology & therapeutics*, 111(1), pp.260–71. Available at: http://linkinghub.elsevier.com/retrieve/pii/S016372580600009X [Accessed October 10, 2017].

Dhingra, A. & Vardi, N., 2012. mGlu receptors in the retina. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling*, 1(5), pp.641–653. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24003403 [Accessed September 11, 2017].

Dobrovetsky, E., Khutoreskaya, G., Seitova, A., Cossar, D., et al., Metabotropic glutamate receptor mglur1 complexed with LY341495 antagonist. *To be Published*. Available at: http://www.rcsb.org/pdb/explore.do?structureId=3ks9 [Accessed July 19, 2017a].

Dobrovetsky, E., Khutoreskaya, G., Seitova, A., Cossar, D., et al., Metabotropic Glutamate receptor mGluR5 complexed with glutamate. *To be Published*. Available at: http://www.rcsb.org/pdb/explore.do?structureId=3lmk [Accessed July 19, 2017b].

Dobrovetsky, E., Khutoreskaya, G., Seitova, A., He, H., et al., mGluR7 complexed with LY341495. *To be Published*. Available at: http://www.rcsb.org/pdb/explore.do?structureId=3mq4 [Accessed July 19, 2017].

Dogrul, A. et al., 2000. Peripheral and spinal antihyperalgesic activity of SIB-1757, a metabotropic glutamate receptor (mGLUR(5)) antagonist, in experimental neuropathic pain in rats. *Neuroscience letters*, 292(2), pp.115–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10998562 [Accessed July 13, 2017].

DOHERTY, A.. et al., 1997. (RS)-2-Chloro-5-Hydroxyphenylglycine (CHPG) Activates mGlu5, but not mGlu1, Receptors Expressed in CHO Cells and Potentiates NMDA Responses in the Hippocampus. *Neuropharmacology*, 36(2), pp.265–267. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0028390897000014 [Accessed August 29, 2017].

Doherty, A.J., Collingridge, G.L. & Jane, D.E., 1999. Antagonist activity of alpha-substituted 4-carboxyphenylglycine analogues at group I metabotropic glutamate receptors expressed in CHO cells. *British journal of pharmacology*, 126(1), pp.205–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10051137 [Accessed August 29, 2017].

Dölen, G. et al., 2007. Correction of Fragile X Syndrome in Mice. *Neuron*, 56(6), pp.955–962. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18093519 [Accessed July 12, 2017].

Doré, A.S. et al., 2014. Structure of class C GPCR metabotropic glutamate receptor 5 transmembrane domain. *Nature*, 511(7511), pp.557–562. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25042998 [Accessed July 25, 2017].

Doria, J. et al., 2013. Metabotropic glutamate receptor 5 positive allosteric modulators are neuroprotective in a mouse model of Huntington's disease. *British Journal of Pharmacology*, 169(4), pp.909–921. Available at: http://doi.wiley.com/10.1111/bph.12164 [Accessed July 14, 2017].

Eaton, S.A. et al., 1993. Competitive antagonism at metabotropic glutamate receptors by (S)-4-carboxyphenylglycine and (RS)-alpha-methyl-4-carboxyphenylglycine. *European journal of pharmacology*, 244(2), pp.195–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8381746 [Accessed August 29, 2017].

Eberhardt, W. et al., 1996. Molecular Cloning of the Rat Inducible Nitric Oxide Synthase Gene Promoter. *Biochemical and Biophysical Research Communications*, 223(3), pp.752–756. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8687469 [Accessed October 13, 2017].

Eberhart, D.E. & Warren, S.T., 1996. Nuclease sensitivity of permeabilized cells confirms altered chromatin formation at the fragile X locus. *Somatic cell and molecular genetics*, 22(6), pp.435–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9131013 [Accessed July 11, 2017].

Elias-Mir, M., Jimnez-Castro, M.B. & Peralt, C., 2012. Ischemia-Reperfusion Injury Associated with Liver Transplantation in 2011: Past and Future. In *Liver Transplantation - Basic Issues*. InTech. Available at: http://www.intechopen.com/books/liver-transplantation-basic-issues/ischemia-reperfusion-injury-associated-with-liver-transplantation-in-2011-past-and-future [Accessed September 6, 2017].

Elimadi, A. & Haddad, P.S., 2001. Cold preservation-warm reoxygenation increases hepatocyte steady-state Ca(2+) and response to Ca(2+)-mobilizing agonist. *American journal of physiology. Gastrointestinal and liver physiology*, 281(3), pp.G809-15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11518693 [Accessed October 25, 2017].

Emaus, R.K., Grunwald, R. & Lemasters, J.J., 1986. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochimica et biophysica acta*, 850(3), pp.436–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2873836 [Accessed October 18, 2017].

Emmitte, K.A., 2017. mGlu $_5$ negative allosteric modulators: a patent review (2013 - 2016). *Expert Opinion on Therapeutic Patents*, 27(6), pp.691–706. Available at: https://www.tandfonline.com/doi/full/10.1080/13543776.2017.1280466 [Accessed October 5, 2017].

Fabbrini, G. et al., 2007. Levodopa-induced dyskinesias. *Movement Disorders*, 22(10), pp.1379–1389. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17427940 [Accessed October 16, 2017].

Fagni, L., Worley, P.F. & Ango, F., 2002. Homer as Both a Scaffold and Transduction Molecule. *Science Signaling*, 2002(137), p.re8-re8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12072556 [Accessed July 31, 2017].

Favaro, E. et al., 2012. Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells. *Cell metabolism*, 16(6), pp.751–64. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1550413112004512 [Accessed October 13, 2017].

Ferguson, S.S., 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacological reviews*, 53(1), pp.1–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11171937 [Accessed October 10, 2017].

Ferraguti, F. & Shigemoto, R., 2006. Metabotropic glutamate receptors. *Cell Tissue Res*.

Ferrigno, A., Di Pasqua, L.G., et al., 2015. Liver plays a central role in asymmetric dimethylarginine-mediated organ injury. *World journal of gastroenterology*, 21(17), pp.5131–7. Available at: http://www.wjgnet.com/1007-9327/full/v21/i17/5131.htm [Accessed October 13, 2017].

Ferrigno, A. et al., 2017. Localization and role of metabotropic glutamate receptors subtype 5 in the gastrointestinal tract. *World journal of gastroenterology*, 23(25), pp.4500–4507. Available at: http://www.wjgnet.com/1007-9327/full/v23/i25/4500.htm [Accessed September 11, 2017].

Ferrigno, A., Vairetti, M., et al., 2015. Selective blockade of mGlu5 metabotropic glutamate receptors is protective against hepatic mitochondrial dysfunction in 6-OHDA lesioned Parkinsonian rats. *Clinical and Experimental Pharmacology and Physiology*, 42(6), pp.695–703. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25904005 [Accessed October 18, 2017].

Ferrigno, A., Richelmi, P. & Vairetti, M., 2013. Troubleshooting and improving the mouse and rat isolated perfused liver preparation. *Journal of Pharmacological and Toxicological Methods*, 67(2), pp.107–114. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23079697 [Accessed October 18, 2017].

Finkbeiner, S., 2011. Huntington's Disease. *Cold Spring Harbor perspectives in biology*, 3(6). Available at: http://www.ncbi.nlm.nih.gov/pubmed/21441583 [Accessed July 17, 2017].

Fisher, K. & Coderre, T.J., 1996. Comparison of nociceptive effects produced by intrathecal administration of mGluR agonists. *Neuroreport*, 7(15–17), pp.2743–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8981459 [Accessed July 12, 2017].

Fitzjohna, S.M. et al., 1996. Activation of group I mG1uRs potentiates NMDA responses in rat hippocampal slices. *Neuroscience Letters*, 203(3), pp.211–213. Available at: http://linkinghub.elsevier.com/retrieve/pii/0304394096123016 [Accessed August 30, 2017].

Flint, A.C., Dammerman, R.S. & Kriegstein, A.R., 1999. Endogenous activation of metabotropic glutamate receptors in neocortical development causes neuronal calcium oscillations. *Proceedings of the National Academy of Sciences of the United States of America*, 96(21), pp.12144–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10518590 [Accessed July 24, 2017].

Fonseca, S.G. et al., 2003. TNF-α mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis. *European Journal of Immunology*, 33(8), pp.2297–2306. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12884305 [Accessed October 25, 2017].

Förstermann, U. & Sessa, W.C., 2012. Nitric oxide synthases: regulation and function. *European heart journal*, 33(7), p.829–37, 837a–837d. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21890489 [Accessed October 2, 2017].

Fourgeaud, L. et al., 2003. The Metabotropic Glutamate Receptor mGluR5 Is Endocytosed by a Clathrin-independent Pathway. *Journal of Biological Chemistry*, 278(14), pp.12222–12230. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12529370 [Accessed October 10, 2017].

Francesconi, A. & Duvoisin, R.M., 2004. Divalent cations modulate the activity of metabotropic glutamate receptors. *Journal of Neuroscience Research*, 75(4), pp.472–479. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14743430 [Accessed October 11, 2017].

Francesconi, A. & Duvoisin, R.M., 2000. Opposing effects of protein kinase C and protein kinase A on metabotropic glutamate receptor signaling: selective desensitization of the inositol trisphosphate/Ca2+ pathway by phosphorylation of the receptor-G protein-coupling domain. *Proceedings of the National Academy of Sciences of the United States of America*, 97(11), pp.6185–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10823959 [Accessed July 31, 2017].

Francesconi, A., Kumari, R. & Zukin, R.S., 2009. Regulation of Group I Metabotropic Glutamate Receptor Trafficking and Signaling by the Caveolar/Lipid Raft Pathway. *Journal of Neuroscience*, 29(11), pp.3590–3602. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19295163 [Accessed October 10, 2017].

Franke, T.F. et al., 1997. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science (New York, N.Y.)*, 275(5300), pp.665–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9005852 [Accessed July 24, 2017].

Frisby, C.L. et al., 2005. Inhibition of transient lower esophageal sphincter relaxation and gastroesophageal reflux by metabotropic glutamate receptor ligands. *Gastroenterology*, 129(3), pp.995–1004. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0016508505013570 [Accessed September 11, 2017].

Fu, Y.H. et al., 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell*, 67(6), pp.1047–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1760838 [Accessed July 11, 2017].

Fujiwara, N. et al., 1987. Effects of hypoxia on rat hippocampal neurones in vitro. *The Journal of physiology*, 384, pp.131–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2443657 [Accessed August 2, 2017].

