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Therapeutic approaches to counteract inflammation of Central Nervous System: role of SUN11602 in brain trauma and Parkinson's disease and DdPAC light stimulation for Alzheimer's disease

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ABSTRACT

The Central Nervous System (CNS) is an important control centre of the total human body and with complex formation and for this reason is particularly susceptible to disorders, which today represent pathological conditions affecting more than a billion people in the world. One most common CNS disorders is represented by Traumatic Brain Injury (TBI), like an external mechanical force, such as falls, vehicle accidents and sports injuries, causing damage to the brain. Head trauma triggers a cascade of events, starting from microglia activation and the formation of astrocytes reactive, that activate several signalling pathways involved in inflammation of the CNS, named neuroinflammation. The main pathways include NF-κB, MAPK, JACK/STAT/SOCS pathways and others, leading neurons towards apoptotic way through the involvement of the Bcl2/Bax signalling pathway. Numerous cohort studies of clinical research demonstrated that neuroinflammation generated by TBI represents an important predictive factor for the development of neurodegenerative diseases (NDDs), which are the most common causes of death worldwide in people over 60 years of age. They are characterized by a progressive neuron deterioration that affects different brain regions. The most well-known include Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS). The etiopathogenesis of neurodegenerative diseases is multifactorial and, nowadays, the triggering cause of these diseases is not entirely clear. PD and AD are among the most frequent NDDs, and their development is thought to be based on an inflammatory process involving the CNS, named neuroinflammation, which involves specific cells of the CNS, such as astrocytes and microglia, modulating the inflammatory processes. Moreover, in this context, there is a dysregulation of cyclic Adenosine monophosphate (cAMP) signalling, an important second messenger, that plays a pivotal role in the CNS in physiological conditions. Great strides have been made in research to seek new therapeutic opportunities for PD and AD. Here, we focused on both pharmacological, based on the use of bFGF, and optogenetic approaches, based on photoactivable adenylate cyclases (PAC). Firstly, bFGF exerts neuroprotective effects in the CNS-damaged supporting neuronal survival, counteracting the neuroinflammation and apoptotic process, contributing to neuronal death, and repairing damaged tissues. Therefore, a new mimic of bFGF, SUN11602, has been synthesized. It has been demonstrated that SUN11602 interacts with the FGFR1 receptor similarly to bFGF, thus enhancing its phosphorylation and stimulating neuronal growth

and survival in pathological conditions. SUN11602 administration significantly reduced the alteration of PD hallmarks, attenuating the neuroinflammatory and reducing nigrostriatal degeneration. Secondly, another novel approach is represented by an optogenetic model using PAC. Especially in this study, we used a variant of the *Deinococcus deserti* bacteriophytochrome (BphP), *Dd*PAC, that can increase cAMP levels under red light stimuli (λ 685 nm), providing insights into the role of cAMP increases in various neuronal processes, such as the study of neuroplasticity. To study cAMP signalling, an Adeno-Associated Virus (AAV) construct was built, containing DdPAC under neuronal or astrocyte-specific promoters, and was injected directly into the hippocampus area of WT and the 5xFAD Alzheimer mouse model. The DdPAC stimulation, of the WT and 5xFAD mice, reduced the GFAP expression, a marker of astrogliosis, and β-Amyloid (Aβ) deposition and the number of Aβ plaques in the hippocampus when DdPAC was stimulated specifically in astrocytes, while minor effects were observed when DdPAC was stimulated in neurons, suggesting that cAMP increases in astrocyte might be a better therapeutic opportunity for AD. In conclusion, the results obtained by both approaches, pharmacological with SUN11602 and optogenetic by DdPAC stimulation specifically in astrocytes may offer promising therapeutic opportunities for NDDs.

CHAPTER 1: INTRODUCTION

1.1. The characteristics and importance of the Central Nervous System

The central nervous system's (CNS) responsibilities include receiving, processing, and responding to sensory information, and consists of the brain and spinal cord. The brain is constituted by two hemispheres, left and right, which are in constant communication and responsible for several brain functions[1] such as awareness, movement, thinking, speech, and the 5 senses[2]. While the spinal cord is an extension of the brain and its main function is to carry information between the brain and the rest of the body via peripheral nerves. Moreover, the brain is made up of gray matter, consisting of nerve cell bodies and blood vessels, and white matter, constituted by axons. The CNS is protected by the brain and spinal cord through cerebrospinal fluid (CSF), the meninges and the skull and backbone. In more detail, inside the brain it is possible to distinguish different portions:

- *Cerebrum*: is the largest part of the brain and controls intelligence, memory, emotion, speech and move
- *Cerebellum*: sends signals for the body to adjust and move
- *Thalamus*: controls memory and movements
- *Hypothalamus*: controls appetite, body temperature, produces hormones and their release in the pituitary gland
- *Brainstem*: responsible for breathing, consciousness, blood pressure, heart rate, and sleep

The CNS is responsible for various functions[3], in particular, it is involved in sensory processing, receiving sensorial information from the external environment (sight, hearing, touch, smell) and processing them to provide a correct response. Moreover, the CNS controls voluntary and involuntary movements by sending signals from the brain to the muscles. It is responsible for cognitive processes, such as thought, memory and language. In this regard, there are specific brain regions that are involved, such as the prefrontal cortex, which regulates emotions, amygdala and hippocampus are involved in emotional responses and memory formation.

The cellular component of the CNS is divided into *neuroepithelial*, formed by neurons and glia, and *mesenchymal*, including blood vessels and microglia[4].

1.1.1. Neuronal cells

The neurons transmit information to the body through chemical and electrical signals[5]. They are constituted by dendrites, soma and axons[6]. The connection point between one neuron and another is called a synapse[7], of which it is possible to distinguish a presynaptic terminal (the point where the electrical signal is converted into a chemical signal[8], therefore by releasing neurotransmitters and a postsynaptic terminal containing specialized receptors to which the neurotransmitter will bind, to obtain the previously elaborated response[9].

Other main cells in the CNS are represented by *glial cells*[9] or *neuroglia*[10], which are non-neuronal cells of the CNS. There are four types of these cells, astrocytes, microglia, oligodendrocytes and macrophages, all of these are involved in the regulation of brain plasticity, protection of neurons, and homeostasis[11 12].

1.1.2. Microglia

Microglia cells occupy all regions of the CNS and colonize the brain during development[13] and play a vital role in maintaining brain health and function[14 15]. Morphologically, microglia share structural and functional parts with macrophages[16], the main cells of the Immune System, highlighting their role in immune responses within the CNS[17]. In the first line, microglia secrete factors that influence the proliferation, differentiation and survival of neural progenitor cells, aiding in brain development and repair[18 19]. These cells are involved in the processes underlying learning and memory, by modulating synaptic plasticity[20]. Not only, because microglia are involved in the processes called synaptic pruning[21], the elimination of excess or dysfunctional synapses (with phagocytose), maintaining of neural networks. In this way, these cells control the homeostasis of the CNS through ionic balance and excitotoxicity prevention[22]. With other cells, astrocytes and oligodendrocytes, the microglia can influence neuronal activity and synaptic plasticity, releasing molecules, like cytokines and growth[23–25]. The majority of microglial functions are necessary to maintain the CNS in good condition[26] through the release of anti-inflammatory and trophic molecules enhancing the survival of surrounding neurons[27,28], and through interaction with neural circuits[29]. Studies showed that changes in microglia activity have been linked to behavioral outcomes such as anxiety, depression and social behaviour [29 30].

1.1.3. Astrocytes

Astrocytes, together with microglia, are the main cells of the CNS that play an important role in maintaining the health and functionality of neurons and offering homeostasis. They are present prevalent in glial cells and the spinal cord. Astrocytes are involved in different physiological processes, especially in neurotransmitter regulation and trophic support through regulation of the metabolism of Glutamate and GABA in the synaptic space and secreting growth factors and neurotrophins preventing excitotoxicity and supporting neuron survival. Moreover, astrocytes can maintain the ion balance by absorbing excess potassium ions (K^+) released during neuronal activity and are involved in intracellular calcium signalling to modulate synaptic activity. The integrity of the blood-brain barrier (BBB) is maintained thank regulation of nutrient transport from blood to neurons and the release signalling molecular that maintain the tight junction between endothelial cells. Astrocytes promote synaptogenesis, participating in the pruning of unnecessary synapses, like microglia cells. They, also, modulate synaptic transmission by releasing gliotransmitters, such as ATP, glutamate and D-Serine (D-Ser). Finally, in the same as microglia cells, following injury, they produce cytokines and chemokines, modulating the immune and inflammatory response.

1.1.4. Oligodendrocytes and Macrophages

Other important cells that make up the CNS are the Oligodendrocytes, responsible for myelination, processes that produce myelin around CNS axons, allowing rapid signal transmission[31]. Moreover, following injury, they participate in repairing and modulating immune cell responses[32]. The cells of the CNS able to control and maintain healthy cerebral functions are represented by Macrophages, distinguishable in *perivascular macrophages*[33], located around the blood vessels in the CNS, monitoring the BBB. They produce cytokines and chemokines to regulate the immune responses, and *meningeal macrophages*, located in the meninges, exert immune surveillance function and control cytokine production.

Therefore, thanks to the presence of these cells, the CNS is considered an "immune-privileged" site, in which the immune responses are regulated to prevent damage to neuronal cells[34]. According to the World Health Organization (WHO), a study released

by *The Lancet Neurology* showed that, in 2021, more than 3 billion people are affected by neurological conditions, the leading cause of illness and disability worldwide[35].

1.2. CNS functional alterations: inflammation of CNS as promotion of neurodegenerative diseases

Neurodegenerative diseases, such as PD, and AD[36], also events like TBI, chronic infections, or autoimmune disorders, are conditions that cause chronic CNS damage and, a particular event that occurs at this level and which starts a cascade of processes, is represented by neuroinflammation[37,38]. Acute and chronic injuries can increase the levels of systemic neuroinflammatory responses by affecting the CNS[39] and, in this regard, neuroinflammation represents a critical component in NDDs[40].

Neuroinflammation means the activation of the brain's immune response, primarily involving microglia, astrocytes, and other immune cells, which could influence these disorders[15]. Although the main role of microglia cells is to act as the brain's immune defence against disease and injury[15] in the presence of pro-inflammatory stimuli as lipopolysaccharide (LPS)[41,42], pesticides (Paraquat, Dieldrin, etc.) and pollution, disease proteins (β-amyloid, or α-synuclein (α-syn), or human immunodeficiency virus (HIV)-Tat)[43,44], there is a shift to a pro-inflammatory phenotype of microglia becoming neurotoxic[45–47]. In this state, microglia change their conformation and release cytotoxic factors, tumour necrosis factor-α (TNF-α), nitric oxide (NO) and reactive oxygen species (ROS) and inflammatory prostaglandins (PGE_2), aimed at destroying the invading pathogens[48], leading to neuronal degeneration. Activated microglia are observed in nearly all kinds of neurological diseases, including AD, PD, ALS and HD[49–51]; infectious and TBI[52,53].

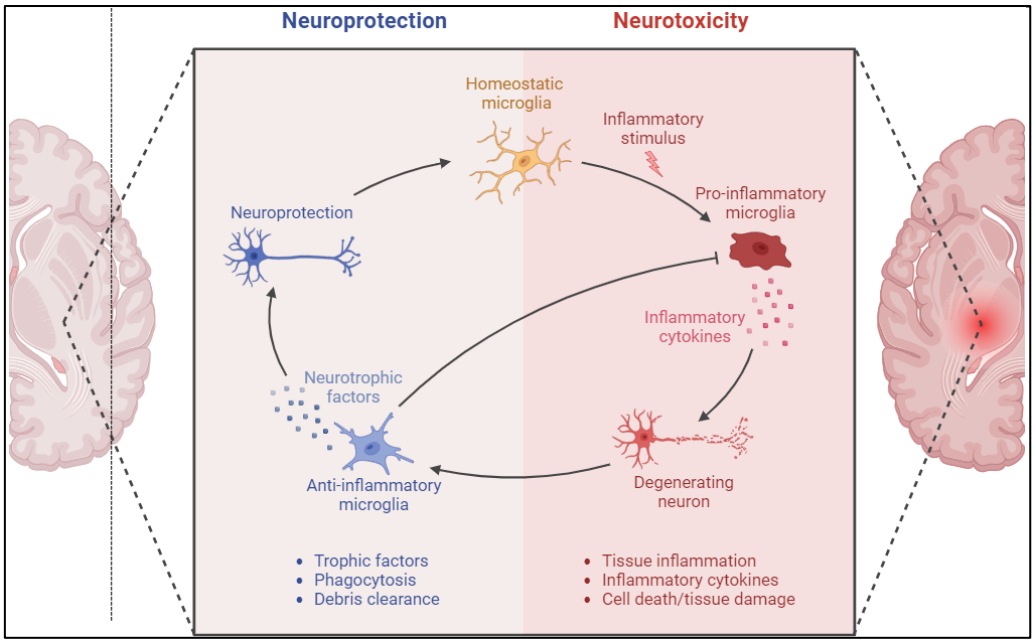

Figure 1. Activation of microglia support neuroinflammation
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Moreover, during brain injury, astrocytes become reactive and they are involved in the gliosis process, better known with the name of *Astrogliosis*. In the same way as microglia cells, also astrocytes change their morphology and functionality. In the neuroinflammation state, reactive astrocytes release pro-inflammatory cytokines and chemokines, activating the immune cells of the CNS[54]. In this case, astrocytes contribute to the progression not also of NDDs, but of other conditions, such as TBI, stroke, epilepsy, etc., exacerbating dopaminergic neuron loss, increasing oxidative stress and pro-inflammatory factors levels and disrupting the BBB and altering neurotransmitter clearance and ionic balance[55]. Although microglia and astrocytes exhibit structural, functional and original differences, are common some characteristics. In the first line, both cells are activated during the injury and disease, and reactive astrocytes may participate in signalling pathways (astrogliosis) and replace damaged neurons, while reactive microglia cells play a role in phagocytosis, removing damaged tissue[56]. Therefore, the interconnection between chronic CNS damage and neuroinflammation, which contribute to the progression of various neurological disorders, is of considerable importance.

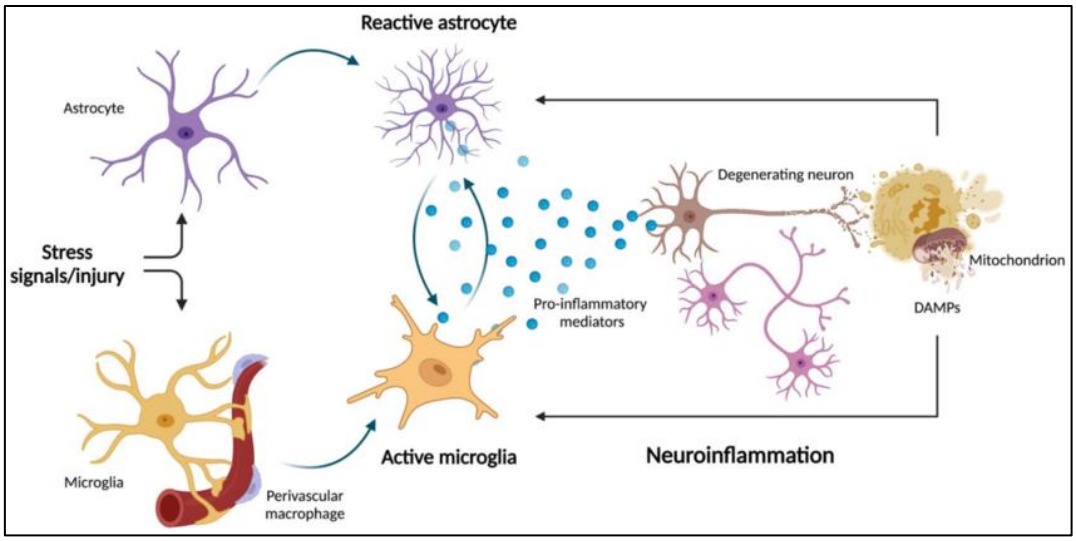

Figure 2. Formation of astrocyte reactive during neuroinflammation
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During the inflammatory process, a key role, together with microglia and astrocytes, is played by cytokines and chemokines, released by these same cells[57]. Cytokines and chemokines contribute to a sustained inflammatory state, which therefore characterizes not only chronic damage to the CNS but also the basis of NDDs[58]. They would lead to further neuronal dysfunction with consequent cell death. The presence of chemokines in the CNS causes the recruitment of microglia and immune cells, thus amplifying the inflammatory response and therefore causing further damage to cells and tissues. Neuroinflammation has been shown to exacerbate the misfolding and aggregation of proteins, such as Aβ and tau in AD. Furthermore, a consequence of persistent neuroinflammation is the disruption of synaptic function[59], and thus communication between different neurons. Furthermore, neuroinflammation is also a crucial factor for neurotoxicity because it can determine the release of neurotoxic factors, such as nitric oxide and ROS, which directly damage neurons and aggravate cell death as in PD.

1.2.1. Main pathways modulating CNS inflammation: from NF-κB to apoptosis

An event that characterizes the neuroinflammation process is represented by cascade activation of several pathways involved in this response to injury[60]. This means a complex, multi-step process involving various cellular and molecular steps that amplify the inflammatory response within CNS[61]. Following microglia and astrocyte activation, the pro-inflammatory cytokines produced by these cells generate positive feedback that strongly amplifies the inflammatory response[62,63], causing a chronic inflammatory state

and tissue damage and facilitating neurodegenerative progression[64]. One of the main pathways involved in this event is the NF-κB pathway (Nuclear factor kappa-light-chain-enhancer of activated B cells)[61]. Briefly, the NF-κB signalling pathway is a transcription factor that regulates several processes, such as apoptosis, immune response, inflammation and cellular stress responses, modulating the cytokine release[65]. Evidence reported that the NF-κB pathway is implicated in various disorders, including neurodegenerative diseases, cancer, autoimmune and metabolic disorders[61].

The NF-κB signalling can exert its activity in Canonical (Classical) and Non-Canonical (Alternative) Pathways, as bellow reported[61]: the Canonical Pathway is defined as the regulator of the inflammatory response[66], immediately activated from acute inflammatory cytokines, such as TNF-α, Interleukin-1β (IL-1β) and ROS, and controls the innate immune response[67], determining also the oxidative stress processes. These events lead to the activation of the IκB Kinase (IKK) complex, which phosphorylates the inhibitor of NK-κB (IκB). In this way, NF-κB translocase into the nucleus, which binds κB site in the DNA and allows the transcription of target genes involved in these events before cited[68]. Contrary, the Non-Canonical Pathway is slower and is associated with an adaptative immune response[69]. Generally, this pathway involves the activation of NF-κB-inducing kinase (NIK) determining the activation of IKKα and p52, which translocate to the nucleus regulating the gene expression[70].

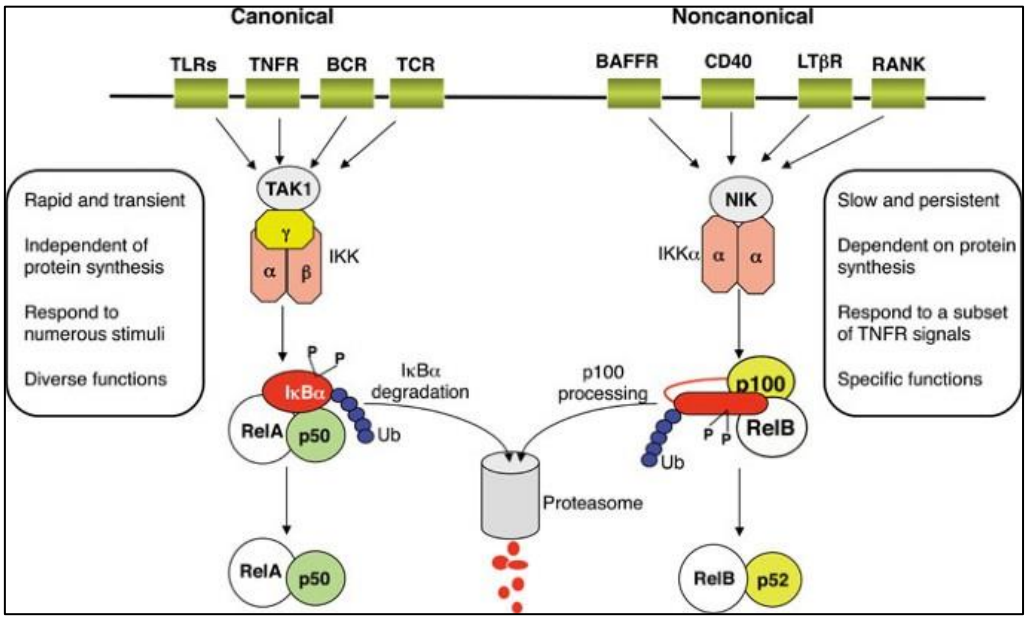

Figure 3. Canonical and non-canonical of NF-κB pathway[70]

In the context of inflammation, NF-κB plays a key role as the mediator that influences the activation of microglia, astrocytes and neurons, respectively[65,71]

Concerning microglia activation, NF-κB upregulates the pro-inflammatory cytokines and chemokines[72,73], recruiting additional immune cells to the site of injury and enhancing the phagocytic activity of microglia cells[74]. Astrogliosis exerted by activated astrocyte cells also relies on NF-κB signalling, a hallmark of CNS disorders and that involves astrocyte proliferation and hypertrophy[75]. Finally, as previously described, the NF-κB pathway can promote neuronal survival by inducing anti-apoptotic genes and it is implicated in synaptic plasticity and memory formation[76,77], but its dysregulation can contribute to upregulation of pro-apoptotic factors, leading to neuronal apoptosis and cognitive deficits[78,61]. As previously mentioned, the NF-κB pathway plays an important role in the inflammatory response and immune regulation, and, in the NNDs context, is activated in response to β-amyloid plaques and Tau tangles, for AD[79]. While PD is implicated in the chronic activation of microglia and aggregation of α-syn[80], contributing to neurotoxicity and the degeneration of neurons, thus to the progression of the pathogenesis of these diseases[81,82].

Toll-like receptors (TLRs) are signal transduction membrane proteins highly expressed at the microglial level[83]. They belong to the innate immune system, representing the first line of defence against pathogens, eliminating them and are involved in the inflammatory response. They recognize specific ligands, such as Myeloid differentiation factor 88 (MyD88), initiating the inflammatory process, activating signalling molecules such as NF-κB and then promoting the release of cytokines, the process of phagocytosis and the expression of co-stimulatory molecules necessary for adaptive immune responses. MyD88 plays a crucial role in CNS infection and subsequent astrocyte activation has been demonstrated in PD and AD[84,85]. Another pathway that, following microglia activation, induces NF-κB translocation is the PI3K/AKT pathway[86] (phosphatidylinositol-3 kinase/protein kinase B), which directs a series of processes, such as inflammatory response, apoptosis and cellular activation. Another member of this family is the mammalian target of rapamycin (mTOR). Once PI3K is activated, it will consequently also activate AKT, determining not only the activation but also the phosphorylation of mTOR, which is important for microglia activation[86]. Active mTOR leads to increased

NF-κB activity, thereby promoting the expression of inflammatory molecules including Inducible nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX-2)[87].

The NF-κB pathway is not the only one involved during neuroinflammation, microglia and astrocytes activation induce, in turn, the promotion of another pathway that, similarly to the NF-κB pathway, directs the inflammatory response within CNS, the MAPK (Mitogen-Activated Protein Kinase) family, including p38 MAPK and SAPK/JNK (Stress-activated protein kinases/Jun amino-terminal kinases)[60,86].Several external stimuli activate members of this family, for example, p38 MAPK is activated by stress and inflammatory signals, in turn regulating the production of pro-inflammatory cytokines and mediators and expressing iNOS and COX-2 in LPS-induced microglia[88,60]. While, SAPK/JNK activation is stimulated by external signals such as environmental stress and inflammatory cytokines and, once activated, the SAPK/JNK pathway translocates to the nucleus regulating the expression of inflammatory mediators, such as iNOS and COX-2[87]. At the level of microglia and astrocytes, active MAPK further stimulates the production of ROS, contributing to oxidative stress and therefore amplifying the inflammatory response. At the neuronal level, however, the effect given by the activation of this pathway is slightly different[60]. The activation of p38 MAPK and JNK leads to a response to cellular stress ending with the process of apoptosis[89].

A chronic inflammatory response involves a series of events, cellular and molecular, that lead to apoptosis (programmed cell death). As previously described, activation of microglia and astrocytes[56], following different external stimuli, determines the release of pro-inflammatory cytokines and chemokines (IL-1β, IL-6, TNF-α) and ROS, which make neurons more susceptible to apoptotic signals. Furthermore, the produced ROS damages proteins, lipids and even DNA, causing mitochondrial dysfunction and directing neurons to undertake the apoptotic pathway. At this point, once the neurons have undertaken the apoptosis pathway, it is possible to distinguish two types of pathways: *intrinsic pathway* (mitochondrial pathway), characterized by the activation of pro-apoptotic proteins, Bax/Bak, thus interrupting the existing balance with anti-apoptotic proteins, bcl-2. This imbalance induces the activation of a protein that plays a key role in the apoptosis pathway, Caspase-9, which determines the cascade activation of Caspases. Caspase-9 can activate a series of effector caspases downstream, such as Caspase-3 and Caspase-7, cleaving cellular proteins and leading to apoptosis. The e*xtrinsic pathway* (death receptor

pathway) is characterized by the interaction of TNF-α with its receptors present on the neuronal membrane, leading to the activation of the extrinsic apoptotic pathway. The consequence of this is the recruitment of adaptor proteins such as FADD (FAS Associated protein with Death Domain) and the activation of Caspase-8, either directly activating executioner caspases or enhancing the intrinsic pathway by cleavage of Bid, another pro-apoptotic member of the Bcl-2 family.

1.2.2. The role of neuroinflammation in brain trauma

Traumatic Brain Injury (TBI), causes metabolic and cellular complications[90], distinguishable into a primary lesion, like the direct consequence of the injury, which is subsequently followed by a cascade of biochemical and physiological events, which characterizes the secondary lesion, supporting an inflammatory state of the CNS (*neuroinflammation*), with axonal damage and neuronal death[91]. These post-traumatic neurological disorders are mediated by inflammatory markers activated and released by microglia activation and astrocytes reactive. Cellular damage, as well as the inflammatory response, developed acutely after TBI, determine the release of endogenous factors (RNA, DNA, heat shock proteins) that act as damage-associated molecular patterns (DAMPs), binding specific receptors, such as TLRs and activating the NF-κB and MAPK pathways, which have as a final event the release of pro-inflammatory mediators, such as cytokines and chemokines (IL-1β, IL-6, TNF-α, CXCL1-5 and CXCL8-10) and immune cells[92], thus supporting the inflammatory response. The increased release of pro-inflammatory cytokines[93] and chemokines, after TBI, are induced by the chronic activation of microglia and the formation of reactive astrocytes, as previously mentioned[94]. In this regard, the main pathways that induce the activation of microglia triggering inflammatory response are: Janus kinase-signal transducer and activator of transcription suppressors of cytokines (JAK/STAT/SOCS), closely associated with brain injury and is activated after TBI[95]; PI3K/AKT/mTOR, AKT/GSK-3β/CREB, with GSK-3β associated with neurodegeneration, is known to affect transcription factors involved in inflammatory activation[96]. The TLR4/NF-κB pathway, Calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ-dependent) AMPK/PGC-1α, whose activation regulates microglia-induced neuroinflammation[97]. Another signalling pathway involved in the pro-inflammatory response is Mitogen-activated protein kinase/ extracellular signal-regulated protein kinase (MEK/ERK), which is required for MAPK signal transduction[98]. An important receptor activated following TBI is the purinergic

2X7 receptor (P2X7R), an ATP-gated nonselective cation channel, present in microglia, astrocyte and oligodendrocyte cells and its expression increases after CNS damage[99]. Finally, the spleen tyrosine kinase (SYK) is a non-receptor protein tyrosine kinase, that modulates immune signals in various cells, such as mast cells, B cells, and macrophages[100], and its activation leads to activation of MAPK, PI3K, AKT, and NF-κB. Therefore, activation of the inflammatory response following head trauma can be harmful, leading to events and consequences already mentioned, and it can be beneficial[101]. Studies report that activation of the inflammatory response is necessary to eliminate the damage caused by the insult to the CNS[102], such as glutamate from astrocytes to reduce neuronal damage from excitotoxicity[103].

1.2.3. Neuroinflammation role in neurodegenerative diseases

As previously described, neuroinflammation, although a physiological process, is activated following different extracellular stimuli, aimed to protect the CNS[104], is at the same time a key element in the pathogenesis of many neurodegenerative diseases, such as PD, AD, HD and ALS, which show different neurodegeneration pathways that affect specific portions of the CNS[105]. More specifically, different studies have been conducted that confirm the link between neuroinflammation and the development of NDDs[106]. This chronic inflammatory state is associated with an increase in peripheral blood levels of pro-inflammatory markers, such as IL-1β, IL-6, IL-18, TNF–α and TGF-β[107–109]. Further evidence of this correlation is also demonstrated by the fact that the CNS has a unique immune response compared to the peripheral immune system[105]. An inflammatory response in the peripheral system, triggered by several factors, including systemic exposure to LPS and/or viral infections, causes immune cells from the periphery to migrate to the CNS[110,111]. This infiltration generates neuroinflammation, through the activation of microglia that, in turn, causes the BBB to release pro-inflammatory markers, weakening, and ultimately destroying nerve cells[112]. Neurodegenerative diseases associated with neuroinflammation affect both the spinal cord and the brain, with progressive degeneration of neural tissue and mortality[113]. As will be described below, each NDD is characterized by specific symptoms, depending on the portion of the CNS involved, but all of them share the neuroinflammatory process, a key role in their pathophysiology.

1.3. From trauma to degeneration: the predictive role of brain trauma in PD and AD

Numerous cohort studies of clinical research, which included the different severity of TBI (mTBI, moderate and severe TBI)[114] demonstrated that head trauma might represent a risk factor for the development of neurodegenerative diseases, such as PD and AD[115,116]. Memory loss and cognitive deficits are common events that emerge in individuals who survive a long time after the trauma[117,118]. About AD, meta-analysis studies have been conducted on over two million patients, showing that the development of dementia is 1.6 times higher after trauma[119,120]. Furthermore, to better understand how TBI triggers chronic neurodegeneration, further preclinical and clinical studies have been conducted, highlighting abnormalities in the main AD proteins, β-amyloid and Tau, generating neurotoxicity and directly contributing to neuronal loss[121] In the same way, an mTBI could generate behavioural problems, characteristic of PD. Raquel C. Gardner et. al.[122] examined PD patients through the VA's database and identified that the risk of developing PD in patients with mTBI is 56%. Moving from a moderate TBI to a severe TBI, the percentage reaches approximately 80% compared to healthy subjects.

1.3.1. Trauma Brain trauma (TBI)

TBI results from the application of an external force and represents the common cause of death among young in industrialized societies[123], around 30-40%. Since 2020, 212.110 TBI-related hospitalizations have been recorded, while in 2021 over 69.473 TBI-related deaths have been recorded[124]. Just in the United States, every year, over 50.000 people die as a result of a TBI[125], while around 80.000 to 90.000 people a year experience a life-long disability as a result of a TBI. Following the head trauma, if the intervention is not timely, the resulting damage, especially neuronal, cognitive and behavioral deficits, becomes irreversible[126]. Various mechanisms might cause head trauma including:

- Falls, especially among young children and older adults (> 65 y/o), make up around 47% of head injuries.
- Blunt force trauma makes up around 15% of head injuries.
- Motor vehicle accidents make up around 14% of all head injuries (65% are the result of car accidents, 15-20% are the result of motorcycle accidents and 10% are from bicycle accidents).

- Assaults (including child abuse) make up around 9% of head injuries.

The most common symptoms that occur following head trauma are initial confusion, followed by headache, amnesia, mood swings, vomiting or nausea, memory problems and the onset of seizures, agitation and combativeness, poor attention, concentration and behavioral and personality changes.

To determine the severity of the TBI, three criteria are used:

1. **The Glasgow Coma Scale (GCS)**: evaluates patients based on the reactivity of: best eye response (E), best verbal response (V) and best motor response (M). The levels of response in the components of the Glasgow Coma Scale are 'scored' from 1, for no response, up to normal values of 4 (Eye-opening response) 5 (Verbal response) and 6 (Motor response)[127]
2. **Loss Of Consciousness (LOC)**
3. **Post-Traumatic Amnesia (PTA):** is a state of confusion and memory loss right after a TBI. PTA occurs because there is an impairment in attention and concentration, which are required to place new information into memory storage.

These latest indicators could be influenced by factors directly or indirectly correlated to trauma.

Moreover, there are three types of TBI:

- *Mild TBI (mTBI):* makes up around 75-80% of cases and it is the equivalent of a concussion and the typical signs are loss of concussions in less than 30 minutes. After this trauma, people recover very quickly, with a timeline from 1 to 3 weeks
- *Moderate TBI*: represents 10-15% of all TBI cases and the recovery is within 6 months. The consequences of moderate TBI concern the impact on daily life.
- *Severe TBI:* makes up around 10% of cases and occurs following complications found in moderate TBI. People with severe TBI have worse outcomes 6-12 months post-injury compared with people with mTBI.

Classification System For Traumatic Brain Injury			
Classification	Duration Of Unconsciousness	Glasgow Coma Scale	Post-Traumatic Amnesia
Mild	<30 Minutes	13-15	<24 Hours
Moderate	30 Minutes-24 Hours	9-12	1-7 Days
Severe	>24 Hours	3-8	>7 Days

Figure 4. Classification System For Traumatic Brain Injury (CSFTBI)

As previously described, in TBI the external force represents the cause of the primary lesion, like the direct consequence of the injury, which is subsequently followed by a secondary event, characterized by a cascade of biochemical and physiological events, leading to axonal damage and neuronal death[91]. Moreover, the neuronal damage suffered determines the activation of microglia and astrocytes[128,129], generating a high amount of inflammatory chemokines and cytokines, including the main IL-1β, TNF-α, IL-6, CXCL1-5, CXCL8-10[130,131]. In turn, as a consequence, the recruitment and activation of immune cells, neutrophils, occurs, thus triggering an inflammatory response, excitotoxicity and production of ROS[132], but not only, because TBI causes an alteration of the intracellular calcium concentration, contributing to excitotoxicity and neuronal damage[133]. Nowadays, it is not yet clear whether this cascade of events can be beneficial, for the survival of neurons, through the release of neuroprotective factors, or harmful since it secretes a high number of inflammatory markers[134]. A prolonged state of these events promotes neurodegeneration[135]. Specifically, depending on the type of TBI, axonal damage may occur, with interruption of transport, thus influencing the molecular mechanisms underlying both the motor deficits that characterize PD[135], increasing the probability of developing this disease early, and the formation of pathological proteins, such as hyperphosphorylated tau and Aβ, with the formation of Aβ plaques and pTau-positive neurofibrillary tangles and dystrophic neuritis, thus correlating TBI to the development of AD[114].

1.4. Neuroinflammation affecting CNS and causing neurodegeneration: Parkinson's and Alzheimer's diseases

NDDs are a heterogeneous group of complex diseases, characterized by irreversible and progressive degeneration and neuron loss of specific areas of the CNS[136,137], destroying the cells responsible for controlling associative and motor functions, causing cognitive, behavioural and motor disorders with hypo or hypermobility, which can be accompanied by speech, swallowing and breathing disorders[138]. However, in most of these pathologies, the presence of insoluble protein aggregates in the brain of patients has been observed[139,140]. The main NDDs include PD, AD, ALS, HD and Frontotemporal Dementia Disease[11]:

NDDs represent one of the main public health problems affecting the entire world population. Epidemiological data show a high prevalence with a progressively increasing trend, especially in aging populations[141]. Additionally, there are cross-cultural variations, with higher prevalence reported in Europe (3rd position, after cardiovascular disease and cancer)[142], North America, and South America compared with African, Asian and Arabic countries[143]. In the United States, more than 7 million people are affected by NDDs, especially AD counting for 6.2 million people followed by PD[144]. Among NDDs, this thesis focused mainly on Parkinson's and Alzheimer's.

