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Ciências

Role of Striatal-Enriched Protein Tyrosine Phosphatase (STEP) in the Nigrostriatal Pathway

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"Alea jacta est"

Júlio César

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RESUMO

A fosforilação proteica através dos resíduos de tirosina tem um papel importante em vários processos neuronais. Esta tem sido implicada no crescimento axonal, em interações célula-célula, célula-matriz extracelular e na diferenciação. Para regular estes processos existe um equilíbrio entre o nível de fosforilação provocado pelas cinases de resíduos de tirosina e a ação oposta provocada pelas fosfatases de resíduos de tirosina.

A proteína fosfatase de resíduos de tirosina enriquecida no estriado (STEP – *Striatal Enriched Protein Tyrosine Phosphatase)* é uma fosfatase específica do cérebro e encontra-se envolvida na transdução de sinal neuronal. A STEP está presente em níveis elevados nos neurónios espiculados médios do estriado - neurónios dopaminoceptivos - que são regulados pelos recetores da dopamina. O ARN mensageiro da STEP origina alternadamente a $STEP_{61}$, uma isoforma associada à membrana e a $STEP_{46}$, uma isoforma citosólica. Ambas as isoformas são expressas no estriado enquanto que outras regiões do cérebro expressam apenas a isoforma $STEP_{61}$.

A via da STEP está alterada em algumas doenças neurodegenerativas, no entanto, não há informação sobre a expressão da STEP na Doença de Parkinson (DP). A DP é caracterizada pela progressiva degeneração dos neurónios dopaminérgicos da *substantia nigra* que projetam para o estriado, resultando em níveis reduzidos de dopamina no estriado, sendo esta considerada um neurotransmissor regulador da atividade da STEP. Assim, com este estudo pretendemos avaliar se alterações na sinalização dopaminérgica, resultantes de uma lesão dopaminérgica seletiva em modelos da Doença de Parkinson, influenciam a expressão de STEP na via nigroestriatal.

Apesar da forte expressão da STEP no estriado, observou-se que esta também é expressa pelos neurónios dopaminérgicos do mesencéfalo e que a sua expressão varia ao longo do desenvolvimento. No entanto, o perfil de expressão da STEP é diferente em ambas as regiões. Com este trabalho, pretendeu-se aprofundar se a expressão de STEP é regulada por uma lesão dopaminérgica em modelos de DP. Recorrendo a modelos, *in vitro* e *in vivo*, foi possível observar que os níveis de STEP estão aumentados em modelos da DP. Para determinar este efeito, culturas mistas de neurónios e astrócitos do mesencéfalo foram previamente estimuladas com MPP^+ e, em paralelo, ratos C57BL/6 adultos foram submetidos a uma injeção intraperitoneal de MPTP. A extensão da lesão foi determinada em ambos os modelos pela quantificação dos neurónios dopaminérgicos e pelos níveis de tirosina hidroxilase. Investigou-se ainda se a lesão neuronal promovia um aumento da reatividade dos astrócitos e se este aumento de reatividade poderia afetar a expressão de STEP.

In vitro, não detetamos alterações da expressão de STEP nos astrócitos. No entanto, *in vivo*, observaram-se níveis aumentados do marcador de reatividade astrocitária na *substantia nigra* em paralelo com níveis mais elevados de STEP.

Estes resultados indicam que existe uma relação entre a expressão de STEP e a lesão dopaminérgica e talvez esta relação possa ser crítica para a patogénese da DP e consequentemente possa ser um alvo terapêutico.

PALAVRAS-CHAVE

STEP, proteina fosfatase de resíduos de tirosina, neurónios dopaminérgicos, MPTP, MPP^+, Doença de Parkinson

ABSTRACT

Protein tyrosine phosphorylation plays a central role in numerous neuronal processes. It has been implicated in axonal growth, synapse formation, cell-cell and cell-extracellular matrix interactions and differentiation. To regulate these processes, there is a balance between the level of phosphorylation caused by protein tyrosine kinases and the opposing action of protein tyrosine phosphatases.

The striatal-enriched protein tyrosine phosphatase (STEP) is a brain-specific phosphatase involved in neuronal signal transduction. STEP is present in high levels in medium spiny neurons of the striatum - the dopaminoceptive neurons - where it is regulated by dopamine receptors. STEP mRNA is alternatively spliced into the membrane-associated $STEP_{61}$ and the cytosolic $STEP_{46}$. Both isoforms are expressed in the striatum, whereas the other brain areas only express $STEP_{61}$.

STEP pathway is altered in some neurodegenerative diseases, however, there is no information on the expression of STEP in Parkinson's disease (PD). PD is characterized by the progressive degeneration of dopaminergic neurons from the *substantia nigra* that project to the striatum resulting in reduced striatal levels of dopamine, a neurotransmitter that regulates STEP activity. In this way, the main goal of the present research activity was to determine if changes in dopaminergic signaling, resultant from a selective dopaminergic lesion in Parkinson's disease models, can influence the expression of STEP in the nigrostriatal pathway.

Besides the strong expression of STEP in the striatum, we observed that STEP is also expressed by midbrain dopaminergic neurons and its expression varies along development, however, the expression profile is different in both regions. With this work we intended to deepen if STEP expression is regulated by the dopaminergic lesion using PD models. Recurring to a cellular and mouse model of the disease, we observed that levels of STEP are increased in PD. For the determination of this effect, neuron-astrocytes midbrain co-cultures were previously stimulated with MPP^+ and, in parallel, young adult C57BL/6 mice were submitted to an intraperitoneal injection of MPTP. The extension of the lesion was determined in both models by assessing both the number of dopaminergic neurons and the levels of tyrosine hydroxylase. Since MPTP is converted to MPP^+ in astrocytes, we further investigated if midbrain astrocytes can be modified by these stimuli and we also evaluated if the changes in STEP expression were associated with increased reactivity of astrocytes. *In vitro*, we did not observe STEP expression in astrocytes. However, *in vivo*, we observed increased levels of a

marker of astrocyte reactivity in the *substantia nigra* in parallel with increased levels of STEP.

These studies indicate that there is a relation between STEP expression and dopaminergic lesion and, perhaps this relation may be critical for the pathogenesis of PD and therefore it can be considered a potential therapeutic target.

KEYWORDS

STEP, protein tyrosine phosphatase, dopaminergic neurons, MPTP, MPP^+, Parkinson's disease

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
Aß	Beta Amiloyde Peptide
cAMP	Adenosine monocyclic Phosphatase
CNS	Central Nervous System
D1Rs	Dopamine Receptors D1
D2Rs	Dopamine Receptors D2
DA	Dopamine
DARP-32	Dopamine-and cAMP-dependent phosphoprotein of 32 kDa
DAT	Dopamine transporter
ERK1/2	Extracellular Signal-regulated Kinase 1 and 2
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acid Protein
GPi	Globus Pallidus internal
HD	Huntington's disease
i.p	Intraperitoneal
IHQ	Immunohistochemistry
KIM	Kinase Interaction Motif
LBs	Lewy Bodies
LTP	Long-term Potentiation
MAPKs	Mitogen-Activated protein Kinases
mhtt	Mutant hungtintin
$MPDP^+$	1-methyl4-phenyl2,3- dihydropyridinium
MPP^+	1-methyl4-phenylpyridinium
MPTP	1-methyl4-phenyl1,2,3,6-phenyl1,2,3,6-tetrahydropyridine
NMDARs	N-methyl-D-aspartate receptors
OD	Optical density
p38	Stress-activated Protein Kinase p38
PBS	Phosphatase Buffer Saline
PBS-T	Phosphatase Buffer Saline - Tween
PD	Parkinson's disease
PFA	Paraphormaldeyde
PKA	Protein Kinase A
PP	Poliproline regions
PP1	Protein Phosphatase 1
PP2B	Protein Phosphatase 2B
PSA	Ammonium persulphate
PTP's	Protein Tirosine Phosphatases

PVDF	Polyvinylidene Difluoride
ROS	Reative Oxygen Species
SDS	Sodium Dodecyl Sulfate
SNCA	α-synuclein
SNpc	Substantia Nigra pars compacta
STEP	Striatal Enriched Protein Tirosine Phosphatase
TEMED	Tetramethylethylenediamine
TH	Tirosine Hidroxilase
VMAT	Vesicular Monoamine Transporter

Chapter 1

INTRODUCTION

Protein phosphorylation-dephosphorylation reactions play important role in the regulation of a wide variety of physiological processes such as cell proliferation, neuronal development or differentiation and signal transduction (*Hasegawa, 2000*).

Many neuronal proteins are regulated by phosphorylation including neurotransmitter and growth factor receptors, ion channels and synaptic vesicles (*Boulanger et al., 1995*). However, tyrosine phosphorylation is used for communication between and within cells, for the shape and motility of cells, for decisions to proliferate *versus* differentiate, for cellular processes like regulation of gene transcription, for mRNA processing and transport of molecules in or out of cells (*Price & Mumby, 1999*). Tyrosine phosphorylation also plays an important role in the coordination of these processes and also in embryogenesis, organ development, tissue homeostasis, and the immune system. These processes are regulated by a balance that exists between two major classes of enzymes: *Protein Tyrosine Kinases* (**PTK's**) and the *Protein Tyrosine Phosphatases* (**PTP's**) (*Boulanger et al., 1995; Tonks & Neel, 1996; Alonso et al., 2004; Mustelin et al., 2005*).

The Protein tyrosine phosphatases are subdivided into **receptor-like PTP's** or **nonreceptor cytoplasmatic PTP's**. Receptor-like PTP's have an extracellular receptor domain, transmembrane domain and usually two catalytic domains. In contrast, the nonreceptor cytoplasmatic PTP's contain a single catalytic domain and additional amino acid sequences (*Oyama et al., 1995; Vactor, 1998*). A variety of PTP's have been identified by Polymerase Chain reaction (PCR) using the DNA sequence homology of their catalytic domain (*Alonso et al., 2004*).

Abnormalities in tyrosine phosphorylation play a role in the pathogenesis of numerous inherited or acquired human diseases from cancer to neuropsychiatric diseases (*Braithwaite et al., 2012; Goebel-Goody et al., 2012*). Investigating whether PTP's are regulated by specific neurotransmitter systems in the brain is an important step in understanding the underlying signaling pathways.

1.1. STRIATAL-ENRICHED PROTEIN TYROSINE PHOSPHATASE

STriatal-Enriched Protein Tyrosine Phosphatase designated as STEP is encoded by the *Ptpn5* gene and is a member of the family of intracellular tyrosine-specific phosphatases (*Lombroso et al., 1993*). It is a brain specific protein and it has recently been identified as a critical player in the regulation of neuronal signal transduction (*Venkitaramani et al., 2011)*.

STEP is specifically expressed in neurons of the central nervous system (CNS). As its name indicates, it presents the highest expression levels in the striatum (*Lombroso et al., 1991*) but it is also expressed in multiple brain regions including the amygdala, neocortex, hippocampus and related regions of the brain that are involved in motor control and cognitive activities (*Boulanger et al., 1995; Pelkey et al., 2002)*.

STEP mRNA is alternatively spliced into two main variants. The proteins products are termed **$STEP_{46}$** and **$STEP_{61}$** based on their electrophoretic mobility (*Boulanger et al., 1995*). **$STEP_{46}$** is enriched in cytosolic fractions while **$STEP_{61}$** is membrane-associated and is targeted to endomembranes such as the endoplasmic reticulum and the postsynaptic densities (PSD) by the presence of an extra 172 aminoacids at its N-terminus (*Lombroso et al., 1993; Oyama, 1995*). Both isoforms are expressed in the striatum, central nucleus of the amygdala and the optic nerve, whereas other brain areas such as hippocampus, neocortex, spinal cord and lateral amygdala only express the $STEP_{61}$ isoform (*Boulanger et al., 1995*).