Fukui, M. et al., 2009. Mechanism of glutamate-induced neurotoxicity in HT22 mouse hippocampal cells. *European Journal of Pharmacology*, 617(1–3), pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19580806 [Accessed September 19, 2017].

Fuxe, K. et al., 2014. Moonlighting proteins and protein-protein interactions as neurotherapeutic targets in the G protein-coupled receptor field. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 39(1), pp.131–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24105074 [Accessed October 6, 2017].

Garber, K.B., Visootsak, J. & Warren, S.T., 2008. Fragile X syndrome. *European Journal of Human Genetics*, 16(6), pp.666–672. Available at: http://www.nature.com/doifinder/10.1038/ejhg.2008.61 [Accessed July 11, 2017].

Gasparini, F. et al., 1999. 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology*, 38(10), pp.1493–503. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10530811 [Accessed September 21, 2017].

Gelmers, H.J., 1985. Calcium-channel blockers: effects on cerebral blood flow and potential uses for acute stroke. *The American journal of cardiology*, 55(3), p.144B–148B. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2578724 [Accessed September 20, 2017].

Gereau, R.W. & Heinemann, S.F., 1998. Role of protein kinase C phosphorylation in rapid desensitization of metabotropic glutamate receptor 5. *Neuron*, 20(1), pp.143–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9459450 [Accessed July 31, 2017].

Gill, S. et al., 2007. Human Heart Glutamate Receptors—Implications for Toxicology, Food Safety, and Drug Discovery. *Toxicologic Pathology*, 35(3), pp.411–417. Available at: http://journals.sagepub.com/doi/10.1080/01926230701230361 [Accessed August 30, 2017].

Gladding, C.M., Fitzjohn, S.M. & Molnar, E., 2009. Metabotropic Glutamate Receptor-Mediated Long-Term Depression: Molecular Mechanisms. *Pharmacological Reviews*, 61(4), pp.395–412. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19926678 [Accessed July 24, 2017].

Godler, D.E. et al., 2010. Methylation of novel markers of fragile X alleles is inversely correlated with FMRP expression and FMR1 activation ratio. *Human molecular genetics*, 19(8), pp.1618–32. Available at: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddq037 [Accessed July 11, 2017].

Golwala, N.H. et al., 2009. Vascular responses to nitrite are mediated by xanthine oxidoreductase and mitochondrial aldehyde dehydrogenase in the ratThis article is one of a selection of papers published in a special issue on Advances in Cardiovascular Research. *Canadian Journal of Physiology and Pharmacology*, 87(12), pp.1095–1101. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20029546 [Accessed September 7, 2017].

Gomeza, J. et al., 1996. The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G-proteins. *The Journal of biological chemistry*, 271(4), pp.2199–205. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8567679 [Accessed July 25, 2017].

Gorman, M.W. et al., 2003. Measurement of adenine nucleotides in plasma. *Luminescence : the journal of biological and chemical luminescence*, 18(3), pp.173–81.

Goudet, C. et al., 2004. Heptahelical domain of metabotropic glutamate receptor 5 behaves like rhodopsin-like receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 101(1), pp.378–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14691258 [Accessed July 25, 2017].

Gourdin, M. & Dubois, P., 2013. Impact of Ischemia on Cellular Metabolism. In *Artery Bypass*. InTech. Available at: http://www.intechopen.com/books/artery-bypass/impact-of-ischemia-on-cellular-metabolism [Accessed September 6, 2017].

Gravius, A. et al., 2008. Investigation on tolerance development to subchronic blockade of mGluR5 in models of learning, anxiety, and levodopa-induced dyskinesia in rats. *Journal of Neural Transmission*, 115(12), pp.1609–1619. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18690408 [Accessed October 16, 2017].

Gray, J.A. & Roth, B.L., 2007. The pipeline and future of drug development in schizophrenia. *Molecular Psychiatry*, 12(10), pp.904–922. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17667958 [Accessed August 30, 2017].

Green, M.D. et al., 2006. In vitro metabolic studies on the selective metabotropic glutamate receptor sub-type 5 (mGluR5) antagonist 3-[(2-methyl-1,3-thiazol-4-yl) ethynyl]-pyridine (MTEP). *Neuroscience letters*, 391(3), pp.91–5. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304394005009572 [Accessed October 6, 2017].

Gregory, K.J. et al., 2011. Allosteric modulation of metabotropic glutamate receptors: Structural insights and therapeutic potential. *Neuropharmacology*, 60(1), pp.66–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20637216 [Accessed August 31, 2017].

Guzy, R.D. & Schumacker, P.T., 2006. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Experimental Physiology*, 91(5), pp.807–819. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16857720 [Accessed September 7, 2017].

Hammond, A.S. et al., 2010. Discovery of a Novel Chemical Class of mGlu(5) Allosteric Ligands with Distinct Modes of Pharmacology. *ACS chemical neuroscience*, 1(10), pp.702–716. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20981342 [Accessed October 4, 2017].

Hansen, A.J., Hounsgaard, J. & Jahnsen, H., 1982. Anoxia increases potassium conductance in hippocampal nerve cells. *Acta physiologica Scandinavica*, 115(3), pp.301–10. Available at: http://doi.wiley.com/10.1111/j.1748-1716.1982.tb07082.x [Accessed August 2, 2017].

Harrison, A.A., Gasparini, F. & Markou, A., 2002. Nicotine potentiation of brain stimulation reward reversed by DH beta E and SCH 23390, but not by eticlopride, LY 314582 or MPEP in rats. *Psychopharmacology*, 160(1), pp.56–66. Available at: http://link.springer.com/10.1007/s00213-001-0953-6 [Accessed October 16, 2017].

Harvey, P.D., 2014. Assessing Disability in Schizophrenia. *The Journal of Clinical Psychiatry*, 75(10), pp.e27–e27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25373132 [Accessed August 30, 2017].

Havlickova, M. et al., 2003. The Second Intracellular Loop of Metabotropic Glutamate Receptors Recognizes C Termini of G-protein α-Subunits. *Journal of Biological Chemistry*, 278(37), pp.35063–35070. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12829705 [Accessed July 25, 2017].

Hersh, J.H., Saul, R.A. & Committee on Genetics, 2011. Health supervision for children with fragile X syndrome. *Pediatrics*, 127(5), pp.994–1006. Available at: http://pediatrics.aappublications.org/cgi/doi/10.1542/peds.2010-3500 [Accessed July 11, 2017].

Hou, L. & Klann, E., 2004. Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 24(28), pp.6352–61. Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.0995-04.2004 [Accessed July 18, 2017].

Hovelsø, N. et al., 2012. Therapeutic potential of metabotropic glutamate receptor modulators. *Current neuropharmacology*, 10(1), pp.12–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22942876 [Accessed October 16, 2017].

Hu, H.Z. et al., 1999. Functional group I metabotropic glutamate receptors in submucous plexus of guinea-pig ileum. *British journal of pharmacology*, 128(8), pp.1631–5. Available at: http://doi.wiley.com/10.1038/sj.bjp.0702980 [Accessed September 11, 2017].

Huang, H. & van den Pol, A.N., 2007. Rapid Direct Excitation and Long-Lasting Enhancement of NMDA Response by Group I Metabotropic Glutamate Receptor Activation of Hypothalamic Melanin-Concentrating Hormone Neurons. *Journal of Neuroscience*, 27(43), pp.11560–11572. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17959799 [Accessed October 19, 2017].

Huber, K.M. et al., 2002. Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proceedings of the National Academy of Sciences of the United States of America*, 99(11), pp.7746–50. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.122205699 [Accessed July 11, 2017].

Huber, K.M., Kayser, M.S. & Bear, M.F., 2000. Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science (New York, N.Y.)*, 288(5469), pp.1254–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10818003 [Accessed July 11, 2017].

Imanishi, H., Scales, W.E. & Campbell, D.A., 1997. Tumor Necrosis Factor Alpha Alters the Cytotoxic Effect of Hydrogen Peroxide in Cultured Hepatocytes. *Biochemical and Biophysical Research Communications*, 230(1), pp.120–124. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0006291X9695901X [Accessed September 7, 2017].

Irving, A.J. et al., 1990. 1S,3R-ACPD stimulates and L-AP3 blocks Ca2+ mobilization in rat cerebellar neurons. *European Journal of Pharmacology*, 186(2–3), pp.363–365. Available at: http://linkinghub.elsevier.com/retrieve/pii/001429999090462F [Accessed August 30, 2017].

Jaeschke, G. et al., 2015. Metabotropic Glutamate Receptor 5 Negative Allosteric Modulators: Discovery of 2-Chloro-4-[1-(4-fluorophenyl)-2,5-dimethyl-1 *H* -imidazol-4-ylethynyl]pyridine (Basimglurant, RO4917523), a Promising Novel Medicine for Psychiatric Diseases. *Journal of Medicinal Chemistry*, 58(3), pp.1358–1371. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25565255 [October 5, 2017].

Jaeschke, H., 1991. Reactive oxygen and ischemia/reperfusion injury of the liver. *Chemico-biological interactions*, 79(2), pp.115–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1884426 [Accessed September 8, 2017].

Jaeschke, H. & Lemasters, J.J., 2003. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology*, 125(4), pp.1246–1257. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0016508503012095 [Accessed September 8, 2017].

Jeffrey Conn, P., Christopoulos, A. & Lindsley, C.W., 2009. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nature Reviews Drug Discovery*, 8(1), pp.41–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19116626 [Accessed July 25, 2017].

Jesse, C.R. et al., 2009. Selective blockade of mGlu5 metabotropic glutamate receptors is hepatoprotective against fulminant hepatic failure induced by lipopolysaccharide and D-galactosamine in mice. *Journal of Applied Toxicology*.

Ji, R.-R. et al., 2003. Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends in neurosciences*, 26(12), pp.696–705. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0166223603003370 [Accessed July 13, 2017].

Jia, H., Rustioni, A. & Valtschanoff, J.G., 1999. Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. *The Journal of comparative neurology*, 410(4), pp.627–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10398053 [Accessed September 11, 2017].

Jiang, C., Agulian, S. & Haddad, G.G., 1992. Cl- and Na+ homeostasis during anoxia in rat hypoglossal neurons: intracellular and extracellular in vitro studies. *The Journal of physiology*, 448, pp.697–708. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1593484 [Accessed August 2, 2017].

Jingami, H., Nakanishi, S. & Morikawa, K., 2003. Structure of the metabotropic glutamate receptor. *Current opinion in neurobiology*, 13(3), pp.271–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12850210 [Accessed July 19, 2017].