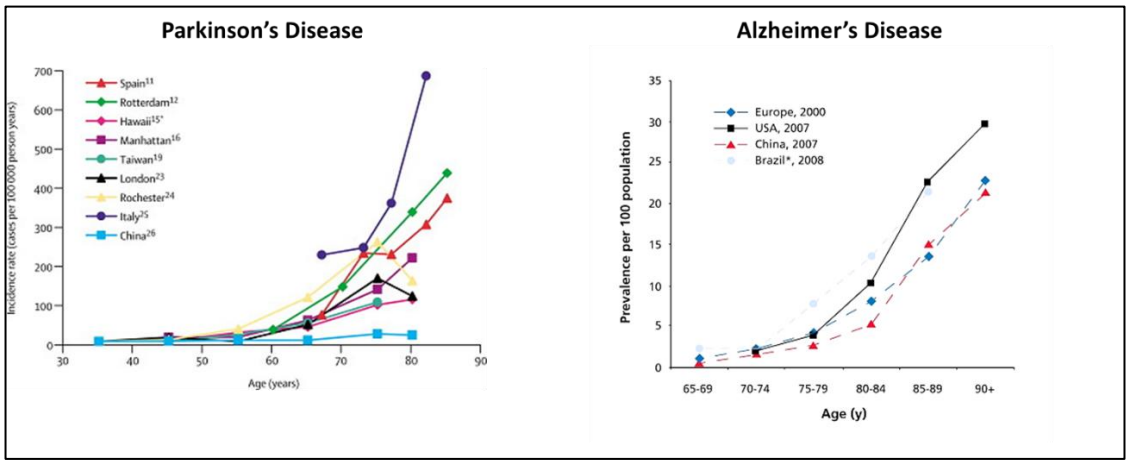

Figure 5. Epidemiology of PD[145] and AD[146]

1.4.1. Parkinson's Disease (PD)

PD is a brain disorder in adult people characterized by progressive and slow neurons and nerves loss that control the parts of the body. Moreover, PD is also named "*movement disorder*" because the first symptom may be a barely noticeable tremor in just one hand.

PD is the most common neurodegenerative disorder in the world after AD, which increases with aging. The incidence of PD increases after 60 years of age, rising sharply to more than 3% among individuals over 80 years old. The male population is more affected by PD than the women population, in rapport 3:2, with first onset at age 65 (10% of cases before 50 y/o)[147–149]. Age represents the first bigger risk factor; also, incorrect lifestyle (dietary habits, smoking, sedentary lifestyle, etc), and environmental toxins are examples of risk factors that may trigger PD symptoms[150,151].

Clinically, PD is characterized by motor symptoms and non-motor symptoms. Patients with PD have several motor symptoms including bradykinesia, muscle stiffness, rest tremor, and postural and gait difficulties[152,153]. Often, these symptoms are preceded by many non-motor symptoms for years or decades before[154,155]. The main non-motor symptoms include hyposmia, olfactory dysfunction, cognitive decline, constipation and rapid eye movement sleep problems, depression, constipation and urinary dysfunction, autonomic dysfunction and pain[155,156].

Moreover, it is possible to distinguish non-tremor-dominant PD and tremor-dominant PD, which, last, is often linked to a slower pace of development and less functional impairment[157]. Clinical studies have shown that tremor-dominant PD patients do not have other motor symptoms typical of PD and dopamine replacement therapy is generally effective. Then, patients with a non-tremor dominant PD are characterized by akinetic-rigid syndrome and a postural instability disorder, increasing the incidence of non-motor features.

1.4.1.1. Etiopathogenesis

The etiopathogenesis of PD is multifactorial, involving a complex interplay of genetic, environmental, and cellular mechanisms[158,159] that lead to neuronal degeneration and clinical manifestations. Genetic predisposition plays a significant role in PD. Several genes are implicated in both familial and sporadic forms of this disorder.

SNCA: the gene that encodes the α-syn protein and is highly expressed in the brain, as [153,157] well as in the heart, skeletal muscle, and pancreas[160]. However, its function is not yet fully understood, based on its properties, structure, and interaction with its specific targets, several hypotheses have been developed regarding its function. Under physiological conditions, it is involved in the regulation of dopamine release and transport

and exerts a neuroprotective phenotype in non-dopaminergic neurons[161,162], by inhibiting the activation of the caspase cascade (Caspase-3 and Caspase-7), acting by reducing the expression of p53 and pro-apoptotic factors[160,163]. Furthermore, it directs the fibrillation of the tau protein (an emerging role also in AD)[164]. Conversely, the presence of missense mutations, such as A53T[165], A30P[166] and E46K[167], in SNCA abolish its neuroprotective effect, instead favouring oxidative processes and directing neurons towards an apoptotic pathway[162,163]. These types of mutations are typical of familial Parkinson's and cause an overproduction or a structural change of the α-syn protein, thus promoting its incorrect misfolding, forming Lewy bodies, which accumulate inside the neurons corresponding to the substantia nigra, thus, exerting a toxic effect. To better understand the type of SNCA mutation, studies have been conducted analysing the evolution of its structure[164]. The results obtained have highlighted that the protagonist of these mutations that cause a structural evolution is the N-terminal domain of SNCA[168], thus determining its involvement in the development of familial PD.

LRRK2: LRRK2 encodes a protein called dardarin and is active in the brain and other tissues[169]. Physiologically, LRRK2 regulates mitochondrial function, autophagy, vescile trafficking and ensures proper neuronal function. Like the SNCA gene, the LRRK2 gene is also subject to mutations, which are associated with sporadic forms of PD. In particular, the mutation of its kinase domain, G2019S, has been identified, developing PD in some ethnic groups and familial PD. The mutation of this domain leads to an increase in kinase activity, responsible for an abnormal phosphorylation of substrate proteins, thus contributing to neurodegeneration. Furthermore, mutations affecting LRRK2 cause mitochondrial dysfunction, which results in a disruption of vesicular trafficking and therefore neuronal death. Other notable LRRK2 mutations include R1441C, R1441G, Y1699C and I2020T[170,171], each of which affects the kinase activity of LRRK2, altering its normal function. Furthermore, mutations in this gene (dardarin, OMIM 609007) lead to late-onset autosomal dominant PD and are the most common genetic cause of PD[169,172], accounting for 4% of familial PD and 1% of sporadic PD in all populations[173,174] . Neuropathological studies revealed that LRRK2 potentiates α-syn-mediated cytotoxicity in gray substantia neurons, an age-dependent factor[175].

PINK1, **Parkin (PARK2), DJ-1:** These three genes, are involved in mitochondrial function and responses to oxidative stress[176], ensuring cellular homeostasis. It is well

known that their mutations can lead to autosomal inherited early-onset PD[176]. A mutation affecting PINK1 (nonsense, missense and frameshift mutations) determines a loss of kinase activity, counteracting mitophagy processes[177], leading to the formation of both truncated and/or non-functional proteins and the accumulation of non-functional mitochondria, increasing oxidative stress[176]. Similar to PINK1, Parkin mutations[178] cause the accumulation of damaged mitochondria, compromising their removal[179]. This results in cellular dysfunction and therefore death of dopaminergic neurons. Unlike PINK1, PD generated by Parkin mutation has a slow onset with a positive response to therapies[180].

Finally, DJ-1 mutations, less common[178] than PINK1 and Parkin, are responsible for the development of early autosomal recessive PD[181] . The consequence of this mutation is the inability of the cell, by DJ-1, to respond adequately to oxidative stress and therefore to neutralize ROS[182]. This event makes dopaminergic neurons vulnerable, increasing the probability of their death[176].

GBA: This gene encodes the lysosomal enzyme glucocerebrosidase (GCase), which is involved in the metabolism of glycosphingolipids[183], breaking them down into glucose and ceramide. Mutations in this gene cause a reduction in the enzyme's activity, with a misfolding of the GCase protein, which results in cellular dysfunction[184]. This is a rare inherited genetic disease called Gaucher disease, which causes the accumulation of lipids in the spleen and liver[185]. GBA mutation and Gaucher disease represent an important and common genetic risk factor for the development of PD[183,186]. The most common mutations in this gene are N370S and L444P. Furthermore, a correlation between GCase levels and α-syn levels has been reported[187]; when Gcase levels are low, an accumulation of the α-syn protein has been recorded in dopaminergic neurons[188]

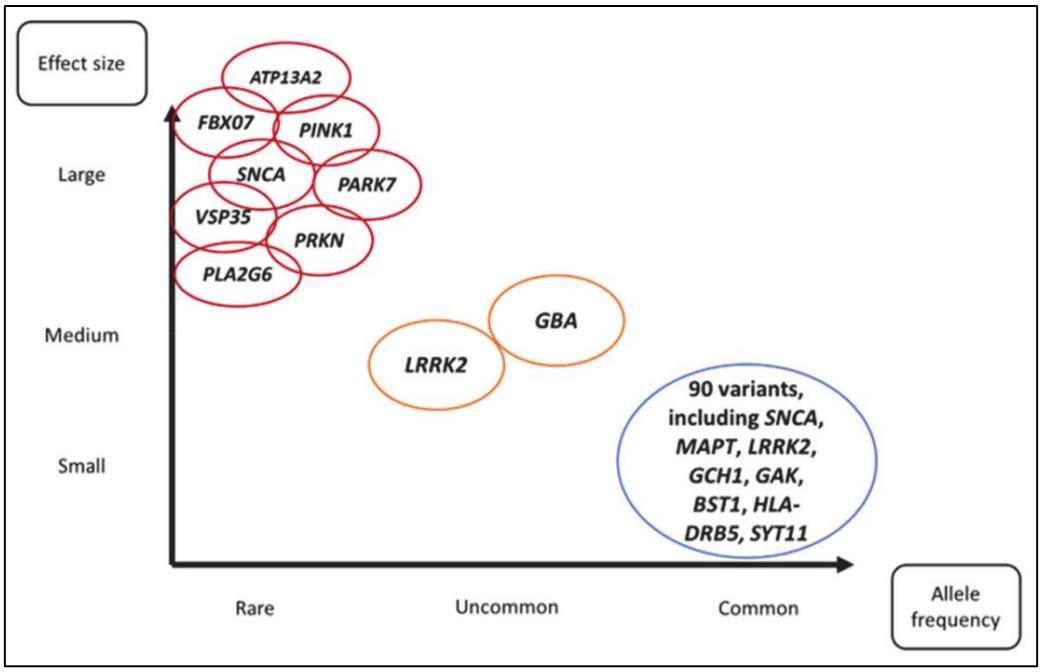

Figure 6. Mutations of the main genes involved in Parkinson's disease[189]

1.4.1.2. Dopaminergic neurons vulnerability

In the PD context, dopaminergic neurons play a pilot role and specific dopamine transporter, DAT, because their dysfunction is central in the pathophysiology of the PD. Dopamine neurons, those of the substantia nigra[190], are involved in several functions, including motor control, reward processing, and even cognitive functions[190]. These neurons release dopamine, a neurotransmitter that modulates several functions (motor control, reward and motivation and cognitive functions). DAT is a protein that regulates dopaminergic neurotransmission and is thus responsible for the reuptake of released dopamine in presynaptic neurons[191], terminating the dopamine signal and recycling it for the next stimulus. Furthermore, it manages the amount of neurotransmitter available for a possible binding with its receptors present on the postsynaptic neurons. In this way, the homeostasis of dopamine is guaranteed. The DAT, being a specific transporter of dopamine, can bind substances, such as the neurotoxin MPTP, allowing it to enter the neurons and therefore cause damage and death of the dopaminergic neurons, just as in the case of Parkinson's. Not only, dopaminergic neurons are highly susceptible to oxidative stress, as ROS are generated from the oxidative metabolism of dopamine[192], contributing to neuronal toxicity.

Pathologically, PD is characterized by the loss of dopaminergic neurons in the area of the brain called substantia nigra pars compacta (SNpc), in the lateral ventral tier[193]. There is a correlation between aging and striatal dopamine loss. S. J. Kish et. al.[194] studied the timing of the gradual loss of striatal dopaminergic markers[194,195], SNpc neurons with age[196] in the dorsal tier of the SNpc, whereas in PD it is exponential and predominantly in the lateral ventral tier[195,196]. Although the crosstalk between aging and loss of dopamine remains unclear what precise role plays in pathogenesis[197].

The clinical symptoms of PD appear when the loss of neurons is over 60%. This event leads to dopamine depletion in the striatum, the dorsolateral putamen[198]. In physiological conditions, dopamine works with other neurotransmitters to coordinate the millions of nerve and muscle cells involved in movement. If the brain lacks dopamine, appear the hallmark symptoms of Parkinson's[199].

1.4.1.3. α-synuclein aggregation

Not only, in the brain of patients with PD has it been discovered the abnormal accumulation of protein and lipidic inclusions, named Lewy bodies, constituted by fibrillary aggregated α-syn protein and rich of β-sheets[200], which represents a hallmark of neuropathology. α-syn is a small molecule and is highly expressed in the CNS and it is a pre-synaptic neuronal protein and, in presynaptic terminals, it is present close, but not within, synaptic vesicles[201,202]. In physiological conditions regulates neuronal development, determining the neuronal phenotype and establishing the synaptic connections[203,204], allowing it to bind to phospholipids and synaptic vesicles. Nowadays, it is not clear the exact mechanism, although is believed to involve impaired protein degradation pathways such as the ubiquitin-proteasome system and autophagy-lysosome pathway[160]. α-syn is involved in synaptic plasticity, through the modulation and maintenance of synaptic connections; furthermore, its function is also essential for learning and memory. At the mitochondrial level, α-syn influences cellular energy metabolism. Finally, evidence demonstrates the correlation between α-syn and lipid metabolism, ensuring cholesterol homeostasis. As described above, Gaucher disease is a common risk factor in the development of Parkinson's.

In pathological conditions, like PD, its α-syn molecular structure changes, from a normal monomeric state to form oligomers and fibrils[205], which are considered toxic and are believed to disrupt cellular functions[206]. This structure is the core of Lewy bodies and

Lewy neurites, which are dense, spherical inclusions that disrupt normal cellular architecture and function[207]. Lewy bodies interact with non-dopaminergic neurons in other brain regions, such as the limbic and neocortical regions[208]. Moreover, the advanced stage of PD involves also the neurons outside of the CNS, such as the olfactory bulb and mesenteric system[209].

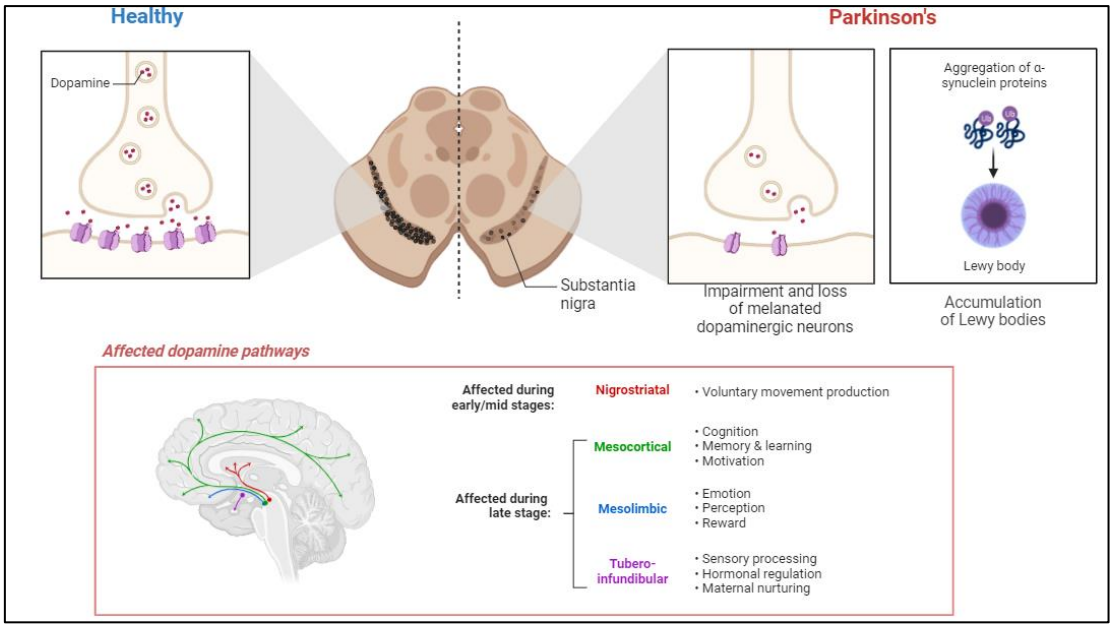

Figure 7. Dopamine neuron function and α-syn aggregation
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1.4.1.4. Calcium homeostasis alteration

An emerging role, both in the correct functioning of the CNS and in the onset of PD, is represented by the Calcium ion (Ca^{2+})[210]. Ca^{2+} is an essential element for the correct functioning of the CNS and survival of dopaminergic neurons in the substantia nigra. These neurons maintain a high basal Ca^{2+} load due to their regular activity. An adequate balance of Ca^{2+} concentration, inside and outside the cell (calcium homeostasis), is important because it ensures the release of neurotransmitters (in this context dopamine) into the synaptic space. Furthermore, it is the second messenger in the various signals that regulate cell survival, gene expression and synaptic plasticity, and it is also involved in the activity of transcription factors and proteins, important for the correct neuronal activity. The correct balance of intracellular Ca^{2+} concentrations is ensured by the presence of calcium-binding proteins (CaBPs). These proteins control calcium signalling pathways, protecting cells from calcium overload, which can be fatal to cells. The main CaBPs are Calbindin-D28k (Calb-1), widely expressed in the brain, especially in

dopaminergic neurons of the substantia nigra. It buffers free Ca^{2+} ions, eliminating them once neuronal activity has ended; it also protects neurons from possible excitotoxicity from Calcium. Another emerging role is played by Parvalbumin present in GABAergic intraneuronal neurons. This protein also buffers intracellular Ca^{2+} ions, preventing excessive accumulation of calcium and ensuring correct synaptic functionality. The S100β protein is involved in cellular biological processes, including cell growth, differentiation and regulation of enzymatic activity. This protein is expressed mainly in astrocytes and, like other CaBPs, regulates calcium-dependent signalling pathways.

As just written, dopaminergic neurons continuously use calcium flux for their activities and dysregulation of calcium homeostasis leads to a series of consequences, such as mitochondrial dysfunction, with oxidative damage (ROS release) with neuronal excitotoxicity, thus directing neurons towards a degenerative path, thus representing a key factor in the pathogenesis of PD. This alteration of calcium homeostasis is mainly linked to an alteration of the expression or function of CaBPs, thus interrupting this equilibrium of calcium ion concentration, causing neuronal dysfunction and therefore death. Furthermore, it has been demonstrated that Calcium binds α-syn, regulating its secretion and formation of protein aggregates[211] and consequently, α-syn can influence Calcium signalling.

1.4.2. PD's involved molecular pathways

From a molecular point of view, several pathways contribute to both the onset and progression of PD, some of which have already been mentioned above, such as the nigrostriatal pathway, which is characterized by degeneration of dopaminergic neurons in this portion of the CNS, responsible of motor deficits, tremors, bradykinesia and rigidity. Another pathway mentioned is the α-syn pathway, represented by the misfolding and aggregation of this protein into Lewy bodies into dopaminergic neurons, disrupting cellular homeostasis. The mitochondrial pathway, characterized by PINK1 and Parkin mutations in genes, is responsible for the reduction of ATP production and increase of ROS levels and initiating the apoptotic. Another important pathway involved in the progression of PD is represented by neuroinflammation through microglia and astrocyte activation, which release pro-inflammatory cytokines and chemokines, through activation of NF-κB pathway, production of ROS and nitric oxide, contributing to dopaminergic neuron damage and death. To confirm, studies conducted by post-mortem brain tissues

and from CSF demonstrated elevated levels of pro-inflammatory proteins[212], including IL-1β, IL-2, IL-4, IL6, IL-10, IL-12, IL-13, CCL2 and CXCL1, together with TNF-α, TGF-β, etc, responsible of motor symptoms[212]. Although pro-inflammatory cytokine production plays an important role in PD etiopathogenesis, their participation mechanism remains unclear[62]. Recent discoveries demonstrated how the Sympathetic Nervous System (SNS) can modulate the release of pro-inflammatory cytokines produced by microglia and involved in NDDs. In particular, it has been demonstrated that the adrenergic receptors (α and β receptors) activation regulates the production of pro-inflammatory proteins[62].

1.4.3. Therapeutic approaches in ameliorating PD

Although research is making great strides towards these NDDs, with the development of new therapeutic approaches, there are currently no treatments capable of acting in a targeted way on the degeneration of neurons, definitively blocking the progression of PD. The most common therapeutic approaches aim to relieve symptoms and slow the progression of the disease. It is possible to divide the treatments into different categories, pharmacological treatments and non-pharmacological treatments.

1.4.3.1. Pharmacological treatments of PD

This category includes all treatments that aim to improve dopamine levels by increasing or maintaining constant. Major drugs include Levodopa (L-DOPA)[213], a dopamine precursor capable of crossing the BBB and subsequently being converted into dopamine. Administered in association with Carbidopa[214] or Benserazide to increase its half-life, because it is rapidly metabolized at the peripheral level. Dopamine agonists, such as Pramipexole[215], Ropinirole[216] and Rotigotin[217], are dopamine mimetics used to stimulate dopamine receptors. Generally, these compounds are used either in the early stages of PD or in association with L-DOPA when the disease has already progressed. Entacapone and Opicapone, Catechol-O-methyltransferase (COMT) inhibitors[218], and Selegiline and Rasagiline, inhibitors of MAO-B[219], enzymes responsible for the metabolism and therefore degradation of dopamine. The use of these drugs causes dopamine to be eliminated slowly from the brain, prolonging the effect of both endogenous and exogenous dopamine. Drugs that act mainly on the symptoms, especially tremors, bradykinesia and muscle rigidity, include anticholinergic drugs, such as trihexyphenidyl

and benztropine[220], reduce symptoms such as tremor and rigidity by blocking the action of acetylcholine, but their use is limited due side effects, such as confusion and dry mouth. The use of Amantadine[221] is useful in reducing dyskinesias (involuntary movements) in patients with PD undergoing long-term therapy with levodopa. Amantadine binds to the NMDA receptor, antagonizing it and thus reducing neuronal excitotoxicity from glutamate.

1.4.3.2. No-pharmacological treatments

In the treatment of PD, it is also possible to choose a surgical approach through deep brain stimulation (DBS), at the level of the subthalamic nucleus (STN) and internal globus pallidus (GPi). This type of therapy involves the use of electrodes implanted in specific brain regions, such as those just mentioned, and is used in patients whose disease stage is advanced, reducing symptoms such as tremor, rigidity and motor fluctuation, through modulation of neuronal activity. This type of therapy would represent the last resort for the treatment of PD when drugs are no longer effective or the patient does not respond correctly to pharmacological treatment. In recent decades, research has been focused on the development of targeted therapies, including immunotherapy, which targets α-syn, reducing or preventing its aggregation, through the use of monoclonal antibodies or vaccines. Currently, this type of therapy is still in the clinical trial phase, but they are giving promising results to reduce or stop the progression of the disease. Another promising therapy in the context of Parkinson's is Gene Therapy, which involves the use of Adeno-associated virus 2 (AAV2) to target neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) (AAV2-GDNF)[222] or neurturin (AAV2-Neurturin), as well as the gene for aromatic L-amino acid decarboxylase (AADC) (AAV2-AADC)[223], to specific brain areas to restore dopamine neuron function and survival and/or improve the efficacy of L-DOPA therapy. Finally, another therapeutic strategy, to enhance the treatment of PD, is the one that targets CaBP, given their crucial role in calcium homeostasis. These are either pharmacological treatments, using drugs that improve the function of these proteins or mimic their buffering effect on Ca ions, thus providing a correct Calcium homeostasis, or Calcium agonist drugs, such as Isradipine[224], which blocks calcium channels, reducing the influx into dopaminergic neurons and thus ensuring their survival from calcium excitotoxicity. Gene therapy has also been developed to increase the expression of CaBP, using Calbindin as a target, to improve its

buffering action in dopaminergic neurons of the substantia nigra and protect them from calcium excitotoxicity.

1.4.4. Alzheimer's Disease (AD)

AD is a neurodegenerative disease that occurs in the whole world and is the cause of Dementia (a gradual decline in memory, thinking, behaviour and social skills)[225]. AD begins with mild memory loss and possibly leads to loss of the ability to carry on a conversation and respond to the environment[226]. The main brain parts involved in this pathology are the brain that controls thought, memory, and language[227]. AD is a public health problem affecting the entire world population and represents the seventh cause of death. The WHO reported in the Global Action Plan 2017-2025 that people affected by dementia will reach 75 million people in 2030, compared to 47 thousand people registered in 2015, with an increase to 131.5 million by 2050[228]. As a result, treatment costs will increase to over a trillion dollars per year.

The prevalence of the development of the pathology is different depending on the geographical areas. For example, Europe and North America have been reported to have the highest rate of people with dementia[229], compared to the regions of Asia and Africa, which have significantly lower rates, with an increase in the rate of dementia life quality[230]. AD affects the female population more, with a 2:1 ratio compared to men, at the same age. Although there are conflicting studies on this matter, this does not exclude the possibility that gender is not a risk factor as each sex has some risk factors determining the development of AD[231].

1.4.4.1. Etiopathogenesis

AD causes a slow and progressive loss of brain function, causing degeneration of brain tissue and neuronal cells[232]. Like PD, AD is a multifactor disease, characterized by various factors that contribute to the progression of the disease. However, the causes triggering AD are not entirely clear[233]. The main risk factor is represented by genetics, in which 5-15% of the disease is hereditary, involving some hereditary genetic anomalies. The onset of the disease is mainly linked to the type of genetic mutation. This genetic landscape includes deterministic (causal) mutations, rare but directly triggering the disease. For early-onset AD (EOAD)[234], the mutation involved is autosomal dominant, meaning that a single copy of the mutated gene can cause the disease. These mutations

lead to the overproduction of β-amyloid (Aβ), a protein that accumulates to form plaques in the brain, a hallmark of AD. The mutation of the amyloid precursor protein (APP) gene[235], encoding the amyloid precursor protein, is responsible for the increased production of the Aβ-42 isoform of the Aβ peptide, leading to the early formation of plaques. Presenil 1 and 2 (PSEN1 and PSEN2)[236] belong to the γ-secretase complex, an enzyme that cleaves the APP protein to produce Aβ. Mutations in this gene alter the enzyme activity leading to increased production of Aβ-42. PSEN1 mutations are very common in familial AD with early onset and aggressive progression, while, PSEN2 mutations are rarer, with slightly later onset, but still early compared to the characteristic age for the development of AD. In late-onset Alzheimer's disease (LOAD)[237], mutations in genes are responsible, the mutation of which does not directly cause the development of the disease, but affects the probability of developing it, following the influence of certain factors, such as genetic, environmental and lifestyle factors. These genes include apolipoprotein E (apo E)[238], a protein component of some lipoproteins that transport cholesterol into the circulation. Depending on the type of mutated ApoE it is possible to distinguish the onset of AD:

- *Epsilon-4*: Early and frequent development of AD
- *Epsilon-2*: Immunity to AD
- *Epsilon-3*: Neither protection nor development tendency of AD

The TREM2 gene[239] (myeloid cell-expressed activation receptor 2) can also be subject to mutation. This gene is expressed in microglia and is physiologically involved in the immune, inflammatory and phagocytic response. Mutations in this gene compromise the normal function of microglia, thus reducing the clearance of Aβ and stimulating neuroinflammation to a greater extent. Genome-wide association studies (GWAS)[240] are underway that have identified the presence of additional genes, whose mutations can increase the risk or directly develop AD.

1.4.4.2. Aβ cascade hypothesis

Aβ plays a central role in the pathophysiology of AD. Generally, Aβ is a peptide obtained from the cleavage of the APP protein[241], a transmembrane protein expressed in many tissues, including the brain. APP can be cleaved through two different pathways, the first involves a non-amyloidogenic pathway, in which APP is first cleaved by α-secretase,

which cuts specifically at the Aβ region, preventing the formation of Aβ; the products obtained from this cleavage are not toxic to the health of the CNS. APP can also take the amyloidogenic pathway, where it is cleaved by β-secretase (BACE1)[242] producing a soluble fragment (sAPPβ), which is subsequently cleaved by γ-secretase, with the release of Aβ peptides, of different lengths, and of these products obtained, those most susceptible to aggregation are 40 and 42 amino acids long (Aβ-40 and Aβ-42), with particular attention to Aβ-42[243,244]. At this point, the formed Aβ-42 peptide undergoes misfolding, first forming small soluble oligomers, which are thought to be the most neurotoxic form, disrupting synaptic plasticity. Subsequently, neurofibrillary plaques[238] are generated and accumulate in the hippocampus and cerebral cortex, areas of the brain responsible for memory and cognition. Aβ plaques[245] are then formed by dystrophic neurites, activated microglia, and reactive astrocytes, thus contributing to neuronal death.

1.4.4.3. Amyloid-Tau cascade hypothesis

Along with the APP protein, another protein directly involved in the development of Alzheimer's is represented by the Tau protein, which is responsible for a series of pathologies called Tauopathies[246], including Alzheimer's. The tau protein is expressed mainly in neurons[247] and is the major microtubule-associated protein (MAP) of a mature neuron[248]. Its function is to stabilize microtubules, structures responsible for maintaining the shape of the cell, allow adequate intracellular transport and provide support to the architecture of axons and dendrites. Under normal conditions, tau protein activity is regulated both by the degree of its phosphorylation, ensuring an adequate balance for correct neuronal function, and by alternative splicing of tau pre-mRNA, producing six soluble isoforms of this protein[249], different in the number of aminoacids. In the presence of CNS disorders, such as AD, an abnormal hyperphosphorylation of the tau protein occurs, characterizing a series of CNS pathologies called tauopathies[248], including frontotemporal dementia, Pick's disease, corticobasal degeneration, dementia pugilistica, and progressive supranuclear palsy, as well as AD. In particular, in AD, all six tau isoforms are abnormally hyperphosphorylated[250], and present in three distinct states, soluble, oligomeric and fibrillated[251], with a prevalence of the oligomeric state (up to 40%) and sedimented. This hyperphosphorylation therefore determines conformational changes, especially of oligomeric tau, which leads to aggregation into paired-helical filaments (PHF)[252] and subsequently to the formation of intracellular neurofibrillary tangles (NFT)[253,] within neurons of the hippocampus and cerebral cortex, and are

abundant in the brain of AD patients. In the context of AD, the two main protagonists are the aggregation of Aβ plaques and abnormal hyperphosphorylation resulting in the formation of insoluble neurofibrillary tau protein. The interesting correlation between Aβ and tau protein has led to the development of a theory, called the "A*myloid-Tau cascade hypothesis*"[254], according to which the accumulation of Aβ leads to hyperphosphorylation of tau and the formation of NFTs, through activation of kinases responsible for hyperphosphorylation of tau protein or disruption of cellular processes responsible for Tauopathies. Therefore, the presence of both Aβ plaques and tau tangles in neurons determines a more aggressive progression of AD. Additional risk factors, although common with other conditions, include high blood pressure, diabetes, high cholesterol levels and smoking. At the brain level, a low level of Acetylcholine (ACh), a neurotransmitter, that in physiological conditions is involved in memory, learning and concentration, has been highlighted in Alzheimer's subjects.

1.4.4.4. AD's involved molecular pathways

AD is a complex neurodegenerative disorder involving numerous interconnected molecular pathways that lead to the hallmarks of the disease, including Aβ plaques, neurofibrillary tangles, neuroinflammation[255], synaptic dysfunction, and ultimately neuronal death. Most of the molecular processes directly involved in the development of AD have just been described, including the amyloidogenic pathway, through the formation of Aβ-42 plaques[256], hyperphosphorylation of the tau protein[257], with the formation of insoluble neurofibrillary tangles. Another important molecular process that contributes to the progression of AD is neuroinflammation, especially a chronic neuroinflammation response[255], which seems to have an emerging role in neurodegeneration[258,259]. As with PD, also in AD, the prolonged inflammatory state favours the release and therefore increase of pro-inflammatory cytokines and chemokines[260] (IL-1β, IL-6, IL-18), harmful to the health of neurons. A high concentration of these pro-inflammatory mediators determines an alteration of mitochondrial functionality, with increased ROS[215,216] production and weakening of the BBB[261,262]. This entire process is favoured in turn by the presence of Aβ plaques formed inside the neurons. Furthermore, low levels of the neurotransmitter 5HT (Serotonin)[263], DA (Dopamine)[264] and NE (Noradrenaline)[265] and their respective receptors have been

reported in AD subjects, which are mainly responsible for cognitive disorders such as depression, anxiety[266], insomnia, learning and memory[255,267].

1.4.4.5. Cyclic adenosine monophosphate (cAMP) pathway

An important role in the CNS is represented by cyclic Adenosine monophosphate (cAMP). cAMP is a ubiquitous second messenger formed from intracellular Adenosine Triphosphate (ATP) by Adenylate cyclase enzyme (AC) and degraded by Phosphodiesterases (PDEs)[268–270]. Many stimuli activate cAMP, such as hormones and neurotransmitters[271], such as adrenaline, dopamine and glucagon, which bind AC receptors, such as G protein-coupled receptors (GPCRs) present on the cell membrane[272], translating the extracellular signal into an intracellular response, such as metabolism regulation, gene expression and others[273]. More specifically, these first messengers[274], once bound to their specific GPCRs, determine their conformational change and subsequent interaction with an adjacent G protein activating it[275]. The latter will bind AC, which enzyme is responsible for converting ATP into cAMP, increasing the level of the latter[276]. The biological effects of this nucleotide are manifested through the activation of a family of enzymes known as protein kinase A (PKA)[277,278] which can phosphorylate other enzymes, structural proteins and/or transcription factors, present on the membranes or inside the cells, thus evoking the appropriate cellular response[269].

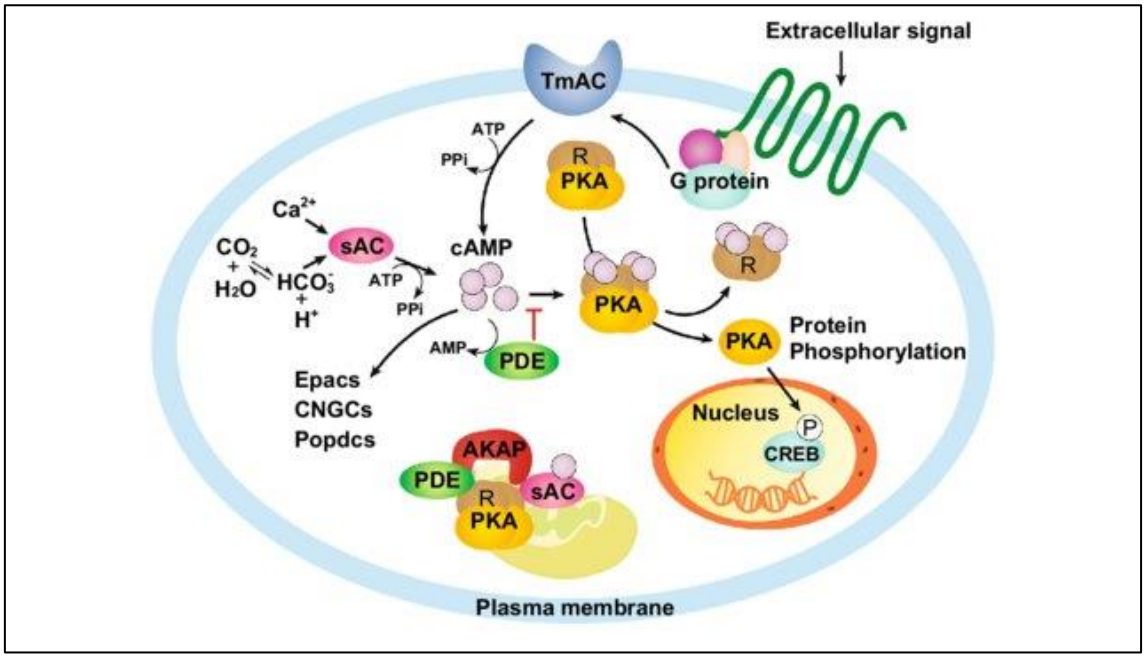

Figure 8. cAMP signalling pathway [279]

The final effect of cAMP production is not the same in all cell types but differs by the GPCR subtype present in the target cell and the specific targets of PKA present within the cell. Therefore, cAMP signalling is said to be compartmentalized[280,281], precisely because it can lead to different outcomes between different cells. This is due to the presence of A-kinase anchoring proteins (AKAPs)[282–284], that localize PKA to specific subcellular regions[277]. All this translates into a faster and more specific response to signals transmitted by first messengers, thus regulating a wide range of physiological processes such as metabolism, cardiac function and neural activity.