Both $STEP_{46}$ and $STEP_{61}$ contain a C-terminal domain of approximately 280 amino acids that has a catalytic site with the consensus sequence (I/V) HCXAGXXR (S/T) and a Kinase-Interacting Motif (KIM) (*Bult et al., 1996*). This domain is necessary for interaction of STEP with its substrates and plays an essential role in many important biological processes such as the stress response, cell proliferation, apoptosis and tumorigenesis (*Paul et al., 2003*). Only the $STEP_{61}$ isoform contains two polyproline (PP) rich regions in the N-terminal domain, two hydrophobic transmembrane domains (TM) and two proline-glutamic acid-serine-threonin (PEST) sequences. These sequences are potential sites for proteolytic cleavage (*Nguyen et al., 1999*). One function of the PP regions is to impart substrate specificity for STEP isoforms. For example, the affinity of Fyn (STEP's substrate) to associate with $STEP_{61}$ is ten-fold greater than with $STEP_{46}$, the isoform that does not contain the PP regions. A series of mutational analysis demonstrates that the first PP region (in addition to the KIM domain) is the region necessary for the interaction of $STEP_{61}$ with Fyn (*Nguyen et al., 2002*).

In addition to $STEP_{46}$ and $STEP_{61}$, two minor alternatively spliced variants of STEP include $STEP_{38}$ and $STEP_{20}$. These variants do not contain the consensus PTP domain and are therefore catalytic inactive (*Brathwaite et al., 2006*). The functions of these two inactive variants are not known, although it is possible that in the absence of a catalytic domain, they may act as a dominant-negative STEP isoforms that compete with the active isoforms for substrate binding

and, by binding to these substrates, prevent their tyrosine dephosphorylation. These variants also have a unique 10-amino acid sequence at their carboxyl terminus that is introduced during splicing of a single gene (*Bult et al., 1996; Saibal, 2011; Goebel-Goody et al., 2012*).

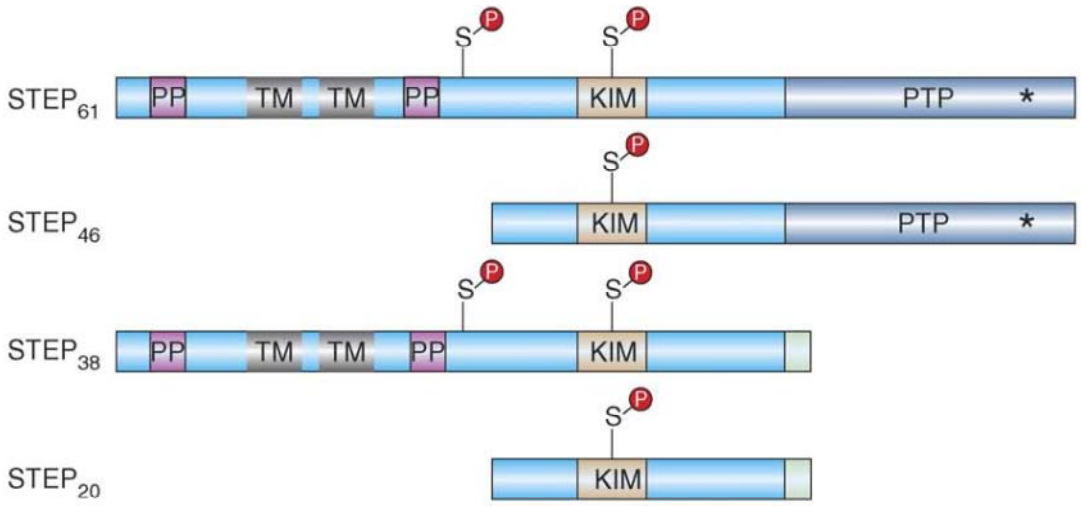


Figure 1. STEP structure. There are four alternatively spliced variants of STEP ($STEP_{46}$, $STEP_{61}$, $STEP_{38}$ and $STEP_{20}$). $STEP_{46}$ and $STEP_{61}$ are the major isoforms expressed in the CNS and differ by an additional 172 amino acids at the N terminus of $STEP_{61}$. This domain contains two transmembrane (TM) domains and two regions (PP) each formed by a polyproline-rich domain and an adjacent PEST domain (adapted from *Braithwaite et al., 2006b*).

1.2. STEP SUBSTRATES

The current model for STEP function is that it opposes the development of synaptic strengthening by dephosphorylating and inactivating key signaling molecules that include the the Mitogen-Activated Protein Kinase Family, the Glutamate receptors and the Tyrosine Kinase **Fyn** (*Nguyen et al.,2002; Paul et al., 2002; Braithwaite et al., 2006; Baum et al., 2010; Goebel-Goody et al., 2012*).

1.2.1. MITOGEN-ACTIVATED PROTEIN KINASE FAMILY

Mitogen-Activated protein Kinases (MAPKs) are important mediators of signal transduction from the cell surface to the nucleus and have been implicated in a wide variety of cellular processes, such as proliferation, differentiation and apoptotic cell death (*Keyse, 2000; Pearson et al., 2001*). Several studies reviewed in Braithwaite et al., (2006b), implicate STEP in the regulation of the MAPKs.

MAPKs are serine/threonine kinases that, when activated, phosphorylate a large number of substrate proteins in the cell, including transcription factors (*Sweatt, 2001; Sharma & Carew, 2004*).

Three major groups of distinctly regulated groups of MAP kinase cascades are known in humans that lead to altered gene expression: the Extracellular Signal-regulated Kinase 1 and 2 (ERK1/2), c-Jun NH_2 terminal kinase (JNK) and Stress-activated Protein Kinase p38 (p38) (*Pearson et al., 2001; Hommes et al., 2003; Qi & Elion, 2005*).

Two members of the MAPK family are known STEP substrates: the ERK1/2 and the p38 (*Paul et al., 2003; Muñoz et al., 2003; Poddar et al., 2010*). The p38 MAPK account for the cell response to stress and inflammation conditions, whereas ERK1/2 are mainly activated by growth and differentiation factors. Active ERK is required for synaptic plasticity in all brain regions tested to date (*Paul et al., 2003; Ivanov et al., 2006*; *Poddar et al., 2010)*. In its activated state, ERK phosphorylates cytoskeletal proteins, regulates backpropagating action potentials, stimulates protein synthesis, and activates transcription (*Braithwaite et al., 2006b*). These processes work in parallel to promote synaptic plasticity (*Barr & Knapp, 2006*).

STEP binds ERK1/2 and p38 through its KIM domain and this association leads to phosphorylation/dephosphorylation events between these two enzymes, which have a regulatory role in the signaling through the MAPK pathways (*Qi & Elion, 2005*). Whereas ERK1/2 promotes learning and cell survival, p38 plays an important role in excitotoxic cell death. The activation of p38 initiates the cell death signaling cascades by phosphorylating the cell survival regulator Bcl-2 and regulating other proapoptotic proteins and transcription factors (*Hopf & Bonci, 2009*).

Venkitaramani et al., (2009), show that ERK1/2 phosphorylation is significantly elevated in the striatum, hippocampus, and central/lateral amygdala of STEP KO mice compared with WT littermates, providing additional support for the regulation of ERK1/2 by STEP (*Venkitaramani et al., 2009*).

1.2.2. GLUTAMATE RECEPTORS

The ionotropic glutamate N-methyl-D-aspartate receptors (NMDARs) are heteromeric ligand-gated ion channels assembled from two subunit families: NR1, which consists of eight recognized isoforms that are generated from alternative splicing of a single gene, and NR2, composed of NR2A, NR2B, NR2C and NR2D, encoded by four distinct genes (*Orlando et al., 2002; Jiang et al., 2011*).

Three distinct components of NMDA receptor trafficking have been identified, each with their own regulatory features: synthesis and assembly of receptor subunits within the endoplasmic reticulum; packaging and delivery of vesicles containing assembled receptors to spines; and insertion of receptor complexes into the synaptic site (*Leveille et al., 2008; Zorumski & Izumi, 2012*).

The NMDA receptor is a second potential STEP substrate (*Pelkey et al., 2002; Snyder et al., 2005; Braithwaite et al., 2006a; Gladding & Raymond, 2011*). STEP regulates NMDA receptor trafficking by controlling the level of tyrosine phosphorylation of the NR2B subunit. Phosphorylation of NR2B at Tyr^{1472} by Src-family members, including Fyn, is required for the movement of NMDA receptors into membranes (*Trepanier et al., 2012*). However, dephosphorylation of the NR2B subunit at this residue leads to endocytosis of NMDA receptors through a mechanism mediated by clathrin and adaptor protein 2 (*Braithwaite et al., 2006b*). Current studies are investigating whether this is a direct effect, through dephosphorylation of the NMDA receptor by STEP, or an indirect effect, through the ability of STEP to reduce Fyn activity and thus decrease NMDA receptor phosphorylation levels, or whether both mechanisms work together in a cooperative way (*Braithwaite et al., 2006a; Hardingham & Bading, 2010*).

STEP seems to directly affect the conductance properties of NMDA receptors in addition to regulating NMDA receptor trafficking, and together these mechanisms oppose the development of synaptic plasticity (*Snyder et al., 2005; Hicklin et al., 2011*).

1.2.3. TYROSINE KINASE Fyn

Src family kinase is a family of non-receptor tyrosine kinases that includes: Src, Yes and Fyn. Src family kinases interact with a variety of cellular cytosolic, nuclear and membrane proteins, modifying these proteins by phosphorylation of tyrosine residues. A number of substrates have been discovered for these enzymes (*Ohnishi et al., 2011*).

Mutations of PTPs in their catalytic domain create inactive variants that can be used as substrate-trapping proteins. Such an inactive STEP protein was used to identify the non-receptor tyrosine kinase Fyn as a third STEP substrate. STEP interacts with Fyn through its KIM domain, although the first polyproline sequence present in $STEP_{61}$ is also involved in Fyn binding (*Jiang et al., 2011*).

Ngueyn et al., (2002), demonstrate that STEP specifically catalyzes the dephosphorylation of Fyn at Tyr^{420}, leading to inactivation. These results suggest that $STEP_{61}$ participates in regulating Fyn activity and that this is one mechanism by which STEP regulates signaling events at excitatory synapses (*Ngueyn et al., 2002*).

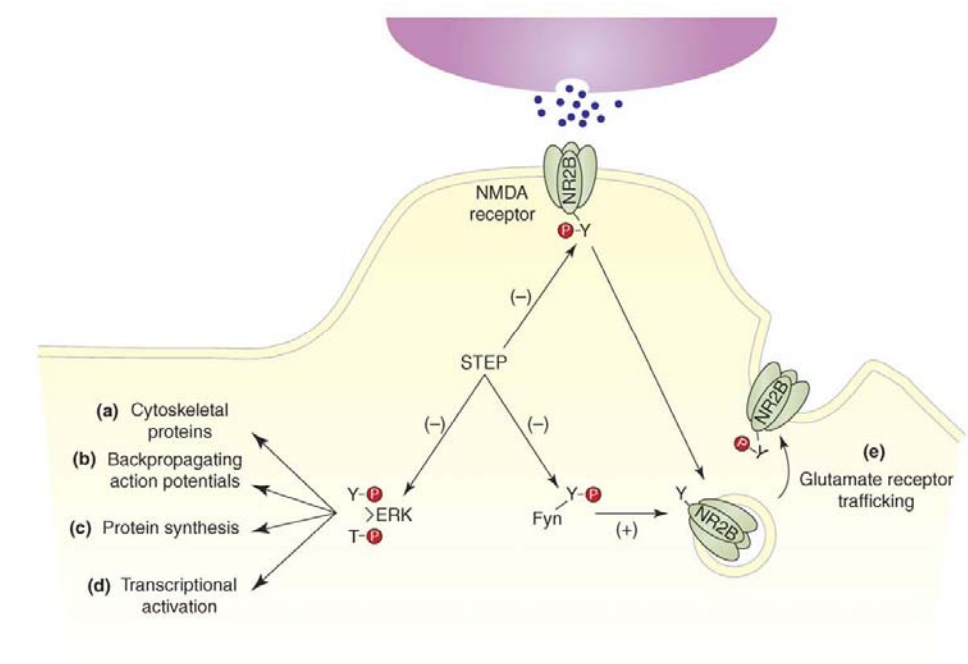


Figure 2. STEP substrates. STEP dephosphorylates ERK, Fyn and the NMDA receptor complex. ERK, Fyn and the NR2B subunit of the NMDA receptor are potential STEP substrates (adapted from *Braithwaite et al., 2006b*).

