Università degli Studi di Palermo

AREA RICERCA E TRASFERIMENTO TECNOLOGICO
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Settore Scientifico Disciplinare BIO/09 - Fisiologia

A TETRAHYDRO-ISOQUINOLINE DERIVATIVE AS A NOVEL $mGlu_{2/3}$ AGO/PAM WITH THERAPEUTIC POTENTIAL IN EXPERIMENTAL MODELS OF PARKINSONISM

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CICLO XXXVIII
ANNO CONSEGUIMENTO TITOLO 2026

"Indeed, the underlying precept of the new science of mind is that all mental processes are biological — they all depend on organic molecules and cellular processes that occur literally 'in our heads'."

Eric R. Kandel,
"In Search of Memory: The Emergence of a New Science of Mind"

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LIST OF ABBREVIATIONS

AADC, aromatic L-amino acid decarboxylase

AD, Alzheimer's disease

Ago/PAM, agonist/positive allosteric modulator

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

AMs, allosteric modulators

ATP, adenosine triphosphate

AU, arbitrary units

Aβ, amyloid-beta

BBB, blood-brain-barrier

BDNF, brain-derived neurotrophic factor

cAMP, cyclic AMP

CNS, central nervous system

COMT, catechol-O-methyltransferase

CRD, cysteine rich domain

CREB, cAMP response element-binding protein

DAG, diacyl glycerol

DAT, dopamine transporter

DMEM, Dulbecco's Modified Eagle Medium

DMEM/F12, Dulbecco's Modified Eagle Medium/F12

DMF, N,N-dimethylformamide

DMSO, dimethyl sulfoxide

DOPAC, 3,4-dihydroxyphenylacetic acid

DTT, dithiothreitol

DUSP1, dual specific phosphatase 1

DUSP6, dual specific phosphatase 6

EC_{20}, effective concentration 20%

EC_{50}, half maximal effective concentration

ECD, extracellular N-terminal domain

ERK1/2, extracellular signal-regulated kinase 1 and 2

FBS, fetal bovine serum

FGF2, fibroblast growth factor 2

GBA, glucosylceramidase beta

GDNF, glial-derived neurotrophic factor

GDP, guanosine-5-diphosphate

GFRα, GDNF-family receptor-alfa

GPCRs, G-protein-coupled receptors

GSK-3-β, glycogen synthase kinase-3-beta

GTP, guanosine-5-triphosphate

H_2O_2, hydrogen peroxide

hAMSCs, human adipose-derived mesenchymal stem cells

HD, Huntington's disease

HRP, horseradish peroxidase

i.p., intraperitoneally

ICD, intracellular C-terminal domain

IL-10, interleukin-10

IL-1β, interleukin-1beta

IL-2, interleukin-2

IL-6, interleukin-6

IP1, inositol monophosphate

IP3, inositol trisphosphate

JNK, Jun kinase

KO, knock-out

LRRK2, leucine-rich repeat kinase 2

LTD, long-term depression

LTP, long-term potentiation

MAO-A, monoamine-oxidase A

MAO-B, monoamine-oxidase B

MAPK, mitogen-activated protein kinase

MAPK/ERK, mitogen-activated protein kinase/extracellular receptor kinase

mBDNF, mature BDNF

$mGlu_1$, metabotropic Glutamate Receptor subtype 1

$mGlu_2$, metabotropic Glutamate Receptor subtype 2

$mGlu_3$, metabotropic Glutamate Receptor subtype 3

$mGlu_4$, metabotropic Glutamate Receptor subtype 4

$mGlu_5$, metabotropic Glutamate Receptor subtype 5

$mGlu_6$, metabotropic Glutamate Receptor subtype 6

$mGlu_7$, metabotropic Glutamate Receptor subtype 7

$mGlu_8$, metabotropic Glutamate Receptor subtype 8

mGluRs, metabotropic Glutamate Receptors

$MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridium

MPP^+, 1-methyl-4-phenyl-pyridinium

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

3-MT, 3-methoxytyramine

mTOR, mammalian target of rapamycin

N.C., negative control

NAMs, negative allosteric modulators

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NGF, nerve growth factor

NMDA, N-methyl-D-aspartate

Nrf2, nuclear factor erythroid 2-related factor 2

OD, optical density

6-OHDA, 6-hydroxydopamine

3-OMD, 3-O-methyl-dopamine

p-AKT, phosphorylated-AKT

p-ERK1/2, phosphorylated-ERK1/2

PAMs, positive allosteric modulators

PBS, phosphate-buffered saline

PD, Parkinson's disease

PI3K, phosphatidylinositol 3-kinase

PI3K–AKT, phosphatidylinositol 3-kinase-Akt

Pink1, PTEN-induced kinase 1

PIP2, phosphatidylinositol 4,5-bisphosphate

PKC, protein kinase C

PLC-β, phospholipase C-beta

RIPA, radioimmunoprecipitation assay

ROS, reactive oxygen species

RTKs, receptor tyrosine kinases

SN, substantia nigra

SNCA, synuclein alpha

SNpc, substantia nigra pars compacta

SOD, superoxide dismutase

TH, tyrosine hydroxylase

7TMD, seven-helix transmembrane domain

TNF-α, tumor necrosis factor-alfa

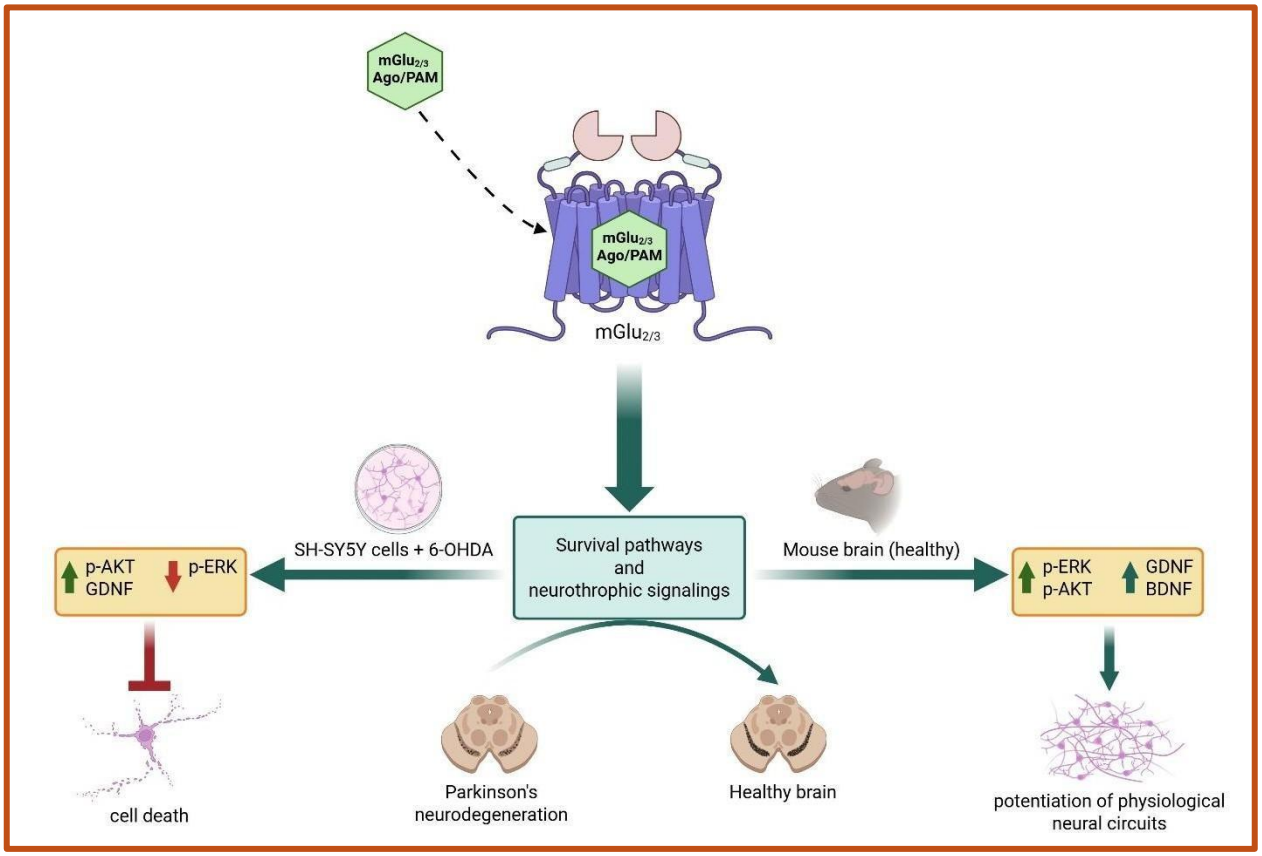
TrkB, tropomyosin receptor kinase B

VFD, Venus flytrap domain

VTA, ventral tegmental area

GRAPHICAL ABSTRACT

Illustration of the binding between the new agonist/positive allosteric modulator (Ago/PAM) and the $mGlu_{2/3}$ receptors, and the resulting effects of potentiation of the main intracellular survival pathways and neurotrophic signaling cascades in a cellular model of Parkinson's disease and in healthy mouse brain. The new molecule, thus, may represent a promising therapeutical agent against Parkinsonian-like neurodegeneration.



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ABSTRACT

Parkinson's disease (PD) is a complex and multi-factorial neurodegenerative disorder marked by the loss of dopaminergic neurons in the nigrostriatal circuit, with the progressive development of motor and non-motor symptoms. Currently, the gold-standard within the available drug therapies are dopamine-based therapies, which only help to mitigate symptoms and are also associated with several side-effects. Indeed, no disease-modifying pharmacological treatments have been available so far. In this context, indirect evidence has suggested that activation of the metabotropic Glutamate receptor 3 ($mGlu_3$) exerts neuroprotective effects in animal models of PD, but, however, the lack of selective agonists for this receptor has hindered more in-depth investigations. Based on this background, the present study characterized, for the first time, a tetrahydro-isoquinoline derivative as an agonist/positive allosteric modulator (Ago/PAM) for the $mGlu_{2/3}$, demonstrating its neuroprotective properties in a cellular model of PD and its neurotrophic effects in healthy mice. Despite showing equal affinity for $mGlu_2$ and $mGlu_3$, the novel Ago/PAM displays greater efficacy at $mGlu_3$ receptors in recombinant cells. It protects the human neuroblastoma cell line SH-SY5Y against death induced by 6-hydroxydopamine (6-OHDA), a drug widely used to induce dopaminergic degeneration, involving the modulation of survival signaling pathways, such as the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) and the phosphatidylinositol 3-kinase (PI3K)–AKT pathways, together with the regulation of glial-derived neurotrophic factor (GDNF). Interestingly, the observed neuroprotection is lost in the presence of ML 337, a selective negative allosteric modulator (NAM) for $mGlu_3$, suggesting the key role played by this mGluRs subtype. Moreover, *in vivo* treatment with the new $mGlu_{2/3}$ Ago/PAM up-regulates the expression of GDNF and brain-derived neurotrophic factor (BDNF) and modulates the activation of MAPK/ERK and PI3K–AKT pathways in mouse brain regions potentially involved in PD pathophysiology. Overall, though preliminary, our results provide new insights into $mGlu_3$ specific function and suggest the therapeutic potential of the new $mGlu_{2/3}$ Ago/PAM in the management of PD.

1. INTRODUCTION

1.1 Pathophysiology of Parkinson's disease

Nowadays, Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer's disease (AD), affecting up to 2% of individuals over the age of 60, especially in the most developed countries (Tenchov, Sasso et al. 2025). In PD, neurodegeneration primarily affects the so-called *diffuse dopaminergic system* and progressively extends to additional brain regions. The pathological process typically begins with the selective degeneration of dopaminergic neurons in the midbrain, specifically within the substantia nigra pars compacta (SNpc), and ultimately evolves into a more widespread neurodegenerative condition involving multiple neural circuits supported by various neurotransmitter systems, including the noradrenergic network (Espay, LeWitt et al. 2014) as well as the adenosinergic, glutamatergic, GABAergic, serotonergic, and histaminergic systems (Kouli, Torsney et al. 2018). The diffuse dopaminergic system refers to a network of neural circuits characterized by dopamine-mediated neurotransmission. As illustrated in *Figure 1*, it comprises three main pathways: I) the nigrostriatal pathway, which connects dopaminergic neurons of the SNpc to the striatum and regulates voluntary movements; II) the mesolimbic pathway consisting of projections from the ventral tegmental area (VTA) to the limbic structures such as the nucleus accumbens, amygdala, and hippocampus, and is primarily involved in motivation and reward; III) the mesocortical pathway, which links VTA to the prefrontal cortex and contributes to the regulation of cognitive functions and working memory (Ledonne and Mercuri 2017) (Samson, Ramesh et al. 2024). The death of dopaminergic neurons within the nigrostriatal circuit, the consequent reduction in circulating dopamine levels, the progressive disruption and atrophy extending from the basal ganglia to the broader dopaminergic network, and the deterioration of other neural projections and brain regions collectively result in a heterogeneous clinical condition characterized by both motor and non-motor symptoms. Motor manifestations include tremors, bradykinesia, rigidity, gait and balance disturbances, fatigue, impaired ocular motor control, difficulties in performing fine motor tasks, and speech deficits (Moustafa, Chakravarthy et al. 2016). Non-motor symptoms encompass mood and sleep disorders, autonomic dysfunctions (such as abnormalities in blood pressure regulation

and thermoregulation), cognitive and affective impairments, and neuropsychiatric comorbidities including anxiety and depression (Batzu, Podlewska et al. 2024).

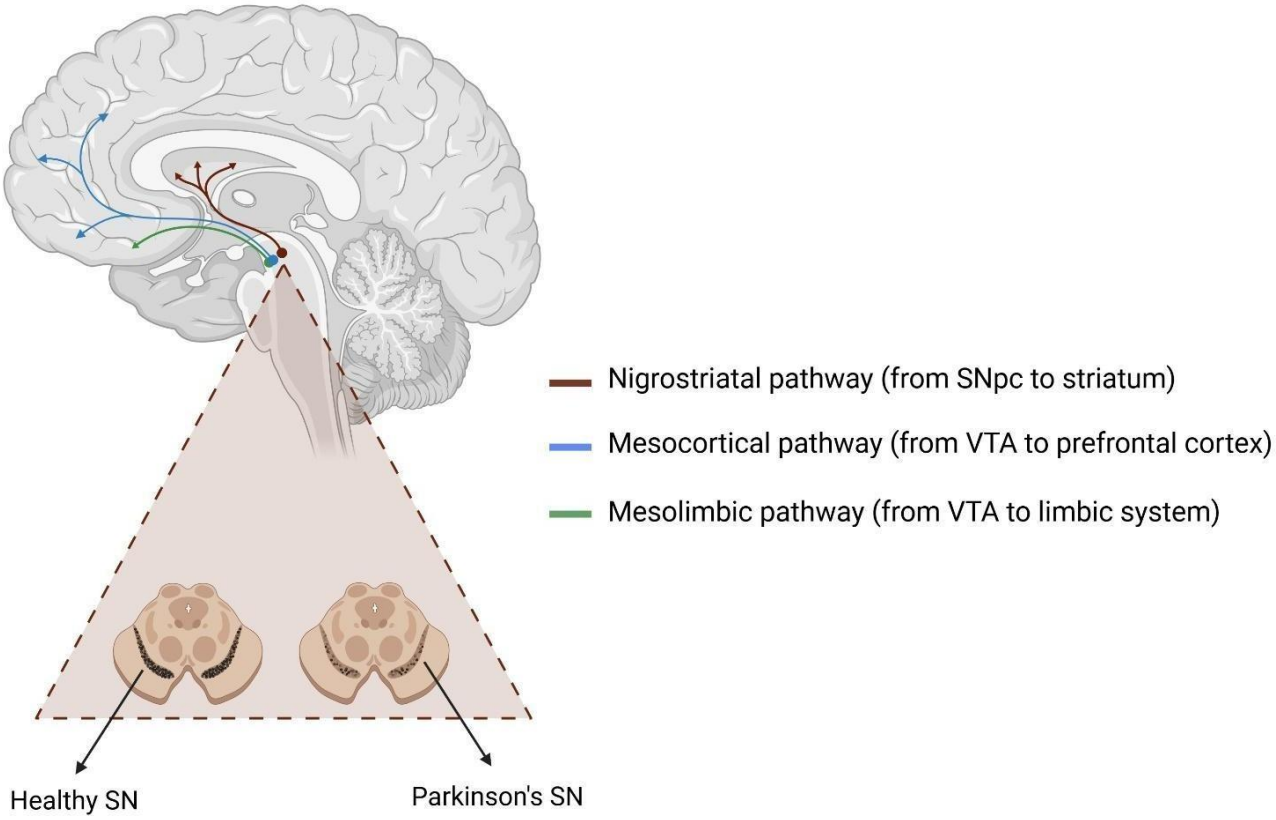


Figure 1: The diffuse dopaminergic system.

Schematic illustration of the three main pathways constituting the diffuse dopaminergic system in the brain. The nigrostriatal pathway (brown) spreads dopamine from SNpc to the striatum and is responsible for the initiation and control of voluntary movements; the mesocortical pathway (blue) releases dopamine from the VTA to the prefrontal cortex, controlling memory and attention; the mesolimbic pathway (green) connects the VTA to the limbic system, regulating motivation and reward processing. At the bottom (triangular inset), representative histological sections of the substantia nigra (SN) from a healthy brain and a PD-affected brain are shown. In PD, the density of pigmented (brown) nuclei, corresponding to dopaminergic neurons containing neuromelanin accumulated during dopamine synthesis, is markedly reduced compared to the healthy SN, reflecting the degeneration and loss of this neuronal population. (Created in https://BioRender.com)

The etiology of PD remains incompletely understood; however, it is generally accepted to result from a complex interplay between genetic and environmental factors. In most cases PD is a sporadic condition arising from a combination of inherited or sporadic genetic mutations, aging, and environmental exposures, such as pathogens, metals, pesticides, and herbicides (particularly in rural settings), as well as lifestyle factors including smoking and physical inactivity. Nonetheless, many environmental risk factors contributing to PD onset remain unidentified (Warner and Schapira 2003) (Sahai and Saxena 2025). Although genetic inheritance can contribute to disease onset, familial forms of PD are relatively uncommon, accounting for only about 10% of cases (Klein and Westenberger 2012). Monogenic forms, in which a single pathogenic mutation is sufficient to cause PD, is even rarer, representing approximately 5–10% of cases (Klein and Westenberger 2012) (Sardi, Cedarbaum et al. 2018). More frequently, PD appears to arise from the combined influence of multiple genetic variants, referred to as *genetic PD risk factors*. These genes are typically involved in maintaining essential cellular homeostatic mechanisms within the brain, including cell-to-cell communication, synaptic function and plasticity, redox balance, protein folding and degradation, and mitochondrial integrity and activity (Wood-Kaczmar, Gandhi et al. 2006). It is important to emphasize that not all individuals carrying pathogenic mutations in PD-associated genes manifest the disease, reflecting the incomplete penetrance that characterizes the genetic contribution to PD (Tenchov, Sasso et al. 2025). The PD-related genes include, for example, the coding gene for PTEN-induced kinase 1 (Pink1) that has a role in sensing and labeling damaged mitochondria. Pink1 subsequently recruits the E3 ubiquitin ligase named “Parkin” that targets mitochondria with lower membrane potential and, upon translocation, initiates the mitophagy process. Thus, mutations in *PARKIN* and *PINK1* genes cause accumulation of misfolded proteins and mitochondrial impairment, leading to cellular death (Narendra and Youle 2024). Another known to be involved in PD pathophysiology is *PARK7* which encodes for the protein DJ-1, a sensor of oxidative stress level. Despite being ubiquitously expressed within different cells and tissues, DJ-1 is a crucial factor for the health of dopaminergic neurons. Because of their high metabolic activity and dopamine metabolism, these neurons are highly exposed to oxidative stress and easily develop redox imbalances that lead to toxic free radical accumulation. DJ-1 increases transcription and synthesis of antioxidant enzymes via activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, promotes mitochondrial quality control by collaborating with Parkin and Pink1, regulates neuroinflammation through modulation of nuclear

factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, and controls protein homeostasis (Alshammari 2025). The *LRRK2* gene, which encodes for the cytoplasmic protein leucine-rich repeat kinase 2 (LRRK2), is also linked to PD. While its exact role remains unclear, mutations in LRRK2 are thought to cause aberrant kinase activity and anomalous interactions with other proteins, leading to activation of death pathways and over-transcription of α-Synuclein (Rui, Ni et al. 2018). α-Synuclein is normally involved in the synaptic vesicles trafficking and release of neurotransmitters in the synaptic area (*Figure 2*). Mutations on its gene, named *SNCA* (synuclein alpha), cause a toxic gain of function, as the protein acquires the ability to form abnormal bonds and structural conformations. These changes favor the formation of stable β-sheets instead of the physiological α-helices, resulting in toxic effects. Indeed, the misfolded α-Synuclein aggregates into fibrillar structures and, in the attempt to be confined and discarded by cells, will be accumulated into cytoplasmic inclusions called "Lewy bodies", a typical histological hallmark of PD in humans, triggering toxic pathways and establishing protracted neuroinflammation (Soni and Shah 2025). Moreover, mutations on the gene *GBA* (glucosylceramidase beta) for the lysosomal enzyme β-glucocerebrosidase, typically associated with lipid storage disorders, also contribute to PD pathogenesis. The resulting enzymatic dysfunction leads to the accumulation of glucocerebroside, which stabilizes toxic α-Synuclein oligomers, further exacerbating neuronal toxicity (Mazzulli, Xu et al. 2011) (Sardi, Cedarbaum et al. 2018).

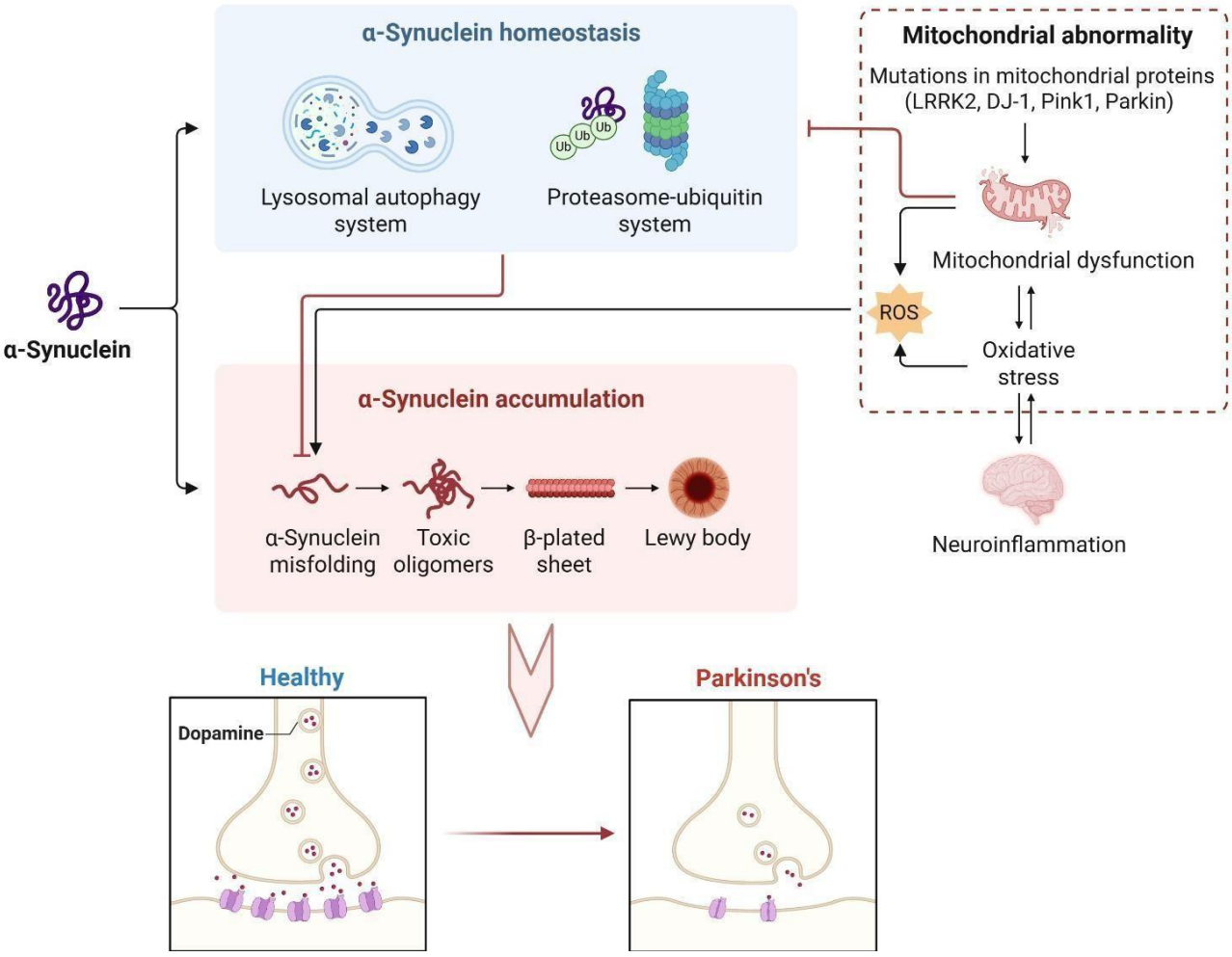


Figure 2: α-Synuclein accumulation in PD.

Representation of α-Synuclein homeostatic degradation through lysosomal or proteasomal systems, contrasted by the pathological process of misfolding and accumulation within Lewy body inclusions. The formation of these aberrant protein aggregates can also be triggered by oxidative stress and neuroinflammation, often as a consequence of mitochondrial dysfunction. Conversely, the loss of function of α-Synuclein impairs both dopamine packing and release in the synaptic space, resulting in the lack of dopaminergic transmission and the subsequent atrophy of dopaminergic circuits. ROS: reactive oxygen species. (Created in https://BioRender.com)

The establishment of a chronic neuroinflammatory condition may represent both the cause and the consequence of PD pathogenesis. Indeed, as shown in *Figure 3*, immune-system cells can cross the blood-brain-barrier (BBB) (for example after exposure to PD-related pathogens) and release pro-inflammatory molecules within the central nervous system (CNS). These molecules are sensed by astrocytes, which contribute to the polarization of resting microglial cells into activated auto-aggressive microglia that further amplifies the release of pro-inflammatory mediators. This self-perpetuating inflammatory loop ultimately results in neuronal death. Notably, increased levels of tumor necrosis factor (TNF)-α, interferon-γ, interleukin (IL)-1β, IL-2, IL-6, and NF-κβ have been observed as pro-inflammatory markers, along with a concomitant decrease in the anti-inflammatory cytokine IL-10, in a mouse model of PD (Goes, Jesse et al. 2018). At the same time, neuroinflammation can be also triggered by microglial activation in response to danger signals, released from dying neurons, such as misfolded α-Synuclein or excessive free radicals, which bind Toll-like receptors on resting microglia. This interaction promotes astrogliosis and microgliosis leading to excessive secretion of pro-inflammatory cytokines through the pathway linking the mitogen-activated protein kinase (MAPK) p38 to NF-κB (Troncoso-Escudero, Parra et al. 2018) (Cinar, Tel et al. 2022).