It has been reported that the brain contains high levels of AC and it is present in both types of cells, neurons and glial cells[285]. In the brain, cAMP has a pivotal role: in learning and memory formation[282,286], mood regulation, neuronal function, synaptic plasticity and overall brain health[282]. More specifically, cAMP is involved in gene expression[287]: cAMP activates PKA, which phosphorylates cAMP Response Element-Binding Protein (CREB)[288], which binds to cAMP response elements (CRE) in the DNA, promoting the transcription of genes[289] (such as brain-derived neurotrophic factor, BDNF) involved in neuron survival, differentiation and synaptic plasticity[289]. Also, a study reported that the cAMP-CREB cascade could influence the morphological maturation of newborn neurons in the adult hippocampus[290]. Moreover, another role of cAMP includes the modulation of the release of neurotransmitters in presynaptic space, controlling the responsiveness of postsynaptic neurons to neurotransmitters[291]. cAMP is involved in neurogenesis processes[292], influencing the development of specific neuronal types and the formation of neuronal circuits during brain development. Moreover, it exerts an anti-apoptotic effect promoting neuronal survival[293,294], modulating the inflammatory response[295] and its levels increase in response to neuronal damage[296], activating the repair mechanism and promoting regeneration and reducing neurodegeneration[295].

In the main CNS disorders, such as NDDs, there is a dysregulation of cAMP signalling[297] and its abnormal levels might impair synaptic plasticity, neurodegeneration and cognitive deficits[298]. While, in the context of AD, cAMP dysregulation can affect the activity of PKA, involved in the phosphorylation of tau protein, which is hyperphosphorylated[299] forming neurofibrillary tangles. Furthermore, this leads to the accumulation of β-amyloid promoting plaque formation and causing neuronal death[300].

1.4.5. Current AD treatments

Even for Alzheimer's, there is no treatment able to act specifically at the level of damaged neurons, definitively stopping the progression of the disease[301]. Nowadays, research is mostly focused on slowing the progression and symptoms of the disease, but not on curing the patient with Alzheimer's[301.]

1.4.5.1. Pharmacological approaches

The first drugs developed involved the use of cholinergic inhibitors, including Donepezil[302], Galantamine[303] and Rivastigmine[304]. These drugs targeted Acetylcholinesterase, an enzyme responsible for the metabolism of the neurotransmitter Acetylcholine, present in the cholinergic neurons of the CNS, involved in learning and memory processes[305–307]. The use of these inhibitors improves attention in AD patients[301] by restoring the cholinergic pathway in the synaptic space and thus reducing ACh hydrolysis. Unfortunately, their use is limited both because they act only at the symptomatology level, improving it, and because they are poorly tolerated by the patient.

Subsequently, agonists of the glutamate neurotransmitter receptor, NMDA, were synthesized, which is also involved in memory and learning and whose excessive quantity is toxic to neuronal health. Among these, the best known is Memantine[308], whose action consists of reducing abnormal brain activity, thus helping to optimize or slow down memory loss. Drugs that target neuroinflammation are also useful in the treatment of AD. One example is the compound AL002[309], which targets the myeloid cell-expressed activation receptor 2 (TREM2). It was synthesized to modulate the activity of microglia, enhancing them, brain immune cells, reducing neuroinflammation, and improving the ability to clear Aβ plaques and neurofibrillary tangles. Rapamycin [310], for example, induces autophagy to clear Aβ and tau aggregates from neurons, reducing cellular stress and, in turn, slowing neurodegeneration. Significant progress has been made in recent years in research, obtaining promising results through the development of immune and gene therapies[311], using monoclonal anti-amyloid antibodies, which specifically target Aβ plaques. The most well-known monoclonal antibodies, including Aducanumab and Lecanemab[312], are designed to help the immune system recognize, bind to, and remove amyloid aggregates from the brain, thereby slowing the progression of Alzheimer's disease. Vaccines have also been developed, such as ACI-24[313], which stimulates the

immune system of the patient with AD, to produce antibodies against Aβ. Along with gene therapy targeting Aβ plaques, specific gene therapy for the tau protein has also been developed, through the development of monoclonal antibodies such as Gosuranemab[314] and Tilavonemab[315], which bind to extracellular tau, preventing its diffusion and therefore the formation of insoluble tangles inside neurons. In addition to gene therapy, genome editing using CRISPR/Cas9[316] has also been developed, which is still being tested to modify genes associated with AD, such as those involved in the production of Aβ or hyperphosphorylation of tau. Another new approach consists of gene silencing with siRNA[317] and ASO[318]. These are small interfering RNAs (siRNA) and antisense oligonucleotides (ASOs) that are effective in silencing genes involved in the production of Aβ or tau, targeting the mRNA of the proteins, reducing their levels in the brains of AD patients.

1.4.5.2. Novel Optogenetic Approaches

To date, pharmacological agents have poor cell type selectivity, resulting in their inability to interfere with molecular signalling pathways in AD. Therefore, the research is focusing towards new selective approaches on pathways mainly involved in AD, such as the cAMP pathway, allowing new non-pharmacological tools have been developed, such as the optogenetic model with Photoactivated Adenylyl Cyclase (PAC)[319–321], capable of better understanding the role of these pathways in the disease. Photoactivated Adenylyl Cyclase (PACs) are a class of proteins activated by light and catalyse the conversion of ATP in cAMP[320,322]. The PAC activation under light stimulation allows the control of cellular functions and the cAMP levels within cells [323].

Briefly, PAC is composed of a light-sensing domain, a flavin adenine dinucleotide (FAD)-binding, able to absorb blue light (λ 450 nm), with conformational changing activating the catalytic domain[324], which this latter converts ATP to cAMP. Most of the currently available PACs are activated by blue light, which is abundantly absorbed by animal tissues due to the presence of two compounds, flavins and porphyrins, producing unwanted side effects[325]. Furthermore, it has also proven to be advantageous for use in association with genetic and pharmacological tools that target components of the cAMP pathway[326], as the combination between PAC and the Adeno-Associated virus (AAV)[327]. AAV is a small and nonenveloped single-stranded DNA (ssDNA) virus belonging to the *Dependovirus genus* of the *Parvoviridae* family[328]. AAV is a non-pathogenic vector, with

high infection efficiency and long-term expression, both in dividing and non-dividing cells and is well tolerated by the immune system. This method has been known and well-established for some time and the use of AAV to express light-sensitive proteins, such as opsins, in specific brain areas[329], has had positive outcomes for human treatments. This study used AAV serotype 9 (AAV9), a common vector choice for gene therapy[330] and its targets are represented by neurons and astrocytes[331]. The use of AAV9 technology has been well established for some time and is the first FDA-approved for the treatment of spinal muscular atrophy (SMA), a genetic disorder characterized by loss of motor neurons[332]. Therefore, the development of transgenic PAC has proven to overcome the obstacle of cell no-selectivity of the pharmacological agents, both because it is expressed under the control of cell type-specific promoters, and because under light stimulation, it increases cAMP synthesis, offering a cell-selectivity.

1.4.5.3. Deinococcus deserti Photoactivated Adenylyl Cyclase (DdPAC): A Novel PAC Variant

Research on several PACs is ongoing, with potential application in developing novel therapeutic strategies and advancing the field of synthetic biology. Therefore, while the use of blue light to stimulate the activation of opsins has a high probability of causing harmful effects due to the high intensity of blue light, the use of red light has been shown to have a reduced harmful effect[333]. This study focused especially on *Deinococcus deserti* Photoactivated Adenylyl Cyclase (DdPAC)[321], which refers to a specific PAC, identified for the first time in *Deinococcus deserti* is a Gram-negative, extremely radio-tolerant and exhibits an ability to withstand ionizing radiation[334]. Moreover, DdPAC exposed to red light (λ 660-760 nm)[335], catalyses the conversion of ATP to cAMP (Figure 9), controlling the levels of cAMP within cells and tissues, and providing insights into the role of cAMP in various neuronal processes, such as the study of neuroplasticity, modelling diseases (AD, HD, etc).

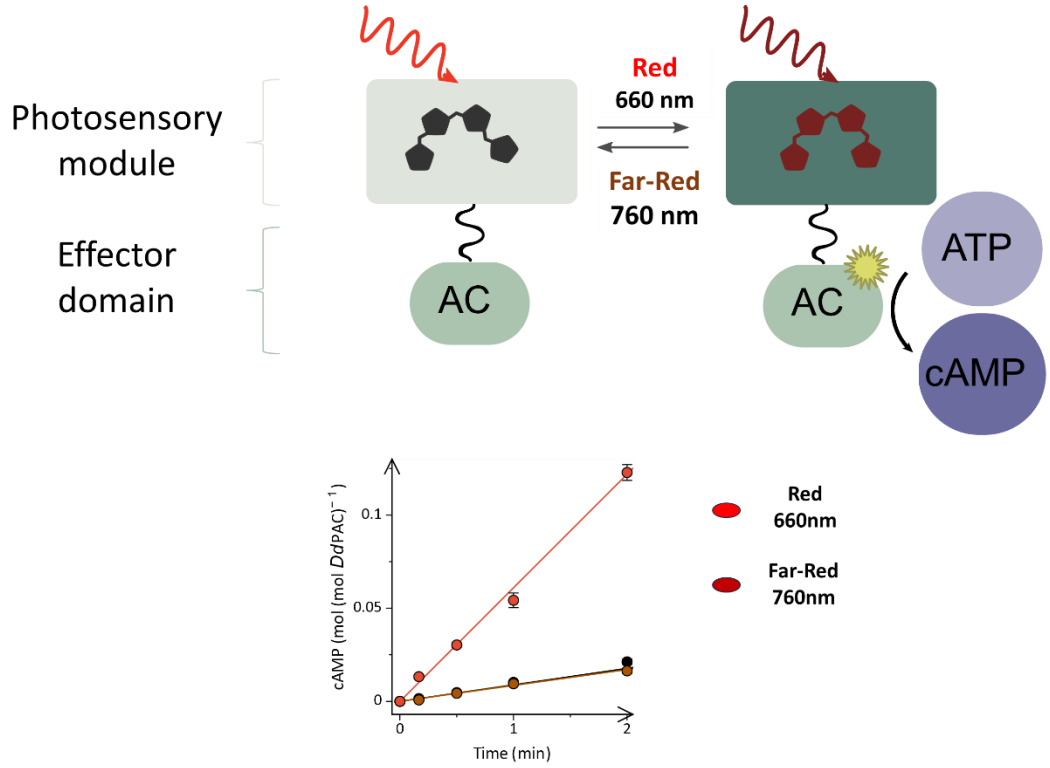

Figure 9. The exposition of DdPAC under red light catalyses the conversion of ATP to cAMP[321]. Retrieved from https://app.biorender.com/biorender-templates

Evidence reports a correlation between DdPAC activity and cAMP levels influencing the organism's behaviour and developmental processes. The activation of DdPAC with red light is instantaneous, allowing a temporal control of the cAMP production[319]. When the red light is turned off (end of stimulus), the FAD domain returns to its original shape by stopping the cAMP production (Figure 9). Therefore, based on the long-established optogenetic technique, which generally involves the expression of light-sensitive proteins such as opsin, this study used similar and innovative strategies using phytochromes, such as DdPAC, for the selective stimulation and spatial-temporal control of proteins in specific cells and brain regions, modulating their activity using red light. The use of phytochromes in neurons and glial cells might provide unprecedented insights into the role of cAMP in various brain processes, such as the study of neuroplasticity and modelling diseases (AD, HD, etc). The use of phytochromes to modulate brain cells is fairly recent and limited to blue PACs[307]. Thus, the implementation of DdPAC, activated by red light, offers better options for its use as a valid therapeutic agent for neurodegenerative disorders, such as AD.

CHAPTER 2: FIBROBLAST GROWTH FACTORS (FGFs) ROLES IN NEURODEGENERATION

Fibroblast growth factors (FGFs) are a family of growth factors involved in a variety of biological processes, including cell growth, tissue repair, angiogenesis and neuronal survival. They were discovered in pituitary extracts in 1973 and they are expressed in cells and tissues of invertebrates and vertebrates[336]. In general, FGFs are polypeptides and consist of 150-300 amino acids. It is possible to group the FGFs based on their functions:

- *Paracrine FGFs*: Development and repair processes and act locally.
- *Endocrine FGFs*: regulation of metabolic processes and their function is systematically
- *Intracrine FGFs*: participation in intracellular signalling and act inside the cells:

It was initially identified as a protein capable of promoting the proliferation of fibroblasts[337]. The FGF family comprises 23 members (Figure 10) and many FGF genes have been identified in vertebrates, such as Acidic FGF (FGF1) and basic FGF (FGF2)[338], which were first isolated from the brain and pituitary gland as growth factors for fibroblasts[339]. The other ones are FGF4, FGF6, FGF8, FGF10, FGF17a, FGF17b, FGF18, FGF24, etc.[340] (Figure 10). All FGFs exert their effects by binding to receptors called FGF receptors (FGFRs). In particular, there are four FGFRs, FGFR1-FGFR4, and each of them has a different isoform[341].

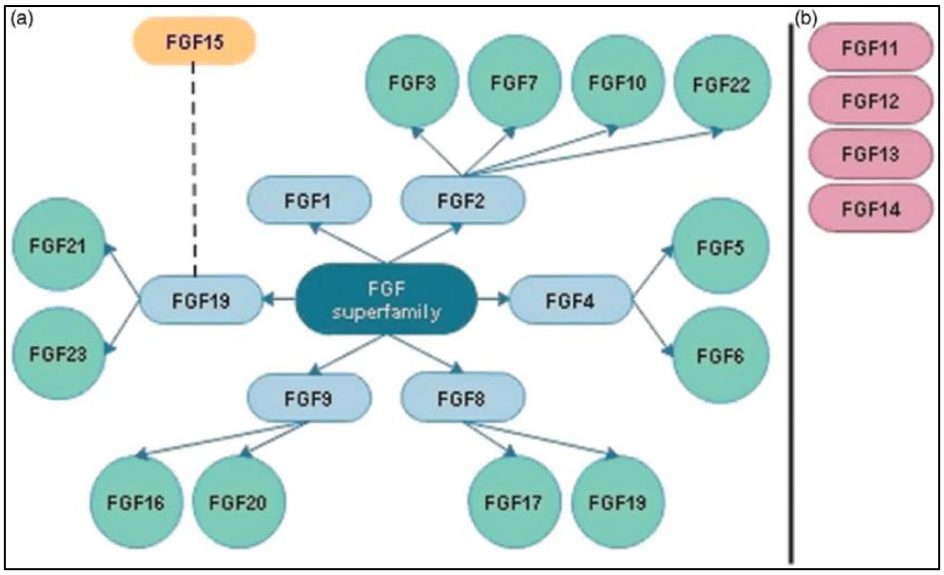

Figure 10. Fibroblast growth factor (FGF) family and subfamily[342]

Each of these FGFs is involved in different roles, for example, FGF2 and FGF20 exert a neuroprotective role, while FGF21 can intervene in neuroinflammation. These FGFs are involved in PD, directly or indirectly. FGF2, together with FGF20 supports neuronal survival, by reducing oxidative stress. FGF20 is expressed in dopaminergic neurons of the substantia nigra, and other brain regions. It ensures neurons' correct functionality and maintenance, protecting them from the persistent presence of toxins and promoting neuronal regeneration. Finally, FGF21 is known metabolic regulator of energy homeostasis, glucose metabolism and insulin sensitivity. It has been shown to have anti-inflammatory properties, thus alleviating CNS neuroinflammation, antioxidant properties and reducing ROS production. Furthermore, it improves mitochondrial function and energy metabolism, improving systemic metabolic health and therefore offering benefits for brain health.

Of these three FGFs, the one of greatest interest in this study is Fibroblast Growth Factor Basic (bFGF), also known as FGF2, which is a member of the fibroblast growth factor family[343] and plays a critical role in various biological processes, particularly in cell growth, tissue repair, and development. From the structural point of view, bFGF is a single-chain polypeptide that typically consists of 146 amino acids, although isoforms with additional N-terminal extensions exist due to alternative initiation of translation. It is known for its heparin-binding properties, essential for its biological activity[344]. bFGF contains 12 antiparallel β-sheets constituting a trigonal pyramidal structure. In this regard,

studies highlight those residues 13–30 and 106–129 represent the binding sites of the receptors[345,346], FGFRs, of which 4 types have been identified (FGFR1-FGFR4). The FGFRs are the Tyrosin-Kinase receptors binding to the membrane (RTK)[347,348], and are encoded by separate genes, but the four receptors share a high level of homology[349]. Structurally, they are three different domains, highly conserved, an extracellular ligand binding domain, a transmembrane domain and an intracellular domain, that interact with cytoplasmic molecules and transduce FGFR signalling[350,351]. Each domain binds a specific ligand, the extracellular domain can bind FGF ligands, heparan sulfate (HS) and extracellular matrix molecules, acting as a scaffold and allowing receptor binding of specific FGFs[352,353]. Through alternative splicing that codes for the extracellular domain it is possible to obtain many isoforms of FGFR, thus modifying the affinity and sensitivity towards FGF ligands[354–356]. The presence of the transmembrane domain serves to transfer the signal from the extracellular domain to the intracellular one[357,358], which is made up of a juxtamembrane domain, two Tyrosine kinase domains and the C-terminal tail[358]. All FGFRs, depending on the tissue and cell type on which they are expressed, have distinct functions, they are involved in both biological processes[359], such as the development and maintenance of tissues[360,361], and the progression of diseases, such as cancer[362,363], metabolic diseases, etc. As initially mentioned, FGFs are the native ligands for FGFRs, and their activity differs depending on the tissue on which these receptors are expressed. In the context of CNS and bFGF, among the four FGFRs, the one with which bFGF exerts a neuroprotective activity is represented by FGFR1.

The interaction between bFGF and FGFRs determines the conformational change of FGFRs, going from monomeric form to dimeric form[364,365], activating multiple signal transduction pathways[361] (Figure 11), which induce the expression of specific genes related to the physiological activity of cell proliferation, survival and degradation of FGFR[356], as will be reported below. Briefly, once activated and phosphorylated FGFR1, it determines the recruitment and also phosphorylation of PLCg leading to the hydrolysis of PIP2 (Phosphatidylinositol Biphosphate) to IP3 (Inositol triphosphate), important for calcium release and cytoskeletal regulation[366] (Figure 11). Furthermore, the RAS-MAPK (mitogen-activated protein kinase) pathway, which is important for proliferation activity, is also activated, as is the PI3K/AKT pathway[365] for survival[347,366] (Figure 11).

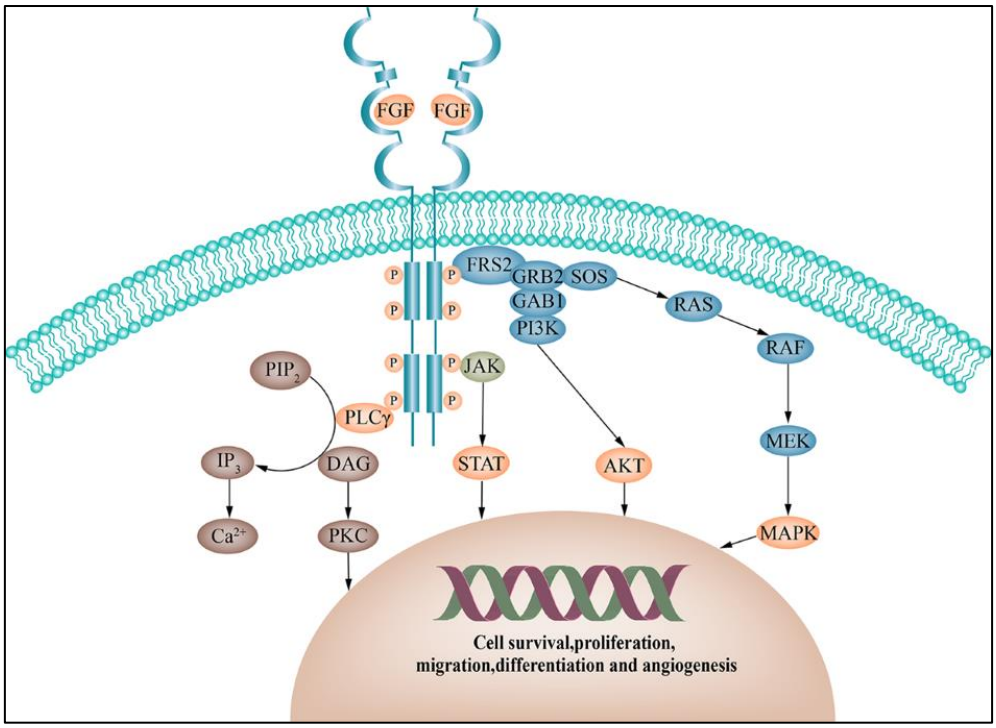

Figure 11. FGFR activation upon binding to FGF [367]

2.1. bFGF/FGFR signalling in CNS

The mechanism of action of bFGF involves several key steps, beginning with interaction with its FGFRs that bring to the activation of signalling pathways. As mentioned, there are four FGFRs, each of them with specific isoforms, but all with high affinity towards bFGF. This bind could be influenced by various stimuli, modifying the ligand-receptor binding specificity[368] and the crosstalk between bFGF/FGFRs[369]. Several mechanisms regulate the bFGF/FGFRs signalling, especially their temporal and spatial expression and/or orientation[370,371] Moreover, different tissue distribution and expression levels influence the function of bFGF and, as a consequence, tissue development and maintenance[360,372]. Furthermore, phosphorylation[373], glycosylation[374] and cellular trafficking are involved in the regulation of bFGF/FGFRs signalling, influencing the signal specificity and intensity[375,376].

In this regard, an important transmembrane receptor group, Cell adhesion molecules (CAMs), modulates the bFGF/FGFRs signalling[365]. In this group belong cadherins, integrins, the Ig superfamily of CAMs (IgCAMs) and the superfamily of C-type of lectin-like domains proteins[377]. Not only, because also GPCRs[378] , especially in the NCS, influence the bFGF/FGFRs signalling[379], for example, regulating the proliferation,

migration, survival and differentiation of neurons[380]. In the CNS, in physiological conditions, bFGF plays an important role in brain development, maintenance and tissue repair; influences also the synapse and the astrocytes and microglia activities[381] and axons growth. Evidence showed that FGFR1 and FGFR2 work in synergism with this growth factor, inducing neural tissue regeneration using planarians and vertebrate embryos[382] and the zebrafish retina as models[383].

The bFGF activity is important during embryonic development, for the proliferation and differentiation of neural progenitor cells into neurons and glial cells[384]. With interaction with its FGFR1, it influences the expression of other growth factors involved in neural development[385]. While, in the adult brain, bFGF promotes neurogenesis in the hippocampus region, important for memory and learning, supporting the proliferation of neural stem cells in the dentate gyrus, an important zone of the hippocampus[386], and the generation of neurons that migrate to the olfactory bulb. In the same way, bFGF influences neural growth and differentiation, it can enhance synaptic plasticity, modulating synaptic transmission and network activity[387]. In this regard, bFGF exerts neuroprotective effect on CNS damaged inducted by TBI, neurodegenerative disease and ischemia and/or stroke[388], inhibiting apoptosis response and promoting the proliferation and migration of glial cells and repairing tissues[389]. Furthermore, bFGF reduces oxidative stress, upregulating antioxidant response[390], and controls neuroinflammation through modulation of the astrocytes' activation, supporting thus neuronal survival[391].

There is scientific evidence that the neuroprotective activity of bFGF is strictly related to intracellular Ca^{2+} metabolism and Ca-binding proteins[341], such as Calb[392], Calpain, S100-β. Among these proteins, Calb is mainly involved in maintaining Ca^{2+} homeostasis[393]. Both Calb and bFGF have a protective action on neurons, especially in NDDs and conditions such as excitotoxicity, causing neuronal death. The activity of Calb consists in maintaining intracellular Ca^{2+} levels, ensuring its homeostasis, and thus protecting neurons from Ca^{2+} ion overload, which can cause cell death by excitotoxicity[394]. While bFGF exerts its neuroprotective effect by regulating the processes of neurogenesis. bFGF interacts with Ca-proteins at the level of glial cells, thus influencing how they govern Ca^{2+} within them. In this context, an important role is always played by Calb, which protein interacts with bFGF at the level of glial cells, ensuring homeostasis and the correct concentration of intracellular Ca^{2+} [395]. Following neuronal damage, such as NDDs or TBI,

etc., the levels of bFGF and Calbindin-D28k are altered, resulting in increases in the brain regions of interest, triggering mechanisms of protection and neuronal survival[396]. Some studies report how the increase in bFGF determines the increase in Calb concentration following neuronal damage[397]. Especially in the context of NDDs, low levels of Calb[398] have been observed in the initial stages of these diseases, making neurons more susceptible to Ca-mediated damage. While, it is well established that in NDDs, bFGF promotes cell survival and repair processes[399]. Therefore, both Calb and bFGF act through complementary mechanisms, as a sort of synergism, where bFGF supports Calb activity in maintaining calcium homeostasis, thus counteracting neuronal death and thus offering cell repair and survival.

Considering that bFGF plays a crucial role in the CNS, it is obvious that its alteration or dysregulation is related to CNS disorders, such as NDDs and Psychiatric disorders, such as Schizophrenia[400,401].

2.2. Therapeutic use of bFGF

The remarkable ability of bFGF to act in different pathological contexts means that it can be used in different clinical conditions[402].

2.2.1. Application in CNS

Known for its neuroprotective properties, bFGF has been widely used in the treatment of neurological conditions[338], such as stroke[339], spinal cord injury (SCI)[340], TBI[341], and neurodegenerative diseases, although studies to date have focused only on PD[328] and AD[342]. In the SCI context, A study was conducted in which rats with moderate spinal cord injury were treated with bFGF and recovered (within 2 to 6 weeks) motor deficits that had developed after SCI[343]. Still, in the context of its neuroprotective effect, another *in vivo* study has instead demonstrated that bFG administered as pretreatment can play a neuroprotective role in the initial phase of mild head trauma, reducing the cerebral edema formed following the trauma[344].

2.2.2. Roles of bFGF in PD and AD

As mentioned, PD is a neurodegenerative disease, characterized by a progressive neuronal cell loss in the substantia nigra and on the base[403]. Nowadays, the studies on bFGF have increased and all of them proved that the bFGF/FGFRs axis plays a crucial

role in the neurogenesis of PD[361]. In this regard, an *in vivo* study[404] showed that reactive astroglia induced by 6-hydroxydopamine (6-OHDA) bring to increase of bFGF, which regulated dopaminergic neurons and nigrostriatal pathway[405], enhancing neuronal survivability[406,407]. Moreover, numerous *in vitro* and *in vivo* experiments demonstrated that bFGF exerts a protective role for dopaminergic neurons, in the nigrostriatal zone[408], subjected to toxin damage by 6-OHDA[406] and/or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[409]. To confirm the importance of bFGF in PD and its involvement in survival and plasticity, another study used bFGF-deleted mice showing a decrease in dopaminergic neuron survival after 6-OHDA injection[403,406].

Concerning AD, the accumulation of Aβ plaques and neurofibrillary tangles may involve bFGF[410]. In this regard, the first studies on bFGF in AD were conducted on rodent brain sections, demonstrating how neurofibrillary tangles and Aβ-plaque present sites of binding for bFGF[411]. Moreover, these studies showed that in the dentate gyrus, there was a stronger bFGF immunoreactivity in plaques, suggesting that bFGF plays an important role in plaque biogenesis[410]. In the same way as PD, also for AD bFGF exerts a neuroprotective effect reducing Aβ accumulation[412] and modulating Tau phosphorylation[413,414] . Following brain damage AD induced, bFGF promotes synaptic plasticity, enhancing and supporting neurotransmitter release and countering synaptic loss[415]. These two neurodegenerative diseases have in common the mechanism of action of bFGF. To exert its neuroprotective effect bFGF, through interaction with FGFRs, actives the MAPK/ERK pathway, the PI3K/AKT pathway and the PLCg pathway, as before mentioned, and each of them contributes to cell survival, helping to protect neurons from degenerative processes[403].

2.2.3. Vascular application

Given its angiogenic activity, bFGF has found use in cardiovascular diseases, enhancing blood flow following ischemia and stimulating the formation of new blood vessels. Studies conducted on animals[416,417], bFGF has been shown to improve myocardial perfusion, offering cardio protection during ischemia-perfusion injury. More in details, the study was conducted on a murine model ex vivo FGF2 transgenic (Tg) mice of ischemia/reperfusion injury[417], demonstrating that cardiac-specific overexpression of FGF2[418], through downstream involvement of the MAPK signalling pathway,

significantly improved recovery of contractile function and reduced infarct size following ischemia-reperfusion injury[417].

2.2.4. Oncology application

In oncology, the role of bFGF has a boomerang effect: on the one hand, it is involved in processes that promote tumour growth, such as proliferation, angiogenesis and metastasis, as it is secreted by tumour cells themselves[419], on the other hand, it is used as a supportive therapy to counteract the side effects of chemotherapy treatments, such as tissue damage caused by radiation[420]. The dualistic behaviour of bFGF in oncology represents a major research challenge, where in-depth clinical studies are needed to balance its two activities. In cancer therapy, the signalling pathway modulated by bFGF/FGFR1 would represent a molecular target for bFGF/FGFR inhibitors, which exert an antiangiogenic action[421].

2.3. Therapeutic limitations of the use of bFGF

Despite the countless therapeutic applications of bFGF due to its biological activities, unfortunately its use, as an exogenous treatment, presents several limitations due to the activities it exerts. Regarding the proliferative activity, although it is exploited in wound healing, bFGF can cause excessive tissue growth even causing fibrosis, compromising the function of organs and tissues. Its therapeutic limitation is due not only to the activity exerted, but also to its pharmacokinetic profile. In fact, in this context, bFGF has a very short half-life, limiting the therapeutic efficacy. This translates into an increase in the frequency of administration, with the possibility of increasing the dose of administration to maintain the efficacy of the pharmacological effect. To bypass this drawback, alternative administration systems are required, such as nanoparticle and/or hydrogel formulations, and prolonged release formulations, however increasing the complexity of its use in therapeutic applications. Since FGF receptors are present throughout the body, this is a limitation regarding its administration, because a systemic administration, for example, produces side effects such as unwanted cell proliferation, angiogenesis, or even inflammation in uninvolved areas. Therefore, a targeted administration of bFGF is difficult. Considering that the treatment involves the exogenous administration of bFGF, this could trigger the activation of the immune and inflammatory response by the organism, which develops neutralizing antibodies, causing allergic reactions and therefore reducing the effectiveness of the drug. The therapeutic limitations of bFGF

concern not only its side effects but also its development and production, because they require a certain cost.

2.4. 4-[[4-[[2-(4-amino-2,3,5,6-tetramethylanilino)acetyl]-methylamino]piperidin-1-yl]methyl]benzamide known as SUN11602

The new synthetic compound, 4-[[4-[[2-(4-amino-2,3,5,6-tetramethylanilino)acetyl]-methylamino]piperidin-1-yl]methyl]benzamide, named SUN11602, is a novel aniline derivative and has been designed to mimic the biological activity of bFGF (Figure12).

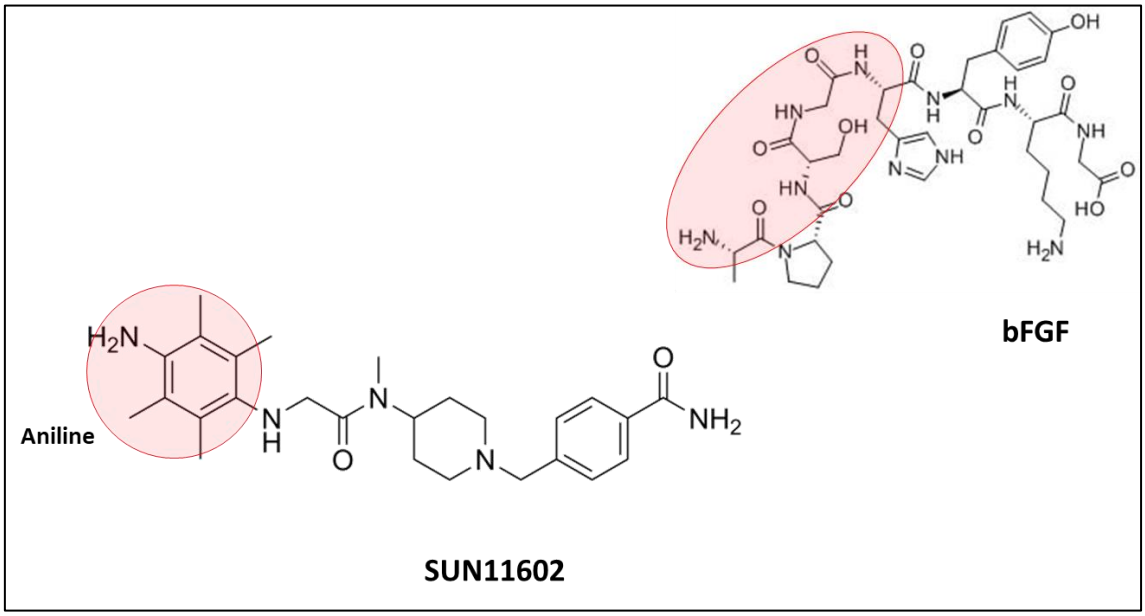

Figure 12. Structure of SUN11602, aniline derivate and mimetic of bFGF.

Pharmacokinetic studies on SUN11602 were performed on rat and dog (beagle) models and in various contexts, about its main use as an antidepressant. These parameters, although some are not publicly available, have given notable results regarding its oral bioavailability, which is greater than 65%, precisely to have a rapid absorption into the bloodstream. Although more in-depth studies are needed regarding its metabolism, it is believed that, like most chemotherapeutics, SUN11602 is metabolized at the hepatic level by CYP450, with a longer half-life than its parent compound bFGF and above all a better penetration of the BBB.

2.4.1. Therapeutic applications of SUN11602

The study on the properties of SUN11602 dates back a few decades, and although more in-depth preclinical and clinical studies are needed to better understand its activity, this

new compound has been studied for its potential therapeutic application in various fields. For example, SUN11602 is believed to act by selectively enhancing the reuptake of serotonin (SSRI), thus falling into the category of antidepressants with greater efficacy and safety. SUN11602 increases serotonin levels by improving its release and reuptake, alleviating the symptoms of depression. In the treatment of depression, SUN11602 is chosen especially when patients do not appear to respond positively to other antidepressants, including SSRIs. However, the use of SUN11602 as an antidepressant is currently at the preclinical stage, because further, more in-depth studies are needed to better understand its efficacy and safety. Not only, acting as an antidepressant on serotonin reuptake, the positive effects of SUN11602 are found in the stabilization of the patient's mood, offering it the opportunity to also be used as an anxiolytic. Therefore, SUN11602 could be used in combination with other antidepressant or psychotropic drugs to improve the treatment in all those patients who are resistant to conventional therapies.

2.4.2. Protective effects of SUN11602 in ameliorating CNS diseases

In this context, the property of SUN11602 to ensure neuronal survival and repair damaged tissues is exploited. In this regard, its use has also been extended to neurodegenerative diseases, in particular PD and AD. In particular, SUN11602 can reduce the neuroinflammatory process that characterizes these CNS pathologies, reducing oxidative stress and offering neuronal survival as it reduces apoptosis. Although further preclinical studies are needed, SUN11602 appears to be a promising candidate for adjunctive therapy in these diseases. Its neuroprotective activity is not confined only to neurodegenerative diseases, but to all those CNS disorders that involve neuronal damage with subsequent cell death. In particular, preclinical *in vivo* studies have been conducted in which the ability of SUN11602 to restore neuronal activity following subacute SCI has been tested.

Although, indeed, SUN11602 is widely used in the context of NDDs, it must be said that it was born mainly as an antidepressant drug[422], given its ability to act at the CNS level by regulating the activity of the neurotransmitters serotonin, dopamine and noradrenaline, stabilizing mood and therefore offering a neuroprotective effect. Not only that, precisely because of its brain activity, the study of this new compound has also been extended to neurodegenerative diseases[423]. In this regard, there are several studies, albeit few, that have highlighted its potential effects. In confirmation of this, a study was conducted on mouse hippocampal neuronal cells, in which the neuroprotective activity of SUN11602

against cell death by excitotoxicity was evaluated[395]. From this study, it emerged that SUN11602 positively influenced neuronal survival by activating the FGFR1/MEK/ERK signalling pathway[395], molecules involved in the frontline of neuroprotection[392]. To better understand the neuroprotective effect of SUN11602, further studies on the MEK/ERK signalling pathway were conducted to clarify their association with cell survival[392]. The study involved the use of primary cultures of cerebrocortical neurons, stimulated for 20 minutes with SUN11602 (10 and 100 mM)[392] , in which the extracellular signal-regulated protein kinase 1/2 (ERK1/2) was phosphorylated, highlighting its neuroprotection. Subsequently, regarding the MEK protein, a specific inhibitor, PD98059[392], was used, turning off the activation of this signalling pathway (MEK/ERK)[392], thus confirming that these signalling molecules are mainly involved in the neuroprotection induced by SUN11602.