1.3. REGULATION OF STEP ACTIVITY

STEP expression and ability to bind to and dephosphorylate its substrates is regulated by known mechanisms such as phosphorylation, dimerization and proteolytic cleavage (*Braithwaite et al., 2006b*; *Fitzpatrick & Lombroso, 2011; Goebel-Goody et al., 2012*).

Studies on the regulation of STEP activity have focused upon the striatum where it is expressed in medium spiny neurons that make up about 90% of the neuronal cell types within this brain region. This type of neurons receives two main synaptic inputs: a glutamatergic input from the neocortex and a dopaminergic inputs from the midbrain close to the glutamatergic contacts (*Paul et al., 2000; Chen et al., 2009*). The proximity of these two synaptic inputs suggests a functional consequence, namely, the modulation of one neurotransmitter pathway by the other (*Cepeda & Levine, 1993*). The presence of STEP in the same subcellular compartment raised the possibility that it may regulate or be itself regulated by one of these neurotransmitter systems. Both dopamine (DA) and glutamate are critical in the regulation of neuronal function trough activation of the two chief intracellular signals: **Calcium** (Ca^{2+}) and **cyclic Adenosyne Monophosphate** (cAMP) (*Cepeda & Levine, 1993; Dunah & Standaert, 2001; Hallett et al., 2006*).

$STEP_{46}$ and $STEP_{61}$ activity is regulated by the phosphorylation of a Serine (Ser) residue (Ser^{49} and Ser^{221}, respectively) within its KIM domain. Phosphorylation at this site prevents STEP from binding to all of its substrates (*Paul et al., 2000; Paul et al., 2003*).

Stimulation of D1 and D5 dopamine receptors (DRs) or blockade of DRs D_2 (D_2S and D_2L) D_3 and D_4 is coupled to adenylciclase through Gαs leading to increased cAMP levels, which in turn activates the Protein Kinase A (PKA) pathway. In your turn, PKA phosphorylates $STEP_{46}$ and $STEP_{61}$, inactivating them. In contrast, glutamate stimulation of NMDARs results in the influx of Ca^{2+}, activation of the serine/threonine phosphatase calcineurin and also activation of Protein Phosphatase 1 (PP1) (*Paul et al., 2000; Valjent et al., 2005*) that dephosphorylates STEP. Once activated, STEP dephosphorylates the glutamate receptor subunit NR2B (*Pelkey et al., 2002; Braithwaite et al., 2006a)* leading to endocytosis and also to the inactivation of the kinases ERK1/2, p38, and Fyn (*Nguyen et al., 2002; Paul et al., 2007*).

The ability of PP1 to dephosphorylate and activate STEP is also controlled by dopamine-and cAMP-dependent phosphoprotein of 32 kDa (DARP-32) (*Fernandez et al., 2006; Dupre, 2008*). A prominent aspect of the distribution of DARPP-32 in the brain is its high enrichment in dopaminoceptive neurons in the striatum (*Svenningsson et al., 2005*). When phosphorylated by cAMP-dependent PKA, DARP-32 inhibits PP1 activity so that it no longer dephosphorylates /activates STEP. In this way two parallel pathways converge to regulate STEP phosphorylation and substrate binding: ***1)*** Direct Phosphorylation of STEP by PKA; ***2)*** Indirect regulation of STEP phosphorylation via PKA-induced phosphorylation and activation of DARP-32, which in turn, inhibit PP1 activity.

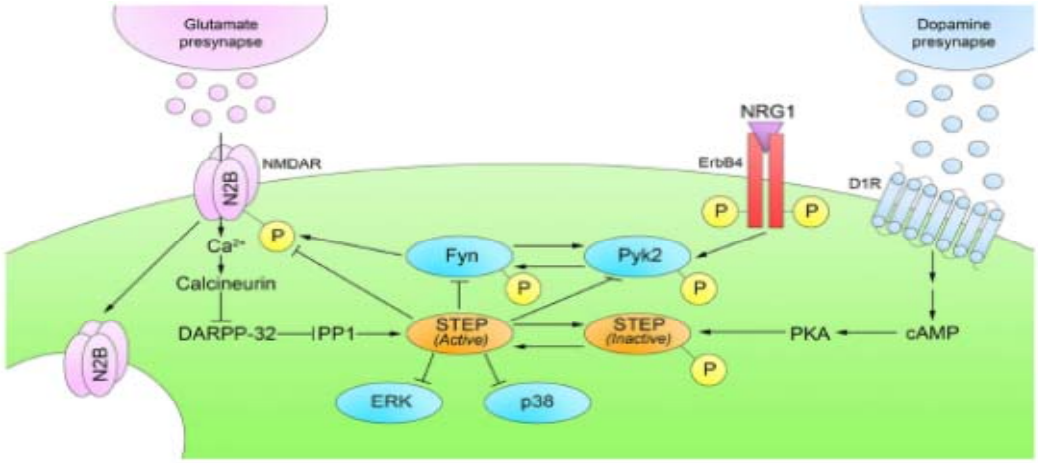


Figure 3. Regulation of STEP phosphorylation. D1R stimulation phosphorylates and inactivates STEP through a cAMP/PKA pathway. Glutamatergic stimulation of NMDARs reverses this process by dephosphorylation and activation of STEP through a calcineurin/PP1 pathway. Active STEP dephosphorylates regulatory tyrosines within STEP substrates. STEP dephosphorylation of Fyn, Pyk2, ERK1/2 and p38 leads to their inactivation; however, STEP dephosphorylation of GluN2B subunit results in internalization of NMDARs from synaptosomal surface membranes (adapted from *Fitzpatrick & Lombroso, 2011*).

Deb et al., (2011), showed that $STEP_{61}$ can also form dimers under basal conditions both in neurons and in transfected cells. This basal dimerization involves intermolecular disulfide bond formation between two cysteine residues (Cys^{65} and Cys^{76}) present in a hydrophobic region of the amino terminus of $STEP_{61}$. Dimerization of $STEP_{61}$, but not $STEP_{46}$, occurs basally and results in substantial loss of phosphatase activity. Oxidative stress induced by hydrogen peroxide (H_2O_2) leads to a significant increase in the formation of dimers and high-order oligomers of $STEP_{61}$ and results in even less phosphatase activity. Although $STEP_{46}$ does not dimerize under basal conditions, oxidative stress caused by exposure to H_2O_2 also leads to formation of oligomers. The loss of phosphatase activity of PTP's in response to various oxidants, including H_2O_2, has been attributed, at least in part, to the oxidation of the catalytic site cysteine residue. These studies may suggest a class of non-competitive STEP inhibitors (*Deb et al., 2011; Braithwaite et al., 2006b; Goebel-Goody et al., 2012*).

Another regulatory mechanism of STEP is the proteolytic cleavage mediated by a Ca^{2+} - dependent protease calpain. $STEP_{61}$ is rapidly cleaved between residues Ser^{224} and Leu^{225} in the KIM domain and produces a smaller isoform ($STEP_{33}$) that cannot associate with or dephosphorylate its substrates. This cleavage frequently occurs after strong glutamatergic stimulation such as during excitotoxic or ischemic insult (*Nguyen et al., 1999; Xu et al., 2009*). The extrasynaptic NMDAR stimulation results in the activation of one of STEP's substrates, p38, and initiates the cell death signaling cascade. These findings suggest that treatments preventing the cleavage of $STEP_{61}$ may be useful in stroke/ischemia (*Braithwaite et al., 2008; Xu et al., 2009)*. Similar to the phosphorylation site the cleavage site resides within the KIM domain and has been shown to negatively affect substrate binding, thereby prolonging substrate activity (*Paul et al., 2007*).

1.4. INVOLVEMENT OF STEP IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are a group of chronic, progressive disorders characterized by the gradual loss of neurons in discrete areas of the CNS (*Ross & Poirier, 2004*).

Many studies show that STEP deregulation contributes to the pathophysiology of neurodegenerative diseases. In some cases, STEP protein and/or activity is up-regulated, this is the case of Alzheimer's disease (AD), Schizophrenia (SZ) and Fragile X Syndrome (FXS). In these cases the learning process is compromised. Moreover, there are some diseases where STEP protein and/or activity is down-regulated such as Huntington's disease (HD) and in the stroke/ischemia (*Hasegawa et al., 2000; Snyder et al., 2005; Braithwaite et al., 2008; Kurup et al., 2010; Zhang et al., 2010; Saavedra et al., 2011; Goebel-Goody et al., 2012*).

A large amount of data presented in the next section support the STEP's role in some neurodegenerative diseases as Alzheimer's disease and Huntington's disease.

1.4.1. ALZHEIMER'S DISEASE

Alzheimer's disease is the most common neurodegenerative disorder worldwide (*Tanzi, 2005*). It is characterized by the presence of intracellular neurofibrillary tangles of hiperphosphorylated tau protein, accumulation of ß-amyloid peptide (Aß) and the formation of amyloid plaques, all of which have been implicated in synaptic loss, in the blockade of Long-Term Potentiation (LTP), a form of synaptic plasticity closely associated with learning and memory, and in the cognitive decline (*Wetzel et al., 2004; Nixon et al., 2005; Venkitaramani et al., 2007; Hu et al., 2012*).

Snyder et al., (2005), showed that Aß modulates STEP via two parallel pathways:

1) Dephosphorylation Pathway: the Aß peptide binds to NMDARs leading to a Ca^{2+} influx into neurons and activates the Protein Phosphatase 2B (PP2B)/calcineurin and PP1 to dephosphorylate $STEP_{61}$, thereby increasing the affinity of $STEP_{61}$ for its substrates (*Snyder et al., 2005; Mansuy et al., 2006*);

2) Degradation Pathway: the Aß peptide inhibits the Ubiquitin Proteasome System (UPS) and prevents degradation of $STEP_{61}$ (*Snyder et al., 2005; Kurup et al., 2010*).

Both pathways showed that in AD there is an accumulation of unphosphorylated and active $STEP_{61}$ protein levels which leads to internalization of NMDARs and consequently decreases LTP and facilitates Long-term Depression (LTD), an opposing form of synaptic plasticity (*Snyder et al., 2005; Venkitaramani et al., 2007; Kurup et al., 2010; Zhang et al. 2011*).

1.4.2. HUNTINGTON'S DISEASE

In some diseases, STEP protein and/or activity is up-regulated but reduced protein levels can also have a negative impact on neuronal function and contribute to neurodegenerative disorders. One example is Huntington's disease (*Saavedra et al., 2011*), a progressive neurodegenerative disorder with an established genetic origin and characterized by poor muscle coordination, mood disorders and dementia (*Ross & Margolis, 2002*). HD is an autossomal dominantly inherited disorder caused by an abnormal CAG codon expansion in exon-1 of the *huntingtin (htt)* gene (*The Huntington's Disease Collaborative Research Group, 1993*). This results in a neurodegenerative disorder that specifically affects striatal projection neurons, which constitute the vast majority of striatal neurons (*Reiner et al., 1988*).