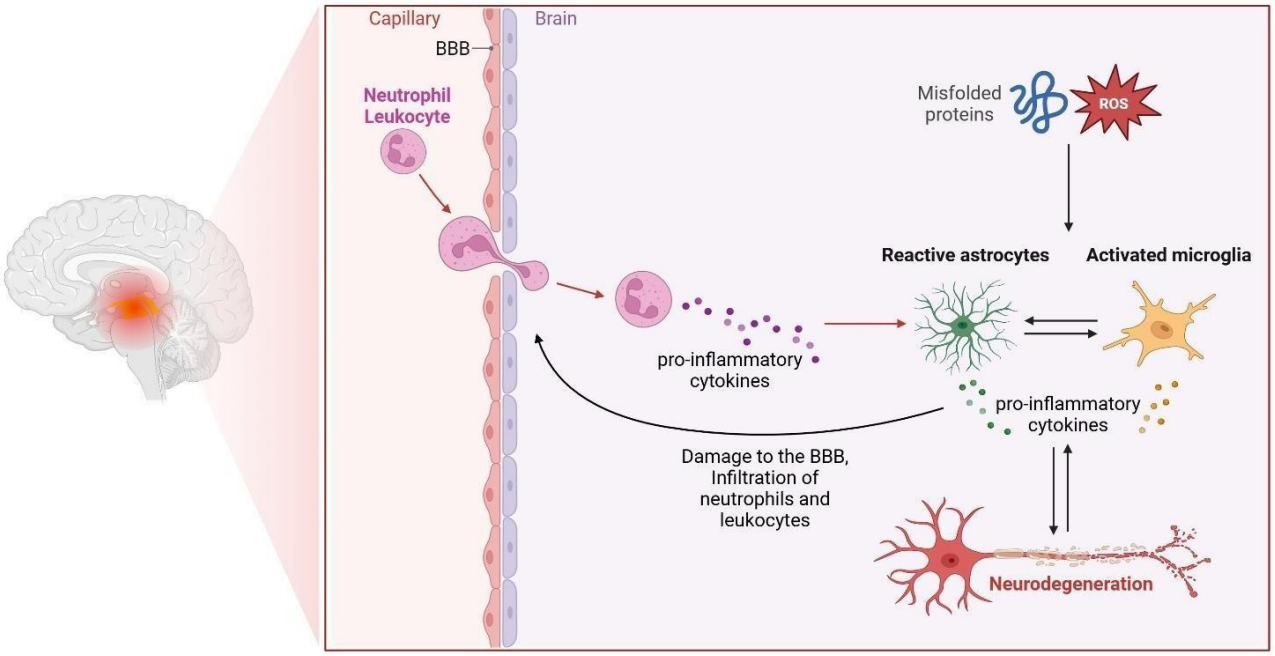


Figure 3: Neuroinflammation in PD.

Representation of the main mechanisms underlying neuroinflammation in PD. Neuroinflammation is initially triggered in the midbrain (highlighted in yellow) and subsequently spreads to other brain regions. Several insults can damage the BBB, allowing the infiltration of immune-system cells that release pro-inflammatory cytokines within the brain, resulting in the activation of glial cells and neuronal death. Astrocytes and microglia can also be activated by the accumulation of misfolded proteins or excessive amounts of reactive oxygen species (ROS). Conversely, glial-released pro-inflammatory cytokines can further damage the BBB, facilitating the migration of neutrophiles/leukocytes into the CNS, fueling the degenerative loop. (Created in https://BioRender.com)

1.2 Most common experimental models of PD: MPTP and 6-OHDA-induced neurotoxicity

Given the complex pathophysiology of PD, the scientific community has long struggled to develop experimental models that faithfully replicate the key features of the human disease, enabling deeper understanding of PD mechanisms and the identification of effective therapeutic strategies. Among toxic chemical compounds, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) emerged in the 1980s as a reliable tool for PD research, after individuals who self-injected this synthetic drug developed severe Parkinsonism (Nonnekes, Post et al. 2018). Indeed, as a lipid-

soluble compound, MPTP easily cross the BBB and it is converted by the enzyme monoamine-oxidase B (MAO-B) of astrocytes and serotoninergic neurons into 1-methyl-4-phenyl-2,3-dihydropyridium ($MPDP^+$) and, subsequently, into 1-methyl-4-phenyl-pyridinium (MPP^+). MPP^+ is selectively taken up by dopaminergic neurons via the dopamine transporter (DAT), resulting in: I) impairment of mitochondrial complex I, which leads to reduced adenosine triphosphate (ATP) production (followed by general inhibition/collapse of energy-dependent processes) and increased intracellular calcium, which causes DNA and protein damage; II) elevated reactive oxygen species (ROS) and oxidative stress, causing lipid peroxidation, disruption of mitochondrial and cellular membranes, DNA damage, and protein modification/fragmentation (Sian, Youdim et al. 1999). Moreover, mitochondria damage triggers Cytochrome C release, activating Caspase-9-depending apoptosis in the nigrostriatal circuit (*Figure 4*) (Lal, Singh et al. 2024). Although MPTP exposure reproduces many biochemical and symptomatic features of PD, some hallmarks, such as Lewy body formation and resting tremor, are absent or rarely observed in non-human primates and rodents (Sian, Youdim et al. 1999). This limitation highlights the need for alternative toxins to model specific aspects of PD molecular pathology and to test potential interventions.

Alternatively, *in vitro* and *in vivo* models of PD can be generated using 6-hydroxydopamine (6-OHDA). This compound is a structural analogue of catecholamines (dopamine and noradrenaline) that cannot cross the BBB. When stereotactically injected into SNpc, striatum or medial forebrain bundle of rodent brain, it is selectively up-taken by dopaminergic neurons via DAT and oxidized by monoamine-oxidase A (MAO-A) into catecholamine quinones and hydrogen peroxide (H_2O_2). Both metabolites are cytotoxic and serve as substrates for ROS production. The resulting oxidative stress causes the depletion of cellular antioxidant defenses, triggering the molecular damages analogous to those observed in PD and culminating in dopaminergic neuronal death (*Figure 4*) (Simola, Morelli et al. 2007).

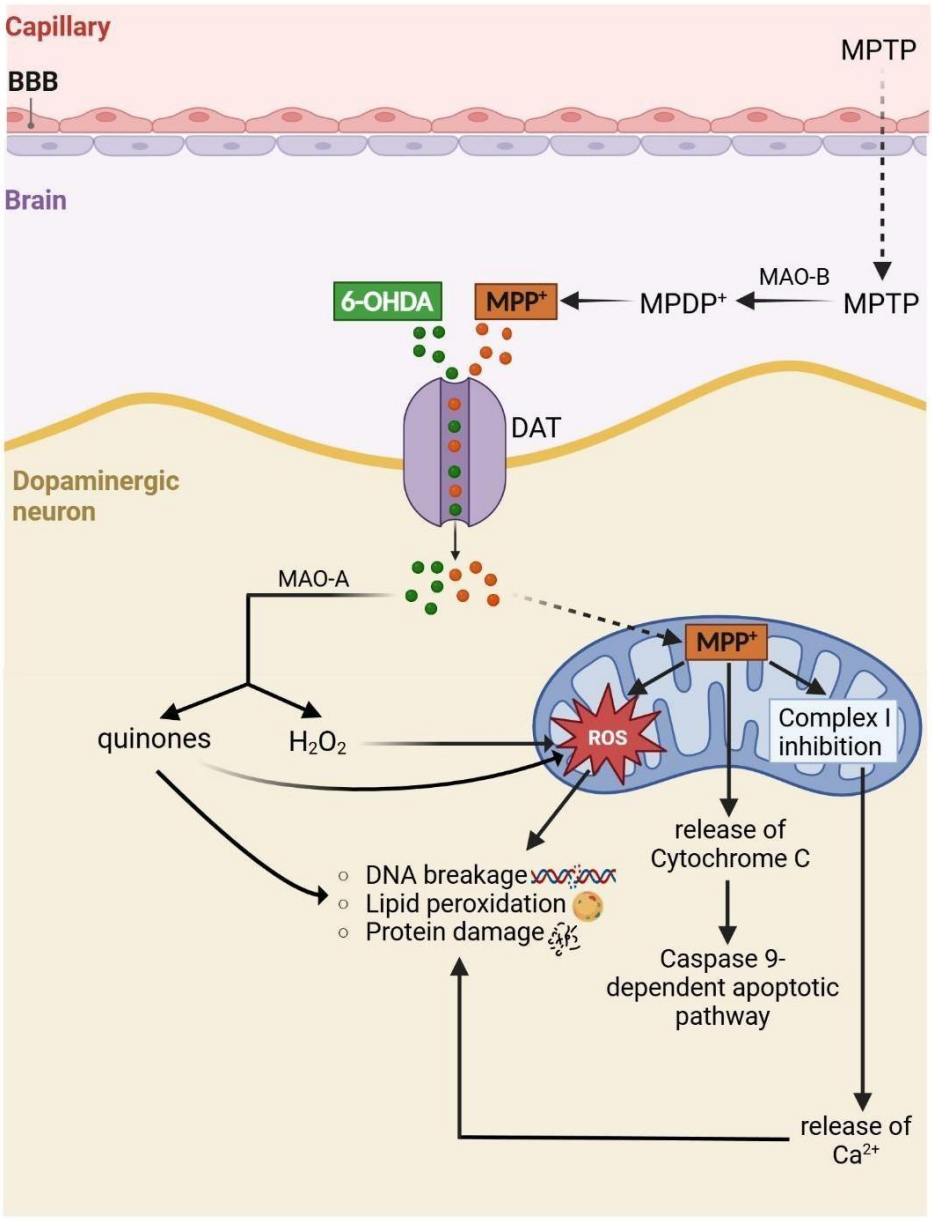


Figure 4: MPTP and 6-OHDA neurotoxic effects.

Oxidized MPP^+ and 6-OHDA are up-taken selectively by dopaminergic neurons of substantia nigra (SN) via DAT protein. Once inside, MPP^+ crosses the mitochondrial membrane and inhibits energy production by blocking complex I of the cellular respiratory chain, leading to unbalanced overproduction of ROS and activation of pro-apoptotic pathways. On the other hand, 6-OHDA is converted into quinones and H_2O_2, which are intrinsically cytotoxic and contribute to ROS-mediated cellular damage and neuronal death. Arrows legend = Dashed arrows: transfer of the molecule between compartments; Solid arrows (shaded at origin): biochemical transformation; Solid arrows (unshaded): resulting effect. (Created in https://BioRender.com)

However, 6-OHDA-induced animal models of PD primarily reproduce the motor and autonomic deficits of the disease, whereas the expected cognitive impairments are generally observed only in intracisternally injected neonatal rats or in adult rats injected into the ventrolateral striatum. Moreover, some evidence has pointed out that 6-OHDA-induced dopaminergic neuronal damage and dopamine depletion are mainly confined to specific brain regions, failing to produce Lewy bodies or to recapitulate the more complex pattern of neurodegeneration seen in humans (Simola, Morelli et al. 2007). Taken together, these findings indicate that 6-OHDA is most suitable for studying *in vitro* models of dopaminergic degeneration. Indeed, 6-OHDA-induced neurotoxicity is rapid, reproducible, and well-suited to simplified *in vitro* systems, such as cell lines, particularly for investigating dose-response kinetics and time-course effects. In contrast, MPTP requires astrocyte-mediated conversion into its active toxic form, making it more appropriate for complex systems, such as animal models, where the full spectrum of PD pathology can be studied.

1.3 Pharmacological therapies available against PD

Since the etiology of PD remains incompletely understood, current therapeutic approaches, primarily dopamine-based therapies, are not only limited but also insufficiently effective in contrasting the underlying pathological molecular mechanisms, preventing progressive neurodegeneration, or enabling meaningful recovery in patients (Tenchov, Sasso et al. 2025) (Stocchi, Bravi et al. 2024). This limitation is particularly evident because, by the time clinical symptoms appear, patients have already lost around half of the dopamine-producing neurons in the substantia nigra (SN) (Heng, Malek et al. 2023). Consequently, current treatments are largely symptomatic, providing temporary relief while often being associated with multiple side effects (Tenchov, Sasso et al. 2025) (Stocchi, Bravi et al. 2024). *Figure 5* shows the major pharmacological approaches currently available for PD. The first-line medication is Levodopa, which is particularly effective in managing motor symptoms because it is converted into dopamine in the brain, serving as an exogenous source of this neurotransmitter. However, long-term Levodopa is associated not only with side effects, such as confusion, hallucinations, and dyskinesia, but also with motor fluctuations as the disease progresses (Chakraborty, Brauer et al.

2020). Levodopa is often administered in combination with aromatic L-amino acid decarboxylase (AADC) inhibitors, which prevent its conversion to dopamine in the periphery, thereby reducing the required dose of Levodopa and minimizing peripheral side effects. Other strategies include early use of dopamine receptor agonists (such as Ropinirole, Pramipexole and Rotigotine), which mimic dopamine activity and help prevent Levodopa-associated motor complications; however, long-term use is still associated with adverse effects (Stocchi, Bravi et al. 2024). Motor fluctuations in treated patients are primarily due to incomplete restoration of the dopaminergic circuit and the rapid metabolism of dopamine. To address this, therapies have been complemented with drugs that prolong dopamine action by inhibiting enzymes responsible for catecholamine degradation, such as MAO-B and catechol-O-methyltransferase (COMT). These drugs, used as monotherapy or combined with Levodopa, enhance dopamine availability by preventing its conversion into 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), or 3-O-methyl-dopamine (3-OMD) (Stocchi, Bravi et al. 2024). Additional adjunctive therapies include anticholinergic drugs, which block acetylcholine activity to reduce motor symptoms, though they are associated with significant cognitive side effects, including dementia (Barrett, Sargent et al. 2021). Adenosine A2A receptor antagonists represent another class of adjunctive treatment. Adenosine A2A receptors are localized in the basal ganglia, and their activation is required to obtain fine control of movement. In PD, balance between dopamine and adenosine is disrupted, leading to an excessive activation of A2A receptors with consequent bradykinesia. Blocking A2A receptor signaling with antagonists can therefore help counteract dopaminergic loss (Tenchov, Sasso et al. 2025). Beyond motor symptoms, PD-related cognitive deficits significantly impact patients' quality of life; consequently, pharmacological approaches also include antidepressants, anxiolytics, and cognitive enhancers (Tenchov, Sasso et al. 2025).

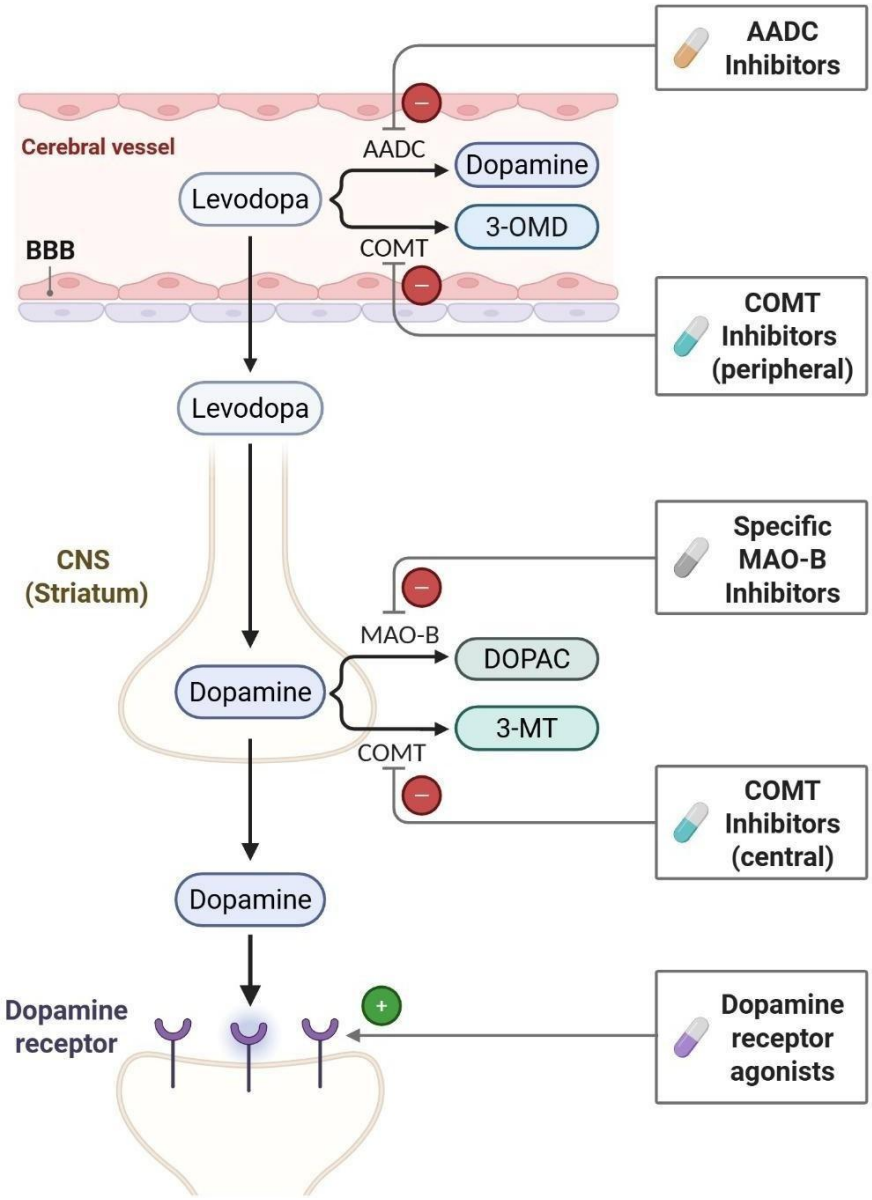


Figure 5: Available pharmacological strategies against PD.

Schematic illustration of the mechanism of action in the CNS of commonly used drugs for PD. AADC: aromatic L-amino acid decarboxylase; COMT: catechol-O-methyltransferase; MAO-B: monoamine-oxidase B; 3-OMD: 3-O-methyl-dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; 3-MT: 3-methoxytyramine. (Created in https://BioRender.com)

Given the palliative nature of current medical treatments for PD, gaining novel insights into the molecular mechanisms of the disease, identifying novel pharmacological targets, and developing innovative therapeutic approaches, particularly disease-modifying therapies, remain critical unmet goals that must be urgently addressed.

1.4 Metabotropic Glutamate Receptors (mGluRs)

Metabotropic Glutamate Receptors (mGluRs) are a family of receptors belonging to the G-protein-coupled receptors (GPCRs) superfamily, widely distributed throughout the CNS. Briefly, after ligand binding, GPCRs undergo a conformational change within the G-protein: the α subunit exchanges guanosine-5-diphosphate (GDP) for guanosine-5-triphosphate (GTP), dissociates from the βγ dimer, and both units then activate distinct intracellular signaling cascades. The cycle concludes when GTP is hydrolyzed back to GDP and the α, β, and γ subunits reassociate, returning the G-protein to its inactive state. Owing to their highly structured conformation and their ability to interact with multiple downstream effectors, GPCRs play a central role in finely regulating numerous physiological processes, making them crucial for appropriate cellular responses to both external and internal stimuli, particularly within the CNS environment (Wong, Li et al. 2023). mGluRs belong to subtype C of human GPCRs, the subclass selectively responsive to glutamate, and function as dimers composed of two monomeric subunits. Each monomer is characterized by a large extracellular N-terminal domain (ECD), formed by the so-called “Venus flytrap domain” (VFD) and a cysteine rich domain (CRD), followed by a seven-helix transmembrane domain (7TMD) and an intracellular C-terminal domain (ICD). Each VFD consists of two lobes hosting the binding site for the orthosteric ligand (glutamate) in a cleft between them (opened-opened conformation, inactive). Evidence has demonstrated that, when a single molecule of glutamate binds to one VFD, a conformational change happens, causing the increase in the affinity between the VFD of the second monomer and another molecule of glutamate, finally resulting in the closure of the two VFDs together. This causes the reorientation of the ECD, the CRD, and the 7TMD, bringing the two monomers into closer proximity (closed-closed conformation, active) (*Figure 6*). Ultimately, the conformational change propagates to the ICD, leading to activation of the

associated G-protein and the initiation of intracellular second messenger signaling cascades (Niswender and Conn 2010) (Cao, Quast et al. 2021).

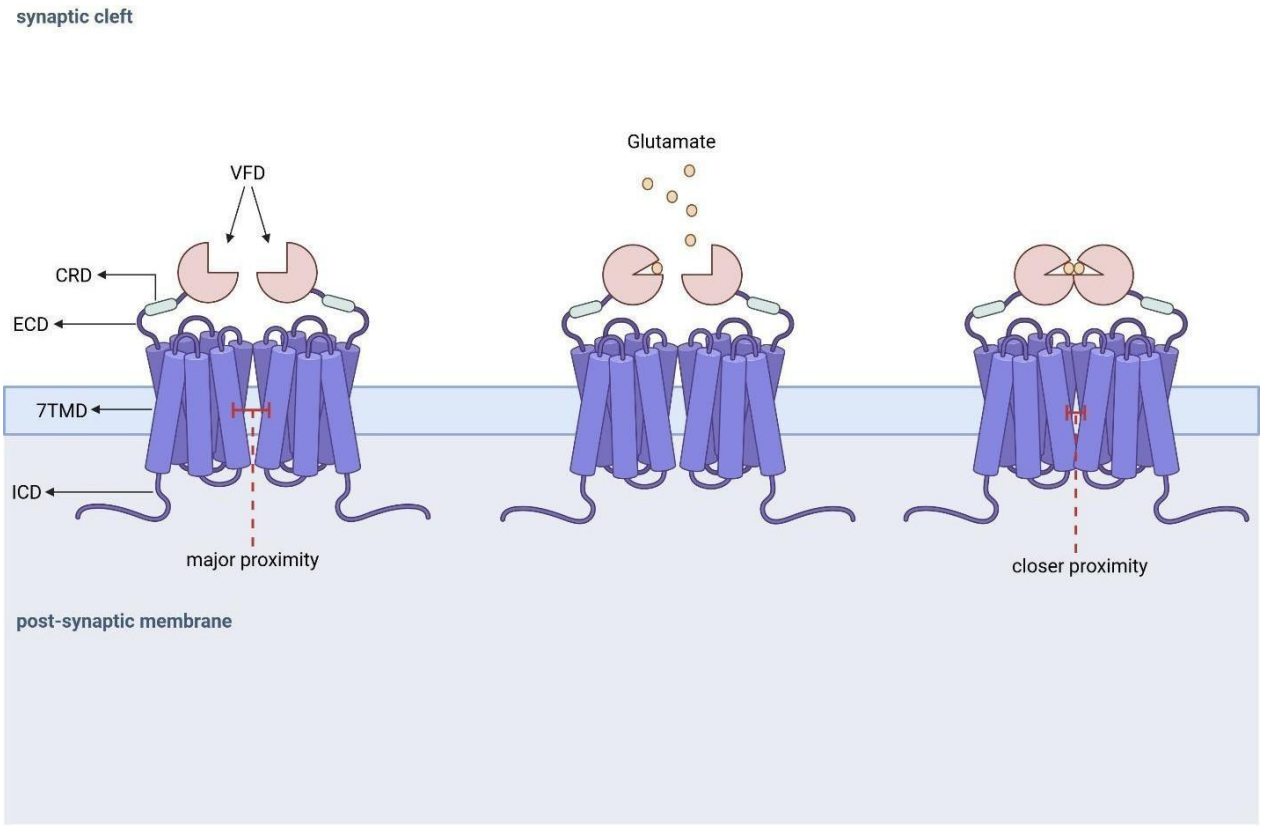


Figure 6: Structural features of mGluRs.

mGluRs appear as dimers with each monomer comprising: I) a large ECD at N-terminal, characterized by a CRD and a VFD with a cleft hosting a single molecule of glutamate; II) a 7TMD; III) an ICD at C-terminal. In the absence of glutamate, mGluRs adopt the resting conformation ("opened-opened" conformation, left), in which the monomers are maximally separated (red line). When glutamate is released from the pre-synaptic cell in the synaptic space, it binds to one VFD, inducing conformational changes that propagate to the second monomer and increase its glutamate affinity ("closed-opened" conformation, middle). Consequently, a second molecule of glutamate binds to the other VFD, causing the rotation of the 7TDM and the stabilization of the active "closed-closed" conformation (right), which brings the two monomers into close proximity. This conformational shift activates downstream signaling pathways mediated by the associated G-protein. (Created in https://BioRender.com)

mGluRs are subclassified into three groups based on sequence homology, the type of coupled G-protein and the associated signaling pathways (*Table 1*).

Group	Subtype	Coupled G- protein	Associated pathways/effects
I	**$mGlu_1$** **$mGlu_5$**	$G_{q/11}$	• PLC-β activation → Ca^{2+} release → PKC activation; • MAPK/ERK, JNK, mTOR-p70-S6K modulation; • L-type Ca^{2+} channels potentiation; • K^+ channels inhibition; • NMDA receptors interaction.
II	**$mGlu_2$** **$mGlu_3$**	$G_{i/o}$	• Adenylyl cyclase inhibition → ↓cAMP; • K^+ channels activation; • Pre-synaptic Ca^{2+} channels inhibition.
III	**$mGlu_4$** **$mGlu_6$** **$mGlu_7$** **$mGlu_8$**	$G_{i/o}$	• Adenylyl cyclase inhibition → ↓cAMP; • K^+ channels activation; • Pre-synaptic Ca^{2+} channels inhibition.

Table 1: Table summarizing mGluRs groups, subtypes, coupled G-protein and associated pathways/effects.

The table lists mGluRs subtypes, divided by groups, and summarizes the main associated pathways/effects.

Group I includes mGluRs subtypes 1 and 5, which are primarily localized post-sinaptically in different brain regions. $mGlu_1$ are particularly expressed in the cerebellar cortex, olfactory bulb, lateral septum, globus pallidus, ventral pallidum, magnocellular preoptic nucleus, and thalamic nuclei (Ribeiro, Vieira et al. 2017), whereas $mGlu_5$ are highly expressed in the cerebral cortex, hippocampus, striatum, and dorsal horn of the spinal cord (Benarroch 2008). mGluRs from group I are coupled to the $G_{q/11}$ protein, whose activation triggers phospholipase C-β (PLC-β) which hydrolyzes phosphatidylinositol 4,5- bisphosphate (PIP2) to produce: diacyl glycerol (DAG), that will release stored calcium (Ca^{2+}) from endoplasmic reticulum into the cytoplasm, and inositol trisphosphate (IP3), which activates protein kinase C (PKC). This cascade can then activate several downstream effector pathways, including Jun kinase (JNK), mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK), and the mammalian target of rapamycin (mTOR)-p70-S6 kinase. Functionally, group I mGluRs potentiate L-type Ca^{2+} channel activity, inhibit potassium (K^+) channels, and interact with the ionotropic glutamate receptors, specifically N-methyl-D-aspartate (NMDA) receptors, thereby modulating neuronal excitability and contributing to long-term potentiation (LTP) and depression (LTD). Group II includes mGluRs subtypes 2 and 3, while group III consists of subtypes 4, 6, 7 and 8. Both groups are pre-synaptically localized, with specific expressions as follows: $mGlu_2$ is found in the cerebellar cortex, striatum, and olfactory bulb; $mGlu_3$ is widely expressed throughout the CNS, including the olfactory tubercle, cerebral cortex, dentate gyrus, lateral septal nucleus, striatum, nucleus accumbens, amygdaloid nuclei, SN pars reticulata and cerebellar cortex (Ribeiro, Vieira et al. 2017). $mGlu_4$ is primarily located in the cerebellum, $mGlu_6$ is mainly restricted to the retina, and $mGlu_8$ is expressed in the olfactory bulb, certain regions of the cerebral cortex, pontine nuclei and lateral reticular nucleus of the medulla oblongata (Ribeiro, Vieira et al. 2017). Both group II and III mGluRs are coupled to $G_{i/o}$ protein which inhibits adenylyl cyclase activity, leading to a reduction in cyclic AMP (cAMP) production and the inhibition of the downstream kinases. Moreover, mGluRs from groups II and III are responsible for the activation of K^+ channels and the inhibition of pre-synaptic Ca^{2+} channels, which helps to prevent neuronal hyperexcitability and calcium-induced toxicity (Niswender and Conn 2010) (Benarroch 2008) (*Figure 7*). Interestingly, while most mGluRs are expressed in neurons, subtypes 3 and 5 are also found in glial cells, especially in astrocytes. In astrocytes, $mGlu_5$ plays a role in promoting Ca^{2+} release into the cytoplasm, while $mGlu_3$ is involved in glia-driven mechanisms of inflammation and neuroprotection (Benarroch 2008).

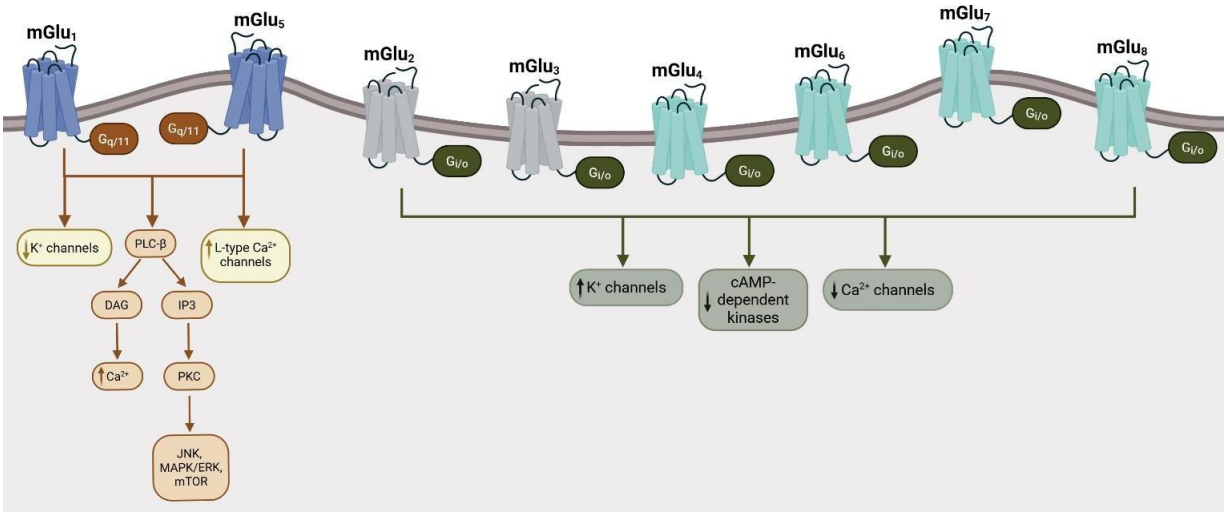


Figure 7: Signaling pathways mediated by mGluRs.

