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# Group I metabotropic glutamatergic receptors regulating glutamate release and microglia phenotype in a murine model of amyotrophic lateral sclerosis

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# ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by the death of upper and lower motor neurons. Although the aetiology of the disease is still unclear, glutamate (Glu)-mediated excitotoxicity is a major cause. Our previous studies demonstrated that presynaptic Group-I metabotropic Glu receptors (mGluR1 and mGluR5) are over-expressed in spinal cord synaptosomes of 120-day-old $SOD1^{G93A}$ mice, that represent the late stage of the disease, and that their activation by the selective mGluR1/5 agonist (S)-3,5-Dihydroxyphenylglycine (3,5-DHPG) produced abnormal Glu release. The aim of the present study was to investigate whether mGluR1 and mGluR5 also affect Glu release during the pre- and early-symptomatic time-course of the pathlogy (30, 60 and 90 days), in the same animal model.

Our results showed that the mGluR1/5 agonist 3,5-DHPG evoked the release of glutamate in a concentration-dependent way and the effects were almost superimposable between 30/60-day-old WT and $SOD1^{G93A}$ mice. At variance, 0.3 μM 3,5-DHPG significantly increased Glu release (25%, $p<0.05$) in 90-day-old $SOD1^{G93A}$ mice but not in WT aged controls. The involvement of both metabotropic glutamate receptor subtypes was demonstrated using mGluR1 and mGluR5 selective antagonists/negative allosteric modulators (LY367385, MPEP, respectively). The analysis of the molecular mechanisms underlying the 3,5-DHPG-evoked Glu release revealed that it was of vesicular origin and induced by $Ca^{2+}$ released from intra terminal stores. Confocal imaging confirmed that both mGluR1 and mGluR5 were co-localized onto glutamatergic nerve terminals and their expression was increased in $SOD1^{G93A}$ mice at the onset of the disease.

We have also set up a method to isolate extracellular vesicles enriched in exosomes to investigate whether EVs derived from cultured activated astrocytes, treated with a mGluR5 antagonist, were able to change the inflammatory pattern of microglia.

# ABBREVIATION LIST

AD: Alzheimer’s disease

ADAR2: Adenosine Deaminase Acting on RNA

ALS: Amyotrophic lateral sclerosis

Aβ: β-amiloide

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

2-APB: 2-aminoethoxydiphenyl borate

APP: Amyloid Precursor Protein

Arg1: arginase 1

ASO: antisense oligonucleotide

ATD: extracellular amino-terminal domain

ATP: adenosine triphosphate

BAPTA-AM: 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester

BBB: brain blood barrier

Bcl-2: B-cell lymphoma 2

BDNF: brain-derived neurotrophic factor

BiP: immunoglobulin-binding protein

BMAA: B-N-methylamino-L-alanine

B2M: Beta-2-Microglobulin CaMKII: Ca 2+ /calmodulin-dependent protein kinase II

CD86: cluster of differentiation 86

CD163: cluster of differentiation 163

CD206: cluster of differentiation 206

CHCHD10: coiled-coil-helix-coiled-coil-helix domain-containing protein 10

CNS: central nervous system

COX-2: cyclooxygenase-2

CTD: intracellular C-teminal domain

CTEP: 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridi- ne

C21orf2: chromosome 21 open reading frame 2

C9orf72: Chromosome 9 open reading frame 72

DAG: diacylglycerol

DMSO: Dimethyl sulfoxide

$EAAC_1$: Excitatory Amino Acid Carrier 1

$EAAT_{1-5}$: Excitatory Amino Acid Transporters

EAE: Experimental Autoimmune Encephalomyelitis

EDTA: Ethylenediamine tetraacetic acid

ERK: extracellular signal-regulated kinase

EVs: extracellular vesicles

FALS: familial ALS

FBS: fetal bovine serum

FDA: Food and Drug Administration

Fizz1: the cysteine-rich secreted protein Fizz1

FUS/TLS: Fused in Sarcoma/Translocated in Liposarcoma

GABA: aminobutyric acid

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GDNF: glial cell-derived neurotrophic factor

GFAP: glial fibrillary acidic protein

GLAST: Glutamate/Aspartate Transporter

GLT1: Glutamate transporter 1

Glu: glutamate

GPCR: G-protein-coupled receptor

HIV: Human immunodeficiency virus

hNSCs: human neural stem cells

HSPs: heat shock proteins

IGF-1: Insulin-like growth factor 1

IL-1: interleukin-1

IL-1β: interleukin-1 beta

IL-6: interleukin-6

IL-10: interleukin-10

iNOS: inducible nitric oxide synthase

iNPCs: induced neural progenitors cells

iPSCs: induced pluripotent stem cells

IP3: inositol 3 phosphate

KARS: alteration of lysyl-tRNA synthetase

LBD: ligand binding domain

L-BMAA: amino acid beta-N-methylamino-L-alanine

LTD: long term depression

LTP: long term potentiation

LY367385: (S)-(+)-a-amino-4-carboxy-2-methylbenzeneacetic acid

MAM: methylazoxymethanol

MATR3: Matrin-3

mGluR: metabotropic Glutamate receptors

mGluR1: metabotropic Glutamate receptors 1

mGluR5: metabotropic Glutamate receptors 5

MN: motor neuron

MPEP: 2-methyl-6-(phenylethynyl)pyridine

mPrp: mouse prion protein

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS: Multiple sclerosis

MSC: Mesenchymal stem cell

MVs: microvesicles

NEK1: NIMA-related kinase 1

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NAM: negative allosteric modulator

NMDA: N-methyl-D-aspartate

PAM: positive allosteric modulators

PBS: Phosphate Buffered Saline

PD: Parkinson's disease

PDC: Parkinsonism Dementia Complex

PKC: protein kinase C

PLC: phospholipase C

PPIA: peptidylprolyl isomerase A

PPMS: primary progressive

RIPA: radio immunoprecipitation

ROS: reactive oxygen species

RPLP0: Large Ribosomal Protein

RRMS: relapsing-remitting

SALS: sporadic ALS

SEM: standard error of mean

SOD1(2,3): Cu/Zn superoxide dismutase 1 (2,3)

$SOD1^{G93A}$: mutant human SOD1 carrying a glycine substituted to alanine at position 93

SPMS: secondary progressive

SNARE: SNAP REceptor

TARDBP: TAR DNA binding protein

TBK1: TANK Binding Kinase 1

TDP-43: Transactive Response DNA-Binding Protein 43

TGF-β: transforming growth factor beta

Thy-1.2: Anti-Mouse CD90.2

TMD: transmembrane domain

TNF-α: Tumor necrosis factor-alpha

TRAP-δ: delta-associated translocation protein

TUBA4A: Tubulin Alpha 4a

U73122: 1-[6-[((17)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione

VGLUT1-3: Vesicular glutamate transporters 1-3

VEGF: Vascular endothelial growth factor

WT: wild type

YM1: the macrophage protein YM1

2-AG: 2-arachidonoylglycerol

[$^3$H]D-Asp: [$^3$H]D-Aspartate

3,5-DHPG: (S)-3,5-Dihydroxyphenylglycine

6-OHDA: 6-hydroxydopamine

# 1

# INTRODUCTION

## 1.1 Amyotrophic Lateral Sclerosis: the pathology

Amyotrophic lateral sclerosis (ALS) is a progressive, neurodegenerative disorder that leads to degeneration of both upper and lower motor neurons (Norris et al., 1993; Rowland & Shneider, 2001; Logroscino et al., 2008; Renton et al., 2014). Patients may present motor manifestations that usually begins in one region of the body, mainly related to dysfunction of upper motor neurons, such as spasticity and weakness, with involvement of the lower motor neurons which becomes evident in the advanced stages of the disease (van Den Berg-Vos et al., 2003; Ravits et al., 2007; Tartaglia et al., 2007; Al Chalabi et al., 2016). Several evidence demonstrated that ALS has a cortical onset that can precede clinical symptoms by 3-6 months (Vucic et al., 2008; Bakulin et al., 2016). In contrast, other patients may present symptoms related to dysfunction of the lower motor neurons, including fasciculations, cramps and muscle atrophy. More than 5% of patients have respiratory problems and, in these cases, patients may also present an unjustified weight loss, probably linked with an accelerated metabolism (Kiernan et al., 2011). Patients may have a pure motor phenotype of ALS and have normal cognition and behavior, but some of them present an exclusively cognitive or behavioral phenotype or a mixed phenotype.

Peak age at onset is in the range 58-63 years and there are several studies showing that after 80 years of age the incidence decreases (Cronin et al., 2007). However, when the onset occurs in late adolescence or in early years of adulthood, it is usually indicative of familial ALS.

This pathology was described for the first time by the French neurologist Jean-Martin Charcot and became sadly known when the pathology affected several famous sportsmen,

such as the American baseball player Lou Gehrig.

***Jean-Martin Charcot and Lou Gehrig***
Adapted and extracted by Wikipedia http://resource.nlm.nih.gov/101425121; Heritage Auctions.

The incidence of ALS in Europe is about 2.16 per 100000 person-years with male more affected compared to women (1:350 for men and 1:400 for women; Logroscino et al., 2010).

Primary motoneurons project from the cortex to the brainstem and spinal cord, while the secondary motor neurons project from the brainstem or spinal cord directly to the skeletal muscles. The loss of function of corticospinal motor neurons causes muscle rigidity and spasticity, while the loss of function of spinal motor neurons leads to an excessive electric dysfunction, causing spasms of involuntary muscles or spontaneous and rapid contractions of groups of muscle fibers.

More specifically, based on the nature of the prodromal symptoms and the location of the districts most affected by the pathology, two main forms of ALS can be distinguished: the spinal form and the bulbar one.

The first is manifested by muscle weakness focused on both upper (cervical symptoms) and lower limbs (lumbar symptoms), local atrophy of the muscles of the hands, forearms, shoulders, legs with a marked reduction in the ability to walk. Furthermore, a subpopulation of patients also showed cognitive and sensory dysfunctions (Phukan et al., 2007; Hammad et al., 2007).

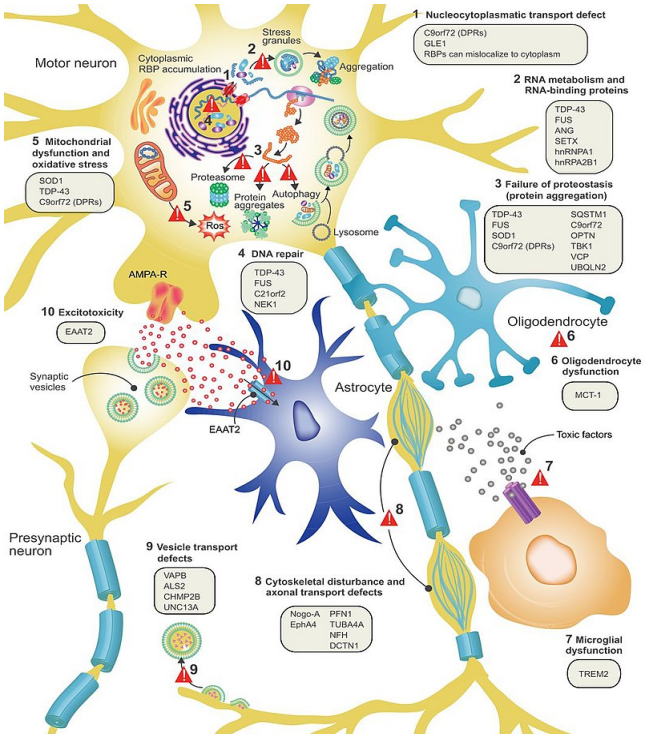
On the other hand, the bulbar form is more common in women and can cause both bulbar paralysis associated with dysarthria, atrophy of the tongue and dysphagia, and pseudo-bulbar paralysis characterized by laughter or uncontrollable crying caused by an increased sensitivity to emotions (Haverkamp et al., 1995; Louvel et al., 1997; Forbes et al., 2004; Beghi et al., 2007). Regardless of the form of ALS, death occurs mainly due to paralysis of the respiratory muscles. The neurons that innervate eyes and sphincter muscles are usually not affected up to an advanced stage of the pathology.

In order to improve the quality of life of patients there are several health support interventions, including artificial ventilation, motor and respiratory rehabilitation, the maintenance of swallowing and speaking (Chiò & Calvo, 2011; Ash et al., 2013; Da Costa Franceschini & Mourao, 2015; Valadi, 2015; Macpherson & Bassile, 2016; Tabor et al., 2016).

Neurodegeneration characterizes ALS progression and it has been shown that it can spread from the original sites to adjacent regions of the CNS by cell-to-cell transmission of pathological aggregates of SOD1, TDP-43 and C9orf72, but only the first two are transmissible also in vivo (Grad et al., 2011, 2014; Nonaka et al., 2013; Ayers et al., 2014, 2016; Feiler et al., 2015; Porta et al., 2018). Although ALS can spread between synaptically connected motor neurons, this does not seem to be the case in all patients, suggesting the existence of different mechanisms (Ding et al., 2015; Westergard et al., 2016). In addition, it has been reported that larger motor neurons are more vulnerable to neurodegeneration in $SOD1^{G93A}$ mice, the most used murine model of the human pathology, and that their size increases during disease progression (Le Masson et al., 2014; Dukkipati et al., 2018).

However, ALS is not only a motor neurons disease and there are evidences about the role of other cell types, such as astrocytes and microglia (Al Chalabi & Hardiman, 2013; Poppe et al., 2014). As a matter of fact, both microglia and astrocytes are involved in disease

progression, while oligodendrocytes seem to drive disease initiation (Ragagnin et al., 2019).




***Proposed pathomechanisms of ALS***

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In SOD1$^{G93A}$ mice, microglial cells increase during disease progression and their activation is characterized by the switch from the neuroprotective M2 phenotype to the inflammatory M1 phenotye (Chiu et al., 2013; Geloso et al., 2017). As recently reported, microglia ablation in mice did not affect the onset of the pathology, but delayed its progression (Boillée et al., 2006b).

As for astrocytes, in physiological conditions they provide neurotrophic factors for motor neurons, but in ALS they become vulnerable and release toxic factors causing cellular death. In fact, it has been found that astrocytes expressing mutant SOD release soluble neurotoxic factors that kill motor neurons (Nagai et al., 2007). In addition, neuroinflammatory microglia can induce A1 reactive astrocytes capable of causing neuronal death and that have been found abundant in post mortem tissues from patients

with different neurodegenerative diseases, including ALS (Liddelow et al., 2017).

Also oligodendrocytes seem involved in ALS pathogenic mechanism and TDP-43 and FUS aggregates have been detected in this cell population in patients (Arai et al., 2006; Mackenzie et al., 2007, 2011; Tan et al., 2007; Philips et al., 2013). Interestingly, oligodendrocytes derived from induced pluripotent stem cells (iPSCs) or from induced neural progenitors cells (iNPCs) of ALS patients (both sporadic and familial cases) have been shown to induce motor neuron death via both soluble factors and cell-to-cell contact, but with different mechanisms (Ferraiuolo et al., 2016) Regarding Schawnn cells, after the observation that myelin is altered in peripheral nerve in ALS patients, there are conflicting studies reporting their implication in the pathology (Perrie et al., 1993).

In addition, there are studies reporting the immunologic hypothesis as one of the causes of ALS. In particular, typical hallmarks of autoimmunity are present during ALS, such as the presence of circulating immune complexes, higher frequency of specific histocompatibility types, the degree of T-lymphocitic infiltration in the anterior horn of the spinal cord and aberrant macrophage activity (Oldstone et al., 1976; Graves et al., 2004). Moreover, there are evidences about the ability of immunoglobulins from ALS patients to cause apoptosis of motor neurons in primary spinal cord cultures (Demestre et al., 2005; Ralli et al., 2019).

Regarding the diagnosis, unfortunately there are no tests to identify the pathology with certainty, due to the heterogeneity of both the symptomatic manifestations and the variable rate of progression. The diagnosis is based on clinical examinations associated with electromyography, to confirm the extent of degeneration, and simultaneously with laboratory tests to exclude other disorders that might resemble the course of ALS (Rowland & Shneider, 2001; Al-Chalabi et al., 2016). One of the main problems is that 8-15 months are required to have a definitive diagnosis of ALS (Paganoni et al., 2014). A key clinical symptom of ALS is the presence of fasciculations, result of aberrant and spontaneous discharges of the inferior motor neurons on the muscle fibers (Eisen, 2009).

Motor symptoms are the first signs of ALS, but more than 50% of patients develop cognitive and behavioral alterations similar to frontotemporal dementia and 13% of patients have concomitant frontotemporal dementia (Elamin et al., 2013; Turner et al., 2013; Swinnen & Robberecht, 2014).

## 1.2 The pathology variants

ALS presents a complex and still poorly understood etiology, where genetic and environmental factors are interconnected in generating a wide spectrum of clinical variants and phenotypes (Al Chalabi & Hardiman, 2013; Swinnen & Robberecht, 2014; Van Rheenen et al. , 2016; Al Chalabi et al., 2017). ALS can occur in a sporadic form, which affects 90-95% of cases, and in a familial form, which affects 5-10% of patients and can occur in an autosomal dominant or recessive form (Horton et al., 1976).

### *1.2.1 Sporadic ALS*

Sporadic ALS has an unknown etiology, but as in other sporadic forms of neurodegenerative pathologies, it seems to be due to the interaction between genetic and environmental factors (neurotoxins, heavy metals, foods, electromagnetic fields, traumas, etc). Studies on populations of Western Pacific islands, where there was a high incidence of ALS/PDC (Parkinsonism Dementia Complex), have provided important information on possible environmental factors involved in the pathophysiology of ALS (Spencer, 2019). One possible factor, common to the different populations of this geographic area, is the exposure to the neurotoxic seeds of cycad plants (e.g. Cycas Circinalis) containing cycasin as major neurotoxic chemical and the amino acid beta-N-methylamino-L-alanine (L-BMAA) in lower concentrations (Spencer et al., 2016). Cycasin is the glycone of methylazoxymethanol (MAM), a molecule able to induce DNA damage and to cause neurotoxic effects, whereas L-BMAA has been shown to stimulate NMDA, AMPA and mGlu5 glutamatergic receptors, and to induce a motor-system disorder in adult primates

(Spencer et al., 2015). Other compounds that have been involved in the development of ALS as environmental factors are nitrosamines and hydrazines, used as herbicides and pesticides, that have genotoxic and neurotoxic effects similar to MAM.

### *1.2.2 Familial ALS*

Regarding familial ALS, two different types of familial ALS have been identified: the autosomic dominant form and a rare recessive pathology. The autosomic dominant form manifests in adulthood and is symptomatologically and pathologically indistinguishable from the sporadic form, suggesting that they share common mechanisms of degeneration (Mulder et al., 1986). The recessive ALS, on the contrary, shows symptoms in early adulthood, but it is characterized by a longer survival (Ben Hamida et al., 1990). The genetic component responsible for the familial form of the pathology is identified within more than 30 genes (Renton et al., 2014; Cirulli et al., 2015), but about 70% of all familial cases are due to mutations affecting four main genes coding respectively for SOD1 (Copper-zinc dependent superoxide-dismutase type 1; Rosen et al., 1993), C9orf72 (Chromosome 9 open reading frame 72; DeJesus-Hernandez et al., 2011; O'Rourke et al., 2015), TDP-43 (Transactive Response DNA-Binding Protein 43; Arai et al., 2006; Mackenzie et al., 2007) and FUS/TLS (Fused in Sarcoma/Translocated in Liposarcoma; Vance et al., 2009).

Following the identification of the mutated genes responsible for the familial forms, it was possible to develop transgenic mouse models for the study of the pathogenetic mechanisms. However, all these animal models have limitations since no one completely reflects all the features of the human disease.

## 1.3 ALS therapies

As introduced in the previous chapters, ALS is a fatal, multi-subtype syndrome, rather than a single disease, that occurs in adulthood and cause a fatal paralysis within 3-5 years after

onset; only 4% of patients survives 10 years or more (Turner et al., 2003; Testa et al., 2004; Kumar et al., 2016). The rapid disease process is also due to the lack of a therapy able to stop the progressive neurodegeneration.

### *1.3.1 Riluzole, edaravone and other drugs*

Until now, FDA has approved only two molecules for the treatment of ALS, riluzole and edaravone. Unfortunately, there is no effective cure for the disease, nor even a way to stop its progression and the therapeutic approaches used until now are only symptomatic and physical.

Riluzole has a modest efficacy on disease symptoms and prolongs survival probability by only 3-6 months without changing the course of the pathology; even if its mechanism of action has not yet been fully clarified, several studies indicate that it is able to inhibit voltage-dependent $Na^+$ channels, to reduce the release of glutamate from presynaptic terminals, to increase its re-uptake and to antagonize NMDA glutamatergic ionotropic receptors (Distad et al., 2008). Different riluzole prodrugs have been proposed which are metabolically inert, possess a high stability and allow steady riluzole plasma levels (McDonnel et al., 2012; Pelletier et al., 2018).

Edaravone, a small antioxidant molecule that has been used for many years to treat stroke, was shown to slightly slow the progression of ALS only when the pathology is at an early stage. The delay between symptoms onset and the diagnosis becomes problematic if the success of the therapy requires an early intervention, like for edaravone (Scott, 2017).

Today, the medical support given to patients is based above all on a series of interventions aimed at counteracting the disabilities occurring during the course of the disease in order to improve their quality of life. A common practice is artificial ventilation, but those who are kept alive by such ventilation reach a state in which they show problems also with the movement of the eyes (Hayashi & Kato, 1989; Sasaki et al., 1992). Other important aspects are represented by motor and respiratory re-education (Valadi, 2015; Macpherson

& Bassile, 2016), the maintenance of swallowing (Chiò & Calvo 2011; Tabor et al., 2016) and speaking (Ash et al., 2013; Da Costa Franceschini & Muorao, 2015). The development of new neuroprotective therapies is made difficult by the lack of knowledge about the causes of neuronal death in ALS and the mechanisms by which ALS develops.

In the last decades, there have been many drugs trialed in the clinical phase, but no one demonstrated a significant therapeutic efficacy to be approved by FDA. Several clinical trials with minocycline (Leigh et al., 2008; Gordon et al., 2007), lithium carbonate (Chiò et al., 2010; Morrison et al., 2013) or pioglitazone (Dupuis et al., 2012) have failed efficacy tests and it has been hypothesized that the neuroprotective effect of these molecules could be even antagonized by the action of riluzole (Yañez et al., 2014).

On the basis of their protective action on MNs, other drugs, such as despramipexole and rasagiline, have been proposed to reduce oxidative stress in ALS. Preclinical studies showed a reduction in neuronal death after administration of despramipexole and it was observed its ability in maintaining or increasing ATP synthesis, decreasing oxygen consumption and stabilizing the cellular metabolism profile. In addition, it has been observed that despramipexole has a protective effect also against proteasome inhibition (Alavian et al., 2012). In clinical trials on ALS patients, despramipexole turned out as a well tolerated drug in phase II studies, but the efficacy end point was not reached in phase III trials (Cudkowicz et al., 2013). Rasagiline is an irreversible inhibitor of monoamine oxidase B that has shown anti-apoptotic effects and the ability to slow down neuronal loss by regulating the transition of mitochondrial permeability and increasing mitochondrial survival (Weinreb et al., 2010). Following promising results obtained in animal models (Waibel et al., 2004), the association of rasagiline toghether with riluzole was studied in a phase II clinical trial (Ludolph et al., 2018). The treatment was safe and well tolerated in ALS patients but there was no difference with the placebo group in terms of survival; however, a post-hoc analysis revealed that rasagiline could be able to slow down the

disease progression in a subset of patients with normal to fast progression. Mitochondrial coenzime Q10 was also tested for its antioxidant properties and showed prolonged survival in $SOD1^{G93A}$ transgenic mice, but it did not produce any significant effect in clinical trials on ALS patients (Kaufmann et al., 2009; Miquel et al., 2014).

Olesoxime, a drug with cholesterol-like structure, showed neuroprotective effects in ALS models, probably with actions at the level of mitochondria and microtubules, causing a delay in the onset of motor dysfunction and weight loss, and prolonging survival probability in $SOD1^{G93A}$ mice (Bordet et al., 2007; Martin, 2010; Rovini et al., 2010). However, a phase II-III clinical trial in ALS patients on riluzole treatment did not show any significant beneficial effect (Lenglet et al., 2014).

Starting from the evidence that the inflammatory processes amplify neurodegenerative mechanisms, involving microglia and immune cells, many compounds acting on these cell populations have been tested with the aim of reducing neuroinflammation in ALS patients (Evans et al., 2013; Philips & Rothstein, 2014; Rizzo et al., 2014).

Fingolimod, a drug approved for multiple sclerosis, is a sphingosine-1-phosphate receptor agonist that is able to block T lymphocytes within the lymph nodes, thus preventing their access to the CNS (Chun et al., 2011). A first phase II trial has shown that this drug is well tolerated, has a favorable safety profile and reduces circulating lymphocytes in ALS patients (Berry et al., 2017). However, studies of its efficacy in this disease have not carried out yet.

Regarding macrophages activation, two different substances have been proposed to reduce it: NP001 and tocilizumab. The first is a novel immune regulator of inflammatory monocytes/macrophages that has been shown to regulate macrophage functions by down-regulating inflammatory pathways activated by NF-kB. In a phase I study, NP001 was shown to be generally safe and well tolerated and able to reduce some markers of inflammation (Miller et al., 2014). Regretfully, a phase II trial showed that NP001 did not

overall slow disease progression, although a beneficial effect was observed at a high dose in the group of ALS patients with greater inflammation at baseline (Miller et al., 2015).

Tocilizumab is a humanized monoclonal antibody against the IL-6 receptor, which is able to reduce the production of pro-inflammatory cytokines (Mizwicki et al., 2012). A phase II trial to assess tocilizumab safety and tolerability has been completed (NCT02469896) but data are not yet available. However, a preliminary study on 10 patients with sporadic ALS has shown that the effects of tocilizumab could be dependent on the individual level of inflammation; in fact, in a group of patients with a strong inflammatory state, the drug down-regulated the markers of inflammation, whereas in patients with weak inflammation an up-regulation was observed (Fiala et al., 2013).