As previously described, the neuroprotective activity of bFGF is strictly linked to Ca^{2+} metabolism[424]. Considering that SUN11602 is a mimetic compound of bFGF, which therefore exerts the same action by binding to the same receptor, also for SUN11602, its activity is linked to the Ca^{2+} ion[392]. In confirmation of this, experimental studies in vitro have been conducted on rat cerebrocortical cells, the results of which have demonstrated, and therefore confirmed, that in the same way as its parent compound, SUN11602 increases the levels of the calcium-binding protein, Calbindin, suppressing the excess of Ca^{2+} ions at the intracellular level[392,424] . Moreover, *in vitro* studies have been conducted demonstrating that stimulation of hippocampal cells with SUN11602[395] increases Calb concentration, preventing neuronal death. One hypothesis for this could be that SUN11602 indirectly enhances Calb activity, reducing cellular stress caused by high Ca^{2+} concentration in neurons and ensuring proper Ca^{2+} homeostasis. Furthermore, knowing that SUN11602 binds the same receptor as bFGF, thus ensuring a neuroprotective action, the last step was to verify whether these activities were due to the activation of the same signalling pathways activated by bFGF. In this regard, PD166866, a specific inhibitor of the tyrosine kinase FGFR1, was used in neuronal cultures[392]. The results showed that the neuroprotective effect of SUN11602, as well as of bFGF, was blocked in the presence of the inhibitor PD166866 at a concentration of 0.3 mM[425]. Since the neuroprotective effect occurs following the phosphorylation of the tyrosine kinase domain of FGFR1, this study demonstrated that SUN11602 does not compete with bFGF to bind the same receptor. Therefore, it can be assumed that SUN11602 can, directly and indirectly, trigger the

phosphorylation of the tyrosine kinase domain of FGFR1, although how this phosphorylation occurs still needs to be explored.

Therefore, based on what has just been reported, bFGF and SUN11602, although having in common the portion of the structure responsible for the pharmacological activity, are distinct compounds used in different biological and pharmacological fields, remembering that bFGF is a ubiquitous protein, present in all living organisms, while SUN11602 was synthesized in the laboratory as an antidepressant drug.

Regarding their mechanism of action, as mentioned above, bFGF mainly binds its FGFRs on the cell surface, activating several intracellular pathways, such as the MAPK/ERK and PI3K/AKT pathways, exerting its action on biological processes, such as cell proliferation, differentiation and survival; moreover, bFGF is also involved in neurogenesis, angiogenesis and repair of damaged tissues. bFGF acts on various cells, including CNS, skin and connective tissue cells. Although SUN11602 is a bFGF mimetic compound, its precise mechanism of action is not entirely clear to date. It is known that SUN11602 interacts at the CNS level by modulating the activity of neurotransmitters, such as serotonin, adrenaline and noradrenaline, granting it an antidepressant activity. Therefore, bFGF has been widely studied in clinical practice and regenerative medicine, its use is limited to cell cultures, where its proliferative activity towards stem cells and other cell types is exploited, and its study has also been extended to neurodegenerative and cardiac diseases. SUN11602, although a promising candidate in research, has not yet been approved as a drug. This limitation will most likely be due to its insufficient efficacy and/or problems encountered during clinical trials. Therefore, SUN11602 is used in research mainly to identify new treatments for depression or mood stabilizers. Although bFGF and SUN11602 are similar compounds, their mechanisms of action and pharmacological applications are fundamentally different.

CHAPTER 3: AIM OF THE THESIS

In recent decades, traumatic brain injury (TBI) has become a global concern due to the increasing number of reported incidents, among young adults (18-30 years) mainly as a result of motor vehicle and sports accidents and violence, but also in older subjects (>65 y/o) with falls and domestic accidents, thus highlighting the need for increased safety. Failure to intervene promptly following a TBI leads to a cascade of physiological and biochemical events (neuroinflammation, α-synuclein aggregation and dementia) that result in neuronal degeneration and death, which could trigger progressive neurodegeneration, characteristic of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases.

Therefore, the present project aims to find therapeutic strategies for the treatment of TBI, Parkinson's and Alzheimer's diseases. In particular, to understand the role of SUN11602, a novel aniline derivate, designed to mimic the biological activity of bFGF, by interacting with the same receptor FGFR1, in reducing neuroinflammation and degeneration developed following a head injury, also offering an improvement in motor and cognitive deficits that occur in PD, and support to the neuron, stimulating its neurogenesis and therefore allowing neuronal survival. Additionally, an opportunity for optogenetic therapeutic intervention is offered by the increase in cAMP synthesis through stimulation with DdPAC at the hippocampal level with light, to better control the chronic activation of microglia and reactive astrocytes, reducing the formation and deposition of β-amyloid plaques, favouring its elimination in AD.

CHAPTER 4: MATERIALS AND METHODS

4.1. TBI study

4.1.1. Materials

SUN11602 was purchased by Tocris Bioscience (Bristol, UK). Unless otherwise indicated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

4.1.2. In vivo model of TBI

Adult male CD1 mice (25-30 g; 6-8 weeks old; Envigo, Italy) were accommodated in a controlled environment and provided with standard rodent chow and water. Mice were housed in stainless steel cages in a room maintained at 22 °C ± 1°C with a 12 hrs light and 12 hrs dark cycle. This study was approved by the University of Messina Review Board for the care of animals, in compliance with Italian regulations on the protection of animals (n° 617/2017-PR released on 02/08/2017). Animal experiments comply with Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116192) as well as EU regulations (OJ of EC L 358/1 12/18/1986).

4.1.3. Controlled cortical impact (CCI) experimental TBI

The animals were anesthetized, and TBI was induced by a controlled cortical impact (CCI) by using the controlled impactor device Impact One™ Stereotaxic impactor for CCI (Leica, Milan, Italy), as previously described[426]. A craniotomy was induced in the right hemisphere, with a Micromotor handpiece and drill (UGO Basile SRL, Comerio Varese, Italy), among the sagittal suture and the coronal ridge. The bone flap was removed and the craniotomy enlarged additionally with cranial rongeurs (New Adalat Garh, Roras Road, Pakistan). A cortical contusion was made on the exposed cortex using the controlled impactor device Impact One™ Stereotaxic impactor for CCI (Leica, Milan, Italy). Concisely, the impacting shaft was extended and the impact tip was lowered over the craniotomy site until it touched the dura mater. Subsequently, the rod was retracted and the impact tip was advanced to produce a brain injury of moderate severity for mice (tip diameter: 4mm; cortical contusion depth: 3mm; impact velocity: 1.5m/sec).

immediately after injury, the skin incision was secured with nylon sutures, spreading 2% lidocaine jelly to the lesion site to reduce pain. Sham mice underwent the same surgical procedure including anaesthesia and craniotomy but were not injured[427].

4.1.4. Experimental design

Animals were casually allocated into the following groups:

- *Group 1:* Sham + vehicle: mice were subjected to the surgical procedures, without CCI and vehicle solution (saline) was orally administered at 1 and 4 hours after craniotomy (n = 10)
- *Group 2:* Sham + SUN11602 (1mg/kg): like Sham + vehicle, in addition, mice were orally administrated with SUN11602 (1 mg/kg in 5% DMSO) at 1 and 4 hours after craniotomy (n=10)
- *Group 3:* Sham + SUN11602 (2.5mg/kg): like Sham + vehicle, in addition, mice were orally administrated with SUN11602 (2.5 mg/kg in 5% DMSO) at 1 and 4 hours after craniotomy (n=10)
- *Group 4:* Sham + SUN11602 (5mg/kg): like Sham + vehicle, in addition, mice were orally administrated with SUN11602 (5 mg/kg in 5% DMSO) at 1 and 4 hours after craniotomy (n=10)
- *Group 5:* TBI + vehicle: mice were subjected to CCI, and saline solution was orally administered at 1 and 4 hours after craniotomy (n = 10)
- *Group 6:* TBI + SUN11602 (1mg/kg): mice were subjected to CCI, and SUN11602 (1 mg/kg in 5% DMSO) was administered orally 1 and 4 hours after craniotomy (n = 10)
- *Group 7:* TBI + SUN11602 (2.5mg/kg): mice were subjected to CCI, and SUN11602 (2.5 mg/kg in 5% DMSO) was administered orally 1 and 4 hours after craniotomy (n = 10)
- *Group 8:* TBI + SUN11602 (5mg/kg): mice were subjected to CCI, and SUN11602 (5 mg/kg in 5% DMSO) was administered orally 1 and 4 hours after craniotomy (n = 10)

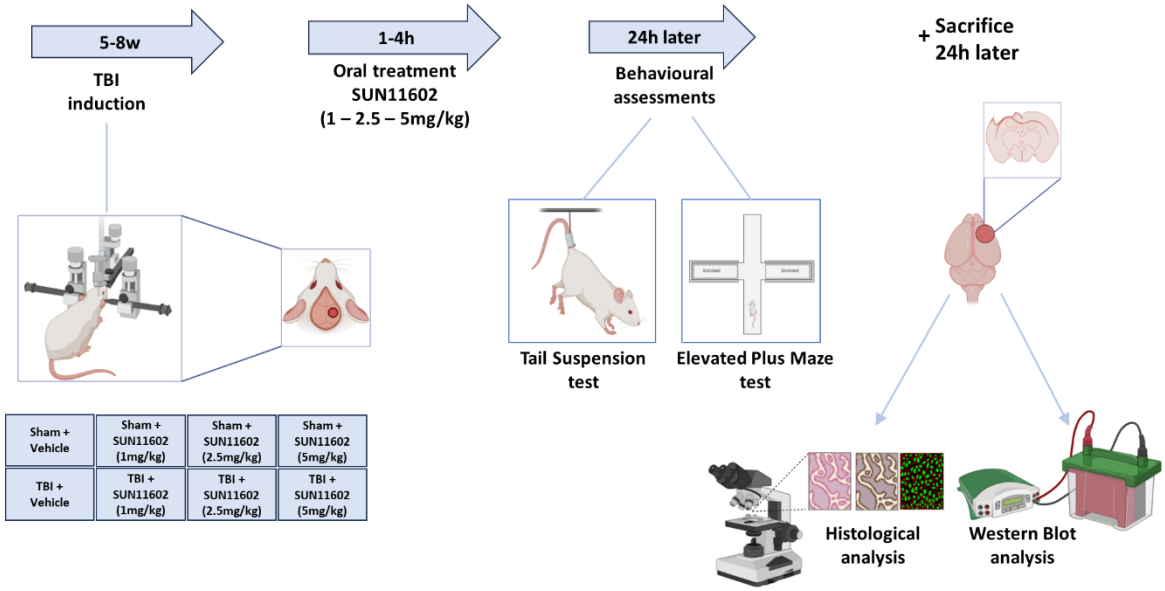

Figure 13. Timeline Traumatic Brain Injury Retrieved from https://app.biorender.com/biorender-templates

The doses (1, 2.5 and 5mg/kg) and the oral administration route of SUN11602 used in this study were based on previous *in vivo* studies[423,393]. Furthermore, numerous studies have illustrated the importance of intervening promptly, within 4 hours of the head injury, to obtain the best neuroprotective effect. In addition, animal experimental data from the Sham + SUN11602 groups were not shown because the vehicle and administration of SUN11602 did not demonstrate toxicity or other effects compared to sham control animals.

4.1.5. Behavioural tests

Behavioural assessments on each mouse were made 24h post-CCI. All behavioural testing was conducted during the light cycle phase and in enclosed behaviour rooms (50–55 dB ambient noise) within the housing room. The behavioural tests were conducted by three different reliable expert observers blinded to the injury status of the animals[428].

4.1.5.1.Tail Suspension test (TST)

The experiment was carried out based on previous research[429]. Each mouse was suspended from the tail with duct tape attached to a 50 cm high stand and filmed for 5 minutes. The latency with which the mice appeared immobile and the total duration of immobility were evaluated; the total duration of the test for each mouse was 5 minutes.

4.1.5.2.Elevated Plus Maze test (EPM)

Anxiety-like behaviour in mice was evaluated using Elevated Plus Maze (EPM) system. We used the same methodology employed in our previous study and described below[430]. The EPM apparatus consisted in two open arms (30 × 5 × 0.25 cm), two enclosed arms (30 × 5 × 15 cm) and a centre area (5 × 5 cm); the entire apparatus was elevated by single central support to a height of a 60 cm above floor level. Mice were placed individually in the open arm and the time spent in each arm was measured. Anxiety reduction was indicated by an increase in the proportion of time spent in the open arms[431]. Behavioural representatives of the animal's emotional state were: latency, frequency and duration of visits in the open and closed arms. The total number of arm entries and the number of closed-arm entries were used as measures of general activity. Data were analysed by One-Way analysis of variance, with the percentage of time spent in the closed arms as a percentage of the total.

4.1.6. Histological analysis

Coronal sections of 7 µm thickness from the perilesional brain area of each animal were evaluated by Hematoxylin and Eosin (H&E) staining[432,433]. The histopathologic changes of the grey matter were scored on a six-point scale. The scores from all the sections of each brain were averaged to give a final score for individual mice. The results from every section of the spinal cord were averaged to obtain a final score (1 to 5) for distinct mice. The results of histological examinations were acquired by using a Leica QWin V3 and showed at 20× (50 µm scale bar) and 40× magnifications (20 µm scale bar). Histological studies were performed in a blinded fashion by experienced histopathologists.

4.1.7. Immunohistochemical localization of iNOS, nNOS, Bcl-2 and Bax antibodies

Brain tissues containing the lesion were fixed in 10% (w/v) buffered formaldehyde 24 hours after TBI and 7 um sections were prepared from paraffin-embedded tissues[434]. Briefly, after deparaffinization, endogenous peroxidase was quenched with 0.3% hydrogen peroxide in 60% methanol for 30 min. Sections were incubated overnight (O/N), at 37°C, with the following antibodies: anti-iNOS (1:100; BD Biosciences), anti-nNOS (1:100; Cell Signaling), anti-Bcl2 (1:100; sc-7382; Santa Cruz Biotechnology) and anti-Bax (1:100; sc-7480; Santa Cruz Biotechnology). After washing with PBS, the sections were incubated with a secondary antibody for 1 h. The signal of binding with the

antibodies was amplified by using the peroxidase Avidin-Biotin complex (Vector Lab. Inc., Burlingame, CA). The reaction was revealed by a chromogenic substrate (brown DAB), and counterstaining with Nuclear Fast Red. To prove the binding specificity for different antibodies, some sections were also incubated with only primary antibodies or secondary antibodies, no positive staining was observed in these sections. Images were collected using a Zeiss microscope and Axio Vision software. For the graphic display of densitometric analyses, the % of positive staining (brown staining) was measured by computer-assisted colour image analysis (Leica QWin V3, UK). All stained sections were observed and analysed in a blinded manner. For immunohistochemistry, 20× (50 μm scale bar) and 40× (20 μm scale bar) were shown[435].

4.1.8. Western blot analysis for Inflammatory, Apoptosis and Ca-dependent pathways

At 24 h after post-CCI, brains were surgically removed. Tissue samples were processed and cytosolic and nuclear fractions were obtained, respectively[436]. The membranes were incubated, at 4°C overnight, with specific primary antibodies: anti-NF-κB p65 (1:500; sc-8008; Santa Cruz Biotechnology) anti-IκB-α (1:500; sc-1643; Santa Cruz Biotechnology), anti-IL-6 (1:500; sc-57315; Santa Cruz Biotechnology) for NF-κB pathway; anti-Calbindin-D28K (Calb-1) (1:500; sc-28285; Santa Cruz Biotechnology) and anti-S100-β (1:500; sc-393919; Santa Cruz Biotechnology) for Ca-dependent pathway; anti-COX-2 (1:500; sc-28285; Santa Cruz Biotechnology), anti-iNOS (1:500; sc-8310; Santa Cruz Biotechnology); anti-bcl-2 (1:500; sc-7382; Santa Cruz Biotechnology), and anti-p53 (1:500; sc-126; Santa Cruz Biotechnology), for apoptosis pathway, in 1 × PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 at 4°C, overnight. The densitometric values are normalized with Lamin A/C (nuclear fraction 1:500 Sigma-Aldrich Corp.) and β-actin (cytosolic fraction 1:500; sc:8432; Santa Cruz Biotechnology) and expressed as % of the control. Finally, membranes were incubated for 1 h at room temperature with a secondary antibody and bands were obtained using a chemiluminescence detection system (ECL) according to the manufacturer's instructions (Thermo, Waltham, MA, USA). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDocTMXRS + software.

4.1.9. Immunofluorescence staining of NT-3 and BDNF in brain tissue

Brain sections were processed for immunofluorescence staining as previously described[123]. Sections were incubated with antineurotrophin-3 (NT-3) (1:100, sc-518099, Santa Cruz Biotechnology) and anti-brain-derived neurotrophic factor (BDNF) (1:100, sc-65514; Santa Cruz Biotechnology) antibodies in a humidified chamber O/N at 4°C. Sections were then incubated for 24h after incubation, the sections were washed with PBS and then incubated with conjugated anti-mouse Alexa Fluor-488 secondary antibody #A32731 (1:1000 in PBS, vol/vol Molecular Probes, Monza, Italy) for 1 h at room temperature. Nuclei were stained by adding 2 μg/mL 4′,6′-diamidino-2-phenylindole (DAPI; Hoechst, Frankfurt, Germany) in PBS. Sections were observed at 40× magnification using a Leica DM2000 microscope (Leica, Milan, Italy). Contrast and brightness were established by examining the most brightly labelled pixels and applying settings that allowed clear visualization of structural details while keeping the highest pixel intensities close to 250. The same settings were used for all images obtained from the other samples that had been processed in parallel.

4.1.10. Calcium colourimetric assay kit

Calcium concentration was evaluated on brain tissue extracts for each experimental group following the manufacturer's instructions (BioVision, K380-250).

4.1.11. Statistical analysis

Experimental data are expressed as mean ± standard error of the mean (SD) of N observations, in which N represents the number of animals studied. Data are representative of at least three independent experiments. The results were examined by One-Way ANOVA analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons. Only a *p-value* < 0.05 was considered significant.

4.2. PD study

4.2.1. In vivo model of PD

Adult male CD1 mice (30–35 g; 6–8 weeks old; Envigo, Italy) were accommodated in a controlled environment and provided with standard rodent chow and water. Mice were housed in stainless steel cages in a room maintained at 22 °C ± 1 °C with a 12 h light and 12 h dark cycle. Animal experiments comply with Italian regulations on the protection of

animals used for experimental and other scientific purposes (DM 116,192) as well as EU regulations (OJ of EC L 358/1 12/18/1986) and the ARRIVE guidelines. Moreover, the study was approved by the OPBA of Messina with authorization number 537/2018-PR

4.2.2. MPTP-induced nigrostriatal degeneration

Adult male CD1 mice received four intraperitoneal injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (20 mg/kg; Sigma-Aldrich, St. Louis, MO) in saline at 2 h intervals in 1 day, the total dose for each mouse was 80 mg/kg. Starting 24 h after the first MPTP injection, animals received oral administration of SUN11602 at doses of 1–2.5–5 mg/kg (in 10% DMSO), respectively; thereafter, oral administration was given once daily until 7 days after the MPTP injection. Mice were killed 7 days after MPTP injection and their brains were harvested, sectioned, and processed. The dose of MPTP (80 mg/kg) used was based on previous in vivo studies[428,437].

4.2.3. Experimental design

Animals were randomly distributed into the following groups:

- *Group 1*: Sham + vehicle; vehicle solution (saline) was injected intraperitoneally during the first day like the MPTP group, in addition, starting 24 h after vehicle injection, saline was administered orally for 7 consecutive days (n= 10);
- *Group 2:* Sham + SUN11602 1 mg/kg; like Sham + vehicle group, in addition, mice was administered orally with SUN11602 1 mg/kg for 7 consecutive days starting 24 h after vehicle solution injection (n=10);
- *Group 3*: Sham + SUN11602 2.5 mg/kg; like Sham + vehicle group, in addition, mice was administered orally with SUN11602 2.5 mg/kg for 7 consecutive days starting 24 h after vehicle solution injection (n= 10);
- *Group 4*: Sham + SUN11602 5 mg/kg; like Sham + vehicle group, in addition, mice was administered orally with SUN11602 5 mg/kg for 7 consecutive days starting 24 h after vehicle solution injection (n= 10);
- *Group 5*: MPTP + vehicle: MPTP solution was administered intraperitoneally during the first day, as described before, plus saline oral administration for 7 consecutive days starting 24 h after MPTP injection (n= 10);

- *Group 6:* MPTP + SUN11602 1 mg/kg; like MPTP + vehicle group, in addition, mice was administered orally with SUN11602 1 mg/kg for 7 consecutive days starting 24 h after MPTP injection (n= 10);
- *Group 7*: MPTP + SUN11602 2.5 mg/kg; like MPTP + vehicle group, in addition, mice was administered orally with SUN11602 2.5 mg/kg for 7 consecutive days starting 24 h after MPTP injection (n= 10);
- *Group 8*: MPTP + SUN11602 5 mg/kg; like MPTP + vehicle group, in addition, mice was administered orally with SUN11602 5 mg/kg for 7 consecutive days starting 24 h after MPTP injection (n=10).

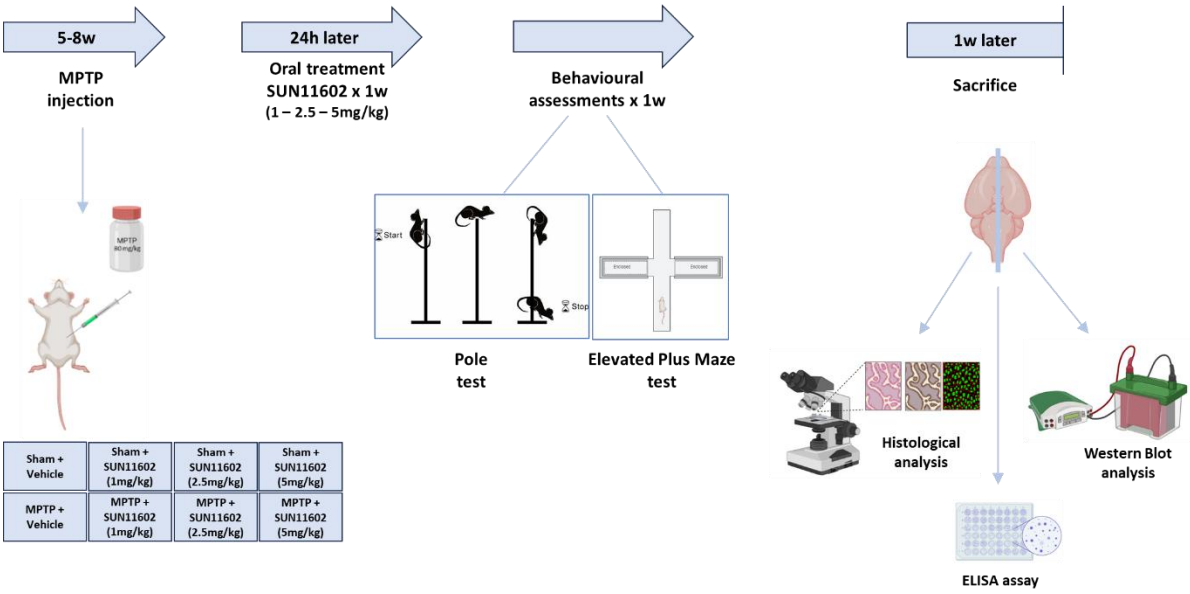

Figure 14. Timeline Parkinson induction Retrieved from https://app.biorender.com/biorender-templates

The dose and route of administration of SUN11602 were based on previous *in vivo* studies[423,428], on large-scale dose studies performed in our laboratory, and considering the mice body surface area-based dosing.

The experimental data relating to the Sham groups treated with SUN11602 were reported and did not determine either toxicity or improvement compared to the Sham + vehicle group.

4.2.4. Behavioural tests

Behavioural assessments on each mouse were made 7 days after the last MPTP injection. The mice were placed in the behaviour room for 5 min for 2 days for acclimation before the onset of behavioural testing[428]

4.2.4.1.Pole test

The pole test was performed to assess movement disorders. The test consisted of a 50 cm high, gauzed pole (1 cm in diameter). The animals were placed on top of a vertical pole, directed towards their cages. Under natural conditions, the mice will be oriented downwards along the length of the pole. The parameters evaluated in this test were: the time until the animal turned by 180°, called the turning time and the time until the animal dropped to the floor, the total time[428].

4.2.4.2.Elevated plus maze test (EPM)

Anxiety deficits were evaluated using an EPM system. We used the same methodology employed in our previous study[428] and briefly described below. The elevated plus-maze test is one of the most used tests to measure anxiety-related behaviour in rodent animals. The apparatus consists of two open arms, two closed arms and a centre area. Mice were placed individually in the open arm and the time allowed to explore was 5 min. Behavioural representatives of the animal's emotional state were: latency, frequency and duration of visits in the open and closed arms. The total number of entries in the arms was used as a general index of activity. Data were analysed by One-way analysis of variance, with the percentage of time spent in the closed arms as a percentage of the total.

4.2.5. Immunohistochemical localization of Tyrosine Hydroxylase (TH), DAT, α-syn and microtubule-associated protein-2 (MAP-2)

Immunohistochemical staining was performed as previously described[438,439] and reported in paragraph 4.1.8. Sections were incubated overnight (O/N) with the following antibodies: anti-tyrosine hydroxylase (TH) (1:100; sc25269; Santa Cruz Biotechnology), anti-dopamine transporter (DAT) (1:100; sc-14002; Santa Cruz Biotechnology), pan-anti α-syn (α-syn; affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of α-syn of human origin) (1:100; sc-7011; Santa Cruz Biotechnology) and anti-MAP-2 (1:100; SB5622; Millipore). Images were collected

using a Zeiss microscope and Axio Vision software. For the graphic display of densitometric analyses, the % of positive staining (brown staining) was measured by computer-assisted colour image analysis (Leica QWin V3, UK). All stained sections were observed and analysed in a blinded manner. For immunohistochemistry, 20 × (50 µm scale bar) and 40 × (20 µm scale bar) were shown.

4.2.6. Immunofluorescence staining of glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (IBA-1), β3-tubulin and p-53

Brain sections were processed for immunofluorescence staining as previously described[123] and reported in paragraph 4.1.10. Sections were incubated with primary anti-GFAP antibody (1:100; sc-33673; Santa Cruz Biotechnology), anti-IBA1 antibody (1:100; sc-32725; Santa Cruz Biotechnology), anti-β3-Tubulin antibody (1:100; sc-69966; Santa Cruz Biotechnology) and anti-p53 antibody (1:100; sc-126; Santa Cruz Biotechnology) in a humidified chamber for O/N at 37 °C. Sections were observed and acquired at 40 × magnifications using a Leica DM2000 microscope (Leica, UK, EU).

4.2.7. Western blot analysis of NF-κB pathway (of IκB-α, TNF-α, IL-1β, IL-6, IL-18), Calbindin-D28k, S100-β, Bcl-2, Bax and Caspase-3

Brain samples were surgically removed, and the ventral mesencephalon was isolated as previously described by Campolo et al.[428]. Tissue samples were processed as previously described[440] and reported in paragraph 4.1.9. The expression of IκB-α, TNF-α, IL-1β, IL-6, IL-18, Calbindin-D28k, S100-β, Bcl-2, Bax and Caspase-3 were quantified in cytosolic fractions. NF-κBp65 was quantified in the nuclear fraction. The membranes were incubated, at 4 °C overnight, with specific primary antibodies: anti-IκB-α (1:500; sc-1643; Santa Cruz Biotechnology), anti-TNF-α (1:500; sc-52746; Santa Cruz Biotechnology), anti-IL-1β (1:500; sc-32294; Santa Cruz Biotechnology), anti-IL-6 (1:500; sc-130326; Santa Cruz Biotechnology), anti-IL-18 (1:500; sc-7954; Santa Cruz Biotechnology), anti-Bcl-2 (1:500; sc-7382; Santa Cruz Biotechnology), anti-Bax (1:500; sc-7480; Santa Cruz Biotechnology), anti-Caspase-3 (1:500; sc-7272; Santa Cruz Biotechnology), anti-Calbindin-D28k (1:500; sc-28285; Santa Cruz Biotechnology), anti-S100-β (sc-393919; Santa Cruz Biotechnology) and NF-κB (1:500; sc-8008; Santa Cruz Biotechnology) in 1 × phosphate-buffered saline (PBS), 5% (w/v), non-fat dried milk and 0.1% Tween-20. Signals were detected by enhanced chemiluminescence (ECL) detection system reagent according to the manufacturer's instructions (SuperSignal West Pico

Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDoc™XRS + software and standardized to β-actin or LAMIN A/C levels as the internal control.

4.2.8. Measurement of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanilic acid (HVA) levels in the striatum

Measurements were made as previously showed and briefly reported below[437,441]. 7 days after the last MPTP injection, 4 mice per group, were killed and the striatum was dissected, frozen on dry ice and stored at − 70 °C. To measure the levels of Dopamine and its metabolites, DOPAC and HVA, high-performance liquid chromatography (HPLC) with electrochemical detection was used in each sample, using 0.15 M monochloroacetic acid, pH 3.0 and 200 mg/L Sodium octyl sulfate, 0.1 mM EDTA, 4% acetonitrile and 2.5% tetrahydrofuran as mobile phase. Data were collected and processed on a Dynamax computerized data manager (Rainin Instruments).

4.2.9. Calpain activity

Calpain activity was estimated fluorometrically according to previous studies[442,443]. Briefly, the supernatant of the brain samples, containing calpain, was subjected to Ca-dependent fluorescence and non-Ca-dependent fluorescence, to determine calpain activity, using N-succinyl-Leu-Tyr-(N-succinyl-LY)-AMC, cleaved by μ/m-calpain. To measure Ca-dependent fluorescence, samples were incubated at 37 °C in buffer A containing 63 mm imidazole–HCl, pH 7.3, 10 mm B-mercaptoethanol and 5 mm CaCl2 and cleaved with 150um M-succinyl-LY-AMC. The same methodology was performed to measure non-Ca-dependent fluorescence, using Buffer A without calcium, containing 1 mm EDTA and 10 mm EGTA.

4.2.10. Stereological analysis

Unbiased counting of TH^+ dopaminergic neurons within substantia nigra par compacta (SNpc) was performed as described previously[444,445]. Each section was incubated with polyclonal primary antibody mouse anti-TH (1:400, Santa Cruz Biotechnology) O/N and processed with the ABC method (Vector Laboratories, Burlingame, CA). Brain sections were counterstained with cresyl violet, a Nissl stain, and covered. To count the number

of TH^+ cells, StereoInvestigator software was used (Microbrightfield, Williston, VT). Cells were counted with a 10 × and 20 × objective, respectively, using a Leica DM2000 microscope (Leica, UK, EU). The area of interest for counting TH-immunoreactive cells was performed within a 50 × 50 × 5 µm frame on the same side of the brain, with an upper and lower control zone of 1 µm; for Nissl cell counting, the same sections were examined.

4.2.11. ELISA kit

Phospho-α-syn (p-α-syn) and CD68 were evaluated on brain tissue extracts for each experimental group as previously described[439]. p-α-syn and CD68 were measured by ELISA kit, according to the manufacturer's instructions (MyBioSource, respectively), through a colorimetric microplate reader.

4.3. AD study

4.3.1. In vivo model of AD

All animal procedures were approved by local ethical review 356/19 from the University of Barcelona and 11070 from Generalitat de Catalunya and performed according to Spanish RD 53/2013 and current EU legislation. The 3Rs, replacement, refinement and reduction were considered for animal procedure planning.

The transgenic mouse line 5xFAD (MMRRC, Cat#034840-JAX, RRID: MMRRC_034840-JAX) was used from 7-8 months of age to 8-9 months of age (males and females). 5xFAD mice overexpress the 695-amino acid isoform of the human amyloid precursor protein (APP695) carrying the Swedish, London, and Florida mutations under the control of the murine Thy-1 promoter. Besides, they express human presenilin-1 (PSEN-1) carrying the M146L/L286V mutation, also under the control of the murine Thy-1 promoter[446]. Genotypes were obtained by polymerase chain reaction (PCR) from ear biopsy and WT littermates were used as the control group. All animals were housed with access to food and water ad libitum in a colony room at 19–22 °C and 40–60% humidity, under a 12h light and 12h dark schedule.

4.3.2. Adeno-associated virus constructs

Eight recombinant Adeno-Associated Virus serotype 9 (AAV9) constructs were used in this study targeting neurons and astrocytes[331]. AAV9 constructs were designed to express DdPAC in neurons, DdPAC in astrocytes, and their respective control AAVs expressing eGFP in neurons and astrocytes:

- *AAV9 Vector*: includes the inverted terminal repeats (ITRs) for packaging into the AAV capsid. It is designed to introduce a new gene to target cells, neurons and astrocytes.

- *Promoter*s: GFAP to ensure astrocyte-specific expression or CamKII for excitatory neurons specific expression

- *DdPAC gene*: sequence obtained from Dr. Andreas Möglich, Bayreuth University, Germany. Generate the sequence for DdPAC from *Deinococcus deserti*

- *Reporter gene*: eGFP, the enhanced green fluorescent protein (eGFP) gene sequence for fluorescent tagging of control AAV or 3xFLAG-Tag for DdPAC expressing AAV

- *WPRE*: the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) is used for enhancing transgene expression

- *Polyadenylation Signal (PolyA)*: used for proper mRNA stability and translation

Thus, the following constructs were generated: AAV9-CamKII-DdPAC-3xFLAG-WPRE (AAV9-CamKII-DdPAC), AAV9-CamKII-eGFP-WPRE (AAV9-CamKII-GFP), AAV9-GFAP-DdPAC-3xFLAG-WPRE (AAV-GFAP-DdPAC) and AAV9-GFAP-eGFP-WPRE (AAV-GFAP-GFP). The AAV9-GFAP-ddPAC-3xFLAG-WPRE (AAV-GFAP-DdPAC) and AAV9-GFAP-eGFP-WPRE (AAV-GFAP-GFP) were produced following the co-transfection method as described previously and purified through iodixanol gradient ultracentrifugation[447].Concentration and buffer exchange were carried out using PBS containing 0.001% Pluronic. The titers of AAV vector stocks were subsequently determined using the real-time quantitative PCR titration method[448] with SYBR Green (Thermo Fischer Scientific). Titers of all virus were ~1-3x10^{14} vg/ml.

4.3.3. Experimental design

The WT and 5xFAD mice (females and males) were divided into eight groups:

Neuronal group:

- WT: AAV9-CamKII-DdPAC
- 5xFAD: AAV9-CamKII-DdPAC
- WT: AAV9-CamKII-GFP
- 5xFAD: AAV9-CamKII-GFP

Astrocytic group:

- WT: AAV9-GFAP-DdPAC
- 5xFAD: AAV9-GFAP-DdPAC
- WT: AAV9-GFAP-GFP
- 5xFAD: AAV9-GFAP-GFP

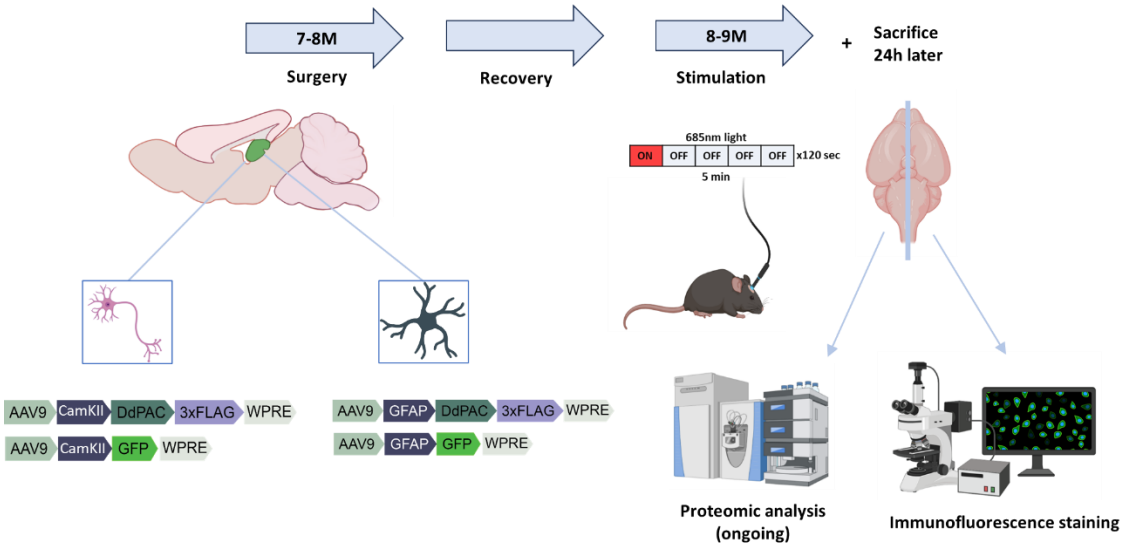

Figure 15. Timeline of constructs injection and DdPAC light stimulation in neurons and astrocytes. Retrieved from https://app.biorender.com/biorender-templates

Each group received the AAV9-CamKII-DdPAC infection directly in the hippocampus region by stereotaxic surgery 4 weeks after surgery the mice received the DdPAC stimulation (λ 685 nm) for 10 min (1sec ON and 4sec OFF) and then underwent euthanasia by cervical dislocation. The Hippocampus was dissected, one hemisphere was post-fixed and underwent sucrose gradient, while the other was dissected and snap-frozen for proteomics analysis.