Schematic representation of the intracellular signaling pathways of the different mGluRs. Group I mGluR ($mGlu_1$ and $mGlu_5$) are coupled to $G_{q/11}$ proteins, leading to activation of PLC-β, production of DAG and IP_3, intracellular Ca^{2+} mobilization, and engagement of downstream signaling cascades including PKC, MAPK/ERK, JNK, and mTOR pathways. In contrast, group II ($mGlu_2$, and $mGlu_3$,) and III mGluRs ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) are coupled to $G_{i/o}$ proteins and negatively regulate neurotransmitter release through inhibition of adenylyl cyclase, reduction of cAMP-dependent kinases activity, and modulation of Ca^{2+} and K^+ channels. (Created in https://BioRender.com)

1.4.1 Neuroprotective role of group II mGluRs and the emerging potential of $mGlu_3$ as a novel therapeutic target in PD

Due to their role in the fine regulation of excitability and synaptic plasticity within the CNS, mGluRs are deeply involved in physiological processes and CNS disorders. Depending on the context, their activation can trigger either protective or harmful pathways. Indeed, several studies have pointed out the connection between mGluRs and a range of neurodegenerative and psychiatric disorders, including AD, PD, Huntington's disease (HD), schizophrenia, depression,

anxiety, and addiction. For example, hippocampal deletion of $mGlu_1$ in mice has been associated with deficits in the induction of LTP and impairments in associative and context-dependent learning (Niswender and Conn 2010). Additionally, other studies have described how the binding of amyloid-beta (Aβ) aggregates to $mGlu_1$ can trigger neurodegeneration (Ribeiro, Vieira et al. 2017). Regarding $mGlu_5$, studies have shown that knock-out (KO) mice lacking this receptor exhibit deficits in prepulse inhibition, a characteristic often impaired in patients with schizophrenia (Niswender and Conn 2010). On the other hand, either genetic ablation of $mGlu_5$ or treatment with a specific antagonist has proven protective in mouse models of AD, leading to improvements in cognitive function and a reduction in Aβ plaques (Ribeiro, Vieira et al. 2017). In addition, decreasing the activation of $mGlu_5$ has been shown to ameliorate symptoms and cellular degeneration in different animal models of PD, though the data regarding its role in HD remain controversial (Ribeiro, Vieira et al. 2017). Regarding group II mGluRs, some studies reported that $mGlu_2$ KO mice display increased responsiveness to cocaine (Niswender and Conn 2010). Other investigations demonstrated that $mGlu_2$ activation can enhance Aβ-induced neurotoxicity *in vitro*, whereas general inhibition of group II mGluRs produces anxiolytic effects in animal models of AD (Ribeiro, Vieira et al. 2017). In contrast, other evidence demonstrated that activation of group II ameliorates motricity and neuronal survival in mouse models of HD (Ribeiro, Vieira et al. 2017), as well as mitigates PD-related motor deficit in rats following selective stimulation of $mGlu_2$ (Shaqfah, Kang et al. 2025). With respect to group III mGluRs, it has been reported that KO mice for $mGlu_4$ exhibit reduced cerebellar synaptic plasticity, impaired motor learning, and decreased spatial memory (Niswender and Conn 2010), while $mGlu_4$ activation was protective in animal models of PD (Ribeiro, Vieira et al. 2017). Additionally, mice lacking $mGlu_7$ expression display epileptic characteristics, problems in learning and increased levels of anxiety and depression, while $mGlu_8$ KO mice exhibit a marked anxiety phenotype (Niswender and Conn 2010).

Over the years, from *in vitro* to *in vivo* explorations, researchers have increasingly focused on targeting $mGlu_{2/3}$ to achieve protective effects against PD neurodegeneration. Interest in them arises from their physiological role in reducing glutamate transmission at the pre-synaptic level in cortico-striatal circuits. Specifically, they inhibit voltage-dependent calcium channels and activate potassium channels, thereby modulating glutamate flux and preventing excitotoxicity. For example, Jantas, Gredal et al. pointed out that activating $mGlu_{2/3}$ with the dual orthosteric ligand LY354740 promoted recovery of undifferentiated SH-SY5Y cell viability after MTPT-induced toxicity and reduced MPP^+ up-take, highlighting a neuroprotective role for group II mGluRs

(Jantas, Greda et al. 2014). Furthermore, brief local application of LY354740 in rat brain slices decreased excitatory post-synaptic currents between subthalamic nucleus and SNpc, suggesting that targeting group II mGluRs might be an efficient strategy to counteract the overactivation of this circuit, which otherwise contributes to PD-related hypokinetic motor impairment (Bradley, Marino et al. 2000). Consistently, intraperitoneal injection of LY354740 reduced motor deficits and catalepsy in rat PD models (Bradley, Marino et al. 2000). Other studies demonstrated that activation of group II receptors with specific agonists such as LY379268 and DCG-IV attenuated glutamatergic transmission and aberrant cortico-striatal activity in brain slices from 6-OHDA-lesioned rats (Picconi, Pisani et al. 2002). Similarly, treatment with the $mGlu_{2/3}$ agonist 2R,4R-APDC increased both the number of tyrosine hydroxylase (TH)-positive cells in the SNpc and the content of dopamine in the striatum of 6-OHDA-lesioned rats (Vernon, Palmer et al. 2005). Moreover, using intraperitoneal injections of LY379268, Murray et al. further confirmed the neuroprotective activity of group II mGluRs activation, showing increased TH-positive neurons, improved dopamine turnover, and reduced rotational asymmetry following 6-OHDA injection (Murray, Messenger et al. 2002). In line with this data, Chan et al. observed that 2R,4R-APDC treatment decreased glial fibrillary acidic protein and OX-42 immunoreactivity in the SNpc of 6-OHDA-lesioned rats, indicating reduced astrocytic and microglial activation and highlighting the anti-inflammatory properties of group II mGluRs. Behavioral analysis revealed improved motor performance, including decreased preferential use of the ipsilateral forelimb relative to vehicle-treated animals (Chan, Paur et al. 2010). More recently, studies in MPTP-lesioned non-human primates showed that treatment with the $mGlu_{2/3}$ agonists LY354740, LY404039, and the selective $mGlu_2$ agonist CBiPES, when administered alongside Levodopa, improved bradykinesia, alertness, and posture (Frouni, Kwan et al. 2024). Given the substantial evidence supporting group II mGluRs stimulation as beneficial in PD, considerable effort has been directed toward disentangling the specific contributions of $mGlu_2$ versus $mGlu_3$. Due to the absence of selective agonists for $mGlu_3$, direct activation of this subtype has not been feasible. Therefore, researchers have relied on indirect approaches, including: I) the use existing selective $mGlu_2$ positive allosteric modulators (PAMs) (Valenti, Rekawek et al. 2025) (Augier, Dulman et al. 2016) (Jin, Semenova et al. 2010); II) pharmacological blockade of $mGlu_2$ with negative allosteric modulators (NAMs) combined with group II pan-agonists (Alonso de Diego, Linares et al. 2024); III) selective inhibition of $mGlu_3$ with NAMs displaying different grades of potency/selectivity for $mGlu_3$ over $mGlu_2$, such as RO4491533, LY2389575, ML 289, ML 337 (Wenthur, Morrison et al. 2013) and VU0650786 (Engers, Rodriguez et al. 2015); IV) genetic knock-out of either $mGlu_2$ or $mGlu_3$

combined with pan-group II activation *in vitro* or *in vivo*. Through these indirect strategies, several studies highlighted the neuroprotective role of $mGlu_3$. A remarkable aspect is that $mGlu_3$ activation leads to the increase in glial-derived neurotrophic factor (GDNF) expression levels. GDNF is an essential neurotrophin supporting dopaminergic neuron survival and differentiation, which represents a promising therapeutic factor against PD (Barker, Bjorklund et al. 2020). In rodent and non-human primate PD models, intracerebral injection of GDNF rescued nigrostriatal neurons (D'Anglemont de Tassigny, Pascual et al. 2015). However, despite promising preclinical results, clinical trials have been limited by surgical delivery requirements, immune responses, and other adverse effects. Consequently, research has shifted toward disease-modifying pharmacological strategies that can promote endogenous GDNF production. In this context, early investigation showed that systemic administration of LY379268, a group II agonist non-selective between $mGlu_2$ and $mGlu_3$, induced up-regulation of GDNF mRNA and protein in mouse striatum and cortex, an effect absent in $mGlu_3$ KO mice (Battaglia, Molinaro et al. 2009). Moreover, this treatment protected the nigrostriatal circuit from MPTP-induced neurodegeneration in mice (Corti, Battaglia et al. 2007) (Battaglia, Molinaro et al. 2009). Additional studies comparing wild-type and $mGlu_3$ KO mice demonstrated that $mGlu_3$ activation counteracts neuroinflammation and gliosis by promoting astrocytic release of trophic factors, including transforming growth factor-beta, nerve growth factor (NGF) and S100 calcium-binding protein beta (D'Antoni, Berretta et al. 2008). Supporting the positive role of $mGlu_3$, Aronica et al. reported that its activation by the non-selective group II mGluRs agonist DCG-IV in cultured human astrocytes, lacking $mGlu_2$ expression, leads to the up-regulation of glutamate transporter protein expressions, reinforcing the receptor anti-excitotoxic role in clearing extracellular glutamate (Aronica, Gorter et al. 2003). Notably, recent evidence indicates that genetic deletion of $mGlu_3$ exacerbates MPTP- induced nigrostriatal degeneration and gliosis, whereas deletion of $mGlu_2$ does not. Moreover, the same study also correlates different polymorphisms of the gene encoding $mGlu_3$ to different PD symptomatic phenotypes observed in patients, thus deepening the knowledge on the association between PD and this specific receptor (Di Menna, Alborghetti et al. 2025). On the contrary, $mGlu_2$ activation has been mainly described as pro-excitotoxic. In fact, under stimulation with NMDA, cortical neurons from $mGlu_3$ KO mice exhibited increased excitotoxicity and cell death, which could only be rescued when $mGlu_2$ was also deleted, when LY379268 was applied, or when co-cultured with astrocytes expressing $mGlu_3$ but not $mGlu_2$. This experimental setting indirectly revealed that $mGlu_2$ presence and activation drives glutamate-mediated neurotoxicity pathways,

also through communication with NMDA receptors, and that $mGlu_3$ is a key receptor in the regulation of the tripartite synapse. The same study also revealed that *in vivo* administration of LY379268 protects striatal neurons from NMDA-induced damage only in wild-type or $mGlu_2$ KO mice, not in $mGlu_3$ KO mice, and preserves the nigrostriatal pathway from MPTP toxicity in $mGlu_2$ KO but not $mGlu_3$ KO mice (Corti, Battaglia et al. 2007). In addition, it is known that the activation of $mGlu_2$ in microglia induces a pro-inflammatory phenotype promoting the release of TNF-α and the induction of apoptotic pathways driven by Fas ligand (D'Antoni, Berretta et al. 2008). Collectively, these findings indicate the beneficial aspect of selective $mGlu_3$ activation for a positive regulation of the neuronal-glial homeostasis, which is detrimentally changed in many CNS disorders including PD. Despite challenges posed by the high sequence homology between $mGlu_2$ and $mGlu_3$, ongoing research continues to pursue selective $mGlu_3$ ligands to better characterize and exploit its therapeutic potential. Recently, a tetrahydro-isoquinoline derivative, described as a selective $mGlu_3$ PAM (Potnick 2014), has been synthesized.

1.5 Allosteric Modulators (AMs): general mechanism of action, functional advantages and overview on known AMs for mGluRs

Allosteric modulators (AMs) are molecules that bind a receptor using a site which is topographically different from the binding-site of the natural ligand, called "orthosteric ligand", resulting in the modification of the receptor's conformation (May and Christopoulos 2003) (Kenakin 2011). This modification leads to either a positive or negative or even neutral modulation of the orthosteric ligand affinity, potency, and efficacy, the partial activation or blockade of the receptor, and the orientation of the receptor toward a specific signaling pathway, thereby affecting the kinetic of interaction (association or dissociation) between the native ligand and the target receptor (Cao, Quast et al. 2021).

From a pharmacological perspective, AMs, in particular PAMs and NAMs, have been emerging as molecules of great interest because basically they don't produce direct effects on the given receptor, but they only exert a fine regulatory/modulatory action of the receptor-orthosteric ligand system, potentiating (PAM) or reducing (NAM) the existing response. Moreover, generally,

receptor allosteric sites are less evolutionally conserved than orthosteric sites, resulting in more chances to design selective drugs for a specific target, allowing to differentiate activities from similar receptors and avoiding side-effects coming from off-target stimulation (Wakefield, Bajusz et al. 2022). For all these reasons, the exploitation of PAMs or NAMs represents a particularly advantageous therapeutic strategy for pathological conditions characterized by dysregulation of complex signaling pathways, such as those occurring in CNS disorders (Kenakin 2011). More specifically, a PAM, upon binding its site, generally increases the efficacy of the receptor response to the orthosteric ligand and/or the affinity of the orthosteric ligand for the receptor. When efficacy is potentiated, this results in an increase of the reached maximal receptor response. On the other hand, increase in the affinity results in a maximal receptor response at lower concentrations of the orthosteric ligand (reported as a shift to the left in the dose-response curve), thereby reducing ligand-related side effects, such as toxicity and receptor desensitization, caused by a direct long- lasting stimulation (Kenakin 2011) (Kenakin 2017) (Gregory and Goudet 2021). On the contrary, a NAM will either reduce the system efficacy by depressing the maximal receptor response or decrease the affinity between the orthosteric ligand and the receptor (the dose-response curve is shifted to the right) resulting in the requirement for higher doses of ligand to reach full receptor activation (*Figure 8*).

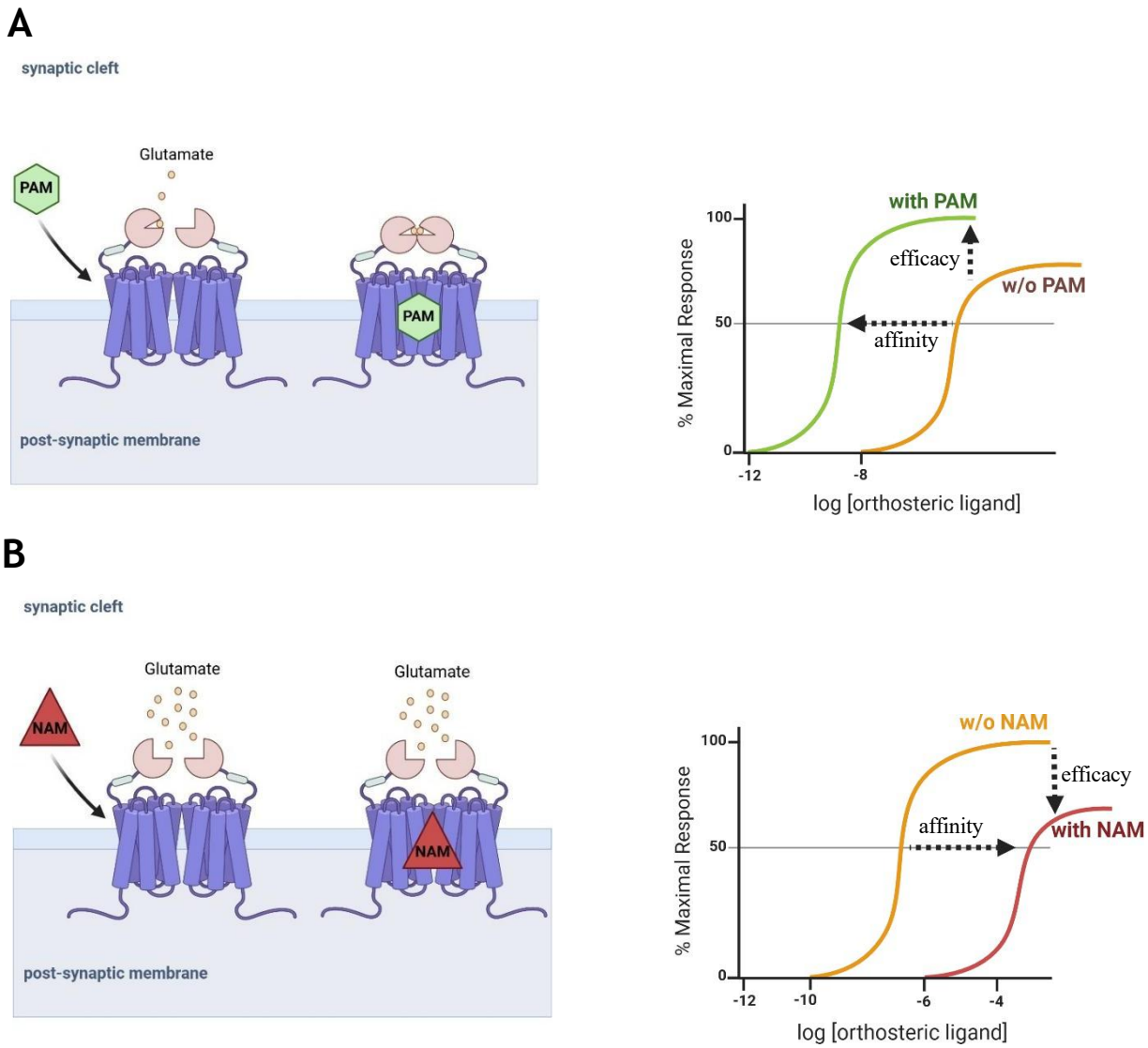


Figure 8: Representation of PAM and NAM mechanism of action.

When a PAM binds to the 7TDM of the receptor, it either increases the binding affinity between the receptor and its orthosteric ligand (e.g., glutamate) or enhances the efficacy of the maximal receptor response. As a result of modulation in the affinity, the receptor responds earlier to its orthosteric ligand, even when only a small amount of ligand is present in the synaptic cleft (panel A, left). Indeed, this is reflected in a leftward shift of the dose-response curve, since less ligand is required to reach activation (panel A, right). Additionally, PAM effect on efficacy is described as a potentiation of the maximal response reached (panel A, right). On the contrary, when a NAM binds to the allosteric site of the receptor, the binding affinity between the receptor and glutamate decreases, causing a slower and delayed response of the receptor to the orthosteric ligand (panel B, left) and a shift to the right of the dose-response curve, indicating that more ligand is needed to achieve activation (panel B, right). Furthermore, if the NAM affects efficacy, it causes a depression of the maximal response curve (panel B, left). (Created in https://BioRender.com)

Because of their highly modular structure, GPCRs are particularly susceptible to allosteric modulations. In the case of mGluRs, a modulator binding at the 7TMD can trigger a reconfiguration of the receptor, for example, inducing local "rolling" or rearrangement of the transmembrane helices, which in turn alters the orientation of the ECD and the VFD, thereby modifying the receptor affinity for glutamate (Caprioli, Justinova et al. 2018) (Strauss, Gonzalez-Hernandez et al. 2024). In the specific case of positive modulation, a PAM not only enhances the effect of an orthosteric agonist on its receptor but may also activate the receptor in the absence of that agonist, thereby functioning as an agonist/positive allosteric modulator (Ago/PAM). For example, investigations into $mGlu_4$ PAMs reveal that agonistic activity (i.e., receptor activation independent of the orthosteric ligand) arises when the PAM binds a shallower pocket of the 7TMD. In contrast, pure cooperativity (i.e., the enhancement of agonist binding or effect without intrinsic agonist activity) occurs when the PAM binds a deeper pocket within the 7TMD. Thus, two different mechanisms of action appear to depend on which structural site within the receptor 7TMD is occupied (Rovira, Malhaire et al. 2015). Interestingly, various binding sites within the 7TMD have also been described for $mGlu_5$, together with the bivalent mechanism of action of its PAMs (Dalton, Gomez-Santacana et al. 2014). Furthermore, it is noteworthy that a recent cryoEM study on $mGlu_5$ has revealed how pure PAM and Ago/PAM, when bound to the 7TMD, differently affect the conformational structure of the receptor and differently impact on the global equilibrium of the dimeric structure towards the active state (Cannone, Berto et al. 2025), thereby potentially leading to distinct pharmacological outcomes.

Given the broad and diversified roles of mGluRs within the CNS, the development of multiple AMs targeting different mGluRs subtypes has progressed over the years, with the aim of identifying new therapies for CNS disorders. For example, a bunch of PAMs (VU6046980, VU6005649, VU6027459) and NAMs (MMPIP, XAP044, VU6019278) selective for $mGlu_7$ are currently under preclinical evaluation to better understand $mGlu_7$ potential role in deficits of synaptic plasticity characterizing animal models of AD (Hunjan and Aran 2025). Regarding other mGluRs, some NAMs for $mGlu_5$, such as Basimglurant, demonstrated efficacy in alleviating neuropathic pain in animal models and have progressed into clinical trials (Li, Zhu et al. 2024). On the other hand, the $mGlu_5$ PAM CDPPB has shown neuroprotective effects against Aβ-induced neurodegeneration by protecting mouse hippocampal neuronal cultures from death, preventing hippocampal neuronal loss and memory deficits and reversing gliosis in two different mouse models of AD (Bellozi, Gomes et al. 2019). Additional evidence supports the neuroprotective role

of the $mGlu_4$-selective PAM VU0155041, in protecting animals from effects caused by unilateral injection of 6-OHDA. Specifically, VU0155041 was able to counteract both histological/biochemical damages in striatum-SNpc (such as loss of cells expressing TH, and impaired balance between dopa decarboxylase and dopamine levels) and motor deficits induced by the toxin (Betts, O'Neill et al. 2012). In the context of group II mGluRs, a recent study has proven that administration of the selective PAM for $mGlu_2$ LY487379 is able to reverse hypodopaminergia of VTA caused by abstinence from amphetamine in a mouse model of addiction (Valenti, Rekawek et al. 2025). Moreover, additional research shows that $mGlu_2$ PAM AZD8529 protects rats from alcohol relapse and seeking-like behaviors (Augier, Dulman et al. 2016), while $mGlu_2$ PAM BINA is able to stop cocaine self-administration and cocaine-induced brain reward in rats (Jin, Semenova et al. 2010). Additional studies on BINA revealed that it is able to reduce psychotic-like traits, such as head twitches and immobility, in preclinical model of schizophrenia and psychosis (Luessen and Conn 2022). Furthermore, AZD8529 was also shown to ameliorate dyskinesia in 6-OHDA-lesioned rats (Shaqfah, Kang et al. 2025). Concerning substance use disorders, $mGlu_2$ PAM AZD8529 and AZD8418 have shown efficacy, both in acute and chronic treatment, in reducing nicotine self-administration and seeking behavior in rats. Interestingly, AZD8529 has been tested in clinical trial recruiting female smokers (Luessen and Conn 2022). Finally, in a schizophrenia-oriented study, activation of $mGlu_2$ with PAM LY487379 improved norepinephrine and serotonin transmission in the medial prefrontal cortex of rats, thereby promoting cognitive flexibility and control over schizophrenia-like behavioral impulses (Nikiforuk, Popik et al. 2010). On the other hand, a preliminary clinical study with the $mGlu_2$ PAM AZD8529 showed, using functional magnetic resonance imaging, that acute administration of the drug was associated with modest but measurable changes in frontostriatal activation during working memory tasks in patients with schizophrenia, which correlated with individual reductions in negative symptoms, despite the absence of robust group-level clinical effects (Wolf, Zheng et al. 2022).

To date, only selective PAMs for $mGlu_2$ or NAMs for $mGlu_2$ (Alonso de Diego, Linares et al. 2024) and for $mGlu_3$ (Wenthur, Morrison et al. 2013) are available. This means that, so far, the only chance to distinguish $mGlu_3$ effects from those of $mGlu_2$ has been either to pharmacologically block $mGlu_3$ using selective NAMs or to genetically silence $mGlu_3$ expression, thereby indirectly associating downstream pathways. More specifically, a few studies in literature have documented the biological effects after inhibition/depotentiation of $mGlu_3$ by its selective NAMs, unveiling $mGlu_3$ contribution and role in CNS homeostasis and disorders, relative to $mGlu_2$. For example, it

has been discovered that the selective $mGlu_3$ NAM VU0650786 inhibits the marble burying behavior associated with anxiety-like and obsessive-compulsive disorder, thereby implicating $mGlu_3$ as a key mediator of behaviors previously ascribed to combined $mGlu_{2/3}$ blockade (Engers, Rodriguez et al. 2015). Another recent study has proven that selective pharmacological blockade of $mGlu_3$ with VU0650786 is able to counteract memory impairment in mice treated with methamphetamine, although it should be noted that similar results were also obtained by blocking $mGlu_2$, thus leaving the dominant receptor in question unspecified (Busceti, Di Menna et al. 2024). Further, VU0650786 has demonstrated antidepressant potential in a mouse model of major depressive disorder, via inhibition of LTD normally mediated by active $mGlu_3$, though again, comparable effects were seen with $mGlu_2$-selective NAMs (Joffe, Santiago et al. 2020). Using a combined pharmacology–genetics approach, another study sought to distinguish $mGlu_3$-mediated effects from those driven by $mGlu_2$. Specifically, in brain slices, the $mGlu_{2/3}$ agonist LY379268 strongly potentiated β-adrenergic receptor–evoked cAMP accumulation and promoted adenosine-dependent signaling, which in turn inhibited β-adrenergic enhancement of hippocampal LTP via A1 receptor activation. This effect was blocked by the $mGlu_3$ NAM VU0469942, not enhanced by the $mGlu_2$ PAM BINA, and was lost in $mGlu_3$ KO but preserved in $mGlu_2$ KO mice. Moreover, *in vivo*, systemic administration of LY379268 disrupted contextual fear memory reconsolidation similarly to the β-adrenergic antagonist propranolol, an effect reversed by the $mGlu_3$ NAM VU0650786. Taken together, these data indicate that $mGlu_3$ is the mGluRs subtype engaged in astrocyte-dependent signaling mechanisms to fine-tune β-adrenergic receptor–mediated modulation of hippocampal synaptic plasticity and cognition (Walker, Sheffler et al. 2017). Finally, a study by Dogra et al. used VU0650786 as proof-of-concept to elucidate $mGlu_3$ function: they found that the pharmacological blockade of $mGlu_3$ impaired the partnership between $mGlu_3$ and $mGlu_5$ in enhancing hippocampal cognitive ability and plasticity in a mouse model of schizophrenia-like disorders (Dogra, Stansley et al. 2021). A comprehensive table of all pharmacological ligands cited in this thesis for mGluRs is presented in *Table 2*.

However, a major gap remained: the absence of a direct selective agonist or PAM for $mGlu_3$. Recently, the synthesis of a novel tetrahydro-isoquinoline compound, described in the patent by Potnick, J. (Potnick 2014), has opened new possibilities for the selective activation of $mGlu_3$, paving the way for mechanistic studies and potential therapeutic applications in diseases such as PD.