Celecoxib, an inflammatory agent that inhibits COX-2 reduced astrogliosis and microgliosis, thus positively affecting weight loss in ALS animal models, but it showed no efficay in ALS patients as demonstrated in a clinical trial (Drachman et al., 2002; Cudkowicz et al., 2006).

It is well known that the endocannabinoid system plays an important role in the modulation of various biological processes and that there are many evidences supporting its antioxidant, anti-inflammatory and neuroprotective activity. Therefore, it has been hypothesized that the use of compounds acting on the cannabinoid system can improve ALS symptoms. The first study was performed by administering marijuana to ALS bedridden patients and it was observed a moderate improvement in appetite and reduction of spasticity, depression, pain and excessive salivation; nevertheless, it was ineffective on difficulties with speech and swallowing (Amtmann et al., 2004). Recently a combination of phytocannabinoids ($\triangle^9$-tetrahydrocannabinol- and cannabidiol-enriched extracts) have been studied in $SOD1^{G93A}$ mice with encouraging results (Moreno-Martet et al., 2014).

Another therapeutic strategy tested in ALS was to stimulate the degradation of protein aggregates, which characterize this pathology, by activating the proteasome system and the

autophagic pathway. In this view, arimoclomol is a drug that induces the expression of heat shock proteins (HSPs) under conditions of cell stress and that has shown neuroprotective effects in experimental models of ALS (Kieran et al., 2004; Kalmar et al., 2008; Phukan, 2010). Two clinical trials have shown that arimoclomol is safe and well-tolerated in ALS patients and could have beneficial effects, although these studies were not powered to assess therapeutic efficacy (Cudkowicz et al., 2008; Benatar et al., 2018). In this scenario, also lithium carbonate has been shown to reduce SOD1 aggregates in MNs, to delay disease onset and to increase survival of $SOD1^{G93A}$ mice (Fornai et al., 2008a). In a first clinical trial on patients with sporadic ALS, lithium in association with riluzole was able to slow down the disease progression in comparison with patients treated with riluzole alone (Fornai et al., 2008b). Unfortunately, a successive randomised, double-blind, placebo-controlled trial on 84 ALS patients did not confirm the beneficial effects of the combined therapy with lithium and riluzole (Aggarwal et al., 2010).

Off label use of some drugs has shown positive effects: the treatment with dextromethorphan/quinidine showed an improvement in emotional sensitivity (Brooks et al., 2004), treatment with modafinil led to improvements of insomnia and fatigue (Rabkin et al., 2009) and the administration of botulinum toxin led to an improvement in patients' sialorrhoea without particular adverse effects (Gilio et al., 2010).

### *1.3.2 Gene therapy*

As a promising therapeutic approach, gene therapy was also tested with the aim of enhancing the expression of neurotrophic factors (e.g. IGF-1, VEGF and GDNF) or anti-apoptotic proteins (e.g. Bcl-2), or of blocking the expression of proteins involved in neurotoxic effects, such as mutated SOD1. Indeed, this therapeutic strategy lead to very encouraging results in ALS preclinical models (Kaspar et al., 2003; Azzouz et al., 2000, 2004; Kumar et al., 2016).

Following the positive results obtained in preclinical studies, an antisense oligonucleotide

(ASO) against SOD1 was trialed in ALS patients with the familial form of the disease and was found to be well-tolerated and safe following intrathecal infusion (Miller et al., 2013)

### *1.3.3 Cell therapy*

In this regard, recent pre-clinical and clinical studies have focused on the transplantation of different cell types, such as neural stem cells, mesenchymal stem cells or glial progenitors. In ALS animal models, the transplantation of different stem cells has been shown to induce neuroprotective effects through different mechanisms, including the secretion of neurotrophic factors, the dampening of glutamate excitotoxicity and the modulation of neuroinflammation (Teng et al., 2012). However, since cell transplantation can involve a great number of biological factors, it is very difficult to identify which is/are the key mechanisms that can confer neuroprotection.

The complex scenario of the different cell therapies that can be adopted include neural, mesenchymal or induced pluripotent stem cells transplantation.

- Neural stem cells. A large number of preclinical studies have been carried out with transplantation of human neural stem cells (hNSCs) that were grafted in the spinal cord of $SOD1^{G93A}$ rats and mice. These hNSCs extensively differentiated into neurons that were able to synaptically contact host neurons and to integrate in the motor circuits of the spinal cord segment; moreover, the grafts also secreted GDNF and BDNF (Xu et al., 2006, 2009, 2011; Yan et al., 2006; Hefferan et al., 2012; Teng et al., 2012; Zalfa et al., 2019). This procedure resulted in the delayed onset and progression of the pathology together with a prolonged survival. Although the transplantation of such cells did not replace degenerated motor neurons, the positive effects of this therapeutic approach suggested that neurotrophic factors, produced by transplanted cells, could protect motor neurons from death and mediate the observed improvements.

Phase I clinical studies on hNSCs transplantation in ALS patients have been carried out so far, demonstrating that this approach is safe and well tolerated for up to 60 months after surgery, and may have positive effects on disease progression (Glass et al., 2012; Riley et al., 2012; Feldman et al., 2014; Mazzini et al., 2015, 2019).

In other studies, neuronal stem cells have been engineered to produce growth factors, such as GDNF, BDNF VEGF and IGF-1, that can help neuron survival (Klein et al., 2005; Hwang et al., 2009; Park et al., 2009).

- Mesenchymal stem cells. Mesenchymal stem cells (MSCs) represent a heterogeneous cellular population capable of repairing damaged tissues due to their ability to differentiate into other cell lines and that have been experimented in a large number of preclinical and clinical studies for ALS (Gugliandolo et al., 2019). The systemic or central administration of MSCs is now known to prevent astrocyte and microglia activation with reduction of neuroinflammation, to normalize the abnormal glutamate release, thus possibly reducing excitotoxicity, to diminish oxidative stress, all effects that lead to the delay of motoneurons death, disease onset and progression, and to the improvement of motor skills and increased survival of $SOD1^{G93A}$ rodents, also when administered after the onset of clinical symptoms (Zhao et al., 2007; Vercelli et al, 2008; Boucherie et al., 2009; Kim et al., 2010; Uccelli et al., 2012; Boido et al., 2014). However, MSCs seem not to transdifferentiate into neurons and replace lost MNs and it has been shown that only a small number of these cells migrate into the spinal cord, thus suggesting that the beneficial effects observed could be due to their bystander role with the secretion of a variety factors (the secretome) that ultimately lead to protection of neurons from death.

  MSCs have been also administered to ALS patients in a large number of phase 1/2 clinical trials, but results are available only for some of them. Overall, these clinical

studies have demonstrated that intravenous, intrathecal or intraspinal administration of MSCs of different origin is safe and well-tolerated with no serious adverse events; in same cases, also beneficial effects on disease progression have been reported.

- Induced pluripotent stem cells. Induced pluripotent stem cells (iPSCs) are produced by converting adult fibroblasts in pluripotent stem cells, using retroviral transduction of specific genes. These cells can be then differentiated in many cell types, including neurons and glial cells. Since both the donor and the transplanted is the same person, ethical concern and problems of graft-versus-host disease should be overcome. Many studies have shown the feasibility of producing MNs from human fibroblast-derived iPSCs using different protocols (Trawczynski et al., 2019). So far, a few preclinical studies have investigated the effects of grafts with iPSCs-derived neural stem cells or neural progenitors in ALS models. In a first study, neural progenitor cells, derived from human iPSCs, were transplanted in the spinal cord of $SOD1^{G93A}$ rats (Popescu et al., 2013). These cells survived in large number and showed efficient differentiation into cells with a neuronal phenotype and motor neuron-like morphology. Nizzardo and co-workers carried out two studies using intrathecal or intravenous administration of a specific sub-population of neural stem cells, obtained from human iPSCs, to ALS mice (Nizzardo et al., 2014, 2016). These cells engrafted well into cervical and lumbar spinal cord regions and differentiated into neuronal and glial cells, although a significant part maintained an undifferentiated phenotype. More importantly, this transplantation resulted in a significant reduction of motor neuron loss, increase of muscle strength, improvement of motor activity and increase of survival. However, this kind of approach with autologous transplantation needs to be extensively studied before starting its use in clinical trials as the iPSCs obtained from ALS patients may present alterations (e.g. mutations at the level of the SOD1 gene or

other genes) that can lead to cell malfunctioning, thus reducing the beneficial effect of the therapy itself.

### *1.3.4 Immunotherapy and vaccine approaches*

The knowledge that gene mutations are responsible for the production of aberrant proteins involved in the neurodegenerative processes, especially in the familial forms of ALS, has led to experiment whether immunotherapies and vaccines against those proteins could exert beneficial effects.

Gros-Louis and collaborators have reported that the intracerebroventricular administration of D3H5, a monoclonal antibody against the misfolded form of the mutated SOD1, was able to delay mortality in a murine model of ALS (Gros-Louis et al., 2010).

Some vaccines against SOD1, such as WT-Apo, tgG-DES2lim or tgG-DSE5b, were tested in ALS animal model and significantly postponed disease onset and increased lifespan (Takeuchi et al., 2010; Zhao et al., 2019). It has been reported that *Amorfix* in collaboration with *Biogen Idec*. is developing a vaccine against specific epitopes to eliminate the misfolded and aggregated forms of mutant SOD1 (Kumar et al., 2016).

# 2

# FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS (FALS)

As introduced above, although the familial forms of ALS account for only 10% of all cases of this pathology, their discovery has permitted to develop different rodent models that have undoubtedly contributed to a better understanding of the pathophysiology of this neurodegenerative disease.

## 2.1 SOD1 mutations

Among the most common genetic modifications, the alterations in SOD1 protein were the first identified in the study of ALS and, in fact, transgenic mice bearing mutations in the SOD1 gene are the most used animal models in research (Rosen et al., 1993). SOD1 is a metal-enzyme of 153 amino acids, which is expressed in the mithocondria of all eukaryotic cells and it belongs to the SOD family, which also includes SOD2, mitochondrial superoxide dismutase manganese dependent, and the SOD3, extracellular superoxide dismutase copper-zinc dependent. SOD1 is encoded by five exons and forms a globular protein (Levanon et al., 1985). In each monomer, there is the active site containing a copper ion, the catalyst, and a zinc ion, which is necessary for the stability of the protein (Kunst, 2004). Its main function is the detoxication of the free-radical superoxide anion by dismutation into oxygen and hydrogen peroxide ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). Hydrogen peroxide is then detoxified to form water through the action of glutathione peroxidase or catalase enzymes.

The animal models with SOD1 mutations are characterized by a rapid and aggressive course of the disease, and mimics the clinical and anatomo-pathological characteristics of ALS in human patients. Among the SOD1 transgenic models, one of the most used in ALS research is the $SOD1^{G93A}$ that is characterized by the over-expression of a mutated form of

human SOD1 bearing the replacement of alanine with glycine in position 93 (Gurney et al., 1994).

The mutation in the SOD1 gene is responsible for ALS in about 20% of familial cases and in 3% of total cases. To date, more than 180 mutations related to the SOD1 gene have been identified in ALS (Andersen et al., 2011; Felbecker et al., 2010). Most of these mutations (such as glycine-93-alanine, glycine-37-arginine, glycine-85-arginine substitutions) are dominant, except for the D90A mutation (replacement of an alanine for aspartate in position 90), which is the most common in the world and is inherited both in a dominant and recessive manner (Andersen, 2006; Robberecht et al., 1996).

The cause of familial ALS was initially attributed to the loss of function of SOD1, but it was soon clear that this was not the case. In fact, many SOD1 mutations, identified in human ALS, do not alter the activity of the enzyme and only some of them compromise the antioxidant functions of the protein, causing an accumulation of oxidized species (Bowling et al., 1993; Rosen et al., 1993; Robberecht, 2000). Furthermore, it was observed that the total elimination of this enzyme in SOD1 knock-out mice does not lead to the development of neurodegenerative phenomena (Reaume et al., 1996). These evidences lead to the hypothesis that the pathology could derive from a gain of some cytotoxic functions of the enzyme. Indeed, several studies reported that SOD1 mutations lead to an increased nitration capacity of tyrosine residues through the formation of peroxynitrite ($ONOO^-$), increased peroxidation reactions with production of reactive oxygen species (ROS) and alteration of lysyl-tRNA synthetase (KARS) and delta-associated translocation protein (TRAP-δ; Kunst et al., 1997; Shibata et al., 2000). However, new functions of SOD1 are emerging such as interaction with the endoplasmic reticulum and the Golgi complex, roles as a transcription factor and RNA binding protein that, altered by mutations, could cause neurodegeneration of motor neurons (Bunton-Stasyshyn et al., 2015).

Since protein aggregates are frequently associated with neurodegenerative diseases, several

studies focused on this observation also in ALS pathology and proposed possible mechanisms through which SOD1 aggregates. An acquisition of function could lead the protein to lose its structural complexity, forming aggregates of mutated protein, as it happens in age-related neurodegenerative disorders (Durham et al., 1997; Cleveland & Liu, 2000). Furthermore, copper ions, when not complexed with proteins, are cytotoxic, thus a possible mechanism of neurotoxicity in ALS could be a reduced ability of the mutated SOD1 to bind the ion, causing the increase of its free intracellular concentration with the binding of the ion by sites that normally do not bind it. Starting from this observation, it has been reported that the administration of copper chelators in presence of mutated SOD1 was demonstrated to increase the survival of spinal motor neurons in $SOD1^{G93A}$ mice almost 200% (Azzouz et al., 2000). As a matter of fact, several lines of evidence have suggested that copper dyshomeostasis could be one relevant mechanism of neurotoxicity by mutant SOD1 in ALS (Gil-Bea et al., 2017).

In addition, the mutated SOD1 protein is known to induce stress of the endoplasmic reticulum through different pathways. In fact, mutant SOD1 has been shown to accumulate in fractions enriched in endoplasmic reticulum membranes in affected tissues, a phenomenon that intensifies as the disease progresses; at this level, mutant SOD1 forms high molecular weight species and bind to the immunoglobulin-binding protein (BiP) present in the lumen (Kikuchi et al., 2006). On the other hand, it has been recently reported that endoplasmic reticulum stress favors the aggregation of SOD1, thus possibly representing a risk factor to develop ALS (Medinas et al., 2018).

Other studies showed that mutated SOD1 aggregates inhibit the proteosomal system, therefore reducing its capability in removing abnormally formed proteins from the cytoplasm, as demonstrated in the lumbar part of the spinal cord of mutated SOD1 mice (Hoffman et al., 1996; Kabashi et al., 2004; Cheroni et al., 2009).

### 2.2 C9orf72 mutation

The C9orf72 gene, whose function is still unknown, is located on chromosome 9 and recently, C9orf72 animal models have been developed to study ALS (Liu et al., 2016). This protein is located in different areas of the CNS where it can be found especially in neuron citoplasm and in nerve terminals. The prevalence of the mutation is variable in various countries; 40% of familial ALS and 7-11% of sporadic ALS are ascribed to this mutation (Renton et al., 2011; Dejesus-Hernandez et al., 2011; Gijselinck et al., 2015).

The expansion of the hexanucleotide GGGGCC sequence from hundreds to thousands of repeats in the C9orf72 gene is considered one of the most important genetic causes both in ALS and in frontotemporal dementia, considering that the majority of healthy individuals have less than 11 repeats. It has been proposed that the mutation can give rise to a loss or a gain of function with a significant impact on a variety of cellular pathways, including RNA processing and splicing, impairment in the proteasomal system and autophagy, alteration of axonal transport and induction of glutamate excitotoxicity (Dejesus-Hernandez et al., 2011; Renton et al., 2011; Balendra & Isaacs; 2018).

### 2.3 TARDBP mutation

The Transactive Response DNA Binding Protein (TDP-43) gene TARDBP is located on chromosome 1 and encodes for the nuclear protein TDP43 that binds both DNA and RNA. Approximately 30 mutations of this gene have been found in 5% of patients with familial ALS and in 1% with sporadic ALS (Tan et al., 2007; Sreedharan et al., 2008; Kabashi et al., 2008; Van Deerlin et al., 2008; Yokoseki et al., 2008; Rutherford et al., 2008; Mackenzie et al., 2010). TDP-43, a protein with a molecular weight of 43 kDa, consists of 414 amino acid residues, possesses two specific regions for the recognition of DNA and RNA, named RRM1 and RRM2, a nuclear localization sequence (NLS) and a C-terminal region, rich in glycine, responsible for protein-protein interaction; in the nucleus, TDP-43

is involved in the regulation of mRNA splicing and transcription, and in miRNA processing (Van Deerlin et al., 2008; Strong, 2010; Lagier-Tourenne et al., 2010; Lee et al., 2015; Scotter et al., 2015; Feneberg et al., 2018).

In the presence of TARDBP mutations, TDP43 is abnormally cleaved, hyperphosphorylated and ubiquitinated to form insoluble aggregates in the cytoplasm of motor neurons and glial cells that can be found both in the familial and sporadic forms of ALS (Neumann et al., 2006; Sasaki et al., 2010; Scotter et al., 2015; Feneberg et al., 2018). Also in this case, it is still not clear whether a loss of function in the capacity of TDP-43 to bind RNA and modulate splicing processes or a gain of toxic properties following the translocation and aggregation of the protein in the cytoplasm, or both, are the causes of the pathocascade leading to ALS.

Following the discovery of TARDBP mutations, animal models with TDP-43 mutations have been created using the mPrp (mouse prion protein), the Thy-1.2 or the CaMKII promoter-based strategy to induce overexpression of TDP-43 (Wegorzewska & Baloh, 2011). These murine models recapitulate some features of ALS, although to a different extent, showing for instance loss of nuclear TDP-43 (but rare phospho-TDP-43 inclusions), C-terminal TDP-43 fragments, motor neuron degeneration, muscle denervation, astrocytic and microglial activation, neuronal apoptosis and show variable symptom onset and lifespan.

Despite these models, it is still not clear whether a loss of function in the capacity of TDP-43 to bind RNA and modulate splicing processes or a gain of toxic properties, following the translocation and aggregation of the protein in the cytoplasm, or both are the causes of the pathocascade leading to ALS.

**2.4 FUS/ mutation**

Fused in Sarcoma/Translocated in Liposarcoma (FUS/TLS) is a gene on chromosome 16

encoding for the protein FUS/TLS that mediates many physiological functions involved in DNA repair, transcription, mRNA stability, transport, splicing and translation, miRNA processing mRNA translation (Sama et al., 2014; Ratti & Buratti, 2016). Autosomal dominant mutations in the FUS gene accounts for 3-5% of patients affected by ALS familial forms and their identification has expanded the genetic and neuropathological landscape of ALS. Similar to TDP43, FUS proteins form aggregates in the cytoplasm and it is indeed worth noting that most of the FUS mutations occur in the C-terminus region that is critical for its nuclear localization (Kwiatkowski et al., 2009; Vance et al., 2009).

Different rodent models of FUSopathies have been developed but none of them completely recapitulate the characteristics of human ALS, although they have certainly contributed to provide evidence supporting that both loss- and gain-of function pathomechanisms are associated with FUS mutations (Nolan et al., 2016)

**2.5 Other mutations**

In addition to the main genes identified as cause of familial ALS forms, seven new ALS-associated genes have been identified: MATR3, CHCHD10, TUBA4A, TBK1, NEK1 and C21orf2.

MATR3 is a nuclear protein with domains binding DNA and RNA (Coelho et al., 2015). The observed symptoms related to this genetic variant are mainly dysfunctions of vocal cords and pharynx (Johnson et al., 2014).

CHCHD10 is a mitochondrial protein, whose function is important for the maintenance of the mitochondrial activity and cellular bioenergetics (Genin et al., 2016). This mutation seems to be more frequent in patients with frontotemporal dementia rather than in ALS patients (Dols-Icardo et al., 2015); it has been found in a wide range of neurodegenerative disease, including ALS, frontotemporal degeneration (Zhang et al., 2015), Parkinson's disease (Dols-Icardo et al., 2015; Perrone et al., 2017), Alzheimer's disease (Xiao et al.,

2017), adult-onset spinal muscular atrophy (Penttilä et al., 2015) and Charcot-Marie Tooth disease type 2 (Auranen et al., 2015).

TUBA4A protein mutation is considered a rare cause of ALS and in primary motoneurons it interferes with tubulin dimerization leading to a weakening of the microtubule network (Smith et al., 2014; Perrone et al., 2017).

TBK1 is a protein involved in cellular processes, including neuroinflammation and autophagy, and also implicated in 1% of both sporadic and familial ALS patients (Freischmidt et al., 2015; Van Rheenen et al., 2016; Oakes et al., 2017).

On the contrary, NEK1 mutation is implicated only in sporadic ALS (Cirulli et al., 2015). It interacts with two proteins involved in lipid trafficking and associated with the development of ALS (Gijselinck et al., 2015). Simultaneously it has been observed that another mutated protein, C21orf2, is associated with the increased risk of ALS. NEK1 and C21orf2 interact each other and are involved in microtubule assembly, DNA damage and repair response, and mitochondrial functions (Fang et al., 2015; Wheway et al., 2015).

# 3

# GLUTAMATE INVOLVEMENT IN ALS

## 3.1 Glutamate neurotransmission: a brief overview

Glutamate is the most important excitatory neurotransmitter of the central nervous system and it is involved in almost all physiological functions of the brain. In neurons, it is mainly produced from glutamine by a reaction catalyzed by glutaminase, a phosphate-dependent enzyme. Once synthesized, it is stored in synaptic vesicles through three different types of transporters (VGLUT1-3; Santos et al., 2009). After its exocytotic release in the synaptic cleft, glutamate signals activates two different groups of membrane receptors, ionotropic (iGlu) and metabotropic (mGlu) glutamate receptors (Reiner & Levitz, 2018).

The ionotropic group is composed by the N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and the kainate receptors that assemble as cation permeable homo- or hetero-tetramers with each subunit being formed by an extracellular amino-terminal domain (ATD), a ligand binding domain (LBD), a transmembrane domain (TMD) forming the ion channel and an intracellular C-teminal domain (CTD).

The NMDA receptor is the only ligand- and voltage-gated receptor known to date and it is composed by GluN1, GluN2 (A, B, C, D) and GluN3 (A, B) subunits that assemble into obligatory heterotetramers, which usually comprise two GluN1 subunits for the binding of glycine or D-serine and two GluN2 subunits for glutamate binding; however, during development and in particular cell types, GluN2 can be substituted by GluN3 to form a triheteromeric NMDA receptors with atypical properties (Perez-Otano et al., 2016).

The subunits composing AMPA receptors are GluA1,2,3 and 4, whereas kainate receptors are formed by GluK1, 2, 3, 4 and 5 (Greger et al., 2017; Mollerud et al, 2017).

In general, the subunit composition of the iGluRs and the post-transcriptional modifications (e.g. RNA editing) of specific subunits (i.e. GluA2, GluK1) significantly affect the ligand binding affinity, channel kinetics, ion selectivity ($Na^+$ and $Ca^2$) as well as receptor trafficking.

Glutamate metabotropic receptors belong to the G-protein-coupled receptor (GPCR) family that can exist as homo- or heterodimers with each subunit being composed by a long extracellular region at the N-terminal domain, seven transmembrane domains and a C-terminal portion in the intracellular side that is necessary for the coupling to second messenger systems via heterotrimeric G-proteins. mGlu receptors consist in eight different subtypes classified in three different groups: group I (mGluR1,5), group II (mGlu2,3) and group III (mGluR4,6,7,8). Group I receptors are coupled to $G_q/G_{11}$ proteins and stimulate phospholipase C (PLC), leading to a subsequent increase in diacylglycerol (DAG) and inositol 3 phosphate (IP3) production, elevation of intracellular $Ca^{2+}$ and activation of protein kinase C (PKC). In addition, they also induce phosphorylation of the extracellular signal-regulated kinase (ERK) both in a transient manner, dependent on PLC activity, and in a sustained manner, induced by β-arrestin. As for their localization, they are predominantly expressed at postsynaptic sites but their presence on presynaptic nerve terminals, where they regulate neurotransmitter release, has been also demonstrated (Raiteri, 2008; Pittaluga, 2016). Group II and Group III receptors are associated with $G_i/G_o$ proteins and inhibit adenylate cyclase (AC), causing a cAMP reduction and inactivation of PKA (Suh, 2018). They are localized at presynaptic level; in particular Group II mGlu receptors are located away from the glutamate release sites, whereas Group III mGlu receptors are typically localized at the presynaptic active zones and act as autoreceptors by inhibiting glutamate release (Shigemoto et al., 1997; Pinheiro & Mulle, 2008; Niswender & Conn, 2010; Nicoletti et al., 2011).

Glutamate signaling is then terminated mainly by its neuronal and astrocytic reuptake

operated by five different transporters that in humans are named $EAAT_{1-5}$ (Excitatory Amino Acid Transporters), while in rodents the nomenclature is GLAST (Glutamate/Aspartate Transporter), GLT1 (Glutamate transporter 1), EAAC1 (Excitatory Amino Acid Carrier 1).

$EAAT_1$/GLAST and $EAAT_2$/GLT1 are predominantly localized on astrocytes in many regions of the CNS but are also expressed in some neurons, $EAAT_3$/EAAC1 is the neuronal transporter localized mainly to soma and dendrites, EAAT4 is almost exclusively present of cerebellar Purkinje cells and EAAT5 is confined to the retina with very low levels in the brain (Olivares-Banuelos, 2019).

### 3.2. Excitotoxicity

Among all the different mechanisms proposed for neurodegenerative processes in ALS, a large body of evidence indicates that excessive excitatory neurotransmission, a phenomenon known as excitotoxicity, plays a key role in the disease progression (Van Den Bosch et al., 2006; King et al., 2016).