Jong, Y.-J.I. et al., 2005. Functional Metabotropic Glutamate Receptors on Nuclei from Brain and Primary Cultured Striatal Neurons. *Journal of Biological Chemistry*, 280(34), pp.30469–30480. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15958386 [Accessed July 24, 2017].

Jong, Y.-J.I. et al., 2014. Location-Dependent Signaling of the Group 1 Metabotropic Glutamate Receptor mGlu5. *Molecular Pharmacology*, 86(6), pp.774–785. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25326002 [Accessed October 10, 2017].

Jong, Y.-J.I. & O'Malley, K.L., 2017. Mechanisms Associated with Activation of Intracellular Metabotropic Glutamate Receptor, mGluR5. *Neurochemical research*, 42(1), pp.166–172. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27514643 [Accessed October 10, 2017].

Julio-Pieper, M. et al., 2011. Exciting times beyond the brain: metabotropic glutamate receptors in peripheral and non-neural tissues. *Pharmacological reviews*.

Kahn, R.S. et al., 2015. Schizophrenia. *Nature Reviews Disease Primers*, p.15067. Available at: http://www.nature.com/articles/nrdp201567 [Accessed August 30, 2017].

Kalogeris, T. et al., 2012. Cell biology of ischemia/reperfusion injury. *International review of cell and molecular biology*, 298, pp.229–317. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22878108 [Accessed September 7, 2017].

Kamal, M. & Jockers, R., 2009. Bitopic ligands: all-in-one orthosteric and allosteric. *F1000 biology reports*, 1, p.77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20948611 [Accessed October 30, 2017].

Kammermeier, P.J., 2012. The orthosteric agonist 2-chloro-5-hydroxyphenylglycine activates mGluR5 and mGluR1 with similar efficacy and potency. *BMC pharmacology*, 12(1), p.6. Available at: http://bmcpharma.biomedcentral.com/articles/10.1186/1471-2210-12-6 [Accessed August 29, 2017].

Karachot, L. et al., 2001. Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein. *Journal of neurophysiology*, 86(1), pp.280–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11431509 [Accessed July 11, 2017].

Karim, F., Wang, C.C. & Gereau, R.W., 2001. Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(11), pp.3771–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11356865 [Accessed July 13, 2017].

Kawabata, S. et al., 1996. Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature*, 383(6595), pp.89–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8779726 [Accessed July 31, 2017].

Kettunen, P. et al., 2002. Signaling mechanisms of metabotropic glutamate receptor 5 subtype and its endogenous role in a locomotor network. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(5), pp.1868–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11880516 [Accessed July 24, 2017].

Keywood, C., Wakefield, M. & Tack, J., 2009. A proof-of-concept study evaluating the effect of ADX10059, a metabotropic glutamate receptor-5 negative allosteric modulator, on acid exposure and symptoms in gastro-oesophageal reflux disease. *Gut*, 58(9), pp.1192–1199. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19460767 [Accessed October 16, 2017].

Kim, J.-S., He, L. & Lemasters, J.J., 2003. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochemical and Biophysical Research Communications*, 304(3), pp.463–470. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0006291X03006181 [Accessed October 17, 2017].

Kim, Y.S. et al., 2009. Expression of metabotropic glutamate receptor mGluR5 in human dental pulp. *Journal of endodontics*, 35(5), pp.690–4. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0099239909001411 [Accessed August 30, 2017].

Kingston, A.E. et al., 2002. Inhibition of group I metabotropic glutamate receptor responses in vivo in rats by a new generation of carboxyphenylglycine-like amino acid antagonists. *Neuroscience letters*, 330(2), pp.127–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12231428 [Accessed August 29, 2017].

Kingston, A.E. et al., 1995. Pharmacological analysis of 4-carboxyphenylglycine derivatives: comparison of effects on mGluR1 alpha and mGluR5a subtypes. *Neuropharmacology*, 34(8), pp.887–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8532170 [Accessed October 12, 2017].

Kitano, J. et al., 2002. Tamalin, a PDZ domain-containing protein, links a protein complex formation of group 1 metabotropic glutamate receptors and the guanine nucleotide exchange factor cytohesins. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(4), pp.1280–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11850456 [Accessed July 31, 2017].

Kocerha, J. et al., 2014. microRNA-128a dysregulation in transgenic Huntington's disease monkeys. *Molecular Brain*, 7(1), p.46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24929669 [Accessed October 6, 2017].

Kolber, B.J. et al., 2010. Activation of Metabotropic Glutamate Receptor 5 in the Amygdala Modulates Pain-Like Behavior. *Journal of Neuroscience*, 30(24), pp.8203–8213. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20554871 [Accessed July 12, 2017].

Kordi-Tamandani, D.M., Dahmardeh, N. & Torkamanzehi, A., 2013. Evaluation of hypermethylation and expression pattern of GMR2, GMR5, GMR8, and GRIA3 in patients with schizophrenia. *Gene*, 515(1), pp.163–166. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23149219 [Accessed October 6, 2017].

Krieger, P. et al., 2000. Interaction between metabotropic and ionotropic glutamate receptors regulates neuronal network activity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 20(14), pp.5382–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10884323 [Accessed August 30, 2017].

Kritis, A.A. et al., 2015. Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/study. *Frontiers in cellular neuroscience*, 9, p.91. Available at: http://www.frontiersin.org/Cellular_Neuroscience/10.3389/fncel.2015.00091/abstract [Accessed October 25, 2017].

Kronquist, K.E., Sherman, S.L. & Spector, E.B., 2008. Clinical significance of tri-nucleotide repeats in Fragile X testing: a clarification of American College of Medical Genetics guidelines. *Genetics in medicine : official journal of the American College of Medical Genetics*, 10(11), pp.845–7. Available at: http://www.nature.com/doifinder/10.1097/GIM.0b013e31818b0c8a [Accessed July 11, 2017].

Kuang, D. et al., 2003. Molecular Similarities in the Ligand Binding Pockets of an Odorant Receptor and the Metabotropic Glutamate Receptors. *Journal of Biological Chemistry*, 278(43), pp.42551–42559. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12912984 [Accessed October 11, 2017].

Kubo, Y., Miyashita, T. & Murata, Y., 1998. Structural basis for a Ca2+-sensing function of the metabotropic glutamate receptors. *Science (New York, N.Y.)*, 279(5357), pp.1722–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9497291 [Accessed October 11, 2017].

Kunishima, N. et al., 2000. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature*, 407(6807), pp.971–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11069170 [Accessed July 25, 2017].

Lacza, Z., Pankotai, E. & Busija, D.W., 2009. Mitochondrial nitric oxide synthase: current concepts and controversies. *Frontiers in bioscience (Landmark edition)*, 14, pp.4436–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19273361 [Accessed October 17, 2017].

Lea, P.M. et al., 2005. Neuroprotective activity of the mGluR5 antagonists MPEP and MTEP against acute excitotoxicity differs and does not reflect actions at mGluR5 receptors. *British journal of pharmacology*, 145(4), pp.527–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15821750 [Accessed October 6, 2017].

Lea, P.M. & Faden, A.I., 2006. Metabotropic Glutamate Receptor Subtype 5 Antagonists MPEP and MTEP. *CNS Drug Reviews*, 12(2), pp.149–166. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16958988 [Accessed October 6, 2017].

Lee, H.J. et al., 2006. Peripheral mGluR5 antagonist attenuated craniofacial muscle pain and inflammation but not mGluR1 antagonist in lightly anesthetized rats. *Brain Research Bulletin*, 70(4–6), pp.378–385. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17027773 [Accessed August 30, 2017].

Lee, J. & Ro, J.Y., 2007. Differential regulation of glutamate receptors in trigeminal ganglia following masseter inflammation. *Neuroscience letters*, 421(2), pp.91–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17560028 [Accessed August 30, 2017].

Lee, J.H. et al., 2008. Calmodulin dynamically regulates the trafficking of the metabotropic glutamate receptor mGluR5. *Proceedings of the National Academy of Sciences of the United States of America*, 105(34), pp.12575–80. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.0712033105 [Accessed July 31, 2017].

Lemasters, J.J., 1999. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. *The American journal of physiology*, 276(1 Pt 1), pp.G1-6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9886971 [Accessed September 6, 2017].

Lentsch, A.B. et al., 1999. Requirement for interleukin-12 in the pathogenesis of warm hepatic ischemia/reperfusion injury in mice. *Hepatology*, 30(6), pp.1448–1453. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10573524 [Accessed September 7, 2017].

Li, W. & Neugebauer, V., 2003. Differential Roles of mGluR1 and mGluR5 in Brief and Prolonged Nociceptive Processing in Central Amygdala Neurons. *Journal of Neurophysiology*, 91(1), pp.13–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13679408 [Accessed July 13, 2017].

Li, X. et al., 2006. Metabotropic Glutamate 5 Receptor Antagonism Is Associated with Antidepressant-Like Effects in Mice. *Journal of Pharmacology and Experimental Therapeutics*, 319(1), pp.254–259. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16803860 [Accessed October 16, 2017].

Lindsley, C.W. et al., 2004. Discovery of Positive Allosteric Modulators for the Metabotropic Glutamate Receptor Subtype 5 from a Series of *N* -(1,3-Diphenyl-1 *H* - pyrazol-5-yl)benzamides That Potentiate Receptor Function in Vivo. *Journal of Medicinal Chemistry*, 47(24), pp.5825–5828. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15537338 [Accessed October 4, 2017].

Liu, F. et al., 2008. ADX47273 [S-(4-Fluoro-phenyl)- -methanone]: A Novel Metabotropic Glutamate Receptor 5-Selective Positive Allosteric Modulator with Preclinical Antipsychotic-Like and Procognitive Activities. *Journal of Pharmacology and Experimental Therapeutics*, 327(3), pp.827–839. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18753411 [Accessed October 4, 2017].

Liu, M.-G. et al., 2012. Metabotropic glutamate receptor 5 contributes to inflammatory tongue pain via extracellular signal-regulated kinase signaling in the trigeminal spinal subnucleus caudalis and upper cervical spinal cord. *Journal of Neuroinflammation*, 9(1), p.750. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23181395 [Accessed August 30, 2017].

Liu, M. & Kirchgessner, A.L., 2000. Agonist- and reflex-evoked internalization of metabotropic glutamate receptor 5 in enteric neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 20(9), pp.3200–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10777784 [Accessed September 11, 2017].

López-Bendito, G. et al., 2002. Differential distribution of group I metabotropic glutamate receptors during rat cortical development. *Cerebral cortex (New York, N.Y. : 1991)*, 12(6), pp.625–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12003862 [Accessed September 11, 2017].