Group I ($mGlu_1$, $mGlu_5$)	Group II ($mGlu_2$, $mGlu_3$)	Group III ($mGlu_4$, $mGlu_6mGlu_7$, $mGlu_8$)
NAM: Basimglurant *(PMID: 39368605).* **PAMs:** • CDPPB *(PMID: 31541651)*; • VU0092273 *(PMID: 31600563);* • VU0360172 *(PMID: 31926033).*	**Agonists:** • LY354740 *(PMID: 10777772), (PMID: 24713472), (PMID: 25661514), (PMID: 38900249)*; • LY379268 *(PMID: 12429591), (PMID: 12117601), (PMID: 17670976), (PMID: 19672295), (PMID: 21619889), (PMID: 19909793), (PMID: 28664928)*; • LY404039 *(PMID: 38900249)*; • DCG-IV *(PMID: 12429591), PMID: 12786977*); • 2R,4R-APDC *(PMID: 16197521), (PMID: 20948891).* **PAMs:** • LY487379 *(PMID: 40337518), (PMID: 20739457)*; • AZD8529 *(PMID: 27339394), (PMID: 39841218), (PMID: 35710132), (PMID: 34667261)*; • AZD8418 *(PMID: 35710132);* • CBiPES *(PMID: 38900249)*; • BINA *(PMID: 20555310), (PMID: 35710132), (PMID: 28664928), (PMID: 16608916).* **NAMs:** • 6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one derivatives: e. g., VU0650786 *(PMID: 26335039), (PMID: 38969501), (PMID: 31735403), (PMID: 33965197), (PMID: 28664928)*; • VU0469942 *(PMID: 28664928);* • RO4491533 *(PMID: 23718281);* • LY2389575 *(PMID: 23718281);* • ML 289 *(PMID: 23718281);* • ML 337 *(PMID: 23718281).*	**PAMs**: • VU0155041 *(PMID: 22404342);* • VU6046980 *(PMID: 40316832);* • VU6005649 *(PMID: 40316832);* • VU6027459 *(PMID: 40316832).* **NAMs:** • MMPIP *(PMID: 40316832);* • XAP044 *(PMID: 40316832);* • VU6019278 *(PMID: 40316832).*

Table 2: Overview of the pharmacological ligands targeting mGluRs.

The table lists the pharmacological ligands for the different mGluRs cited in the present thesis.

2. AIMS

Given the challenging complexity of PD pathophysiology and the limitations of currently available therapies, coupled with the regulatory role of group II mGluRs in CNS disorders, growing attention has been directed toward $mGlu_3$ as a potential neuroprotective target. Although evidence supporting the protective role of $mGlu_3$ against dopaminergic neurodegeneration is still largely indirect, the recent synthesis of a novel putative $mGlu_3$ PAM offers a valuable opportunity to further investigate this mechanism.

In this context, the present work aims to first characterize the pharmacological profile of the new compound *in vitro* by assessing its selectivity for $mGlu_2$ and/or $mGlu_3$. This will be achieved by exploiting recombinant cell systems and pharmacological assays based on the quantification of inositol monophosphate (IP1) production. Subsequently, the neuroprotective potential of the compound will be evaluated *in vitro* using SH-SY5Y cells exposed to the dopaminergic neurotoxin 6-OHDA, with cell viability assessed through MTT assay.

To gain mechanistic insights into the observed effects, the modulation of intracellular neuroprotective signaling pathways will be investigated in SH-SY5Y cells by western blot analyses. In parallel, the ability of the compound to modulate the expression of GDNF protein will be also assessed, in order to ensure consistency with the existing literature on group II mGluRs and $mGlu_3$-mediated neuroprotection (Battaglia, Molinaro et al. 2009).

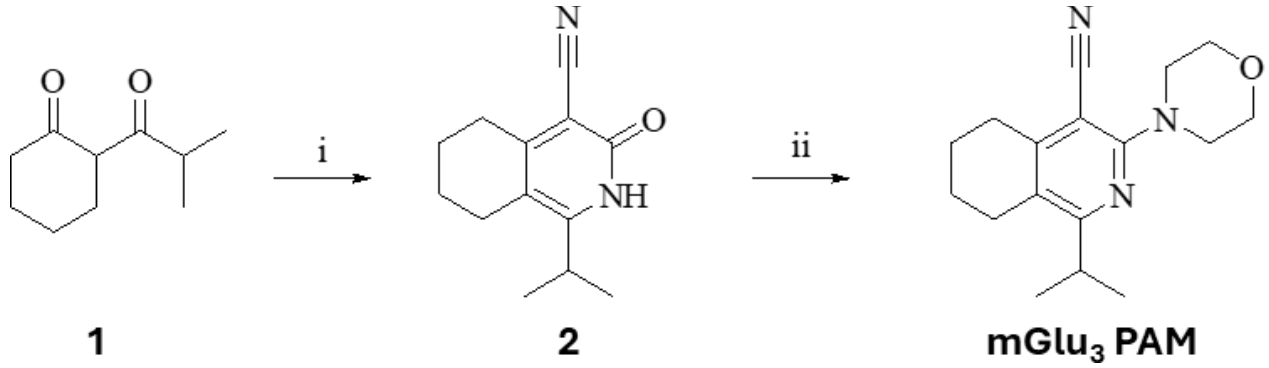
Finally, a preliminary *in vivo* study will be conducted in healthy control mice to investigate the neuromodulatory effects of the compound on major neurotrophic factors and associated signaling pathways. These analyses, performed by western blotting, will provide a first evaluation of the compound ability to affect or potentiate brain circuitry in naïve animals, thereby establishing a foundation for subsequent testing in PD animal models.

3. MATERIALS AND METHODS

3.1 Preparation of the new putative selective $mGlu_3$ PAM

3.1.1 Chemistry

The novel putative selective $mGlu_3$ PAM, developed under patent by Merck, was synthesized by the research unit of chemists at the University of Palermo, led by Prof. Virginia Spanò, following previously published methodological guidelines (Potnick 2014). Briefly, 2-isobutyrylcyclohexanone **1** reacted with 2-cyanoacetamide in the presence of a catalytic amount of piperidine in ethanol, as solvent, to give the intermediate **2**. Later, morpholine was added together with the BOP reagent (to activate the carbonyl group) and diisoprilethylamine as base, and the reaction was run in presence of N,N-dimethylformamide (DMF). Finally, the product of interest **3** was isolated (*Scheme 1*).

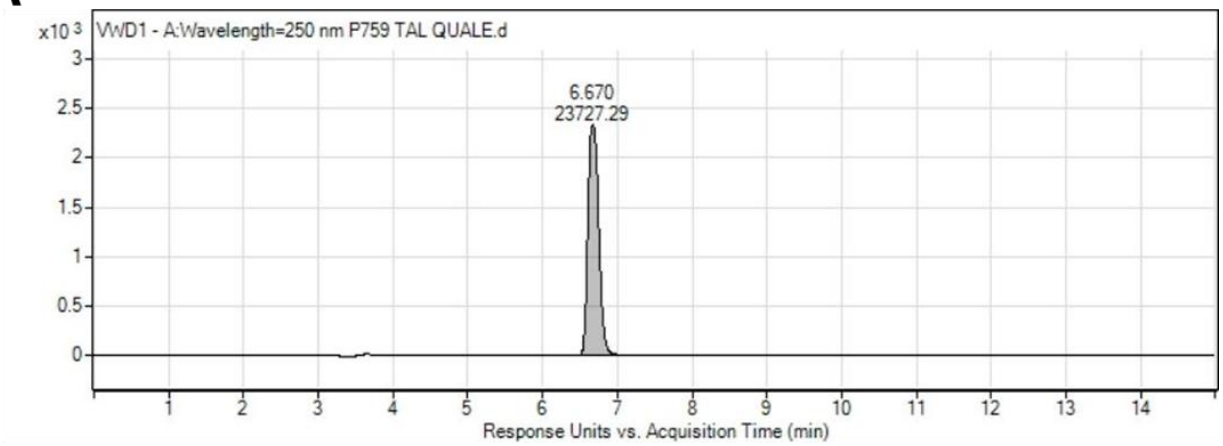


Scheme 1: Synthesis of 1-isopropyl-3-morpholin-4-yl-5,6,7,8-tetrahydroisoquinoline-4-carbonitrile (putative $mGlu_3$ PAM). (i) 2-cyano acetamide, piperidine, ethanol, rt; (ii) BOP reagent, diisopropylethylamine, morpholine, DMF, 50 °C.

For purity assessment of the compound of interest, Reversed Phase HPLC/ESI/Q-TOF mass spectrometry was performed as follows. Water and acetonitrile were of HPLC/MS grade.

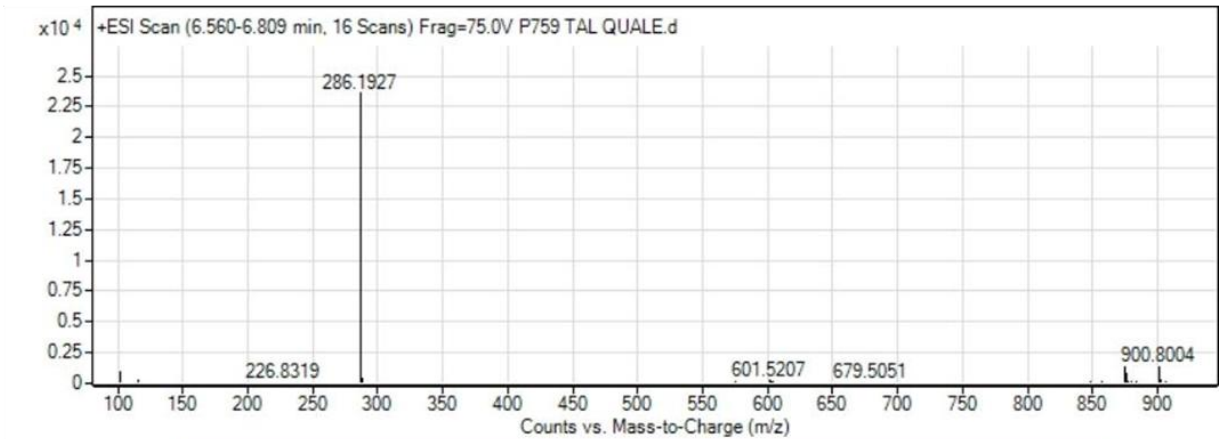
Formic acid was of analytical quality. The HPLC system was an Agilent 1260 Infinity. A reversed-phase C18 column (Luna C18) with a Phenomenex C18 security guard column was used. The flow-rate was 0.4 mL/min and the column temperature was set to 30 °C. The eluents were formic acid–water (0.1:99.9, v/v) (phase A) and formic acid–acetonitrile (0.1:99.9, v/v) (phase B). The run was performed under isocratic conditions with 70% B for 15 min. Injection volume was 10 mL. The eluate was monitored through UV absorbance at 250 nm. Mass spectra were obtained on an Agilent 6540 UHD accurate-mass Q-TOF spectrometer equipped with a Dual AJS ESI source working in positive mode. N_2 was employed as desolvation gas at 300 °C and a flow rate of 8 L/min. The nebulizer was set to 45psig. The Sheat gas temperature was set at 400 °C and a flow of 12 L/min. A potential of 3.5 kV was used on the capillary for positive ion mode. The fragmentor was set to 175 V. MS spectra were recorded in the 150–1000 m/z range (*Figure 9*). The final spectroscopic data agreed with literature (Potnick 2014).

A



Peak	RT	Area Sum %	Area	Area %	Height	Max Y
1	6.67	100	23727.29	100	2330.67	2330.92

B



HRMS (ESI$^+$/Q-TOF) calculed for $[C_{17}H_{23}N_3O+H]^+$: 286.1914, found: 286.1927

Figure 9: HPLC trace of mGlu$_3$ PAM.

3.1.2 Biology

For biological assays, the new putative $mGlu_3$ PAM was first solubilized in a minimal volume of dimethyl sulfoxide (DMSO) and then diluted with phosphate-buffered saline (PBS) to achieve a final stock concentration of 5 mM for *in vitro* experiments.

3.2 Other drugs

6-hydroxydopamine hydrochloride (6-OHDA, H-6507) and LY294002 (440202) were purchased from Merck KGaA, Darmstadt, Germany. ML 337 (4982) and BINA (4048) were purchased from Tocris Bioscience.

6-OHDA solution was freshly prepared each time by dissolving the compound in basal cell culture medium to obtain a 2 mM stock concentration. LY294002, ML 337 and BINA were solubilized in DMSO to a final concentration of 10 mM, and stock aliquots were stored at -20°C.

3.3 Cell cultures

3.3.1 HEK293 cells

HEK293 cells (ATCC-CRL1573), regularly tested to be mycoplasma-free, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Cergy Pontoise, France), supplemented with 10% of fetal bovine serum (FBS), at 37°C and 5% CO2. Cells were transiently transfected by electroporation with human clones of $mGlu_2$ and $mGlu_3$ receptors, together with the high-affinity glutamate transporter EAAC1 (in order to minimize extracellular glutamate concentration) and a chimeric Gqi9 protein to allow coupling to the PLC pathway. Cells were seeded at a density of 1×10^5 cells/well in a 96-well plate previously coated with poly-ornithine. Prior to experiments, plates were kept in the incubator at 37°C and 5% CO2 for 24 h.

3.3.2 SH-SY5Y cells

Human SH-SY5Y neuroblastoma cell line was grown in T25 flasks in a humidified atmosphere of 95% air and 5% CO2 at 37 °C, using a medium composed by Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, Thermo Fisher Scientific), 10% FBS (Thermo Fisher Scientific), Penicillin-Streptomycin (100 U/mL, Thermo Fisher Scientific) and 2 mM L-Glutamine (Thermo Fisher Scientific). The cell culture medium was replaced every three days, and cells were sub-cultured once they reached 90% confluence. All treatments were performed 48 h after plating, using cells up to passage 14.

3.4 *In vitro* experimentation design

3.4.1 HEK293 cell treatment

For the evaluation of the pharmacological activity on $mGlu_2$ and $mGlu_3$ clones, HEK293 cells were exposed for 30 min to different concentrations of the compound of interest, described in literature as a putative selective $mGlu_3$ PAM, ranging from 0.1 nM to 100 μM, alone or in presence of 3 nM of the $mGlu_{2/3}$ agonist LY379268.

3.4.1.1 Cell based pharmacological assays

The monitoring of receptor activity was performed by quantification of the production of IP1 in HEK293 cells transiently transfected with the $mGlu_2$ or $mGlu_3$ and the chimeric Gqi9 protein, using the IP-One HTRF kit (Revvity) according to the manufacturer's recommendations.

24 h after transfection, the cell medium was replaced by DMEM Glutamax (Life Technologies, Cergy Pontoise, France) for 90 min and preincubated with 1 μM of the group II mGluRs antagonist LY341495 during the last 10 min, to reduce the influence of the extracellular glutamate concentration on receptor activity. Cells were stimulated with different concentrations of the novel $mGlu_3$ PAM to induce IP1 accumulation, in presence or absence of 3 nM, known as

the effective concentration 20% (EC_{20}), of the group II mGluRs agonist LY379268. Cells were incubated for 30 min, at 37 °C. After stimulation, the medium was removed from the plate and 28 µL of lysis buffer were added to each well. Cell lysates (14 µL) were transferred to a white 384-well plate in which d2-labeled IP analog and terbium cryptate-labeled anti-IP antibodies (6 µL) were added using a repetitive pipette, and the plate was incubated for 1 h. The fluorescence at 665 nm and at 620 nm was measured with a pherastar HTRF HTS microplate reader (BMG Labtech). Afterwards, the HTRF ratios (665/620 nm) were transformed to IP concentration produced by the cells, using a standard IP1 curve.

From this experiment, the compound functions as a PAM/agonist, with slight preference for $mGlu_3$ selectivity, and will henceforth be referred to as $mGlu_{2/3}$ Ago/PAM.

3.4.2 SH-SY5Y cell treatment

For the $mGlu_{2/3}$ Ago/PAM dose-effect and time-course evaluation on cell viability, SH-SY5H cells were exposed for 24 h to three different concentrations (1 µM, 10 µM and 100 µM). The highest dose (100 µM) was then used to prolong treatment up to 72 h.

For 6-OHDA dose-effect evaluation on cell viability, cells were exposed for 24 h to different concentrations, ranging from 1 µM to 1 mM. Then, based on the obtained half maximal effective concentration (EC_{50}) value, 50 µM dose was selected for all subsequent experiments.

To evaluate the putative neuroprotective effect of the new $mGlu_{2/3}$ Ago/PAM on cell viability, cells were treated for 24 h with different concentrations of the compound (1 µM, 10 µM and 100 µM) 10 min before exposure (co-treatment) to 50 µM 6-OHDA. Based on these results, the $mGlu_{2/3}$ Ago/PAM concentration of 100 µM, which partially inhibited 6-OHDA-induced neurotoxicity, was selected for subsequent experiments.

To assess a dose-effect response of cell viability to $mGlu_3$ NAM (ML 337), cells were exposed for 24 h to different concentrations of ML 337 (from 5 µM to 100 µM). For all subsequent experiments, ML 337 was used at 10 µM, the highest non-toxic concentration that produced no effect on $mGlu_1$, $mGlu_2$, or $mGlu_{4–8}$ (Wenthur, Morrison et al. 2013).

In experiments assessing the blockage of $mGlu_{2/3}$ Ago/PAM neuroprotective effect on cell viability given by ML 337, cells were pre-treated with ML 337 (10 μM) for 30 min, followed by co-treatment with $mGlu_{2/3}$ Ago/PAM (100 μM) and 6-OHDA (50 μM) for 24 h.

In dose-effect experiments on cell viability with the $mGlu_2$ PAM (BINA) (Galici, Jones et al. 2006), cells were treated for 24 h with different concentrations of BINA (ranging from 1 μM to 100 μM), based on the compound's EC_{50} and the previously explored doses for $mGlu_{2/3}$ Ago/PAM.

To evaluate BINA potential neuroprotective effects against 6-OHDA-induced cell death, cells received 10 μM or 100 μM of BINA added 10 min before (co-treatment) 6-OHDA 50 μM, for 24 h.

In experiments investigating the putative role of phosphatidylinositol 3-kinase-Akt (PI3K–AKT) in $mGlu_{2/3}$ Ago/PAM-induced neuroprotection against cell death caused by 6-OHDA, cells were pre-treated for 30 min with 10 μM of LY294002, based on previous validation (Scordino, Urone et al. 2024), followed by addition of $mGlu_{2/3}$ Ago/PAM (100 μM) and 6-OHDA (50 μM) for 24 h.

In experiments exploring $mGlu_{2/3}$ Ago/PAM-induced modulation of GDNF and PI3K-AKT and MAPK/ERK pathways, cells received $mGlu_{2/3}$ Ago/PAM (100 μM) 10 min before 6-OHDA treatment (50 μM) for 24 h. In contrast, when measuring the regulation of PI3K–AKT and MAPK/ERK protein levels after a short-term exposure to $mGlu_{2/3}$ Ago/PAM, cells were treated with $mGlu_{2/3}$ Ago/PAM only (100 μM) for 5 min, 15 min, and 30 min.

In all the experiments, control condition cells received an equal amount of the solvent only.

3.4.2.1 RNA extraction and RT-PCR

SH-SY5Y cells were plated in 96-well plates, at a density of 2×10^4 cells/well and in a final volume of 100 μL/well. After reaching confluence, cells were mechanically detached, and the content of 7 wells was pooled into one sample. Samples were centrifuged at 4000 rpm for 5 min at 4 °C to remove medium. Then, RNA extraction was performed using Qiagen RNeasy mini kit (74104, Qiagen, Hilden, Germany) and the manufacturer's instructions. RNA concentration was detected with a MultiskanGO Microplate Spectrophotometer (Thermo Scientific Waltham,

MA USA) and 1 µg of RNA was reverse-transcribed in 20 µL, as final volume, of the following mix: 5× first strand buffer (18080-044, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µM random hexamers (N112470, Roche, Basel, Switzerland), 2.5 mM dithiothreitol (DTT) (18080-044, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 0.5 mM dNTPs (18427-013, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 40 U RNAse inhibitor (N8080119, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and 200 U Superscript III Reverse Transcriptase (18080-044, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The mix was first incubated for 2 h at 50 °C and, later, for 15 min at 70 °C. 2 µL of the obtained cDNA were amplified using $mGlu_3$ specific primers (F: GGTGGCTCTTATAGCAGTGTTTC; R: CCTGGCAAAGTAATCATAGCGCG) and the following protocol: first denaturation step at 95°C for 3 min, followed by a cyclic denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 1 min repeated for 40 cycles, and a final elongation at 72 °C for 5 min. 10 µL of amplified product were run on a 2% agarose ethidium bromide stained gel and a 129 bp band was visualized with a Biorad Gel Doc XR+.

3.4.2.2 MTT assay

SH-SY5Y cells were grown in 96-well plates at a density of 2×10^4 cells/well and in a final volume of 100 µL/well. At the end of treatment, the medium was removed and replaced with 100 µL/well of 0.5 mg/mL tetrazolium salt (MTT) solution. After 3 h incubation at 37°C, MTT solution was replaced with 100 µL/well of DMSO and the presence of purple formazan granules, produced only by metabolically active cells, was quantified by measuring absorbance at 570 nm. Cell viability was expressed as arbitrary units (AU), with the control group set to 1. Data were collected from a minimum of three independent experiments, each with at least three biological replicates per treatment.

3.5 Animals

2-months old male C57BL/6 mice, purchased from Envigo S.r.l (Indianapolis, Indiana), were housed under controlled environmental conditions (22–24 °C, 50 ± 10% humidity) in a 12 h dark-light cycle (8:00–20:00 h) with food and water ad libitum. Before starting all experimental procedures, animals underwent a two-weeks acclimation period.

All experiments were conducted in agreement with the ARRIVE guidelines and the European Directive (2010/63/EU), following the 3Rs principles. The experimental procedures were approved by the animal welfare committee of the University of Palermo and were authorized by the Ministry of Health (Rome, Italy; Authorization Number 573/2022-PR). All efforts were made to minimize both the suffering and the number of animals used, including the design of a pilot experiment with a small sample size to identify the most effective $mGlu_{2/3}$ Ago/PAM dose and treatment duration (*see section 3.5.1*).

3.5.1 *In vivo* experimentation design

For the pilot experiment, animals were divided into two groups and then injected intraperitoneally (i.p.) with two different doses of $mGlu_{2/3}$ Ago/PAM (1 mg/kg and 5 mg/kg) for two different time points (24 h and 72 h) (*Figure 10*), based on previous evidence about *in vivo* administration of group II mGluRs agonists (Battaglia, Molinaro et al. 2009).

Subsequently, a second experiment was performed using $mGlu_{2/3}$ Ago/PAM at 1 mg/kg for 24 h. Control condition animals received an equal amount of the solvent only (DMSO and saline solution). Potential behavioral changes or acute side effects associated with $mGlu_{2/3}$ Ago/PAM exposure were excluded by visual inspection and pain assessment through the Grimace Scale. At the end of treatment, all animals were sacrificed by cervical dislocation after 2% isoflurane anesthesia, and the brain was quickly removed. Hippocampus, prefrontal cortex and striatum were dissected under stereomicroscopy with refrigerate support. Collected tissues were quickly frozen and stored at −80 °C for protein extraction.

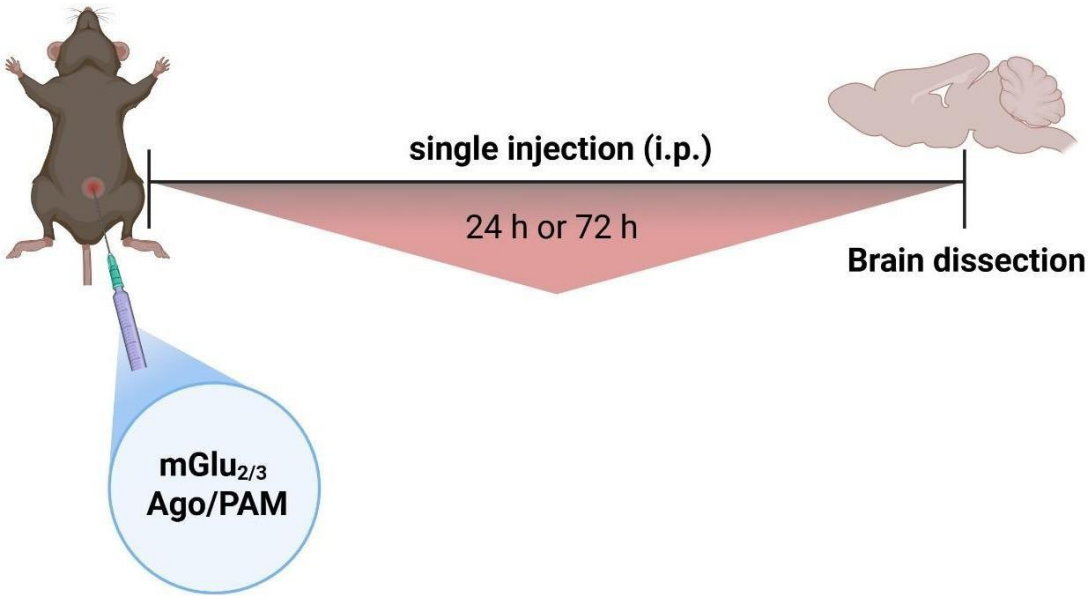


Figure 10: In vivo experimental design.

Illustration of the *in vivo* experimental scheme. Healthy mice, wild type for $mGlu_2$ and $mGlu_3$ expression, received a single injection of $mGlu_{2/3}$ Ago/PAM i.p. For the pilot study, sacrifice and brain dissection were done after 24 h or 72 h from administration of a dose of 1 mg/kg or 5 mg/kg, while further investigations were performed administering the new $mGlu_{2/3}$ Ago/PAM only at 1 mg/kg for 24 h. (Created in https://BioRender.com)

3.6 Protein extraction, protein electrophoresis and Western blotting

SH-SY5Y cells were plated in 96-well plates, at a density of 2 x 10^4 cells/well and in a final volume of 100 µL/well. At the end of treatment, cells were mechanically detached, and the content of 4 wells was pooled into one sample. Cell pellet as well as animal tissue were homogenized in a cold radioimmunoprecipitation assay (RIPA) buffer containing: 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, SDS 0.1% and a cocktail of protease inhibitor (Sigma-Aldrich P8340) and phosphatase inhibitor (P5726 Sigma-Aldrich Merck KGaA, Darmstadt, Germany). Homogenates were then sonicated (30 pulsations/min) and protein amount was quantified via the Lowry method (Lowry, Rosebrough et al. 1951).

Protein samples (30 µg for cell homogenates and 50 µg for tissue homogenates) and the molecular weight marker (PageRuler Plus Prestained Protein Ladder, 26619 ThermoFisher Scientific, Waltham, MA USA) were run on an 8%-12% polyacrylamide gel and transferred onto a nitrocellulose membrane (10600002, Amersham Protran, Cytiva). Later, membranes were incubated for 1 h at 4 °C with a blocking buffer (1× TBS, 0.1% Tween−20, 5% w/v nonfat dry milk), and subsequentially overnight with the following specific primary antibodies: rabbit anti-GDNF (1:1000 diluted, sc-328, Santa Cruz Biotechnology, Dallas, TX, USA) or goat anti-GDNF (1:1000 diluted, AF-212-NA, Bio-Techne, Minneapolis, MN, USA), rabbit anti-BDNF (1:1000 diluted, sc-546, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-FGF2 (1:1000 diluted, sc-79, Santa Cruz Biotechnology, Dallas, TX, USA), rat anti-DAT (1:1000 diluted, sc-32258, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-p-ERK1/2 (1:1000, 9101, Cell Signaling Technology, Beverly, MA, USA), rabbit anti-p-Akt (1:1000, 4060, Cell Signaling Technology, Beverly, MA, USA). Membranes were then washed three times for 10 min with TBS-T and incubated for 1 h with the following secondary antibodies: rabbit anti-goat IgG-HRP (1:6500, sc-2768), goat anti-rat IgG-HRP (1:6500, sc-2006), or mouse anti-rabbit IgG-HRP (1:8000, sc-2357), all purchased from Santa Cruz Biotechnology, Dallas, TX, USA. For normalization, membranes were exposed to a horseradish peroxidase-conjugated β-Actin primary antibody (1:8000 diluted, sc-47778 Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. After three washings with the TBS-T, the immunocomplexes were visualized by a chemiluminescence reagent (SuperSignal West Pico Plus, ThermoFisher Scientific, Waltham, MA, USA) and the signal was captured using an autoradiography film (28-9068-36 Amersham Hyperfilm ECL, GE Healthcare Europe, Milano, Italy) exposed to a Kodak D19 developer and fixer (1900984 and 1902485, Kodak GBX, Rochester, NY, USA), or using the iBright FL1000 Imaging System (ThermoFisher Scientific, Waltham, MA, USA). Optical density (OD) was measured using ImageJ Software (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA) or iBright Analysis Software (ThermoFisher Scientific, Waltham, MA, USA), with results expressed as AU. For cell samples, data were collected from at least three independent experiments, with at least 3 biological replicates per treatment, and the average was calculated. For tissue samples, data were collected from two independent experiments, with 3 biological replicates per treatment. Electrophoresis and western blotting were repeated three times for each sample (technical replicates), and the results were averaged before averaging the results from independent experiments.