Excitotoxicity may occur due to an excessive increase of extracellular glutamate (but also other endogenous ligands such as aspartate or quinolinic acid) concentrations or following exposure to environmental neurotoxins, such as domoic acid or B-N-methylamino-L-alanine (BMAA), that act as glutamate receptor agonists. On the other hand, excitotoxicity can be due to an abnormal responsiveness of the postsynaptic neuron to excitatory stimuli. In the first case, the glutamate excess can be caused by an increase in release or a reduction in re-uptake. In the second case, even if the neurotransmitter concentrations are sub-toxic, excitotoxicity may be mediated by an altered inhibitory transmission, a modified expression or function of the glutamatergic receptors, functional changes of ion channels or a varied cellular excitability. The excitotoxicity phenomena lead to neuronal death mainly by inducing a pathological increase in intracellular level of calcium that determines mitochondrial dysfunctions, oxidative and nitrosative stress, anomalous proteases,

phospholipases and/or endonucleases activation (Van Damme et al., 2005a).

### *3.2.1 Direct excitotoxicity in ALS*

Elevated levels of extracellular glutamic acid were detected in plasma and CSF of patients with both familial and sporadic forms and it was found that glutamate reuptake was impaired due to a dramatic reduction in CNS levels of EAAT2, including the cerebral cortex and the spinal cord (Perry et al., 1990; Rothstein et al., 1990; Rothstein et al., 1995; Fray et al., 1998; Sasaki et al., 2000; Spreux-Varoquaux et al., 2002; Fiszman et al., 2010) and it has been hypothesized that such a reduction could be due to alterations of transcription, translation or post-translation mechanisms of EAAT2 expression, possibly caused by an aberrant processing of RNA synthesis or oxidative damage (Lin et al., 1998; Rao et al., 2003; Boehmer et al., 2006; Van Landeghem et al., 2006). It has to be noted that EAAT2, which is mainly present on astrocytes, is the most expressed during adult life and responsible for more than 90% of glutamate clearance at the synaptic level (Maragakis & Rothstein, 2004).

In studies carried on in ALS animal models, it was found that GLT1 (rodent ortholog of human EAAT2) expression is remarkably reduced and its induced overexpression in $SOD1^{G93A}$ mice delayed the onset of the disease but did not prevent the fatal outcome (Bruijn et al., 1997; Howland et al., 2002; Guo et al., 2003). Moreover, the β-lactam antibiotic ceftriaxone was able to selectively increase GLT1 levels and its reuptake function, and when administered to $SOD1^{G93A}$ mice resulted in the delay of motor neuron loss and muscle strength but it did not prevent death, although it enhanced survival (Rothstein et al., 2005). Actually, a multi-stage, randomized, double-blind, placebo-controlled, phase 3 clinical trial failed to show any beneficial effect in ALS patients (Cudkowicz et al., 2014).

These results suggest that other neurotoxic mechanisms, in addition to impairment of glutamate reuptake, contribute to determine motor neurons death in ALS. This conclusion

is also supported by the observation that spinal infusion of GLT1 inhibitors does not result in significant neuronal death which, instead, occurs if they are administered in association with non-toxic glutamate doses (Hirata et al., 1997; Corona & Tapia , 2004; Tovar-Y-Romo et al., 2009; King et al., 2016).

Beside decreased glutamate uptake, other studies have shown that also glutamate release is pathologically increased in ALS.

The research group of Prof. Bonanno, with which I have collaborated as PhD student, demonstrated that the spontaneous release of glutamate from spinal cord nerve terminals (synaptosomes) was more elevated in pre-symptomatic (30 days of life) and symptomatic (70-90 and >130 days) $SOD1^{G93A}$ mice than in age-matched controls. In addition, the increase of glutamate release, induced by the activation of glycine/GABA hetero-transporters, KCl-mediated depolarization or ionomycin, was significantly more elevated in spinal cord synaptosomes obtained from pre-symptomatic and symptomatic $SOD1^{G93A}$ mice than in controls (Raiteri et al., 2003, 2004, 2005; Milanese et al, 2011; Bonifacino et al, 2016). Further insights into the molecular mechanisms have demonstrated that, at the synaptic bouton level, ALS transgenic mice showed higher cytosolic $Ca^{2+}$ concentrations, increased activation of calcium-calmodulin dependent kinase II (CaMKII) and augmented phosphorylation of synapsin-I and glycogen synthase kinase-3; moreover, an increased number of SNARE complexes, together with enhanced expression of synaptotagmin-I and β-actin were observed in $SOD1^{G93A}$ spinal cord nerve terminals. All these factors are known to favour neurotransmitter exocytosis and fit well with the functional observation that the boosted glutamate release in this ALS model is likely due to the increased size of the vesicle ready-releasable pool of glutamate (Milanese et al., 2011; Bonifacino et al., 2016). These results are also in agreement with data showing that genetic reduction of the expression of the vesicular glutamate transporter 2 (VGLUT2), which is responsible for the storage of glutamate into synaptic vesicles competent for exocytotic release, in ALS mice

that was able to protect motor neurons in the spinal cord and brain stem, as well as neuromuscular junctions (Wootz et al., 2010). However, despite the obvious reduction of glutamate release, this procedure neither affected disease onset nor increased lifetime, thus further indicating that the abnormal release of glutamate is not the only cause of degeneration in ALS.

In addition to alterations of endogenous glutamate levels, direct excitotoxicity in ALS could also be linked to environmental factors, such as bacteria-produced toxicants that can contaminate different freshwater, marine and terrestrial habitats. Indeed, following the observation of a high incidence in the Chamorro population of the pacific island of Guam of what has been named the ALS/PD complex (also known as Guam disease), data have accumulated suggesting that the neurotoxic amino acid β-methyl-amino-L-alanine (BMAA), which is produced by symbiotic cyanobacteria, can activate NMDA, AMPA and mGlu5 receptors and induce oxidative stress. Human exposure to this neurotoxin could be an environmental factor that increases the risk of developing ALS and other neurodegenerative diseases (Bradley & Mash, 2009; de Munck et al., 2013; Stommel et al., 2013; Sher, 2017; Cox et al., 2018; Lance et al., 2018). As a matter of fact, significant levels have been measured in CNS tissues of North American ALS patients (Pablo et al., 2009) and exposure to BMAA, following cyanobacteria blooms, may represent a risk factor for ALS in different populations (Sabel et al., 2003; Field et al., 2013; Torbick et al., 2014).

### *3.2.2 Indirect excitotoxicity*

Several studies suggested that AMPA receptors may have an excitotoxic role in ALS. Both in vitro and in vivo studies have shown that motor neurons are more sensitive to toxic effects of the AMPA/kainate agonist kainic acid (Rothstein et al., 1993; Carriedo et al., 1996; Ikonomidou et al., 1996 ; Bar-Peled et al., 1999; Sun et al., 2006; King et al., 2016),

an effect that could be due to low levels of the GluA2 subunit in the composition of the receptor. In fact, it has been amply demonstrated that with this subunit in the tetrameric composition of the AMPA receptor, the channel is permeable to sodium ions, while its absence leads to the entrance of calcium ions causing cytotoxic effects linked with an excessive increased in intracellular $Ca^{2+}$ concentration. It was observed that in ALS patient primary and secondary motor neurons have a reduced expression of the GluA2 subunit compared to other neuronal populations (Tomiyama et al., 1996; Shaw et al., 1999; Takuma et al., 1999). Interestingly, expression of GluA2 subunits with reduced calcium permeability in $SOD1^{G93A}$ significantly delayed disease onset and mortality, whereas the genetic ablation of the GluA2 subunit in these mice increased motor neuron degeneration and shortened survival (Tateno et al., 2004; Van Damme et al., 2005b). At variance with these evidences, no changes in the expression levels of the GluA2 subunit were observed in another mouse model of ALS (G86R SOD1; Morrison et al., 1998).

Impairment of the post-transcriptional GluA2 Q/R editing process, which produces sodium permeable, calcium impermeable AMPA receptors, has been also involved in ALS. This mRNA editing process is mediated by the action of the ADAR2 (Adenosine Deaminase Acting on RNA), an enzyme that transforms the adenosine of mRNA into inosine, thus leading to the expression of arginine instead of glutamine in the second transmembrane domain of the GluA2 subunit. Under physiological conditions, it has been estimated that less than 1% of AMPA receptors throughout the CNS are unedited and, therefore, permeable to calcium (Kawahara et al., 2003).

In this view, it has been reported that ADAR2 mRNA expression is reduced in the gray matter of the ventral spinal cord and in motor neurons of patients with sporadic ALS and when silenced in motor neurons of mice an ALS-like phenotype could be observed (Takuma et al., 1999; Kawhara et al., 2004; Kwak & Kawahara, 2005; Aizawa et al., 2010; Hideyama et al., 2010; Kwak et al., 2010). However, such ADAR2 alterations in GluA2

editing are not observed in SOD1 mice and rats (Kawahara et al., 2006).

### 3.3 Role of group I metabotropic glutamate receptors in neurodegeneration/ neuroprotection

While initial preclinical studies showed that, besides iGluRs, activation of group I glutamate metabotropic receptors mGlu1 and mGlu5 was involved in mediating the excitotoxic effects of abnormal glutamatergic neurotransmission characterizing almost all neurodegenerative disorders, it is now clear that the activation of these receptors can cause neurotoxic or neuroprotective effects, thus making the biological scenario on their roles in neurodegenerative disorders much more complex. Indeed, the new concept of "ligand bias", which applies to many GPCRs, add further complications to a clear-cut picture of the effects of mGluR1/5 under pathological conditions (Rajagopal et al., 2010; Bruno et al., 2017). The ligand bias implies that a GPCR can signal by activating its classical G-protein linked effectors (i.e. PLC, IP3 and DAG) or by stimulating a non-canonical GRK/β-arrestin dependent pathway (e.g. MAPK, PI3K, AKT), and a given receptor agonist can cause neurotoxicity by activating the PLC pathway linked to calcium mobilization, while another agonist is able to confer neuroprotection through stimulation of the other pathway (e.g. MAPK).

Finally, it has to be taken into account that mGluRs are present also on glial cells that, therefore, can have a significant influence on the overall effect of a selective agonist on neurotoxicity or neuroprotective mechanisms.

With this premise in mind, several preclinical studies have evaluated the role of mGluR1/5 in different models of neurodegenerative disorders.

A large number of in vivo and in vitro studies have shown that selective mGluR1 antagonists have a protective activity against hypoxia-induced and ischemia-induced neuronal damage (De Vry et al., 2001; Cozzi et al., 2002; Meli et al., 2002; Moroni et al.,

2002; Pellegrini- Giampietro et al., 2003; Makarewicz et al., 2006; Kohara et al., 2008; Murotomi et al., 2008, 2010; Landucci et al., 2009).

In rats with induced transient global ischemia, mGluR1 antagonists were able to protect CA1 pyramidal neurons of the hippocampus from ischemic damage and neuroprotection was likely mediated by the increase in GABA release from interneurons (Cozzi et al., 2002). On the other hand, Murotomi and collaborators (2008) have suggested that the anti-ischemic effects of mGluR1 antagonists were due to the decrease of tyrosine phosphorylation of the NMDA receptors. Interestingly from a mechanistic point of view, it has been shown that the activation of the mGluR1 stimulates the production of DAG that is converted by a DAG lipase into the endocannabinoid 2-arachidonoylglycerol (2-AG). 2AG then diffuses back to the presynaptic terminal and activates the cannabinoid receptor type 1 (CB1), which in turn inhibits GABA release. Therefore, mGluR1 antagonists would cause neuroprotection by reducing the increase in 2-AG produced during ischemia and favouring the release of GABA (Landucci et al., 2009).

The role of mGluR5 in ischemic neurodegeneration has been more debated, although it is well known that these receptors are physically and functionally coupled to NMDA receptors and their antagonism results in neuroprotection against NMDA neurotoxicity. A first study on transient forebrain ischemia in the gerbil has found that the selective mGluR5 negative allosteric modulator MPEP significantly protected neurons from death (Rao et al., 2000). In a model of a 2-hour transient focal ischemia, both MPEP and the selective mGluR5 agonist CHPG reduced the brain damage when administered soon after the induction of ischemia but no significant neuroprotection was observed when given after reperfusion. On the basis of in vitro data, it was concluded that MPEP neuroprotective effects appeared to be due to its antagonistic activity at NMDA receptors, whereas the agonist CHPG might have activated anti-apoptotic pathways (Bao et al., 2001). However, in another study no neuroprotection by MPEP was observed (Meli et al., 2002). Recently,

using an in vitro model of cerebral ischemia, it has been reported that mGluR5, but not mGluR1, positive allosteric modulators (PAM) significantly protected hippocampal neurons from ischemic-induced death by activating the PI3K/Akt pathway and reducing the down-regulation of the GluA2 AMPA receptor subunit (Cavallo et al., 2019).

Reducing the mGluR5 activation seems also a promising neuroprotective strategy in Parkinson's disease. In fact, administration of MPEP has been shown to reduce the methamphetamine-induced toxic effects on striatal dopaminergic nerve terminals as well as the loss of dopaminergic neurons in rats and mice treated with MPTP or 6-OHDA, a result that has been replicated also with the genetic deletion of mGluR5; similar results have been obtained with other mGluR5 NAMs (Bruno et al., 2017 and references therein).

mGluR5 are also involved in the pathological processes leading to Alzheimer's disease (AD). In fact, these receptors have been found to mediate the synaptotoxic effect of β-amyloid oligomers on the synaptic plasticity phenomena of LTP and LTD, thus disrupting memory formation and consolidation, and mGluR5 NAMs or genetic deletion are able to prevent such dysfunctions (Bruno et al., 2017 and references therein). However, increasing evidence indicate that mGluR5 positive allosteric modulators (PAMs) have beneficial effects on memory deficits that could be exploited for the symptomatic treatment of AD (Bruno et al., 2017). Therefore, targeting mGlu5 receptors as a novel therapeutic strategy in this neurodegenerative pathology could be difficult to pursue.

### 3.4 Group I metabotropic receptors in ALS

Under physiological conditions, both group I subtypes are expressed in human spinal cord neurons. mGluR1 immunolabeling has been observed on neurons, including motor neurons, in both the dorsal and ventral horns, with a more intense localization in the latter; similarly, mGluR5 immunoreactivity was present in both areas, but the strongest signal was present in the dorsal horns (Aronica et al., 2001). In both FALS and SALS patient

spinal cord, a decreased neuronal immunoreactivity was evident but the overall distribution of the two receptor subtypes was comparable to controls. Besides neurons, these receptors are also expressed by glial cells in the spinal cord of control subjects, although to a low level, but they show an important upregulation in reactive spinal cord glial cells of ALS patients (Aronica et al., 2001). A recent PET study has reported a consistent increase of mGluR5 binding signal in the striatum, hippocampus and frontal cortex of $SOD1^{G93A}$ mice, with an average increase of approximately 50% in whole brain that was further enhanced (23%) during progression of the pathology; the receptors increased also in the spinal cord, although to a lesser extent (Brownell et al., 2015). Moreover, prolonged treatment of cultured motor neurons and reactive astrocytes with MPEP reduced the experimental AMPA-mediated neurotoxicity, an effect that was absent when motor neurons were co-cultured with a reduced number of astrocytes, thus indicating a key role of these latter cells in the excitotoxic effects of mGluR5 (D'Antoni et al., 2011). Neuroprotection by systemic treatment with MPEP has also been demonstrated in $SOD1^{G93A}$ mice which showed delayed disease onset, slower astrocytic degeneration and a slight increase of their lifespan (Rossi et al., 2008).

Although group I mGluRs have been long considered as post-synaptic receptors, several studies have demonstrated their presence also onto presynaptic nerve terminals where they induce the release of neurotransmitters, including glutamate, thus acting as positive feedback autoreceptors (Gereau & Conn, 1995; Chu & Hablitz, 1998; Chen & van den Pol, 1998; Schwartz & Alford, 2000; Marino et al., 2001; Muly et al., 2003; Tan et al., 2003; White et al., 2003; Park et al., 2004; Luccini et al., 2007; Musante et al., 2008; Pittaluga, 2016; Raiteri 2008; Xie et al., 2017; Vergassola et al., 2018)

Professor Bonanno's research group investigated the modulation of glutamate release by the activation of Group I metabotropic glutamate receptors in the $SOD1^{G93A}$ murine model of ALS at the late phase of the pathology corresponding to 120 days in comparison with

age-matched SOD1 mice. Using lumbar spinal cord synaptosomes in superfusion, they tested different concentrations of 3,5-DHPG (from 0,001 to 30 μM) and the results showed that low concentrations of the agonist (≤ 0,3 μM) enhanced the basal release of glutamate in $SOD1^{G93A}$ but not in control mice demonstrating that group I metabotropic autoreceptors were much more active in pathological conditions; higher concentrations potentiated the basal release both in ALS and control animals. Experiments with selective antagonists for the two different subtypes (i.e. MPEP and CPCCOEt) showed both receptors were involved in the enhancement of glutamate release by high 3,5-DHPG concentrations whereas mGluR5 was preferentially involved in the high potency effects and its expression level was also 50% increased in $SOD1^{G93A}$ mice, as assessed by western blot analysis. Furthermore, using immunocytochemistry techniques and confocal microscopy analysis, they demonstrated that in mGluR1 and mGluR5 were co-localized onto spinal cord synaptosomes (Giribaldi et al., 2013). In a subsequent study, they reported that the genetic knocking down of mGluR1 in $SOD1^{G93A}$ mice, resulting in 50% decrease of the receptors, caused a significant reduction of motor neurons loss in the lumbar spinal cord that was associated to improved motor performances, delayed the onset of clinical symptoms, slowed disease progression and increased survival (Milanese et al., 2014). A more detailed analysis also showed that these mice had lower levels of damaged mithocondria, reduced oxidative stress, decreased activated astrocytes and microglia and a normalization of glutamate release. Interestingly, this genetic halving of mGluR1 also induced a significant reduction of mGluR5 expression. Finally, the mechanisms leading to the amelioration of the disease picture in these mutant mice was demonstrated to be independent of GLT1 because no modifications in its expression were found in the spinal cord (Milanese et al., 2014).

Using the same genetic approach, it has been reported that 50% reduction of mGluR5 in $SOD1^{G93A}$ mice also led to preservation of motor neurons, a significant decrease of

astrocytosis and microgliosis, possibly diminishing neuroinflammation, normalization of intracellular calcium concentrations and glutamate release. These changes were paralleled by a significant delay in the onset of clinical symptoms, an improvement in disease progression and a significant increase of lifespan (Bonifacino et al., 2017). Surprisingly enough, halving mGluR5 expression slowed down the motor symptoms only in males mice, but it is worth noting that $SOD1^{G93A}$ females had baseline motor performances higher than males, which could have minimized the beneficial effects of the mGluR5 genetic down regulation. At variance with what was observed in the previous study on mGluR1 ablation, halving mGluR5 expression did not affect mGluR1 (Bonifacino et al., 2017). However, complete ablation of mGluR5 in $SOD1^{G93A}$ resulted in the same beneficial biochemical and clinical effects reported above both in males and females; on the contrary, mGluR1 knocking out produced a very negative phenotype, characterized by reduced dimensions, ataxia, progressive motor deficits and early death (Bonifacino et al., 2019a).

# 4

# CELL TO CELL COMMUNICATION AND EXTRACELLULAR VESICLES

During my PhD, I spent three months for a research stage at Département de Physiologie, Université de Lausanne, in the laboratories directed by Prof. Rosa Chiara Paolicelli. During this period, I carried out a pilot project on the role of astrocytes-derived exosomes on microglial activation.

Cell to cell communication can occur through direct contact between cells or by paracrine action mediated by secreted molecules or organelles, including extracellular vesicles (EVs), and different types of neural cells are largely involved in this novel mechanism of intercellular communication both under physiological and pathological conditions (Frühbeis et al., 2013).

## 4.1 Extracellular vesicles

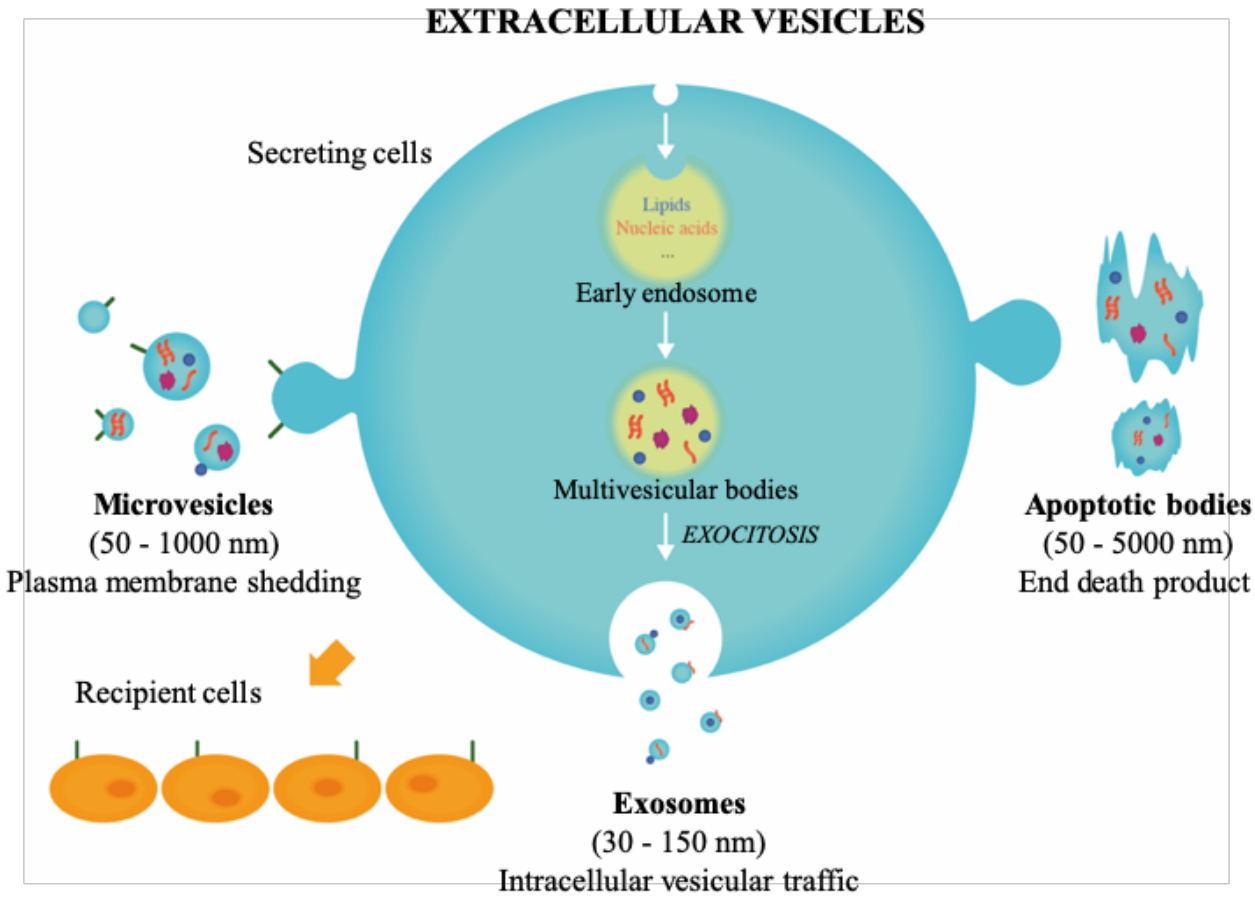
EVs are a heterogeneous population of membrane structures with the classical phospholipid bilayer, secreted by different cell types, released in the extracellular space and they play a pivotal role in intercellular communication (Abels & Breakfield, 2016; Paolocelli et al., 2019; Smith et al., 2015). EVs are classified on the basis of their origin and size in three main categories:

- Exosomes (30 - 150 nm): they have an endocytic origin, deriving from the inward budding of the limiting membrane of multi-vesicular bodies and could be degraded through fusion with lysosomes or released in the extracellular space upon fusion with the plasma membrane (Colombo et al., 2014; György et al., 2011). Exosomes are released by practically all healthy cells. Commonly they are enriched in proteins, such as flotillin and Alix, that are normally used as exosome markers. Tetraspanins (e.g. CD63, CD81, CD9)

are a family of membrane proteins known to cluster into microdomains at the plasma membrane. These proteins are abundant in exosomes and used as well as specific marker.

- Microvesicles or ectosomes (MVs; 50 nm - 1 µm): they originated from the outward budding of plasma membrane. Their release occurs in physiological conditions or after specific stimuli, such as ATP.

- Apoptotic bodies (50 nm - 5 µm): dimensionally, they are the largest vesicles produced by cells and their release is linked to the apoptosis process of the cells.




***Extracellular vesicles classification.***

In the last decade, some oncologist described specific EVs derived from cancer cells, having 1 - 10 μm dimension, called oncosomes (Al-Nedawi et al., 2008; Minciacchi et al., 2015).

EVs have been isolated from biological fluids using a variety of methods, such as ultracentrifugation, ultrafiltration and immunologically-based separation by means of magnetic beads (Momen-Heravi et al., 2013; Konoshenko et al., 2018).

Extracellular vesicles are secreted by progenitor cells, of which they maintain some similar characteristics (for example the lipid membrane), and are able to elicit diverse responses in recipient cell types. Indeed, once released, they exert their effects on specific recipient cells, after being taken up. Uptake mechanisms include endocytosis, fusion with the plasma membrane of recipient cells or uptake through specific surface proteins binding (i.e. tetraspanins). They can transport bioactive molecules to the target cells, such as specific proteins, lipids, messenger RNA, non-coding RNA and genomic DNA (Théry et al., 2002; Henderson & Azorsa, 2012; Rufino-Ramos et al., 2017). EVs content is protected from the RNAse digestion thanks to the lipid coating and could be internalized by recipient cells acting also on specific cells functions (Mulcahy et al., 2014). In addition, the presence of the lipid membrane confers stability in the bloodstream, protection against the immune system and facilitate the transition through physiological barriers, such as the BBB (Rajendran et al., 2014; Rufino-Ramos et al., 2017). RNA transported by EVs can modulate gene expression in recipient cells, where mRNA is translated in new proteins and can also inhibit the expression of other proteins (Skog et al., 2008; Zhang et al., 2010).