López, A. et al., 2004. Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: Effect on survival in patients with septic shock*. *Critical Care Medicine*, 32(1), pp.21–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14707556 [Accessed October 13, 2017].

Lubs, H.A., 1969. A marker X chromosome. *American journal of human genetics*, 21(3), pp.231–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5794013 [Accessed July 12, 2017].

Maione, S. et al., 2000. Periaqueductal gray matter metabotropic glutamate receptors modulate formalin-induced nociception. *Pain*, 85(1–2), pp.183–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10692617 [Accessed July 13, 2017].

Makarewicz, D. et al., 2006. Neuroprotective potential of group I metabotropic glutamate receptor antagonists in two ischemic models. *Neurochemistry International*, 48(6–7), pp.485–490. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16513218 [Accessed October 11, 2017].

Malaspina, D. et al., 2001. Advancing paternal age and the risk of schizophrenia. *Archives of general psychiatry*, 58(4), pp.361–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11296097 [Accessed August 30, 2017].

Malherbe, P. et al., 2006. Comparison of the binding pockets of two chemically unrelated allosteric antagonists of the mGlu5 receptor and identification of crucial residues involved in the inverse agonism of MPEP. *Journal of neurochemistry*, 98(2), pp.601–15. Available at: http://doi.wiley.com/10.1111/j.1471-4159.2006.03886.x [Accessed September 26, 2017].

Malherbe, P. et al., 2003. Mutational Analysis and Molecular Modeling of the Binding Pocket of the Metabotropic Glutamate 5 Receptor Negative Modulator 2-Methyl-6-(phenylethynyl)-pyridine. *Molecular Pharmacology*, 64(4), pp.823–832. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14500738 [Accessed September 26, 2017].

Malherbe, P. et al., 2003. Mutational analysis and molecular modeling of the binding pocket of the metabotropic glutamate 5 receptor negative modulator 2-methyl-6-(phenylethynyl)-pyridine. *Molecular pharmacology*, 64(4), pp.823–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14500738 [Accessed July 25, 2017].

Manzoni, O. et al., 1990. (trans)-1-amino-cyclopentyl-1,3-dicarboxylate stimulates quisqualate phosphoinositide-coupled receptors but not ionotropic glutamate receptors in striatal neurons and Xenopus oocytes. *Molecular pharmacology*, 38(1), pp.1–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2164627 [Accessed August 30, 2017].

Mao, L. et al., 2005. The scaffold protein Homer1b/c links metabotropic glutamate receptor 5 to extracellular signal-regulated protein kinase cascades in neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(10), pp.2741–52. Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.4360-04.2005 [Accessed July 18, 2017].

Mao, L.-M. et al., 2008. Phosphorylation of group I metabotropic glutamate receptors (mGluR1/5) in vitro and in vivo. *Neuropharmacology*, 55(4), pp.403–408. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18585398 [Accessed July 31, 2017].

Marin, J.C.A. & Goadsby, P.J., 2010. Glutamatergic fine tuning with ADX-10059: a novel therapeutic approach for migraine? *Expert opinion on investigational drugs*, 19(4), pp.555–61. Available at: http://www.tandfonline.com/doi/full/10.1517/13543781003691832 [Accessed July 13, 2017].

Martin, E.I. et al., 2009. The neurobiology of anxiety disorders: brain imaging, genetics, and psychoneuroendocrinology. *The Psychiatric clinics of North America*, 32(3), pp.549–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19716990 [Accessed October 16, 2017].

Martin, J.P. & Bell, J., 1943. A PEDIGREE OF MENTAL DEFECT SHOWING SEX-LINKAGE. *Journal of neurology and psychiatry*, 6(3–4), pp.154–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21611430 [Accessed July 12, 2017].

Masuoka, T. et al., 2016. Long-Term Activation of Group I Metabotropic Glutamate Receptors Increases Functional TRPV1-Expressing Neurons in Mouse Dorsal Root Ganglia. *Frontiers in Cellular Neuroscience*, 10, p.79. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27064319 [Accessed October 19, 2017].

Mathiesen, J.M. et al., 2003. Positive allosteric modulation of the human metabotropic glutamate receptor 4 (hmGluR4) by SIB-1893 and MPEP. *British journal of pharmacology*, 138(6), pp.1026–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12684257 [Accessed October 6, 2017].

Matosin, N. et al., 2017. Shifting towards a model of mGluR5 dysregulation in schizophrenia: Consequences for future schizophrenia treatment. *Neuropharmacology*, 115, pp.73–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26349010 [Accessed October 6, 2017].

Medeiros, R. et al., 2007. Connecting TNF- Signaling Pathways to iNOS Expression in a Mouse Model of Alzheimer's Disease: Relevance for the Behavioral and Synaptic Deficits Induced by Amyloid Protein. *Journal of Neuroscience*, 27(20), pp.5394–5404. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17507561 [Accessed October 13, 2017].

Mela, F. et al., 2007. Antagonism of metabotropic glutamate receptor type 5 attenuates l-DOPA-induced dyskinesia and its molecular and neurochemical correlates in a rat model of Parkinson?s disease. *Journal of Neurochemistry*, 101(2), pp.483–497. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17359492 [Accessed October 16, 2017].

Michalon, A. et al., 2012. Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron*, 74(1), pp.49–56. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627312002723 [Accessed July 12, 2017].

Minakami, R., Jinnai, N. & Sugiyama, H., 1997. Phosphorylation and calmodulin binding of the metabotropic glutamate receptor subtype 5 (mGluR5) are antagonistic in vitro. *The Journal of biological chemistry*, 272(32), pp.20291–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9242710 [Accessed July 31, 2017].

Mion, S. et al., 2001. Bidirectional Regulation of Neurite Elaboration by Alternatively Spliced Metabotropic Glutamate Receptor 5 (mGluR5) Isoforms. *Molecular and Cellular Neuroscience*, 17(6), pp.957–972. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11414786 [Accessed October 6, 2017].

Mølck, C. et al., 2014. mGluR5: Exploration of Orthosteric and Allosteric Ligand Binding Pockets and Their Applications to Drug Discovery. *Neurochemical Research*.

Momeni, H.R., 2011. Role of calpain in apoptosis. *Cell journal*, 13(2), pp.65–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23507938 [Accessed September 7, 2017].

Montana, M.C. et al., 2009. The metabotropic glutamate receptor subtype 5 antagonist fenobam is analgesic and has improved in vivo selectivity compared with the prototypical antagonist 2-methyl-6-(phenylethynyl)-pyridine. *The Journal of pharmacology and experimental therapeutics*, 330(3), pp.834–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19515968 [Accessed July 13, 2017].

Morikawa, K. et al., 2000. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature*, 407(6807), pp.971–977. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11069170 [Accessed July 19, 2017].

Morimoto, R. et al., 2006. Secretion of L-glutamate from osteoclasts through transcytosis. *The EMBO journal*, 25(18), pp.4175–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16957773 [Accessed August 30, 2017].

Mühlemann, A. et al., 2006. Determination of key amino acids implicated in the actions of allosteric modulation by 3,3'-difluorobenzaldazine on rat mGlu5 receptors. *European journal of pharmacology*, 529(1–3), pp.95–104. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16352303 [Accessed October 4, 2017].

Muntané, J. et al., 2000. TNF-alpha dependent production of inducible nitric oxide is involved in PGE(1) protection against acute liver injury. *Gut*, 47(4), pp.553–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10986217 [Accessed October 25, 2017].

Murphy, J.M., Murch, S.J. & Ball, R.O., 1996. Proline is synthesized from glutamate during intragastric infusion but not during intravenous infusion in neonatal piglets. *The Journal of nutrition*, 126(4), pp.878–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8613891 [Accessed October 11, 2017].

Muto, T. et al., 2007. Structures of the extracellular regions of the group II/III metabotropic glutamate receptors. *Proceedings of the National Academy of Sciences*, 104(10), pp.3759–3764. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17360426 [Accessed July 19, 2017].

Nandi, J. et al., 2010. TNF-α modulates iNOS expression in an experimental rat model of indomethacin-induced jejunoileitis. *Molecular and Cellular Biochemistry*, 336(1–2), pp.17–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19802525 [Accessed October 24, 2017].

Nasser, Y. et al., 2007. Expression of a functional metabotropic glutamate receptor 5 on enteric glia is altered in states of inflammation. *Glia*, 55(8), pp.859–872. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17405149 [Accessed August 30, 2017].

Nastos, C. et al., 2014. Global consequences of liver ischemia/reperfusion injury. *Oxidative medicine and cellular longevity*, 2014, p.906965. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24799983 [Accessed September 8, 2017].

Négyessy, L. et al., 1997. Light and electron microscopic demonstration of mGluR5 metabotropic glutamate receptor immunoreactive neuronal elements in the rat cerebellar cortex. *The Journal of comparative neurology*, 385(4), pp.641–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9302110 [Accessed September 11, 2017].

Neki, A. et al., 1996. Metabotropic glutamate receptors mGluR2 and mGluR5 are expressed in two non-overlapping populations of Golgi cells in the rat cerebellum. *Neuroscience*, 75(3), pp.815–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8951875 [Accessed September 11, 2017].

Newsholme, P. et al., 2003. Glutamine and glutamate?their central role in cell metabolism and function. *Cell Biochemistry and Function*, 21(1), pp.1–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12579515 [Accessed October 11, 2017].

Nicoletti, F. et al., 2011. Metabotropic glutamate receptors: from the workbench to the bedside. *Neuropharmacology*, 60(7–8), pp.1017–41. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0028390810002923 [Accessed July 12, 2017].

Nicoll, R.A., 2003. Expression mechanisms underlying long-term potentiation: a postsynaptic view. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 358(1432), pp.721–726. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12740118 [Accessed July 13, 2017].

Nieminen, A.L. et al., 1990. Protection by acidotic pH and fructose against lethal injury to rat hepatocytes from mitochondrial inhibitors, ionophores and oxidant chemicals. *Biochemical and biophysical research communications*, 167(2), pp.600–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2322245 [Accessed September 6, 2017].

Nijveldt, R.J., Teerlink, T. & van Leeuwen, P.A.M., 2003. The asymmetrical dimethylarginine (ADMA)-multiple organ failure hypothesis. *Clinical nutrition (Edinburgh, Scotland)*, 22(1), pp.99–104. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12553957 [Accessed October 13, 2017].