4.3.4. Stereotaxic surgeries

Stereotaxic surgeries were performed at 7-8 mounths of mice age and 4 weeks before the start of the DdPAC stimulation[449]. The vectors AAV9-CamKII-DdPAC and AAv9-CamKII-GFP; AAV9-GFAP-DdPAC and AAV9-GFAP-GFP were injected in the hippocampus region of WT and 5xFAD mice. The surgery was performed using Isoflurane anaesthesia (5% induction, and 1.5% maintenance) and Meloxicam (2mg/kg s.c.) that was injected before the surgery to avoid pain and reduce inflammation. A volume of 0.5 µL of corresponding viral constructs was injected through a Hamilton syringe with a 33-gauge needle at 0.1 µL/min following the coordinates from bregma and from the skull surface: AP (anterior-posterior): -2 mm from bregma; ML (medial-lateral): ± 1.25 mm from bregma and DV (dorsal-ventral): -2 mm. After the injection, we waited for 5-10 minutes to allow the viral vector to spread while avoiding reflux. Then, fiber-optic cannulas (AP: -2.0; ML: ± 1.25 and DV: -2), with 2 mm length and 400 µM diameter, were implanted bilaterally, and secured using dental cement. DdPAC stimulation was performed four weeks after surgery.

4.3.5. DdPAC stimulation

After stereotactic surgery, DdPAC stimulation was performed using red light from a single 685 nm LD fiber light source (Doric lenses, specs) delivered 5-20 mW (measured at the tip of the patch cord) for 10 minutes (1 second at 685 nm ON and 4 seconds at 685 nm OFF) and controlled by the free study software Doric Neuroscience. During DdPAC stimulation, animals were free to move around inside a cage and 24h after stimulation, the animals were sacrificed.

4.3.6. Immunohistochemistry localization of DdPAC, GFAP, GFP, DAPI and Aβ

After DdPAC stimulation, mice were sacrificed by cervical dislocation. One brain hemisphere was post-fixed with 4% PFA and dehydrated in a PBS/sucrose gradient [from 15% (48h postmortem) to 30% (32 h postmortem)] with 0.02% sodium azide and finally stored at 4ºC. Sections (30µm) were cut on a vibratome (Leica VT1000S) and stored in cryopreservation solution (30ml Ethylene glycol; 30ml Glycerol; 25ml TB (1M Tris HCl pH 7,5); 15ml H2O miliQ for 1 liter of solution) at -20ºC. For immunohistochemistry analysis, three hippocampal sections were selected from each animal. Sections were washed twice in PBS 0.01M for 5min at RT and then treated with 50 mM NH4Cl for

15min at RT. Subsequently, the free-floating sections were permeabilized with 0.01M PBS containing 0.5% Triton X-100 and blocked for 2hrs with a solution of 0.01M PBS with azide 0.02%, 0.3% Triton X-100, BSA 0.2% and 5% normal goat serum (NGS) at RT. Sections were incubated O/N at 4°C with anti-Flag Ab (#F1804; Sigma-Aldrich; St. Louis, Missouri, USA), or anti-GFP Ab (#132006; SYSY Antibodies; Göttingen, Germany) and anti-GFAP Ab (NBPs-62566; NOVUS Biologicals; Mòstoles, Madrid) was used to assess GFAP-positive astrocytes in AAV-GFAP-GFP or AAV-GFAP-DdPAC injected mice, respectively. A second IHC was performed to detect the presence and localization of Aβ plaques on the hippocampal section. We incubated the sections O/N at 4°C with primary antibody anti-Aβ (6E10 antibody) (#NBP2-62566; NOVUS Biologicals; Móstoles. Madrid). All primary antibodies were diluted in a solution of 0.01M PBS with azide 0.02%, 0.5% Triton X-100, BSA 0.2% and 5% NGS. 24h after, sections were washed with 0.01M PBS and incubated for 1:30h at RT with AlexaFluorTM 488 goat-anti mouse IgG (A32723; Invitrogen; Waltham, Massachusetts, USA) for anti-Flag; AlexaFluorTM 647 anti-rabbit (#711-605-152; Jackson Immune Research; Paese) for anti-GFAP; AlexaFluorTM 594 anti-rabbit (#111-585-144; Jackson Immune Research; Paese) for anti-Aβ (6E10 antibody) and 488 anti-chicken (#A11039; Invitrogen; Waltham, Massachusetts, USA), diluted in 0.01M PBS. Sections were washed in 0.01M PBS and mounted on microscope slides using DAPI Fluoromount-G (Southern Biotechnology) and kept in the dark at 4°C.

4.3.7. Acquisition and analysis of immunofluorescence images

Fluorescence images were acquired by a confocal microscope (AF6000 Leica). Mosaic images to identify the virus-infected zone for all GFAP images were acquired with 5x and 10x objective lenses. All the quantification analyses were conducted using 10x objective lenses, and the mean of three sections was obtained for each animal. For GFAP quantification, firstly, GFAP mean intensity was analysed in the whole hippocampus area, using the ImageJ software (v1.54j). Secondly, as GFAP expression changes along the different layers of the hippocampus, we further analysed the GFAP intensity levels in each layer in the different groups (Hilus, S. Stratum granulosum (S.g), Stratum Moleculare (S.m), Stratum lacunosum-moleculare (S.l-m), Stratum radiatum (S.r) and Stratum Oriens (S.o)). For the Aβ plaques expression quantification, using ImageJ software (v1.54j), we calculated either the deposition of the Aβ plaques and the number

of them. Firstly, for Aβ plaques deposition expression, we created the masks starting by selecting the hippocampus portion and measuring the total area (expressed in microns) and subtracting the background. Then, using the Threshold tool, the masks were created and analysed the area of a single plaque was expressed in pixels. Subsequently, we normalized to the area of the hippocampus to obtain the Aβ deposition expressed in percentage (%Aβ). The creation of the mass allowed us to count automatically the number of plaques present in the portion of the hippocampus and normalize for the total hippocampal area of each section to obtain N° Aβ plaques/mm^2.

4.3.8. Statistical analysis

All the results were expressed as mean ±SEM. Data from an individual mouse was represented by single points Statistical analyses were performed using the Two-way ANOVA with genotype and AAV construct as factors, or groups and layers as factors, followed by Fisher's LSD post hoc test, and indicated in Results and/or figure legends. For Aβ plaques, statistical analysis was performed using a Student t-test between 5xFAD GFP and 5xFAD DdPAC groups. Values of $p < 0.05$ were considered statistically significant. All statistic tests were performed on GraphPad Prism version 8.0.1 Software.

CHAPTER 5: RESULTS

5.1. TBI study

5.1.1. Recovery of psychomotor function exerted by SUN11602

The behavioural tests were performed to investigate the relationship between neurological deficits in TBI. Considering anxiety, a critical component of behavioural change after brain damage traumatic[126], TBI-induced mice were subjected to the EPM at 24h after TBI (Figures 16A and B), assessing the animals' aversion to open spaces. . Considering anxiety, a critical component of behavioral change after TBI[125], TBI-induced mice were subjected to EPM 24 hours after TBI (Figures 16A and B) evaluating the animals' aversion to open spaces. The results obtained from the test reported the cognitive improvement that oral treatment with SUN11602, increasing doses (1 - 2.5 and 5mg/kg) offers to mice after TBI (Figures 16A and B), improving their latency. Graph A reports a reduction in permanence in the closed arms of the maze, consequently increasing that in the open arms. In addition, SUN11602 reduced the total time of entries in the closed arms, as reported in graph B. Anxious behavior is almost always accompanied by another cognitive disorder, represented by depression, which seems to be a frequent mental disorder in people with TBI. Therefore, TST was performed, as a measure of depression-like behavior (Figure 16C), by measuring the animal's immobility time. TBI mice show significantly longer immobility time (Figure 16C) than healthy mice. Oral treatment with SUN11602, at increasing doses (1 - 2.5 and 5 mg/kg), significantly reduced immobility time (s) at 24 hours after TBI (Figure 16C), thus reducing the depressive component affecting TBI animals.

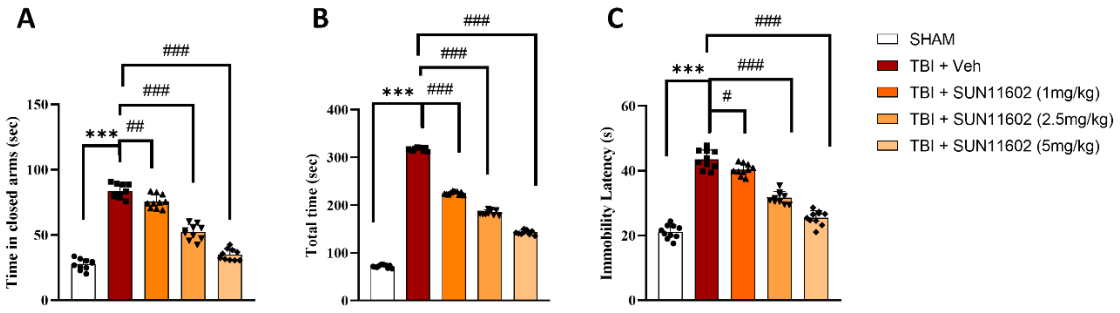

Figure 16. Effect of SUN11602 treatment on the psychomotor function in mice with TBI. The behavioural test reported the change after brain damage in mice TBI-induced **(A-C)**. Mice with brain trauma without the treatment of SUN11602 showed an increase in the total time spent in the closed arms **(A)**. The treatments with SUN11602, at the doses of 1, 2.5 and 5 mg/kg, significantly decreased permanence in closed arms and by an increment in the number of entries in open arms **(A)**. Figure 16B reported how SUN11602 at increased doses can reduce the total time. The tail suspension test has been performed as a measure of depression-like behaviour **(C)**. We demonstrated that the mice subjected to TBI **(C)** had a longer time of immobility than mice treated with SUN11602, at the doses of 1, 2.5 and 5mg/kg **(C)**, which reduced the immobility time (s) at 24h after TBI **(C)**. Data are expressed as mean ± SEM from N= 10 mice for each group. One-way ANOVA test (P <0.05) followed by Bonferroni post-hoc test for multiple comparisons. ***p <0.001 vs sham; # p < 0.05 vs TBI; ## p <0.01 vs TBI; ### p <0.001 vs TBI.

5.1.2. Protective effect of SUN11602 to reduce neuronal damage TBI-induced.

To evaluate the trauma-prone area in brain tissue, histological analysis of the periwound area was performed by H&E staining (Figures 17A-E). The staining detected major white matter alteration and inflammation in the perilesional area of TBI mice, compared to the control group (Figure 17A, score F). SUN 11602 at a dose of 1 mg/kg didn't demonstrate an improvement in brain tissue (Figure 17C, score F). Instead, SUN11602 treatments, at doses of 2.5 and 5 mg/kg, were able to significantly reduce brain tissue damage 24 hours after head injury (Figures 17D-E, score F).

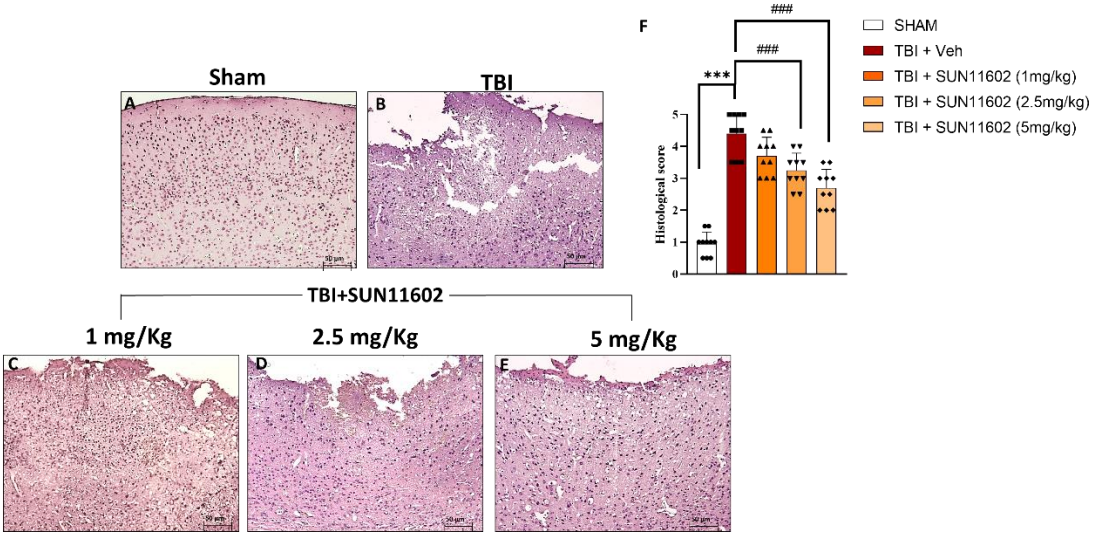

Figure 17. SUN11602 treatment restored tissue damage brain after TBI. The histological examination showed a tissue lesion in the prefrontal cortex of the right hemisphere, with structural alteration and inflammation of the white matter in the parenchyma **(A-E)**. The group of animals that received treatment with SUN11602, 1h and 4h after TBI, demonstrated a reduction in the severity of the damage **(D-E, F)**. SUN 11602 at a dose of 1 mg/kg did not demonstrate a noticeable improvement in brain tissue **(C, F)**. Data are expressed as mean ± SEM from N= 10 mice for each

group. One-way ANOVA test ($p<0.05$) followed by Bonferroni post-hoc test for multiple comparisons. ***p <0.001 vs sham; # p <0.05 vs TBI; ### p <0.001 vs TBI.

5.1.3. Neuroprotective effect of the SUN11602 treatment on the inflammatory response.

Inflammation represents the common biological process triggered following brain injury[450]. To determine the neuroprotective effect of SUN11602 treatment on inflammatory processes, we also demonstrated by western blot analyses to evaluate the expression of nuclear factor kappa-light-chain-enhancer of activated B cells p65 (NF-κB p65) pathway, such as IκB-α, IL-6. The data showed how, in the TBI group, there was a significant degradation of IκB-α levels in the cytosolic portion, compared to the control group, in which basal levels expression of IκB-a was found (Figure 18A, densitometric analysis A1). Contrarily, the NF-κB p65 expression, in the nuclear fraction, was increased in mice with TBI compared to the sham group (Figure 18C, densitometric analysis C1). The data obtained with oral treatment with SUN11602 reported that, at the doses of 2.5 and 5 mg/kg, the compound restored the IκB-α levels, reducing, at the same time, the NF-κB p65 expression levels (Figure 18C, densitometric analysis C1). SUN11602 at the dose of 1mg/kg minimally reduced the levels of NF-κB (Figure 18C, densitometric analysis C1), but not for the anti-inflammatory protein IκB-α (Figure 18A, densitometric analysis A1). Finally, considering that the activation of the NF-κB pathway led to the pro-inflammatory cytokines, by western blot analysis we evaluated the expression levels of IL-6 in the TBI group, which showed higher levels of this cytokine, than the control group (Figure 18B, densitometric analysis B1). The doses of 2.5 and 5mg/kg were able to counteract the release of IL-6 (Figure 18B, densitometric analysis B1), modulating the inflammation process after head trauma. Oral treatment with SUN11602, at the dose of 1 mg/kg was not significantly effective (Figure 18A, densitometric analysis A1).

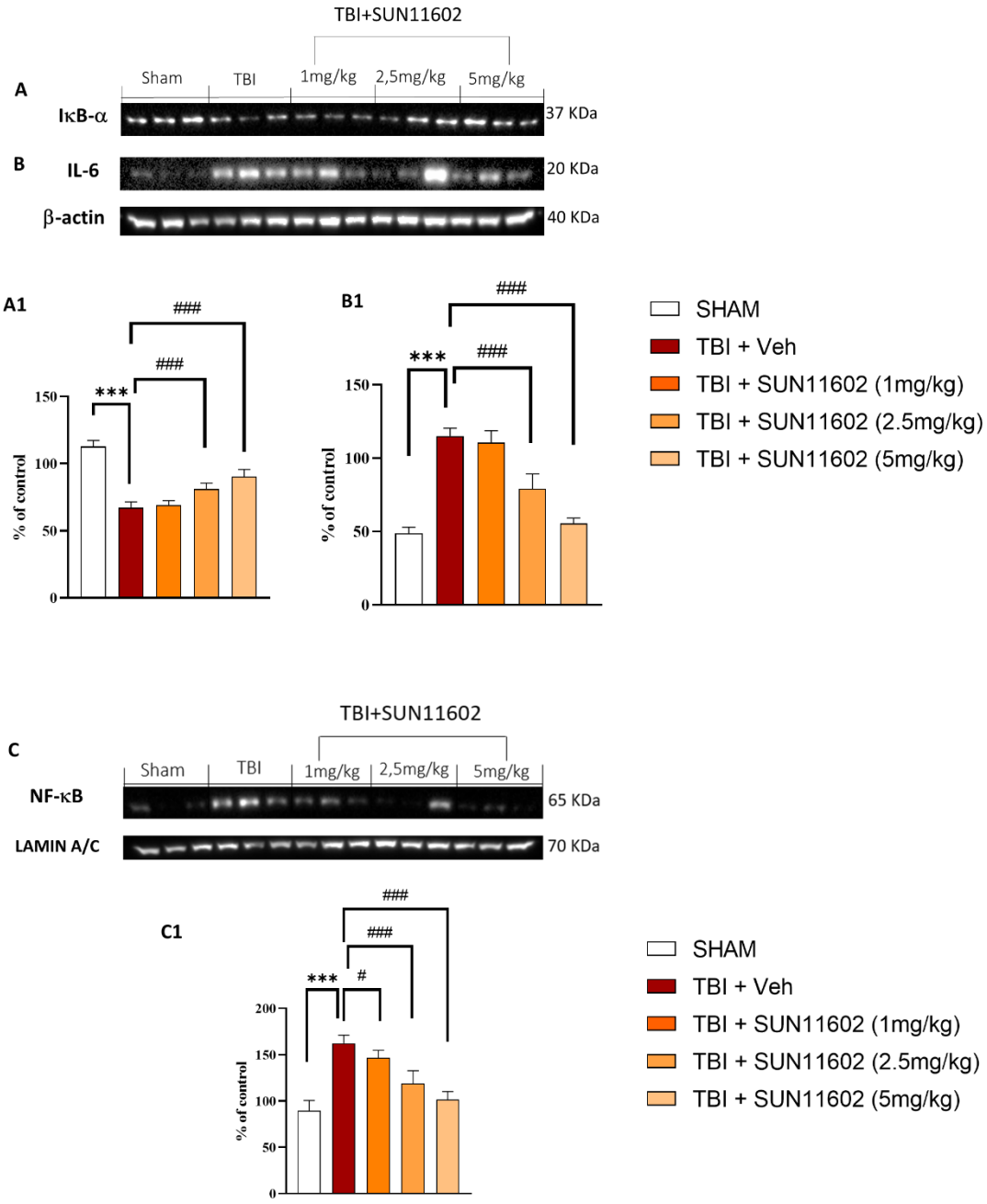

Figure 18. Neuroprotective effect of SUN11602 on inflammatory response after TBI. Western blot analysis reported the expression of inflammatory markers, such as NF-κB, IκB-α and IL-6 **(A-C, densitometric analysis A1-C1)**. SUN11602 at the doses of 2.5 and 5 mg/kg restored basal levels of these proteins compared with the TBI group **(A-C, densitometric analysis A1-C1),** increasing the expression levels of IkB-a and decreasing NF-kB and IL-6. Instead, SUN11602 was not significantly effective in modulating the inflammation response **(A-C, densitometric analysis A1-C1)**. Data are expressed as mean ± SEM from N=10 mice for each group. One-way ANOVA test ($p < 0.05$) followed by Bonferroni post-hoc test for multiple comparisons. ***$p < 0.001$ vs sham; ### $p < 0.001$ vs TBI.

5.1.4. SUN11602 reduced lipid peroxidation and nitrosative stress following TBI.

Another important mediator highly expressed during the inflammatory response and associated with delayed neuronal death following TBI is COX-2, whose upregulation is controlled by NF-κB. The western blot analysis for COX-2 showed that, after 24h of head trauma, there is an increase of the expression of this pro-inflammatory protein (Figure 19A, densitometric analysis A1) compared to the control group, in which the COX-2 level is the baseline (Figure 19A, densitometric analysis A1). Oral treatment with SUN11602, at doses of 2.5 and 5 mg/kg, reduced COX-2 expression, modulating the inflammatory process. Moreover, to determine the role of nitric oxide (NO) produced during TBI, iNOS and neural nitric oxide synthase (nNOS) expression were evaluated by western blot and immunohistochemistry analysis. In the western blot analyses, the control group showed a low expression of iNOS (Figure 19E, densitometric analysis E1) compared to the mice subjected to TBI (Figure 19E, densitometric analysis E1). Steal, the treatment with SUN11602, at doses of 2.5 and 5 mg/kg, modulated the iNOS expression (Figure 19E, densitometric analysis E1) and, this analysis was confirmed by immunohistochemistry staining for iNOS, showing a lack of cells positive staining for iNOS in the sham group (Figure 19A-A1, score F), as well as in the group treated with SUN11602, at the doses of 2.5 and 5 mg/kg (Figure 19D-E1, score F), compared to the TBI group (Figure 19B-B1, score F). Regarding the 1 mg/kg dosage of SUN11602, its effect on the modulation of inflammation caused by lipid peroxidation and nitrosative stress, is ambiguous in the analyses performed, as it does not show the same efficacy among the proteins examined. Similarly, immunohistochemical analysis for nNOS, demonstrated that SUN11602 at the doses of 2.5 and 5 mg/kg was able to reduce the immunopositivity for nNOS (Figure 19J-K1, score L) compared to the TBI group which showed a high grade of positive staining for nNOS (Figure 19H-H1, score L).

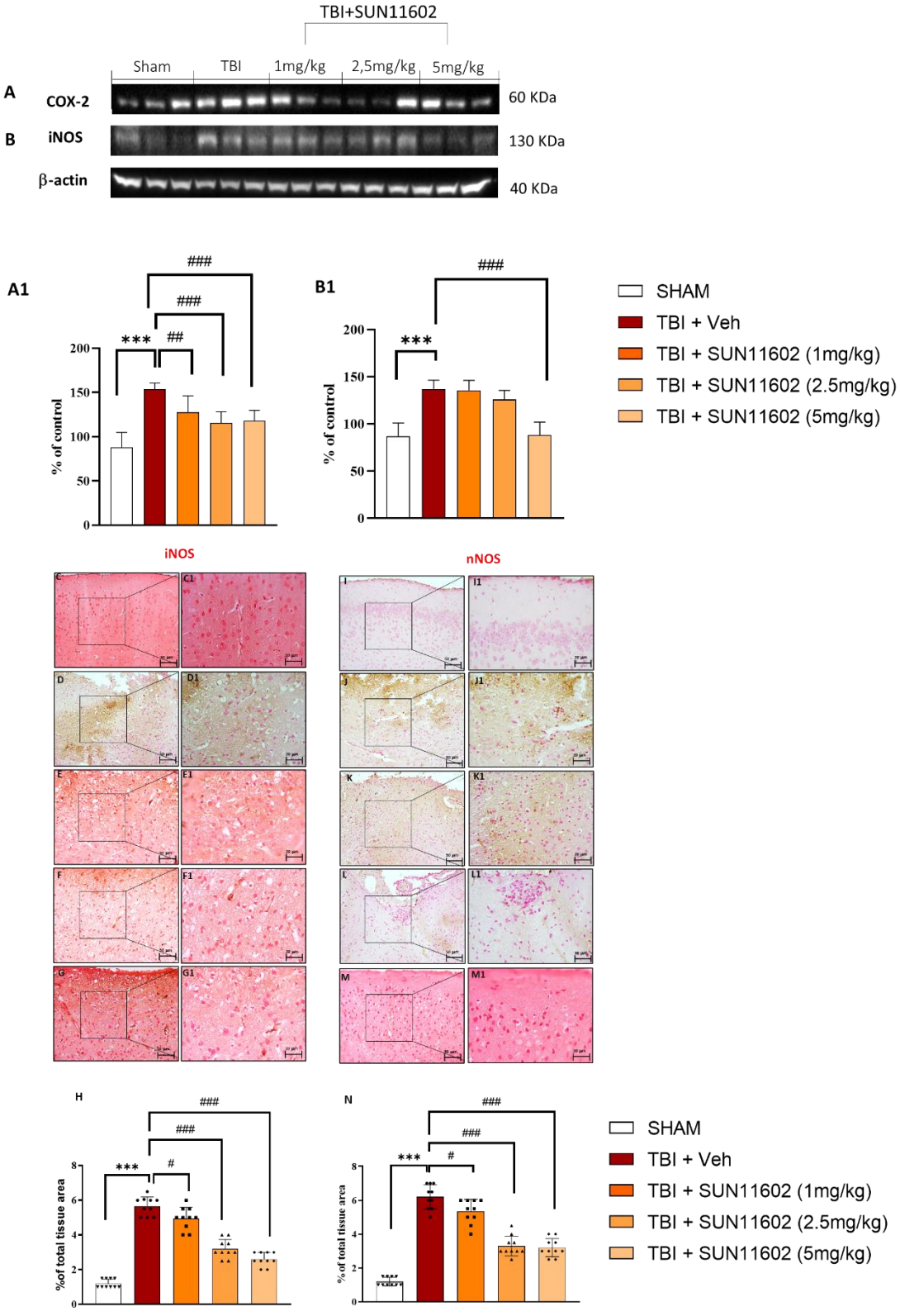

Figure 19. SUN11602 reduced lipid peroxidation and nitrosative stress following TBI. In the western blot analysis, COX-2 and iNOS expressed were highly elevated in the TBI group than in the control group **(A-B, densitometric analysis A1-B1)**. SUN11602 oral treatment, at doses of 2.5 and 5 mg/kg reduced COX-2 and iNOS levels, but not for SUN11602 at the dose of 1mg/kg, which exerted its effect only on COX-2 levels expression **(A-B, densitometric analysis A1-B1)**. To confirm the effect of SUN11602 on the lipid peroxidation and nitrosative stress, the

immunohistochemical analysis demonstrated that the animal group subjected to TBI showed significantly increased iNOS and nNOS reactivity **(D-D1, score H; J-J1, score N)**, compared with the control group **(C-C1, score H; I-I1, score N)**. SUN11602 treatment, at doses of 2.5 and 5 mg/kg, is able to reduce the inflammatory response by TBI-induced **(F-G1, score H; L-M1, score N)**. SUN11602 at the dose of 1 mg/kg has reduced only slightly iNOS and nNOS positive cells staining **(E-E1, score H; K-K1, score N).** Data are expressed as mean ± SEM from N = 10 mice for each group. One-way ANOVA test ($p < 0.05$) followed by Bonferroni post-hoc test for multiple comparisons. ***$p < 0.001$ vs sham; # $p < 0.05$ vs TBI; ## $p < 0.01$ vs TBI; ### $p < 0.001$ vs TBI.

5.1.5. Protective effect of SUN11602 treatment to reduce apoptosis TBI-induced.

Apoptosis generally represents a defense mechanism that the cell implements to safeguard adjacent cells, allowing their survival[451]. Although the damage induced by TBI is acute, this represents a springboard to trigger a series of processes that end with neuronal death, among these precisely apoptosis[452]. Therefore, the neuroprotective effect of SUN11602 on the apoptotic process was assessed by immunohistochemical staining on bcl-2 and bax proteins, followed by western blot analysis on the levels of bcl-2 and p53 proteins as further confirmation. Firstly, the immunohistochemical analysis detected that at 24h after TBI it is possible to observe a decrease of bcl-2 positive staining (Figure 20B-B1, score F), compared to the sham group (Figure 20A-A1, score F). Oral administration of SUN11602, at doses of 2.5 and 5 mg/kg, increased bcl-2 cells positive staining (Figure 20D-E1, score F), while SUN11602, at the dose of 1 mg/kg, did not exert a protective effect on the apoptosis process (Figure 20C-C1, score F). Moreover, western blot analysis, of this anti-apoptotic protein, showed a low expression of bcl-2 in mice with TBI (Figure 20G, densitometric analysis G1), compared with the sham group (Figure 5G, densitometric analysis G1). As confirmation of IHC staining, the dose 1mg/kg of SUN11602 did not reduce the apoptosis process (Figure 20G, densitometric analysis G1) than SUN11602 at doses of 2.5 and 5 mg/kg showed a restoration of bcl-2 expression also in western blot analysis (Figure 20G, densitometric analysis G1). Similarly, we evaluated the immunopositivity of the pro-apoptotic protein, bax, in the TBI group (Figure 20I-I1, score M) compared with the sham group (Figure 20H-H1, score M). SUN11602 at the dose of 1mg/kg has reduced in a slightly insignificant way (Figure 20J-J1) and the doses 2.5 and 5mg/kg, of the ora treatment with SUN1102, reduced bax-positive cells staining (Figure 20K-L1, score M). Finally, we evaluated pro-apoptotic protein expression p53 by western blot analysis, which showed an increased expression in mice subjected to head trauma (Figure 20H, densitometric analysis H1) compared with the control group (Figure

20H, densitometric analysis H1). Through oral treatment with SUN11602, at doses of 2.5 and 5 mg/kg, we demonstrated a significant decrease of p53 expression, especially at the dose of 5 mg/kg; SUN11602 at the dose of 1 mg/kg, was not able to contain apoptosis process (Figure 20N, densitometric analysis N1).

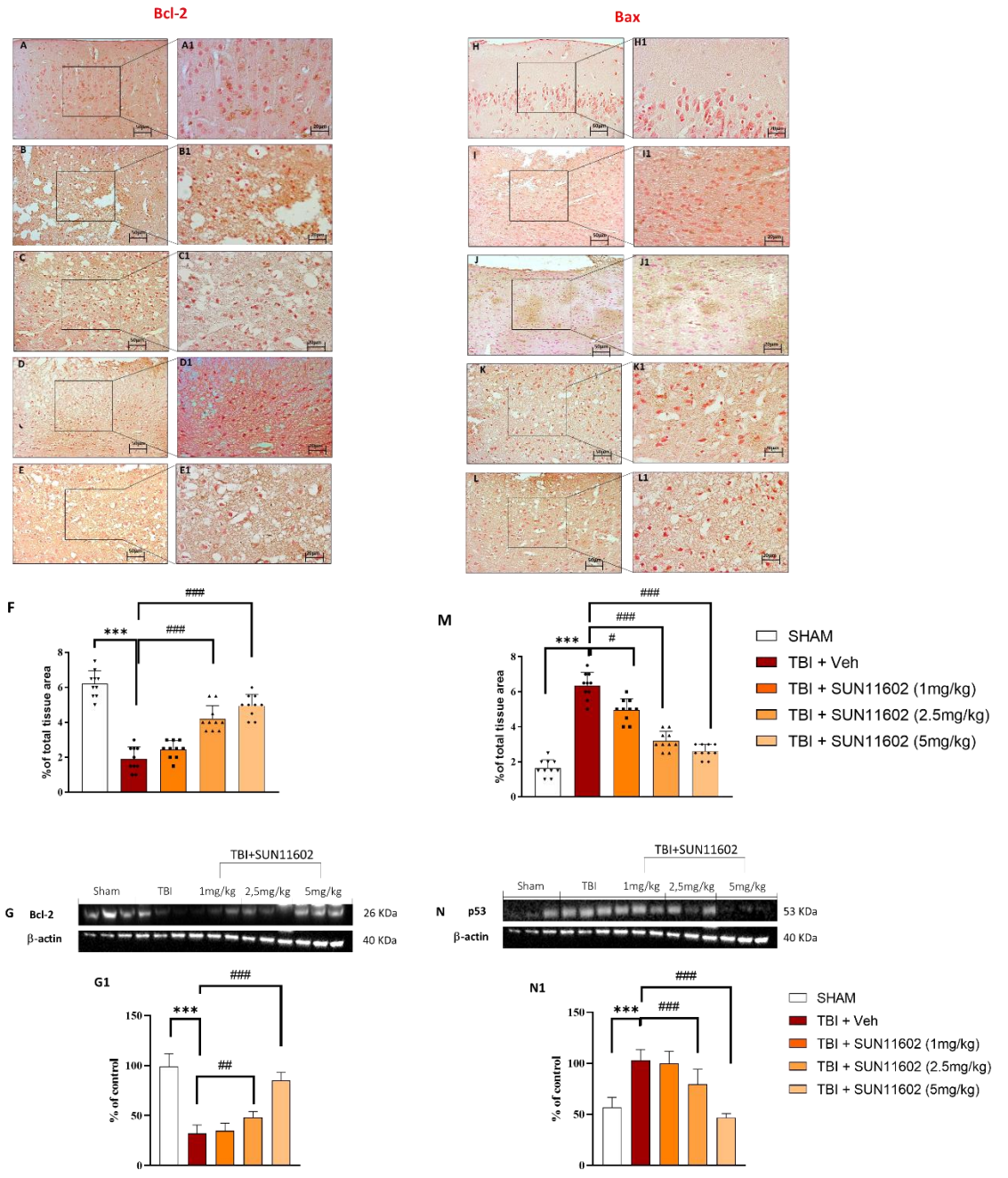

Figure 20. Effects of SUN11602 on apoptosis after TBI. The immunohistochemical analysis reported that the positive staining for bcl-2 was significantly reduced in TBI mice **(B-B1, score F)** compared to the control group **(A-A1, score F)**. Whereas SUN11602 treatment, at doses of 2.5 and 5 mg/kg, significantly restored bcl2 expression levels **(D-E1, score F)**. The treatment w ith SUN11602 at the dose of 1mg/kg was not effective to modulate the apoptotic process **(C-C1, score F).** Western blot analysis reported that, at 24h after TBI, mice showed a decrease of bcl-2

levels in the group (**G, densitometric analysis G1**). bcl-2 expression increased with SUN11602 oral treatment, in particular the doses of 2.5 and 5 mg/kg **(G, densitometric analysis G1)**. Similarly, immunohistochemical analysis demonstrated that in the TBI group, bax expression is elevated (**I-I1, score M**) than in the control group that reported basal levels of pro-apoptotic protein Bax (**H-H1, score M**). SUN11602 at doses of 2.5 and 5 mg/kg can restore the bax expression (**K-L1, score M**), while SUN11602 at the dose of 1 mg/kg has reduced in a slightly insignificant way (**J-J1, score M**). Moreover, western blot analysis showed that p53 expression was observed in TBI-induced mice compared to the sham group **(N, densitometric analysis N1)**. Treatment with SUN11602 reduced the expression of p53 **(N, densitometric analysis N1)**. Data are expressed as mean ± SEM from N=10 mice for each group. One-way ANOVA test ($p < 0.05$) followed by Bonferroni post hoc test for multiple comparisons. ***$p < 0.001$ vs sham; # $p < 0.05$ vs TBI; ## $p < 0.01$ vs TBI; ### $p < 0.001$ vs TBI.

5.1.6. SUN11602 regulated calcium homeostasis into CNS after TBI

Calcium plays an important role in the development of the CNS and in neuron growth. For the correct functioning of this ion, Ca^{2+}-dependent proteins, such as Calbind-D28K (Calbindin), S100-β and Calmodulin, maintain Ca^{2+} homeostasis. Several studies indicate that brain trauma generates an alteration in the Ca^{2+} homeostasis[453], specifically causing an increase in intracellular Ca^{2+} flux. This imbalance heightens the vulnerability to neuronal damage, ultimately resulting in cell death. Following the TBI, the expression of these proteins is altered; in this regard, by western blot analysis, our results showed an increase in the expression of Calbindin and S100-β (Figure 21A-B, densitometric analysis A1 and B1, respectively), compared to the control group (Figure 21A-B, densitometric analysis A1 and B1, respectively). Oral treatment with SUN11602, at doses of 2.5 and 5 mg/kg, seems to contain excessive levels of intracellular Ca^{2+} (Figure 21A-B, densitometric analysis A1 and B1, respectively), contributing to restoring Ca^{2+} homeostasis. Instead, SUN11602 at the dose of 1 mg/kg, has a less effective action than the higher doses used in this study (Figure 21A-B, densitometric analysis A1 and B1, respectively), still managing to contain the high amount of intracellular calcium. For further confirmation of the control of SUN11602 on the excessive intracellular calcium flux, the colorimetric calcium kit that we performed to evaluate the calcium concentration (Figure 21C). As just described, mice that had suffered TBI showed an increase in intracellular Ca concentration (Figure 21C). Oral treatment with SUN1160 at increasing doses (1- 2.5 and 5mg/kg) re-establishes a balance of Ca^{2+} levels, by binding to Calcium-coupled proteins (Figure 21C), thus restoring its homeostasis altered following TBI.