Recent findings from Saavedra et al., (2011), demonstrated that STEP plays a role in the pathophysiology of HD. R6/1 mice that express mutant huntingtin (*mhtt*), display reduced STEP protein levels in the striatum and cortex and increased phosphorylation levels in the

striatum, cortex and hippocampus. Both of these events would decrease the ability of STEP to dephosphorylate its substrates. The early increase in striatal STEP phosphorylation levels correlates with a dysregulation of the PKA pathway that together with reduced calcineurin activity at later stages further contributes to an enhancement of STEP inactivation. Consistent with decreased STEP expression and activity, the phosphorylation of both ERK1/2 and p38, two targets of STEP, are elevated in R6/1 mice striatum at advanced stages of the disease (*Saavedra et al., 2011*).

The same group has also shown that decreased STEP activity could, through the regulation of its targets, be involved in the development of resistance to excitotoxicity in the striatum of R6/1 mice. Intrastriatal injections of quinolic acid (NMDAR agonist) induce excitotoxic cell death and result in lesion formation, which is dependent on calcineurin activation. After the injection, they detected higher phosphorylated STEP levels in R6/1 than in wild-type mice, suggesting that STEP inactivation could mediate neuroprotection in R6/1 striatum (*Xifró et al., 2009*).

Together, these findings demonstrate that STEP increases the vulnerability of striatal neurons to undergo cell death after an excitotoxic insult and establish that the down-regulation of STEP in HD mouse models may be responsible for their increased resistance to excitoxicity (*Xifró et al., 2009*; *Saavedra et al., 2011*).

Although STEP has been associated to several neurodegenerative diseases there is no information on the expression of STEP in Parkinson's disease (PD).

1.5. PARKINSON'S DISEASE

Parkinson's disease is the most prevalent movement disorder and second to Alzheimer's disease the most prevalent neurodegenerative disorder. PD is named after James Parkinson who described the clinical symptoms in 1817 in his monograph "Essay on the Shaking Palsy" (*Dauer & Przedborski, 2003*).

PD is a progressive illness particularly prevalent in the elderly population, with a typical clinical onset after 60–65 years of age. There are four cardinal features of PD that can be grouped under the acronym TRAP: Tremor at rest, Rigidity, Akinesia (or bradykinesia) and Postural instability. Non-motor symptoms are a common and underappreciated feature of PD. These include autonomic dysfunction, cognitive/neurobehavioral disorders, and sensory and sleep abnormalities (*Jankovic, 2008*).

This disorder is caused by the progressive degeneration of the neuronal connection between the *Substantia Nigra pars compacta* (*SNpc*) and the striatum, two brain regions essential for motor function (*Nussbaum & Ellis, 2003*).

Braak et al., (2003), suggested that the pathology of PD starts in the brainstem and olfactory regions and gradually spreads to affect other brain areas. Only when cell death occurs in the *SNpc* and motor symptoms appear is a diagnosis of PD proposed (*Braak et al., 2003*).

The first symptoms appear when DA levels in the striatum are reduced by 80% or when 60% of the nigrostriatal dopaminergic neurons are lost, what may indicate that the underlying cause of Parkinson's disease happens, years if not decades, before symptoms start to become visible (*Bossy et al., 2004*).

Besides dopaminergic neurons from the nigrostriatal pathway other neurotransmitter systems (e.g. cholinergic, adrenergic, and serotoninergic) are also affected and cell loss is seen in other brain stem nuclei and in the cortex. This non-dopaminergic degeneration is a major cause of the non-motor symptoms of PD such as cognitive decline and autonomic dysfunction (*Giasson & Lee, 2001*).

The pathology of the disease is characterized by the accumulation of a protein called α-synuclein into neuronal eosinophilic intracytoplasmatic inclusions known as *Lewy bodies* (LBs). These inclusions are the pathological hallmark of PD. They are α-synuclein-immunoreactive inclusions made up of a number of neurofilament proteins together with proteins responsible for proteolysis. These include ubiquitin, a heat shock protein which plays an important role in targeting other proteins for breakdown (*Chung et al., 2001; Taylor et al., 2002*). Mutations in the α-synuclein gene (SNCA) are responsible for some familial forms of PD in which LBs are also seen. Mutations in the parkin protein produce a Parkinsonian syndrome without LBs in juvenile cases suggesting that the parkin protein plays an important role in the development of these inclusions. It has been shown that parkin facilitates the binding of ubiquitin to other proteins leading to the formation of LBs. Lewy bodies are found in PD and dementia but are not a pathological hallmark of any other neurodegenerative disease (*Lim & Tan, 2007*). The anatomical distribution of the Lewy bodies is often directly related to the expression and degree of the clinical symptoms of each individual (*Davie, 2008*).

1.5.1. NIGROSTRIATAL PATHWAY

Dopamine, also a catecholamine, is an important neurotransmitter, especially in the CNS, where it is the most abundant catecholamine neurotransmitter (*Girault & Greengard, 2004*).

Catecholamines are synthesized at the nerve terminal and stored in vesicles. Dietary phenylalanine is converted to L-tyrosine, which is taken up into the cytoplasm of the nerve terminal and hydroxylated to form L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) (*Jakowec et al., 2004*). This is the rate-limiting step of catecholamine biosynthesis. Tyrosine hydroxylase requires iron, molecular O_2 and tetrahydrobiopterin as cofactor. The cofactor helps to keep TH active in a reduced state (*Nass & Przedborski, 2008*).

DA, once synthesized is stored in synaptic vesicles that protect the dopaminergic neurons from its auto-oxidative effects. In dopaminergic neurons, sequestration of cytosolic DA into synaptic vesicles by Vesicular Monoamines Transporters (VMATs) is essential for the neurons to avoid the neurotoxicity of DA. There are two types of VMATs: **$VMAT_1$** that is localized predominantly in the neuroendocrine cells and the **$VMAT_2$** that is widely distributed in monoaminergic terminals and dendrites (*Cartier et al., 2009*). DA that is not incorporated into the vesicles gets oxidized, either spontaneously or by Monoamine Oxidase type A (MAO-A), leading ultimately to the formation of Reactive Oxygen Species (ROS) that are neurotoxic. The dopaminergic synaptic transmission is terminated by the transportation of 95% of synaptic dopamine into the nigrostriatal terminals by the dopamine transporter (DAT) (*Lyons & Pahwa, 2007*).

There are four major dopaminergic pathways in the brain: the **mesolimbic pathway** that originates in the ventral tegmental region of the midbrain, near the *SNpc*, and projects to several higher centers of the limbic system; the **mesocortical pathway** projects from the ventral tegmentum to the frontal cortex; the **tuberoinfundibular pathway** is a short but important DA projection and, last but not least, the **nigrostriatal pathway** that projects from the *SNpc* to the putamen and caudate nucleus, which are implicated in the control of fine motor function (*Greenstein & Greenstein, 2000*). The dopaminergic nigrostriatal pathway is the largest brain pathway using dopamine as neurotransmitter, and has been extensively studied because of its importance in degenerative brain diseases (*Waxman, 2005*).

In the nigrostriatal pathway, the cell body of dopaminergic neurons localize in the *SNpc*, and the axons project to the striatum (caudate-putamen). In general, these neurons regulate the motor integration, neuroendocrine hormone function, sexual activity, cognition, emotional behavior and a sense of reward (*Prensa et al., 2009*).

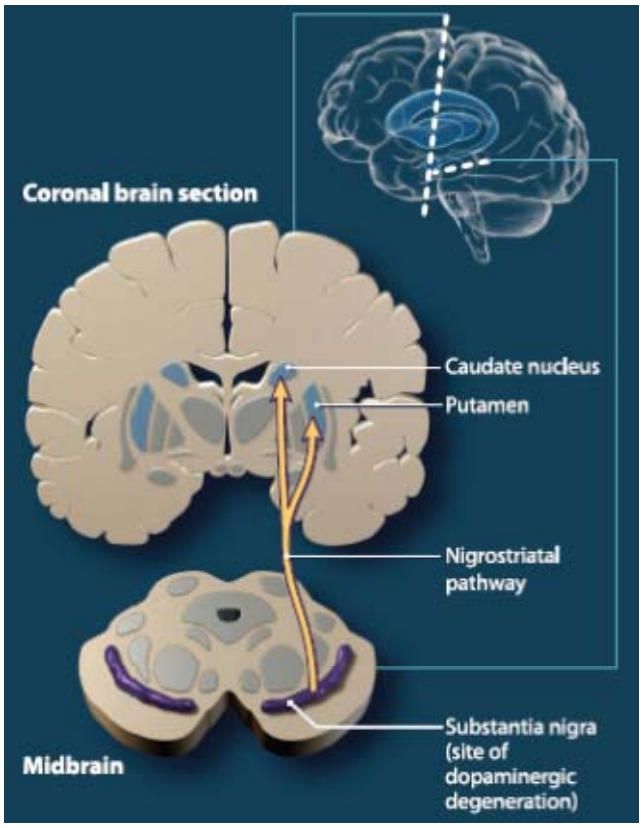


Figure 4*:* Coronal section of the brain showing nigrostriatal pathway and location of selective dopaminergic degeneration (adapted from *Guttman, 2003*).

As related above, one of the affected areas in Parkinson's disease is the basal ganglia, an important area related to motor, cognitive and emotional symptoms. The basal ganglia are a group of nucleus of varied origin in the brain of vertebrates that act as a cohesive functional unit (*Graybiel, 2000*). They are situated at the base of the forebrain and are strongly connected with the cerebral cortex, thalamus and other brain areas. The main components of the basal ganglia are the **striatum** (composed of the caudate and putamen), the **globus pallidus** (composed of globus pallidus external segment (GPe) and globus pallidus internal segment (GPi)), the ***substantia nigra*** (composed of both *substantia nigra pars compacta* and *substantia nigra pars reticulata* (*SNpr*)) and the **subthalamic nucleus** (STN) (*Haber & Gdowsky, 2004)*. The largest component, the striatum, receives input from many brain areas but sends output only to other components of the basal ganglia (*Graybiel, 2005*).

Clinical and experimental work on the basal ganglia has been inspired by the idea that this region can release or inhibit movement by the opposing influences of two main pathways originating in the striatum and extending through the GP and *SN*: the movement-releasing ***Direct Pathway*** and the movement-inhibiting ***Indirect Pathway*** (*Graybiel, 2005*). The direct pathway is related to GABAergic cell bodies in the striatum which contain on their surface D_1 dopamine receptors while in the indirect pathway those cell bodies contain mainly D_2 dopamine receptors. DA activates the D1Rs and consequently activates Gαs-proteins that

stimulate the production of cAMP. In the other hand, the D2Rs act through different G-proteins, inhibiting cAMP production. Depletion of dopamine in the striatum, characteristic in PD, leads to increased activity in other basal ganglia nucleus and promotes neuronal inhibition and difficult to initiate movement (*Prensa & Parent, 2001; Hurley & Jenner, 2006; Nass & Przedborski, 2008*).

1.5.2. ETIOLOGY OF PD

Previously, Parkinson's disease has been considered a nongenetic disorder. However, recent data increasingly implicate genetic factors in its etiology (*Goedert, 2001*). There are growing number of genes that have been associated with the disease: α-synuclein, ubiquitin C-terminal hydrolase like 1 (UCH-L1), parkin (PRKN), LRRK2, PINK 1 and DJ-1 genes (*Napolitano et al., 2002; Davie, 2008*). This type of PD is referred to as inherited PD and is often characterized by an early onset of the disease. The rest of cases are referred to as sporadic PD. Although a number of genetic polymorphisms are linked to PD, it is likely that the majority of PD cases are not inherited but related to environmental factors (*Brown et al., 2005*).

Studies in human *postmortem* material indicate that ROS are important in the pathogenesis of sporadic PD (*Zhang et al., 2000*). There are also consistent findings of decrements in mitochondrial complex I function. Impaired complex I activity leads to free radical stress and makes neurons vulnerable to glutamate excitotoxicity (*Dawson & Dawson, 2003*). Several epidemiologic studies suggest that pesticides and other environmental toxins that inhibit complex I, such as, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat, are involved in the pathogenesis of PD (*Dauer & Przedborski, 2003*).