3.7 Statistical analysis

For *in vitro* experimentation, data were collected from three independent experiments, each with at least 3 biological replicates per treatment. For *in vivo* experimentation, data were collected from two independent experiments, with 3 animals per group (6 animals per group in total).

Data analysis was performed using GraphPad Prism 9.0.2 software (GraphPad Software, La Jolla, CA, USA). The normal distribution of data was assessed via the Shapiro–Wilk test, while outliers were identified using the Rout method (Q = 5%). For data normally distributed, results were analyzed by one-way ANOVA, followed by Tukey's Post-hoc test, or unpaired two-tailed t-test. The relative results were presented as the mean ± SEM. For non-normally distributed data, statistical evaluations were performed by Kruskal–Wallis test, followed by Dunn's multiple comparison test, or Mann-Whitney test. The relative results were displayed as median with interquartile range. For $mGlu_{2/3}$ Ago/PAM dose- and time-dependent data from the *in vivo* pilot experiment, a two-way ANOVA followed by Tukey's post-hoc test was applied. Differences in p-value less than 0.05 were considered statistically significant.

4. RESULTS

4.1 Characterization of the pharmacological activity of the new compound on $mGlu_2$ and $mGlu_3$

As a first step, the ability of the compound to enhance the activity of $mGlu_2$ and $mGlu_3$ receptors, either alone or in presence of an agonist, was evaluated. To that aim, HEK293 cells were transiently transfected to express the human $mGlu_2$ and $mGlu_3$ receptors and subsequently stimulated with increasing concentrations of the compound, applied either alone or together with a low concentration (3 nM; approximately EC_{20}) of the group II mGluRs agonist LY379268 (*Figure 11*). The compound displayed a dose-dependent potentiation of agonist-induced activity of both $mGlu_2$ and $mGlu_3$ receptors, with a pEC_{50} of 4.25 ± 0.09 (n=4) and 4.99 ± 0.14 (n=5), respectively. When applied alone, it dose-dependently activated both $mGlu_2$ and $mGlu_3$ receptors, with slightly lower pEC_{50} values of 3.27 ± 0.06 (n=3) and 4.64 ± 0.22 (n=4), respectively. The possibility that, at least in part, the agonist activity of the compound on $mGlu_2$ and $mGlu_3$ observed in absence of added agonist could result from a potentiation of ambient glutamate cannot completely ruled out, despite the experimental conditions being designed to reduce the presence of glutamate in the medium. Thus, the compound appears as an Ago/PAM presenting a slight preference for $mGlu_3$ over $mGlu_2$ receptors ($mGlu_{2/3}$ Ago/PAM).

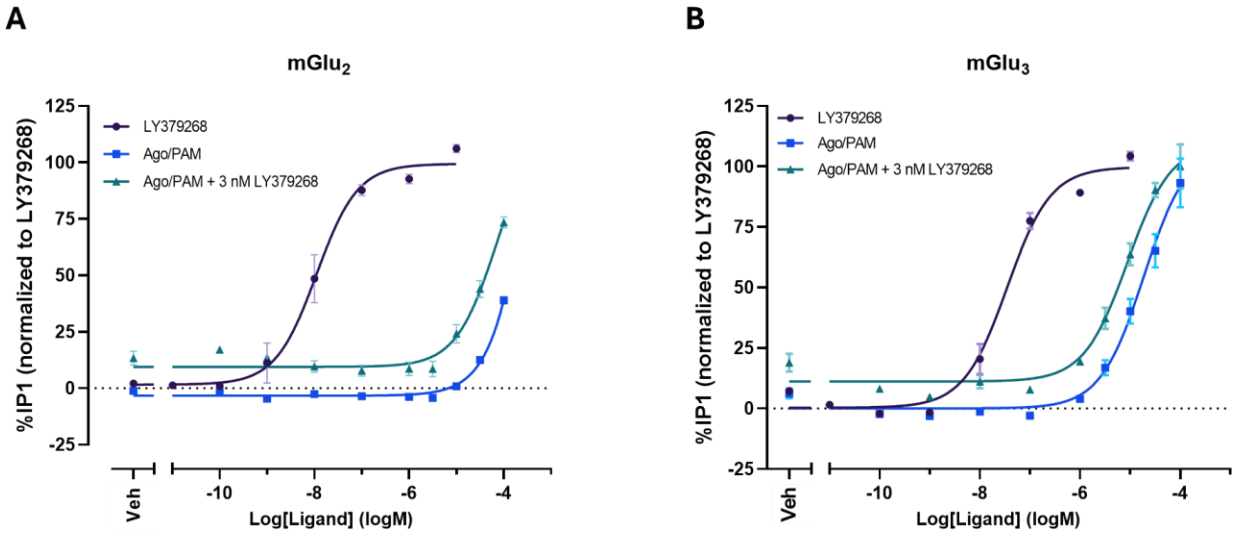


Figure 11: Effect of $mGlu_{2/3}$ Ago/PAM on $mGlu_2$ and $mGlu_3$ receptors signaling in HEK293 cells.

A) Dose-dependent potentiation of $mGlu_2$ receptor signaling, in absence or in presence of a low concentration (3 nM ≈ EC_{20}) of the group II mGluRs agonist LY379268. **B)** Dose-dependent potentiation of $mGlu_3$ receptor signaling, in absence or in presence of a low concentration (3 nM ≈ EC_{20}) of the group II mGluRs agonist LY379268. Receptor activity was monitored by quantification of the production of IP1 in HEK293 cells transiently transfected with the $mGlu_2$ or $mGlu_3$ and the chimeric Gqi9 protein.

4.2 $mGlu_{2/3}$ Ago/PAM effects on SH-SY5Y cells viability

Before exploring the neuroprotective effects of $mGlu_{2/3}$ Ago/PAM on human neuroblastoma SH-SY5Y cell line, we first checked $mGlu_3$ gene expression through RT-PCR. *Figure 12A* shows a 129 bp band in the cell sample, whereas no signal was detected in the negative control (N.C.) sample, in which the cDNA template was replaced with water in the reverse transcription mix.

Consequently, MTT assay was performed to verify and, eventually, exclude any possible effects on cell viability given by the exposure to the newly synthesized compound. *Figure 12B* shows that 24 h treatment with 1 µM, 10 µM and 100 µM of the new $mGlu_{2/3}$ Ago/PAM did not affect cell viability, while *Figure 12C* shows that, even when exposure to the highest $mGlu_{2/3}$

Ago/PAM dose of 100 µM was prolonged up to 72 h treatment, the new tetrahydro-isoquinoline derivative did not exert any cytotoxic effects on SH-SY5Y cells.

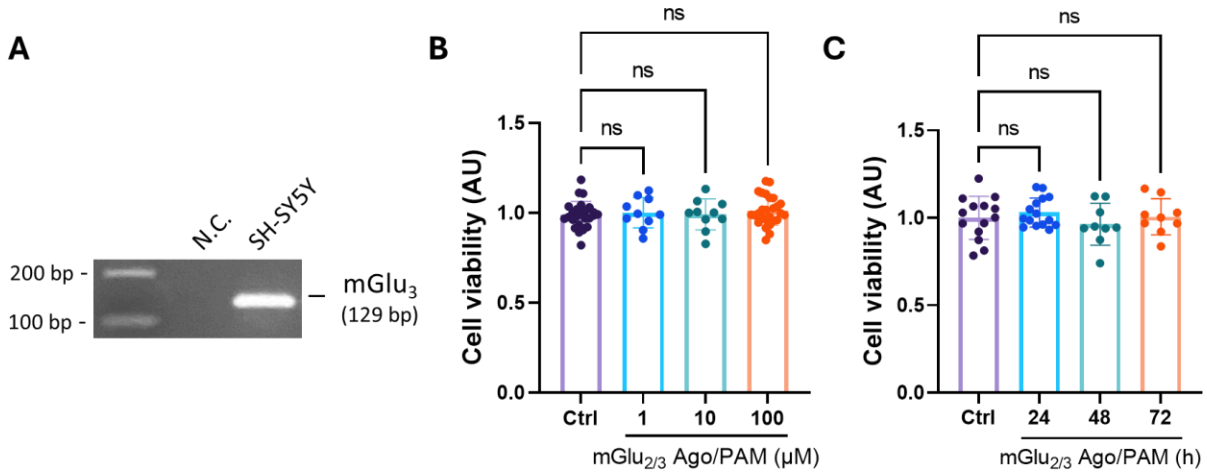


Figure 12: Effects of $mGlu_{2/3}$ Ago-PAM on SH-SY5Y cell viability.

A) RT-PCR validation of $mGluR_3$ gene expression from SH-SY5Y cDNA compared to negative control (N.C.) sample without DNA template. **B)** MTT assay on the dose-effect of $mGlu_{2/3}$ Ago/PAM (1 µM, 10 µM or 100 µM) on SH-SY5Y cell viability at 24 h. **C)** Time-course evaluation of 100 µM $mGlu_{2/3}$ Ago/PAM on SH-SY5Y cell viability at 24 h, 48 h, and 72 h, assessed via MTT assay. ANOVA followed by Tukey's test: ns (not significant).

4.3 Susceptibility of SH-SY5Y cells to 6-OHDA and neuroprotective effect of the new $mGlu_{2/3}$ Ago/PAM

Next, SH-SY5Y cells sensitivity to the 6-OHDA, a selective neurotoxin for dopaminergic cells, was checked. *Figure 13A* shows western blot bands supporting protein expression of DAT by SH-SY5Y cells, compared to tissue sample used as positive control (mouse striatum). Based on this, a dose–response curve for 6-OHDA toxicity was generated by exposing cells to concentrations ranging from 1 µM to 1 mM, and cell viability was assessed 24 h after treatment using the MTT assay. As shown in *Figure 13B*, 6-OHDA gradually reduced SH-SY5Y viability,

with an EC_{50} of approximately 50 μM, resulting in about 40% cell death (*Figure 13B,C*). This concentration was therefore selected for subsequent experiments.

Finally, the putative neuroprotective effect of the new $mGlu_{2/3}$ Ago/PAM on SH-SY5Y cell viability was investigated. Cells were exposed to a combination of 6-OHDA 50 μM and $mGlu_{2/3}$ Ago/PAM 1 μM, 10 μM or 100 μM for 24 h, and MTT was carried out. *Figure 13D* shows that lower doses of the new $mGlu_{2/3}$ Ago/PAM were not sufficient in rescuing cells after exposure to 6-OHDA, while *Figure 13E* indicates that 100 μM of the compound partially, yet significantly, counteracted 6-OHDA-induced cell death.

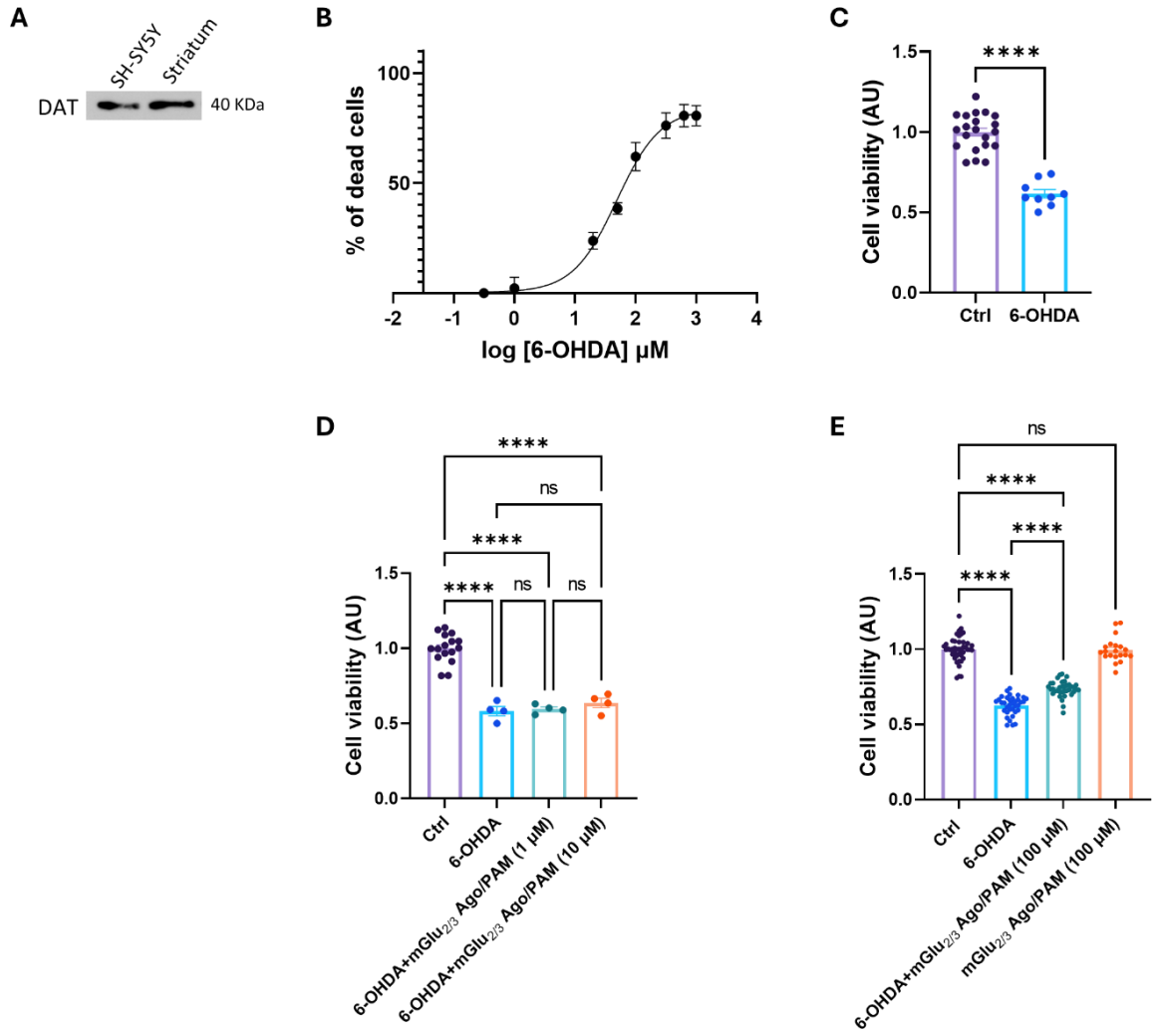


Figure 13: 6-OHDA effects on SH-SY5Y cell viability and neuroprotection of the new mGlu$_{2/3}$ Ago/PAM.

A) Western blot bands of DAT protein expression by SH-SY5Y and mouse striatum under basal condition. **B**) Dose-response curve showing percentage of SH-SY5Y dead cells in relation to the logarithmic concentration of 6-OHDA (expressed in µM), evaluated via MTT assay. **C)** Column graph showing that 6-OHDA 50 µM, corresponding to the drug's EC_{50}, caused 40% death circa in cell population at 24 h. **D)** Column graph displaying the failure of both 1 µM and 10 µM mGlu$_{2/3}$ Ago/PAM (24 h) in counteracting 6-OHDA-induced cell death (50 µM, 24 h) in SH-SY5Y cells, evaluated through MTT assay. **E)** Column graph showing the rescue effect of the mGlu$_{2/3}$ Ago/PAM (100 µM, 24 h) on SH-SY5Y cell viability following 6-OHDA-induced cell death (50 µM, 24 h), as assessed by the MTT assay. t-test in C, ANOVA followed by Tukey's test in D and E: **** $p < 0.0001$, ns (not significant).

4.4 Pharmacological evidence for the involvement of $mGlu_3$ in the activity of the newly synthesized Ago/PAM

At this stage, confirmation of $mGlu_3$ involvement in the Ago/PAM-induced neuroprotection observed in SH-SY5Y cells was required. To achieve this aim, a commercially available NAM (ML 337) (Wenthur, Morrison et al. 2013) selective for $mGlu_3$ was used. First, a dose–response analysis of NAM (ML 337) on SH- SY5Y cell viability was performed using the MTT assay. Cells were exposed for 24 h to increasing NAM (ML 337) concentrations (5–100 μM), and a reduction in cell viability was observed at concentrations ≥ 20 μM (*Figure 14A*). Consequently, 10 μM NAM (ML 337), the highest concentration showing no cytotoxic effect *per se*, was selected for further experiments. When co-administered for 24 h with 6-OHDA (50 μM) and the $mGlu_{2/3}$ Ago/PAM (100 μM), NAM (ML 337) completely abolished the neuroprotective effect previously observed for the new $mGlu_{2/3}$ Ago/PAM, as shown by the MTT assay results (*Figure 14B*).

In addition to this, a commercial PAM selective for $mGlu_2$ (BINA) (Galici, Jones et al. 2006) was also tested to further determine whether the observed neuroprotective effect on SH-SY5Y cell viability was specifically associated with $mGlu_3$ activation. A preliminary dose–response experiment revealed no toxic effects of the $mGlu_2$ PAM (BINA) on SH-SY5Y cells (*Figure 14C*). Subsequently, concentrations of 10 μM and 100 μM were co-applied for 24 h with 6-OHDA (50 μM), but neither dose was able to restore cell viability (*Figure 14D*), in contrast to the significant neuroprotection elicited by the novel $mGlu_{2/3}$ Ago/PAM.

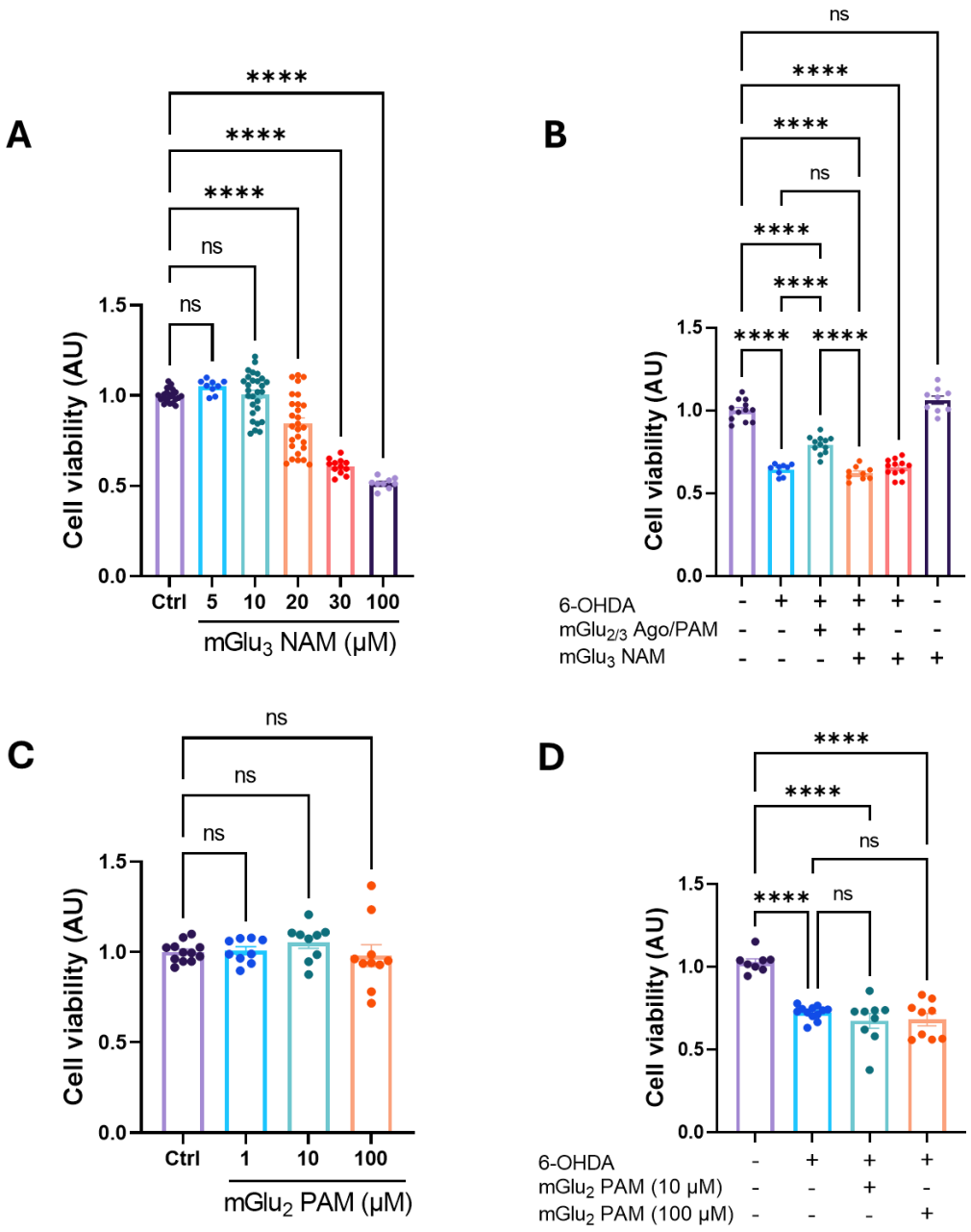


Figure 14: Evidence for the involvement of mGlu$_3$ in the neuroprotection induced by the new Ago/PAM.

A) Dose-effect of mGlu$_3$ NAM (ML 337) (5 µM, 10 µM, 20 µM, 30 µM, or 100 µM) on SH-SY5Y cell viability after 24 h exposure, assessed by MTT assay. **B)** Column graph showing that mGlu$_3$ NAM (ML 337) (10 µM, 24 h) abolished the cell viability recovery induced by the novel mGlu$_{2/3}$ Ago/PAM (100 µM, 24 h) following 6-OHDA (50 µM, 24 h)-induced cell death, as evaluated by MTT assay. **C)** Dose-effect of mGlu$_2$ PAM (BINA) (1 µM, 10 µM or 100 µM) on SH-SY5Y cell viability after 24 h exposure, evaluated by MTT assay. **D)** Column graph showing that mGlu$_2$ PAM (BINA) (10 µM or 100 µM, 24 h) failed in counteracting 6-OHDA (50 µM, 24 h)-induced cell death, as assessed by MTT assay. ANOVA followed by Tukey's test: **** $p < 0.0001$, ns (not significant).

4.5 *In vitro* evaluation of neuroprotective pathways activated by the new $mGlu_{2/3}$ Ago/PAM: investigation on PI3K-AKT modulation

Western blot analyses were performed in SH-SY5Y samples to identify protein pathways underlying the protective effect of the novel $mGlu_{2/3}$ Ago/PAM against dopaminergic damage. *Figure 15A* shows that short-term exposure (5, 15, and 30 min) to the novel $mGlu_{2/3}$ Ago/PAM (100 μM) induced up-regulation of phosphorylated-AKT (p-AKT), indicating activation of the PI3K–AKT pathway. *Figure 15B* shows that 24 h co-treatment with the Ago/PAM (100 μM) reversed the 6-OHDA (50 μM)-induced suppression of PI3K–AKT signaling. Notably, when cells were treated for 24 h with 6-OHDA (50 μM), Ago/PAM (100 μM), and 10 μM LY294002, a PI3K–AKT pathway inhibitor, the recovery of cell viability was abolished, highlighting the essential role of this signaling cascade in Ago/PAM-mediated neuroprotection (*Figure 15C*).

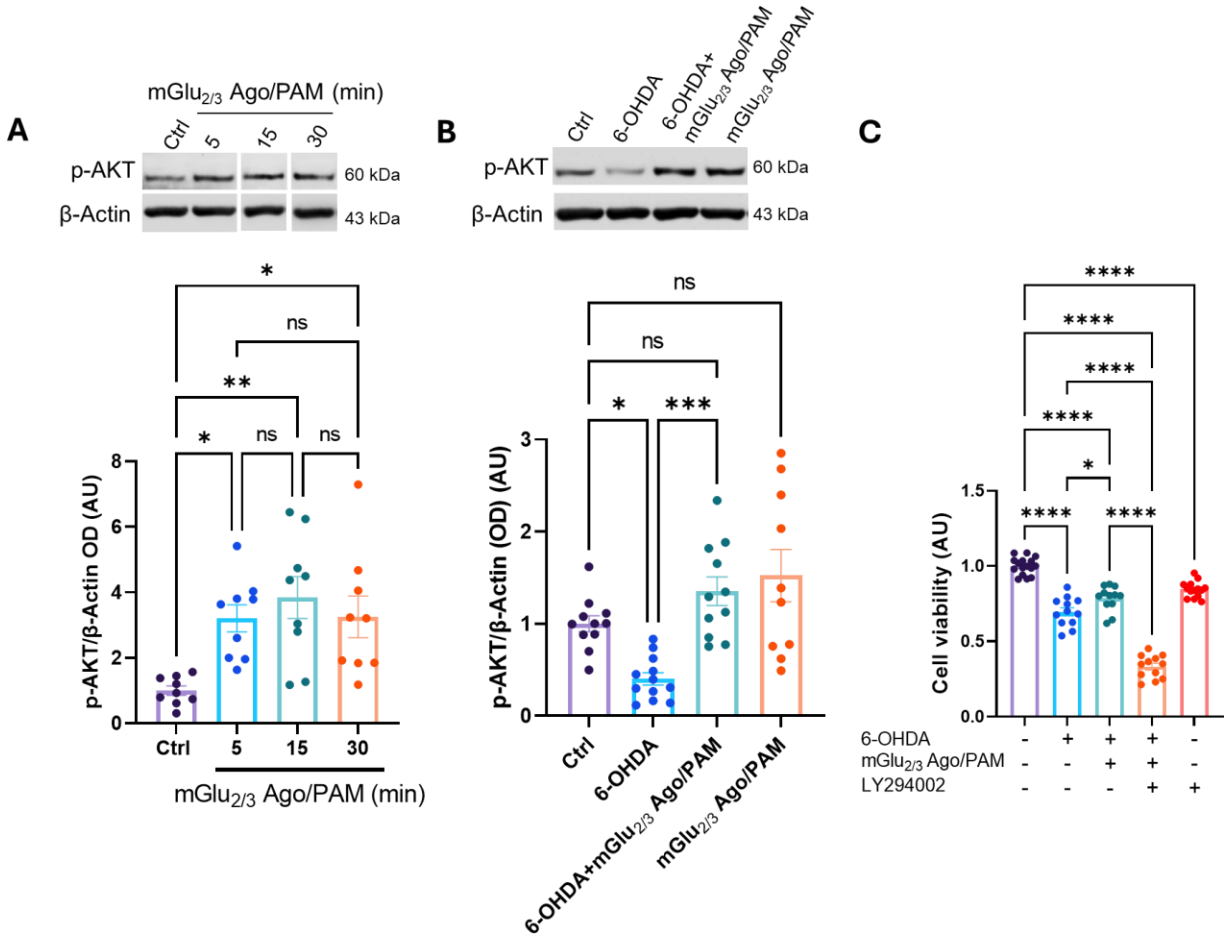


Figure 15: Modulation of PI3K–AKT protein pathway in SH-SY5Y cells by mGlu$_{2/3}$ Ago/PAM.

A) Up-regulation of p-AKT in SH-SY5Y cells after short-term exposure (5, 15, and 30 min) to mGlu$_{2/3}$ Ago/PAM (100 µM). Representative western blot bands (top) and quantification of p-AKT normalized to β-Actin optical density (OD) (bottom). **B)** p-AKT modulation in untreated control cells (Ctrl) and cells treated for 24 h with 6-OHDA 50 µM, 6-OHDA 50 µM + mGlu$_{2/3}$ Ago/PAM 100 µM, or mGlu$_{2/3}$ Ago/PAM 100 µM only. Western blot bands (top) and quantification of p-AKT normalized to β-Actin optical density (OD) (bottom). **C)** Column graph showing that inhibition of the PI3K–AKT pathway with LY294002 (10 µM, 24 h) abolishes the protective effect of mGlu$_{2/3}$ Ago/PAM (100 µM, 24 h) on SH-SY5Y cell viability against 6-OHDA (50 µM, 24 h), as assessed by MTT assay. ANOVA followed by Tukey's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns (not significant).