All brain cells (neurons, astrocytes, microglia and oligodendrocytes) can release EVs (Fauré et al., 2006; Taylor et al., 2007; Krämer-Albers et al., 2007; Bakhti et al., 2011; Hooper et al., 2012; Glebov et al., 2015). In neurons, there were evidences of the involvement of glutamate in the regulation of EVs release (Lachenal et al.,2011).

There are controversial evidences about the role of EVs in brain disease: they can play the important role of removing toxic proteins and aggregates from releasing cells or they can contribute in the spreading of the pathology, transferring their cargo of toxic elements to healthy cells (Croese & Furlan, 2018).

EVs may also represent specific biomarkers for neurodegenerative diseases, since they are released in the extracellular space in the brain and can be found in biological fluids, such as blood (Garcia-Contreras et al., 2017). The possibility to evidentiate suitable biomarkers could lead to early diagnosis and systemic monitoring of the progression of the disease, for instance in clinical trials.

Once their release from neural cells was documented, EVs were studied as a potential source of information about neural cells involved in the pathogenesis of neurodegenerative disease (Scolding et al., 1989; Rajendran et al., 2006; Verderio et al., 2012). Furthermore, besides neurons, astrocytes, microglia and oligodendrocytes, blood-infiltrating cells, which contribute to neuroinflammation during neurodegenerative processes, can also represent another source of EVs.

However, many aspects of EVs still remain unknown, such as what kind of vesicles have the ability to move into the biological liquids, why they are released, which types of message they carry and which are the target cells.

Despite the lack of information, extracellular vesicles may represent a novel therapeutic approach in brain diseases and a way to deliver drugs to specific targets.

Interestingly, it is also possible to engineer the surface of EVs with a small peptide derived from the rabies virus glycoproteins to better pass the BBB (Kumar et al., 2007). An alternative way to reach the brain is the intranasal administration of exosomes, an administration route tested in a Parkinson's disease animal model that showed neuroprotective effects (Haney et al., 2015).

The research on the therapeutic use of EVs concentrated the efforts on the possibility to load them with therapeutic elements. The internalization of specific molecules can be performed by incubation with drugs or transfection with specific miRNA (Pascucci et al., 2014; Kosaka et al., 2010). However, the most common method is the drug-loading through passive or active incubation (Ingato et al., 2016). Among the active loading strategies, electroporation was demonstrated to maintain the integrity of EVs (Kooijmans et al., 2013). Other approaches are incubation at room temperature, permeabilization with saponin, freeze-thaw cycles and sonication.

The wide range of EVs content identified may have numerous applications in the treatment of brain diseases, also because their use could be safer than the use of the progenitor cells. For example, mesenchymal stem cells are the progenitor cells most commonly used in therapy, but since they are able to differentiate in-vivo they can exhibit undesirable effects (Rani et al., 2015).

### 4.2 Extracellular vesicles as therapeutic agents

Many studies have focused on EVs as potential tools for therapeutic intervention in neurological and neurodegererative diseases (Croese & Furlan, 2018), which are reported in the following sections.

#### *4.2.1 Alzheimer's disease*

Alzheimer's disease is a progressive neurodegenerative disease characterized by dementia, memory and cognitive decline and affects 35 millions of people usually after 65 years old, with a 2/3 incidence in women (Brookmeyer et al., 2011). The main features of this pathology are the presence of extracellular amyloid senile plaques, intracellular neurofibrillary tangles containing abnormally phosphorylated tau protein, neuroinflammation, excessive microglia activation and the downregulation of pre- and post-synaptic proteins that lead to neurodegeneration (Reddy et al., 2005).

As in other brain pathologies, there are evidences regarding the dual role of EVs, both beneficial or detrimental, also in AD. Particular attention was given to the discovery of proteins and peptides associated with AD, such as Amyloid Precursor Protein (APP) and β-amiloid (Aβ), in association with exosomes. In addition, also exosomal specific proteins, such as flotillin, have been found in Aβ plaques (Agosta et al., 2014; Rajendran et al., 2006; Vingtdeux et al., 2007; Sharples et al., 2008; Perez-Gonzalez et al., 2012).

Another protein involved in Alzheimer etiology is Tau and there are several studies demonstrating that it spreads via exosome secretion and that the inhibition of EVs release significantly reduces Tau propagation both in vitro and in vivo (Asai et al., 2015). In vitro studies demonstrated that EVs derived from Aβ-stimulated astrocytes and from Tau-treated microglia are involved in Aβ aggregation and Tau propagation, respectively (Asai et al., 2015; Dinkins et al., 2016; Fiandaca et al., 2015; Xiao et al., 2017).

### *4.2.2 Parkinson's disease*

Parkinson's disease is a progressive neurodegenerative disorder especially, but not exclusively, affecting movements. In general, the main cardinal symptoms of this disease resting tremors, bradykinesia/akinesia and muscle stiffness (rigidity) that are caused by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta due to different mechanism of cellular toxicity, including excitotoxicity, mitochondrial dysfunctions and oxidative stress. Another main pathological characteristic of PD is the accumulation of insoluble alpha-synuclein protein in neurons (Capriotti & Terzakis, 2016).

Similarly to what reported for AD, alpha-synuclein oligomers associated with EVs can enter in the recipient cells inducing a more serious toxicity compared to EVs free from alpha-synuclein oligomers, contributing with this mechanism to Parkinson's spreading (Emmanouilidou et al., 2010; Danzer et al., 2012; Chang et al., 2013).

### *4.2.3 Multiple Sclerosis*

Multiple sclerosis (MS) is a chronic, autoimmune and inflammatory neurodegenerative disease destroying myelinated axons in the central nervous system. There are three main forms of MS that are classified as relapsing-remitting (RRMS), primary progressive (PPMS) and secondary progressive (SPMS). The most common form is the RRMS that affects 80-85% of patients, followed by SPMS, whereas PPMS affects 15% of patients (Thompson et al., 2018).

Due to the important role of EVs in immune regulation, the growing interest of their involvement in MS is not surprising. In this scenario, endothelial and platelet-derived extracellular vesicles from MS patients have been shown to increase the permeability of endothelial layers, suggesting their involvement in the disruption of the BBB (Sheremata et al., 2008; Marcos-Ramiro et al., 2014; Alexander et al., 2015).

One of the most used animal model for Multiple Sclerosis is the Experimental Autoimmune Encephalomyelitis (EAE) mouse (Constantinescu et al., 2011). The brain injection of microglia-derived exosomes in EAE mice resulted in enhanced inflammation and exacerbated disease (Jy et al., 2004). It has been reported that EVs are higher in MS patients than controls and that their level in plasma or CSF was shown to reflect disease progression, but the molecular mechanisms remain unknown. In accordance with these results, mice in which the secretion of microvesicles was inhibited were more resistant to EAE (Verderio et al., 2012).

### *4.2.4 Amyotrophic Lateral Sclerosis*

As largely reported in the first part of this thesis, ALS is a multifactorial and multicellular neurodegenerative disease characterized by upper and lower motor neurons death.

In experiments with the Neuro2a cell line and with primary neurons, TDP- 43 was detected in exosomes isolated from the brain, but not in those released by astrocytes or microglia. The exposure of Neuro2a cells to ALS brain-derived exosomes caused cytoplasmic

redistribution of TDP-43, while the exposure to EVs from control brain did not, suggesting that the formers can contribute to propagation of the TDP-43 proteinopathy (Iguchi et al., 2016).

In a recent study, EVs progenitor cells were transfected with mutant $SOD1^{G93A}$ protein and the effects of their derived exosomes (mSOD1 exosomes) were studied on the recipient N9 microglial cell line. The activation and polarization of microglia toward a mixed pro- and antiinflammatory phenotype was detected (Pinto et al., 2017).

Although both exosomes and MVs can transfer biological information between cells, most of the studies on the potential therapeutic role of EVs and ALS have been focused on the use of exosomes since they are the smallest EVs and they are able to cross the BBB (Bonafede & Mariotti, 2017). Bonafede and colleagues demonstrated that exosomes derived from adipose stromal cells may increase NSC-34 cell line viability, protecting from oxidative damage, which is one of the main neurotoxic mechanism of ALS (Bonafede et al., 2016). Moreover, it has been shown that exosomes derived from adipose stromal cells, which are considered to possess therapeutic effects in neurodegenerative diseases, were able to ameliorated the aggregation of SOD1 in neurons derived from $SOD1^{G93A}$ mice (Lee et al., 2016).

Other studies have demonstrated that, besides micropinocytosis of free aggregates, misfolded SOD1 can propagate into recipient cells also both through an exosome-dependent mechanism (Silverman et al., 2016) and CNS-derived EVs are mainly secreted by astrocytes and neurons, rather than by microglia, both in normal and $SOD1^{G93A}$ mice (Silverman et al., 2019). In particular, it has been shown that astrocytes-derived EVs spread SOD1 to spinal neurons, thus inducing motor neuron death (Basso et al., 2013).

In addition to TDP-43 and SOD1, other relevant abnormal for ALS have been characterized in EVs, such as FUS and C9Orf72, as well as non-coding miRNAs characteristic for the pathology.

### 4.3 Neural cells producing EVs

Microglial cells represent the CNS resident macrophages involved in the maintenance of brain homeostasis, repair and defense during insults. Microglial release vesicles (0,1 – 1 μm) with high level of phosphatidylserine on the surface. When stimulated with ATP, microglia released EVs containing pro-inflammatory cytokines (Bianco et al., 2005). These microglial EVs carry inflammatory messages to recipient microglia or neurons, causing in the latter an increased spontaneous and evoked excitatory transmission through the induction of ceramide and sphingosine synthesis that augments release probability (Verderio et al., 2012; Antonucci et al., 2012; Turola et al., 2012).

Under pathological conditions, microglia switch from a surveying ramified state into an activated amoeboid state and migrates to the lesion site, blocking the spread of the damage (Davalos et al., 2005; Teeling & Perry, 2009). Activated microglia is involved in neurogenesis as well as in neurotoxic effects. The activation state can vary between the classical activated M1 phenotype, with pro-inflammatory characteristics, and the alternative M2 state with antiinflammatory characteristics (Tejera & Heneka, 2016). The two phenotypes are not clearly separated.

Astrocytes are present throughout the brain tissue and are constitutive part of the BBB; besides their well-known trophic role to neurons, we now know that they actively participate in cell-to-cell signaling by releasing gliotransmitters and also take part to repair processes. Similarly to microglia, they can display two activation states, A1 and A2. They release EVs with a size of 8 μm or more and also in this case the process can be evoked by ATP stimulation (Bianco et al., 2009). Due to the large dimensions, these EVs can carry also entire mitochondria but they have heterogeneous composition and can produce both positive or negative effects (Falchi et al., 2013). In AD, ALS and HIV, it has been reported that they can negatively contribute to the disease processes by spreading the pathology (Wang et al., 2012; Basso et al., 2013).

In the CNS, oligodendrocytes have long been known to provide to neuronal axons with the myelin sheath to speed up the transmission of action potentials along the axons. Besides this role, it is now clear that they are also crucial in providing trophic support to neurons to maintain axon integrity (Nave & Trapp, 2008). It has been reported that such a function may depend on exosome transfer from oligodendrocytes to neurons triggered by neurotransmitters, in particular glutamate acting at AMPA and NMDA receptors (Frühbeis et al., 2013). On the other hand, oligodendrocyte-derived exosomes were observed to negatively regulate myelin synthesis in an autocrine manner under control of neurons (Bakhti et al., 2011).

### 4.4 Cross-talk between microglia and astrocytes

Microglia and astrocytes take active part in various pathological brain conditions, such as trauma and neurodegenerative disorders, and their activation is proportional with the development of diseases. Astrogliosis consists in the activation and increase in the number of astrocytes to minimize and repair damages (Osborn et al., 2016). Similar modifications take place during microglia activation.

Increasing evidence support the existence of a bidirectional relationship between the activation of astrocytes and the recruitment of microglia (Liu et al., 2011; Jha et al., 2019). Although the cross-talk between astrocytes and microglia is a well-documented process and profoundly influence reciprocally the features of the two cell populations, the two distinct activations seem to occur with a spatially and temporarily delay from each other and there is a general consensus that microglia is activated first and promotes astrocytic activation (Liu et al., 2011).

The time-course of microglia and astrocytes activation depends on the neurodegenerative disease considered for example. In the EAE animal model of MS, microglial cells proliferate at the initial stage while astrocytes activation occurs at the late stage of the disease (Matsumoto et al., 1992). Similarly, astrocytes activate subsequently to microglia

in AD (Frautschy et al., 1998; Gatan & Overmier, 1999). Diminishing reactive gliosis in a mouse model of AD led to migration of microglial cells in plaques and increased the expression of microglia markers (Kraft et al., 2013), whereas activated astrocytes suppressed the recruitment of microglia (Pekny et al., 2014).

There is also evidence for the role of the released inflammatory molecules produced and released by M1 microglia on GFAP-positive and negative astrocytes that, in turn, release other molecules that cause an increased in microglia activation, thus triggering a vicious circle. Proinflammatory cytokines, in particular interleukin-1 (IL-1) produced by activated microglia, may facilitate astrocytes activation (Herx et al., 2000; Herx & Yong, 2001). The pivotal role of IL-1 is due to its fast release in pathological conditions and to its ability in upregulating other inflammatory cytokines, such as IL-6 and tumor necrosis factor alpha (TNF-α; John et al., 2005). It was reported that IL-1 produces astrocytes activation, but it manifests also a protective role (John et al., 2004).

Once astrocytes are activated, the cytosolic $Ca^{2+}$ concentration increases and propagates among astrocytes in form of calcium waves. Following an insult and upon the increase of $Ca^{2+}$ concentration, astrocytes releases ATP, leading to microglia activation and causing rapid changes in microglial morphology and migration, also suggesting that the calcium waves can also spread to microglia (Verderio & Matteoli, 2001; Schipke et al., 2002; Davalos et al., 2005).

Astrocytes-derived ATP can promote microglia activities, enhancing also EVs formation and release by microglia and inducing phagocytosis (Bianco et al., 2005, 2009; Dou et al., 2012; Sieger et al., 2012; Domercq et al., 2013).

EVs released by microglia under ATP stimulation induced a strong inflammatory reaction in glial cells (Verderio et al., 2012). Moreover, ATP modified the EVs content of proteins implicated in adhesion and extracellular matrix organization, autophagy and cellular metabolisms, thus, affecting also astrocytes (Drago et al., 2017). Astrogliosis may improve

activation of distant microglia and inhibit its activities, decreasing for example NO, reactive oxygen species (ROS) and TNF-α production by microglia (Smits et al., 2001; von Bernhardi & Eugenín, 2004; Tichauer et al., 2007).

Thus, the scenario of astrocytes and microglia cell-to-cell communication also by EVs release is quite complex and more studies are needed to better understand the pathophysiological functions and the mechanisms involved.

# 5

# AIM OF THE PROJECT

All the results reported in the previous chapter have shown that glutamate neurotransmission by group I metabotropic receptors is altered in ALS and, therefore, these receptors could represent druggable targets for novel pharmacological strategies effective in the treatment of this neurodegenerative pathology.

In particular, mGluR1 and mGluR5 feedforward autoreceptors modulating glutamate release from spinal cord nerve endings were found to be more active in $SOD1^{G93A}$ mice at the end stage of the pathology (120 days of age) than in control animals, suggesting that the abnormal release of glutamate induced by these receptors could participate in exacerbating excitotoxicity phenomena that contribute to the death of spinal MNs (Giribaldi et al., 2013). However, since these results were obtained at the end stage of the $SOD1^{G93A}$ pathology, it was not possible to evaluate whether the functional alterations of group I autoreceptors were a cause/concause or an effect of the disease.

Therefore, in order to better understand the role of mGluR1 and mGluR5 in the development and progression of the disease, we analysed the effects of the selective agonist 3,5-DHPG on the release of glutamate through the activation of glutamatergic metabotropic autoreceptors of group I in the pre-symptomatic and subsequent phases of the pathology, corresponding to 30, 60 and 90 days of life of $SOD1^{G93A}$ mice.

I carried out this project in collaboration with the research group of Prof. Bonanno, in particular with Dr. Claudia Rebosio and Dr. Tiziana Bonifacino. To study these phenomena, the model of the superfused synaptosomes was adopted, and western blot and confocal analysis were performed to analyse mGluR1 and mGluR5 expression in glutamatergic spinal cord synaptosomes from both WT and $SOD1^{G93A}$.

Another part of my thesis work has been dedicated to the possible cross-talk between astrocytes and microglia via exosomes and the role of mGluR5 in modulating this type of cell-to-cell communication. As a matter of fact, results obtained by the research group of Prof. Bonanno, in particular Dr. Francesca Provenzano, have shown that astrocytes treated with mesenchymal stem cell (MSC)-derived exosomes show a less noxious phenotype and can rescue MNs from death in mixed astrocytes/MNs co-cultures (manuscript in preparation). Thus, exosome-treated astrocytes can support MN viability directly by reducing the toxicity of the extracellular milieu or by transferring protective molecules to MNs by secreted exosomes. Besides this direct effect, astrocytes could indirectly improve MN viability by ameliorating the pro-inflammatory phenotype of microglia, which in turn can positively affect MNs.

As previously reported, it was also showed that mGluR5 are up-regulated in $SOD1^{G93A}$ mice and abnormally stimulated glutamate release in the spinal cord of these animals (Giribaldi et al., 2013; Bonifacino et al., 2019a). Moreover, reduction of mGluR5 activity, by the genetic down-regulation of these receptors in-vivo ameliorated the progression of the disease in $SOD1^{G93A}$ mice (Bonifacino et al., 2018, 2019b). Interestingly, astrocytes derived from $SOD1^{G93A}$ with halved mGluR5 mice were able to reduce in vitro motor neuron death in comparison with astrocytes derived from $SOD1^{G93A}$ mice, and the exposure to a negative allosteric modulator of mGluR5 ameliorated the activation phenotype of astrocytes (manuscript in preparation).

Therefore, for this part of the project, in the laboratory of Prof. Paolicelli, I set up a method to isolate extracellular vesicles secreted from cultured astrocytes and evaluated their characteristics and impact on microglia activation. In particular, I addressed whether the in vitro pharmacological antagonism of mGluR5 present on astrocytes could affect astrocyte-released extracellular vesicles and influence the microglia activation state.

# 6

# METHODS

## 6.1 Animals

B6SJL-TgN SOD1/G93A(þ)1Gur adult mice with a high copy number of mutant human SOD1 carrying a Gly93Ala substitution [SOD1G93A] (Gurney et al., 1994) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals were bred in the animal care facility at the Department of Pharmacy (University of Genova, Genova, Italy) at constant temperature (22 ± 1°C) and relative humidity (50%), with a regular light–dark exposure (light 7 a.m.-7 p.m.). Food and water were available ad libitum.

Transgenic animals for SOD1 gene were crossed with background-matched B6SJL wild-type females to permit the maintenance of each transgene in the hemizygous state. Wild type (WT) non-transgenic animals have been used as controls in each experimental condition and both female and male mice were equally distributed in each series of experiments to limit possible gender influence. In general, death in transgenic mice occured between 120 and 140 days (late phase of disease). To collect the data regarding the time course of the pathology, animals were killed at 30, 60, and 90 days of life, corresponding to pre- (30 and 60 days) and early-symptomatic (90 days) stages of the disease.

Experiments were carried out in accordance with the European Union Directive 2010/63/EU for animal experiments and the ARRIVE guidelines, and were approved by the Ethical Committee of the University of Genoa (protocol n° 09/02/2016 OPBA). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

Similarly, the animals used in Lausanne were C57BL/6J wild-type mice and they were housed on a 12–12 h light cycle (lights on at 7:00) with food and water ad libitum. All procedures aimed to fulfill the criterion of the 3Rs and were approved by the Veterinary Offices of Vaud (Switzerland; license VD3409). P2-P5 (postnatal day) were used for each experiments and all efforts were made to minimize the number of subjects used and their suffering.

**6.2 Animal genetic analysis**

Genotypization was performed on each animal of the colony to detect the presence of the mutant superoxide dismutase gene analyzing tissue extracts from tail tips. Tissues were manually homogenated in PBS Dulbecco, freeze-thawed twice and centrifuged at 23,000 x g for 15 minutes at 4°C. Then, each sample was added with a buffer solution containing 40% glycerol, 250 nM Tris HCl (pH 6.8) and bromophenol blue. The technique consists in analyzing the samples by gel electrophoresis on a vertical polyacrylamide gel. After the electrophoretic run, gels were stained in a solution of 1 mg/ml of nitrotetrazolium blue for 45 minutes. The gel was then immersed in a solution having the following composition: $K_2HPO_4$ 36 mM (pH 7.8), TEMED 28 mM and Riboflavin 37 µM. In this way, nitrotetrazolium blue is able to interact with the superoxide radicals produced by the photo-oxidation of riboflavin giving a violet color. This signal makes it possible to distinguish the samples containing the transgenic human SOD1 that present two characteristic bands with different molecular weights (human and murine SOD1) from the samples containing only the murine SOD1.

Screening was performed before each experiment to discriminate between G93A positive (transgenic mutated) non-transgenic animals and between positive and non-transgenic SOD1 animals.

### 6.3 Preparation of purified synaptosomes

Spinal cords were rapidly removed at 0-4°C after mice euthanasia. Tissues were homogenized in sucrose (0,32 M, 1:10 weight/volume) buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue homogenizer (clearance 0,25 mm, 12 up-down strokes in approximately 1 min). The homogenate was first spun at 1,000 x g for 5 min (4°C) to remove nuclei and debris. Then, purified synaptosomes were prepared by a Percoll® gradient separation (Sigma-Aldrich, St Louis, MO; Nakamura et al., 1993). The supernatant obtained from the first centrifugation was stratified on a discontinuous Percoll® gradient (6, 10, and 20% v/v in Tris-buffered sucrose, pH 7.4) and spun at 33,500 x g for 5 min at 4°C. The enriched synaptosomal fraction (layer between 10 and 20% Percoll) was collected and washed by a third centrifugation.

### 6.4 Release experiments

Spinal cord purified synaptosomes from 30, 60, 90 day-old WT an $SOD1^{G93A}$ mice were resuspended in physiological medium (NaCl 140 mM; KCl 3 mM; $CaCl_2$ 1.2 mM; $MgSO_4$ 1.2 mM; $NaH_2PO_4$ 1.2 mM; HEPES 10 mM; glucose 10 mM; pH 7.4), incubated at 37°C for 15 min with 0.01 μM [$^3$H]D-Aspartate (a metabolism-resistant marker of glutamatergic nerve terminals; Perkin-Elmer, Milan, Italy) and then stratified in identical aliquots on microporous filters that were positioned at the bottom of parallel superfusion chambers (Superfusion System, Ugo Basile, Comerio, Varese, Italy) maintained at 37°C. Once stratified, synaptosomes were superfused with physiological medium (same composition as above) at a flow rate of 0.5 mL/min for a total time of 51 min; five 3-min superfusate samples (t = 36-39, 39-42, 42-45, 45-48, 48-51 min) were collected after the system was equilibrated for 36 min to reach a steady-state level of neurotransmitter release. To study the effects of mGluR1/5 activation, synaptosomes were exposed to different concentrations (0.03-30 μM) of the non-subtype-selective agonist (s)-3,5-Dihydroxyphenylglicine (3,5-

DHPG) from the end of the first sample collected (t = 39 min) to the end of the experiments (t = 51). The selective drugs used to characterize the receptor subtypes involved (LY367385 mGluR1 antagonist and MPEP mGluR5 negative allosteric modulator, both at 1 μM) and the other drugs used to analyse the release mechanisms (the phospholipase C inhibitor U73112 and the IP3 receptor blocker 2-APB) were introduced 9 min before the 3,5-DHPG challenge (t=30 min). To analyse whether the 3,5-DHPG-induced effects were dependent on extracellular calcium, in some experiments $Ca^{2+}$ was omitted from the superfusion medium 19 min before the 3,5-DHPG challenge (t = 20 min). To evaluate whether the effects of 3,5-DHPG involved intracellular calcium and release of glutamate from synaptic vesicles, in some experiments synaptosomes were incubated for 30 min (15 min before and during [$^3$H]D-Asp labelling) in the presence of 100 μM of the calcium chelator BAPTA-AM or with 0.1 μM of the neurotransmitter vesicle depleting agent bafilomycin A1. Appropriate controls were always used in parallel. At the end of the experiment, the radioactivity in the collected fractions and that remaining in synaptosomes were counted by liquid scintillation counting. The efflux of radioactivity in each fraction was expressed as a percentage of the total radioactivity present in synaptosomes at the beginning of the fraction collection (fractional rate, FR).

The effect of 3,5-DHPG was quantified by calculating the ratio between the fractional release of the fifth fraction (t = 48-51 min, $FR_5$, where the maximum effect of the agonist on neurotransmitter release was observed) and the one of the first fraction (t = 36-39 min, $F_1$, basal release). This $FR_5/FR_1$ ratio was then compared to the corresponding one obtained under control conditions. Differences have been analysed by one-way ANOVA followed by Dunnett's or Tukey-Kramer multiple comparison test, where appropriate, and considered significant at the level of $P < 0.05$, at least.

### 6.5 Western blot analysis

Purified synaptosomes or total tissue derived from spinal cord of WT and SOD1$^{G93A}$ mice at the different time points and astrocytes-derived EVs fraction were lysed in ice-cold RIPA buffer containing 1% protease inhibitor cocktail. After lysis, samples were centrifuged at 10,000 x g for 10 min at 4°C and the resulting supernatants were subjected to immunoblot analysis. The concentration of proteins in each sample was in the linear portion of the standard curve.