The main risk factor in sporadic PD is age. Although age itself can not cause PD, over time, accumulation of small mistakes made by biochemical processes inside the cell might eventually pass a threshold causing a positive feedback loop (*Moore et al., 2005*).

1.5.3. EXPERIMENTAL MODELS OF PARKINSON'S DISEASE

The rodent toxin models of Parkinson's disease are among the earliest developed and they remain in wide use today. With so much being learned today about the genetic basis of PD, and with widespread availability of modern techniques of transgenesis, the rodent toxin models continue to be studied because only these neurotoxins models reliably produce neurodegeneration in dopaminergic neurons of the *substantia nigra (Dauer & Przedborski, 2003; Tieu, 2011)*. So, for studies related to neuron death, and with reasonable likelihood of

direct relevance to human PD, rodent neurotoxins models remain indispensable. The more studied dopaminergic neurotoxins are:

- **6-Hydroxidopamine** (6-OHDA), which was the first chemical agent discovered with specific neurotoxic effects on catecholaminergic pathways. 6-OHDA uses the same catecholamine transport system as dopamine and produces specific degeneration on catecholaminergic neurons. Systematically administered this toxin cannot cross the blood-brain barrier. To specifically target the nigrostriatal pathway, 6-OHDA must be injected stereotactically into the *SNpc* and striatum (*Betarbet et al., 2002*).

- **Rotenone** is a naturally occurring complex ketone pesticide. Due to its hydrophobicity rotenone crosses biological membranes easily, including the blood-brain barrier and it does not depend on the dopamine transporter to access the cytoplasm. This toxin is a strong inhibitor of complex I, which is located at the inner mitochondrial membrane and protrudes into the matrix (*Hisahara & Shimohama, 2011*). Betarbet et al., (2000), demonstrated that chronic systemic exposure of rotenone to rats causes many features of PD, including nigrostriatal dopaminergic degeneration (*Betarbet et al., 2000*).

- **Paraquat** or 1,1'-dimethyl-4,4'-bypyridinium is a herbicide that has emerged as a putative risk factor for PD (*Hisahara & Shimohama, 2011*). Paraquat crosses the blood-brain barrier, although slowly, and to a limit extend causes destruction of the dopaminergic neurons in the *SNpc*. The mechanism of action of this neurotoxin is believed to involve oxidative stress and the mitochondria (*Betarbet et al., 2002*).

- **1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine** (MPTP) was identified as a contaminant of the manufacture of a synthetic opiate. Drug users who accidentally injected MPTP developed a syndrome resembling PD. In humans and non-human primates, depending on the regimen used, MPTP can produce an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD, including tremor, rigidity, slowness of movement, postural instability, and freezing (Waxman*, 2005)*.

MPTP has a complex multistep metabolism (*Przedborski et al., 2001*). It is highly lipophilic, and freely and rapidly crosses the blood-brain barrier. Once in the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) in glia and serotonergic neurons, the only cells that contain this enzyme (*Vila et al., 2000*). $MPDP^+$ is then converted (probably by spontaneous oxidation) to the active MPTP metabolite: 1-methyl-4-phenylpyridinium (MPP^+), which is released into the extracellular space, where acts as a "false-substrate" for DAT. MPP^+ accumulates in dopaminergic neurons and inhibits mitochondrial complex I thus leading to energy depletion and the formation of reactive oxygen species (*Petzinger et al., 2006*).

MPTP is usually systemically administered (subcutaneous, intraperitoneal, intravenous or intramuscular). For unknown reasons, rats are resistant to MPTP toxicity and mouse strains vary widely in their sensitivity to the toxin. Several different experimental paradigms have been developed and used over time (*Betarbet et al., 2002; He et al., 2004*).

The MPTP model has become the most commonly used, for at least three reasons. MPTP is the only known dopaminergic neurotoxin capable of causing a clinical picture in both humans and monkeys indistinguishable from PD. Although handling MPTP requires a series of precautions, its use is not otherwise technically challenging: it does not require any particular equipment such as a stereotactic surgery on live animals as for 6-OHDA. Finally, MPTP produces a reliable and reproducible lesion of the nigrostriatal dopaminergic pathway after its systemic administration, which is often not the case for other documented poisons (*Tieu, 2011*).

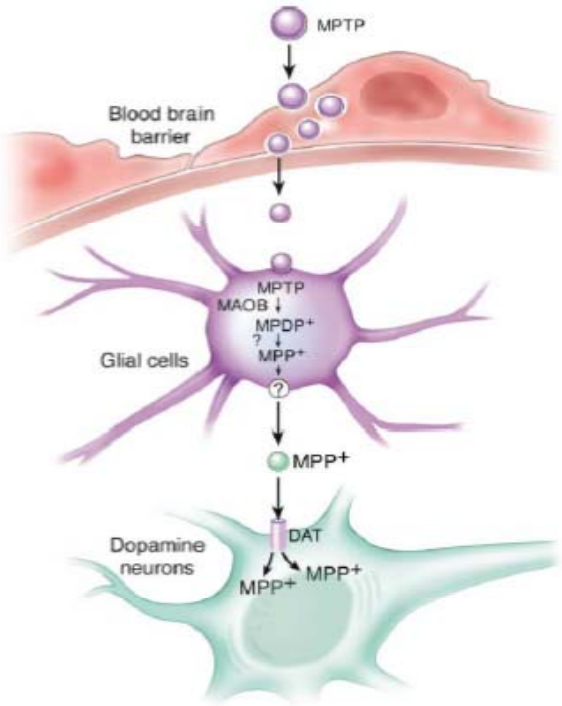


Figure 5: Schematic representation of MPTP Metabolism after systemic administration (adapted from *Dauer & Przedborski, 2003*).

As mentioned above (section 1.3), STEP can be regulated by dopaminergic receptors and dopamine is a neurotransmitter that influences STEP expression. Since PD is characterized by reduced levels of dopamine in the striatum; in this present study we examined if a selective MPTP/MPP^+-dopaminergic lesion in cellular and mouse models influence the expression of STEP in the nigrostriatal pathway.

Chapter 2

OBJECTIVES

The general objective of this work is to determine if changes in dopaminergic signaling, resultant from a selective dopaminergic lesion in Parkinson's disease models, influence the expression of STEP in the nigrostriatal pathway.

The specific objectives are as follows:

- To characterize the expression of $STEP_{61}$ in the nigrostriatal pathway along different developmental stages;
- To determine if midbrain reactive glial cells express this protein;
- To investigate if exposure of ventral midbrain cell cultures to a specific neurotoxin alters the expression and/or activity of STEP;
- To find out, in a Parkinson's disease animal model, if STEP expression and/or activity are regulated by the dopaminergic lesion.

Chapter 3

MATERIALS AND METHODS

3.1. ANIMALS

All experiments related to animals were conducted in compliance with protocols approved by the national ethical requirements for animal research, and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive number 2010-63-EU).

All animals were kept under controlled conditions of temperature and light, with food and water available *ad libitum*.

3.2. *IN VITRO* ASSAY

3.2.1. NEURON-ASTROCYTE MIDBRAIN CO-CULTURES

For the *in vitro* assays we used Wistar pregnant females with 15 or 16 days of gestation. After anesthetization of the pregnant females with Ketamine:Xylazine (2:1), the embryos were removed and the ventral midbrains was dissected.

After dissection of the ventral midbrain, the meninges were carefully stripped of, and the dissected tissue was put in phosphate buffer saline (**PBS**: *NaCl 140 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM and Na_2HPO_4 8.1 mM, pH 7.4*). The tissue was then dissociated by enzymatic digestion (*Trypsin 4.5mg/ml and DNAse 2.5 mg/ml diluted in PBS*) and incubated at 37º C for 5 minutes. The cells were pelleted by centrifugation (*3K18C Bioblock Scientific; Sigma Laboratory Centrifuges*) for 1 minute at 88 g. To stop the enzymatic digestion, a solution containing PBS with 10% Fetal Bovine Serum (FBS) inactivated by heat (*Biochrom, Holliston, USA*), was added to the pellet. The cell suspension was centrifugated for 1 minute at 88 g and the supernatant was rejected. After adding PBS to the pellet, the tissue was mechanically dissociated with a 5 ml pipette, followed by further 5-10 sequential passes with micropipette tips. The suspension was centrifugated one more time for 3 minutes at 405 g.

The cells were resuspended in Neurobasal Medium (*Gibco, Paisley, Scotland, UK*) supplemented with *B27 2%, glutamate 25 μM/mL, glutamine 0.5 mM/mL* and *gentamicine 120 μg/mL*. For cell counting it was performed a 1:1 dilution of the final cell suspension with Trypan blue (0.4% in NaCl 0.9%) and viable cells were counted using a Neubauer chamber.

Finally, the cells were plated into poly-D-lysine (*Sigma-Aldrich, St. Louis, USA)*-coated 12-wells plates or coated-coverslips (*BD Biosciences, San Jose, California, USA)* at a density of 0.8 x 10^6 cells/well. The cultures were kept at 37º C under a 5% CO_2 and 95% air atmosphere during 4-5 days. After cells reach confluence, 5- fluorodeoxyuridine was added to the culture (**FDU**: *uridine 68μM and 5-Fluoro-5′-deoxyuridine 27 μM)* (Sigma-Aldrich, *St. Louis, USA*) to inhibit cell proliferation.

3.2.2. MPP^+ STIMULUS

Twenty four hours before the stimulus, the culture medium was replaced with 1.2 ml of fresh medium.

To induce the dopaminergic lesion, different concentrations (0 μM, 5 μM, 10 μM and 20 μM) of MPP^+ (*Sigma-Aldrich, St. Louis, USA*) were applied. The culture plates were then incubated at 37°C in 5% CO_2, 95% air atmosphere for further 24 hours. At the end of the experiment the cells used in Western blot assays were lysed with 150μl of lysis buffer (*Triton X-100 1%, Tris-HCl 50mM, pH 7.5, EGTA 10 mM, NaCl 150 mM, protease inhibitors (phenylmethylsulphonyl fluoride 2 mM, aprotinin 10 μg/μl, and leupeptin 1 μg/μl), and phosphatase inhibitor (Na_3VO_4 2 mM)*) whereas the cells used for immunocytochemical analysis were fixed with Paraformaldehyde Fixate Solution 4% (PFA) during 10 minutes. The coverslips were kept in PBS until Immunocytochemical processing.

The Figure 6 is representative of the cell cultures treatment.

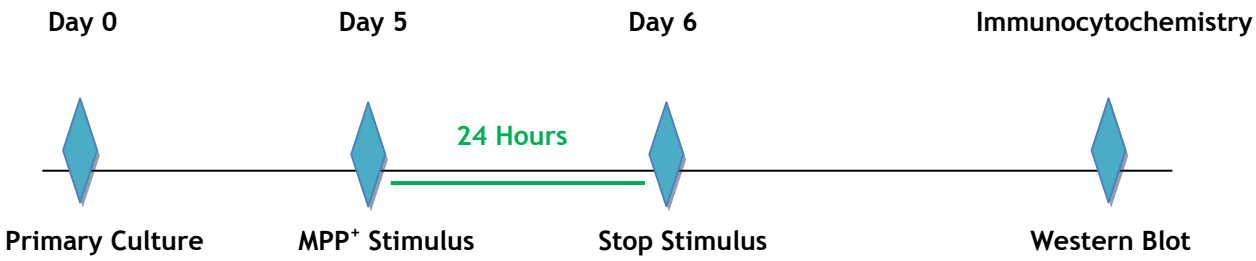


Figure 6. Treatment of the cells cultures within MPP^+. The cells isolated from *SN* and plated in 12-coated wells plates or coated-coverslips, maintained under the appropriate conditions proliferated until they reach confluence (days 4-5). At this stage, fresh medium without serum was added to the cells to avoid the influence of hormones and growth factors. At day 5 we applied different concentrations of MPP^+ (0 μM, 5 μM, 10 μM and 20 μM) to the cultures for 24 hours. The experiment was stopped by adding lysis buffer (Western blot) or fixation of the cells (immunocytochemistry).