Nishimura, Y., Romer, L.H. & Lemasters, J.J., 1998. Mitochondrial dysfunction and cytoskeletal disruption during chemical hypoxia to cultured rat hepatic sinusoidal endothelial cells: The pH paradox and cytoprotection by glucose, acidotic pH, and glycine. *Hepatology*, 27(4), pp.1039–1049. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9537444 [Accessed September 6, 2017].

Nishizawa, Y., 2001. Glutamate release and neuronal damage in ischemia. *Life sciences*, 69(4), pp.369–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11459428 [Accessed August 2, 2017].

Nissim, I., 1999. Newer aspects of glutamine/glutamate metabolism: the role of acute pH changes. *The American journal of physiology*, 277(4 Pt 2), pp.F493-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10516271 [Accessed October 11, 2017].

Niswender, C.M. & Conn, P.J., 2010. Metabotropic Glutamate Receptors: Physiology, Pharmacology, and Disease. *Annual Review of Pharmacology and Toxicology*, 50(1), pp.295–322. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20055706 [Accessed July 28, 2017].

Noetzel, M.J. et al., 2012. Functional Impact of Allosteric Agonist Activity of Selective Positive Allosteric Modulators of Metabotropic Glutamate Receptor Subtype 5 in Regulating Central Nervous System Function. *Molecular Pharmacology*, 81(2), pp.120–133. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22021324 [Accessed September 25, 2017].

Nucifora, F.C. et al., 2001. Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. *Science (New York, N.Y.)*, 291(5512), pp.2423–8. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.1056784 [Accessed July 17, 2017].

O'Brien, J.A. et al., 2003. A Family of Highly Selective Allosteric Modulators of the Metabotropic Glutamate Receptor Subtype 5. *Molecular Pharmacology*, 64(3), pp.731–740. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12920211 [Accessed October 4, 2017].

O'Brien, J.A. et al., 2003. A family of highly selective allosteric modulators of the metabotropic glutamate receptor subtype 5. *Molecular pharmacology*, 64(3), pp.731–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12920211 [Accessed July 25, 2017].

O'Brien, J.A. et al., 2004. A Novel Selective Allosteric Modulator Potentiates the Activity of Native Metabotropic Glutamate Receptor Subtype 5 in Rat Forebrain. *Journal of Pharmacology and Experimental Therapeutics*, 309(2), pp.568–577. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14747613 [Accessed October 4, 2017].

O'Hara, P.J. et al., 1993. The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron*, 11(1), pp.41–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8338667 [Accessed July 19, 2017].

O'Leary, D.M. et al., 2000. Selective mGluR5 antagonists MPEP and SIB-1893 decrease NMDA or glutamate-mediated neuronal toxicity through actions that reflect NMDA receptor antagonism. *British journal of pharmacology*, 131(7), pp.1429–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11090117 [Accessed September 20, 2017].

O'Malley, K.L. et al., 2003. Activation of Metabotropic Glutamate Receptor mGlu5 on Nuclear Membranes Mediates Intranuclear Ca^{2+} Changes in Heterologous Cell Types and Neurons. *Journal of Biological Chemistry*, 278(30), pp.28210–28219. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12736269 [Accessed October 10, 2017].

Olive, M.F., 2009. Metabotropic glutamate receptor ligands as potential therapeutics for addiction. *Current drug abuse reviews*, 2(1), pp.83–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19630739 [Accessed October 16, 2017].

Pacheco, R. et al., 2004. Group I Metabotropic Glutamate Receptors Mediate a Dual Role of Glutamate in T Cell Activation. *Journal of Biological Chemistry*.

Pacheco, R. et al., 2007. Role of glutamate on T-cell mediated immunity. *Journal of Neuroimmunology*.

Pagano, A. et al., 2000. The Non-competitive Antagonists 2-Methyl-6-(phenylethynyl)pyridine and 7-Hydroxyiminocyclopropan[*b*]chromen-1 *a* -carboxylic Acid Ethyl Ester Interact with Overlapping Binding Pockets in the Transmembrane Region of Group I Metabotropic Glutamate Receptors. *Journal of Biological Chemistry*, 275(43), pp.33750–33758. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10934211 [Accessed September 26, 2017].

Pagano, A. et al., 2000. The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *The Journal of biological chemistry*, 275(43), pp.33750–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10934211 [Accessed July 25, 2017].

Palmer, E., Monaghan, D.T. & Cotman, C.W., 1989. Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. *European journal of pharmacology*, 166(3), pp.585–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2553440 [Accessed August 28, 2017].

Palmer, M.J. et al., The group I mGlu receptor agonist DHPG induces a novel form of LTD in the CA1 region of the hippocampus. *Neuropharmacology*, 36(11–12), pp.1517–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9517422 [Accessed July 12, 2017].

Pałucha, A. et al., 2005. Potential antidepressant-like effect of MTEP, a potent and highly selective mGluR5 antagonist. *Pharmacology Biochemistry and Behavior*, 81(4), pp.901–906. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16040106 [Accessed October 16, 2017].

Pałucha, A., Brański, P. & Pilc, A., Selective mGlu5 receptor antagonist MTEP attenuates naloxone-induced morphine withdrawal symptoms. *Polish journal of pharmacology*, 56(6), pp.863–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15662102 [Accessed October 16, 2017].

Papadopoulos, D. et al., 2013. Hepatic ischemia and reperfusion injury and trauma: current concepts. *Archives of trauma research*, 2(2), pp.63–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24396796 [Accessed September 6, 2017].

Park, S. et al., 2008. Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD. *Neuron*, 59(1), pp.70–83. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627308004583 [Accessed July 24, 2017].

Di Pasqua, L.G. et al., 2016. MCD diet-induced steatohepatitis is associated with alterations in asymmetric dimethylarginine (ADMA) and its transporters. *Molecular and Cellular Biochemistry*, 419(1–2), pp.147–155. Available at: http://link.springer.com/10.1007/s11010-016-2758-2 [Accessed October 18, 2017].

Pecknold, J.C. et al., 1982. Treatment of anxiety using fenobam (a nonbenzodiazepine) in a double-blind standard (diazepam) placebo-controlled study. *Journal of clinical psychopharmacology*, 2(2), pp.129–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7042771 [Accessed July 12, 2017].

Pescador, N. et al., 2010. Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. *PloS one*, 5(3), p.e9644. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20300197 [Accessed October 13, 2017].

Petrov, D. et al., 2014. Mavoglurant as a treatment for Parkinson's disease. *Expert Opinion on Investigational Drugs*, 23(8), pp.1165–1179. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24960254 [Accessed October 16, 2017].

Phillis, J.W., Song, D. & O'Regan, M.H., 1997. Inhibition by anion channel blockers of ischemia-evoked release of excitotoxic and other amino acids from rat cerebral cortex. *Brain research*, 758(1–2), pp.9–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9203527 [Accessed August 2, 2017].

Piccinini, F. et al., 2017. Cell Counting and Viability Assessment of 2D and 3D Cell Cultures: Expected Reliability of the Trypan Blue Assay. *Biological procedures online*, 19, p.8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28814944 [Accessed September 13, 2017].

Pieretti, M. et al., 1991. Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell*, 66(4), pp.817–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1878973 [Accessed July 11, 2017].

Piers, T.M. et al., 2012. Translational Concepts of mGluR5 in Synaptic Diseases of the Brain. *Frontiers in Pharmacology*, 3, p.199. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23205012 [Accessed July 31, 2017].

Piira, A. et al., 2013. Effects of a One Year Intensive Multidisciplinary Rehabilitation Program for Patients with Huntington's Disease: a Prospective Intervention Study. *PLoS Currents*, 5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24459605 [Accessed July 17, 2017].

Pin, J.-P., Galvez, T. & Prézeau, L., 2003. Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. *Pharmacology & Therapeutics*, 98(3), pp.325–354. Available at: http://linkinghub.elsevier.com/retrieve/pii/S016372580300038X [Accessed July 25, 2017].

Pin, J.P. et al., 1994. Domains involved in the specificity of G protein activation in phospholipase C-coupled metabotropic glutamate receptors. *The EMBO journal*, 13(2), pp.342–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8313879 [Accessed July 25, 2017].

Pohorecki, R. et al., 1990. Ischemic brain injury in vitro: protective effects of NMDA receptor antagonists and calmidazolium. *Brain research*, 528(1), pp.133–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2147119 [Accessed September 20, 2017].

Porter, R.H.P. et al., 2005. Fenobam: A Clinically Validated Nonbenzodiazepine Anxiolytic Is a Potent, Selective, and Noncompetitive mGlu5 Receptor Antagonist with Inverse Agonist Activity. *Journal of Pharmacology and Experimental Therapeutics*, 315(2), pp.711–721. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16040814 [Accessed October 6, 2017].

Produit-Zengaffinen, N., Pournaras, C.J. & Schorderet, D.F., 2009. Retinal ischemia-induced apoptosis is associated with alteration in Bax and Bcl-x(L) expression rather than modifications in Bak and Bcl-2. *Molecular vision*, 15, pp.2101–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19862336 [Accessed October 19, 2017].

Raymond, C.R. et al., 2000. Metabotropic glutamate receptors trigger homosynaptic protein synthesis to prolong long-term potentiation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 20(3), pp.969–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10648701 [Accessed July 11, 2017].

Reddy, S. et al., 2004. Liver transplantation from non-heart-beating donors: current status and future prospects. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society*, 10(10), pp.1223–32. Available at: http://doi.wiley.com/10.1002/lt.20268 [Accessed September 6, 2017].

Reynolds, G.P., 2004. Receptor Mechanisms in the treatment of Schizophrenia. *Journal of Psychopharmacology*, 18(3), pp.340–345. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15358977 [Accessed August 30, 2017].

Rezzani, R. et al., 2003. Cyclosporine-A treatment inhibits the expression of metabotropic glutamate receptors in rat thymus. *Acta Histochemica*.

Ribeiro, F.M. et al., 2014. Metabotropic glutamate receptor 5 as a potential therapeutic target in Huntington's disease. *Expert Opinion on Therapeutic Targets*, 18(11), pp.1293–1304. Available at: http://www.tandfonline.com/doi/full/10.1517/14728222.2014.948419 [Accessed July 14, 2017].

Ribeiro, F.M. et al., 2014. Metabotropic glutamate receptor 5 knockout promotes motor and biochemical alterations in a mouse model of Huntington's disease. *Human Molecular Genetics*, 23(8), pp.2030–2042. Available at: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddt598 [Accessed July 18, 2017].