Following separation by electrophoresis on SDS-polyacrylamide gels (4 to 20% gradient), proteins were transferred to nitrocellulose membranes using a 25 mM Tris cold buffer, 192 mM glycine and 20% methanol. Membrane were incubated for 12 hours at 4°C in TBS TWEEN (t/TBS: 200 mM Tris, 1,3 M NaCl, pH 7.5, 0.05% tween 20, 5% skimmed milk powder) and electroblotted proteins were monitored using Naphthol blue black staining (Sigma Aldrich, MO, USA). After saturation, membranes were incubated with the following antibodies: Mouse monoclonal anti-mGluR1 (1:500, cat n. 610964; BD Biosciences, San Jose, CA, USA); rabbit monoclonal anti-mGluR5 (1:500, cat n. ab53090; Abcam, Cambridge, UK); mouse monoclonal anti-glyceraldeide phosphate dehydrogenase, GAPDH (1:10000, cat. N. G8795; Sigma Aldrich, MO, USA); mouse monoclonal anti-β-tubulin III (1:1000; cat. N. T8578; Sigma-Aldrich, MO, USA). After washing in t/TBS and incubation with secondary antibodies conjugated to horseradish peroxidase, protein bands were analysed for optical density using an enhanced chemiluminescence substrate (ECL, LiteAblot PLUS, Euroclone, Milan, Italy) and a chemiluminescence system (Alliance 6.7 WL 20M, UVITEC, Cambridge, UK) using the UV1D software (UVITEC). GAPDH or β-tubulin III levels in the same membrane were used to normalise bands of interest.

Regarding EVs samples in loading buffer 1X (10% beta-mercaptoethanol and 90% 4X Protein sample loading buffer LI-COR Biosciences), they were loaded (10 µg protein) on Bio-Rad pre-custom gels 10% and run in running buffer 1X (Bio-Rad®) added with 3 µl

of ladder Precision Plus Protein western (Bio-Rad®) and applying 200 Volt for 30 min. Proteins were transferred to Trans-blot turbo midi nitrocellulose transfert packs (Bio-Rad®) using Trans-blot turbo Transfert System (Bio-Rad®) at 1,3 A, 25 V for 7 min. After 1 hour blocking at room temperature, in TBS TWEEN (TBS + 3% BSA + 0,05% TWEEN), membranes were incubated overnight with rabbit monoclonal anti-Alix (1:1000; cat. N. 12422-1-AP; Proteintech); mouse monoclonal anti-CD9 (1:2500; cat. N. 60232-1-Ig; Proteintech); rabbit polyclonal anti-Calnexin (1:5000; cat. N. 10427-2-AP; Proteintech); mouse monoclonal anti-glyceraldeide phosphate dehydrogenase, GAPDH (1:15000, cat. N. 60004-1-Ig; Proteintech); mouse monoclonal anti-β-actin (1:5000; cat. N. 60008-1-Ig; Proteintech). After three times 10 min washing in TBS-TWEEN, membranes were incubated for 1 hour at room temperature with appropriate secondary antibodies (1:20000). Protein bands were detected and analyzed for optical density using Li-Cor Odyssey and Image Studio Lite.

**6.6 Confocal microscopy**

Spinal cord purified synaptosomes were resuspended in a physiological HEPES buffered solution (NaCl 140 mM, KCl 3 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.2 mM, $NaHCO_3$ 5 mM, $NaH_2PO_4$ 1.2 mM, HEPES 10 mM, glucose 10 mM, pH 7.2-7.4). $SOD1^{G93A}$ and WT spinal cord derived synaptosomes from mice of the same age (40 μg of protein) were stratified onto poly-L-lysine pre-treated coverslips and maintained for 45 min at room temperature to allow setting and sticking to the surface. The preparations were fixed with 2% paraformaldehyde for 15 min, washed with phosphate buffered saline (PBS; 3 × 5 min), permeabilized with 0.05% Triton X-100 for 5 min and incubated overnight at 4°C with primary antibodies diluted in PBS containing 3% bovine serum albumin. The following primary antibodies were used: murine anti-vesicular transporter Glu antibody (anti-vGluT1, 1:1000; Merk Millipore, Billerica, MA, USA), anti-mGluR1 murine antibody

(1:1000; BD Biosciences, NJ, USA) and anti-mGluR5 rabbit antibody (1:500; Abcam, Cambridge, UK). After washing with PBS containing 0.5% BSA (3 x 5 min), the preparations were incubated for 45 min with the following secondary antibodies (diluted 1:2000 in PBS containing 3% albumin): goat anti-guinea pig Alexa Fluor A488-conjugated (cat n. A11073), donkey anti-mouse Alexa Fluor A647-conjugated (cat n. A31571), and goat anti-rabbit AlexaFluor A555-conjugated (cat n. A21428) (Molecular Probes Europe, Leiden, The Netherlands).

For fluorescence image acquisition (512 x 512 x 8 bits) we used a three-channel Leica TCS SP5 confocal laser scanning microscope (excitation lines 458, 476, 488, 514, 543 and 633 nm) using a 63x oil immersion lens. Configuration for light collection was optimized for each combination of fluorochromes and sequential channel acquisition was performed to avoid crosstalk. For acquisition of images, we used the Leica "LAS AF" software package. Co-localized protein estimation was performed by calculating co-localization coefficients (Manders et al., 1993) and is expressed as mean ± SEM of three independent experiments (3 WT and 3 $SOD1^{G93A}$ mice) run in triplicate (3 replicates).

### 6.7 Cytosolic $Ca^{2+}$ concentration measurements

The intracellular concentration of cytosolic calcium $[Ca^{2+}]_c$ was determined in synaptosomes purified from spinal cords derived from 30-, 60-, and 90-day-old WT and $SOD1^{G93A}$ mice using the fluorescent dye fura 2-AM (Laemmli, 1970). Synaptosomes were incubated for 40 min at 37°C in physiological HEPES medium, under gentle stirring, in a medium containing $CaCl_2$ 20 μM and fura-2AM 5 μM (dissolved in 0.5% dimethylsulfoxide: DMSO; Sigma-Aldrich, St Louis, MO, USA). In order to measure auto-fluorescence, we used synaptosomes incubated only with 0.5% DMSO. After incubation, excess of fura 2-AM was removed by centrifugation and synaptosomes were resuspended in 4°C calcium-free, HEPES-buffered medium and then divided into 200 μL aliquots (200

μg proteins/sample) that were kept in ice till the measurement, which were carried out within 2 hours. Each synaptosomes aliquot was diluted in a final volume of 2 ml of physiological medium containing 1,2 mM $CaCl_2$ and equilibrated at 37°C for 15 min. Measurements were made at 37°C in a thermostated cuvette under continuous stirring using a double wavelength RF-5301PC spectrophotofluorimeter (Shimadzu Corporation, Milan, Italy), with the alternation of excitation wavelength between 340 nm and 380 nm, and emission monitored at 510 nm.

After recording basal fluorescence for 1 min, synaptosomes were exposed to 3,5-DHPG at the concentrations of 0.3 or 30 μM for another 10 min. Calibration was performed at the end of each measurement by the addition of ionomycin (10 mM ) together with $CaCl_2$ to obtain Fmax, followed by EDTA (10 mM) at pH 8.0 buffered with Tris (3 mM) to obtain Fmin. After correction for extracellular FURA, $[Ca^{2+}]_c$ was calculated with the equation of Grynkiewicz and collaborators (Grynkiewicz et al., 1985; using the $Ca^{2+}$/ fura-2 complex KD = 224 nM).

### 6.8 Astrocyte and microglia cell cultures and microglia treatment

Primary microglia cultures were obtained from mixed glial cultures prepared from the brains of mice at P2-5. After removing the meninges, brains were enzymatically (TrypLE Express™ Enzyme 1X, Thermo Fisher Scientific®) and mechanically dissociated and then plated on T75 flask in DMEM Gibco® (4.5 g/L D-glucose, L-glutamine, 25 mM HEPES, pyruvate and additioned with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin). After 24 hours, medium was completely replaced with fresh one. In the next 14 days, medium change occurs every 2-3 days. Once astrocytes reached the confluence and microglia grew on top, microglial cells were detached by shaking and seeded at a density of 30000 cells/well on poly-D-lysin (Sigma) pre-coated 96 well plates.

After microglia detaching, astrocytes were mantained in culture and they were exposed

every 2 days to the mGluR5 negative allosteric modulator 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine (CTEP) at 100 nM concentration (provided by Pharmacology and Toxicology Unit, Department of Pharmacy, University of Genoa).

At day 5, astrocytes were activated with TNF-alpha 30 ng/ml (Biologend®) in fresh medium for 24 hours as reported in literature, in the presence or in the absence of CTEP, as appropriate. At day 6, medium was changed with DMEM added with FBS exosomes-depleted medium (Thermo Fisher Scientific®), to eliminate the contribution of exosomes derived from FBS. After 24 hours astrocytes-derived EVs were isolated.

### 6.9 Extracellular vesicle isolation from astrocytes and microglia treatment

The ultracentrifugation protocol used for the extracellular vesicles isolation was obtained by Prof. Nunzio Iraci, University of Catania.

Astrocytes culture medium from three flasks per condition (not-treated astrocytes, astrocytes exposed to CTEP, astrocytes activated with TNF-α, astrocytes activated with TNF-α and exposed to CTEP ) was collected and centrifuged at 1,000 x g for 15 min at 4°C. The supernatant was collected in Thick wall tubes (355631 Beckman Coulter for rotor 70 Ti) and spin down at 100,000 x g for 130 min at 4°C. The pellet obtained was resuspended in PBS in small ultracentrifuge tubes (362305 Beckman Coulter) and spin down at 162,000 x g for 30 min at 4°C. The pellet, constituted by exosome-enriched extracellular vesicles, was resuspended in culture medium. Microglia was exposed to exosomes-containing culture medium for 24 hours. Exosomes originating from 15 astrocytes were used for each microglia cell.

### 6.10 RNA isolation and RT-qPCR

Both astrocytes and microglia cultures were added with trizol overnight at -20°C. Cells were scraped on ice and 20% of sample volume of chloroform was added. After vigorous

shaking for 15 s, samples allowed to stay at room temperature for 5 min. The suspension was centrifuged at 12,000 x g for 15 min at 4°C; the aqueous phase was transferred into other tubes and added with an equal volume of isopropanol, and incubated for 10 min at room temperature. The solution was centrifuged at 12,000 x g for 8 min at 4°C to precipitate the RNA, while the supernatant was discarded. The pellet was twice added with 1 ml of 75% EtOH in nuclease-free water and centrifuged at 7500 x g for 5 min at 4°C. The RNA pellet was air-dried for a few minutes and then 10 min at 37°C. The RNA was resuspended in 20 µl of RNAse free water and solubilized for 10 min at 55°C. RNA was treated with DNAse enzyme for 30 min at 37°C, which was inactivated adding EDTA 5 mM. RNA was quantified and total RNA (20 - 40 ng) was used in 20 - 40 µl of reverse transcription reaction (high-capacity cDNA Reverse Transcription Kit). The ΔΔCt method was applied to determine differences in gene expression levels after normalization to the arithmetic mean of Beta-2-Microglobulin (B2M), Large Ribosomal Protein (RPLP0) and Peptidylprolyl isomerase A (PPIA) used as housekeeping genes. The primer used to determine the pro- and antiinflammatory panel were for: interleukin-6 (IL-6), transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), inducible nitric oxide synthase (iNOS), cluster of differentiation 86 (CD86), interleukin-10 (IL-10), cluster of differentiation 163 (CD163), arginase 1 (Arg1), cluster of differentiation 206 (CD206), the macrophage protein YM1 and the cysteine-rich secreted protein (Fizz1). Real-time PCR was performed using ViiA7 (Applied Biosystems).

### 6.11 Statistics

Data presented in the graphs are expressed as mean ± SEM of the number of experiments reported in the figure legends. Normality of the data was assessed by the Shapiro-Wilk normality test and Grubbs' test was performed to eliminate outlier values. Comparison between two population means was performed by unpaired two-tailed student's t-test,

whereas when comparing more than two population means, data were first analysed with one or two-way ANOVA, as appropriate, and then by Bonferroni's or Dunnet's multiple comparison test with statistical significance set at $P < 0.05$. Analyses were carried out using the SigmaStat software (Systat Software 3.5, Inc., San Jose, CA, USA).

# 7

# RESULTS

As previously mentioned, Giribaldi et al. (2013), reported the effects of metabotropic Glu autoreceptors of group I on glutamate release at the end stage of SOD1$^{G93A}$ mice. They showed that the release of glutamate from purified spinal cord synaptosomes was differently regulated by glutamatergic group I metabotropic autoreceptors in 120 day-old SOD1$^{G93A}$ mice and age-matched wild type controls; in particular concentration of the mGluR1/5 agonist 3,5-DHPG above 0.3 μM stimulated the spontaneous neurotransmitter release both in ALS and control mice, while concentrations of the agonist ≤ 0.3 μM increased glutamate release in SOD1$^{G93A}$ mice only. Then, to understand which receptor subtype was involved in the increase of glutamate release, they performed release experiments in the presence of selective mGluR1 and mGluR5 antagonists that were both able to antagonize the effect of 3,5-DHPG, indicating the implication of both receptors, although to a different extent. In addition, it was demonstrated the vesicular origin of the glutamate release and the involvement of internal calcium. Confocal microscopy imaging analysis on spinal cord synaptosomes highlighted the co-existence of both receptor subtypes on glutamatergic nerve terminals and excluded their possible localization at the postsynaptic levels; indeed only few mGluR1 and mGluR5 positive synaptosomal particles were also positive for PSD95 (a specific postsynaptic density marker). Western blot permitted to quantify the receptors expression and showed that there were no differences for mGluR1 between WT and SOD1$^{G93A}$, whereas mGluR5 levels were significantly higher in ALS mice.

To understand at which age the observed abnormal activity of the release-regulating group I mGlu receptors occurs and if it could be a possible cause/concause of neuronal damage or

a consequence of the disease, we investigated the effects of the mixed mGluR1/5 agonist 3,5-DHPG on the release of glutamate, monitored by using the non-metabolizable analogue of glutamate [$^3$H]D-aspartate, from purified synaptosomes isolated from SOD1$^{G93A}$ and WT mice spinal cord at 30, 60 and 90 days of life, corresponding to the pre-symptomatic and early symptomatic phase of the pathology.

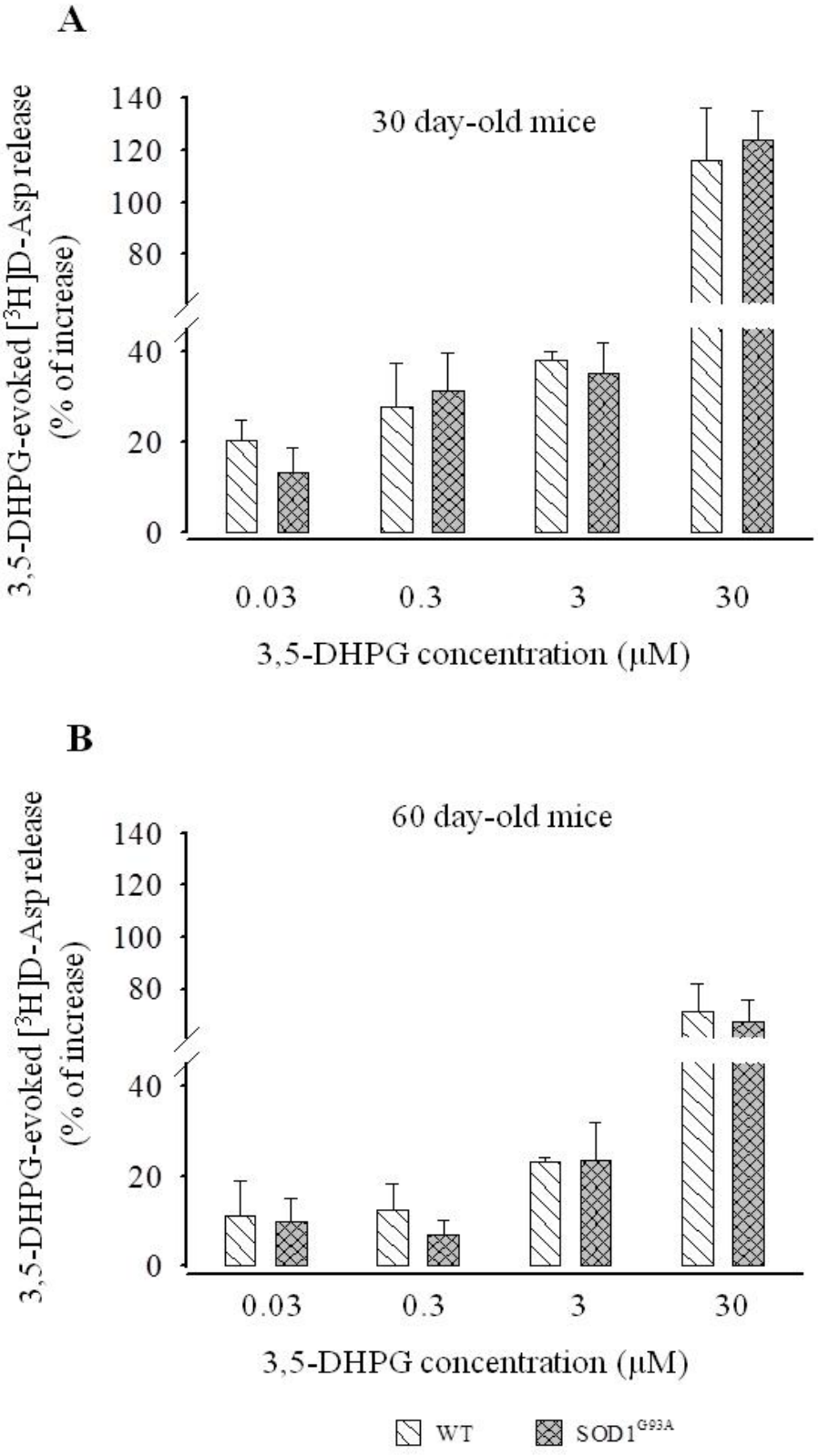
The following results have been published in the article entitled "Enhanced Function and Overexpression of Metabotropic Glutamate Receptors 1 and 5 in the Spinal Cord of the SOD1$^{G93A}$ Mouse Model of Amyotrophic Lateral Sclerosis during Disease Progression" of which I am co-author (Bonifacino et al., 2019b).

### 7.1 Glutamate release induced by 3,5-DHPG during disease progression in SOD1$^{G93A}$ mice

Release experiments were performed on purified spinal cord synaptosomes isolated from SOD1$^{G93A}$ mice and age-matched control animals at the three different stages of the disease described before. When synaptosomes were exposed to increasing concentration of 3,5-DHPG (0.03 - 0.3 - 3 - 30 μM), a concentration-dependent increase of [$^3$H]D-Asp release was observed in both animal groups. Labelling by [$^3$H]D-Asp did not significantly differ between control and SOD1$^{G93A}$ mice when normalized for synaptosomal protein content. During the experiments, five 3 min samples were collected and counted for radioactivity, analyzing variations of the basal release after 3,5-DHPG exposure. No differences in the 3,5-DHPG effects on [$^3$H]D-Asp release were observed in WT and SOD1$^{G93A}$ pre-symptomatic mice at 30 (Figure 1A; $F_{(1,3,3,33)} = 0.189$) or 60 (Figure 1B; $F_{(1,3,3,17)} = 0.0423$) days of life. On the contrary, when the effects of the mixed mGluR1/5 agonist were analysed on the release from purified synaptosomes obtained from 90 day-old mice, we found a significant difference between WT and SOD1$^{G93A}$ mice. In fact, at the concentration of 0.3 μM 3,5-DHPG significantly increased [$^3$H]D-Asp release in SOD1$^{G93A}$ mice by approximately 25%, while it was ineffective in control animals (Figure

1C; * $p < 0.05$; $F_{(1,3,3,48)} = 1.502$).

These data show that glutamate release induced by 3,5-DHPG abnormally increased in $SOD1^{G93A}$ mice concomitantly with early clinical symptoms manifestation, while there are no statistically significant differences during the first stages of the disease, when the pathological signs are still latent and motor functions are still preserved.


A
30 day-old mice
3,5-DHPG-evoked [3H]D-Asp release (% of increase)
140
120
100
80
40
20
0
0.03
0.3
3
30
3,5-DHPG concentration (μM)
B
60 day-old mice
3,5-DHPG-evoked [3H]D-Asp release (% of increase)
140
120
100
80
40
20
0
0.03
0.3
3
30
3,5-DHPG concentration (μM)
WT
SOD1G93A

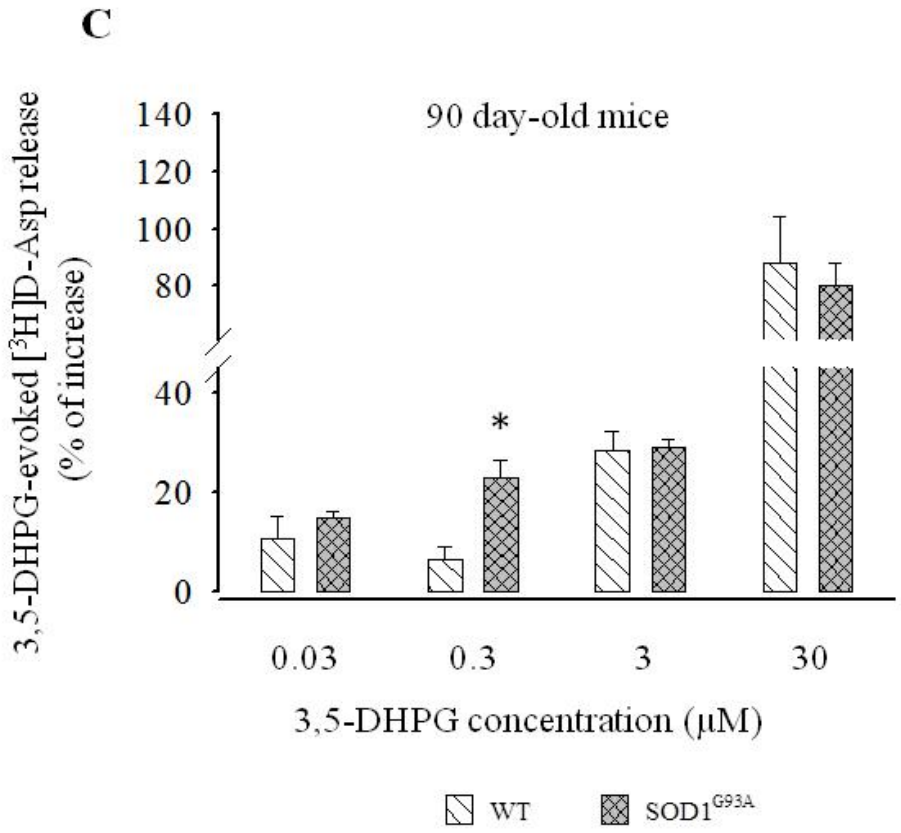



**Figure 1.** ***Effects of 3,5-DHPG on [$^3$H]D-ASP release from spinal cord synaptosomes of SOD1$^{G93A}$ mice at different stages of the pathology.*** The effect of (S)-3,5-dihydroxyphenylglycine (3,5-DHPG) was measured on the spontaneous release of glutamate, monitored as [$^3$H]D-Asp, from spinal cord synaptosomes prepared from SOD1$^{G93A}$ mice at the pre-symptomatic phase (30 and 60 days of life; panels A and B) and at disease onset (90 days of life; panel C). WT animals at the same age were used as controls. Synaptosomes were pre incubated with [$^3$H]D-Asp, to label the intra-terminal releasing pools of glutamate, and exposed during superfusion experiments to increasing concentrations (0.03, 0.3, 3, and 30 μM) of 3,5-DHPG. Bars expressed the percentage increase over control basal release. The data reported are the means ± SEM of five independent experiments (n=5 mice per each type of animals). *$p < 0.05$ vs. wild type mice (two-way ANOVA followed by Bonferroni's post-hoc test).

### 7.2 Pharmacological characterization of the receptor subtypes responsible for the increased release of [$^3$H]D-Asp induced by 3,5-DHPG

Since the abnormal [$^3$H]D-Asp release induced by 0.3 μM 3,5-DHPG just occurred in 90-day-old SOD1$^{G93A}$ mice, the other experiments planned to characterize the mechanisms through which this effect happens, were carried out at disease onset.

In order to identify the subtype of the metabotropic receptors involved in the 3,5-DHPG-evoked release of [$^3$H]D-Asp, we used the competitive mGluR1 antagonist (S)-(+)-a-

amino-4-carboxy-2-methylbenzeneacetic acid (LY367385; Bruno et al., 1999) and the mGluR5 negative allosteric modulator 2-methyl-6-(phenylethynyl)pyridine (MPEP; Gasparini et al., 1999).

Spinal cord synaptosomes from SOD1$^{G93A}$ mice were, therefore, exposed in superfusion to 1 μM of the selective antagonists 8 min before and concomitantly with the 0.3 μM 3,5-DHPG challenge.

As shown in Figure 2, the 0.3 μM 3,5-DHPG-induced glutamate release was strongly reduced by both antagonists (*$p < 0.05$; $F_{(4,9)} = 8.422$), demonstrating that activation of both mGluR1 and mGluR5 triggers the abnormal increase of glutamate release observed at disease onset.