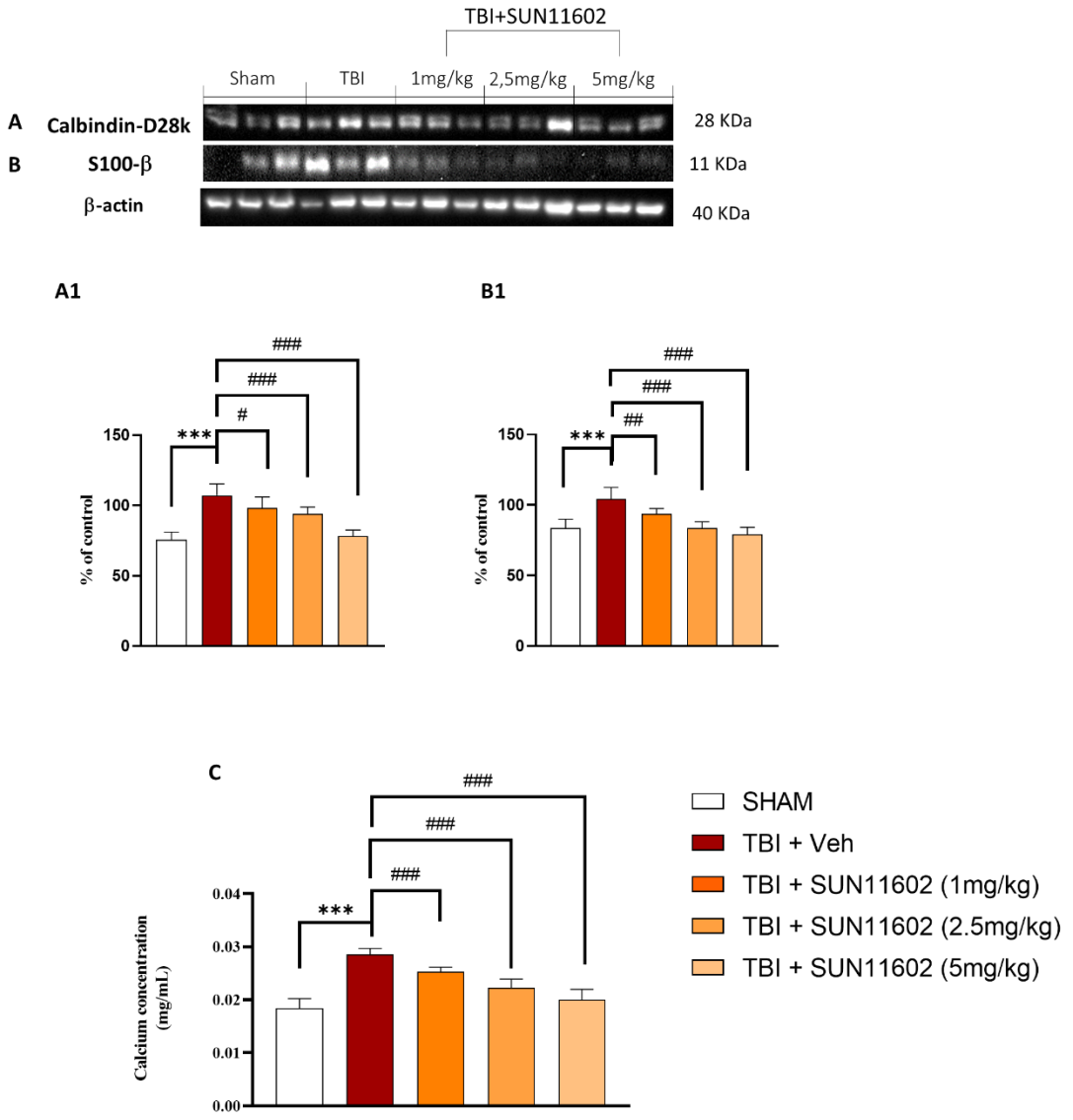

Figure 21. SUN11602 modulated calcium-binding protein levels. Western blot analysis showed an increase of Calbindin and S-100β in mice subjected to TBI-induced **(A-B, densitometric analysis A1-B1)** compared to the sham group, in which the levels of these proteins are basal **(A-B, densitometric analysis A1-B1).** SUN11602 treatment, at the doses of 2.5 and 5 mg/kg, is capable to restore the expression levels of Calbindin and S-100β **(A-B, densitometric analysis A1-B1).** The graph of the colorimetric calcium kit showed an increase in Calcium concentration in mice subjected to TBI-induced **(C)** compared to the sham group **(C)**. Oral treatment of SUN11602, at doses of 2.5 and 5 mg/kg, reduced the calcium concentration **(C),** restoring almost basal levels of calcium. While SUN11602 at a dose of 1mg/kg reduces, although less efficiently, elevated Ca concentrations **(C)**. Data are expressed as mean ± SEM from N = 10 mice for each group. One-way ANOVA test ($p < 0.05$) followed by Bonferroni post hoc test for multiple comparisons. ***$p < 0.001$ vs sham; # $p < 0.05$ vs TBI; ### $p < 0.001$ vs TBI.

5.1.7. SUN11602 treatment modulated neurotrophic factors and neurogenesis process.

The molecular processes triggered following TBI, especially neuroinflammation, alter the levels of neurotrophins, molecules with a related structure[454], reducing them. The

reduction of these factors in turn contributes to neuronal damage, directing the cell towards death. Therefore, based on this knowledge, the aim was to determine the neuroprotective effect of SUN11602 at 24 hours after TBI, through immunofluorescent staining (IF) of nerve growth factor-3 (NT-3)[455] and brain-derived neurotrophic factor (BDNF) (Figure 22). Furthermore, considering the low efficiency of the 1 mg/kg dose of SUN11602 in acting on the molecular mechanisms involved in head trauma, this last analysis was conducted exclusively using only the 2.5 and 5 mg/kg doses of SUN11602. Following TBI, a reduction in the number of NT-3 and BDNF positive cells was observed (Figure 22A-D, score E; F-I, score J), compared to the control group (Figure 22A and F, score E and J). Oral treatment of SUN11602, at doses of 2.5 and 5 mg/kg, restored the number of NT-3 and BDNF-positive cells (Figure 22C-D, score E; H-I, score J). SUN11602 at the dose of 1 mg/kg was not reported in this analysis as it was ineffective in the previous analyses.

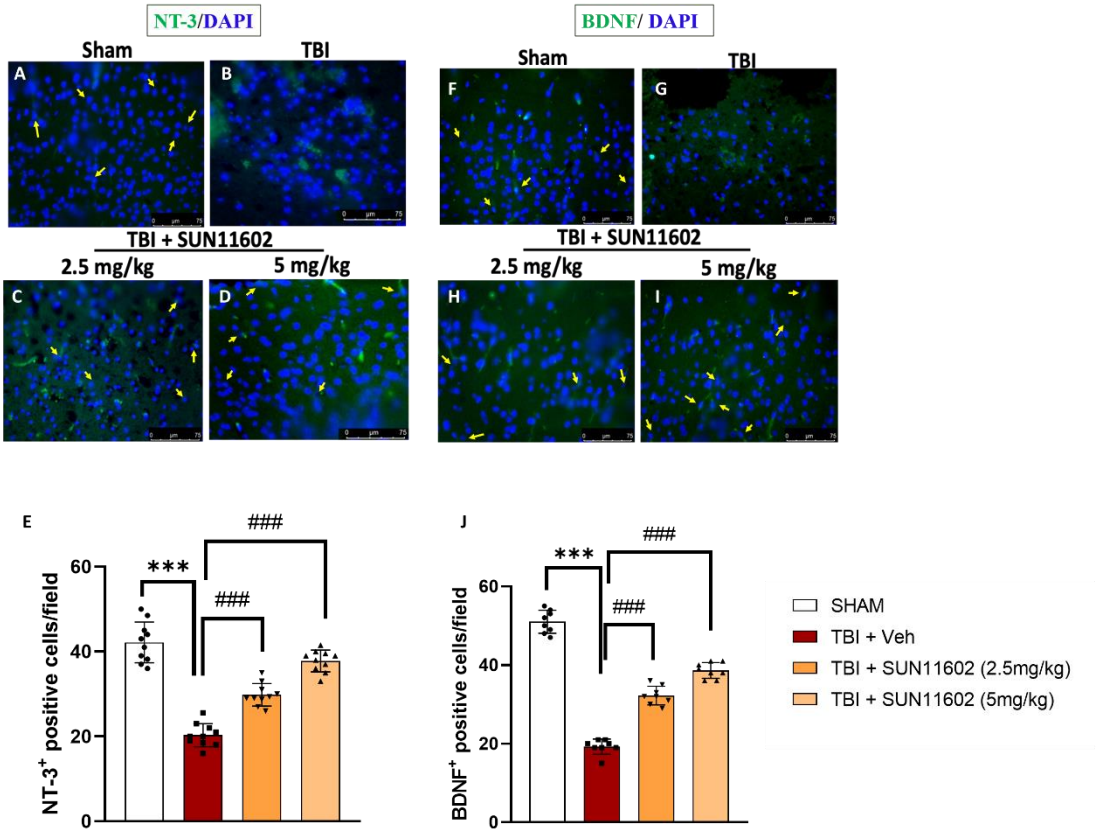

Figure 22. Effect of acute TBI on Neurotrophic factors. Immunofluorescence analysis detected a basal level of BDNF and NT-3 positive staining in brain samples from control mice **(A and F, scores E and J)**. BDNF and NT-3 expression was significantly reduced at 24h after TBI **(B and G, scores E and J)**. SUN11602 treatment, at the doses of 2.5 and 5 mg/kg enhanced the neuroprotective effect post-TBI **(C-D score E; H-I, score J)**. Data are expressed as mean ± SEM

from N= 10 mice for each group. One-way ANOVA test ($p < 0.05$) followed by Bonferroni post-hoc test for multiple comparisons. ***$p < 0.001$ vs sham; ## $p < 0.01$ vs TBI; ### $p < 0.001$ vs TBI.

5.2. PD study

5.2.1. SUN11602 administration ameliorated behavioural impairments induced by MPTP intoxication

Behavioural tests were performed in all the experimental groups. The pole test was performed to evaluate the motor alteration and bradykinesia caused by MPTP intoxication. The pole test showed that "Time to turn" and "Total time" increased in MPTP-injected mice compared to the Sham group (Figure 23A-B). SUN11602 5 mg/kg administration showed an important decrease in "Time to turn" and "Total time" compared to the MPTP group, thus suggesting a strong reduction in bradykinesia (Figure 23A-B). A slight but significant reduction was also found in the SUN11602 2.5 mg/kg group (Figure 23A-B). Contrarily, SUN11602 1 mg/kg did not show considerable improvement in the behavioural test (Figure 23A-B). EMP reported an increase in the percentage of time spent in the closed arm by MPTP-intoxicated mice compared to the Sham group (Figure 23C-D). Instead, it was interesting to note that SUN11602 2.5 mg/kg, and especially at the dose of 5 mg/kg, effectively reduced the time spent in the closed arms compared to MPTP mice (Figures 23C-D). There was no significant improvement with SUN11602 1 mg/kg administration (Figure 23C-D). Sham + SUN11602 administered groups were comparable to Sham + vehicle animals (Figure 23A–D).

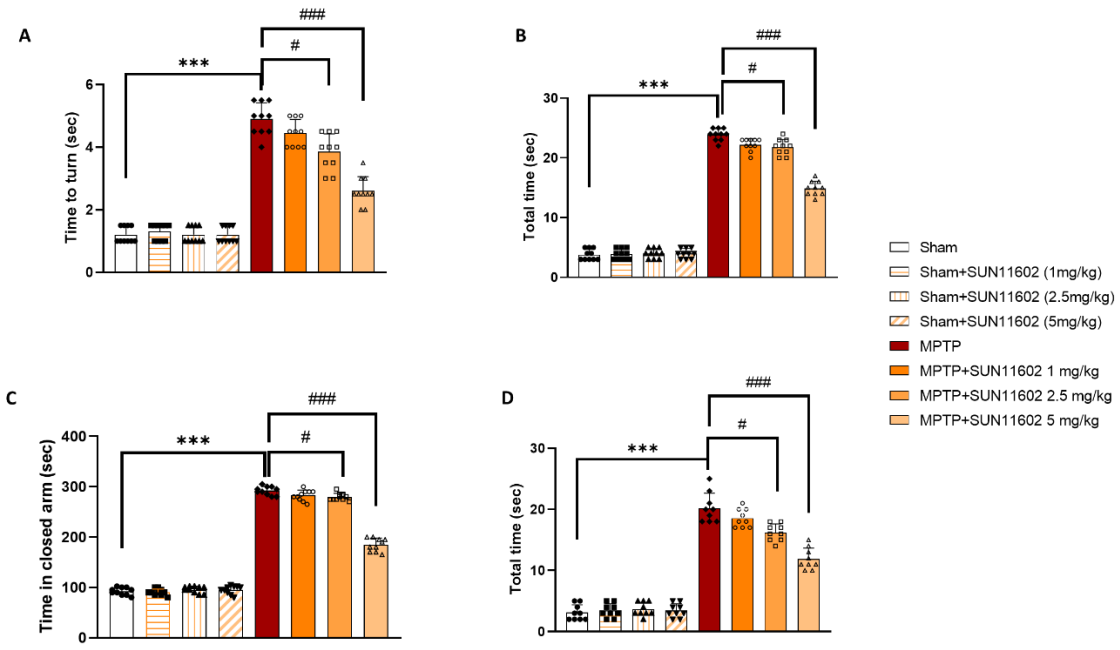

Figure 23. Effect of SUN11602 on behavioural impairments induced by MPTP. 7 days after MPTP injection mice showed a significant increase in behavioural deficits compared to the Sham group **(A–D)**. Contrarily, SUN11602, at the two highest doses, considerably decreases "Time to turn" and "Time in closed arms" **(A-D)**. Sham + SUN11602 administered groups were comparable to Sham + vehicle animals **(A–D)**. Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; $^{\#}p < 0.05$ vs MPTP; $^{\#\#\#}p < 0.001$ vs MPTP

5.2.2. SUN11602 administration reduced loss of TH expression following MPTP injection

To confirm the neuroprotective effect of SUN11602 after MPTP intoxication, we evaluated TH expression by stereological analysis and immunohistochemical staining (Figure 24Panel A). As evidenced by stereological analysis MPTP-injected mice exhibited a substantial loss in the number of TH^+ neurons (Figure 24Panel A: B-B1, score F) compared to the Sham animals (Figure 24Panel A: A-A1, score F). SUN11602 1 mg/kg administration did not induce any considerable increase (Figure 24Panel A: C-C1, score F). Contrarily, SUN11602 treatment at the dose of 2.5 mg/kg (Figure 24Panel A: D-D1, score F), and especially at 5 mg/kg, considerably preserved the number of TH^+ neurons (Figure 24Panel A: E-E1, score F). Figure 24Panel B shows the immunohistochemical analysis, in which is reported a decrease of TH-positive neurons, 7 days after in mice with MPTP injection (Figure 24Panel B: H-H1, score L) compared to the Sham group (Figure 24Panel B: G-G1, score L). SUN11602 1 mg/kg treatment did not show any significant improvement (Figure 24Panel B: I-I1, score L). However,

treatment with SUN11602 at a dose of 2.5 mg/kg, and even more significantly at 5 mg/kg, restored TH expression levels (Figure 24Panel B: J-J1 and K-K1, respectively; score L).

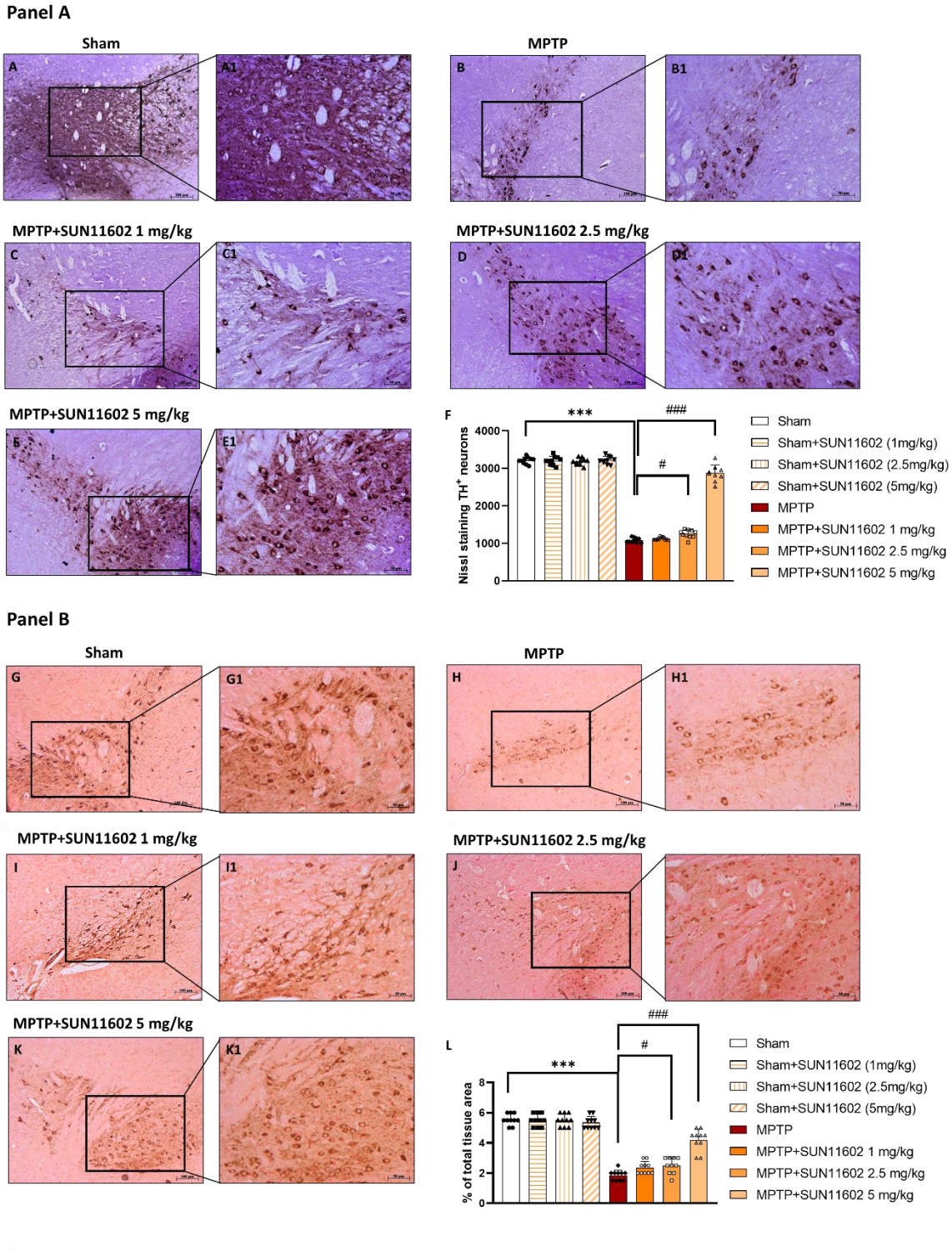

Figure 24. Effect of SUN11602 treatment on TH expression. MPTP mice exhibited an extensive loss of TH^+ neurons **(Panel A: B-B1, score F)**, compared to the Sham group **(Panel A: A-A1, score F)**. SUN11602 2.5 mg/kg treatment, and especially at the dose of 5 mg/kg, restored the numbers of TH^+ neurons **(Panel A: D-D1; E-E1, score F)**. SUN11602 1 mg/kg was ineffective **(Panel A: C-C1, score F)**. MPTP-injured mice revealed a marked loss of TH^+ cells **(Panel B: H-H1, score L)** compared to the Sham group (Panel B: G, G1, score L). SUN11602 2.5 mg/kg, and more powerfully 5 mg/kg, counteracted the degeneration of dopaminergic neurons **(Panel B: J-J1; K-K1, score L)**. SUN11602 1 mg/kg proved to be ineffective **(Panel B: I-I1,**

score L). Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; $^{\#}p < 0.05$ vs MPTP; $^{\#\#\#}p < 0.001$ vs MPTP

5.2.3. SUN11602 prevented dopamine transporter (DAT) depletion from MPTP toxicity

We evaluated DAT expression in the substantia nigra to investigate the neuroprotective effects of SUN11602 treatment on the dopamine pathway, as shown in Figure 25Panel A. A significant loss of DAT-positive staining was detected in MPTP-injected mice (Figure 25Panel A: B-B1, score F) compared to the Sham group (Figure 25Panel A: A-A1, score F). The restoration of DAT levels in this portion was remarkable following SUN11602 2.5 mg/kg administration (Figure 25Panel A: D-D1, score F) and more effective after SUN11602 5 mg/kg treatment (Figure 25Panel A: E-E1, score F), compared to the MPTP group. Meanwhile, SUN11602 1 mg/kg treatment did not improve DAT expression meaningfully (Figure 25Panel A: C-C1, score F). Moreover, considering the striatum a major locus of dopamine action, we also evaluated DAT expression in this brain area by immunohistochemical localization (Figure 25Panel B). MPTP-intoxicated mice exhibited a significant reduction in DAT expression (Figure 25Panel B: H-H1, score M), compared to the Sham group (Figure 25Panel B: G-G1, score M). SUN11602 2.5 mg/kg treatment, (Figure 25Panel B: K-K1, score M) and in a more significant way SUN11602 5 mg/kg administration (Figure 25Panel B: L-L1, score M), showed a considerable restoration of DAT levels. SUN11602 1 mg/kg did not induce significant increases in DAT levels (Figure 25Panel B: J-J1, score M).

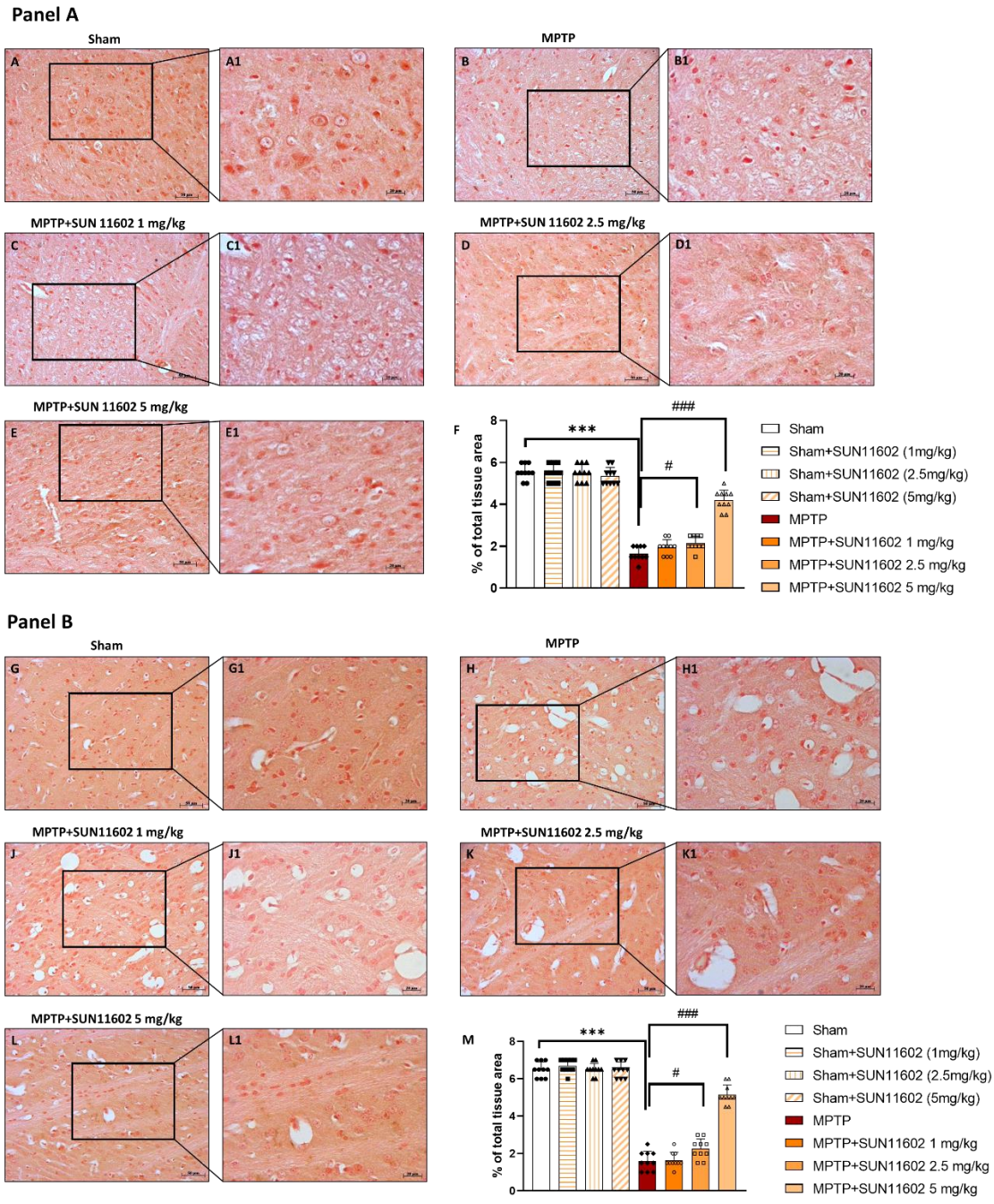

Figure 25. Effect of SUN11602 on DAT expression. MPTP-intoxicated mice showed a significant decrease in DAT expression in substantia nigra **(Panel A: B-B1, score F)** compared to the Sham group **(Panel A: A-A1, score F)**. SUN11602 administration, at the doses of 2.5 **(Panel A: D-D1, score F)** and more meaningfully at 5 mg/kg **(Panel A: E-E1, score F)** resulted in neuroprotective. SUN11602 1 mg/kg didn't demonstrate significant efficacy **(Panel A: C-C1, score F)**. MPTP group showed a significant reduction of DAT expression in the striatum **(Panel B: H-H1, score M)**, compared to the Sham group **(Panel B: G-G1, score M)**. Dose-dependent response to DAT recovery was detected following treatment with SUN11602 **(Panel B: J-J1; K-K1; L-L1, score M)**. Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. $^{***}p < 0.001$ vs Sham; $^{\#}p < 0.05$ vs MPTP; $^{\#\#\#}p < 0.001$ vs MPTP

5.2.4. Effect of SUN11602 on dopamine metabolites after MPTP intoxication

Degeneration of the nigrostriatal innervation leads to the loss of dopaminergic cells. Therefore, we evaluated the effect of SUN11602 on dopamine metabolism, measuring the striatal levels of dopamine and its metabolites: DOPAC and HVA. MPTP intoxication reduced striatal dopamine, DOPAC, and HVA levels which appeared around 20%, respectively. SUN11602 1 mg/kg treatment did not block this MPTP-induced loss. By contrast, SUN11602 at 2.5 mg/kg, particularly at 5 mg/kg, significantly restored dopamine and its metabolite levels to approximately 65% (Figure 26A–C).

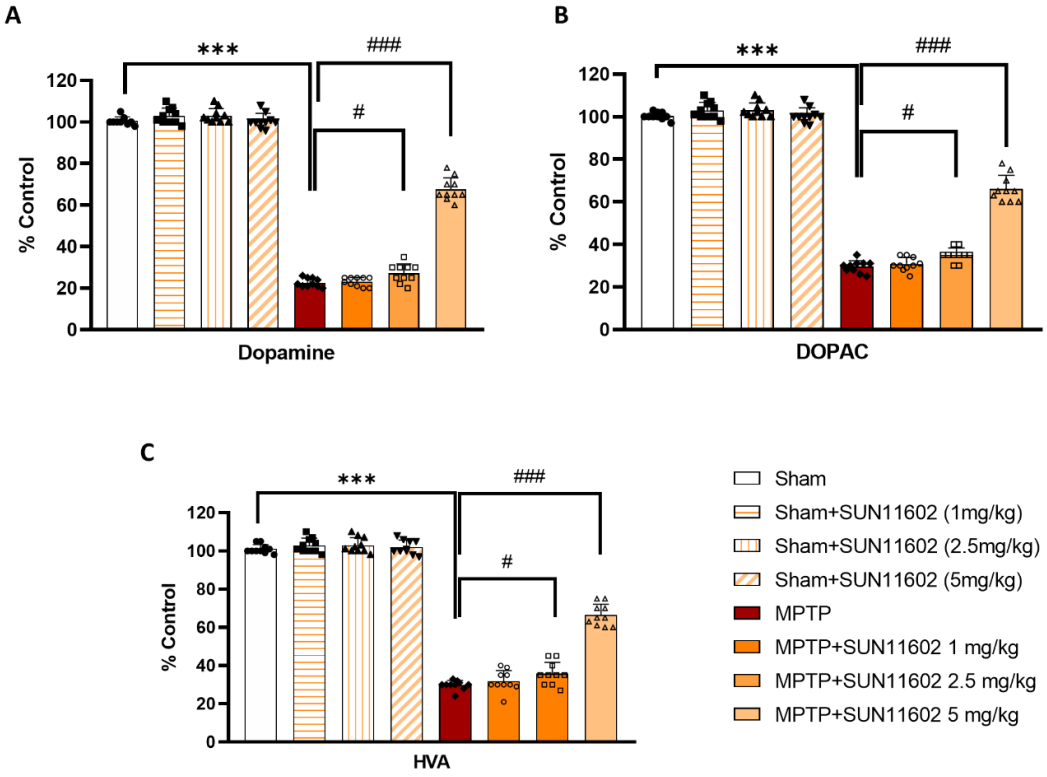

Figure 26. Effect of SUN11602 on dopamine metabolites after MPTP intoxication. MPTP-injected animals exhibited a considerable loss of dopamine and its metabolites, compared to the Sham mice; contrarily, treatment with SUN11602 in a dose-dependent manner increased metabolites levels (**A**–**C**). Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; $^{\#}p < 0.05$ vs MPTP; $^{\#\#\#}p < 0.001$ vs MPTP

5.2.5. Effect of SUN11602 on α-syn accumulation induced by MPTP intoxication

Aggregation of cytoplasmatic α-syn in the dopaminergic neurons of the substantia nigra is a typical PD characteristic[456]. To assess the neuroprotective effect of SUN11602, we performed immunohistochemistry staining. Here, we demonstrated that SUN11602, at

the dose of 2.5 mg/kg (Figure 27D-D1, score F), and more considerably at 5 mg/kg (Figure 27E-E1, score F), was able to counteract the deposition of α-syn in dopaminergic neurons compared to MPTP mice (Figure 27B-B1, score F), thus playing a neuroprotective role. SUN11602 1 mg/kg didn't demonstrate significant efficacy (Figure 27C-C1, score F). The Sham group showed basal levels of α-syn (Figure 27A-A1, score F). To further assess the neuroprotective effect of SUN11602 against α-syn accumulation, we also evaluated p-α-syn form as a peculiar protein implicated in the pathogenesis of PD. Elevated p-α-syn levels were found in the MPTP group compared to the Sham mice (Figure 27G). SUN11602, in a dose-dependent manner, decreased p-α-syn levels, resulting in particularly effective at the dose of 5 mg/kg (Figure 27G). Considering these preliminary results, we decided to continue our experiments analysing only SUN11602 5 mg/kg as the most effective dose in counteracting MPTP-induced nigrostriatal degeneration.

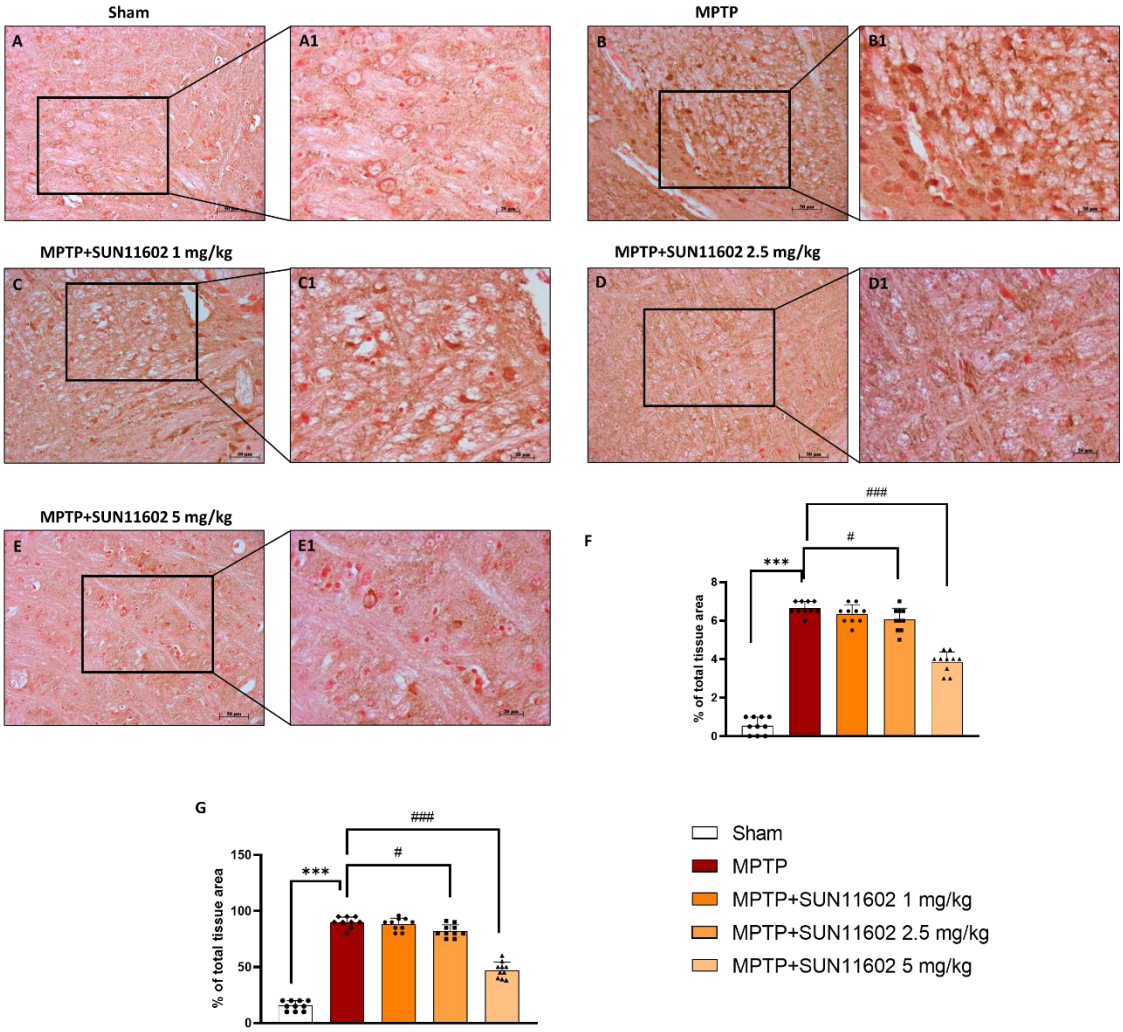

Figure 27. SUN11602 administration preserved α-syn accumulation. MPTP-intoxicated showed an increase in the number of α-syn aggregates in dopaminergic neurons **(B-B1, score F)**, compared to the Sham group **(A-A1, score F)**. The neuroprotective role of SUN11602, at doses of 2.5 mg/kg and 5 mg/kg, in MPTP-injected mice is revealed by the reduction in the number of α-syn aggregates **(D-D1 and E-E1, score F)**. SUN11602 1 mg/kg was ineffective in diminishing α-syn accumulation **(C-C1, score F)**. Concordant results were also obtained from ELISA analysis of p-α-syn **(G)**. Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. *** $p < 0.001$ vs Sham; # $p < 0.05$ vs MPTP; ### $p < 0.001$ vs MPTP

5.2.6. Effect of SUN11602 treatment on GFAP, IBA-1 and CD68 expression

The MPTP-induced nigrostriatal dopaminergic degeneration in neurons is accompanied by a substantial increase of astrocyte and microglia activation, which results in an upregulation of the respective markers GFAP and IBA-1 as well as CD68 (Figure 28). Immunofluorescence staining revealed that GFAP and IBA-1 were significantly higher in the MPTP group (Figure 28B and F, respectively, score D and H) compared to the control group (Figure 28A and E, respectively, score D and H) thus denoting reactive astrocyte and microglia. We investigated whether the neuroprotective effects of SUN11602 were associated with the attenuation of these pro-inflammatory markers. Our data reported that the number of GFAP and IBA-1 positive cells was significantly reduced in SUN11602 -treated mice (Figure 28C and G, respectively, score D and H). Furthermore, we evaluated CD68 levels as an additional marker of reactive microglia through an ELISA kit. The results confirmed the ability of SUN11602 to decrease microglia reactivity (Figure 28I).