Richard Baker, S. et al., 1995. Enzymatic resolution and pharmacological activity of the enantiomers of 3,5-dihydroxyphenylglycine, a metabotropic glutamate receptor agonist. *Bioorganic & Medicinal Chemistry Letters*, 5(3), pp.223–228. Available at: http://linkinghub.elsevier.com/retrieve/pii/0960894X9500013J [Accessed August 30, 2017].

Ritzen, A., Mathiesen, J.M. & Thomsen, C., 2005. Molecular Pharmacology and Therapeutic Prospects of Metabotropic Glutamate Receptor Allosteric Modulators. *Basic <html_ent glyph="@amp;" ascii="&"/> Clinical Pharmacology <html_ent glyph="@amp;" ascii="&"/> Toxicology*, 97(4), pp.202–213. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16176554 [Accessed October 10, 2017].

Rodriguez, A.L. et al., 2005. A close structural analog of 2-methyl-6-(phenylethynyl)-pyridine acts as a neutral allosteric site ligand on metabotropic glutamate receptor subtype 5 and blocks the effects of multiple allosteric modulators. *Molecular pharmacology*, 68(6), pp.1793–802. Available at: http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.105.016139 [Accessed October 5, 2017].

Rodriguez, A.L. et al., 2010. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. *Molecular pharmacology*, 78(6), pp.1105–23. Available at: http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.110.067207 [Accessed October 4, 2017].

Romano, C. et al., 2001. Covalent and noncovalent interactions mediate metabotropic glutamate receptor mGlu5 dimerization. *Molecular pharmacology*, 59(1), pp.46–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11125023 [Accessed July 24, 2017].

Romano, C. et al., 1995. Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. *The Journal of Comparative Neurology*, 355(3), pp.455–469. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7636025 [Accessed September 11, 2017].

Romano, C., van den Pol, A.N. & O'Malley, K.L., 1996. Enhanced early developmental expression of the metabotropic glutamate receptor mGluR5 in rat brain: protein, mRNA splice variants, and regional distribution. *The Journal of comparative neurology*, 367(3), pp.403–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8698900 [Accessed September 11, 2017].

Romano, C., Yang, W.L. & O'Malley, K.L., 1996. Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *The Journal of biological chemistry*, 271(45), pp.28612–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8910492 [Accessed October 6, 2017].

Rondard, P. et al., 2006. Coupling of Agonist Binding to Effector Domain Activation in Metabotropic Glutamate-like Receptors. *Journal of Biological Chemistry*, 281(34), pp.24653–24661. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16787923 [Accessed October 11, 2017].

Ronesi, J.A. & Huber, K.M., 2008. Homer Interactions Are Necessary for Metabotropic Glutamate Receptor-Induced Long-Term Depression and Translational Activation. *Journal of Neuroscience*, 28(2), pp.543–547. Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.5019-07.2008 [Accessed July 24, 2017].

Rong, R. et al., 2003. PI3 kinase enhancer–Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. *Nature Neuroscience*, 6(11), pp.1153–1161. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14528310 [Accessed July 18, 2017].

Rook, J.M. et al., 2013. Unique Signaling Profiles of Positive Allosteric Modulators of Metabotropic Glutamate Receptor Subtype 5 Determine Differences in In Vivo Activity. *Biological Psychiatry*, 73(6), pp.501–509. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23140665 [Accessed October 5, 2017].

Ross, C.A. & Tabrizi, S.J., 2011. Huntington's disease: from molecular pathogenesis to clinical treatment. *The Lancet Neurology*, 10(1), pp.83–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21163446 [Accessed July 17, 2017].

Rouzade-Dominguez, M.-L. et al., 2017. The selective metabotropic glutamate receptor 5 antagonist mavoglurant (AFQ056) reduces the incidence of reflux episodes in dogs and patients with moderate to severe gastroesophageal reflux disease. *Neurogastroenterology & Motility*, 29(8), p.e13058. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28337838 [Accessed October 16, 2017].

Rylander, D. et al., 2010. A mGluR5 antagonist under clinical development improves L-DOPA-induced dyskinesia in parkinsonian rats and monkeys. *Neurobiology of Disease*, 39(3), pp.352–361. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20452425 [Accessed October 16, 2017].

Saab, C.Y. et al., 2006. Microglia: a newly discovered role in visceral hypersensitivity? *Neuron glia biology*, 2(4), pp.271–7. Available at: http://www.journals.cambridge.org/abstract_S1740925X07000439 [Accessed July 13, 2017].

Sacaan, A.I., Santori, E.M. & Rao, T.S., 1998. (S)-4-carboxy-3-hydroxyphenylglycine activates phosphatidyl inositol linked metabotropic glutamate receptors in different brain regions of the neonatal rat. *Neurochemistry international*, 32(1), pp.77–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9460705 [Accessed August 28, 2017].

Sakon, M. et al., 2002. Ischemia-reperfusion injury of the liver with special reference to calcium-dependent mechanisms. *Surgery today*, 32(1), pp.1–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11871810 [Accessed September 7, 2017].

Sauer, I.M., Neuhaus, P. & Gerlach, J.C., 2002. Concept for modular extracorporeal liver support for the treatment of acute hepatic failure. *Metabolic brain disease*, 17(4), pp.477–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12602523 [Accessed September 7, 2017].

Scaccianoce, S. et al., 2003. Endogenous activation of group-II metabotropic glutamate receptors inhibits the hypothalamic-pituitary-adrenocortical axis. *Neuropharmacology*, 44(5), pp.555–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12668041 [Accessed August 30, 2017].

Schanne, F.A. et al., 1979. Calcium dependence of toxic cell death: a final common pathway. *Science (New York, N.Y.)*, 206(4419), pp.700–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/386513 [Accessed September 7, 2017].

Schoepp, D.D. et al., 1991. Comparison of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD)- and 1R,3S-ACPD-stimulated brain phosphoinositide hydrolysis. *European journal of pharmacology*, 207(4), pp.351–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1664338 [Accessed August 30, 2017].

Schoepp, D.D. et al., 1991. In Vitro and In Vivo Pharmacology of *trans*- and *cis* -(±)-1-Amino-1,3-Cyclopentanedicarboxylic Acid: Dissociation of Metabotropic and Ionotropic Excitatory Amino Acid Receptor Effects. *Journal of Neurochemistry*, 56(5), pp.1789–1796. Available at: http://doi.wiley.com/10.1111/j.1471-4159.1991.tb02082.x [Accessed August 30, 2017].

Schoepp, D.D., Jane, D.E. & Monn, J.A., 1999a. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology*, 38(10), pp.1431–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10530808 [Accessed August 29, 2017].

Schoepp, D.D., Jane, D.E. & Monn, J.A., 1999b. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology*, 38(10), pp.1431–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10530808 [Accessed August 28, 2017].

Schwabe, R.F. & Brenner, D.A., 2006. Mechanisms of Liver Injury. I. TNF-α-induced liver injury: role of IKK, JNK, and ROS pathways. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 290(4). Available at: http://ajpgi.physiology.org/content/290/4/G583.long [Accessed September 7, 2017].

Schwendt, M. & McGinty, J.F., 2007. Regulator of G-Protein Signaling 4 Interacts with Metabotropic Glutamate Receptor Subtype 5 in Rat Striatum: Relevance to Amphetamine Behavioral Sensitization. *Journal of Pharmacology and Experimental Therapeutics*, 323(2), pp.650–657. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17693584 [Accessed October 10, 2017].

Selzner, M. et al., 2002. Transgenic mice overexpressing human Bcl-2 are resistant to hepatic ischemia and reperfusion. *Journal of hepatology*, 36(2), pp.218–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11830333 [Accessed September 8, 2017].

Semenza, G.L., 2000. Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochemical pharmacology*, 59(1), pp.47–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10605934 [Accessed October 2, 2017].

Sergin, I. et al., 2017. Sequences within the C Terminus of the Metabotropic Glutamate Receptor 5 (mGluR5) Are Responsible for Inner Nuclear Membrane Localization. *The Journal of biological chemistry*, 292(9), pp.3637–3655. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28096465 [Accessed October 10, 2017].

Sharma, S. et al., 2009. Discovery of Molecular Switches That Modulate Modes of Metabotropic Glutamate Receptor Subtype 5 (mGlu $_5$) Pharmacology in Vitro and in Vivo within a Series of Functionalized, Regioisomeric 2- and 5-(Phenylethynyl)pyrimidines. *Journal of Medicinal Chemistry*, 52(14), pp.4103–4106. Available at: http://pubs.acs.org/doi/abs/10.1021/jm900654c [Accessed October 4, 2017].

Shigemoto, R. et al., 1997. Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(19), pp.7503–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9295396 [Accessed September 11, 2017].

Shigemoto, R. et al., 1993. Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. *Neuroscience letters*, 163(1), pp.53–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8295733 [Accessed July 25, 2017].

Shirasugi, N. et al., 1997. Up-regulation of oxygen-derived free radicals by interleukin-1 in hepatic ischemia/reperfusion injury. *Transplantation*, 64(10), pp.1398–403. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9392301 [Accessed September 7, 2017].

Silva, A.M. & Oliveira, P.J., 2012. Evaluation of Respiration with Clark Type Electrode in Isolated Mitochondria and Permeabilized Animal Cells. In *Methods in molecular biology (Clifton, N.J.)*. pp. 7–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22057558 [Accessed September 13, 2017].

Silver, I.A. & Erecińska, M., 1990. Intracellular and extracellular changes of [Ca2+] in hypoxia and ischemia in rat brain in vivo. *The Journal of general physiology*, 95(5), pp.837–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2163431 [Accessed August 2, 2017].

Silverman, J.L. et al., 2012. Negative Allosteric Modulation of the mGluR5 Receptor Reduces Repetitive Behaviors and Rescues Social Deficits in Mouse Models of Autism. *Science Translational Medicine*, 4(131), p.131ra51-131ra51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22539775 [Accessed July 12, 2017].

Simons, K. & Ikonen, E., 1997. Functional rafts in cell membranes. *Nature*, 387(6633), pp.569–572. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9177342 [Accessed October 10, 2017].

Siriussawakul, A., Zaky, A. & Lang, J.D., 2010. Role of nitric oxide in hepatic ischemia-reperfusion injury. *World journal of gastroenterology*, 16(48), pp.6079–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21182222 [Accessed September 7, 2017].

SLASSI, A. et al., 2007. PIPERAZINES AND PIPERIDINES AS mGluR5 POTENTIATORS. Available at: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2007087135&recNum=171&docAn=US2007000231&queryString=((mGluR)%2520OR%2520(orexin)%2520OR%2520(PRKX)%2520OR%2520(%2522AMPA%2520positive%2520allosteric%2522)%2520OR%2520(GPR52)%2520OR%2520(GlyT)%2520OR%2520(%2522glycine%2520transporter%2522))%2520AND%2520((IC/A61*)%2520OR%2520(IC/C07*))&maxRec=2381 [Accessed October 5, 2017].