3.2.3. IMMUNOCYTOCHEMISTRY

To initiate the immunocytochemistry, cells were washed with PBS, permeabilized with 0.25% Triton X-100 in PBS for 10minutes and then washed with PBS. Once washed, non specific binding was blocked by incubation of cells with solution of 20% FBS in PBS and 0.1% - Tween (PBS-T) for 1 hour at room temperature.

After blocking, the coverslips were washed with PBS-T and incubated with the primary antibodies (diluted in PBS-T 1% FBS, according to Table 1) overnight at 4º C. After overnight incubation, the coverslips were washed 6x during 15 minutes with PBS-T and then were incubated with the corresponding secondary antibodies conjugated to Alexa® 488 or Alexa® 594 fluorophores diluted in the same solution used previously to prepare the primary antibodies (according to Table 1).

After the incubation with the secondary antibodies the cells were washed three times with PBS-T, counterstained with Hoecsht 2µM (*Invitrogen, CA, USA*) during 10 minutes and mounted in a fluorescent mounting medium (*DAKO, Glostrup, Denmark*). The images were acquired with a Zeiss Axio imaging Microscope (*Axiobserver Z1, Zeiss*).

3.2.3.1. CELL COUNTING AND QUANTITIVE ANALYSIS

All immunocytochemical analyses were performed at least in three different preparations using two or three coverslips per experiment and at least 10 snaps per coverslip at the same exposure. TH positive cells were determined by counting for each staining, 40 fields (x63 magnification) of two or three coverslips for the different conditions. The results are represented as the mean value of TH positive cells in relation to total number of cells ± SEM.

Table 1. Primary and secondary antibodies used in Immunocytochemistry.

Protein	Primary Antibody	Dilution	Company	Secondary Antibody	Dilution	Company
STEP	Monoclonal Anti-STEP	1:200	*23E5, Santa Cruz Biotechnology, Germany*	Anti-Mouse conjugated to Alexa® 488	1:1000	*Invitrogen, UK*
Tyrosine - Hydroxylase	Polyclonal Anti-TH	1:500	*ab112, abcam, Cambridge, USA*	Anti-Rabbitt conjugated to Alexa® 594	1:1000	*Invitrogen, UK*
Glial Fibrillary Acid Protein	Polyclonal Anti-GFAP	1:800	*AB5541, Chemicon, Temecula, USA*	Anti-Chicken conjugated to Alexa® 488	1:100	*Invitrogen, UK*

3.3. *IN VIVO* ASSAY

3.3.1. MPTP – INDUCED LESION

For the *in vivo* assay (MPTP model of Parkinson's disease) we used 37 young adult C57BL/6 mice (10-12 weeks-old) and an experimental paradigm described to deplete striatal dopamine by 40–50% in young adult C57BL/6 mice (*Jackson-Lewis et al., 1995*).

This protocol of MPTP (*Sigma-Aldrich, St. Louis, USA*) treatment consisted of a single intraperitoneal (i.p.) injection of MPTP per day (30mg/kg/day) for 5 consecutive days (*Tatton and Kish, 1997*). Injections were always performed at the same time of day. The animals were sacrificed 7 days after the last injection. Control mice were injected with saline (100µL, NaCl 0.9%) instead of MPTP. At the end of experiment, the animals (MPTP-lesioned mice and control mice) were divided into two groups. The first group was transcardially perfused and the brains were used for Immunohistochemistry (IHQ), whereas the second group of mice was killed and the brains were quickly removed, *SN* and striatum were dissected and stored at -80°C for Western blot analysis.

The Figure 7 summarizes the laboratory procedures used after MPTP administration.

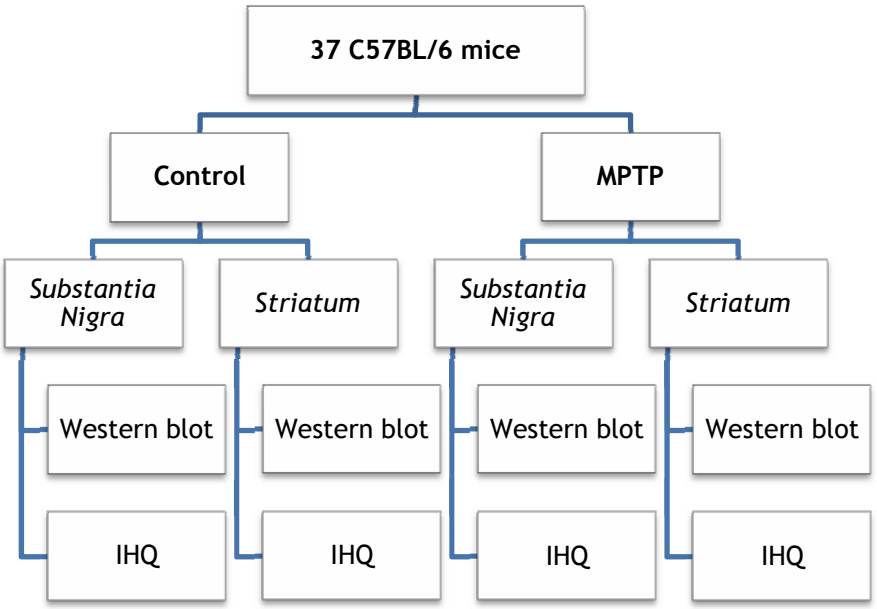


Figure 7. Laboratory procedures used after MPTP administration. The brains are quickly removed and the regions of interest (*SN* and striatum) were dissected for Western blot analysis. For Immunohistochemistry (IHQ) the brains were previously treated with PFA 4%.

All the procedures were based in Jackson-Lewis & Przedborski, (2007), and in Przedborski et al., (2001), were they provide a detailed protocol as well as a list of recommendations and guidelines to produce the MPTP mouse model of PD in a reliable and safe manner.

3.3.2. IMMUNOHISTOCHEMISTRY

3.3.2.1. TISSUE PREPARATION

Animals used for the Immunohistochemistry procedure were transcardially perfused first with 0.1M PBS and, after blood removal, with a 4% PFA solution. The brains were removed and postfixed in 4% PFA overnight at 4º C. After this fixation brains were cryoprotected in 30% sucrose until sinking. Before sectioning, the brains were embedded in optimal cutting temperature (O.C.T.) compound and were cut using a cryostat-microtome (*Leica CM3050S, Leica Microsystems, Nussloch, Germany*) in 35 µm coronal sections. The slices corresponding to the *SN* and striatum of each animal were collected and stored in 24-well plates (*BD Biosciences, San Jose, California, USA),* free-floating in 0.1M PBS supplemented with 0.12 µmol/L sodium azide, at 4° C until immunohistochemical processing.

3.3.2.2. FREE-FLOATING IMMUNOHISTOCHEMISTRY

Free-floating immunohistochemistry was initiated by incubating brain sections on a 10mM citrate solution (pH 6.0) at 80º C for antigen retrieval. The slices, after cooled to room temperature inside the solution, were placed in water for 5 minutes and then, were washed for 10 minutes in PBS-T.

Once washed, the sections were blocked with 10% FBS in PBS containing 0.1% Triton-X-100 (1h at room temperature) and then were washed twice for 10 minutes in PBS-T. For the inhibition of endogenous peroxidases activity brain sections were incubated for 10 minutes with 3% H_2O_2 in water, protecting slices from the light. This step was followed by two washes of 10 minutes with PBS-T.

Incubation with primary antibodies was performed overnight at 4º C: mouse monoclonal anti-TH antibody (1:1000; *T20720, Transduction Lab BD, San Jose, California, USA)* and mouse monoclonal anti-STEP antibody (1:5000; *23E5, Santa Cruz Biotechnology, Heidelberg, Germany*) diluted in 5% FBS in PBS. After being washed three times (10 minutes each) with PBS-T, the slices were incubated for 1h at room temperature with a biotinylated goat anti-mouse antibody (1-200; *Vector Laboratories, Burlingame, CA*) diluted in 1% FBS in PBS,.

The sections were washed three times (10 minutes each) with PBS-T and were then incubated with the avidin–biotin peroxidase complex reagent (*ABC kit from Vector Laboratories, Burlingame, CA*) for 1 h. The sections were washed extensively in PBS-T and developed using 3,3'-Diamine Benzidine (*DAB, Sigma-Aldrich, Saint Louis, MO, USA).* The reaction was stopped by adding Tris-Buffered Saline solution (**TBS:** *Tris 20 mM and NaCl 137 mM*).

Sections were then mounted onto Superfrost precleaned coated slides, dehydrated through a graded-ethanol series, cleared using xylene and coversliped with a permanent mounting

medium (*EntellanR, Merck, NJ, USA*) for light microscopy in the Ziess Axiovert 200 imaging Microscope (*Axiobserver Z1, Zeiss*).

3.3.2.3. CELL COUNTING AND QUANTITIVE ANALYSIS

Since *SN* does not have weel-defined borders with adjacent brain structures, we defined the same boundaries between *SN* and ventral tegmental area (VTA). TH^+-neurons were counted if they were stained perceptibly above the background level and only if they contained a nucleus surrounded by cytoplasm. The total number of TH^+-neurons for each representative mesencephalic section (6-7 sections per animal) was calculated under the magnification of x10.

All immunohistochemical analyses were performed in three animals of each group (control and MPTP). The results are represented as the value of TH cells per section ± SEM. Statistical significance was evaluated using Student's *t*-test.

3.4. TOTAL PROTEIN EXTRACTION

In the first part of the study, 33 C57BL/6 mice were sacrificed at the age of 1, 2, 4, 8, 16 and 60 weeks (5-6 animals/age group) for Western blot analysis.

All brains were quickly removed, and the striatum and *SN* were dissected out and homogenized in cold lysis buffer containing *Triton X-100 1%, Tris-HCl 50 mM, pH 7.5, EGTA 10 mM, NaCl 150 mM, protease inhibitors (phenylmethylsulphonyl fluoride 2 mM, aprotinin 10 µg/µl, and leupeptin 1µg/µl), and phosphatase inhibitor (Na_3VO_4 2 mM)*.

The brain tissue was centrifuged at 16100 g for 20 minutes at 4º C (*Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany*) and the supernatants were collected.

The protein concentration was determined by the method of Bradford using the *DC* Protein Assay kit (*Bio-Rad, Munich, Germany*). The protein levels in each sample were determined using the Bovine Serum Albumine (BSA) as protein standard. The absorbance was determined at 595 nm.

In the *in vitro* assay, the cellular extracts were also lysed in the same lysis buffer and the protein concentration was determined by the same process.

3.5. WESTERN BLOT

After quantification of total protein levels present in the supernatants, the proteins were denatured in 0.5M Tris-HCl, pH 6.8, 10% (w/v) Sodium Dodecyl Sulfate (SDS), 10% glycerol, 140 mM 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue and heated at 100°C for 5 minutes.

The volumes of protein extracts correspondent to 20 µg (cell cultures) or 40 µg (mouse brain tissue) were concentrated in the stacking gel containing 4% acrylamide, 0.5 mM Tris-HCl (pH 6.8), 10% SDS, 0.05% ammonium persulphate (PSA) and 0.05% tetramethylethylenediamine (TEMED). The proteins were then separated in 1.5 mm thick acrilamide resolving gel. The resolving gel was prepared with acrylamide at 12% in 1.5 M Tris-HCl (pH 8.8), containing 10% SDS, 0.05% PSA and 0.05% TEMED.