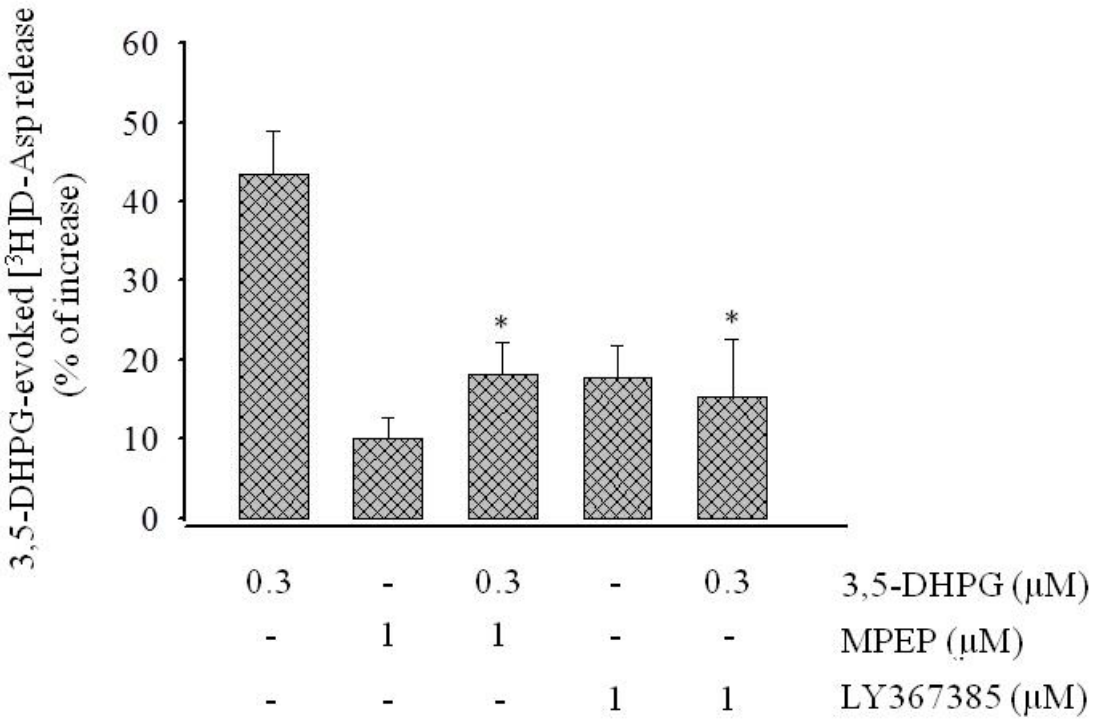



**Figure 2.** ***Effects of the mGluR1 and mGluR5 antagonists on the release of [$^3$H]D-Asp induced by 3,5-DHPG in spinal cord synaptosomes of 90-day SOD1$^{G93A}$ mice.*** The effects of the selective antagonists for mGluR1 (LY367385) and mGluR5 (MPEP) were tested on the increased glutamate release induced by 0.3 μM 3,5-DHPG. During superfusion experiments, synaptosomes were exposed to 1 μM LY367385 or 1 μM MPEP to study the receptor subtypes contribution. The data reported are the means ± SEM of five independent experiments (n=5 mice per group) conducted in triplicate (three superfusion chambers for each experimental condition). The effect of 0.3 μM 3,5-DHPG was reduced by the exposure to both LY367385 and MPEP in SOD1$^{G93A}$ mice (*$p < 0.05$ vs. 3,5-DHPG; one-way ANOVA followed by Bonferroni's post-hoc test).

### 7.3 Effect of 3,5-DHPG on intracellular calcium concentration in SOD1$^{G93A}$ and control WT mice at the onset of the disease

It is well known that Group I metabotropic glutamate receptors acts through the activation of $G_q$ proteins that lead to the hydrolysis of phosphatidylinositol 3-phosphate by Phospholipase C. This process generates the second messengers diacylglycerol and inositol 3-phosphate, the latter rapidly translocating from the membrane to the endoplasmic reticulum where it acts on its own specific receptors, allowing the exit of calcium ions into the cytosol (Pin & Acher, 2002).

Therefore, we wanted to investigate whether the release of glutamate induced by 3,5-DHPG in spinal cord synaptosomes from 90-day-old WT and SOD1$^{G93A}$ mice, was accompanied by an increase in the cytosolic calcium concentration ($[Ca^{2+}]_C$).

The intracellular calcium levels were monitored both under basal conditions and following the exposure to 3,5-DHPG. Synaptosomes were incubated with the fura-2-acetoxymethyl ester (FURA 2-AM) fluorescent dye and were then exposed to increasing concentrations of 3,5-DHPG (0.3, 3 and 30 μM).

The data obtained show that the basal intracellular calcium concentration was significantly more elevated in SOD1$^{G93A}$ with respect to WT mice (Figure 3, first bar group from the left; * $p < 0.001$; $F_{(1,3,3,30)} = 11.154$). When synaptosomes were exposed to 3,5-DHPG, the agonist evoked a $[Ca^{2+}]_C$ increase in a concentration-dependent manner in both animal groups (Figure 4; *$p < 0.001$; $F_{(1,3,3,30)} = 11.154$). However, the increase in cytosolic calcium levels produced by 3,5-DHPG was statistically greater in SOD1$^{G93A}$ mice than in WT mice.

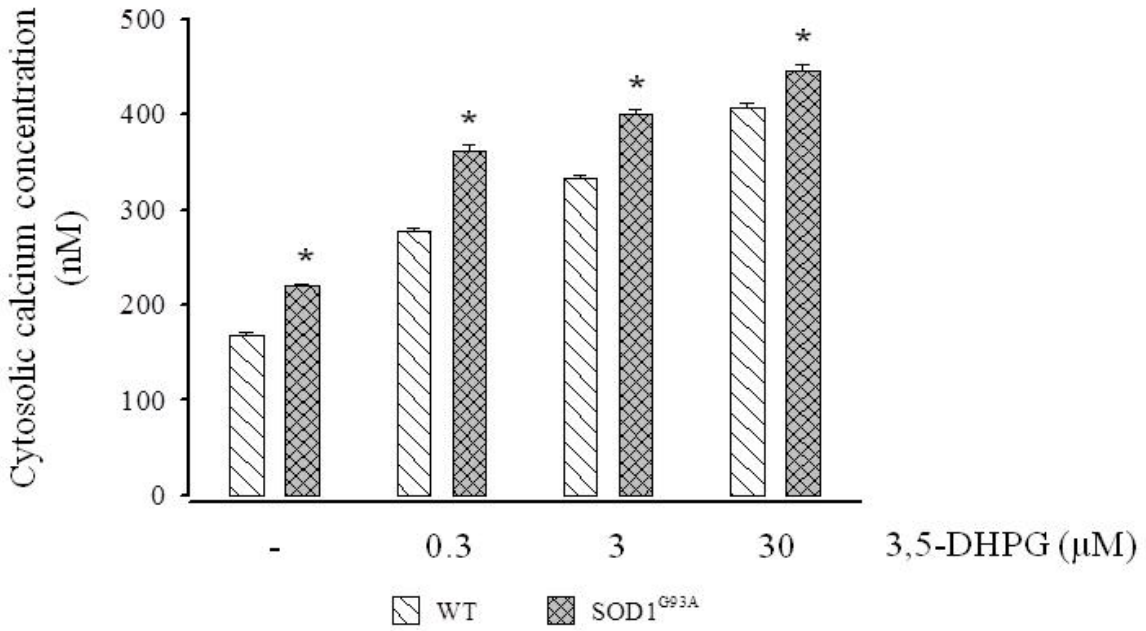



**Figure 3.** ***Effect of 3,5-DHPG exposure on intracellular calcium concentration in spinal cord synaptosomes of 90-day SOD1$^{G93A}$ and WT mice.*** Cytosolic [$Ca^{2+}$] concentrations before and after 3,5-DHPG challenge were measured in purified spinal cord synaptosomes obtained from early symptomatic SOD1$^{G93A}$ and age-matched WT control mice. Following labelling with fura-2-acetoxymethyl ester (Fura 2-AM), synaptosomes, were exposed to standard medium or to 0.3, 3, and 30 μM 3,5-DHPG, as described in the methods. Data are expressed as mean ± SEM of three independent experiments (n=3 mice per group) conducted in triplicate (three replications for each experimental condition). * $p < 0.001$ vs. WT mice (two-way ANOVA followed by Bonferroni's post-hoc test).

### 7.4 Intracellular mechanisms involved in the modulation of the glutamate release induced by 3,5-DHPG in SOD1$^{G93A}$ mice at disease onset

In order to identify the cellular mechanisms underlying the 0.3 μM 3,5-DHPG-induced [$^3$H]D-Asp release from spinal cord synaptosomes of 90 days SOD1$^{G93A}$, we performed additional experiments under the following conditions:

- absence of extracellular $Ca^{2+}$;
- intracellular calcium sequestration by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a $Ca^{2+}$ chelator;
- blockade of phospholipase C (PLC) by the selective inhibitor 1-[6-[((17)-3-

methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122; Bleasdale et al., 1990);

- blockade of the IP3 receptor by the selective antagonist 2-aminoethoxydiphenyl borate (2-APB; Maruyama et al., 1997);
- depletion of the vesicular neurotransmitter content in synaptic vesicles by bafilomycin A1, a vesicular ATPase inhibitor (Bowman et al., 1988).

When synaptosomes were perfused with a $Ca^{2+}$-free perfusion medium, 3,5-DHPG was still able to fully increase the release of [$^3$H]D-Asp (Fig. 4). In contrast, when synaptosomes were pre-incubated with BAPTA-AM (100 μM) to load and entrap the calcium chelator into nerve terminal cytosol, the effect of 3,5-DHPG on [$^3$H]D-Asp release was abolished (Figure 4; $*p < 0.001$; $F_{(5,28)} = 567.116$). Similarly, the 3,5-DHPG-induced increase of release [$^3$H]D-Asp was also prevented by 1 μM U73122 or by 10 μM 2-APB (Figure 5; $*p < 0.001$; $F_{(5,28)}=567.116$). Finally, the effect induced by 3,5-DHPG was also abolished by the pre-incubation of synaptosomes with 0.1 μM bafilomycin A1 ($* p < 0.001$; $F_{(5,28)} = 567.116$).

Overall, these data demonstrate that the abnormal effect of 3,5-DHPG in 90-day-old $SOD1^{G93A}$ mice is mediated by the classical pathway coupled to glutamatergic group I metabotropic receptors, that is activation of PLC, production of IP3, induction of calcium release from endoplasmic reticulum stores and activation of synaptic vesicle exocytosis.

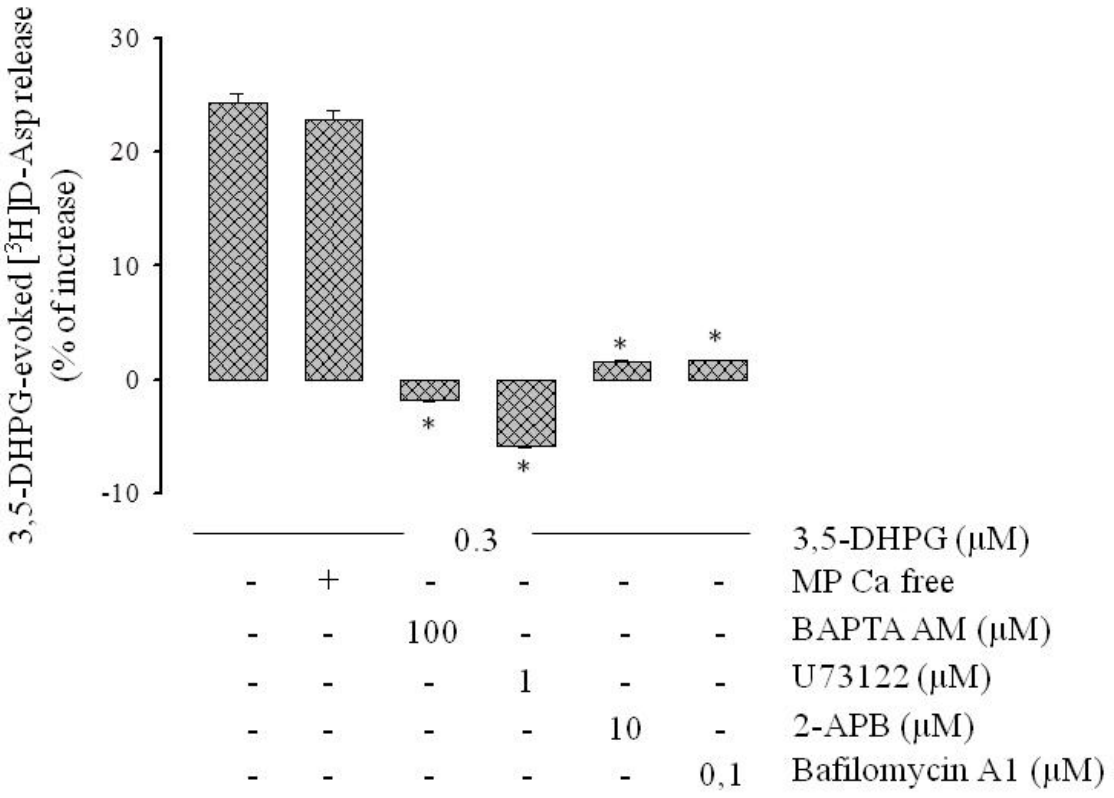



**Figure 4.** ***Study of the mechanisms supporting the [$^3$H]D-Asp release induced by 3,5-DHPG in spinal cord synaptosomes isolated from 90-days SOD1$^{G93A}$ mice.*** The graph reports the effects of 0.3 μM 3,5-DHPG in the absence of extracellular calcium or in the presence of calcium chelator BAPTA-AM (100 μM), of the phospholipase C inhibitor U73122 (1 μM), the IP3 receptor blocker 2-APB (10 μM) or the V-ATPase inhibitor bafilomycin A1 (0.1 μM). The incubation before release experiments with BAPTA-AM or bafilomycin A1 did not significantly affect synaptosomal labelling by [$^3$H]D-Asp. The results are expressed as percent increase with respect to basal glutamate release. Data are expressed as mean ± SEM of four independent experiments (n=4 mice per group) conducted in triplicate (three superfusion chambers for each experimental condition). *$p$ < 0.001 vs. the effects of 3,5-DHPG (one-way ANOVA followed by the Bonferroni post-hoc test).

## 7.5 Group I metabotropic glutamate receptors expression in spinal cord synaptosomes from SOD1$^{G93A}$ mice during disease progression

In order to evaluate changes in mGluR1 and mGluR5 expression during disease progression, we performed Western blot experiments on protein extracts derived from spinal cord synaptosomes prepared from SOD1$^{G93A}$ and age matched WT mice of 30, 60 and 90 days of age.

The results obtained showed no significant changes in the expression of group I metabotropic glutamate receptors. Both mGluR1 (Figure 5) and mGluR5 (Figure 6) did not change in SOD1$^{G93A}$ mice at any of the time points analysed during the development of the disease.

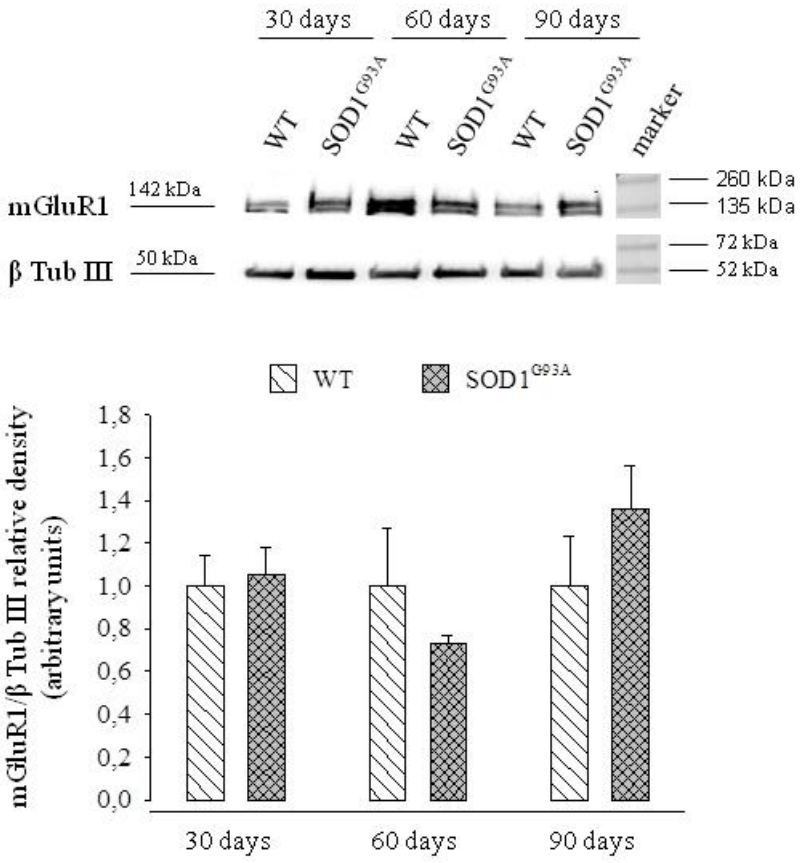



**Figure 5.** ***Expression of mGluR1 in purified spinal cord synaptosomes of SOD1$^{G93A}$ and WT mice at presymptomatic stages and at the clinical onset of the disease.*** The expression of mGluR1 was measured by western blot on purified synaptosomes obtained from the spinal cord of mice at 30, 60 and 90 days of life. Representative immunoreactive bands are shown (top). mGluR1 expression levels were normalized for β-tubulin III in the same blotted membrane and data quantification is reported in the bar graph. Results are expressed as the relative density and the expression of mGluR1 in WT synaptosomes is referred to as 1.00. Data are expressed as mean ± SEM of 3 independent experiments (n=3 mice per group) conducted in triplicate (3 experimental replicates). No significant differences were detected in mGluR1 expression between the two experimental groups (two-tailed Student's t-test).

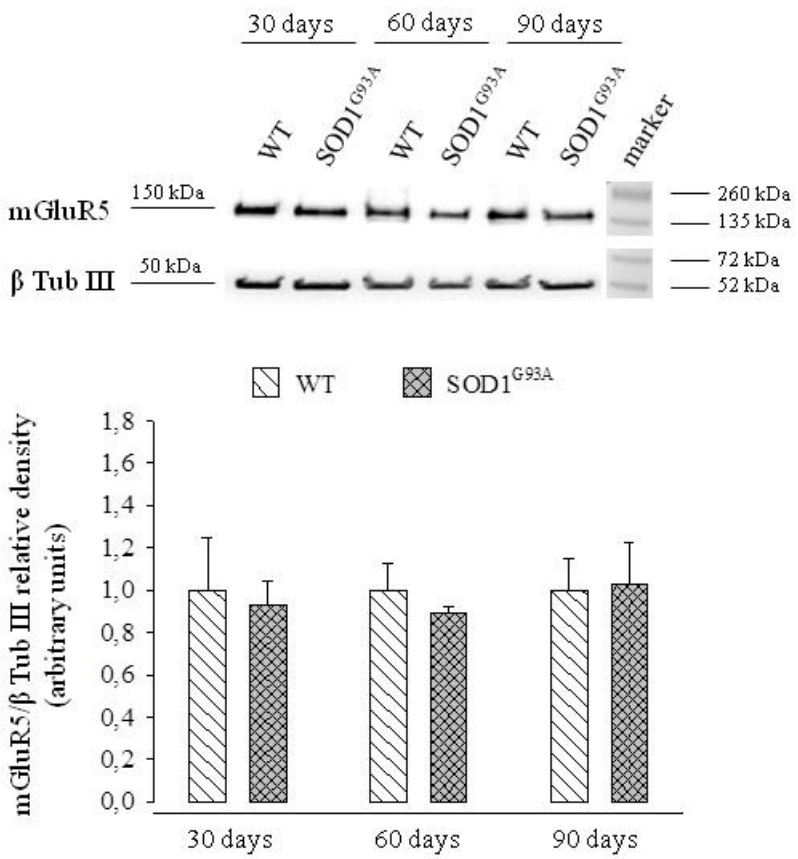



**Figure 6.** ***Expression of mGluR5 in purified spinal cord synaptosomes of SOD1$^{G93A}$ and WT mice at presymptomatic stages and at the clinical onset of the disease.*** The expression of mGluR5 was measured by western blot on purified synaptosomes obtained from the spinal cord of mice at 30, 60 and 90 days of life. Representative immunoreactive bands are shown (top). mGluR5 expression levels was normalized for β-tubulin III in the same blotted membrane and the data qunatification is reported in the bar graph . Results are expressed as the relative density and the expression of mGluR5 in WT synaptosomes is referred to as 1.00. Data are expressed as mean ± SEM of 3 independent experiments (n=3 mice per group) conducted in triplicate (3 experimental replicates). Similarly to mGluR1, no significant differences were detected in mGluR5 expression between the two experimental groups (two-tailed Student's t-test).

However, it must be taken into account that total synaptosomal lysates may include heterogeneous populations of spinal cord synaptic terminals with different expression levels of metabotropic receptors. Therefore, to circumvent this possible bias, we performed immunofluorescence analysis to verify the levels of mGluR1 and mGluR5 expressed onto glutamatergic synaptosomes, herein after referred to as metabotropic autoreceptors, during disease progression. To this purpose, we carried out immunofluorescence labelling of purified spinal cord synaptosomes with antibodies for mGluR1 and mGluR5 and

antibodies for the vesicular glutamate transporter type 1 (vGluT1), and then imaged signal co-localization by confocal microscopy. Since synaptosomes were efficiently labelled by all the antibodies used and the expression of vGluT1 did not vary between control and $SOD1^{G93A}$ mice, a very accurate analysis of the relative fluorescence intensity of the mGluR1 and mGluR5 co-localized with vGluT1 was possible.

The image analysis showed that the expression of both metabotropic autoreceptor subtypes was not significantly changed at the presymptomatic stage in $SOD1^{G93A}$ mice compared to WT controls (Figure 7). In contrast, both mGlu1 and mGlu5 autoreceptors were significantly increased in symptomatic $SOD1^{G93A}$ mice of 90 days of life (Figure 8; mGluR1, $*p < 0.001$, $t_{(4)} = -12.021$; mGluR5, $*p < 0.001$, $t_{(4)} = -12.728$).

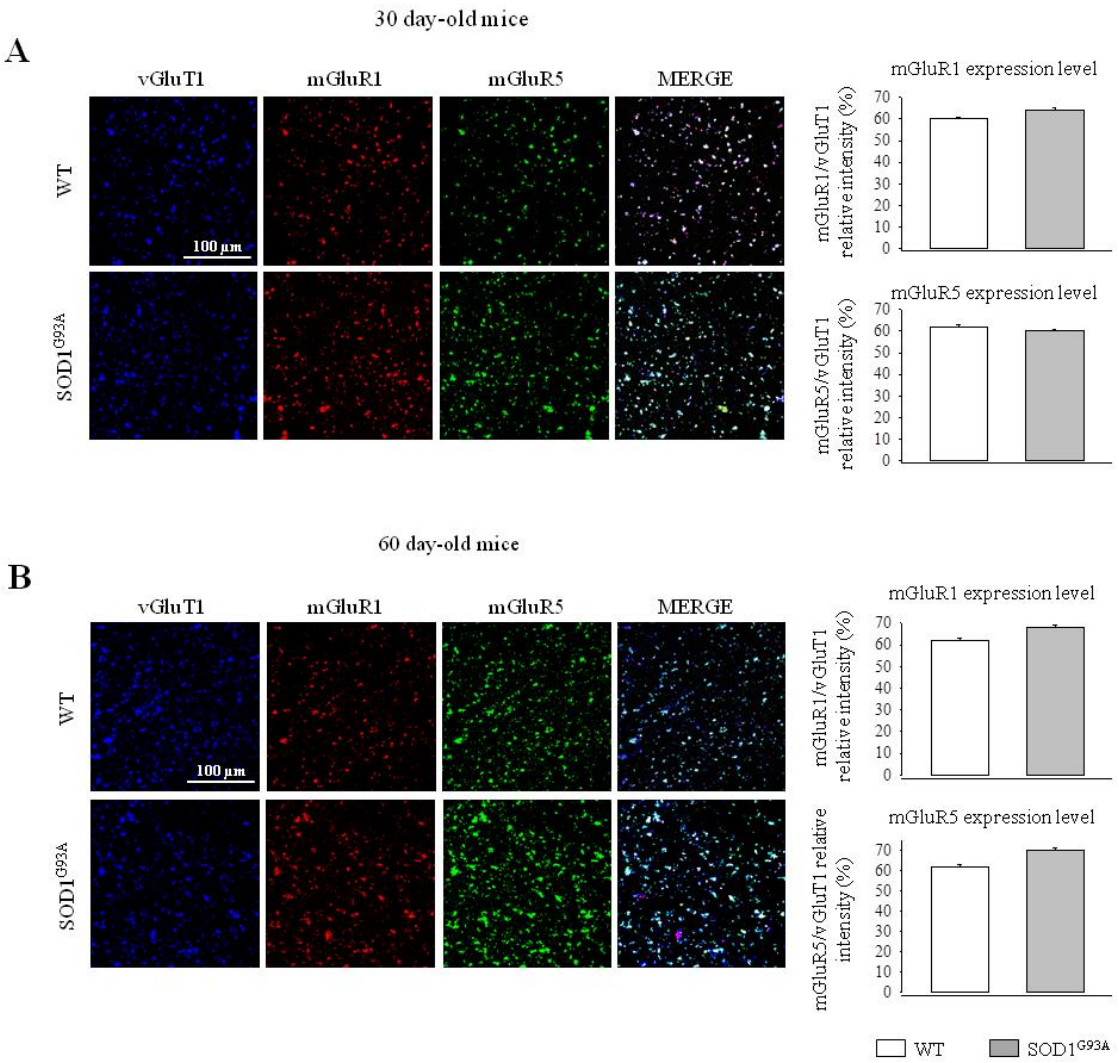



**Figure 7.** ***mGlu1 and mGlu5 autoreceptors in spinal cord synaptosomes of SOD1$^{G93A}$ and WT mice at pre-symptomatic stage of the disease.*** Synaptosomes have been obtained from SOD1$^{G93A}$ and WT mice of 30 (**A**) and 60 (**B**) days of life, corresponding to the pre-symptomatic phases of the pathology. After purification, synaptosomes were stratified on coverslips, fixed with paraformaldehyde, permeabilized with triton X-100, incubated with specific primary and secondary antibodies, and analysed by laser confocal microscopy. These representative images show triple-stained immunopositivity for vGluT1 (blue; representing glutamatergic synaptosomes), mGluR1 (red) and mGluR5 (green). The merge panels show the co-expression of the three proteins. The bar graphs indicate the quantitative relative florescence intensity of mGluR1 or mGluR5 on vGluT1-positive glutamatergic spinal cord synaptosomes. The bars represent the mean ± SEM of 3 independent experiments (n=3 mice per group) conducted in triplicate (3 experimental replicates).