Snyder, M.A. & Gao, W.-J., 2013. NMDA hypofunction as a convergence point for progression and symptoms of schizophrenia. *Frontiers in cellular neuroscience*, 7, p.31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23543703 [Accessed August 31, 2017].

Sorensen, S.D. & Conn, P.J., 2003. G protein-coupled receptor kinases regulate metabotropic glutamate receptor 5 function and expression. *Neuropharmacology*, 44(6), pp.699–706. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12681368 [Accessed October 10, 2017].

Spooren, W.P. et al., 2000. Effects of the prototypical mGlu(5) receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine on rotarod, locomotor activity and rotational responses in unilateral 6-OHDA-lesioned rats. *European journal of pharmacology*, 406(3), pp.403–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11040347 [Accessed October 16, 2017].

Spooren, W.P. et al., 2001. Novel allosteric antagonists shed light on mglu(5) receptors and CNS disorders. *Trends in pharmacological sciences*, 22(7), pp.331–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11431019 [Accessed October 23, 2017].

Stauffer, S.R., 2011. Progress toward positive allosteric modulators of the metabotropic glutamate receptor subtype 5 (mGluR5). *ACS chemical neuroscience*, 2(8), pp.450–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22860171 [Accessed July 25, 2017].

Stepulak, A. et al., 2014. Glutamate and its receptors in cancer. *Journal of neural transmission (Vienna, Austria : 1996)*, 121(8), pp.933–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24610491 [Accessed October 16, 2017].

Storto, M. et al., 2000. Expression of metabotropic glutamate receptors in murine thymocytes and thymic stromal cells. *Journal of Neuroimmunology*.

Storto, M. et al., 2001. Expression of metabotropic glutamate receptors in the rat and human testis. *Journal of Endocrinology*.

Storto, M. et al., 2006. Insulin Secretion Is Controlled by mGlu5 Metabotropic Glutamate Receptors. *Molecular Pharmacology*, 69(4), pp.1234–1241. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16424079 [Accessed October 16, 2017].

Storto, M. et al., 2004. Mouse hepatocytes lacking mGlu5 metabotropic glutamate receptors are less sensitive to hypoxic damage. *European Journal of Pharmacology*.

Storto, M. et al., 2003. Selective blockade of mGlu5 metabotropic glutamate receptors is protective against acetaminophen hepatotoxicity in mice. *J Hepatol*.

Storto, M. et al., 2000. Selective blockade of mGlu5 metabotropic glutamate receptors protects rat hepatocytes against hypoxic damage. *Hepatology (Baltimore, Md.)*.

Sun, J. et al., 1998. Isolation of PSD-Zip45, a novel Homer/vesl family protein containing leucine zipper motifs, from rat brain. *FEBS letters*, 437(3), pp.304–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9824313 [Accessed July 31, 2017].

Sureda, F. et al., 1997. Metabotropic glutamate receptor agonists stimulate polyphosphoinositide hydrolysis in primary cultures of rat hepatocytes. *Eur J Pharmacol*.

Sutcliffe, J.S. et al., 1992. DNA methylation represses FMR-1 transcription in fragile X syndrome. *Human molecular genetics*, 1(6), pp.397–400. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1301913 [Accessed July 11, 2017].

Swulius, M.T. & Waxham, M.N., 2008. Ca2+/Calmodulin-dependent Protein Kinases. *Cellular and Molecular Life Sciences*, 65(17), pp.2637–2657. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18463790 [Accessed July 24, 2017].

Tadokoro, S. et al., 1999. Involvement of unique leucine-zipper motif of PSD-Zip45 (Homer 1c/vesl-1L) in group 1 metabotropic glutamate receptor clustering. *Proceedings of the National Academy of Sciences of the United States of America*, 96(24), pp.13801–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10570153 [Accessed July 31, 2017].

Tanaka, M. & Wanless, I.R., 1998. Pathology of the liver in budd-chiari syndrome: Portal vein thrombosis and the histogenesis of veno-centric cirrhosis, veno-portal cirrhosis, and large regenerative nodules. *Hepatology*, 27(2), pp.488–496. Available at: http://doi.wiley.com/10.1002/hep.510270224 [Accessed September 6, 2017].

Tang, T.-S. et al., 2005. Disturbed Ca2+ signaling and apoptosis of medium spiny neurons in Huntington's disease. *Proceedings of the National Academy of Sciences*, 102(7), pp.2602–2607. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15695335 [Accessed July 18, 2017].

Tappe-Theodor, A. et al., 2011. Homer1a signaling in the amygdala counteracts pain-related synaptic plasticity, mGluR1 function and pain behaviors. *Molecular pain*, 7, p.38. Available at: http://journals.sagepub.com/doi/10.1186/1744-8069-7-38 [Accessed July 13, 2017].

Taylor, C.P., Burke, S.P. & Weber, M.L., 1995. Hippocampal slices: glutamate overflow and cellular damage from ischemia are reduced by sodium-channel blockade. *Journal of neuroscience methods*, 59(1), pp.121–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7475242 [Accessed August 2, 2017].

Teh, J. & Chen, S., 2012. Metabotropic glutamate receptors and cancerous growth. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling*, 1(2), pp.211–220. Available at: http://doi.wiley.com/10.1002/wmts.21 [Accessed October 16, 2017].

Testa, A. et al., 2013. Psychiatric emergencies (part III): psychiatric symptoms resulting from organic diseases. *European review for medical and pharmacological sciences*, 17 Suppl 1, pp.86–99. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23436670 [Accessed October 16, 2017].

Thomas, U., 2002. Modulation of synaptic signalling complexes by Homer proteins. *Journal of neurochemistry*, 81(3), pp.407–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12065649 [Accessed July 31, 2017].

Tilg, H., Kaser, A. & Moschen, A.R., 2006. How to modulate inflammatory cytokines in liver diseases. *Liver International*, 26(9), pp.1029–1039. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17032402 [Accessed October 2, 2017].

Topiol, S., Sabio, M. & Uberti, M., 2011. Exploration of structure-based drug design opportunities for mGluRs. *Neuropharmacology*.

Trenkwalder, C. et al., 2016. Mavoglurant in Parkinson's patients with l -Dopa-induced dyskinesias: Two randomized phase 2 studies. *Movement Disorders*, 31(7), pp.1054–1058. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27214258 [Accessed October 16, 2017].

Trivedi, R.R. & Bhattacharyya, S., 2012. Constitutive internalization and recycling of metabotropic glutamate receptor 5 (mGluR5). *Biochemical and Biophysical Research Communications*, 427(1), pp.185–190. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22995293 [Accessed October 10, 2017].

Tsuchihashi, T. et al., 2000. Metabotropic glutamate receptor subtypes involved in cardiovascular regulation in the rostral ventrolateral medulla of rats. *Brain research bulletin*, 52(4), pp.279–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10856825 [Accessed August 30, 2017].

Tsuchiya, D. et al., 2002. Structural views of the ligand-binding cores of a metabotropic glutamate receptor complexed with an antagonist and both glutamate and Gd3+. *Proceedings of the National Academy of Sciences of the United States of America*, 99(5), pp.2660–5. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.052708599 [Accessed July 19, 2017].

Tsujimoto, Y. & Shimizu, S., 2000. Bcl-2 family: life-or-death switch. *FEBS letters*, 466(1), pp.6–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10648802 [Accessed September 8, 2017].

Tu, J.C. et al., 1999. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron*, 23(3), pp.583–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10433269 [Accessed August 31, 2017].

Tückmantel, W. et al., 1997. Synthesis, molecular modeling, and biology of the 1-benzyl derivative of APDC-an apparent mGluR6 selective ligand. *Bioorganic & Medicinal Chemistry Letters*, 7(5), pp.601–606. Available at: http://www.sciencedirect.com/science/article/pii/S0960894X97000681 [Accessed August 28, 2017].

Uematsu, K. et al., 2015. Protein kinase A directly phosphorylates metabotropic glutamate receptor 5 to modulate its function. *Journal of Neurochemistry*, 132(6), pp.677–686. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25639954 [Accessed July 31, 2017].

Vairetti, M. et al., 2012. Impaired hepatic function and central dopaminergic denervation in a rodent model of Parkinson's disease: a self-perpetuating crosstalk? *Biochimica et biophysica acta*, 1822(2), pp.176–84. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0925443911002687 [Accessed October 17, 2017].

Valenti, O., Conn, P.J. & Marino, M.J., 2002. Distinct physiological roles of the Gq-coupled metabotropic glutamate receptors co-expressed in the same neuronal populations. *Journal of Cellular Physiology*, 191(2), pp.125–137. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12064455 [Accessed October 16, 2017].

Vanhaesebroeck, B. & Alessi, D.R., 2000. The PI3K-PDK1 connection: more than just a road to PKB. *The Biochemical journal*, 346 Pt 3, pp.561–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10698680 [Accessed July 24, 2017].

Vardi, N. et al., 2000. Localization of mGluR6 to dendrites of ON bipolar cells in primate retina. *The Journal of comparative neurology*, 423(3), pp.402–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10870081 [Accessed July 13, 2017].

Varney, M.A. et al., 1999a. SIB-1757 and SIB-1893: selective, noncompetitive antagonists of metabotropic glutamate receptor type 5. *The Journal of pharmacology and experimental therapeutics*, 290(1), pp.170–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10381773 [Accessed July 13, 2017].

Varney, M.A. et al., 1999b. SIB-1757 and SIB-1893: selective, noncompetitive antagonists of metabotropic glutamate receptor type 5. *The Journal of pharmacology and experimental therapeutics*, 290(1), pp.170–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10381773 [Accessed September 21, 2017].

Varney, M.A. & Gereau, R.W., 2002. Metabotropic glutamate receptor involvement in models of acute and persistent pain: prospects for the development of novel analgesics. *Current drug targets. CNS and neurological disorders*, 1(3), pp.283–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12769620 [Accessed July 13, 2017].

Verkerk, A.J. et al., 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell*, 65(5), pp.905–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1710175 [Accessed July 11, 2017].

Vranesic, I. et al., 2014. AFQ056/mavoglurant, a novel clinically effective mGluR5 antagonist: Identification, SAR and pharmacological characterization. *Bioorganic & Medicinal Chemistry*, 22(21), pp.5790–5803. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25316499 [Accessed October 5, 2017].