We carried out electrophoresis of the samples and protein standard (color molecular weight marker) under 160 V for approximately 1 hour, held at room temperature, and using a buffer containing 960 mM Glycine, 125mM Tris and 0.5% SDS.

After the electrophoresis, the stacking gel was removed and the resolving gel was dipped in CAPS Buffer (*10% Ciclohexilamin Acid propanosulphonic, 10% methanol, pH 11*). In order to make the proteins accessible to antibody detection, they were transfered from the gel onto a polyvinylidene difluoride (PVDF - *GE Healthcare, Amersham, UK*) membrane. Before the transference, the PVDF membranes were activated by dipping the membranes for 5 seconds in methanol 100%, followed by 5 minutes in water and 10 minutes in CAPS buffer. Each membrane was placed on top of the respective gel. The electrotransference was carried out at 300 mA for about 1 hour and 30 minutes, at 4° C.

After electrotransference of proteins, the membranes were blocked by incubation in 5% non fat milk (*Regilait, France*) in 0.1% Tween 20 in Tris-buffered saline (TBS-T) for 1 h at room temperature. After blocking, the membranes were incubated with the primary antibodies (diluted in TBS-T according to Table 2), overnight at 4º C.

Blots were washed three times in TBS-T, for 15 minutes each, and incubated with a mouse monoclonal antibody against α-tubulin or against ß-actin to obtain loading controls. After incubation with primary antibodies, the membranes were washed with TBS-T and then incubated for 1 hour at room temperature with the secondary antibodies (diluted in TBS-T according to Table 2).

After washing, bands were visualized with the alkaline phosphatase substrate (*Enhanced ChemiFluorescent substrate, GE Healthcare, Amersham, UK*) and visualized with a Molecular Imager FX system *(Bio-Rad, Munich, Germany*).

The specific OD was then normalized with respect to the amount of α-tubulin or ß-actin loaded in the corresponding lane of the gel. A partition ratio was calculated and expressed as a percentage of values obtained under control conditions.

Table 2. Primary and secondary antibodies used in Western blot.

Protein	Primary Antibody	Dilution	Company	Secondary Antibody	Dilution	Company
STEP	Monoclonal Anti-STEP	1:200	*23E5, Santa Cruz Biotechnology, Germany*	Goat Anti-Mouse	1:10000	*RPN 5781, GE, Amersham, UK*
Tyrosine - Hydroxylase	Monoclonal Anti-TH	1:5000	*T20720, Transduction Lab BD, California, USA*	Goat Anti-Rabbit	1:20000	*RPN5783, GE Amersham, UK*
Glial Fibrillary Acid Protein	Polyclonal Anti-GFAP	1:2500	*Z0334, DAKO, Glostrup, Denmark*	Goat Anti-Rabbit	1:20000	*RPN5783, GE Amersham, UK*
Tubulin	Monoclonal Anti-α-tubulin	1:5000	*T-9026, Sigma-Aldrich, USA*	Goat Anti-Mouse	1:10000	*RPN 5781, GE, Amersham, UK*
Actin	Monoclonal Anti-ß-actin	1:5000	*A5541, Sigma-Aldrich, USA*	Goat Anti-Mouse	1:10000	*RPN 5781, GE, Amersham, UK*

3.6. DATA ANALYSIS AND STATISTICS

Data are expressed as percentages of values obtained in control conditions or as the total number of cells, and are presented as mean ± S.E.M. of at least three independent experiments, performed in triplicate. Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's Multiple Comparison Test or Student's *t*-test. Values of $P<0.05$ were considered significant. All statistical procedures were performed using GraphPad Prism 5 Demo (*GraphPad Sotware Inc., San Diego, CA*).

Chapter 4

RESULTS

4.1. DOES THE EXPRESSION OF STEP VARY ALONG DEVELOPMENT?

The first part of this chapter focuses on characterizing the expression of $STEP_{61}$ in ventral midbrain and in striatum along different mice ages. Protein extracts of ventral midbrain and striatal region were prepared from postnatal tissue of C57BL/6 mice (1, 2, 4, 8, 16 and 60 weeks of age), and then subjected to Western blot to quantify $STEP_{61}$. In the *SN* there is a significant decrease in STEP levels in the first two weeks being the levels maintained during adult age. However, in aged animals (60 weeks) there is a significant increase in STEP levels. In the striatum, the STEP levels are constant during the first four weeks, and then a significant decrease could be observed in adult age. Like in the *SN*, there is a significant increase in STEP levels in aged animals.

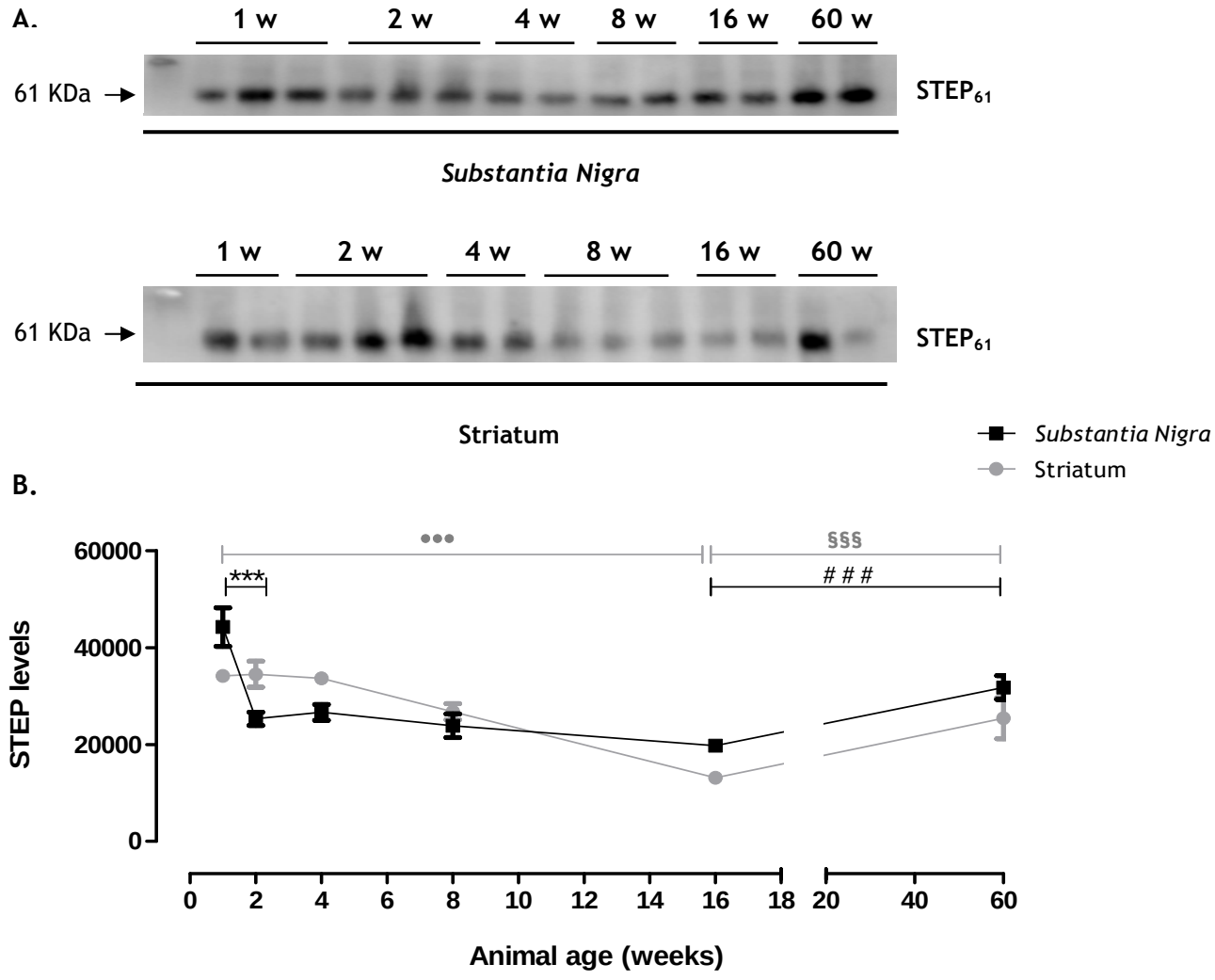


Figure 8. STEP protein levels in mice *SN* and striatum at different developmental stages. (A, B) Western blotting analysis of lysates of *SN* and striatum derived from C57BL/6 mice (5-6 animals/age

group. (A) Representative Western blot probed for STEP (MW: 61KDa) protein. (B) Optical densitometry analysis of *SN* revealed a significant decrease in STEP levels in the first two weeks and a significant increase in aged animals; in the striatum the STEP levels are constant and, like in the *SN*, there is a significant increase in STEP levels in aged animals.. Values are expressed as mean ± SEM. In the *SN*: ***P<0.001, compared to 1 week; ###P<0.001, compared to 16 weeks. In the striatum: ···P<0.001, compared to 1 week; §§§P<0.001, compared to 16 weeks (One-Way ANOVA followed by Bonferroni's Multiple Comparision Test).

4.2. THE CHANGES IN DOPAMINERGIC TRANSMISSION AFFECT STEP EXPRESSION?

4.2.1. *IN VITRO* ASSAY – MPP^+ - INDUCED LESION

It was described that MPP^+ has quite selective abilities to cause neuronal death in dopaminergic cells in the *substantia nigra*. This process occurs through a high-affinity uptake process in nerve terminals normally used to reuptake dopamine after it has been released into the synaptic cleft. The dopamine transporter moves MPP^+ inside the cell (*Dauer & Przedborski, 2003*).

At this stage, neuron-astrocyte midbrain co-cultures were used to evaluate the effect of MPP^+-induced dopaminergic neurodegeneration. They were stimulated with different concentrations of MPP^+ (0 µM, 5 µM, 10 µM and 20 µM) and, by using these cultures, we observed the MPP^+-lesion extension and if this specific stimulus can produce alterations in STEP, TH and Glial Fibrillary Acid Protein (GFAP) levels.

4.2.1.1. Assessment of MPP^+ - induced lesion

In order to determine the lesion extension produced by MPP^+, we performed an Immunocytochemistry assay of these cultures against TH. The extent of the dopaminergic lesion was evaluated by assessing the number of TH^+ cells and also by quantification of TH protein levels by Western blot.

We observed a decrease in the number of TH^+ cells in MPP^+-treated cultures when compared to control (Figure 9A). In addition, Western blotting analysis (Figure 9B) showed that exposure to 10 or 20 µM of MPP^+ induced a significant increase in TH levels when compared to the control.