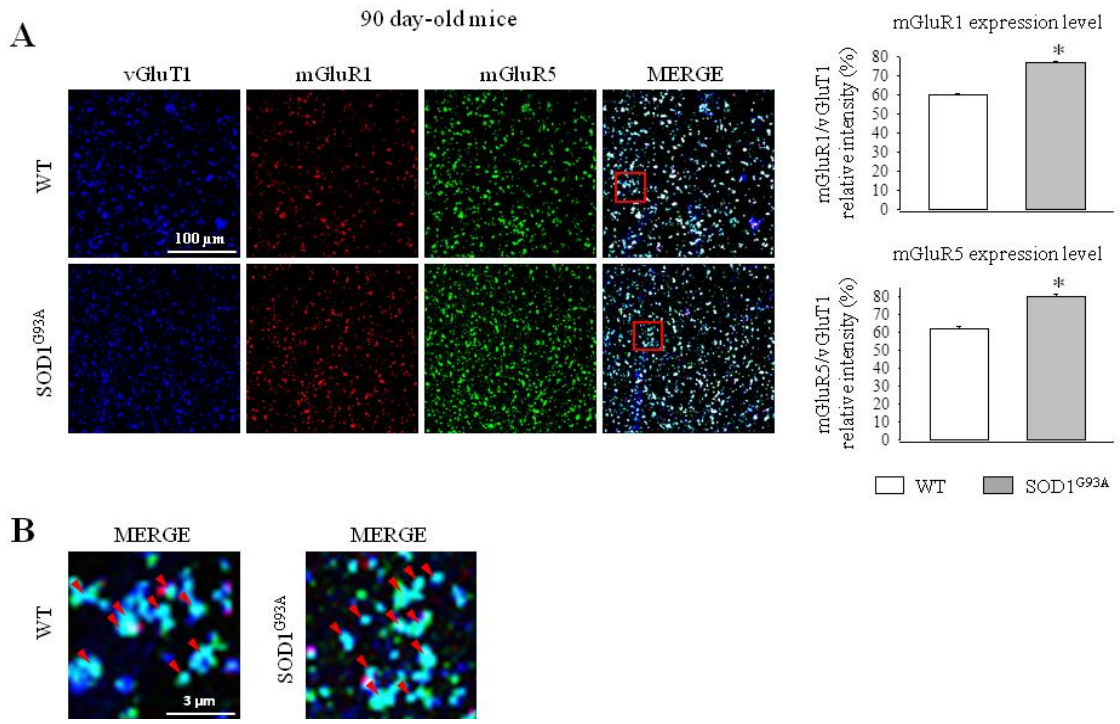



**Figura 8.** ***mGlu1 and mGlu5 autoreceptors in spinal cord synaptosomes of SOD1$^{G93A}$ and WT mice at early symptomatic stage of the disease.*** SOD1$^{G93A}$ and WT mice of 90 days of life, corresponding to the early symptomatic phase of the pathology, were used for these experiments. Experimental details are the same as in figure 7. (**A**) mGluR1 and mGluR5 autoreceptors were significantly over-expressed in synaptosomes purified from 90-day-old SOD1$^{G93A}$ mice (*$p < 0.001$ vs. WT mice; two-tailed Student's t-test). (**B**): magnification of the red frames in merge panels of spinal cord synaptosomes purified from 90-day-old WT and SOD1$^{G93A}$ mice. Red arrowheads point to vGluT1, mGluR1, and mGluR5 triple-stained synaptosomes.

### 7.6 Expression of mGluR1 and mGluR5 in spinal cord total tissue homogenate from SOD1$^{G93A}$ mice during disease progression

It is well known that mGluR1 and mGluR5 are present both in neurons, at pre- and post-synaptic levels, and in non-neural cells, such as astrocytes, microglia and oligodendrocytes (Biber et al., 1999; Luyt et al., 2003; Panatier & Robitaille, 2016).

The data reported above indicate that the expression of mGluR1 and mGluR5 does not change significantly in the total population of synaptosomes obtained from the spinal cord of SOD1$^{G93A}$ mice before disease onset. However, we found that their expression is upregulated at glutamatergic axon terminals, concomitantly with the manifestation of early clinical symptoms.

To verify if these receptors were also over-expressed even in non-presynaptic sites (for

example on post-synaptic terminals and/or non-neuronal cells in the ALS mouse model, we performed western blot experiments to measure their expression on protein extracted from whole spinal cord tissue of both pre- and early-symptomatic SOD1$^{G93A}$ and WT mice.

With regards to mGluR1, the results showed that its expression did not change in SOD1$^{G93A}$ mice of 30 and 90 days of life, whereas a significant increase was observed at 60 days (Figure 9; $*p < 0.05$, $t_{(4)} = -3.148$). Instead, the expression of mGluR5 was significantly over-expressed in SOD1$^{G93A}$ mice both at 60 and 90 days of life (Figure 10; $*p < 0.05$, $t_{(8)} = -2.615$ and $* p < 0.05$, $t_{(6)} = -3.374$, respectively).

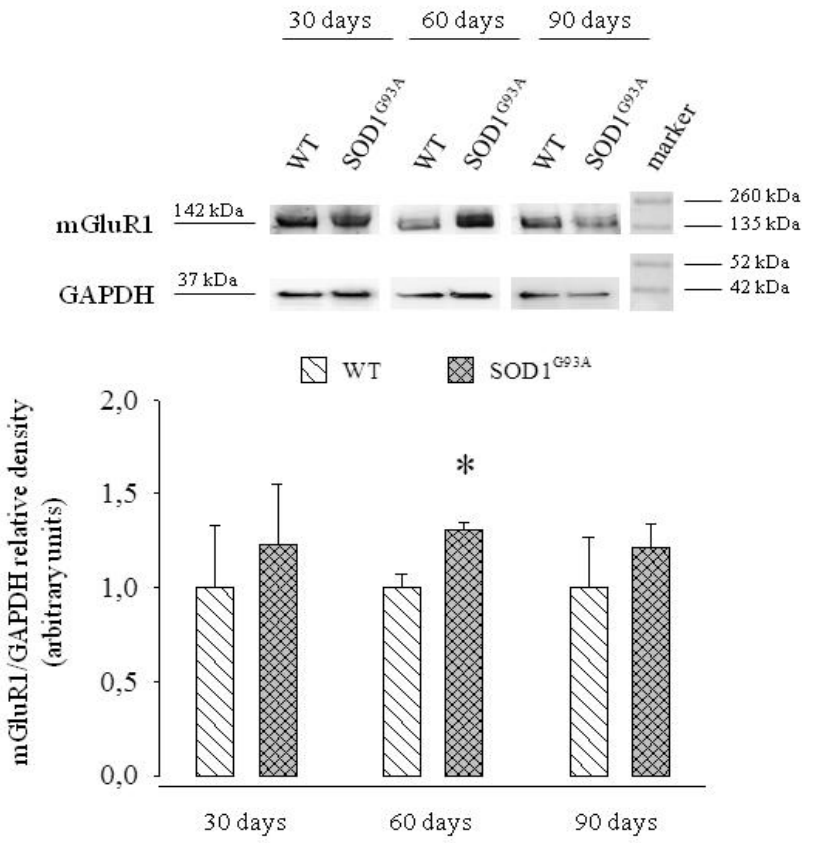



**Figure 9.** ***Changes in the expression of mGluR1 in spinal cord homogenates of SOD1$^{G93A}$ and WT mice at presymptomatic stages and at the clinical onset of the disease.*** Western blot analysis was performed on homogenate samples obtained from spinal cord of 30-, 60- and 90-day-old SOD1$^{G93A}$ and age-matched WT mice. The expression levels of mGluR1 was normalized for GAPDH acquired on the same membrane and the quantification is reported in the bar graph. mGluR1 level is expressed as the relative density of SOD1$^{G93A}$ with respect to WT mouse bands, which is referred to as 1.00. Data are expressed as mean ± SEM of 3 independent experiments (n=3 mice per group) conducted in triplicate (3 experimental replicates). $*p < 0.05$ vs. WT mice (two-tailed Student's t-test).

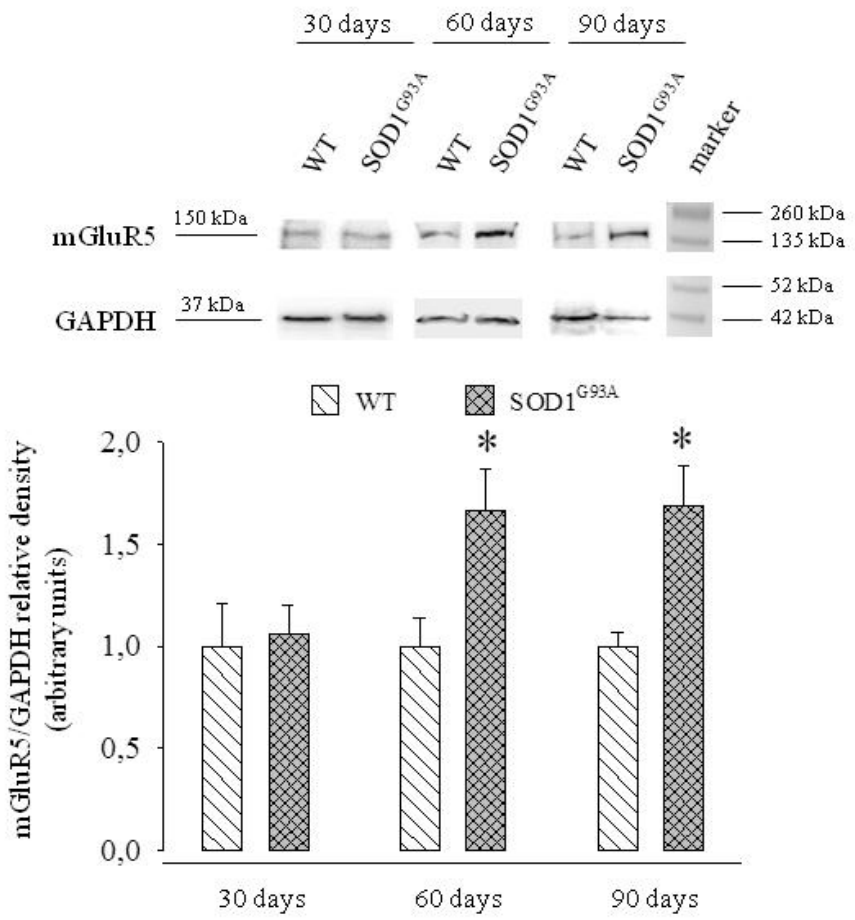



**Figure 10.** ***Changes in the expression of mGluR5 in spinal cord homogenate of SOD1$^{G93A}$ and WT mice at presymptomatic stages and at the clinical onset of the disease.*** Western blot analysis was performed on homogenate samples obtained from spinal cord of 30-, 60- and 90-day-old SOD1$^{G93A}$ and age-matched WT mice. The expression levels of mGluR5 was normalized for GAPDH acquired on the same membrane and the quantification is reported in the bar graph. mGluR5 level is expressed as the relative density of SOD1$^{G93A}$ with respect to WT mouse bands, which is referred to as 1.00. Data are expressed as mean ± SEM of 3 independent experiments (n=3 mice per group) conducted in triplicate (3 experimental replicates). *$p < 0.05$ vs. WT mice (two-tailed Student's t-test).

## 7.7 Preliminary results on the role of astrocytes-derived exosomes on microglial activation

In this section, I have reported the preliminary results obtained during my research stage in Prof. Paolicelli's laboratory at the University of Lausanne, regarding the astrocyte-to-microglia signaling via extracellular vesicles.

### *7.7.1 Efficiency of the EVs isolation protocol*

Western blot analyses were conducted to demonstrate that EVs enriched with exosomes were contained within the isolated fraction. We have used an ultracentrifugation protocol that permits to isolate EVs from a comparable amount of primary cultured astrocytes and BV2 microglial cells in order to evaluate the efficiency of the method in different cell types. To characterize the isolated fraction, we used rabbit monoclonal anti-Alix and mouse monoclonal anti-CD9 antibodies as specific markers for EVs, and rabbit polyclonal anti-Calnexin antibody as a cellular marker.

The analysis showed that the astrocytic EVs presented the characteristic band at the level of molecular weights of the specific marker Alix and CD9, while there were no detectable bands related to Calnexin (Figure 11). This result evidenced the adequacy of the protocol used in producing an EVs sample consisting mainly of exosomes purified from the cellular fraction.

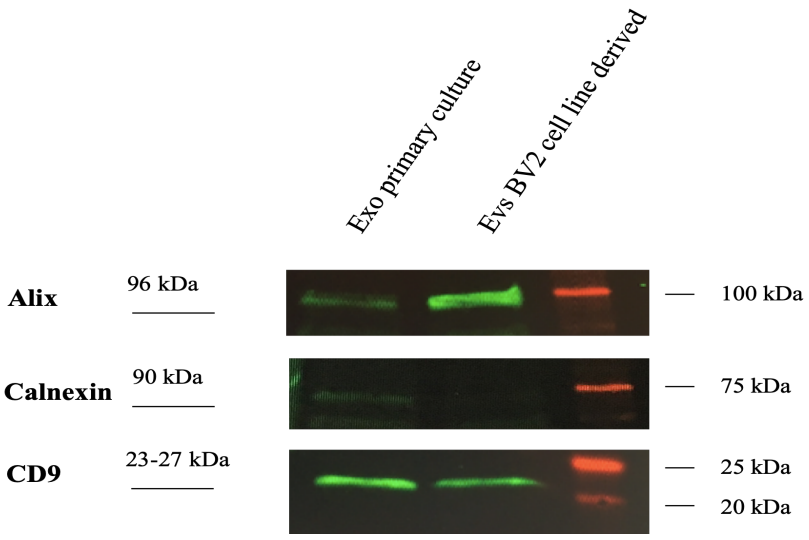



**Figure 11.** ***Expression of EVs and cellular markers in EVs fraction isolated from WT primary astrocytes cultures and BV2 cellular line.*** Western blot was performed on the isolated fraction derived from primary cultured astrocytes and BV2 cell line to demonstrate the efficiency of the isolation protocol for EVs.

### *7.7.2 TNF-α activation and CTEP exposure of primary astrocytes cultures*

WT primary astrocytic cultures were divided in four different experimental groups:

- Controls (no treatment);
- TNF-α activation (30 ng/ml) for 24 hours;
- CTEP (100 nM) treatment for 5 days, after microglia detachment and before EVs isolation;
- CTEP (100 nM) treatment for 5 days and TNF-α activation for 24 hours before EVs isolation.

To verify whether the treatment with TNF-α was able to promote an inflammatory phenotype in astrocytes and whether mGluR5 antagonism by CTEP could change the inflammatory phenotype, RT-PCR was performed to measure a panel of pro- and anti-inflammatory cytokine genes in RNA isolated from the different experimental groups.

However, the results obtained were too variable and difficult to interpret at the moment (Figure 12). Representative in this sense is the fact that in TNF-α-activated astrocytes, the expression of TNF-α itself was not significantly increased. Such a high variability suggests that probably the protocol must be ameliorated to optimize the activation of astrocytes.

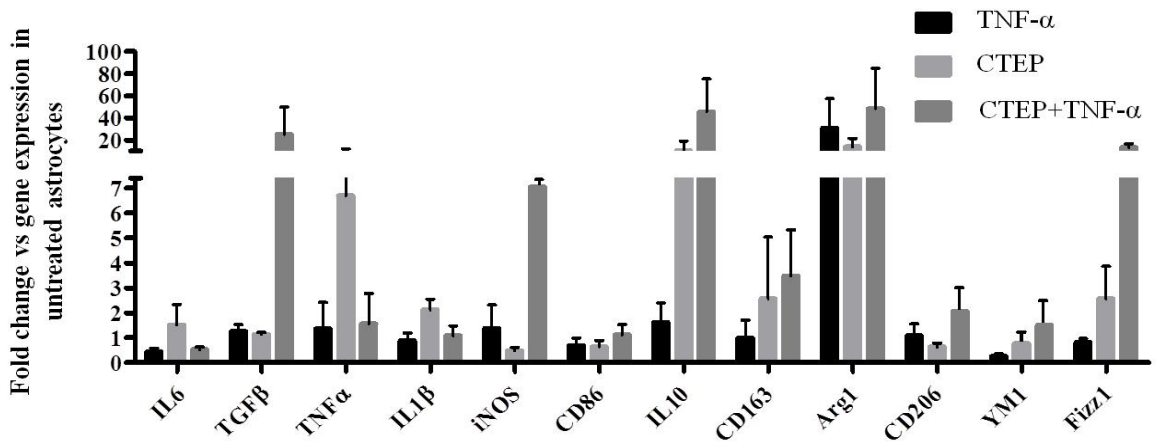



**Figure 12.** ***RT-PCR on the expression of pro and anti inflammatory markers in astrocytes.*** The semi-quantitative PCR analysis was performed to quantify the RNA extracted from WT primary cultured astrocytes, activated with TNF-α (30 ng/ml) for 24 hours, exposed to CTEP 100 nM or both. Results are expressed as fold change with respect to gene expression in RNA derived from untreated primary cultured astrocytes which we refer as 1.

### *7.7.3 Effects of EVs treatment on microglial cells*

Isolated EVs were used to treat microglia to evaluate if there were differences in the microglia inflammatory gene expressions. In particular, we analyzed changes between control microglia, microglia exposed to EVs isolated from untreated astrocytes and microglia exposed to EVs isolated from astrocytes treated with TNFα (Figure 13 A). We also analyzed changes in microglia exposed to EVs derived from astrocytes treated with TNF-α, CTEP or both in comparison to those isolated from untreated astrocytes (Figure 13 B).

It is worth noting that there are differences in the expression of several genes in microglia treated with EVs obtained from untreated astrocytes; in particular, we observed a significant increase in the expression of the pro-inflammatory IL-6, TNF-α, IL-1β and iNOS, while only the expression of Arg1 improved in the panel of anti-inflammatory molecules (Figure 13 A). Disappointingly, no relevant differences were observed between microglia treated with EVs derived from untreated astrocytes or from astrocytes activated with TNF-α (Figure 13 A). Similarly, no significant differences were observed in microglia

cultures treated with EVs isolated from astrocytes exposed to TNF-α, CTEP or the combination of TNF-α + CTEP (Figure 13 B).

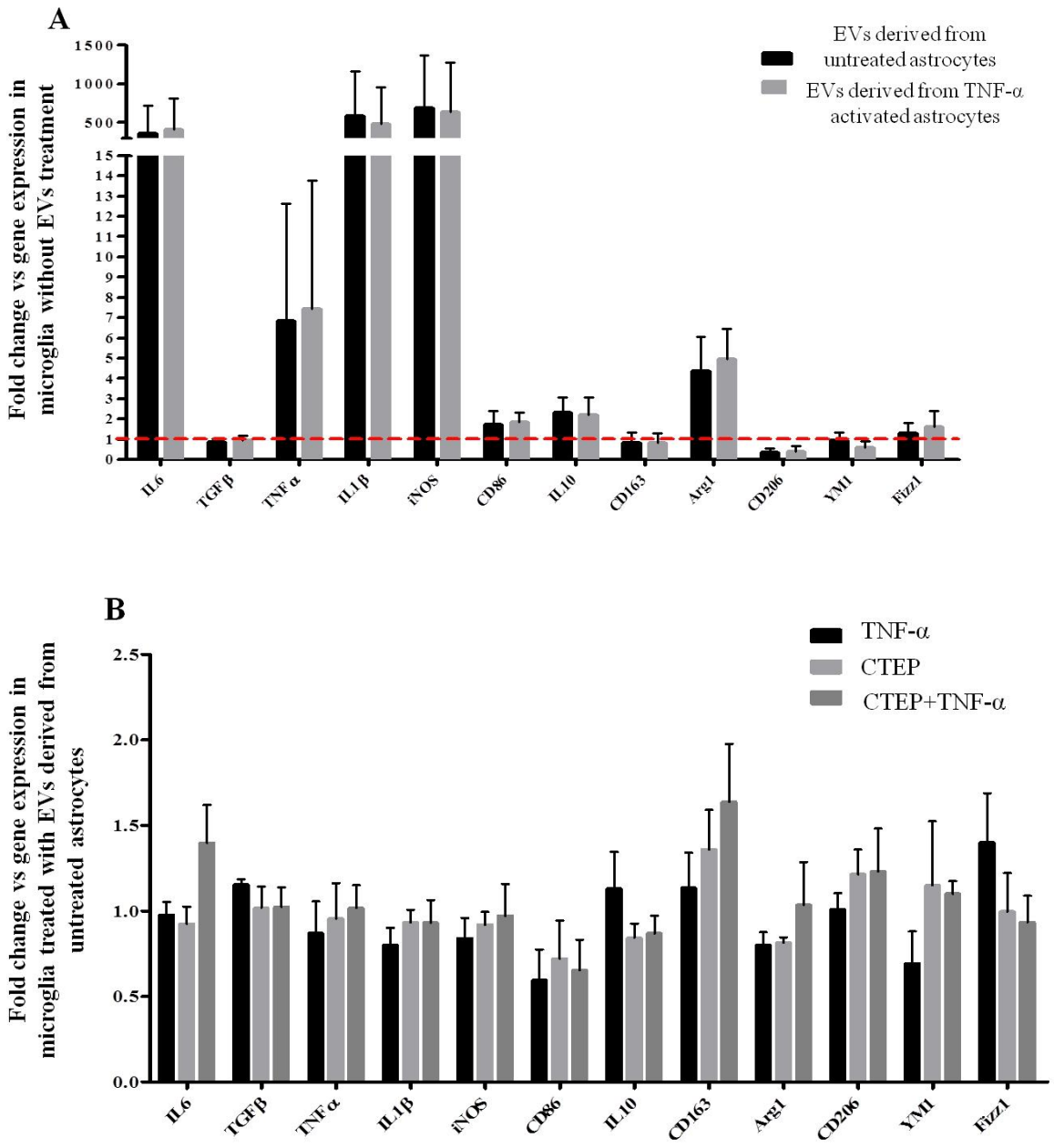



**Figure 13.** ***RT-PCR on the expression of pro- and anti-inflammatory markers in primary microglia from WT mice, after EVs treatment.*** The semi-quantitative PCR analysis was performed to quantify the RNA extracted from WT primary microglia cultures after EVs treatment. (**A**) Results were expressed as fold change of microglia treated with EVs derived from astrocytes activated or not with TNF-α with respect to the gene expression in RNA derived from untreated primary microglia which we refer as 1 (red line). (**B**) Results were expressed as fold change of microglia, treated with EVs derived from astrocytes activated with TNF-α (30 ng/ml) for 24 hours, exposed to CTEP 100 nM for 5 day or activated with TNF-α and exposed to CTEP, respect to the gene expression in RNA derived from primary microglia treated with EVs derived from untreated astrocytes which we refer as 1.

# 8

# DISCUSSION

There are many evidences in the literature showing enhanced extracellular glutamate levels in a large number of patients with both familial and sporadic Amyotrophic Lateral Sclerosis (ALS), as well as in murine models of the disease. These data are in accordance with the important role played by excitotoxicity in motor neuron degeneration (Shaw et al., 1995; Spreux-Varoquaux et al., 2002; Wuolikainen et al., 2011). The first mechanism proposed for causing the neurodegeneration was a reduced expression of type 1 glutamate transporters that provoked an altered reuptake of glutamate from the synaptic cleft (Rothstein et al., 1995; Foran et al ., 2011; Blasco et al., 2014; Rosenblum & Trotti, 2017), but it is now clear that dysfunction of this process is not the only mechanism involved in the increased synaptic levels of this excitatory neurotransmitter.

In search of a possible target to modulate excitatory synaptic transmission, metabotropic glutamate receptors were studied with interest to try to maintain controlled glutamate levels. Previous works reported the important role of Group I metabotropic glutamate receptors (mGluR1 and mGluR5) in the regulation of key cellular processes altered in ALS (Aronica et al., 2001; Anneser et al., 2004; Nicoletti et al., 2011; Vergouts et al., 2018). In this view, Prof. Bonanno's research group demonstrated that various mechanisms of neurotransmitter release could be affected. In particular, both spontaneous and KCl-evoked glutamate release, as well as the release induced by activation of GABA and glycine heterotransporters, is abnormal in the spinal cord of $SOD1^{G93A}$ mice, the most used animal model to study the pathogenetic mechanisms of ALS (Raiteri et al., 2003; Milanese et al., 2010, 2011, 2015). Further studies showed that the excessive and abnormal release of glutamate in $SOD1^{G93A}$ was present even before the onset of clinical symptoms at the early stage of the disease, suggesting that it could be a cause and not just a consequence of the

progression of ALS (Raiteri et al., 2004; Bonifacino et al., 2016;).

In addition, it was also observed that the stimulation of mGlu1 and mGlu5 autoreceptors induced an abnormal increase in the release of glutamate from spinal cord of late symptomatic $SOD1^{G93A}$ mice (Giribaldi et al., 2013). In particular, it has been shown that relatively high concentrations of the non-subtype selective metabotropic group I antagonist 3,5-DHPG (1-3-10 μM) stimulated glutamate release in both control and $SOD1^{G93A}$ mice to the same extent, while concentrations less than or equal to 0.3 μM led to an increase in glutamate release only in $SOD1^{G93A}$ mice. Both metabotropic autoreceptor were involved in this effect as demonstrated using mGluR1 and mGluR5 selective antagonists or negative allosteric modulators, with the latter subtype playing a major role. More recent in vivo studies showed also the positive effect of partial or total knocking-out of mGluR1 and/or mGluR5 $SOD1^{G93A}$ mice that led to both amelioration of motor symptoms and increase of survival probability (Milanese et al., 2014; Bonifacino et al., 2017, 2019a).