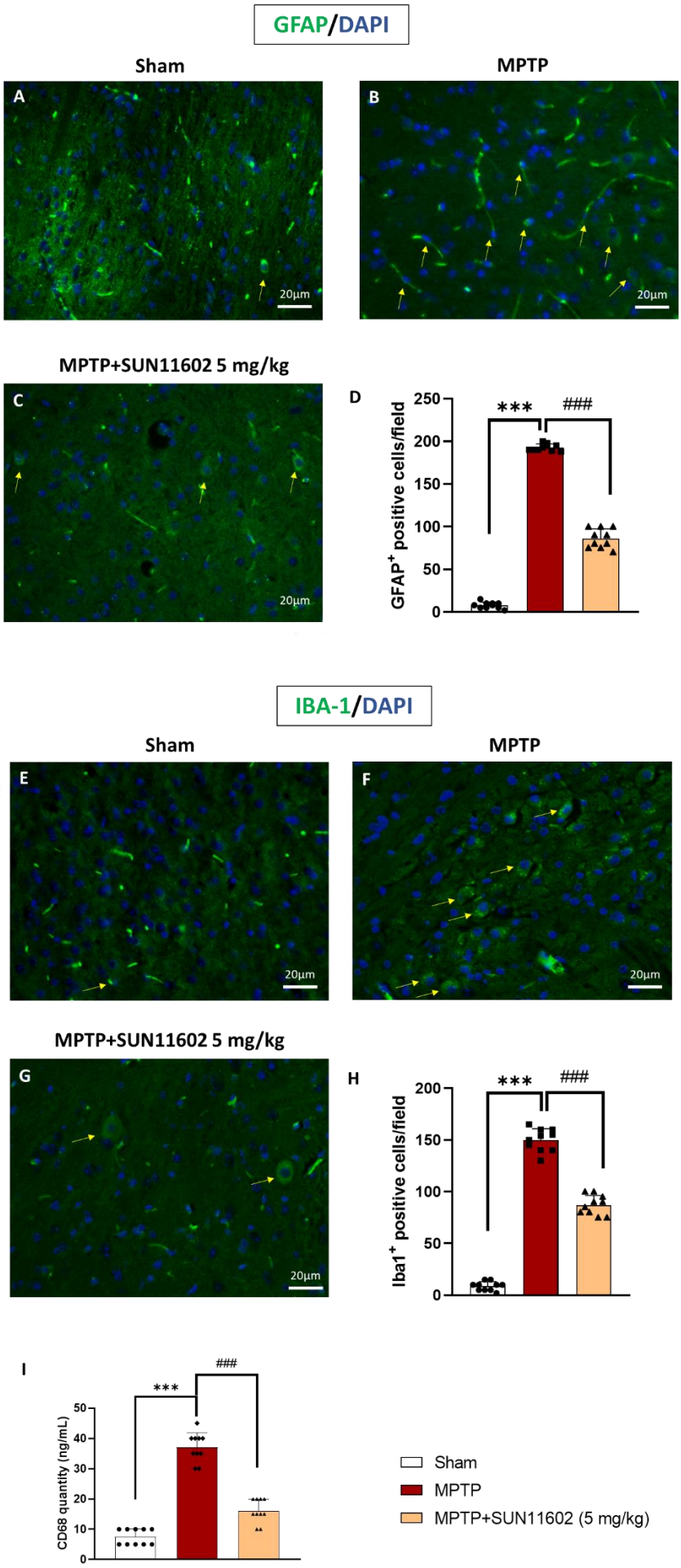
GFAP/DAPI
Sham
MPTP
A
B
20μm
20μm
MPTP+SUN11602 5 mg/kg
C
20μm
D
GFAP+ positive cells/field
250
200
150
100
50
0
***
###
IBA-1/DAPI
Sham
MPTP
E
F
20μm
20μm
MPTP+SUN11602 5 mg/kg
G
20μm
H
Iba1+ positive cells/field
200
150
100
50
0
***
###
I
CD68 quantity (ng/mL)
50
40
30
20
10
0
***
###
Sham
MPTP
MPTP+SUN11602 (5 mg/kg)

Figure 28. Effect of SUN11602 on GFAP, IBA-1 and CD68 expression. Tissues of MPTP-injected mice showed high expression of GFAP (**B, score D**) and IBA-1 (**F, score H**), compared to the Sham groups (**A**, **score D**; **E**, **score H**). GFAP and IBA-1 expression decreased after SUN11602 5 mg/kg administration (**C**, **score D**, and **G**, **score H**, respectively). SUN11602 also decreased CD68 levels (**I**). Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; $^{###}p < 0.001$ vs MPTP

5.2.7. SUN11602 modulated NF-κB pathway and reduced pro-inflammatory cytokines levels induced by MPTP intoxication

In PD, neuroinflammation plays an important role in the neurodegenerative process and the consequent loss of dopaminergic neurons. For this purpose, western blot analysis evaluated the expression of IκB-α and the nuclear translocation of NF-κB p65 (Figure 29). In the MPTP group, we detected an extensive degradation of IκB-α levels, compared to the Sham group, in which basal expression of IκB-α was found (Figure 29A, see densitometric analysis A1). However, SUN11602 5 mg/kg administration significantly restored IκB-α levels (Figure 29A, see densitometric analysis A1). On the other hand, we observed an increase in the nuclear translocation of NF-κB p65 in MPTP-intoxicated mice compared to the Sham group (Figure 29B, see densitometric analysis B1). Such increase was considerably reduced by SUN11602 5 mg/kg treatment (Figure 29B, see densitometric analysis B1). NF-κB is considered crucial for initiating the inflammatory signalling pathway, since it leads to the activation of several pro-inflammatory factors such as IL-1β, IL-6, IL-18 and TNF-α[457]. Our data showed a significant upregulation of IL-1β, IL-6 and IL-18 and TNF-α in MPTP-intoxicated mice compared to the Sham group (Figure 29C-F; see densitometric analysis C1-F1). SUN11602 5 mg/kg administration demonstrated its ability to reduce cytokines levels (Figure 29C–F; see densitometric analysis C1-F1).

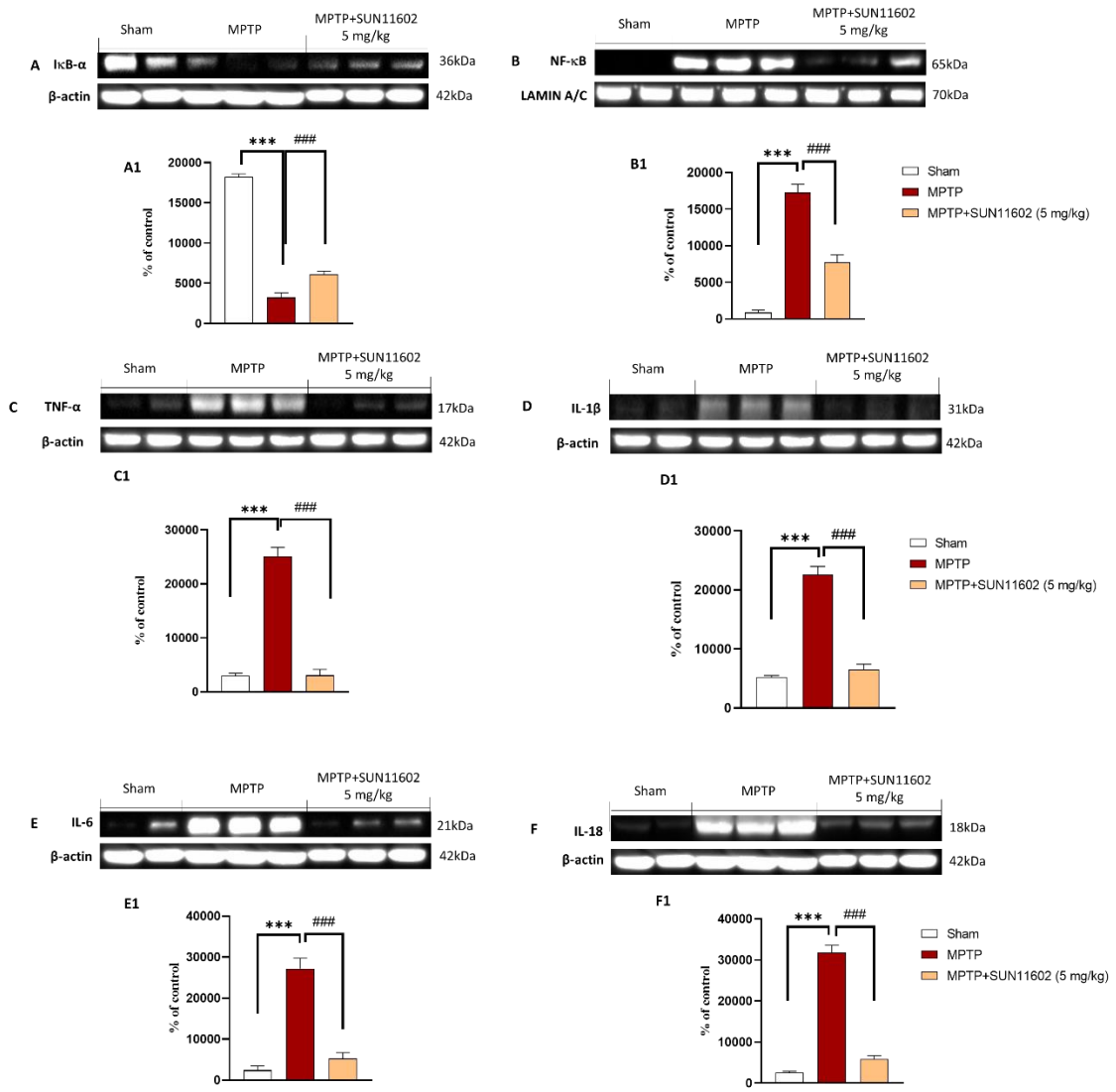

Figure 29. SUN11602 administration reduced neuroinflammation after MPTP injection. Western Blot analysis revealed a decreased expression of IκB-α in MPTP-injected mice, compared to the Sham group **(A, densitometric analysis A1)**. NF-κB levels were elevated in the MPTP-intoxicated mice compared to Sham animals **(B, densitometric analysis B1).** The anti-inflammatory effects of SUN11602 5 mg/kg were confirmed by increased IκB-α levels and at the same time by reduced NF-κB expression **(A-B, densitometric analysis A1-B1)**. A significant upregulation of pro-inflammatory cytokines expression was detected in the MPTP group, compared to the Sham mice **(C–F, densitometric analysis C1–F1)**. These expressions were considerably reduced following SUN11602 5 mg/kg treatment **(C–F, densitometric analysis C1–F1)**. Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; $_{\#\#}p < 0.01$ vs MPTP; $^{\#\#\#}p < 0.001$ vs MPTP

5.2.8. Effect of SUN11602 treatment on microtubule organization and intracellular calcium homeostasis after MPTP intoxication

Calcium is involved in maintaining the basal activity of the CNS, the alteration of its cytoplasmic concentration triggers biochemical events that cause cell damage[458]. Calbindin-D28k and S-100β are important calcium-binding proteins involved in the homeostasis of Ca^{2+} and the regulation of cellular processes, such as cell progression and differentiation. Elevated levels of S-100β and Calpain were reported in neurodegenerative diseases. Accordingly, our results showed an important decrease in Calbindin-D28k expression in MPTP-injected mice (Figure 30A, see densitometric analysis A1). Conversely, SUN11602 5 mg/kg treatment increased, in a significant way, Calbindin-D28k levels (Figure 30A, see densitometric analysis A1). S-100β and Calpain, markers of brain damage, were increased in the MPTP group, compared to the Sham animals (Figure 30B, see densitometric analysis B1 and C, respectively). SUN11602 5 mg/kg treatment was able to significantly reduce S-100β and Calpain levels (Figure 30B, see densitometric analysis B1 and C, respectively), demonstrating a good capability to improve brain damage (Additional file 1). The involvement of two important neuronal markers MAP-2 and β3-tubulin was investigated by immunohistochemical and immunofluorescence staining, respectively. MAP-2 is a cytoskeletal protein, its role is to stabilize the assembly of microtubules, therefore it is considered a marker of synaptic plasticity[459]. Brain tissues from MPTP-injected mice exhibited a reduction of MAP-2 levels (Figure 30E-E1, score G), compared to the Sham group (Figure 30D-D1, score G). SUN11602 5 mg/kg treatment restored the MAP-2 expression (Figure 30F-F1, score G), counteracting the progression of neuronal loss. Moreover, we also examined β3-tubulin expression, as a constitutive protein of the neuronal cell cytoskeleton[460]. We observed an extensive increase in the number of β3-tubulin-positive cells in the MPTP group (Figure 30I, score K), compared to the Sham group (Figure 30H, score K). SUN11602 5 mg/kg administration demonstrated its good capacity to decrease the number of β3-tubulin-positive cells (Figure 30J, score K).

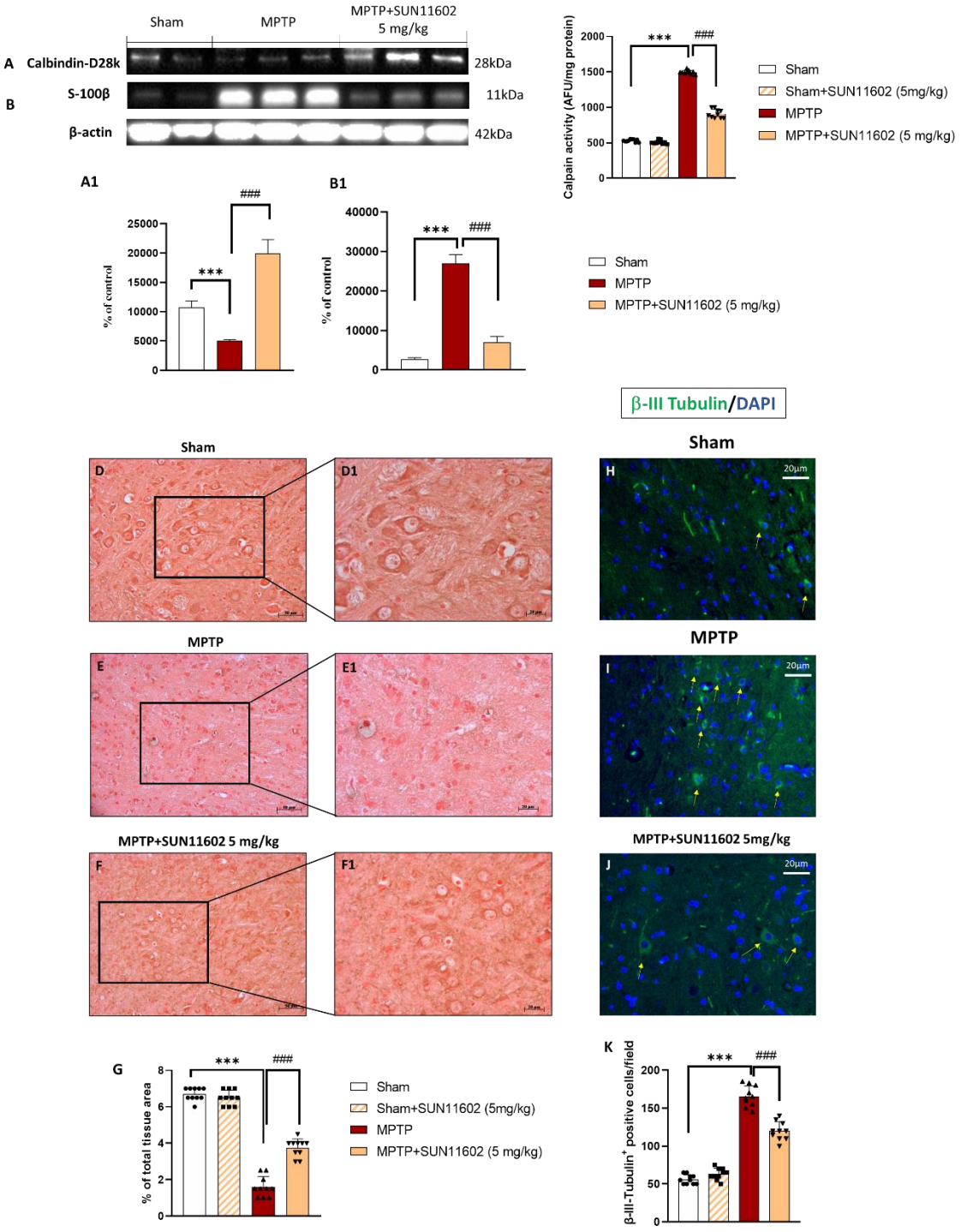

Figure 30. Effect of SUN11602 on neurons architecture and calcium homeostasis following MPTP injection. MPTP-injected mice showed an important decrease of Calbindin expression compared to the Sham group; while SUN11602 induced a significant upregulation of Calbindin (**A, densitometric analysis A1**). S-100β and Calpain levels were increased in MPTP-intoxicated mice, however, SUN11602 administration was able to reduce their levels (**B, densitometric analysis B1; C**). MPTP-intoxicated mice exposed low expression of MAP-2 (**E-E1, score G**) compared to the Sham group (**D-D1, score G**). SUN11602 5 mg/kg was able to restore the levels of MAP-2 protein (**F-F1, score G**). MPTP-injured mice exposed an increase of β3-tubulin-positive cells (**I, score K**) compared to the Sham animals (**H, score K**), SUN11602 5 mg/kg

administration decreased the non-physiological β3-tubulin expression (**J, score K**). Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; $^{\#\#}p < 0.01$ vs MPTP; $^{\#\#\#}p < 0.001$ vs MPTP

5.2.9. Effect of SUN11602 treatment on apoptosis pathway following MPTP injection

Neuroinflammation and Ca^{2+} excitotoxic damage are predisposing factors to cell death, which impact neurodegenerative diseases through apoptosis or necrosis processes[461]. In this context, we evaluated anti/pro-apoptotic markers like Bcl-2, Bax and Caspase-3 by Western Blot analysis and p53 by immunofluorescence analysis. The p53 protein plays a physiological role in cell cycle control. Indeed, following damage or an injury p53 controls the apoptotic cascade, causing neuronal death. Immunofluorescence staining showed an increase of p53 expression in the MPTP-intoxicated mice (Figure 31B, score D), compared to the Sham group in which p53 is physiologically expressed (Figure 31A, score D). SUN11602 5 mg/kg treatment demonstrated a significant reduction in p53 levels, thus suggesting its neuroprotective role against the apoptotic cascade (Figure 31C, score D). Bcl-2 levels are reduced after MPTP injection (Figure 31E, see densitometric analysis E1). Instead, the expressions of Bax (Figure 31F, see densitometric analysis F1) and Caspase-3 (Figure 31G, see densitometric analysis G1) were increased in the MPTP-intoxicated mice compared to the Sham group. SUN11602 5 mg/kg administration was effective in reducing Bax and Caspase expressions while restoring Bcl-2 levels (Figure 31E-F and G; see densitometric analysis E1, F1 and G1, respectively). These data suggest the neuroprotective role of SUN11602 in counteracting the apoptotic processes.

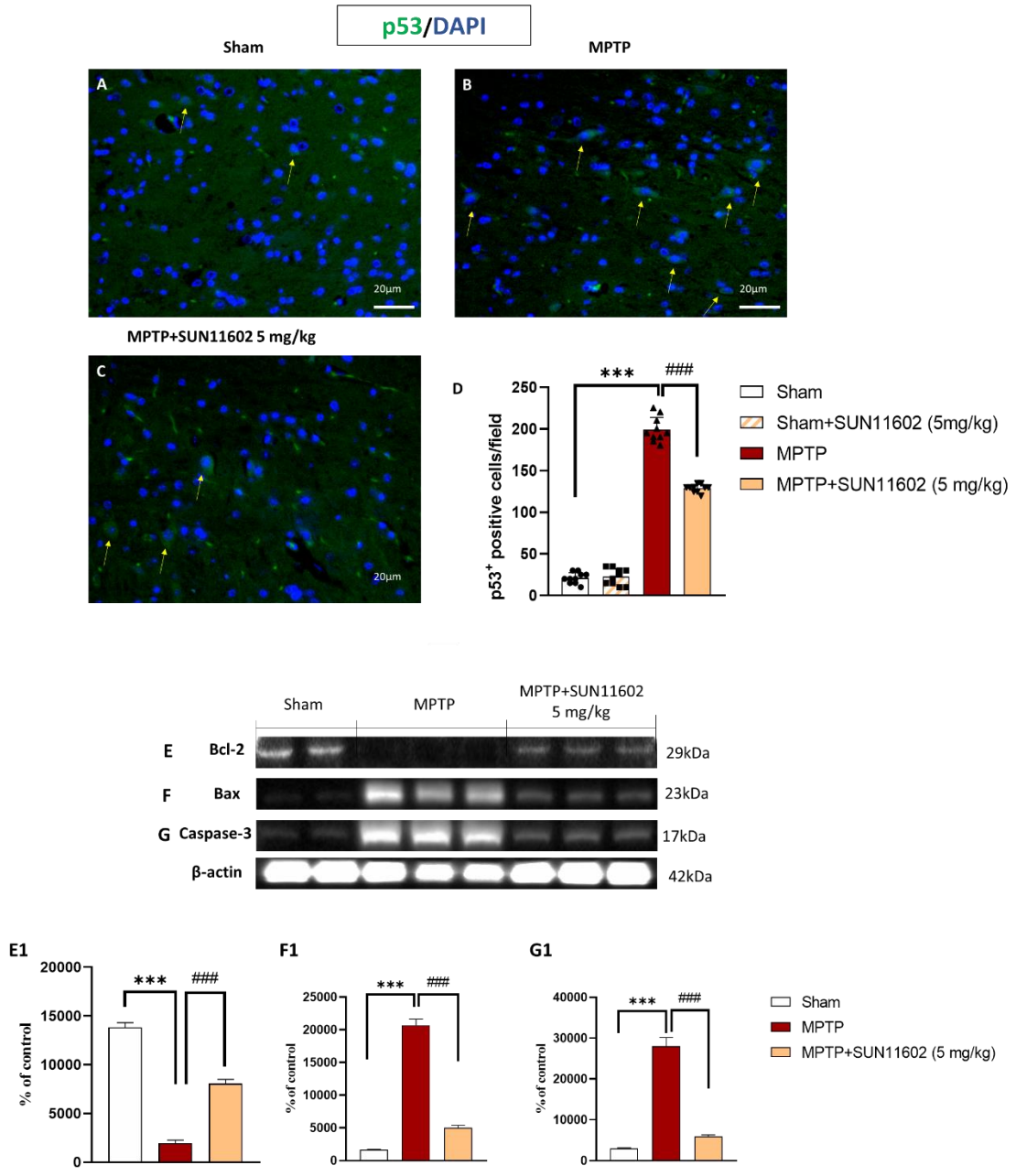

Figure 31. SUN11602 administration modulated apoptosis after MPTP injection. Midbrain section obtained by MPTP-injected mice exposed high expression of p53 **(B, score D)**, compared to the Sham animals **(A, score D)**. SUN11602 5 mg/kg treatment demonstrated a significant reduction in p53 levels **(C, score D)**. MPTP-intoxicated mice displayed increased expression of Bax and Caspase-3 and diminished levels of Bcl-2, compared to the Sham group **(E, densitometric analysis E1; F, densitometric analysis F1; G, densitometric analysis G1)**. SUN11602 5 mg/kg administration reduced the expression of Bax and Caspase-3 while increasing Bcl-2 levels **(E, densitometric analysis E1; F, densitometric analysis F1; G, densitometric analysis G1)**. Data are representative of at least three independent experiments. Values are means ± SEM. One-way ANOVA test. ***$p < 0.001$ vs Sham; ###$p < 0.001$ vs MPTP

5.3. AD study

5.3.1. DdPAC stimulation in hippocampal neurons reduces astrogliosis marker expression, GFAP in 5xFAD mouse model of AD

cAMP is an important second messenger in neurons, playing a critical role in the CNS[462], among memory formation, synaptic plasticity, neuronal survival, modulating microglia and astrocytes activation. In this regard, as previously mentioned, DdPAC induces cAMP synthesis when activated by red light (λ 660-760 nm) and can be also inactivated by far-red light (λ 685nm)[304]. The NDDs, like AD, are characterized by a chronic inflammatory state in CNS (neuroinflammation), with chronic microglial activation and reactive astrocytes, causing an alteration of cAMP level, reducing it. Therefore, the effect of DdPAC stimulation on cAMP levels to counteract neuroinflammation was evaluated by analysing the marker for astrogliosis, GFAP, specifically in the hippocampus (Figure 32). DdPAC was encapsulated into an AAV9 construct, using a CamKII promoter to ensure neuron-specific expression (Figure 32A). To complete the construct, a 3xFlag-Tag has been added to be able to validate the correct expression of the virus in the hippocampus, AAV9-CamKII-DdPAC-3xFlag-WPRE (AAV9-CamKII-DdPAC). Moreover, as a control, the construct containing GFP (AAV9-CamKII-GFP) was injected in WT mice and 5xFAD mice (Figure 32A). Both Flag and eGFP expression were used for localizing the infection of the area. 4 weeks after surgery, the mice received the DdPAC stimulation (λ 685 nm) for 10 min (1 sec ON and 4 sec OFF) and 24h later underwent euthanasia and hippocampal tissue from one hemisphere was obtained and processed for Immunofluorescence and the other hemisphere for proteomics (Ongoing). The DdPAC expression in hippocampal neurons was confirmed by immunohistochemical staining against GFP and 3xFlag (Figure 32B-E1). As reported in our data, GFAP is increased in transgenic mice, 5xFAD, compared to the healthy mice, WT (Figure 32D-D1 and E-E1). Moreover, data shows that DdPAC stimulation in neurons reduces GFAP expression in 5xFAD (Figure 32E-E1). The two-way ANOVA shows the genotype effect ($F_{(1,34)}$ =7.221, p=0.0111), and the genotype/treatment interaction ($F_{(1,34)}$ =0.3427 p=0.5621), but not the treatment effect. Fisher's LSD test post hoc shows that genotypes differences are lost in 5xFAD DdPAC stimulated mice, indicating a recovery of GFAP expression. Moreover, the GFAP expression is not the same in the whole hippocampus, as higher expression of GFAP is observed especially in the Hilus, S.l-m and S.o layers. Thus, mean

GFAP intensity was also quantified in the hippocampal layers, Hilus; S.o; S.m; S.l-m; S.r and S.o (Figure 32G). The two-way ANOVA shows the effect on hippocampal layers ($F_{(5,\ 170)}$ =101.6, p<0.0001), mice group ($F_{(3.34)}$ =2.969, p=0.0455) and group/layers interaction ($F_{(15,\ 170)}$ = p2.476, p=0.0026). Fisher's LSD test post hoc shows that S.r layer is the only one with a treatment effect in 5xFAD mice (DdPAC vs GFP) (Figure 32G).

Therefore, although DdPAC stimulation induces cAMP synthesis in the hippocampus, our results suggest that DdPAC stimulation specifically in hippocampal neurons partially reduces astroglia reactivity in the hippocampus of 5xFAD mice.

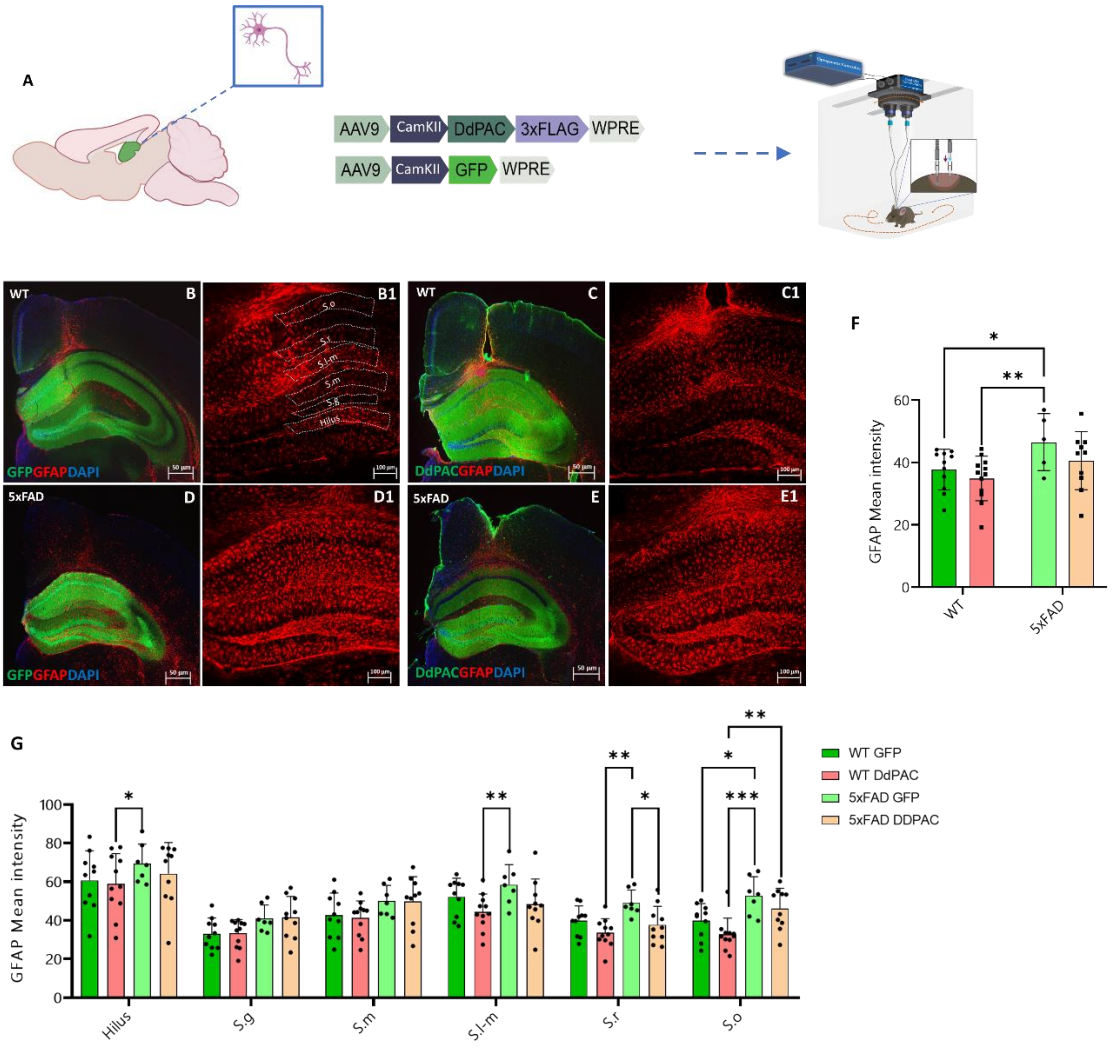

Figure 32. Modulation of inflammation by DdPAC stimulation in hippocampal neurons. Retrieved from https://app.biorender.com/biorender-templates

GFAP expression changes were evaluated upon DdPAC stimulation in hippocampal neurons from control and 5xFAD mice. Mice were injected into the hippocampus with either AAV9-CamKII-DdPAC-Flag or AAV9-CamKII-GFP constructs **(A)**. DdPAC stimulation was performed in vivo for 10 min using 685nm light (1s ON +4s OFF). Representative images of DdPAC (Flag), GFP,

GFAP, and DAPI, in mouse hippocampal slices revealed the co-immunostaining **(B-E1)**. The position of the cannula was also validated. Mean fluorescence intensity was measured and represented (**F**). The graph represents the GFAP mean fluorescence intensity **(F)**. Three images per mouse were obtained and the average from each mouse is represented as an individual value. The two-way ANOVA test with genotype and stimulation as factors showed significant effects on genotype and genotype/treatment interaction. Fisher's LSD post hoc test is shown **(F).** GFAP expression was further quantified in each layer of the hippocampus, as reported in Figure 32B1. Two-way ANOVA showed effects of layer, group and layer/group interaction **(G)**. Fisher's LSD test post hoc effects are shown **(G)**. Data were expressed as mean ±SEM. * $p < 0.05$; ** $p<0.01$; *** $p<0.001$. Scale bar is 50 μm. [N°tot=38 (WT-DdPAC (f)=8; WT-DdPAC (m)=4; 5xFAD-DdPAC (f)=5; 5xFAD-DdPAC (m)=5; WT-GFP (f)=7; WT-GFP (m)=2; 5xFAD-GFP (f)=4; 5xFAD-GFP (m)=3)].

5.3.2. Stimulation of hippocampal astrocytes with DdPAC reverts astrogliosis in the 5xFAD mouse model of AD

Similar to the previous study on hippocampal neurons, the effect of the DdPAC stimulation was detected in the hippocampal astrocytes. Known that, in astrocytes, cAMP plays an important role in their function and regulation, modulating astrocyte reactivity [463], evidence reports that, in the AD context, the cAMP pathway is disrupted, contributing to the chronic inflammation of the CNS and altering the synaptic functions. A construct of the DdPAC was encapsulated into an AAV9 vector, and a GFAP promoter was used to ensure astrocyte-specific expression. Then, the same procedure for hippocampal neurons was performed on hippocampal astrocytes. A 3xFlag-Tag has been added, to be able to validate the correct expression of the virus in the hippocampus, obtaining the complete construct AAV9-GFAP-DdPAC-3xFlag-WPRE (AAV9-GFAP-DdPAC). Moreover, even in this case, the control group is represented by a vector containing GFP (AAV9-GFAP-GFP) in WT mice and 5xFAD mice, used for localizing the infection of the area (Figure 33A). 4 weeks after surgery, the mice received the DdPAC stimulation with red-light (λ 685 nm) for 10 min (1 sec ON and 4 sec OFF) and then, 24h later, underwent euthanasia and hippocampal tissue from one hemisphere was obtained and processed for Immunofluorescence and the other hemisphere for proteomics (Ongoing). The GFAP expression in hippocampal astrocytes was confirmed by immunohistochemical staining against GFP and 3xFlag (Figure 33B-E1). The results obtained from the effect of DdPAC stimulation show a notably response by hippocampal astrocytes. In this case, data showed that GFAP expression is increased in 5xFAD mice compared to WT mice and, in 5xFAD mice, DdPAC stimulation modulated the GFAP expression, measured as the mean intensity of three images/mice (Figure 33D-1; E-E1; score F). The two-way ANOVA test highlighted a genotype effect ($F_{(1,42)}$ =4.916,

p=0.0321), the treatment effect ($F_{(1,42)}$ =4.685, p=0.0362) and genotype/treatment interaction ($F_{(1,42)}$ =1.967, p=0.1681). In the same way as the previous analysis, in hippocampal astrocytes the GFAP expression was quantified in each layer of the hippocampus: Hilus; S.o; S.m; S.l-m; S.r and S.o (Figure 33G). The picture for each representative single layer is reported in Figure 33, B1. The two-way ANOVA test shows layers effect ($F_{(5,199)}$ =54.32, p<0.0001), group effect ($F_{(3,40)}$ =4.045, p=0.0133) and group/layer effect ($F_{(5,199)}$=4.590 p<0.0001). Fisher's LSD test post hoc reports that genotype effect in mice with GFP injection was recorded especially in the Hilus layer (**** $p < 0.00001$), in the S.g and S.o layers (** $p < 0.01$) and the S.m and S.l-m layers (* $p < 0.05$) (Figure 33G). The treatment effect of the DdPAC stimulation in hippocampal astrocytes has been shown in the S.o layer for 5xFAD mice (* $p < 0.05$) and in the Hilus layer for WT mice (*** $p < 0.0001$) (Figure 33G) (Figure 33G). The majority of layers affected the genotype/treatment interaction, to the exclusion of S.l-m and S.r layers, in both groups, 5xFAD GFP – WT DdPAC and 5xFAD DdPAC – WT GFP (* $p < 0.05$ and ** $p < 0.001$, respectively) (Figure 33G).

Thus, the data analysis indicates that DdPAC stimulation effectively enhances cAMP production in hippocampal astrocytes, leading to the modulation of GFAP expression and counteracting neuroinflammation in AD.