4.6 *In vitro* analysis of neuroprotective mechanisms of the novel $mGlu_{2/3}$ Ago/PAM: focus on MAPK/ERK and GDNF signaling

Together with the PI3K–AKT cascade, the MAPK/ERK pathway is also particularly important for the regulation of survival balance in cellular biology. In this perspective, western blotting analyses were performed to check the possible involvement of MAPK/ERK in the cellular neuroprotective mechanisms triggered by the new $mGlu_{2/3}$ Ago/PAM. A short-time exposure from 5 min to 30 min of SH-SY5Y cells to the new compound (100 μM) resulted in the down-regulation of the phosphorylated-ERK1/2 (p-ERK1/2) signal (*Figure 16A*). Moreover, a 24 h exposure of cells to a combination of 6-OHDA 50 μM and $mGlu_{2/3}$ Ago/PAM 100 μM reversed the increase of p-ERK1/2 signal given by 6-OHDA treatment only (*Figure 16B*).

Finally, we evaluated the effect of the novel $mGlu_{2/3}$ Ago/PAM on GDNF protein levels, as this neurotrophic factor plays a key role in the survival and maintenance of dopaminergic neurons. Interestingly, *Figure 16C* shows that, when in co-treatment (6-OHDA 50 μM + $mGlu_{2/3}$ Ago/PAM 100 μM, 24 h), the new compound was able to counteract the decrease in GDNF protein levels caused by 6-OHDA exposure. These findings are consistent with previous *in vivo* observations showing that $mGlu_3$ activation induces GDNF up-regulation, leading to neuroprotection (Battaglia, Molinaro et al. 2009).

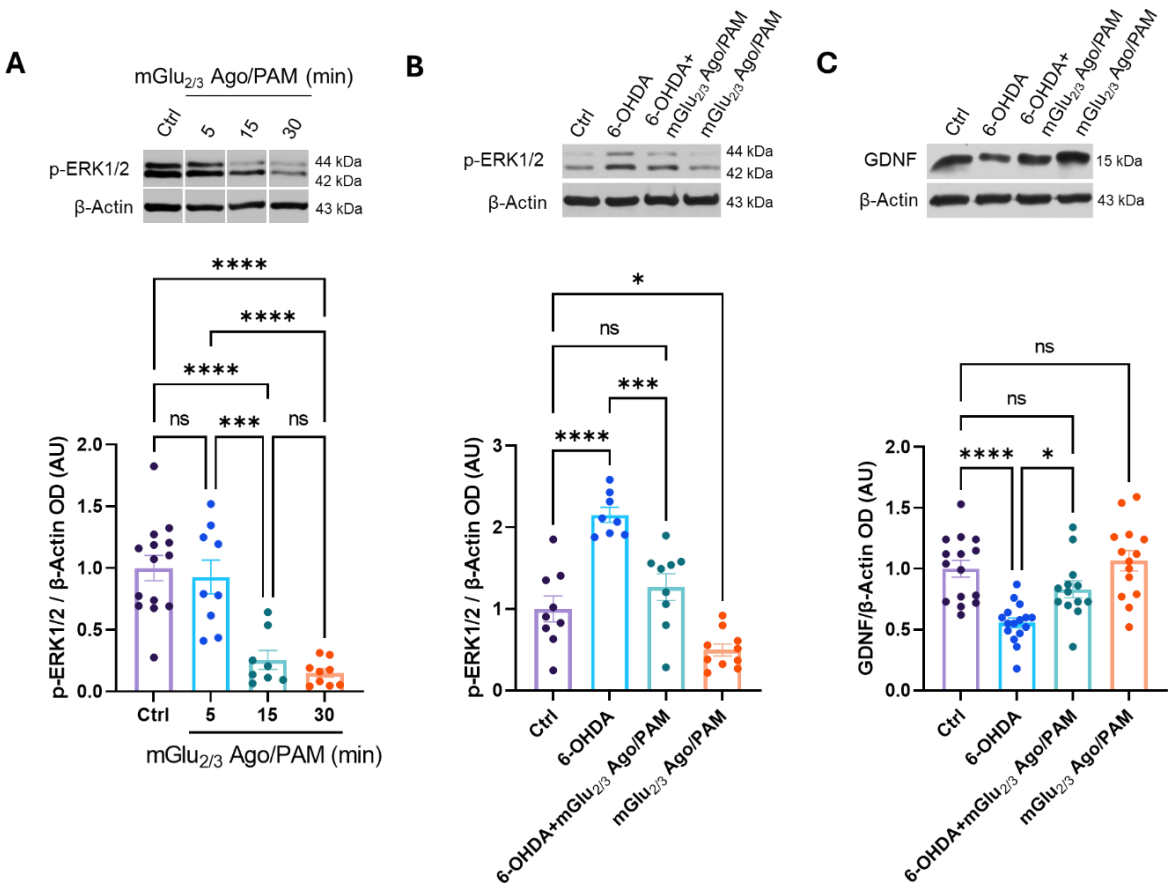


Figure 16: Modulation of MAPK/ERK protein pathway and GDNF protein levels in SH-SY5Y cells by $mGlu_{2/3}$ Ago/PAM.

A) p-ERK1/2 down-regulation in SH-SY5Y cells after short-time exposure (5 min, 15 min, 30 min) to $mGlu_{2/3}$ Ago/PAM (100 µM). Western blot bands (top) and quantification of p-ERK1/2 normalized to β-Actin optical density (OD) (bottom). **B)** p-ERK1/2 modulation in untreated control cells (Ctrl) and cells treated for 24 h with 6-OHDA 50 µM, 6-OHDA 50 µM + $mGlu_{2/3}$ Ago/PAM 100 µM, or $mGlu_{2/3}$ Ago/PAM 100 µM only. Western blot bands (top) and quantification of p-ERK1/2 normalized to β-Actin optical density (OD) (bottom). **C)** GDNF modulation in untreated control cells (Ctrl) and cells treated for 24 h with 6-OHDA 50 µM, 6-OHDA 50 µM + $mGlu_{2/3}$ Ago/PAM 100 µM, or $mGlu_{2/3}$ Ago/PAM 100 µM only. Western blot bands (top) and quantification of GDNF normalized to β-Actin optical density (OD) (bottom). ANOVA followed by Tukey's test: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns (not significant).

4.7 An *in vivo* pilot study: dose- and time-dependent investigation in mouse brain regions

Building on the promising *in vitro* neuroprotective effects of the novel $mGlu_{2/3}$ Ago/PAM against dopaminergic damage, we subsequently evaluated its impact in key brain regions implicated in PD. A preliminary screening of doses and exposure times was conducted in healthy mice to identify the most effective conditions, and protein levels of major neurotrophic factors were assessed via western blotting. As shown in *Figure 17*, in the mouse striatum only brain-derived neurotrophic factor (BDNF) protein levels were significantly modulated. Administration of $mGlu_{2/3}$ Ago/PAM at 1 mg/kg for 24 h resulted in BDNF up-regulation compared to untreated controls; however, this effect was lost after 72 h (*Figure 17B*). In contrast, neither increasing the dose nor extending the exposure time produced significant changes in GDNF or fibroblast growth factor 2 (FGF2) protein levels.

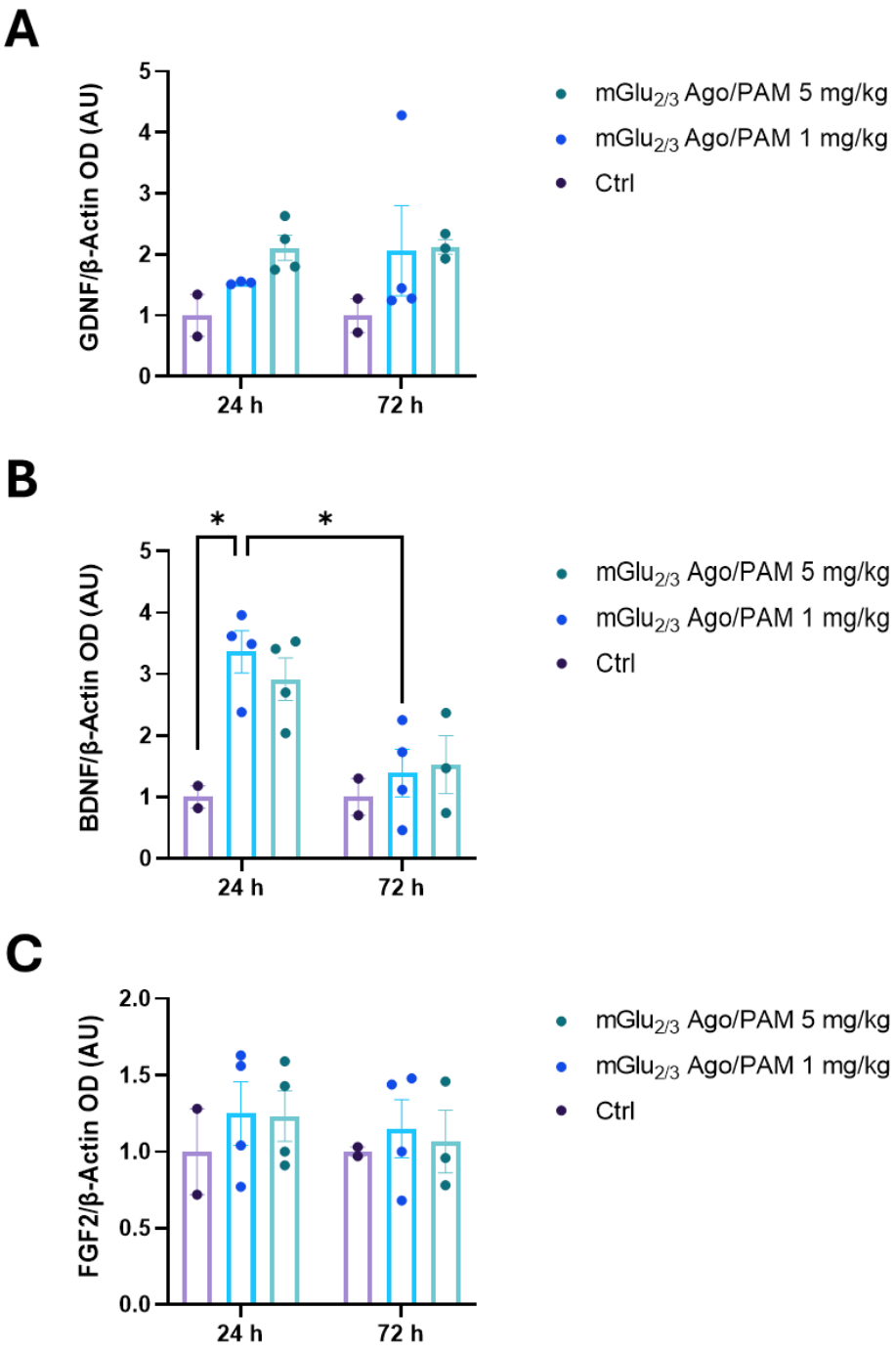


Figure 17: Dose- and time-dependent effects of mGlu$_{2/3}$ Ago/PAM in the mouse striatum.

Column graph showing quantification of GDNF **(A)**, BDNF **(B)**, and FGF2 **(C)** protein levels, normalized to β-Actin optical density (OD), in untreated (Ctrl) mice and mice treated with mGlu$_{2/3}$ Ago/PAM (1 mg/kg or 5 mg/kg, for 24 h or 72 h). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$

The mouse hippocampus was also examined, but no statistically significant changes in GDNF, BDNF, or FGF2 protein levels were observed between low and high doses or short and long exposure times (*Figure 18*).

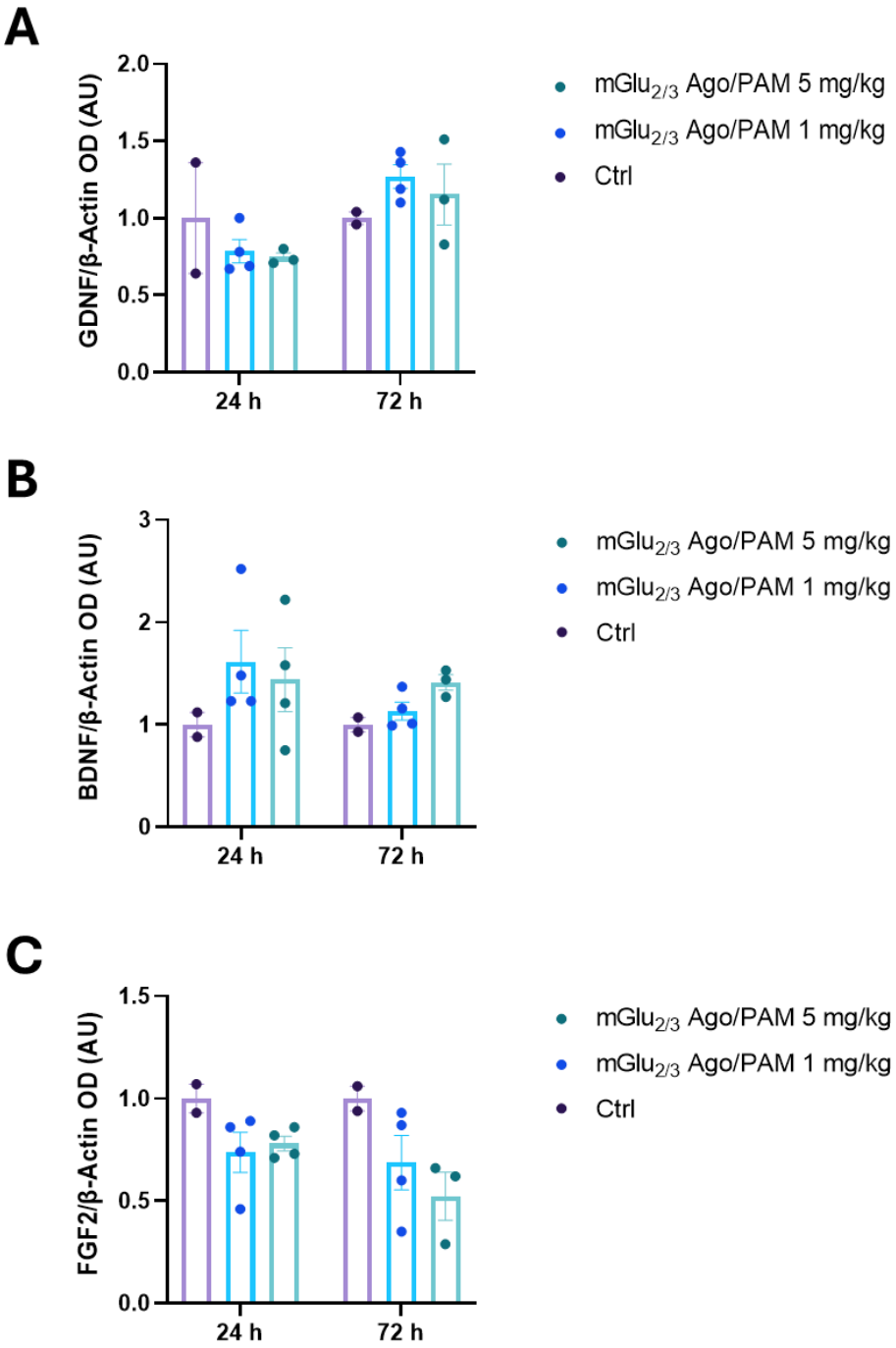


Figure 18: Dose- and time-dependent effects of mGlu$_{2/3}$ Ago/PAM in the mouse hippocampus.

Column graph showing quantification of GDNF **(A)**, BDNF **(B)**, and FGF2 **(C)** protein level, normalized to β-Actin optical density (OD), in untreated (Ctrl) mice and mice treated with mGlu$_{2/3}$ Ago/PAM (1 mg/kg or 5 mg/kg, for 24 h or 72 h). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test.

Finally, analysis of the mouse prefrontal cortex revealed that GDNF protein levels were significantly up-regulated over time in the group receiving $mGlu_{2/3}$ Ago/PAM at 5 mg/kg (*Figure 19A*). No statistically significant changes were observed for BDNF or FGF2 protein levels between low and high doses or short and long exposure times (*Figure 19B,C*). Based on these results, the second experiment was conducted using a single injection of 1 mg/kg, and animals were sacrificed 24 h after administration.

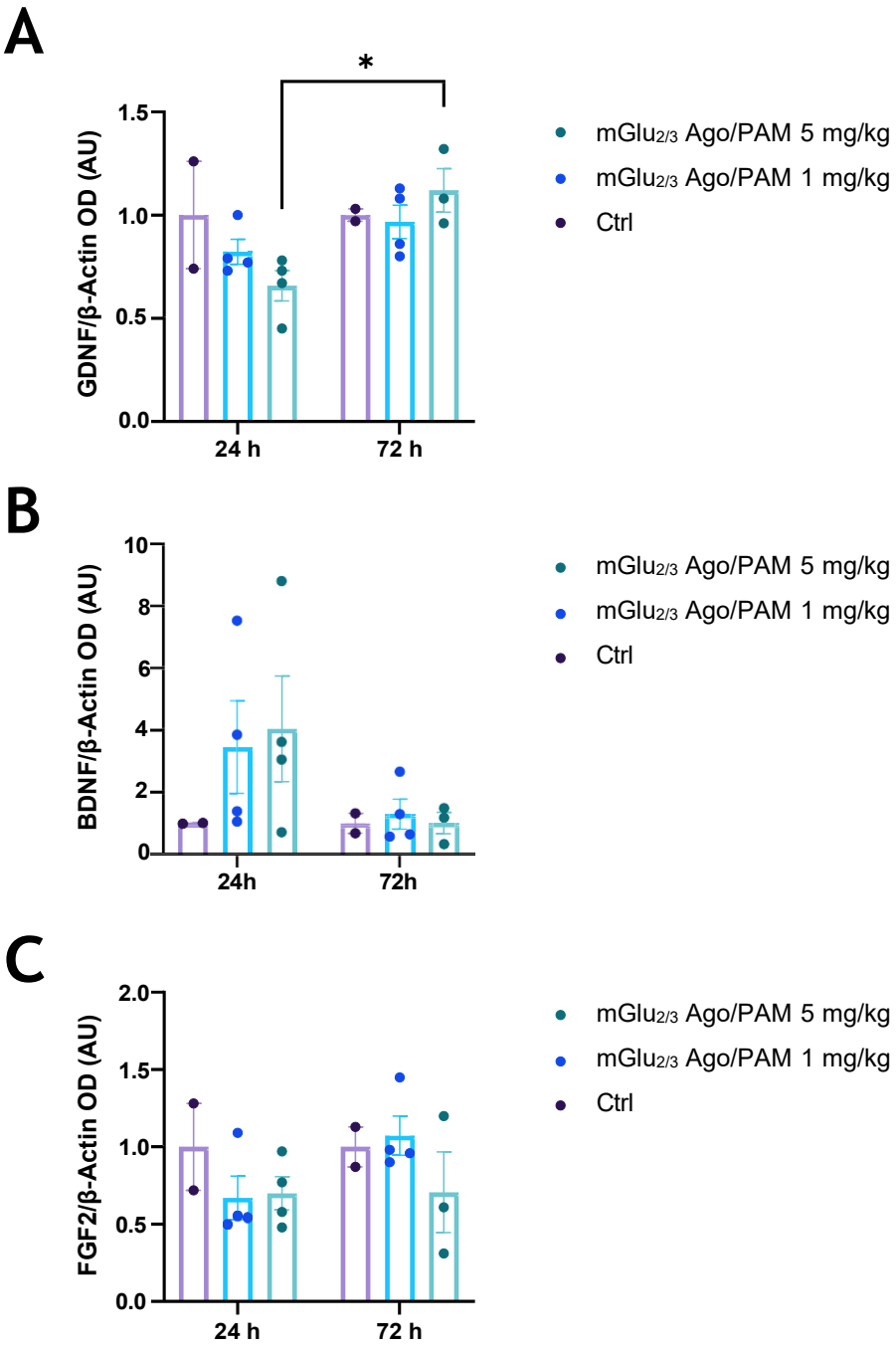


Figure 19: Dose- and time-dependent effects of mGlu$_{2/3}$ Ago/PAM in the mouse prefrontal cortex.

Column graph showing quantification of GDNF **(A)**, BDNF **(B)**, and FGF2 **(C)** protein levels, normalized to β-Actin optical density (OD), in untreated (Ctrl) mice and mice treated with mGlu$_{2/3}$ Ago/PAM (1 mg/kg or 5 mg/kg, for 24 h or 72 h). Data analysed by two-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$

4.8 *In vivo* neuromodulatory effects induced by the new $mGlu_{2/3}$ Ago/PAM: observation in mouse striatum

Starting from the striatum, the primary region affected in PD, 24 h treatment with $mGlu_{2/3}$ Ago/PAM at 1 mg/kg induced a significant up-regulation of both GDNF and BDNF protein levels compared to untreated controls (*Figure 20A,B*), while it did not significantly affect FGF2 protein expression (*Figure 20C)*. Moreover, the main survival pathways were activated by $mGlu_{2/3}$ Ago/PAM, as indicated by increased levels of p-AKT and p-ERK1/2 in treated mice compared to controls (*Figure 20D,E*).

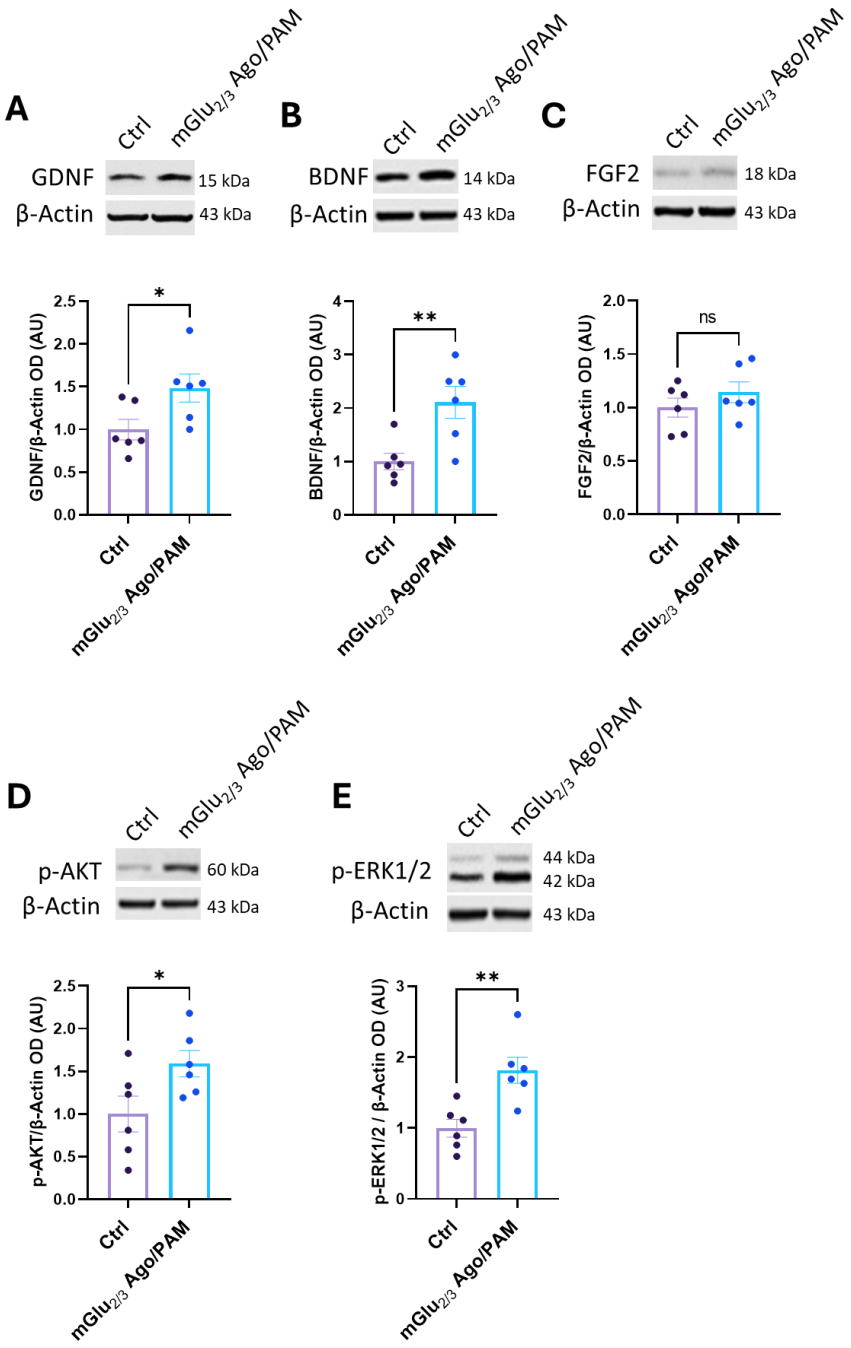


Figure 20: Modulation of neurotrophic factors and signaling pathways by mGlu$_{2/3}$ Ago/PAM in the mouse striatum.

Western blot bands (top) and column graph (bottom) of quantification of GDNF **(A)**, BDNF **(B)**, FGF2 **(C)**, p-AKT **(D)**, p-ERK1/2 **(E)** protein levels, normalized to β-Actin optical density (OD), in untreated mice (Ctrl) and mice treated with mGlu$_{2/3}$ Ago/PAM (1 mg/kg, 24 h). t-test: * $p < 0.05$, ** $p < 0.01$ as compared to Ctrl group.

4.9 *In vivo* neuromodulatory effects induced by the new $mGlu_{2/3}$ Ago/PAM: observation in the mouse hippocampus

Next, western blotting analyses focused on hippocampus, which is typically implicated in the cognitive and affective impairments associated with PD. As shown in *Figure 21A,B,C*, injection of $mGlu_{2/3}$ Ago/PAM 1 mg/kg for 24 h significantly up-regulated BDNF protein levels, while it did not modulate GDNF and FGF2 protein expression. Moreover, it did not significantly activate the PI3K–AKT cascade, but it increased p-ERK1/2 protein levels (*Figure 21D,E*).

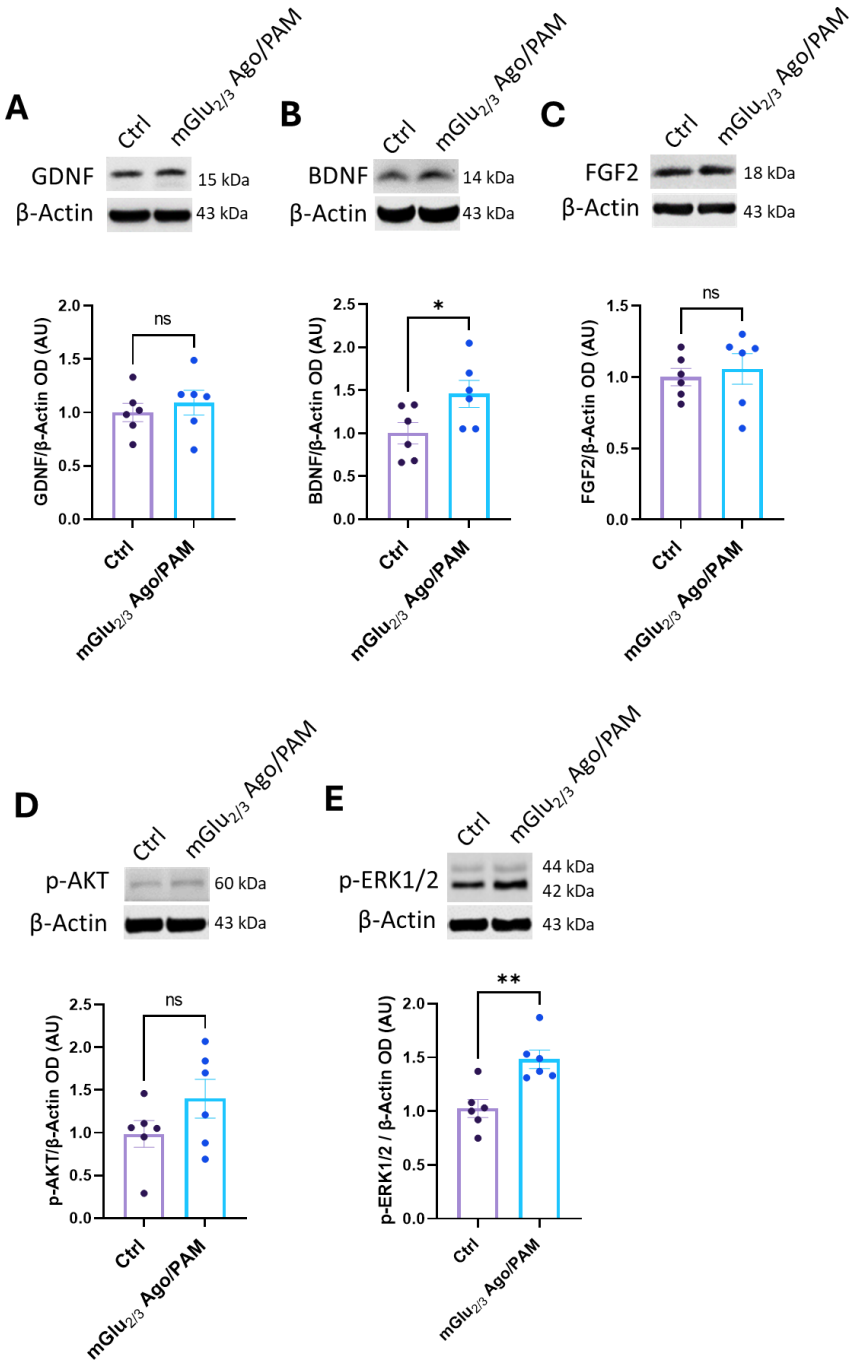


Figure 21: Modulation of neurotrophic factors and signaling pathways by mGlu$_{2/3}$ Ago/PAM in the mouse hippocampus.