However, the possible involvement of mGlu5 and mGlu1 autoreceptors in contributing to the excessive release of glutamate in the early phases of ALS has not been yet studied.

Therefore, the aim of this study was to investigate whether, and to what extent, mGluR1 and mGluR5 activation could modify glutamate release at different time points during the disease course. Therefore, to complete the temporal profile of the possible changes in the functions of glutamatergic metabotropic autoreceptors in $SOD1^{G93A}$ mouse model of ALS, the present study was carried out at the presymptomatic stage, that is at 30 and 60 days of life, and at early symptomatic phase, corresponding to 90 days of life.

### 8.1 Methodological considerations: superfused synaptosomes

Glutamate release has been evaluated using purified synaptosomes in superfusion, according to the original technique described by Raiteri et al. (1974), which still represent

the optimal method to study neurotransmitters release and its modulation by auto- and etero-receptors (Raiteri & Raiteri, 2000).

For those who are not familiar with this technique, synaptosomes are isolated subcellular particles derived from nerve terminals following homogenization of a brain tissue in an isosmotic buffer (Gray & Whittaker, 1962). During the homogenization, synaptic boutons are "pinched-off" from axons and immediately reseals, thus forming viable synaptosomes of 1-2 μm in size that maintain all the neurochemical functions of in situ synaptic nerve endings. In fact, synaptosomes are capable to synthesize, store in vesicles, release and reuptake neurotransmitters and they have auto- and etero-receptors to modulate this release. During tissue homogenization, other subcellular particles are formed, called gliosomes, similar to synaptosomes but derived from astrocytes. They also possess vesicles and the cellular machinery that make them competent for the release of gliotransmitters, especially glutamate, and have also release-regulating membrane receptors (Stigliani et al., 2006; Bonanno et al., 2007; Patti et al., 2007; Raiteri et al., 2008; Milanese et al., 2009, 2010; Cervetto et al., 2015). Purification of synaptosomes from gliosomes is then carried out by centrifugation of the homogenized tissue on a discontinuos Percoll gradient (see, Methods. Dunkley et al., 1988; Nakamura et al., 1993).

An important implementation for experiments with synaptosomes is the superfusion technique that allows to study release-regulating presynaptic receptors in isolation. In this case, synaptosomes are stratified in a quasi-monolayer on filters that are placed at the bottom of thermostated chambers of a superfusion system and are continuously up-down superfused with a physiological solution that immediately removes the released neurotransmitters, thus avoiding the formation of a synaptic biophase and the activation of auto- and etero-receptors on the membrane and the correlated indirect effects. Thus, under these experimental conditions any receptor (auto or hetero) present on any nerve terminal is inactive in the absence of endogenous ligands and can be activated only by adding

selective agonists to the superfusion medium. In addition, antagonists will not affect the neurotransmitter release on their owns but they will be able to block the effects of exogenous agonists. This experimental method, therefore, permits to evaluate and pharmacologically characterize the effects of a receptor activation and its exact localization on a given nerve terminal population.

### 8.2 Group I metabotropic glutamate autoreceptors are functionally hyperactive at the early symptomatic stage of experimental ALS

The data obtained in the present study show that stimulation of group I metabotropic autoreceptors with the non-subtype-selective agonist 3,5-DHPG increased the spontaneous release of glutamate in WT and SOD1$^{G93A}$ at all the time points investigated (30, 60 and 90 days of life). Similarly to what observed in 120-day-old ALS mice, the mGluR1/5 agonist had the same maximal effect at 30 μM, potentiating glutamate release by approximately 100%, but it seemed to possess lower potency. Indeed, when 3,5-DHPG was used at concentrations lower than 30 μM (i.e. 0.03, 0.3 and 3 μM) the potentiation of glutamate release at pre- and early symptomatic phases was always lower than that observed at 120 days (Giribaldi et al., 2013). The present results show that at 30 and 60 days of life, the concentration-dependent effects induced by 3,5-DHPG on glutamate release were similar in ALS and control mice, indicating that there are no differences in the sensitivity of these autoreceptors to the endogenous agonist at the presymptomatic stages. On the contrary, when clinical manifestations occur (disease onset, 90 days of life), the exposure of synaptosomes to 0.3 μM 3,5-DHPG provoked a 25% significant increase of glutamate release in SOD1$^{G93A}$ mice, while no significant effects were observed in age-matched WT mice.

The availability of selective antagonists/NAMs allowed us to identify which subtype of group I metabotropic glutamate autoreceptors was involved in the observed effects on glutamate release. We found that both the mGluR1 antagonist LY367385 and the mGluR5

NAM MPEP were able to prevent the 3,5-DHPG-induced increase of glutamate spontaneous release in spinal cord synaptosomes obtained from early symptomatic $SOD1^{G93A}$ mice, a result that indicate that both mGlu1 and mGlu5 autoreceptors are involved.

Comparing these results with those obtained previously at 120 days of age, two interesting differences emerged. First, in the late phase of the disease, 3.5-DHPG is more effective in stimulating glutamate release, being active already at the concentration of 0.003 μM. Moreover, between the two autoreceptor subtypes of group I, the most involved at late stage appears to be mGluR5, whose expression increased by 50% (Giribaldi et al., 2013).

Present and previous data provide enough evidence showing that group I mGlu feed-forward autoreceptors become hyperactive and increase their expression during the development of ASL, a phenomenon that manifests at the disease onset and progresses to the late stage of the pathology, although with a different pattern for the two receptor subtypes.

Interestingly, it has been reported that the depolarization-evoked glutamate release is almost doubled in $SOD1^{G93A}$ mice compared to control animals at 30 days of age; furthermore, this effect is accompanied by variations of numerous exocytosis molecular determinants, such as CAMKII, synapsin I, synaptotagmin I and SNARE complexes, that can significantly impact the glutamate vesicular ready releasable pool (Milanese et al., 2011; Bonifacino et al., 2016).

On the basis of all these results, we can speculate that the excessive release of glutamate, which occurs at the early presymptomatic phases of the disease, is able to trigger long-term aberrant neuroplastic processes leading to increased expression/hyperactivity of group I mGlu autoreceptors that, in turn, would further enhance glutamate release, thus exacerbating excitotoxic effects on motor neurons from disease onset to the chronic late stage. As a matter of fact, it has been reported that induction of synaptic plasticity (e.g.

chemical LTP) in hippocampal cultured neurones increased mGluR1 through the activation of NMDA receptors, which are known to present onto glutamatergic nerve terminal where they also act as feed-forward autoreceptors (Beretta & Jones 1996; Woodhall et al., 2001; Suarez et al., 2005; Yang et al., 2006; Luccini et al., 2007; Cheyne & Montgomery, 2008; Musante et al., 2011; Summa et al., 2011).

The excessive 3,5-DHPG-induced glutamate release was of vesicular origin and occurred by calcium-dependent exocytosis, as it was abolished by Bafilomicin A1, which depletes vesicular neurotransmitter content (Bowman et al., 1988), and by BAPTA, a chelator of $Ca^{2+}$, suggesting that the calcium involved originated from intracellular deposits. Indeed, the increase of glutamate release by 3,5-DHPG was also prevented by exposing spinal cord synaptosomes to the PLC inhibitor U73122 and the IP3 receptor blocker 2-APB, demonstrating that the effect is mediated by canonical group I metabotropic glutamate receptors that activate PLC and phospholipid hydrolysis, formation of IP3 and calcium release from intracellular stores (Bleasdale et al., 1990; Maruyama et al., 1997).

The hyperactivity of mGluR1 and mGluR5 was also associated to a dysregulation of cytosolic $Ca^{2+}$ homeostasis at the intraterminal level that can activate several pathways, such as the calpain-calpastatin protease system found to be abnormally activated altered in $SOD1^{G93A}$ mice (Stifanese et al., 2010, 2014).

In line with the functional data described above, demonstrating the presence of altered group I mGlu autoreceptors, our immunofluorescence image analysis, performed on purified spinal cord synaptosomes, revealed an increased expression of both mGluR1 and mGluR5 on vGluT1 positive synaptosomes from $SOD1^{G93A}$ at 90 days of life with respect to WT controls. In contrast, the glutamatergic terminals from the spinal cord of 30- and 60-day-old $SOD1^{G93A}$ mice showed no significant changes of mGluR1 or mGluR5 expression, although a trend toward an increase could be observed. Interestingly, when the expression of these receptors was evaluated by western blot analysis on spinal cord synaptosomal

homogenates, no differences between SOD1$^{G93A}$ and control mice was observed at any age tested, indicating that the overexpression of presynaptic mGluR1/5 was confined to glutamatergic nerve terminals. Moreover, this analysis also highlighted the co-existence of the two receptor subtypes on the same glutamatergic nerve terminal, confirming the functional results that showed the involvement of both mGluR1 and mGluR5 in the modulation of glutamate release. It has to be noted that functional interactions between mGluR1 and mGluR5 autoreceptors have also been reported by Musante and collaborators (2008) in mouse cortical synaptosomes, with the two subtypes that may represent the low and the high affinity binding site, respectively.

On the other hand, overexpression of mGluR1 and mGluR5 was observed in total tissue homogenates of spinal cord in SOD1$^{G93A}$ mice at 60 days (mGluR1) and at 90 days (both subtypes) of life, thus suggesting that non-presynaptic receptors located on membranes of neuronal and/or non-neuronal cells (D'Antoni et al., 2008; Scheefhals & MacGillavry, 2018) may also play a role at presymptomatic and early symptomatic stages. Indeed, the possible modulatory roles of group I glutamatergic metabotropic receptors on the different functions of astrocytes, oligodendrocytes and microglia needs to be investigated since it is well known that non-neuronal cells are actively involved in motor neurons degeneration (Boillée et al., 2006b; Yamanaka et al., 2008; Haidet-Phillips et al., 2011; Frakes et al., 2014) .

Overall, the activity of group I metabotropic glutamate autoreceptors at the early onset and late phase of ALS, demonstrated here and in previous studies, suggests the possibility of using antagonists/NAMs for these receptors as a novel neuroprotective therapeutic approach in this motor neuron disease. As a matter of fact, in vitro studies have shown that these pharmacological interventions are effective in protecting spinal cord motor neurons from different neurotoxic insults (Pizzi et al., 2000; Anneser et al., 2006; D'Antoni et al, 2011). Most importantly from a translation point of view, different genetic manipulations,

aimed at decreasing or deleting these mGluR subtypes, have shown several in vivo beneficial neurochemical and clinical effects in the $SOD1^{G93A}$ mouse model, including a significant increase of lifetime (Milanese et al., 2014; Bonifacino et al., 2017, 2019a).

In conclusion, we have demonstrated for the first time that the function of mGlu1 and mGlu5 autoreceptors is altered in $SOD1^{G93A}$ mice starting from the symptom onset of the disease and they result hyperactive in enhancing the release of glutamate in the spinal cord, an effect that was paralleled by the increase of their expression on glutamatergic synaptic boutons. Moreover, we have also found that these receptors are overexpressed in spinal cord total tissue, but not in synaptosomes, even at pre-symptomatic disease stages, suggesting that they may be a cause/concause of ALS when mediating altered glutamate signalling at the postsynaptic levels and/or to astrocytes, oligodentrocytes or microglia. Of course, this latter aspect needs a more detailed analysis to indemnify what type(s) of non-neuronal cells could be involved in triggering the initial phases of the disease.

Overall, our data further support the therapeutic potential of group I metabotropic glutamate receptor antagonist in ALS. In this view, selective and potent antagonists of the mGluR5 subtype (fenobam, AFQ056, CTEP) are available today, which have shown good pharmacokinetic properties, a favorable safety profile and good efficacy in other pathologies (Abd-Elrahman KS et al., 2018; Montana et al. , 2009; Michalon et al., 2014; Lindemann et al., 2011; Levenga et al., 2011; Peterlik et al., 2017; Berry-Kravis et al., 2009; Levandis et al., 2008; Porter et al., 2015). However, a polydrug therapy aimed at blocking both mGluR1 and mGluR5 could turn out to be more effective and could represent a rapid translation application in human.

### 8.3 Role of EVs astrocytes derived in microglia activation

The results obtained in the pilot project carried out in Lausanne in the laboratory of Prof. Paolicelli, although preliminary and related to a limited number of experiments, can represent a promising starting point for the development of this research line.

TNF-α activation in primary astrocytes cultures did not appear effective. This suggests that changes to the protocol, such as exposure time or concentration used, are needed. Another point is the 24-hour washout in medium with FBS depleted from exosomes, performed in the absence of TNF-α. During this time, the effect of TNF-α may have disappeared. Unfortunately, the activation with TNF-α is a key point to observe the effects of CTEP. The treatment with exosomes isolated from astrocytes, exposed or not to CTEP, showed no differences in microglia cytokine expression and this event could be linked to the fact that there is only a little or no activation of astrocytes.

One interesting result, however, is related to the effect of the exposure of microglia cultures to EVs isolated from astrocytes, treated or not with TNF-α, that indeed caused the increase in the expression of several genes, thus confirming that extracellular vesicles could play a crucial role in the cross-talk between astrocytes and microglia.

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Zhao C.P., Zhang C., Zhou S.N., Xie Y.M., Wang Y.H., Huang H., Shang Y.C., Li W.Y., Zhou C., Yu M.J., Feng S.W. (2007) Human mesenchymal stromal cells ameliorate the phenotype of SOD1-G93A ALS mice. Cytotherapy. 9(5):414-26.

Zhao B., Marciniuk K., Gibbs E., Yousefi M., Napper S., Cashman N.R. (2019) Therapeutic vaccines for amyotrophic lateral sclerosis directed against disease specific epitopes of superoxide dismutase 1. Vaccine. 37(35):4920-4927.

# Pubblications

- Roberta Ricciarelli, Chiara Brullo, Jos Prickaerts, Ottavio Arancio, Carla Villa, Claudia Rebosio, Elisa Calcagno, **Matilde Balbi**, Britt T. J. van Hagen, Elentina K. Argyrousi, Hong Zhang, Maria Adelaide Pronzato, Olga Bruno, Ernesto Fedele. "*Memory-enhancing effects of GEBR-32a, a new PDE4D inhibitor holding promise for the treatment of Alzheimer's disease"* Sci Rep. 2017 Apr; 7:46320.

- Claudia Rebosio*, **Matilde Balbi***, Mario Passalacqua, Roberta Ricciarelli, Ernesto Fedele. "*Presynaptic GLP-1 receptors enhance the depolarization-evoked release of glutamate and GABA in the mouse cortex and hippocampus"* *Equally contributed. Biofactors. 2018 Mar; 44(2):148-157.

- Tiziana Bonifacino, Claudia Rebosio, Francesca Provenzano, Carola Torazza, **Matilde Balbi**, Marco Milanese, Luca Raiteri, Cesare Usai, Ernesto Fedele, Giambattista Bonanno. *"Enhanced Function and Overexpression of Metabotropic Glutamate Receptors 1 and 5 in the Spinal Cord of the SOD1$^{G93A}$ Mouse Model of Amyotrophic Lateral Sclerosis during Disease Progression"* Int J Mol Sci. 2019 Sep; 20(18).

## Meetings

***Oral communication:***

- 2018 September: *XXI SIF Seminar for PhD students, Fellows, Post Doc and Specialist Trainees, Bresso, 19th-22nd September 2018.*
  Oral communications: Treatment with exosome-shuttled miRNAs derived from mesenchymal stem cells shifts spinal cord astrocytes isolated from late disease state $SOD1^{G93A}$ mice from a neurotoxic to a neuroprotective phenotype.

***Posters:***

- 2016 July: *10th FENS, Forum of Neuroscience, Copenhagen, Denmark, 2nd-6th July 2016.*
  C. Rebosio, C. Garbarini, M. Balbi, M. Passalacqua, R. Ricciarelli, E. Fedele.
  Poster: Presynaptic GLP-1 receptors modulate glutamate release in mouse cortical and
  ippocampal synaptosomes.
- 2016 September: *XIX National Meeting of PhD student in Pharmacology, Pharmacology Italian Society (SIF) Rimini, 20th-22nd September 2016.*
  C. Rebosio, C. Garbarini, M. Balbi, M. Passalacqua, R. Ricciarelli, E. Fedele.
  Comunicazione orale: Presynaptic GLP-1 receptors modulate glutamate release in mouse cortical and ippocampal synaptosomes.
- 2017 October: *XVII National Meeting of Neuroscience Italian Society (SINS), Ischia, 1st-4th October 2017.*
  M. Balbi, C. Rebosio, M. Passalacqua, R. Ricciarelli, E. Fedele .
  Poster: Presynaptic GLP-1 receptors enhance glutamate and GABA release from purified mouse cortical and ippocampal synaptosomes.
- 2017 October: *38° National Congress of Pharmacology Italian Society (SIF) "Farmaci, salute e qualità della vita", Rimini, 25th-28th October 2017.*
  M. Balbi, C. Rebosio, C. Brullo, J. Prickaerts, O. Arancio, O. Bruno, E. Calcagno, R. Ricciarelli, E. Fedele.
  Poster: GEBR-32a, a new promising PDE4D inhibitor for the treatment of Alzheimer's disease.
  C. Rebosio, M.Balbi, M. Passalacqua, R. Ricciarelli, E. Fedele.
  Poster: Release-regulating GLP-1 receptors are present on cortical and hippocampal glutamatergic and GABAergic nerve terminals.
- 2018 February: *National Meeting of PhD student in Neuroscience, Neuroscience Italian Society (SINS), Napoli, 23rd February 2018.*
  M. Balbi
  Poster: Treatment with CTEP, a mGluR5 negative allosteric modulator, in the SOD1G93A ALS animal model.
- 2018 June: *XXXVI National Conference about Flow Cytometry, Cytometry Italian Society (GIC), Frascati, 6th-8th June 2018.*
- 2018 June: *Glial cells and therapeutic perspectives: from maladaptive plasticity to neurorestoration, Firenze, 29nd June 2018.*
- 2018 June**:** *First brainstorming research assembly for young neuroscientists, Genova, 29nd-30nd June 2018.*

M. Balbi, M. Milanese, T.Bonifacino, C. Rebosio, S. Ravera
Poster: Pharmacological treatment with CTEP, an mGluR5 negative allosteric modulator, in SOD1G93A mice.
S. Ravera, T. Bonifacino, M. Bartolucci, C. Torazza, F. Provenzano, M. Balbi, K. Cortese, I. Panfoli, G. Bonanno
Poster: Characterization of the mitochondrial aerobic metabolism at the pre- and perisynaptic districts of the SOD1G93A mouse model of amyotrophic lateral sclerosis.

- 2018 September: *Focus SLA, Genova, 27th-29th September 2018.*
  M. Balbi, C. Rebosio, T. Bonifacino, M. Milanese, L. Raiteri, M. Nadeem, C. Usai, G. Bonanno
  Poster: The function of release-regulating presynaptic Group I metabotropic glutamate autoreceptors is enhanced in the spinal cord of $SOD1^{G93A}$ mice.
- 2018 December: *Meet the microglia: homeostatic role and harmful contribution to neurological disorders, Milano, 19th December 2018.*
- 2019 July: *XIV European Meeting on Glial Cells in Health and Disease (GLIA 2019), Porto, Portugal, 10th-13 th July, 2019.*
  M. Balbi, T. Bonifacino, M. Milanese, G. Bonanno
  Poster: Partial deletion of mGluR5 affects M1 and M2 phenotypes in microglia acutely isolated from $SOD1^{G93A}$ mice during disease progression.
- 2019 November: *2nd Brainstorming Research Assembly for Young Neuroscientists, Milano, 14th-16th November, 2019.*
  M. Balbi, T. Bonifacino, M. Milanese, G. Bonanno
  Poster: Pro- and anti-inflammatory phenotypes of acute microglia isolated from spinal cord of SOD1G93A mice during disease progression and effects of the partial deletion of mGluR5.
- 2019 November: *39° National Congress of Pharmacology Italian Society (SIF) Firenze, 20th-23rd November, 2019*
  M. Balbi, T. Bonifacino, M. Milanese, G. Bonanno
  *Poster:* Pro- and anti-inflammatory state of microglia is affected by the partial deletion of metabotropic glutamate receptor type 5 in $SOD1^{G93A}$ mice during disease progression.

## Courses:

- 2018 September-December: specialisation course in "Economia del farmaco, della salute e delle tecnologie sanitarie APHEC", University of Genoa.
- 2018 June: Flow Cytometry course organized by Cytometry Italian Society (GIC), Frascati.
- 2017 May: specialisation course in "Nutrition and longevity", Dept. of Medicine (DIMI), University of Genoa.

## Seminars:

- 17.01.2017. Seminar at Dimes, Aula di Fisiologia, Viale Benedetto XV, 3:
  Prof. Emanuele Albano (Dipartimento di Scienze della Salute - Università del Piemonte Orientale) "Stress ossidativo e reazioni immunitarie nell'evoluzione della steatoepatite"

Prof. Giuseppe Poli (Università degli Studi di Torino) "Ossisteroli nella progressione dell'aterosclerosi e nella malattia di Alzheimer"

- 01.02.2017. Seminar at Istituto Giannina Gaslini, Aula Magna:
  Prof. Attilio Bondanza (Ospedale San Raffaele, Milano) "Car-T for cancer, autoimmune and inflammatory disease"
- 03.02.2017. Seminar at DIFAR, Università di Genova, Viale Cembrano, 4:
  Dott.ssa Marta Fumagalli (Dipartimento di Scienze Farmacologiche – Università degli Studi di Milano) "The GPR17 receptor a key player in oligodendrogenesis: physiological roles and involvement in demyelinating disorders"
- 16.02.2017. Seminar at DIMI, Viale Benedetto XV, 6:
  Prof. Alberto Diaspro (Università di Genova e Istituto Italiano di Tecnologia) "The extra microscope"
- 10.03.2017. Seminar at CBA-Torre D, Largo Rosanna Benzi, 10:
  Prof. Dario DiFrancesco (Dipartimento di Bioscienze – Università di Milano) "The funny curret: how to make a heart beat (and excite a brain)"
- 08.06.2017. Seminar at DIMI, Aula A, Viale Benedetto XV, 10:
  Prof. Giorgio Cantelli Forti (Presidente Società Italiana di Farmacologia) "Le farmaco proteine e le grandi rivoluzioni della terapia"
- 11.05.2018. Seminar at DIFAR, Aula C, Viale Cembrano 4:
  Dott.ssa Antonella Gentile (Dipartimento di Medicina dei Sistemi, Laboratorio di Immunopatologia sinaptica - Università Tor Vergata) "Modelli animali di Sclerosi Multipla: dall'indagine comportamentale allo studio dei meccanismi patogenetici"
- 18.05.2018. Seminar at DIFAR, Aula Angelo Ranise, Viale Benedetto XV 3:
  Dr. Andrea Petretto (Istituto Giannina Gaslini) "La proteomica nella Ricerca Traslazionale e Clinica"
  Dr. Ulrich Pfeffer (Istituto San Martino) "Data analytics nei centri di ricerca sanitari"
- 24.05.18. Seminar at DIFAR, Aula Angelo Ranise, Viale Benedetto XV 3:
  Prof.ssa Barbara Anna Bobrowska-Korczak (Warsaw Medical University) "Epigenetic cenge, trace elements and antioxidant status for cancer prevention"
- 3.07.18. Seminar at DIFAR, Aula Angelo Ranise, Viale Benedetto XV 3:
  Dott.ssa Carlota Rangel-Yangui (San Paolo University) "Research & development of nanotechnological alternatives for biological drugs"
- 3.07.18. Inter-doctorate course at Polo Alberti, Aula 6, Via Leon Battista Alberti 4:
  Prof. Rodolfo Quarto, Dott.ssa Barbara Parodi e Dott.ssa Chiara Baldo "Biobanche per diagnosi e ricerca"
- 4.07.18. Inter-doctorate course at Polo Didattico Biomedico, Aula 8, Corso Aldo Gastaldi 161:
  Dott.ssa Paola Pagani, Dott.ssa Cristina Bottazzi, Dott.ssa Alessandra Bo "Le biobanche ad uso terapeutico"
- 4.10.18. Seminar at DIFAR, Aula D, Viale Cembrano 4:
  Miltenyi Biotec "Inspiring technologies for creative neuroscientists"
- 10.10.18. Elsevier on Campus @ Università degli Studi di Genova, Scuola Politecnica, Villa Cambiaso, via Montallegro 1, Salone al Piano Nobile.
  Dott. Massimiliano Bearzot "Come scrivere un articolo scientifico e pubblicarlo su riviste scientifiche ad alto impatto"
- 11.03.2019. Seminar at Department de Physiologie, Seminar room, Rue du Bugnon 7, Lausanne:

Frèdèrique Varoqueaux “Emergence of neuronal communication: peptidergic signaling in placozoans”

- 23.03.2019. Seminar at Department de Physiologie, Seminar room, Rue du Bugnon, 7, Lausanne:
  Bertrand Mollerau “The expanding role of lipid droplets in neurodegenerative diseases”
- 26.03.2019. Seminar at DNF (Departement of Foundamental Neuroscience), Seminar room, Rue du Bugnon, 9, Lausanne:
  Cornelius Gross “Primal Fear- the neural circuitry of instinctive defense”
- 8.04.2019. Seminar at Department de Physiologie, Seminar room, Rue du Bugnon 7, Lausanne:
  Aude Panatier “Glycolysis derived-L-serine contributes to early memory deficits in Alzheimer’s disease”
- 15.04.2019. Seminar at Department de Physiologie, Seminar room, Rue du Bugnon 7, Lausanne:
  Fanny Martineau “Consequences of early neuronal migration failure in the neocortex”
- 3.06.2019. Seminar at Department de Physiologie, Seminar room, Rue du Bugnon 7, Lausanne:
  Juan P. Bolanos “Regulation of brain metabolism and behavior by astrocytic mithocondrial ROS”