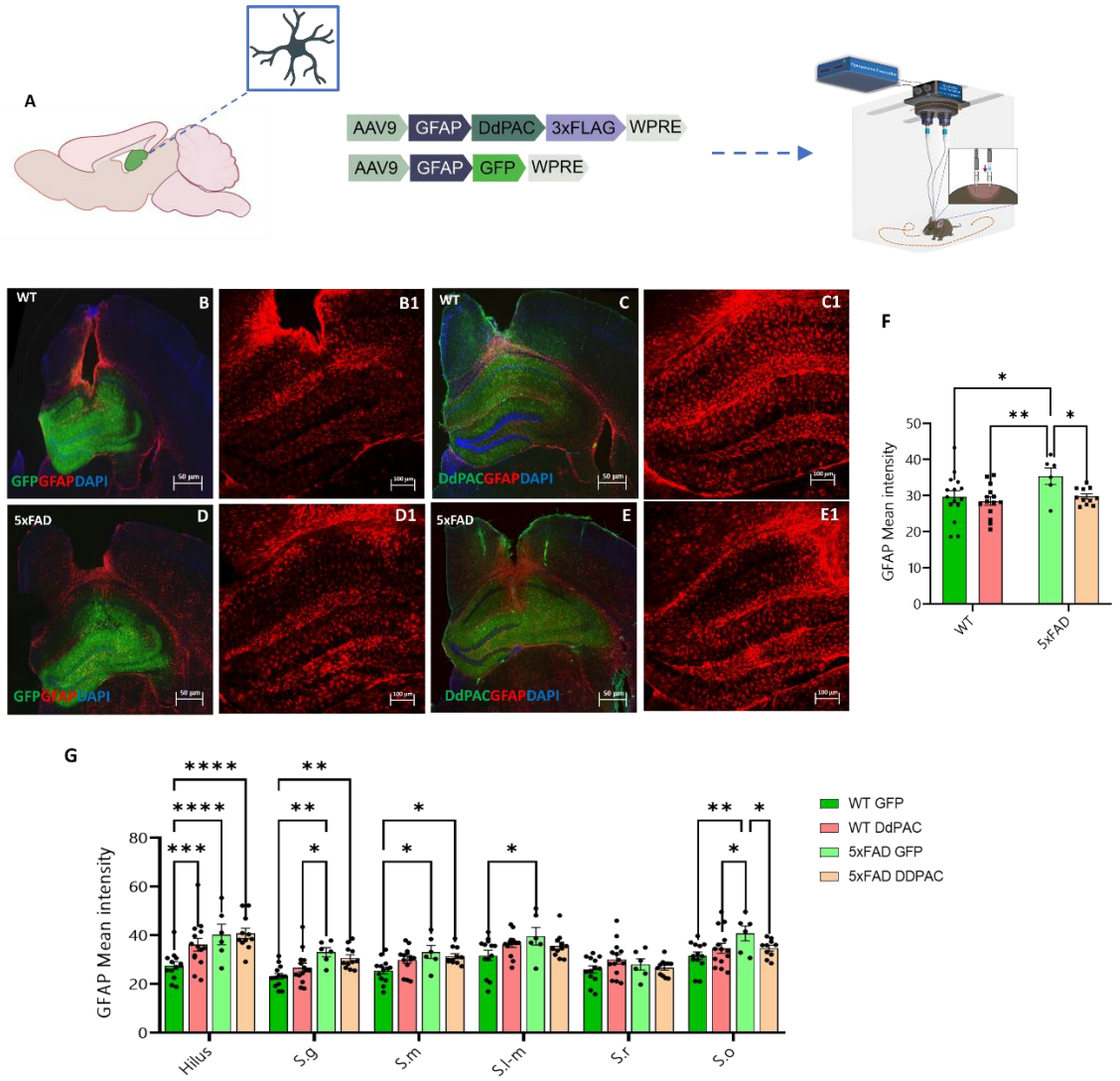

Figure 33. DdPAC stimulation in hippocampal astrocytes reverts GFAP expression in 5xFAD mice. Retrieved from https://app.biorender.com/biorender-templates

The changes in GFAP expression were assessed following DdPAC stimulation in hippocampal astrocytes from both mice, WT and 5xFAD. Mice were injected into the hippocampus with either AAV9-GFAP-DdPAC-Flag or AAV9-GFAP-GFP constructs **(A)**. DdPAC stimulation was performed in vivo for 10 min using 685nm light (1s ON +4s OFF) and mice underwent euthanasia 24 h later **(A)**. The co-localization of DdPAC (flag tag) or eGFP, GFAP and DAPI in astrocytes was revealed in mice hippocampal slides by immunostaining **(B)**. The graph represents the GFAP mean fluorescence intensity of three images/mice in astrocytes, detected in the hippocampus, corresponding to cannula injection **(B-E1; F)**. The two-way ANOVA, using genotype and stimulation as factors, revealed that the genotype effect was clear in mice who had received GFP injection **(F)** (* $p < 0.05$), while the treatment only for transgenic mice, 5Xfad **(F)** (* $p < 0.05$). GFAP expression was quantified into single layers of the hippocampus, as reported for hippocampal neurons, in Figure 32 panel B1. The Hilus layer shows high GFAP expression in mice with GFP injection (**** $p < 0.00001$) (F), followed by S.g and S.o layers (** $p < 0.01$) and in the S.m and S.l-m layers (* $p < 0.05$) **(G)**. Instead, the layers that major affected the effect of DdPAC stimulation have been the S.o layer for 5xFAD mice (* $p < 0.05$) and the Hilus layer for

WT mice (*** $p < 0.0001$) **(G)**. Finally, the group/layer interaction has been shown in both groups, 5xFAD GFP – WT DdPAC and 5xFAD DdPAC – WT GFP (* $p < 0.05$ and ** $p < 0.001$, respectively) **(G)**. Data were expressed as mean ±SEM. Three images per mouse were obtained, with the average from each mouse represented as a single dot. Differences were analysed by two-way ANOVA t-test and Fisher's LSD post hoc test. Scale bar is 50 μm. [N°tot=46 (WT-DdPAC (f)=7; WT-DdPAC (m)=7; 5xFAD-DdPAC (f)=7; 5xFAD-DdPAC (m)=4; WT-GFP (f)=5; WT-GFP (m)=7; 5xFAD-GFP (f)=4; 5xFAD-GFP (m)=2)].

5.3.3. DdPAC stimulation I astrocytes, but not neurons, reduces β-amyloid plaque number and its deposition in the hippocampus of 5xFAD mice

cAMP is known for its role in the CNS, through the control of microglia activity, and involvement in AD due to its dysregulation[464], causing the accumulation of Aβ plaques. Therefore, cAMP production by DdPAC stimulation was exploited in both cells, hippocampal neurons and astrocytes, to reduce and/or contain the deposition of Aβ plaques and consequently their number (Figure 34A and B). In healthy conditions the presence of Aβ plaques is irrelevant; therefore, the fluorescence analysis was performed exclusively on the 5xFAD-GFP and the 5xFAD-DdPAC mice (Figure 34). The Aβ plaque's presence was confirmed by immunohistochemical staining against Aβ (6E10 antibody) (Figure 34A and B). Aβ deposition analysis was performed by calculating the number and the area (expressed in microns) of the Aβ plaques, creating a mask using ImageJ, and normalizing the values to the hippocampus area, to obtain the percentage of Aβ (%Aβ) deposited plaques (Figure 34, graphs C and E) as well as the number of Aβ plaques/mm^2 (Figure 34, graphs D and F). In this case, data showed the %Aβ immunoreactive area and N° Aβ plaques/mm^2 were similar in 5xFAD mice expressing GFP or DdPAC in neurons, analysed by Student t-test ($t=0.01486$; $p=0.9884$) (Figure 34A; graphs C). Regarding the number of Aβ plaques, the Student-t test shows also no significant effect ($t=0.7312$, $p=0.4767$) (Figure 34, graph D). Conversely, in mice where DdPAC was stimulated in astrocytes, the effect of % Aβ deposition in astrocytes is evident, as shown by the Student-t test ($t=3.206$; $p=0.0075$) (Figure 34B; graph E). Also, the reduction of the number of Aβ plaque deposition in hippocampal astrocytes is notable followed by DdPAC stimulation, compared to the control group, GFP (Figure 34, graph F), as shown by the Student t-test ($t=2.491$, $p=0.0319$).

Therefore, in agreement with the previous analysis on neuroinflammation, investigated through immunostaining against GFAP, DdPAC stimulation in hippocampal neurons

does not change Aβ plaques number or area. Instead, DdPAC stimulation on hippocampal astrocytes clearly demonstrates the ability of cAMP induction by DdPAC in astrocytes to counteract neuroinflammation and reduce both the deposition and number of Aβ plaques. Overall, these results point to DdPAC stimulation in astrocytes as a promising therapeutic strategy for AD.

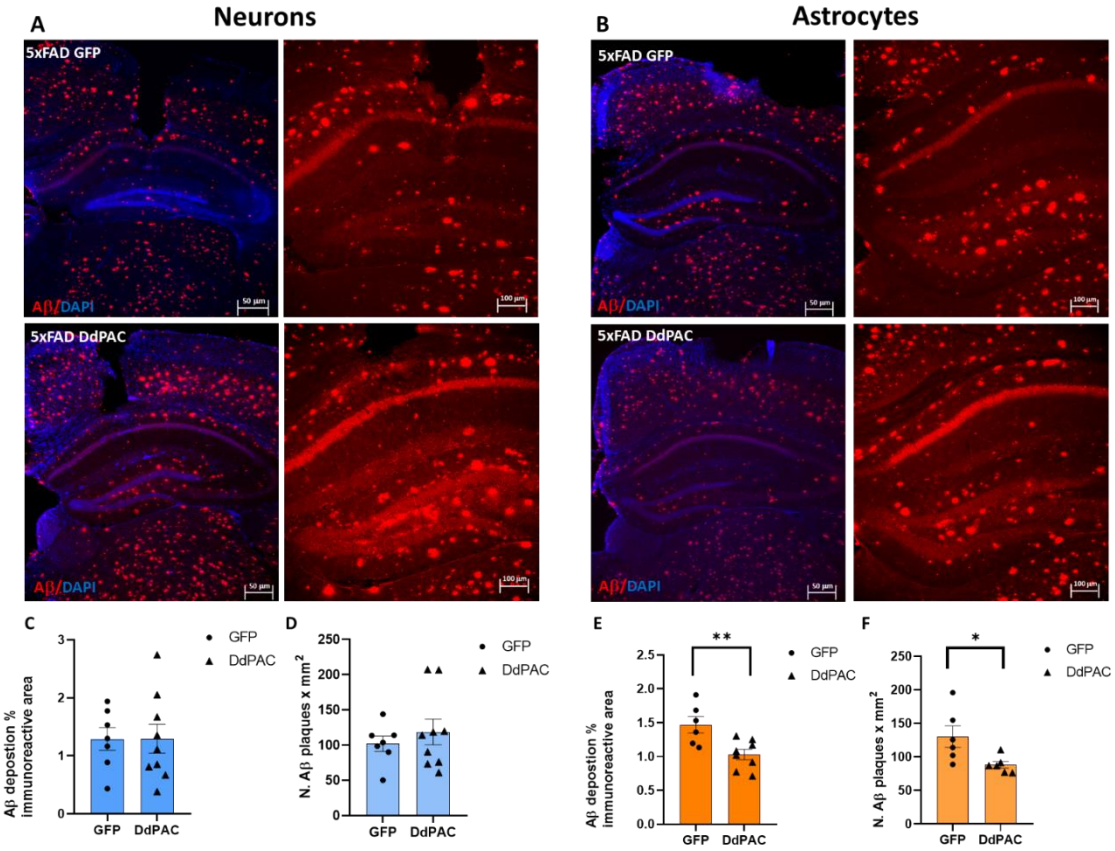

Figure 34. DdPAC stimulation in neurons and astrocytes differently affects Aβ plaques deposition and their number in the hippocampus. Representative images of Aβ and DAPI are shown for 5xFAD mouse hippocampal slices expressing DdPAC or GFP in neurons **(A)** or astrocytes **(B)**, revealed by co-immunostaining. The graphs represent the %Aβ deposited plaques, normalized by the hippocampus area, and the number of Aβ plaques for the total hippocampus area (Aβ/mm^2) of three sections /mice. 5xFAD mice, after DdPAC stimulation on hippocampal neurons, did not show an improvement in the deposition of Aβ plaques **(A; C)**, nor in their number, compared to the control group, GFP **(A; D)**. On the contrary, after DdPAC stimulation on hippocampal astrocytes, the percentage of Aβ deposited plaques was reduced in 5xFAD mice compared to the control group, GFP (** $p < 0.001$) **(B; E)**, and as a consequence, also the number of Aβ plaques was reduced in the hippocampus area (* $p < 0.05$) **(B; F)**. Data were expressed as mean ±SEM. Three images per mouse were obtained, with the average from each mouse represented as a single dot. Differences were analysed by t-test. The scale bar is 50 μm. [N°neurons=16 (5xFAD-DdPAC (f)=4; 5xFAD-DdPAC (m)=5; 5xFAD-GFP (f)=4; 5xFAD-GFP (m)=3); N°astrocytes=14 (5xFAD-DdPAC (f)=4; 5xFAD-DdPAC (m)4; 5xFAD-GFP (f)=4; 5xFAD-GFP (m)=2)].

CHAPTER 6: DISCUSSION

The Central Nervous System (CNS) is a perfect and complex machine, that controls the entire human body, but, at the same time, it is particularly susceptible to disorders, which today represent pathological conditions in industrialized countries, affecting more than a billion people in the world[465]. One of these disorders is represented by brain trauma which occurs when an external mechanical force, such as falls, sports injuries, vehicle accidents and others, causes damage to the brain. Following the head trauma, if the intervention is not timely, the resulting damage, especially neuronal, cognitive and behavioural deficits, becomes irreversible[126]. In this regard, a consequence of trauma is the immediate activation of microglia and astrocyte cells, which determine the release of pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-18, TNF-α, causing a chronic inflammation response, that named neuroinflammation. Moreover, numerous cohort studies of clinical research[114] demonstrated that TBI might represent a risk factor for the development of neurodegenerative diseases, such as PD and AD[115,116]. PD and AD are two of the most common neurodegenerative disorders of the central nervous system, affecting millions of people worldwide and mainly influenced by the aging population (> 65 y/o). Although the brain regions involved in the respective diseases are different, manifesting distinct symptoms, they share similar pathological mechanisms, such as progressive neuronal degeneration, protein misfolding, oxidative stress and neuroinflammation. No treatment has been developed to halt or reverse neuronal decline. It is estimated that by 2022, over 10 million people worldwide will be living with PD, while 55 million people will be living with dementia, with cases expected to double by 2040. Firstly, the clinical symptoms[152] that characterize PD are tremors, bradykinesia, rigidity, and postural instability, secondly, for AD, the main symptoms are memory loss, cognitive decline, and disorientation. Although specific symptoms characterize PD and AD, there is an overlap, as although PD is a predominantly motor disease, it is also characterized by cognitive disorders[117], such as depression, sleep disorders, etc. In contrast, AD, a predominantly cognitive disease, can affect motor functions in the long term. Although research is making progress in developing new therapies to slow the progression of the diseases, improving the quality of life of patients, and attenuating the debilitating effects of PD and AD, they remain, to date, incurable diseases. Current pharmacological treatments for PD include the use of Levodopa, a precursor of Dopamine, which reconstitutes the neurotransmitter, improving motor functions.

Generally used in association with Carbodopa[214], to prolong the half-life of Levodopa; dopamine agonists such as Pramipexole and Ropinirole, are used in the initial phase of the disease and/or in association with Levodopa as a support. Anticholinergics, such as Trihexyphenidyl and benztropine, are also widely used to reduce the activity of Acetylcholine and act on motor symptoms such as tremors and rigidity. Regarding AD, current pharmacological therapies aim to improve memory and cognitive functions. Among these are Cholinesterase inhibitors: Donepezil, Rivastigmine, and Galantamine, preventing the degradation of acetylcholine, involved in memory and learning. Antagonists of the NMDA receptor, Memantine[308], block the receptor hyperactivated by Glutamate; these are used in the moderate/severe phase of the disease. Aducanumab and Lecanemab, are two monoclonal antibodies that reduce the accumulation of Aβ plaques. Non-pharmacological treatments have also been developed for both diseases, ready to stimulate specific brain areas through the use of electrodes, to regulate cognitive functions. It has been known for decades that PD and AD are characterized at the molecular level by a major inflammatory process, called neuroinflammation[64]. The key components of this process are microglia, whose activation causes oxidative stress and the release of proinflammatory molecules, such as TNF-α, IL1β, IL6 and chemokines[65], leading to neuronal death. Other cells, involved in the front line of neuroinflammation are astrocytes, leading to the breakdown of the BBB which allows peripheral immune cells to infiltrate the brain and exacerbate neuroinflammation, contributing to the damage to neurons, mitochondrial dysfunction and apoptosis[69]. An element that plays an essential role in the correct functioning of the CNS and is involved in neuronal damage is the Ca^{2+} ion. Particularly, during neuroinflammation, dysregulation of intracellular Ca^{2+} concentration has been reported, leading to the production of pro-inflammatory cytokines, ROS, and neurotoxic factors. This process emphasizes the role of Ca^{2+} in regulating microglia activity and, consequently, the CNS's response to neuroinflammation. At this stage, elevated Ca^{2+} concentrations have been reported within microglia and astrocytes, which activate Ca^{2+}/calmodulin-dependent protein kinase (CamK)[133] signalling pathways, such as calcineurin and calmodulin. These molecules in turn initiate the transcription of proinflammatory cytokines (IL-1β, IL6 and TNF-α), prostaglandins (PGE2), and ROS production, amplifying and regulating the neuroinflammatory response and contributing to oxidative stress and thus neuronal death. Conversely, a protective role towards the CNS has been reported for cAMP, an important second messenger. It

regulates the immune response triggered by neuroinflammation, modulating the activation of microglia and astrocytes. CAMP relates to energy support in physiological conditions, regulating glutamate absorment[463]. In the presence of stimuli of various origins, cAMP promotes the anti-inflammatory phenotype, modulating cytokines and pro-inflammatory factors[466]. There is a correlation between cAMP and Ca^{2+} ion, regulating its flow through Ca-channel L type voltage-dependent[467]. Astrocytes express a variety of GPCRs[466] that neurotransmitters, neuromodulators, and other signaling molecules can activate. Some of these receptors are linked to transmembrane AC, producing cAMP. β-adrenergic receptors are one of the sources of astrocytic cAMP, increasing its level after the activation of these receptors[468]. Moreover, the protective role of cAMP in the CNS against several pathologies, including NDDs, has been confirmed[469]. In the AD context, as mentioned above, prolonged neuroinflammation and the presence of astrocytes reactive play an important role in this pathology, therefore, a study conducted on Alzheimer's rats reported that β-amyloid plaques activate the NF-κB pathway in astrocyte cells[470], leading to alteration of the morphology of neurons with their lost synapsis. Moreover, internalization of β-amyloid plaques within neurons causes hyperphosphorylation of tau protein, dysregulation of Ca^{2+} homeostasis and production of ROS[471], concluding these events with neuronal death. To investigate on the neuroprotective effect of cAMP, several studies were conducted using cAMP-elevating agents, such as dibutyryl-cAMP, forskolin and rolipram [471], which induced the reduction of cell-surface expression of 67-kDa laminin receptor (67LR) and cellular prion protein (PrP^{C}), leading to the resorption of oligomer β-amyloid plaques[472]. Moreover, interacting with β-adrenergic receptors, cAMP reduces the deposition of β-amyloid plaques and the phosphorylation of tau protein[463], reducing thus the inflammatory response.

cAMP not only acts on inflammatory pathways but, with activation of PKA and the subsequent phosphorylation and activation of CREB[289], promotes the gene expression of neurotrophic factors such as BDNF and GDNF, for cell survival. It is clear that, in the presence of damage in the CNS, the inflammatory response causes a dysregulation of cAMP, by releasing pro-inflammatory cytokines and chemokines, reducing cAMP levels. In support of what has been said above, an *in vitro* study, on the culture of astrocyte cells, exposed these cells to a mix of pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ) increasing the expression of prostaglandins in the culture of astrocyte cells, decreasing instead the cAMP levels[473]. Prolonged treatment with exogenous cAMP or NF-κB

inhibitors demonstrated an increase in the levels of cAMP, which reduced the presence of these cytokines. Moreover, it has been demonstrated that PAC, an optogenetic tool, increases the cAMP levels under blue light stimulation and can be useful to increase the cAMP signaling pathway[474].

Another molecule that promotes neuronal survival and provides neuroprotection is bFGF[457]. Like cAMP, it reduces glial activation, thereby supporting tissue remodeling in the CNS. Activating the anti-apoptotic PI3K/AKT[475] and MAPK/ERK pathways, bFGF protects neurons from the effects caused by inflammatory mediators TNF-α and IL-1β. Moreover, bFGF attenuates the hyperactivation of microglia and astrocytes, it also acts on the NF-κB pathway, inhibiting the transcription of pro-inflammatory genes, and consequent increase in the gene expression of anti-inflammatory molecules such as IL-10. At the astrocytic level, bFGF stimulates astrocytes to secrete neuroprotective factors BDNF and GDNF, supporting neuronal health. Therefore, bFGF promotes a healthy astrocytic response, supporting the regeneration of neurons. Although attempts have been made to maximize the efficacy offered by bFGF through exogenous administration, several clinical studies have reported the onset of side effects, due to its hyperproliferative activity, short half-life and poor permeability to the BBB[392,423], which have limited its use in therapeutic applications.

Considering that, we investigated the ability of SUN11602 to improve, firstly, behavioural deficits developed after TBI, thus providing support to neuronal cells, ensuring their survival through the activation of anti-apoptotic pathways and induction of neurotrophic factors, such as NT-3 and BNDF, responsible for neurogenesis. Secondly, considering TBI as a predictive factor for the development of NDDs, the neuroprotective activity of SUN11602 was examined in a mouse model of PD, induced by MPTP, to evaluate its ability to counteract chronic neuroinflammation, at the basis of these pathologies, and the restabilization of intracellular Ca^{2+} concentration, to ensure its adequate homeostasis, protecting neurons from damage by Ca^{2+} excitotoxicity and activating anti-apoptotic pathways. Thirdly, we enhanced cAMP synthesis through DdPAC stimulation in both hippocampal neurons and hippocampal astrocytes, to sustain the neuroprotective activity physiologically provided by cAMP, counteracting its neuroinflammation and reducing the deposition of β-amyloid plaques and their number, in a 5xFAD transgenic model.

Firstly, an acute TBI, in the absence of immediate intervention, represents a springboard for the development of irreversible damage. Therefore, numerous preclinical and clinical studies highlight how states of anxiety and depression are generated following a TBI[476], inducing neuropsychiatric disorders, even in neurodegenerative diseases such as PD. Literature exposes the neuroprotective capacity of SUN11602 in improving CNS damage[392]. Based on these considerations, we investigated whether the bFGF mimetic, if administered immediately after TBI (1 and 4h after the injury) was able to contain the behavioral deficits developed in mice subjected to trauma. The results of this study confirmed the neuroprotective activity of SUN11602 against the damaged CNS, showing an improvement in behavioral performance [392], with a reduction of anxiety in mice with head trauma, measured by the extent of tissue damage, through reduction of the lesion area, and motor function. The inflammatory response represents one of the main biochemical events triggered after TBI[450]. In this phase, a central role is played by the NF-κB pathway, which, following its activation, induces the release of pro-inflammatory cytokines and mediators (IL-6, COX-2, iNOS and nNOS), exposing cells to lipid peroxidation and nitrosative stress, contributing to clinical decline. In this research, we aimed to extend the ability of SUN11602 to protect neuronal cells from damage induced by NF-κB pathway activation, reducing the inflammatory state [477]. It has long been known that the release of pro-inflammatory cytokines and mediators alters the balance between pro- and anti-apoptotic factors, directing neuronal cells towards a pro-apoptotic pathway due to the increased expression of proteins that promotes cell death[478475]. Therefore, proving to be able to contain the inflammatory response, the protective effect of SUN11602 was also evaluated on the apoptotic pathway. The results of this study reported that the attenuation of brain damage after administration of SUN11602 in mice with TBI was relevant, restoring the expression of the anti-apoptotic protein, bcl-2[479], and decreasing the expression of bax and p53, preserving thus the vitality of neuronal cells that survived the head injury[480].

Numerous studies report the correlation between an active inflammatory state and the alteration of intracellular Ca^{2+} concentrations [481], which occurs as a consequence of the alteration in the concentration levels of CaBPs, with an increase of its concentration within neurons, causing DNA damage and neuronal death for excitotoxicity[482]. Furthermore, studies conducted on both bFGF and SUN11602 have demonstrated how their activity is closely related to calcium metabolism[395,423] through interaction with

CaBPs, such as Calb-1, S100-β and Calpain, modulating their levels [483]. In agreement with what was reported in the literature, in this study the treatment with SUN11602 increased the expression levels of Calb-1, thus protecting the neurons from the excitotoxicity caused by the high concentration of Ca^{2+} while slowing down the activity of the S100-β protein increased following head trauma. finally, studies conducted on humans and mice confirm that following a TBI, even if acute, an alteration of the levels of neurotrophic factors occurs, reducing them [484]. In particular, the main neurotrophic factors examined in this study include NT-3 and BDNF, confirming what has been reported in the literature. Treatment with SUN11602 at 1 and 4 hours after TBI, buffered their decline, consequently stimulating the synthesis of new neurotrophic factors. What has been reported further confirms the neuroprotective activity of SUN11602 towards the CNS following a head injury, promoting neurogenesis and supporting neuronal cells ensuring their survival.

Therefore, considering the results obtained in this first study, where SUN11602 has been shown to counteract the pathophysiological consequences of head trauma, this study has been extended also to the neurodegenerative disease Parkinson. In the mouse model of MPTP-induced nigrostriatal neurodegeneration[485], which characterizes PD, oral administration of SUN11602 significantly reduced the alteration of the hallmarks of the disease, improving motor deficits, particularly tremors[486]. It was also observed that SUN11602 was effective in reducing anxiety in mice with MPTP neurointoxication, thus acting on one of the non-motor symptoms of Parkinson's[393]. Motor and non-motor disorders in PD are nothing but the consequence of the impairment of the dopaminergic system[487]. In these complex circuits, TH plays a key role, as an enzyme that catalyzes the conversion of L-tyrosine to l -3,4-dihydroxyphenylalanine (L-DOPA), which represents the initial and rate-limiting step in catecholamine biosynthesis[488]. Furthermore, as revealed by several post-mortem studies, the alteration of TH^+ levels represents a predictive factor that increases the risk of PD progression and catecholamine dysfunction[489]. Based on the above, our results showed a marked decrease in TH^+ expression in MPTP-injured mice. In contrast, SUN11602 treatment significantly prevented TH^+ expression in neurons, protecting them from MPTP-induced toxicity. The survival of nigral neurons is linked to the preservation of nigrostriatal dopamine homeostasis, provided by nerve cells to synthesize, store and release dopamine[490]. In this context, DAT allows neurons to modulate dopamine clearance following physiological

demands[491]. More specifically, DAT determines a rapid uptake of dopamine from the extracellular space into the presynaptic neuron, essential to regulate the magnitude and duration of dopaminergic signaling[492]. Therefore, in confirmation of this, our results showed that MPTP intoxication caused a substantial decrease in DAT expression in both the substantia nigra and the striatum. Instead, mice treated with SUN11602 revealed a significant upregulation of DAT levels in brain areas, thus supporting the neuroprotective capabilities of this bFGF mimetic. Furthermore, in confirmation of the analysis just mentioned, the levels of dopamine and its metabolites, DOPAC and HVA, were also evaluated in the striatum. We observed that the administration of SUN11602 counteracted the drastic reduction in striatal dopamine, DOPAC and HVA levels after MPTP injection, thus denoting positive results on dopaminergic neural networks[393].

At the biochemical level, it is clear that α-syn aggregates Accumulation of α-syn aggregates in SNpc dopaminergic neurons contributes to their degeneration, thus worsening cognitive decline in PD patients [437,493]. SUN11602 treatment reduced MPTP-induced synucleinopathy. Moreover, it was found that abnormally phosphorylated α-syn represents the toxic form present in the aggregates[494]. Therefore, phosphorylation of α-syn is a critical factor in the pathogenesis of PD, which was evaluated by its expression after SUN11602 treatment, demonstrating a significant decrease of p-α-syn in SUN11602-treated mice and thus validating once again the beneficial effects of this compound[393]. A chronic neuroinflammatory state is characterized by glia activation[495], which is reflected by the increase of reactive astrocytes and microglia, identified through their specific markers, GFAP and IBA-1 respectively, highly expressed in PD brain tissue. In confirmation of this, also our study confirmed a large change in GFAP and IBA-1 expression after MPTP-induced nigrostriatal degeneration. Differently, SUN11602 administration attenuated reactive astrocytes and microglia, upregulating GFAP and IBA-1 levels. Microglia activation is, in turn, controlled by an important biomarker, CD68, used to identify the stages of microglia activation in neuropathological analysis[496]. Treatment with SUN11602 was able to reduce its levels, confirming its ability to decrease microglial activation. As mentioned in the introduction, the transcription factor NF-κB is a key feature in orchestrating the multiple cellular activities underlying inflammation and characterizing the pathophysiology of PD. NF-κB pathway activated, stimulates the release of pro-inflammatory cytokines and mediators that drive disease exacerbation[497]. In the present study, the administration of SUN11602 effectively modulated the NF-κB

pathway and cytokine expression, demonstrating the utility of SUN11602 in attenuating the neuroinflammatory condition[393]. Several papers have elucidated the bidirectional interactions between neuroinflammatory signaling mechanisms and Ca^{2+} dyshomeostasis[498]. Indeed, a chronic inflammatory state compromises intracellular Ca^{2+} homeostasis[499]. Consequently, clinical studies have indicated a compromise of cellular Ca^{2+} regulatory systems in the field of neurodegenerative disorders[500]. Therefore, modulation of CaBPs would represent a promising pharmacological strategy in controlling Ca^{2+} signaling, with neuroprotective action[501]. In particular, overexpression of Calb-1 would protect dopaminergic neurons from the pathological process of PD, inhibiting calcium-mediated calpain activation and rebalancing Ca^{2+} overload in neurons[502,503]. Therefore, considering the potential activity of SUN11602 in increasing Calb-1 levels in neurons, we investigated its ability to restore intracellular calcium homeostasis after MPTP intoxication[393]. From the obtained data, we demonstrated that SUN11602 administration enhanced Calb-1 expression, reduced S100-β levels and inhibited Calpain activity, demonstrating its ability to control intracellular Ca^{2+} efflux through the regulation of CaBPs. Furthermore, it is known that the alteration of neuronal calcium circuits has a great impact on different cytoskeletal systems. For this reason, in this study we analyzed MAP-2, a cytoskeleton-associated protein, which has been shown to limit excessive excitotoxic activity, sparing the architecture of neurons typical of neurodegenerative disorders. Scientific findings have suggested mutations in the structure of tubulin, the major constituent of microtubules essential for intracellular transport[504]. In particular, an increase in the neuron-β3-tubulin isotype was observed after MPTP intoxication[504], leading to their depolymerization and causing significant cellular damage to long projection neurons such as dopaminergic cells of the nigrostriatal pathway[505]. In this study, SUN11602 administration restored the MPTP-induced loss of MAP-2, while reducing the increase in β3-tubulin in the number of labeled cells. Furthermore, dysregulation of Ca homeostasis induces the cell towards an apoptotic pathway[506]. In this regard, p53 is the main modulator of cellular stress responses and its expression increases following several stimuli, including cellular calcium overload and excitotoxicity[507]. In the CNS, p53 activation can trigger apoptosis in neurons by encoding the pro-apoptotic proteins bax and caspase-3. Therefore, based on the evidence in the literature reporting the correlation between the alteration of intracellular Ca concentration and the induction of apoptosis by the cell, and considering that SUN11602 has been shown to positively act on Ca^{2+} flux, we hypothesized that its administration could prevent the apoptosis process,

thus protecting neurons from neurodegeneration. Indeed, in support of our hypothesis, our results revealed that SUN11602 reduced the levels of p53, bax and caspase-3, while bcl-2 expression was significantly increased, thus confirming the modulation of apoptosis after MPTP-induced neuronal death.

Finally, the 5xFAD transgenic model of Alzheimer's, together with WT mice, received the injection of both the AAV9-DdPAC construct[331], with the respective promoters, CamKII for hippocampal neurons, and GFAP for hippocampal astrocytes, and of the construct containing eGFP used as a control. The analyses performed 24h after stimulation with DdPAC (1 sec ON and 4 sec OFF) allowed us to study its effect on the increase of cAMP synthesis. In particular, the analyses reported an increase in the levels of GFAP expression in 5xFAD transgenic mice as previously reported[508]. Moreover, the GFAP levels are layer specific, showing higher expression in Hilus, S.g, S.m and S.o, as also reported in a study from Girard S.D et al.[509], whose demonstrated high GFAP levels close to the dentate gyrus, constituted by Hilus, S.g and S.m, in 5xFAD mice six-months-old[509]. The effect of DdPAC stimulation on neuroinflammation was notable in DdPAC stimulation in hippocampal astrocytes. In contrast, DdPAc stimulation of hippocampal neurons only partially modulated GFAP expression. Scientific evidence indicates that cAMP has different downstream signaling pathways[510], so it should not be surprising that differential effects have been observed between neurons and astrocytes. Furthermore, these first results indicate that cAMP increases in neurons, affecting astrocytes, thus suggesting that cAMP activity is involved in neuron-astrocyte communication. However, to date, we do not know how and by what mechanism cAMP participates in their communication. What is well known is that neurons and astrocytes communicate through the activation of the neurotransmitter GPCR in astrocytes and gliotransmitters[511] from astrocytes to neurons. However, it remains to be explored in depth whether the induction of cAMP would differ in neurons and astrocytes and whether it involves other cells, such as microglia, taking into account that at the CNS level cAMP regulates the activity not only of astrocytes but also of microglia[85], to ensure neuroprotection.

In addition, the effect of DdPAC stimulation was also studied on the deposition of β-amyloid plaques and their number[510], the results of which confirm what was reported in the previous analysis. The t-test between the GFP group and the DdPAC group, reports that the effect of DdPAC stimulation is evident when DdPAC is stimulated in hippocampal astrocytes, on Aβ-plaques depositions and their number. In contrast, when

DdPAC is stimulated in hippocampal neurons, Aβ plaques remain similar. Although DdPAC stimulation can increase the cAMP levels, its effect on hippocampal neurons is not significant, compared to hippocampal astrocytes which appear to be more susceptible to the effect due to stimulation with DdPAC. Therefore, the lack of response by neurons on the reduction of Aβ plaques, when DdPAC is stimulated in hippocampal neurons, could be related to its low capacity to reduce inflammation at this level, although further analyses are needed to support this possible hypothesis.

CHAPTER 7: CONCLUSIONS

In conclusion, the data obtained clarify the advantages provided by the pharmacological treatment with SUN11602, capable of acting on different molecular processes that constitute the etiopathogenesis of head trauma and Parkinson's disease. Specifically, it allowed for restoring the behavioral deficits and the state of anxiety developed, also counteracting the neuroinflammation that afflicts the CNS, acting on the NF-κB pathway, thus reducing the inflammatory cascade represented by pro-inflammatory mediators and cytokines (IL-1β, IL-6, TNF-α, COX2, iNOS and nNOS), and offering a good chance of neuronal survival, reducing the apoptotic process. All this offers a potential neuroprotective effect, both by improving the integrity of the BBB and by interacting with CaBPs, Calb-1 regulating intracellular calcium concentrations, to reduce the risk of neuronal death due to excitotoxicity and, therefore, guarantee their survival. Not only that, the selectivity offered using optogenetic tools, which in this study involved DdPAC stimulation, was also effective in investigating the involvement of specific pathways directly involved in Alzheimer's, such as the cAMP pathway. Furthermore, the ability to increase cAMP synthesis via DdPAC stimulation under red light stimulation (λ 685 nm), is highly advantageous for several reasons, firstly, because it is possible to observe a cell-specific effect, studying the pathways directly involved in these pathologies, secondly, by enhancing the neuroprotective activity of cAMP in astrocytes it is possible to counteract neuroinflammation and the deposition of β-amyloid plaques, as well as their number, offering greater neuronal survival and neuroplasticity. Therefore, the results obtained by both approaches, pharmacological with SUN11602 and optogenetic by DdPAC stimulation specifically in astrocytes, may offer promising therapeutic opportunities for NDDs.

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