Western blot bands (top) and column graph (bottom) of quantification of GDNF **(A)**, BDNF **(B)**, FGF2 **(C)**, p-AKT **(D)**, p-ERK1/2 **(E)** protein levels, normalized to β-Actin optical density (OD), in untreated mice (Ctrl) and mice treated with mGlu$_{2/3}$ Ago/PAM (1 mg/kg, 24 h). t-test: * $p < 0.05$, ** $p < 0.01$ as compared to Ctrl group.

4.10 *In vivo* neuromodulatory effects induced by the new $mGlu_{2/3}$ Ago/PAM: observation in the mouse prefrontal cortex

To conclude, analysis of the mouse prefrontal cortex showed that 24 h administration of the novel $mGlu_{2/3}$ Ago/PAM at 1 mg/kg increased BDNF protein levels (*Figure 22B*) and activated both p-AKT and p-ERK1/2 signaling pathways compared to untreated controls (*Figure 22D,E*), while GDNF and FGF2 protein levels were not significantly affected (*Figure 22A,C*).

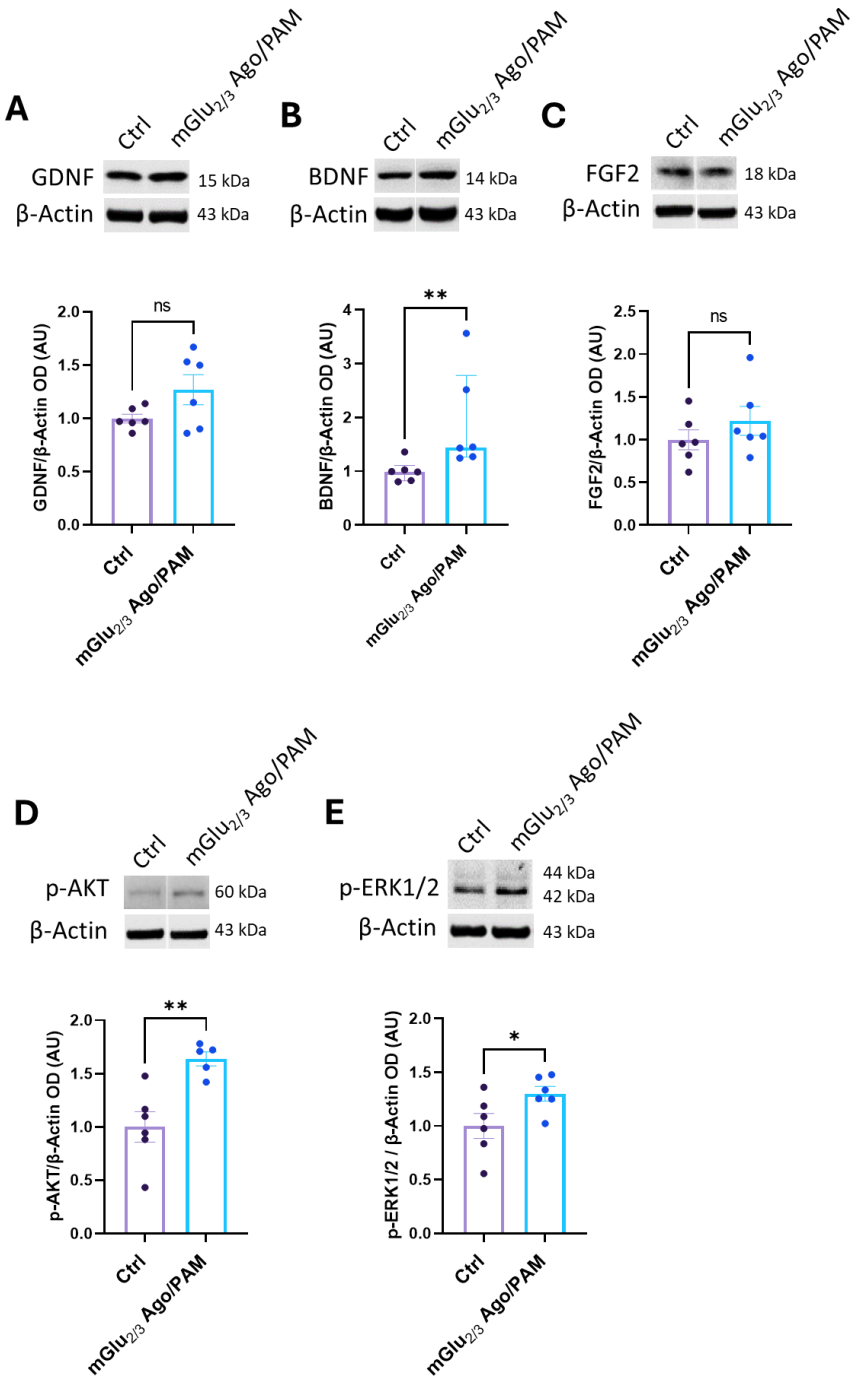


Figure 22: Modulation of neurotrophic factors and signaling pathways by mGlu$_{2/3}$ Ago/PAM in the mouse prefrontal cortex.

Western blot bands (top) and column graph (bottom) of quantification of GDNF **(A)**, BDNF **(B)**, FGF2 **(C)**, p-AKT **(D)**, p-ERK1/2 **(E)** protein levels, normalized to β-Actin optical density (OD), in untreated mice (Ctrl) and mice treated with mGlu$_{2/3}$ Ago/PAM (1 mg/kg, 24 h). t-test: * $p < 0.05$, ** $p < 0.01$ as compared to Ctrl group.

5. DISCUSSION

The present thesis describes, for the first time, the dual agonistic/positive allosteric modulating activity of a recently synthesized tetrahydro-isoquinoline derivative on $mGlu_2$ and $mGlu_3$, unveiling its therapeutic potential in experimental models of PD.

PD has posed a significant challenge to the scientific community for decades due to its intricate, multi-etiologic molecular and cellular mechanisms, along with the phenotypic heterogeneity observed among patients. In addition, the co-occurrence of comorbidities may confound clinical interpretation, further entangling the diagnosis. A recent review of the current state of PD research highlights several advancements in the field, including a deeper understanding of molecular events, the identification of potential biomarkers, progress in translational and precision medicine, and the increasing involvement of patients in cutting-edge clinical trials (Tenchov, Sasso et al. 2025). However, despite this ongoing effort and the evolving landscape, therapeutic progress remains limited and elusive (Tenchov, Sasso et al. 2025). Recent strategies such as drug repurposing, designed to save costs and time, and the development of nanotechnological drug-delivery systems, to enhance drug stability and permeability across the BBB, have yet to demonstrate sufficient effectiveness (Tenchov, Sasso et al. 2025). These challenges underscore the urgent need for a shift in therapeutic strategies and increased investment in research focused on developing disease-modifying therapies. In this context, mGluRs, due to their broad-spectrum activity within the CNS (Niswender and Conn 2010) and their localization in the brain areas critical to PD (Zhang, Zhang et al. 2019), have emerged as promising molecular targets. In particular, the physiological regulatory role of group II mGluRs in attenuating glutamate release at the synaptic level, combined with their capacity to modulate neuroprotective and neuroinflammatory processes, has sparked considerable interest in these receptors (Zhang, Zhang et al. 2019). Substantial *in vitro* and *in vivo* evidence support the neuroprotective role of activated $mGlu_{2/3}$ in mitigating PD-related degeneration, including the restoration of dopaminergic cells viability, preservation or regeneration of the nigrostriatal circuit, reduction of neuroinflammation, enhancement of neurotrophic factors expression and activation of survival signaling pathways, and amelioration of motor symptoms (Battaglia, Busceti et al. 2003) (Jantas, Greda et al. 2014) (Bradley, Marino et al. 2000) (Picconi, Pisani et al. 2002) (Vernon, Palmer et al. 2005) (Murray,

Messenger et al. 2002) (Chan, Paur et al. 2010) (Frouni, Kwan et al. 2024) (Di Liberto, Bonomo et al. 2010) (Di Liberto, Mudo et al. 2011). Among the group II mGluRs, $mGlu_3$ has emerged as a particularly promising therapeutic target for PD. Indirect evidence from KO mice studies suggest that $mGlu_3$ is responsible for up-regulating GDNF in neurons from the striatum and the cortex, positively modulating the MAPK/ERK pathway, and providing neuroprotection against MPTP-induced neuronal death (Battaglia, Molinaro et al. 2009). Furthermore, $mGlu_3$ KO mice have shown that this receptor plays a crucial role in neuroprotection against inflammation associated with Parkinsonism, with a notable effect on potentiating cortical plasticity (Di Menna, Alborghetti et al. 2025). However, the high degree of sequence and structural homology between $mGlu_2$ and $mGlu_3$ has led to the overlap of $mGlu_2$ activity with $mGlu_3$-specific effects, making it difficult to distinguish a unique functional profile for $mGlu_3$. This overlap has been exacerbated by the lack of structurally selective agonists for $mGlu_3$. While selective NAMs for $mGlu_3$, such as ML 337 (Wenthur, Morrison et al. 2013), are available and could help elucidate $mGlu_3$ activity upon inhibition, the downstream effects of $mGlu_3$ following direct positive stimulation or activation remain unexplored in the literature. To the best of our knowledge, only one study has reported recent attempts to synthesize and evaluate selective PAMs for $mGlu_3$. However, the obtained compounds exhibited only moderate potency at $mGlu_3$, while showing stronger potency on $mGlu_2$ (Yamada, Gilliland et al. 2021). In our study, although the pharmacological assay could not exclude potential activity at $mGlu_2$ or confirm a pure selectivity for $mGlu_3$, the new tetrahydro-isoquinoline derivative displayed a greater preference for binding and activating the subtype 3 over the subtype 2. Furthermore, while the compound did not exhibit pure allosteric activity, it potentiated both $mGlu_2$ and $mGlu_3$ receptors in both an agonistic and allosteric manner. The dual activity of some PAMs has already been described in literature and is thought to arise from the highly structured conformation of mGluRs, particularly within the 7TMD (Cannone, Berto et al. 2025). This statement is further supported by structural studies on $mGlu_2$ receptors, which have elucidated the molecular basis of receptor structure and activation upon binding with an agonist/PAM molecule, as well as its interaction with G-proteins (Seven, Barros-Alvarez et al. 2021). Despite speculations about the potential pharmacodynamic advantages of the dual (agonistic and positive allosteric) activity of certain mGluRs ligands, the functional relevance of the intrinsic agonist activity exhibited by some PAMs remains unclear. For example, one study found that a pure PAM and an Ago/PAM for $mGlu_5$ had equivalent efficacy in reversing amphetamine-induced hyperlocomotion

in animal models (Noetzel, Rook et al. 2012). However, another study comparing a pure PAM to an Ago/PAM for $mGlu_5$ reported that, while both compounds alleviated symptoms in a schizophrenia animal model, the Ago/PAM also contributed to seizures-like adverse effects. This could be explained by a disruption in the fine-tuning of glutamatergic signaling, possibly due to an overlapping effect on endogenous glutamate release (Bridges, Rook et al. 2013).

After assessing the receptor selectivity of the new $mGlu_{2/3}$ Ago/PAM, we explored its potential neuroprotective effects against dopaminergic degeneration. Initial *in vitro* screenings utilized SH-SY5Y cells, a widely accepted model due to their morphological and functional similarities to human neurons, especially in PD research (Ioghen, Ceafalan et al. 2023) (Xicoy, Wieringa et al. 2017). SH-SY5Y cells are a human neuroblastoma cell line that exhibits catecholaminergic phenotype, expressing key markers of human dopaminergic neurons, including TH, dopamine-β-hydroxylase, dopamine receptors 2 and 3 (Xicoy, Wieringa et al. 2017) (Kovalevich and Langford 2013), as well as DAT protein (*see Results–4.3 section*). Treatment of SH-SY5Y cells with 6-OHDA is a very well-established method for modeling dopaminergic degeneration *in vitro* (Lal, Singh et al. 2024), as it is selectively taken up by dopaminergic-like neurons through DAT, triggering cell death specifically in this subset. In the presence of the new Ago/PAM, which did not impact cell viability on its own, a partial but significant neuroprotective effect against 6-OHDA-induced cell death was observed. While the compound also exhibited some activity on $mGlu_2$, we excluded any contribution of $mGlu_2$ in the neuroprotection, based on two pharmacological approaches. First, a commercially available $mGlu_2$-selective PAM (BINA) (Galici, Jones et al. 2006), when administered at the same dose (100 μM) of the new Ago/PAM, failed to rescue cells from 6-OHDA-induced toxicity. Second, the neuroprotective effect of the new Ago/PAM was abolished when $mGlu_3$ activity was blocked by a commercially available NAM (ML 337) selective for $mGlu_3$ (Wenthur, Morrison et al. 2013). Notably, high doses of NAM (ML 337) themselves negatively affected cell viability, further highlighting the importance of $mGlu_3$ in dopaminergic-like cell survival. Our *in vitro* findings align with previous studies showing neuroprotection by $mGlu_{2/3}$ agonists, such as LY354740, in undifferentiated SH- SY5Y cells (Jantas, Greda et al. 2015), and *in vivo* studies demonstrating the protective effects of the dual $mGlu_{2/3}$ agonist LY379268 in the nigrostriatal pathway following MPTP-induced dopaminergic damage. In particular, the neuroprotection conferred by LY379268 was shown to depend on $mGlu_3$ activation, as it was lost in $mGlu_3$ KO animals (Battaglia, Molinaro et al. 2009).

We further explored the signaling pathways involved in the neuroprotection mediated by the new Ago/PAM, focusing on the MAPK/ERK and PI3K–AKT pathways. Previous studies have shown that $mGlu_3$ activation by LY379268 increases phosphorylation of both ERK and AKT, which are involved in the up-regulation of neurotrophic factors such as GDNF (Battaglia, Molinaro et al. 2009). In our study, exposure to the new $mGlu_{2/3}$ Ago/PAM led to a rapid increase in phosphorylated AKT levels within 5 min, and 24-h co-exposure with 6-OHDA rescued the AKT phosphorylation levels decreased by the neurotoxin. In contrast, inhibiting PI3K–AKT signaling with LY294002 abolished the neuroprotective effect of the Ago/PAM on cell viability, further confirming the critical role of AKT in the observed neuroprotection. AKT protein is a serine/threonine kinase ubiquitously expressed and highly conserved among mammalians, both in the sequence and in the structure. AKT activity occurs downstream of phosphatidylinositol 3-kinase (PI3K), establishing a signaling pathway that plays a role in the regulation of crucial cellular mechanisms such as metabolism, proliferation, death, growth and regulation of immune response. Upon activation of GPCRs or receptor tyrosine kinases (RTKs), PI3K is activated and converts PIP2 to IP3. Subsequently, AKT is recruited to the membrane, where it binds to IP3 and is phosphorylated by phosphoinositide-dependent kinase-1. Once activated, AKT initiates phosphorylation of various substrates, including glycogen synthase kinase-3β (GSK-3β), which stabilizes pro-survival factors like MCL-1 and c-Myc; forkhead box O transcription factors, which are exported from the nucleus to prevent apoptosis, cell-cycle arrest, and growth inhibition; and tuberous sclerosis complex 2, which activates mTOR complex 1 to suppress autophagy (Manning and Toker 2017). In the CNS, PI3K–AKT axis is activated by neurotrophins and has a prominent role in the promotion of neuronal survival, growth, polarity, synaptic plasticity, and circuit formation. Thus, precise regulation of this pathway is essential for maintaining CNS homeostasis and overall neuronal health (Manning and Toker 2017). It has been already demonstrated that mGluRs PAMs promote the up-regulation of p-AKT, offering protection against degenerative processes within the CNS. For example, the $mGlu_5$ PAM VU0092273 activates the PI3K–AKT pathway, preventing excessive permeability of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and providing neuroprotection against ischemia-induced neuronal death. This effect is blocked by the PI3K inhibitor LY294002, which suppresses AKT signaling (Cavallo, Landucci et al. 2020). Moreover, in a model of traumatic brain injury, the $mGlu_5$ PAM VU0360172 has been shown to increase p-AKT expression, leading to the inhibition

of GSK-3β and the activation of cAMP response element-binding protein (CREB). This, in turn, induces the transcription of anti-inflammatory molecules (Bhat, Henry et al. 2021). Together, these findings highlight that dysregulation of the PI3K–AKT pathway contributes to neurodegenerative disorders, including PD (Goyal, Agrawal et al. 2023). Numerous studies in literature support the neuroprotective role of activated AKT against dopaminergic degeneration. For example, Malagelada et al. reported decreased p-AKT levels in dopaminergic neurons of SNpc in post-mortem brains of PD patients (Malagelada, Jin et al. 2008). Moreover, in line with our results, the same study demonstrated the down-regulation of p-AKT protein levels in neuronal PC12 cells exposed to 6-OHDA, which correlated to increased cell death induced by the toxin (Malagelada, Jin et al. 2008). Similarly, Huang et al. observed that 6-OHDA exposure reduced cell viability, p-AKT levels, and superoxide dismutase (SOD) activity, a key endogenous defense against oxidative stress, typically regulated by AKT activation, in PC12 cells. Notably, treatment with the antioxidant Resveratrol mitigated these neurotoxic effects, emphasizing the protective role of the activated AKT pathway against oxidative stress, which contributes to dopaminergic degeneration (Huang, Huang et al. 2021). In line with our own findings on the restoration of p-AKT levels by the novel Ago/PAM compared to the decrease induced by 6-OHDA, Bergenin, a natural compound, was shown to restore p-AKT levels in a PD mouse model induced by MPTP, resulting in improved motor function, protection of dopaminergic neurons, and reduction of microglia-mediated neuroinflammation (Ji, Wang et al. 2019). Furthermore, studies involving the PI3K–AKT pathway activation by the isoflavonoid Puerarin and the natural compound Miltirone reported recovery of cell viability, decreased ROS and Caspase-3 levels, and increased SOD activity in SH-SY5Y cells exposed to MPTP. In both studies, neuroprotection was lost when the PI3K–AKT pathway was inhibited by LY294002, further supporting our findings (Zhu, Wang et al. 2012). Together, these data underscore the pivotal role of the PI3K–AKT axis in neuroprotection against PD. These findings suggest that therapeutic strategies targeting this signaling pathway could offer novel molecular approaches for combating the disease.

In contrast to AKT modulation, our study demonstrated that while prolonged exposure (24 h) to 6-OHDA up-regulated p-ERK1/2 levels in SH-SY5Y cells, co-treatment with the new $mGlu_{2/3}$ Ago/PAM rescued the basal expression of p-ERK1/2. Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are growth factors-activated kinases, ubiquitously expressed across tissues and highly conserved across species. As members of the MAPK family, ERK1/2 are involved in a cascade of

phosphorylation events that regulate critical processes such as the cell cycle, survival/death balance, and cellular differentiation. Upon activation of RTKs or GPCRs, RAS is activated through GTP loading, which recruits RAF kinase and leads to the phosphorylation of MEK. MEK then activates ERK, which phosphorylates a broad range of cytoplasmic and nuclear substrates involved in cell proliferation, survival, growth, metabolism, migration and differentiation (Lavoie, Gagnon et al. 2020). Notably, the outcome of MAPK/ERK pathway strongly depends on the specific cellular context, revealing the double face (both pro and anti-survival) of this signaling axis, which is particularly relevant in neurodegenerative processes. In our study, we observed that ERK1/2 activation contributed to 6-OHDA-induced neurotoxicity. Accordingly, evidence in literature has already documented the up-regulation of p-ERK levels following exposure to lethal doses of 6-OHDA in another neuroblastoma cell line (B65), and the subsequent loss of toxicity when cells were treated with the ERK inhibitor PD98059 (Kulich and Chu 2001). Moreover, aberrant p-ERK expression has been observed not only in 6-OHDA-exposed B65 cells but also in post-mortem brains of PD patients, where p-ERK aggregates were found around Lewy bodies and in the cytoplasm. These p-ERK aggregates are known to accumulate on the scaffolded surfaces of cellular vesicles, impairing cellular trafficking and organelle function. Interestingly, treatment with the antioxidant enzyme Catalase protected cells from 6-OHDA-induced toxicity and prevented the formation of p-ERK granules, suggesting that oxidative stress plays a key role in dopaminergic degeneration and abnormal ERK activation (Zhu, Kulich et al. 2002). The overactivation of ERK likely results from elevated levels of ROS that oxidize a specific residue in RAF, enhancing the recruitment of RAS to the plasma membrane and ultimately boosting the activation of the MAPK/ERK pathway. Alternatively, ROS can inactivate dual-specificity phosphatases (DUSP1 and DUSP6), which normally dephosphorylate ERK, leading to sustained ERK activation (Cagnol and Chambard 2010). In line with this, treatment of SH-SY5Y cells with MPP^+ also induced an increase in p-ERK levels, α-Synuclein accumulation, and cell death, effects that were reversed by the ERK inhibitor U0126 (Gomez-Santos, Ferrer et al. 2002). Interestingly, the pro-toxic role of ERK activation may depend on the duration of its stimulation. In our experiments, we observed that p-ERK levels increased after 24 h of 6-OHDA exposure. In a similar vein, studies in PC12 cells exposed to varying doses of Forskolin revealed that prolonged ERK activation (following high doses) triggered pro-apoptotic pathways, while transient activation (following low doses) enhanced dopamine synthesis and promoted cell survival (Park, Park et al. 2012). The persistent

activation of ERK pathway has also been detected in *in vivo* experimentation. In particular, aberrant p-ERK levels were found in the striatum of 6-OHDA-injected mice exhibiting dyskinesia after Levodopa treatment (Fiorentini, Savoia et al. 2013). Moreover, chronically activated ERK has been implicated in neurodegeneration in other disorders, including AD and HD (Rai, Dilnashin et al. 2019). Taken together, this evidence suggests that preventing aberrant activation of the MAPK/ERK cascade and accumulation of p-ERK1/2 could be a key molecular target for a potential therapeutic agent against PD neurodegeneration, as proposed by the new $mGlu_{2/3}$ Ago/PAM. Notably, in our study, the Ago/PAM alone caused a decrease in p-ERK1/2 levels, both after 15 min and 24 h of treatment in SH-SY5Y cells. This finding is intriguing because $mGlu_{2/3}$ receptors, which are coupled to $G_{i/o}$ proteins, are typically associated with up-regulation of p-ERK in response to agonist binding. This opposite observation could be explained by several potential mechanisms at the cellular level. For instance, the Ago/PAM might stabilize a distinct conformation of $mGlu_{2/3}$ that preferentially interacts with downstream effectors responsible for the down-regulation of p-ERK, rather than the expected up-regulation. This phenomenon, in which different ligands lead to differential activation of downstream signaling pathways, is referred to as "biased allosterism" and has been described for $mGlu_5$ PAMs (Sengmany, Singh et al. 2017) (Sengmany, Hellyer et al. 2020). One possible explanation is that the Ago/PAM selectively activates DUSP1 and DUSP6, leading to dephosphorylation of ERK and negative regulation of the MAPK/ERK pathway. This mechanism has been explored in cancer research, where DUSP1 and DUSP6 are considered potential targets for inhibiting oncogenic proliferation (Ahmad, Abdollah et al. 2018). It's also important to note that since our *in vitro* studies were conducted in neuroblastoma cells, which are cancerous, our data may be influenced by the inherent characteristics of this experimental model. Another possibility is that GPCRs activation could transactivate other receptors, such as RTKs, leading to intricate signaling crosstalk. Activated GPCRs can enhance RTKs activity, coupling their pathways and resulting in complex signal transduction (Di Liberto, Mudo et al. 2019). In our case, such transactivation might establish a negative feedback loop in which sustained ERK activation promotes phosphorylation of MAPK/ERK pathway components, like SOS and RAF, ultimately desensitizing the signaling cascade (Chandarlapaty 2012). Finally, the complexity of GPCRs signaling, with its numerous effectors, mediators, and isoforms, could contribute to the observed differential activation of the ERK pathway (Belcheva and Coscia 2002). However, since our investigation has not yet explored

all the potential molecular effectors involved in the Ago/PAM-mediated signaling, further studies are needed to fully understand the mechanisms underlying the neuroprotection provided by this novel ligand.

In addition to modulating the two key signaling pathways that govern cell fate, the most significant effect we observed in our *in vitro* dopaminergic degeneration model was the regulation of GDNF protein expression by the new $mGlu_{2/3}$ Ago/PAM. Specifically, this ligand effectively counteracted the down-regulation of GDNF caused by 6-OHDA exposure, restoring its basal expression. This result not only confirmed the dopaminergic phenotype of our *in vitro* system but also highlighted the potential of the new Ago/PAM to block dopaminergic degeneration. The crucial role of GDNF in maintaining dopaminergic neurons homeostasis has been well known for decades already, especially after it was identified as a factor capable of enhancing dopamine uptake in midbrain dopaminergic neurons (Lin, Doherty et al. 1993). GDNF is a neurotrophic factor, a type of protein critical for neuronal survival, proliferation, differentiation, and communication between nerve cells and their targets. Following transcription, the GDNF pre-propeptide undergoes maturation, including proteolytic cleavage, which is necessary to generate the active, smaller form of the protein. As a member of the "GDNF family ligands," mature GDNF binds as a homodimer to the GDNF-family receptor-α (GFRα), which activates the receptor tyrosine kinase RET, thereby promoting neuronal health and survival (Porcari, Cattaneo et al. 2025). GDNF is primarily expressed by dopaminergic neurons in the CNS, but it is also released by other neuronal populations, including serotoninergic neurons (where it enhances both the number of serotonin neurons and their gene expression), hippocampal neurons (where it drives synaptogenesis during early development and maintain synaptic plasticity in adult hippocampal circuits), and astroglial cells (which support tissue regeneration, plasticity, and an antioxidant and anti-inflammatory environment, particularly in the striatum and hippocampus). However, chronic GDNF expression has produced controversial results in some contexts (Porcari, Cattaneo et al. 2025). Additionally, oligodendrocytes and endothelial cells/pericytes in the CNS also contribute to GDNF expression, with released GDNF enhancing the expression of tight junction proteins in the BBB (Porcari, Cattaneo et al. 2025). In the context of dopaminergic function, there is extensive evidence indicating that a reduction in the GDNF-GFRα-RET signaling system, whether due to genetic or pharmacological manipulation, negatively impacts midbrain dopaminergic function and is associated with PD pathophysiology and symptoms (Porcari, Cattaneo et al. 2025). On the other

hand, controlled up-regulation of endogenous GDNF or exogenous administration of GDNF as a therapeutic agent have shown neuroprotective effects. For instance, GDNF has recently been used to enhance the efficacy of mesenchymal stem cell-based regenerative therapies for PD in mice. Specifically, exposure to GDNF promoted the *in vitro* proliferation of human adipose-derived mesenchymal stem cells (hAMSCs) and their differentiation into mature neuron-like cells. Moreover, the injection of hAMSCs and GDNF into the striatum of 6-OHDA-lesioned mice resulted in improved PD-related symptoms and the restoration of striatal TH-positive cells and neuronal antigen nuclei-positive cells (Sun, Zhang et al. 2020). In another study, GDNF treatment demonstrated anti-apoptotic effects against 6-OHDA toxicity in dopaminergic cell lines. Pre-treatment with GDNF enhanced the NF-κB pathway, leading to the up-regulation of anti-apoptotic proteins, like Bcl-2 and Bcl-w, and the down-regulation of pro-apoptotic factors, such as Bax and Bad (Cao, Niu et al. 2013). Despite these promising findings, the poor BBB permeability of GDNF remains a major limitation for its clinical application. Although clinical trials have explored the administration of GDNF (Barker, Bjorklund et al. 2020), pharmacological stimulation of endogenous GDNF through direct activation of group II mGluRs has emerged as one of the most promising approaches for treating PD (Battaglia, Molinaro et al. 2009).