Wakabayashi, Y. et al., 1991. Enzymological evidence for the indispensability of small intestine in the synthesis of arginine from glutamate. II. N-acetylglutamate synthase. *Archives of biochemistry and biophysics*, 291(1), pp.9–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1929439 [Accessed October 11, 2017].

Walker, K. et al., 2001. mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. *Neuropharmacology*, 40(1), pp.10–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11077066 [Accessed July 13, 2017].

Walker, L.M. et al., 2001. Oxidative stress and reactive nitrogen species generation during renal ischemia. *Toxicological sciences : an official journal of the Society of Toxicology*, 63(1), pp.143–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11509754 [Accessed September 7, 2017].

Wang, H. & Zhuo, M., 2012. Group I Metabotropic Glutamate Receptor-Mediated Gene Transcription and Implications for Synaptic Plasticity and Diseases. *Frontiers in Pharmacology*, 3, p.189. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23125836 [Accessed July 24, 2017].

Wang, J.Q., Fibuch, E.E. & Mao, L., 2007. Regulation of mitogen-activated protein kinases by glutamate receptors. *Journal of Neurochemistry*, 100(1), pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17018022 [Accessed July 24, 2017].

Wang, Y. et al., 2017. Selective ATP hydrolysis inhibition in F1Fo ATP synthase enhances radiosensitivity in non-small-cell lung cancer cells (A549). *Oncotarget*, 8(32), pp.53602–53612. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28881834 [Accessed September 13, 2017].

Wang, Y., Lawson, J.A. & Jaeschke, H., 1998. Differential effect of 2-aminoethyl-isothiourea, an inhibitor of the inducible nitric oxide synthase, on microvascular blood flow and organ injury in models of hepatic ischemia-reperfusion and endotoxemia. *Shock (Augusta, Ga.)*, 10(1), pp.20–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9688086 [Accessed September 7, 2017].

Wanner, G.A. et al., 1996. Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. *Shock (Augusta, Ga.)*, 5(1), pp.34–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8821101 [Accessed September 7, 2017].

Watford, M., 2000. Glutamine and glutamate metabolism across the liver sinusoid. *The Journal of nutrition*, 130(4S Suppl), p.983S–7S. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10736366 [Accessed October 11, 2017].

Watkins, J.C. & Evans, R.H., 1981. Excitatory Amino Acid Transmitters. *Annual Review of Pharmacology and Toxicology*, 21(1), pp.165–204. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6112965 [Accessed October 11, 2017].

Watkins, J.C., Krogsgaard-Larsen, P. & Honoré, T., 1990. Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends in pharmacological sciences*, 11(1), pp.25–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2155495 [Accessed August 28, 2017].

Weickert, C.S. et al., 2013. Molecular evidence of N-methyl-D-aspartate receptor hypofunction in schizophrenia. *Molecular psychiatry*, 18(11), pp.1185–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23070074 [Accessed August 31, 2017].

Weiler, I.J. et al., 1997. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proceedings of the National Academy of Sciences of the United States of America*, 94(10), pp.5395–400. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9144248 [Accessed July 11, 2017].

Wernimont, A.. et al., Crystal Structure of Metabotropic glutamate receptor 3 precursor in presence of LY341495 antagonist. *TO BE PUBLISHED*. Available at: http://www.rcsb.org/pdb/explore.do?structureId=3sm9 [Accessed July 19, 2017].

Wieme, R.J. & Demeulenaere, L., 1970. Enzyme assays in liver disease. *Journal of clinical pathology. Supplement (Association of Clinical Pathologists)*, 4, pp.51–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4143914 [Accessed September 13, 2017].

Willard, S.S. & Koochekpour, S., 2013. Glutamate, Glutamate Receptors, and Downstream Signaling Pathways. *International Journal of Biological Sciences*, 9(9), pp.948–959. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24155668 [Accessed October 23, 2017].

Won, H. et al., 2012. Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function. *Nature*, 486(7402), pp.261–265. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22699620 [Accessed August 31, 2017].

Wood, M.R. et al., 2011a. "Molecular Switches" on mGluR Allosteric Ligands That Modulate Modes of Pharmacology. *Biochemistry*, 50(13), pp.2403–2410. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21341760 [Accessed September 25, 2017].

Wood, M.R. et al., 2011b. "Molecular Switches" on mGluR Allosteric Ligands That Modulate Modes of Pharmacology. *Biochemistry*, 50(13), pp.2403–2410. Available at: http://pubs.acs.org/doi/abs/10.1021/bi200129s [Accessed September 26, 2017].

Woolf, C.J., 2011. Central sensitization: implications for the diagnosis and treatment of pain. *Pain*, 152(3 Suppl), pp.S2-15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20961685 [Accessed July 12, 2017].

Woolf, C.J., Evidence for a central component of post-injury pain hypersensitivity. *Nature*, 306(5944), pp.686–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6656869 [Accessed July 13, 2017].

Wu, W.N., McKown, L.A. & O'Neill, P.J., 1995. In Vitro and in Vivo Metabolism of the Antianxiolytic Agent Fenobam in the Rat. *Journal of Pharmaceutical Sciences*, 84(2), pp.185–189. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0022354915497096 [Accessed October 6, 2017].

Xiao, B., Tu, J.C. & Worley, P.F., 2000. Homer: a link between neural activity and glutamate receptor function. *Current opinion in neurobiology*, 10(3), pp.370–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10851183 [Accessed July 31, 2017].

Xu, J. et al., 2014. Role of glycogenolysis in stimulation of ATP release from cultured mouse astrocytes by transmitters and high K+ concentrations. *ASN neuro*, 6(1), p.e00132. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24328680 [Accessed October 26, 2017].

Yamada, H. et al., 1998. Metabotropic glutamate receptors negatively regulate melatonin synthesis in rat pinealocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(6), pp.2056–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9482792 [Accessed August 30, 2017].

Yan, Q.J. et al., 2005. Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. *Neuropharmacology*, 49(7), pp.1053–1066. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16054174 [Accessed July 12, 2017].

Yang, S.-H. & Sharrocks, A.D., 2006. Convergence of the SUMO and MAPK pathways on the ETS-domain transcription factor Elk-1. *Biochemical Society symposium*, (73), pp.121–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16626293 [Accessed July 24, 2017].

Yang, X. & Chen, W., 2005. *In vitro* microsomal metabolic studies on a selective mGluR5 antagonist MTEP: Characterization of *in vitro* metabolites and identification of a novel thiazole ring opening aldehyde metabolite. *Xenobiotica*, 35(8), pp.797–809. Available at: http://www.tandfonline.com/doi/full/10.1080/00498250500230412 [Accessed October 6, 2017].

Yin, S. & Niswender, C.M., 2014. Progress toward advanced understanding of metabotropic glutamate receptors: structure, signaling and therapeutic indications. *Cellular Signalling*, 26(10), pp.2284–2297. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24793301 [Accessed October 23, 2017].

Yu, S.P. et al., 1997. Membrane-delimited modulation of NMDA currents by metabotropic glutamate receptor subtypes 1/5 in cultured mouse cortical neurons. *The Journal of physiology*, 499 (Pt 3), pp.721–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9130168 [Accessed August 30, 2017].

Zalfa, F. et al., 2003. The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell*, 112(3), pp.317–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12581522 [Accessed July 11, 2017].

Zerbib, F., Keywood, C. & Strabach, G., 2010. Efficacy, tolerability and pharmacokinetics of a modified release formulation of ADX10059, a negative allosteric modulator of metabotropic glutamate receptor 5: an esophageal pH-impedance study in healthy subjects. *Neurogastroenterology & Motility*, 22(8), pp.859-e231. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20236248 [Accessed October 16, 2017].

Zhang, Y., Rodriguez, A.L. & Conn, P.J., 2005. Allosteric Potentiators of Metabotropic Glutamate Receptor Subtype 5 Have Differential Effects on Different Signaling Pathways in Cortical Astrocytes. *Journal of Pharmacology and Experimental Therapeutics*, 315(3), pp.1212–1219. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16135701 [Accessed October 4, 2017].

Zho, W.-M. et al., 2002. The group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine induces a novel form of depotentiation in the CA1 region of the hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(20), pp.8838–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12388590 [Accessed July 11, 2017].

Zhuo, X. et al., 2015. Identification of Glutathione Conjugates of Acetylene-Containing Positive Allosteric Modulators of Metabotropic Glutamate Receptor Subtype 5. *Drug Metabolism and Disposition*, 43(4), pp.578–589. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25633841 [Accessed October 13, 2017].

BOOKS

American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. Fourth Edition, 1994. Washington DC: American Psychiatric Press.

Arias IM, Boyer JL, Chisari FV, Fausto N, Schachter D, and Schafritz DA. The liver biology and pathobiology. Fourth Edition, 2001. Chapter 18. Lippincott Williams and Wilkins, Philadelphia, Pennsylvania.

Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association. Fifth Edition, 2013. Arlington: American Psychiatric Publishing.

Kendler KS, Diehl SR. Schizophrenia: Genetics. In: Kaplan and Sadock's Comprehensive Textbook of Psychiatry, by Sadock BJ, Sadock VA, Ruiz P. Sixth Edition, 1985. Baltimore, MD: Williams & Wilkins.

Martin C, Riou B, Vallet B. Physiologie humaine appliqué. First Edition, 2006; Chapter 17: 217-227.

Sponsoring initiatives in the molecular genetics of mental disorders. In: Genetics and Mental Disorders: Report of the NIMH Genetics Workgroup. Bethesda, Md.: NIH 98-4268 1998.

SITOGRAPHY

https://www.nimh.nih.gov/health/topics/anxiety-disorders/index.shtml#pub2

https://adaa.org/about-adaa/press-room/facts-statistics

https://www.ninds.nih.gov/Disorders/All-Disorders/Chronic-Pain-Information-Page

https://medlineplus.gov/magazine/issues/spring11/ articles/spring11pg5-6.html

http://www.prweb.com/pdfdownload/8052240.pdf

http://www.addextherapeutics.com/investors/press-releases/news-details/?tx_ttnews%5Btt_news%5D%20%20=%2088&cHash=d075a6fa63acce925582f8f481fb8dc3

http://www.pdf.org/about_pd

http://www.pdf.org/parkinson_statistics

https://parkinsonsnewstoday.com/parkinsons-disease-statistics/

http://www.pdf.org/about_pd

https://www.merriam-webster.com/dictionary/Parkinson%27s%20disease

https://ghr.nlm.nih.gov/condition/huntington-disease#statistics

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