Our panel of *in vitro* data strongly encouraged us to move forward with *in vivo* investigations. Although we did not assess the ability of the new $mGlu_{2/3}$ Ago/PAM to induce compensatory responses to PD-related degeneration in lesioned animal models, we explored its effects on physiological brain circuitry plasticity in healthy male mice. In this thesis, the compound was therefore evaluated exclusively in naïve animals rather than in a PD model, as this initial study was designed to establish its intrinsic pharmacological and neuromodulatory properties under basal physiological conditions, in line with previous observations on group II mGluR agonists (Battaglia, Molinaro et al. 2009) (Di Liberto, Mudo et al. 2011) (Di Liberto, Bonomo et al. 2010). This approach allowed us to determine whether the compound could elicit detectable molecular or signaling changes within the brain, thereby providing preliminary indirect evidence of its ability to cross the BBB. Assessing these fundamental properties in naïve animals was considered a necessary step to exclude confounding effects arising from disease-related neurodegeneration, inflammation, or compensatory mechanisms. Only after confirming brain exposure and intrinsic biological activity, the compound was considered suitable for subsequent evaluation in disease-relevant models of PD.

Our focus was on the three most relevant brain regions for PD pathophysiology: the striatum, the hippocampus, and the prefrontal cortex. Interestingly, we detected a clear up-regulation of both GDNF and BDNF protein levels, in parallel with increased levels of p-AKT and p-ERK1/2 proteins in the striatum, the first region to undergo degeneration in PD. These findings were accompanied by the positive modulation of BDNF and p-ERK1/2 protein levels in the hippocampus, the central region involved in memory formation, and an increase in BDNF, p-AKT and p-ERK1/2 levels in the prefrontal cortex, which is crucial for cognition and affective behavior. In this physiological context, our results reflect the beneficial action of the new Ago/PAM, which not only boosts endogenous neurotrophic factors through $mGlu_{2/3}$ stimulation, but also optimizes synaptic plasticity and the functionality of brain circuits more broadly. These findings align with previous evidence showing that systemic injection of LY379268, a known $mGlu_{2/3}$ agonist, similarly potentiated neurotrophic signaling and plasticity in healthy mice (Battaglia, Molinaro et al. 2009) (Di Liberto, Mudo et al. 2011) (Di Liberto, Bonomo et al. 2010). The observed differences in protein modulation across the three brain regions likely depend on variations in cellular populations and the specific functions of these areas. Notably, GDNF modulation was most prominent in the mouse striatum, the primary source of endogenous GDNF. In adult rodents, GDNF is predominantly secreted by striatal paravalbumin-positive GABAergic interneurons, as well as cholinergic and somatostatinergic interneurons, in response to dopaminergic input from the SNpc. Consequently, GDNF-producing cells provide trophic support to dopaminergic neurons of the SNpc via retrograde signaling (D'Anglemont de Tassigny, Pascual et al. 2015). Beyond the striatum, GDNF is also expressed by granule cells of the dentate gyrus and pyramidal cells in the hippocampus (Mikroulis, Waloschkova et al. 2022), where it supports neurogenesis and synaptic plasticity (Porcari, Cattaneo et al. 2025). Interestingly, GDNF expression in the adult hippocampus is lower than during developmental stages, when it plays a critical role in synaptic assembly (Porcari, Cattaneo et al. 2025). GDNF is also produced by dopaminergic neurons in the mesolimbic circuit projecting to the prefrontal cortex, though this contribution is comparatively minor (Ortega- de San Luis and Pascual 2016). While enhancing GDNF expression has potential therapeutic benefits, it must be noted that excessive GDNF can lead to dopaminergic signaling abnormalities, which may be linked to neurological disorders, such as schizophrenia-like symptoms (Matlik, Garton et al. 2022) or aberrant sprouting of fibers (Georgievska, Kirik et al. 2002). Given these concerns, our pilot experiment allowed us to identify a non-toxic dose of the new $mGlu_{2/3}$ Ago/PAM that avoids

excessive GDNF up-regulation. However, as our animals were only exposed to a single injection of the compound for a maximum of 72 h, further studies with longer exposure times are needed to evaluate potential neurotoxicity following chronic administration. Additionally, controlled expression or release systems will need to be developed to more accurately model future pharmacological applications of this compound in human PD therapy.

In addition to GDNF, BDNF is another critical neurotrophic factor involved in protecting against dopaminergic degeneration in PD. As a member of the NGF family, BDNF is synthesized and secreted in various regions of the CNS (including hippocampus, striatum and prefrontal cortex). It is first produced as a precursor protein (pre-proBDNF), which is cleaved to form pro-BDNF and mature BDNF (mBDNF). While pro-BDNF plays a role in developmental processes via binding to the p75 neurotrophin receptor, mBDNF binds to tropomyosin receptor kinase B (TrkB) to promote synaptic plasticity, neurogenesis, and neuronal survival in the adult brain. Activation of TrkB by mBDNF triggers downstream signaling pathways, including Ras-MAPK/ERK, PI3K–AKT, and PLC-IP3/DAG, which promote neurogenesis and synaptogenesis (Palasz, Wysocka et al. 2020) (Saeed, Al-Hussainy et al. 2025). Decreased BDNF levels have been linked to the pathogenesis of neurodegenerative diseases, including PD, particularly in patients with motor and cognitive impairments (Palasz, Wysocka et al. 2020) (Saeed, Al-Hussainy et al. 2025). Experimental evidence supports the idea that enhancing BDNF signaling can protect dopaminergic neurons and restore molecular and biochemical balance in PD models, as well as contrast behavioral impairments (Palasz, Wysocka et al. 2020). However, as with GDNF, the challenge of BDNF delivery, due to its short half-life and poor BBB permeability, has limited its clinical utility. Consequently, therapies that target the modulation of endogenous BDNF levels are highly desirable (Chen, Jiang et al. 2025) (Toader, Serban et al. 2025). In this context, our compound appears promising. Our *in vivo* data show that a single dose of the $mGlu_{2/3}$ Ago/PAM induced a significant increase in BDNF levels across all three brain regions, with the striatum showing a two-fold increase. To better understand the observed modulation of BDNF across the targeted brain regions, we considered the distribution of the relevant receptors in these areas, as well as the baseline levels of BDNF protein in the selected tissues. Although BDNF is widely expressed by excitatory neurons throughout the brain, the cortex has been identified as its primary source, with a key role in its release into the striatum. Specifically, BDNF mRNA-expressing neurons are localized in the cortex, not in the striatum. BDNF protein is released from prefrontal cortical terminals and binds to TrkB receptors in the dorsomedial striatum, initiating signaling

pathways that regulate behaviors such as addiction, habits, and stereotyped actions (Ehinger, Soneja et al. 2023). Notably, we observed BDNF up-regulation not only in the striatum but also in the hippocampus, suggesting that our compound may enhance the functioning of interconnected cognitive, affective, learning, and motor circuits in an integrated manner. While we were able to characterize this BDNF modulation in brain tissue after Ago/PAM treatment, we did not observe a similar effect in our *in vitro* experiments, likely due to the very low basal expression of BDNF in SH-SY5Y cells (*data not shown*).

To conduct a comprehensive analysis of protein modulations following *in vivo* administration of the new $mGlu_{2/3}$ Ago/PAM, we also investigated the regulation of FGF2 expression. FGF2 is a member of the fibroblast growth factor (FGF) family, which consists of seven subfamilies, classified based on biochemical functions, evolutionary relationships, and sequence homology (Liu, Deng et al. 2021). FGF2, along with other isoforms in this family, is known for its neuroprotective effects against mechanisms of neurodegeneration in PD, both *in vitro* and *in vivo*. Specifically, FGF2 activates the MAPK/ERK and PI3K–AKT signaling pathways, which help mitigate endoplasmic reticulum stress caused by the accumulation of misfolded proteins, such as α-Synuclein. This process down-regulates the activation of pro-apoptotic effectors in dopaminergic neurons of the nigrostriatal tract (Liu, Deng et al. 2021). Additionally, FGF family members have been shown to counteract oxidative stress, mitochondrial dysfunction, and neuroinflammation, all key factors underlying PD pathology (Liu, Deng et al. 2021). FGF2 treatment has also been tested in PD animal models, where it was found that nasal administration of liposome-loaded FGF2 alleviated behavioral deficits and rescued loss of TH-positive neurons, induced by 6-OHDA, in a rat model of PD (Yang, Zhu et al. 2016). Despite this promising background, our study did not reveal any significant modulations of FGF2 protein levels or effects on physiological circuitry following i.p. injections of the new $mGlu_{2/3}$ Ago/PAM in healthy mice. This suggests that, while our compound modulates key neurotrophic factors like GDNF and BDNF, it may not broadly influence all neurotrophic factors such as FGF2. Furthermore, it remains unclear whether chronic administration of the Ago/PAM would yield a broader modulation of neurotrophic factors. Additionally, similar to our findings with BDNF, we did not detect significant basal levels of FGF2 or any up-regulation in our *in vitro* study, likely due to the very low basal expression of FGF2 in SH-SY5Y cells (*data not shown*).

Beyond its role in regulating neurotrophic factor expression, acute administration of the new $mGlu_{2/3}$ Ago/PAM also modulates key survival-related signaling pathways, as previously

suggested by our *in vitro* tests. Specifically, we observed significant up-regulation of p-AKT and p-ERK1/2 protein levels in both the mouse striatum and prefrontal cortex, as well as p-ERK1/2 up-regulation in the hippocampus. These changes align with the substantial increases in GDNF and BDNF protein levels observed in the brain, supporting our hypothesis that the new $mGlu_{2/3}$ Ago/PAM acts as a potentiator of brain plasticity. These findings are consistent with prior research on the *in vivo* activation of group II mGluRs, further highlighting the potential of these receptors to enhance neuroprotective signaling and promote neural resilience (Di Liberto, Bonomo et al. 2010) (Di Liberto, Mudo et al. 2011) (Battaglia, Molinaro et al. 2009).

6. CONCLUSIONS

Several years of extensive research in the field of PD have clearly highlighted the urgent need for disease-modifying treatments capable of counteracting or arresting PD-related neurodegeneration, rather than providing only symptomatic relief. In this context, $mGlu_3$ has emerged as a promising pharmacological target, primarily through indirect evidence pointing to its involvement in neuroprotective mechanisms. However, the lack of selective agonists has hindered efforts to directly activate $mGlu_3$ and advance related research. In this context, the discovery of a novel tetrahydro-isoquinoline derivative, previously identified as a putative $mGlu_3$ PAM, marks a significant breakthrough in $mGlu_3$-related studies. In the present thesis, we clarified that this new compound acts as an Ago/PAM that activates both $mGlu_2$ and $mGlu_3$, with a stronger preference for $mGlu_3$, as demonstrated through transfection and pharmacological assays. Subsequentially, we investigated the biological relevance of this pharmacological profile by demonstrating the compound neuroprotective effects in a cellular model of dopaminergic degeneration, the hallmark of PD pathophysiology. This effect was accompanied by the regulation of the main survival molecular signaling pathways, including the MAPK/ERK and the PI3K–AKT cascades, together with GDNF modulation. Furthermore, the intrinsic neurotrophic properties of the $mGlu_{2/3}$ Ago/PAM were demonstrated in healthy mice, representing a preliminary step toward evaluating its compensatory potential in PD animal models. The detection of measurable molecular changes in the brains of naïve animals supports the compound's central bioactivity and its capacity to engage neural circuitry *in vivo*.

However, it is important to note that the results presented in this thesis are still preliminary, and several important limitations should be carefully considered. The *in vitro* experiments were conducted using the SH-SY5Y cell line, a well-established and widely employed dopaminergic-like model; however, this system represents a simplified cellular context that does not fully capture the morphological, functional, and molecular complexity of primary dopaminergic neurons, nor does it accurately reflect the intricate cellular interactions occurring within the intact brain. Consequently, the extent to which the observed neuroprotective effects can be extrapolated to more physiologically relevant models remains uncertain. In parallel, the *in vivo* component of the study was limited to acute administration paradigms and involved a relatively small number of animals, which restricts the robustness and generalizability of the conclusions. Such experimental conditions do not allow for the assessment of long-term efficacy,

potential tolerance development, or the emergence of compensatory mechanisms that may arise following chronic modulation of $mGlu_{2/3}$ receptors. Furthermore, although the investigation primarily focused on well-characterized pro-survival signaling pathways, including PI3K–AKT and MAPK/ERK, it is likely that additional intracellular cascades and cross-talk mechanisms contribute to the overall neuroprotective outcome and remain unexplored in the current study. Another important consideration relates to the use of pharmacological inhibitors and negative modulators, which, despite their utility, may exert off-target actions that could partially influence the observed effects and complicate the interpretation of receptor-specific contributions.

Taken together, these findings provide preliminary but encouraging evidence supporting the neuroprotective and neurotrophic properties of this novel $mGlu_{2/3}$ Ago/PAM. Further investigations are clearly warranted and should include long-term *in vivo* treatment paradigms, larger animal cohorts, and genetically modified models, such as $mGlu_3$ KO systems, to definitively dissect receptor-specific mechanisms and enhance the translational relevance of $mGlu_{2/3}$-targeted therapeutic strategies for PD.

In conclusion, this work provides the first pharmacological characterization of the novel $mGlu_{2/3}$ Ago/PAM and offers initial evidence of its neuroprotective and neurotrophic potential, thereby supporting continued investigation into its therapeutic relevance for PD.

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RINGRAZIAMENTI

Credo che il senso di ricerca sia un filo rosso nella mia vita.

Questo percorso trova le sue radici più profonde nella prima educazione all'osservazione attenta e sensibile che due genitori possono offrire: un invito ad interagire con la natura, ad alzare lo sguardo verso i contrasti artistici e culturali di questa città, ad incuriosirsi verso quello che si può trovare al di fuori dei confini di questa parte di terra con l'augurio che, un giorno, gli occhi possano trovare ciò che la mente immagina. Tutto ciò è ordinariamente straordinario, non scontato.

Questo percorso, poi, si svela sempre di più negli anni intensi del liceo. Nell'esercizio delle traduzioni dei testi scritti in latino e greco antico, c'è tutta la potenza dell'educazione al cercare quello che non si palesa: quasi mai è il primo termine quello più adatto alla traduzione, ma è l'esercizio costante alla ricerca della più fine versione che permette di dischiudere tutto il significato incastonato nel testo. Mi chiedo cos'è questo se non ricerca. Nella filosofia, poi, scopro che tutto il sapere proviene da un'unica fonte e che la domanda e il senso di meraviglia sono la forza propulsiva per andare al di là di quello che sembra essere la realtà. Ma come applicare questi strumenti? In un giorno di scuola, studiamo per la prima volta la duplicazione del DNA: pochi concetti, semplici accenni, eppure non riesco a smettere di stupirmi di un processo così articolato che avviene mentre io semplicemente…sono! Da lì, penso alla possibilità di applicare gli strumenti di ricerca e di studio alle biotecnologie e, successivamente, intuisco che, per me, è sorprendente che esista la possibilità di approfondire nel contesto del Cervello quella piccola parte di sapere che ho momentaneamente ottenuto. Entro sul serio per la prima volta in laboratorio durante il periodo del tirocinio alla magistrale e…sento che vorrei far parte di quella realtà. Vorrei saperne di più, essere una mano capace, una mente pensante, esercitare di nuovo il senso di domanda davanti a quello che sembra essere, o che può essere risolto. Ogni giorno in laboratorio è una sfida che richiede volontà, disciplina, confronto, pensiero critico ed autocritica, e che prosegue nel percorso del dottorato in modo sempre più definito, approfondito, ma anche intrecciato. In laboratorio sento che può avvenire la mia personale espressione: negli esperimenti, nello studio, nell'elaborazione dei dati, nelle presentazioni ai congressi e nell'attività di tutoraggio e di insegnamento ci sono io, tra linee guida e identità. In questi anni di dottorato, ho la fortuna di poter spaziare dalla sperimentazione *in vitro* alla sperimentazione

animale e scopro che quest'ultima mi appassiona tanto, rivelandosi un nuovo aspetto di me. In questa "palestra" che è il dottorato mi scontro con le mie rigidità, con i dubbi e le incertezze di un percorso che è tutto da costruire e che, come le cose più belle, richiede impegno. A volte è abbastanza semplice rispondere a tutto questo rimodellandosi, a volte no. A volte prevale l'autocritica, i limiti, l'insicurezza, la necessità di maturare un po' di più sotto le domande che accompagnano un sogno che deve prendere forma e che vorrei rimanesse sempre coerente a me, nella difficoltà di capire cosa sono io. A volte c'è l'errore, ma mai prima d'ora avrei saputo dare valore profondo alla traccia che lascia l'errore. La vita in laboratorio ce lo insegna quotidianamente. Trascorro, infine, un anno all'estero in cui tutto è diverso, tutto è fortemente impattante su di me. Ogni giorno segna un piccolo, ma continuo, cambiamento da cui non posso tirarmi indietro, ma che devo soltanto abbracciare. Per la primissima volta sono io ed io (anche se mai davvero sola), nei tentativi di trovare adattamento e di assorbire il nuovo quanto più possibile. Sono mesi lenti e veloci allo stesso tempo, il tempo si incanta a volte nella natura prorompente dell'Estonia, altre volte galoppa sotto le mille nuove stimolazioni, e io voglio solo sapere quanto altro posso conoscere delle Neuroscienze e di me come persona, studentessa e lavoratrice. Anche lontano, ritrovo comunque il mio modo di vivere il laboratorio, cioè, cercando insegnamento e provando anche a testare la mia graduale autonomia, mentre la passione si espande facendo tesoro del nuovo.

In questo intero percorso non ci sono solo io, ma tante persone che mi hanno permesso di camminare lungo la strada della ricerca scientifica, accompagnandomi, guidandomi, aiutandomi:

-alla Professoressa Valentina Di Liberto, perché lei è la mia prima vera guida scientifica. Tutto quello che ho imparato, l'ho imparato insieme a lei. Dalla teoria durante le lezioni di Neurobiologia Molecolare alla magistrale, alla prima conta cellulare, al primo western blot, alla prima manipolazione animale, alla prima scrittura, al primo poster, alla prima presentazione orale e ai primi passi da "grande", in cui provare a essere meno studentessa e più ricercatrice. In questa nuova, ancora sottilissima, veste la sua guida non è più solo scientifica, ma è una possibilità di confronto e riflessione e questa, per me, è la cosa che conta di più. Non sarei esattamente quello che sono se non tramite tutti questi insegnamenti;
-alla Professoressa Giuseppa Mudò, perché mi ha permesso di studiare ed imparare in questo laboratorio, in particolare dandomi tanta fiducia nei primi tentativi di insegnamento frontale, un'esperienza, per me, bellissima, in cui ho testato la mia autonomia con allo stesso tempo, però,

la sicurezza di avere il suo grande supporto alle spalle;
-a Miriana, per tutti i momenti condivisi. Sappiamo che non è facile trovare dei compagni di lavoro con cui si è in sintonia nell'idea di impegno, dedizione, attenzione, organizzazione che il laboratorio richiede, per questo con Miriana sono stata fortunata. Miriana è la compagna di lavoro che tutti vorrebbero avere. Scherzosamente, ci definiamo due delfini che nuotano insieme, perché ci aiutiamo costantemente, e so che posso contare su di lei anche quando sono più stanca o confusa o sembra che tutto vada male. Con lei ho imparato a muovere i primi passi in laboratorio ed insieme siamo maturate, collaborando sempre con genuinità e supportandoci reciprocamente nelle tappe, dalle più piccole alle più grandi, verso i traguardi più belli. Le parole sono un po' riduttive quando la stima e l'affetto sono una realtà solida. Grazie;
-a Nicolò (Nicolaus) per la gentilezza che regala e che si fa risorsa in mezzo alle difficoltà. Tra ratti che non finiscono mai, una statistica per noi sempre misteriosa e grafici mai definitivi, la tua calma è un valore aggiunto;
-a Giuditta per l'insegnamento scherzoso, la precisione che incontra la risata, il confronto quando ne avevo bisogno;
-a Monica per il sostegno, con dolcezza, durante il percorso;
-a Miriam e a tutti i compagni di laboratorio, per il tempo insieme, la collaborazione, il reciproco impegno, la crescita condivisa;
-a tutti i Professori dell'Istituto di Fisiologia Umana, per le numerose opportunità di confronto e collaborazioni;
-ai Professori Tõnis Timmusk e Cyril Goudet per aver revisionato questa tesi di dottorato con preziosa attenzione e gentilezza;
-ai Professori Tõnis Timmusk e Indrek Koppel, a Flor, Age e a tutti le persone del laboratorio che mi ha accolto in un meraviglioso giro di vita a Tallinn: grazie per le millemila possibilità, l'accoglienza in un grande gruppo, la fiducia riposta nella mia persona e nella mia attività, la considerazione del mio pensiero, l'affetto e la gioia. Sentirmi parte di un contesto così nuovo è stato profondamente edificante e mi ha reso felice;
-a tutti gli affetti che mi sono stati vicini, camminando con me, ispirandomi con evolutissima intelligenza ed umanità. Grazie per essere stati il meglio;
-ai miei genitori e a mio fratello, semplicemente perché non saprei chi sono senza le vostre mani che tengono le mie. E' tutto racchiuso qui, in questa immagine, e non potrebbe essere diversamente. Niente potrà mai cambiarlo.

Recentemente mi sono interrogata sul significato di “genio”, mettendo in discussione quello che mi era stato sempre detto e chiedendomi, quindi, se effettivamente questo fosse talento puro, indisciplinato, che semplicemente esplode e si esprime. Mi sono risposta e convinta più di prima che qualsiasi sia la forma e il significato di talento, nessuna costruzione/idea/progetto dura senza costanza e disciplina e che una mente, per esprimersi, ha bisogno di essere coltivata e che la passione è la guida. Spero che la passione per la ricerca mi accompagni ancora per tanto tempo e di essere sempre più capace a guardare oltre alla forma apparente delle cose, perché è ancora tutto da imparare.

Dedico questo traguardo a chi ci è stato,
grazie a tutti!

Giulia

ACKNOWLEDGEMENTS

I believe that Research runs like a red thread through my life.

This path has its deepest roots in the very first education to careful, sensitive observation that two parents can offer: an invitation to engage with nature, to lift one's eyes toward the artistic and cultural contrasts of this city, to grow curious about what lies beyond the borders of this piece of land, with the hope that one day the eyes might find what the mind imagines. All of this is ordinarily extraordinary, never to be taken for granted.

This path then reveals itself more and more clearly during the intense years of high school. In the exercise of translating texts written in Latin and Ancient Greek, there is all the power of being trained to look for what does not immediately show itself: almost never is the first word the right one, but it is the constant effort to search for the most precise version that allows the full meaning embedded in the text to unfold. What is this, if not research?

Through Philosophy, I then discover that all knowledge comes from a single source, and that questioning and a sense of wonder are the driving forces that push us beyond what seems to be reality. But how can these tools be applied? One day at school, we study DNA replication for the first time: just a few concepts, simple hints, and yet I cannot stop being amazed by such a complex process that happens while I am simply… being. From there, I begin to imagine applying the tools of research to biotechnology, and later I realize that, for me, it is astonishing that there is the possibility of delving deeper, within the context of the brain, into that small piece of knowledge I have momentarily gained.

I truly enter a laboratory for the first time during my master's internship, and… I feel that I want to be part of that world. I want to know more, to be a capable pair of hands, a thinking mind, to once again exercise the habit of questioning in front of what seems to be, or what can be solved. Every day in the lab is a challenge that requires willpower, discipline, dialogue, critical thinking and self-criticism, and this continues through the PhD path in an increasingly defined, deeper, but also more intertwined way. In the lab I feel that my personal expression can take shape: in experiments, in studying, in data analysis, in conference presentations, and in tutoring and teaching activities—there is me, moving between guidelines and identity.

During these PhD years, I have been fortunate enough to range from *in vitro* experimentation to animal experimentation, and I discovered that the latter truly fascinates me, revealing a new side

of myself. In this “training ground” that is the PhD, I come up against my own rigidities, doubts, and uncertainties of a path that still needs to be built and that, like the most beautiful things, demands commitment. Sometimes it is fairly easy to respond to all this by reshaping oneself; sometimes it is not. Sometimes self-criticism, limits, insecurity prevail, along with the need to mature a little more under the weight of questions that accompany a dream that needs to take form, and that I wish to keep always true to myself, within the difficulty of understanding who I am. Sometimes there is error—but never before have I known how to give such deep value to the trace that error leaves behind. Life in the lab teaches us this every single day.
Finally, I spend a year abroad, where everything is different, everything has a powerful impact on me. Every day marks a small but continuous change that I cannot step away from, but can only embrace. For the very first time, it is just me and myself (even if never truly alone), trying to find adaptation and to absorb as much of the new as possible. These are months that feel slow and fast at the same time: time sometimes seems to pause in the overwhelming nature of Estonia, other times it gallops under a thousand new stimuli, and all I want is to know how much more I can learn about Neuroscience and about myself as a person, a student, and a worker. Even far away, I rediscover my own way of living the lab: seeking learning, while also testing my growing autonomy, as passion expands by making the most of what is new.

Throughout this entire journey, it has not been just me, but many people who have allowed me to walk along the path of scientific research, accompanying me, guiding me, helping me:

–to Professor Valentina Di Liberto, because she is my first true scientific guide. Everything I have learned, I have learned together with her. From theory during Molecular Neurobiology classes in my master’s degree, to my first cell count, first western blot, first animal manipulation, first piece of writing, first poster, first oral presentation, and my first steps as a “grown-up,” trying to be less of a student and more of a researcher. In this new, still very delicate role, her guidance is no longer only scientific, but also a space for dialogue and reflection—and for me, this is what matters most. I would not be exactly who I am without all these teachings;
–to Professor Giuseppa Mudò, for allowing me to study and learn in this laboratory, and especially for placing so much trust in me during my first attempts at front-facing teaching—an experience that was beautiful for me, where I tested my autonomy while still having the reassurance of her strong support behind me;
–to Miriana, for all the moments we have shared. We know how hard it is to find coworkers who

are truly aligned in their ideas of commitment, dedication, care, and organization—and in this, I was lucky with Miriana. She is the kind of lab partner everyone would want. Jokingly, we call ourselves two dolphins swimming together, because we constantly help each other, and I know I can rely on her even when I am more tired, confused, or when everything seems to go wrong. With her, I learned my first steps in the lab, and together we have grown, always collaborating with sincerity and supporting one another through every stage, from the smallest to the biggest, toward the most beautiful goals. Words feel a bit inadequate when esteem and affection are such solid realities. Thank you;

–to Nicolò (Nicolaus), for the kindness you give and turn into a resource in the midst of difficulties. Between endless rats, statistics that always remain a mystery to us, and graphs that are never final, your calm is real added value;

–to Giuditta, for precision that meets laughter, and for the dialogue when I needed it.

–to Monica, for her gentle support throughout the journey;

–to Miriam and all my lab mates, for the time spent together, collaboration, shared commitment, and collective growth;

–to all the professors of the Institute of Human Physiology, for the many opportunities for discussion and collaboration;

–to Professors Tõnis Timmusk and Cyril Goudet, for revising this PhD thesis with such care and kindness;

–to Professors Tõnis Timmusk and Indrek Koppel, Flor, Age, and everyone in the lab that welcomed me into a wonderful turn of life in Tallinn: thank you for the countless opportunities, for welcoming me into such a big group, for the trust you placed in me and in my work, for valuing my thoughts, for the affection and joy. Feeling part of such a new environment was deeply formative and made me truly happy;

–to all those I hold dear, who have stayed close to me, walking alongside me, inspiring me with extraordinary intelligence and humanity. Thank you for being the best;

–to my parents and my brother, simply because I would not know who I am without your hands holding mine. Everything is contained in this image, and it could not be otherwise. Nothing will ever change it.

Recently, I have reflected on the meaning of “genius,” questioning what I had always been told and asking myself whether it is pure, undisciplined talent that simply explodes and expresses itself. I answered myself—and convinced myself more than ever—that whatever form or

meaning talent may take, no construction, idea, or project lasts without consistency and discipline; that a mind, in order to express itself, needs to be cultivated; and that passion is the guide. I hope that my passion for research will stay with me for a long time, and that I will become ever more capable of looking beyond the apparent shape of things—because there is still so much left to learn.

I dedicate this milestone to those who were there.
Thank you all!

Giulia