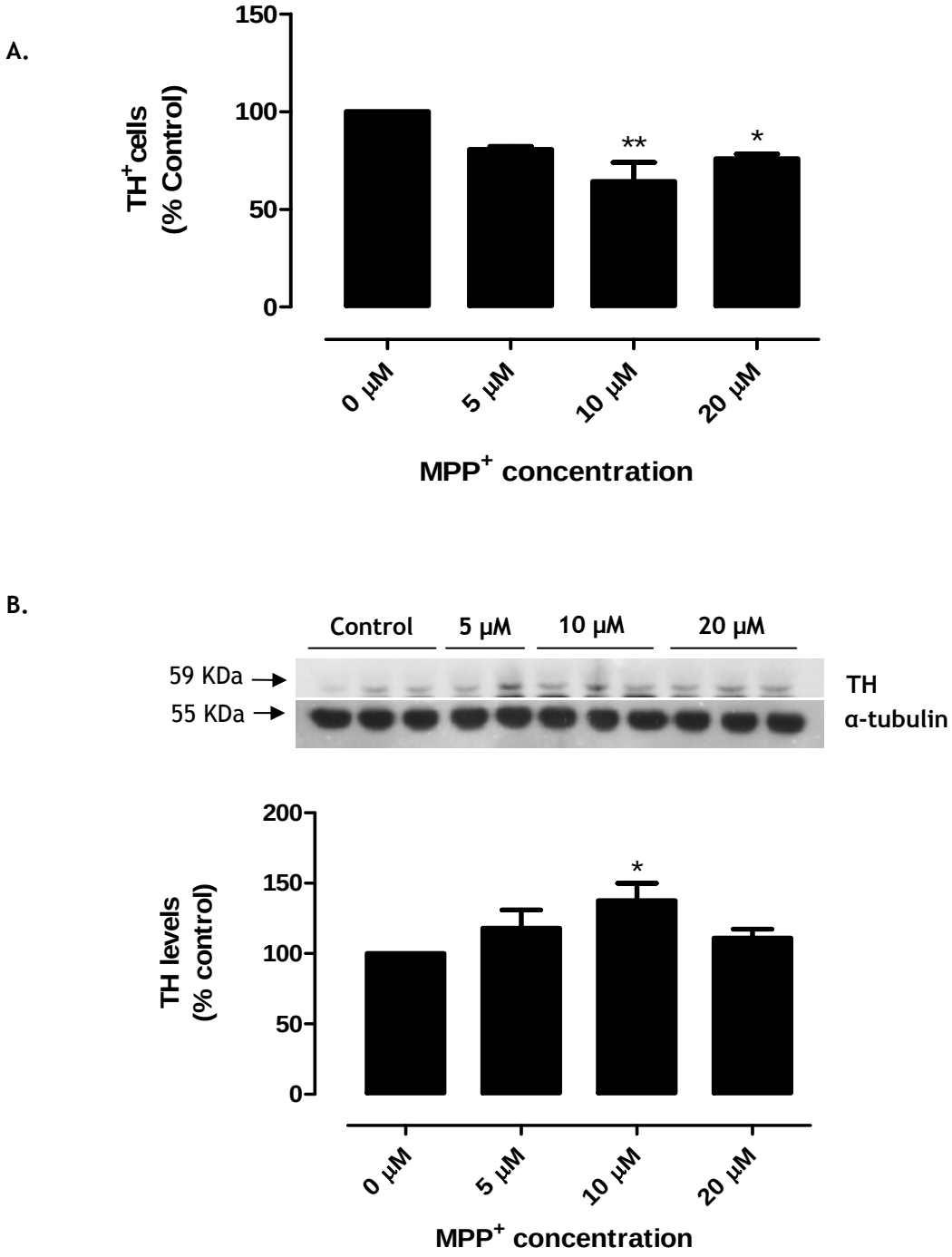


Figure 9. Assessment of MPP^+-induced lesion. (A) Quantification of TH^+ cells through Immunocytochemistry of midbrain co-cultures stimulated with different concentrations of MPP^+ (0 µM, 5 µM, 10 µM and 20 µM). Measurements were performed at three independent experiments with two or three coverslip per condition and results are represented as the mean value of TH positive cells in relation to total number of cells per slide ± SEM. Statistical analysis was performed using One-way ANOVA followed by Dunnett's Test (**P<0.01; *P<0.05). (B) Western blotting analysis of MPP^+ stimulated-cultures (n=4). Representative Western blot probed for mouse Tyrosine-Hydroxylase (MW: 59KDa) and α-tubulin (MW: 55KDa) proteins. Optical densitometry analysis revealed an increase in the TH levels as compared to the control. Each TH lane was normalized according to the α-tubulin band. Results were expressed as TH/ α-tubulin ratio. Values are expressed as mean ± SEM. *P<0.05 (One-Way ANOVA followed by Dunnett's Test).

4.2.1.2. Is STEP expression altered by the dopaminergic lesion?

As mentioned before, the main goal of this project was to understand whether dopaminergic signaling, resultant from a selective dopaminergic lesion in Parkinson's disease models, influences the expression of STEP in the nigrostriatal pathway.

To further investigate if STEP is present in dopaminergic neurons and astrocytes and if its expression was altered by a specific stimulus, we analyzed the levels of STEP in midbrain co-cultures stimulated with MPP^+ through Immunocytochemistry. Cells were stained for nuclei, STEP and TH (Figure 10). Most of TH neurons express STEP. The images suggest a decrease of STEP expression at 5 µM and 20 µM of MPP^+. However, the difference between the Control and the MPP^+ - stimulated cells is not remarkable.

Therefore, the next step was to evaluate STEP levels by Western blot (Figure 11) in the same conditions. Exposure to MPP^+ 10 µM promoted a significant increase of STEP levels, when compared to the control (Figure 11). No significant alterations were detected at other could MPP^+ concentrations.

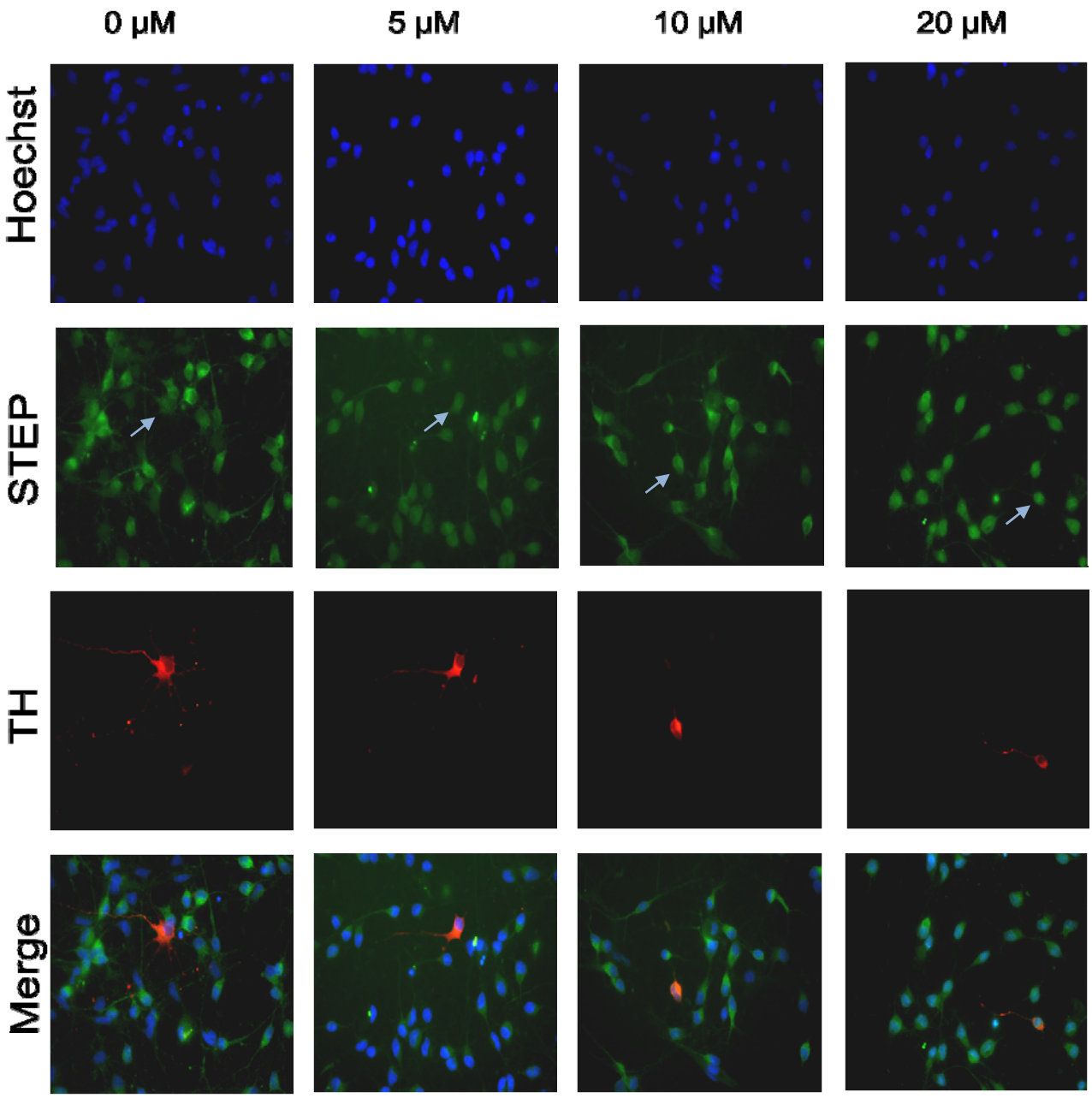


Figure 10. Immunocytochemical staining of STEP in dopaminergic neurons. Representative images of immunocytochemical analysis of midbrain co-cultures stimulated with different concentrations of MPP^+ (0 µM, 5 µM, 10 µM and 20 µM). The Immunocytochemistry was performed against STEP protein (green) and TH (red) and was followed by nuclei staining with Hoechst (blue). Immunocytochemistry was performed in three independent cell preparations. Snaps were taken with the same exposure and at the same time. White arrows indicate STEP staining in dopaminergic neurons.

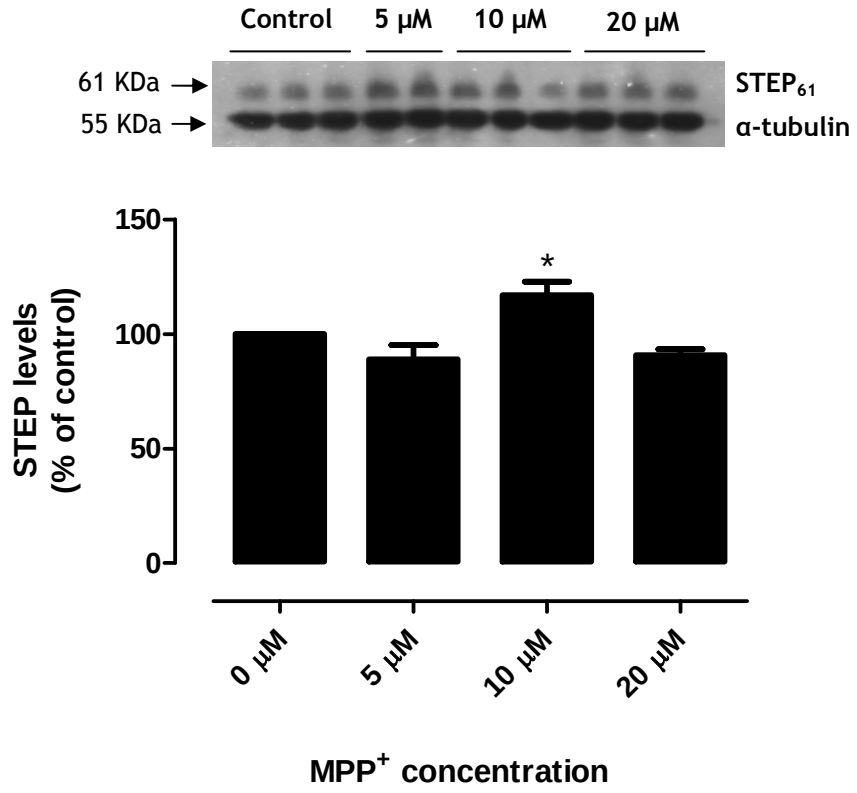


Figure 11. MPP^+ exposure increased STEP expression. Western blotting analysis of MPP^+ stimulated-cultures (n=4). Representative Western blot probed for STEP (MW: 61kDa) and α-tubulin (MW: 55kDa) proteins. Results from the optical densitometry analysis revealed an increase in the STEP levels at concentration of 10 µM, as compared to the control, was observed. Results were expressed as STEP/ α-tubulin ratio. Values are expressed as mean ± SEM. *P<0.05 as compared to the control (o µM), analyzed by One-Way ANOVA followed by Dunnett's Test.

4.2.1.3. Does MPP^+ stimulus influences the reactivity of astrocytes?

STEP is considered neuron-specific. However, Hasegawa and colleagues (2000) show that after transient forebrain ischemia, STEP is also expressed by reactive astrocytes (*Hasegawa et al., 2000*).

Since PD is associated with extensive astrogliosis, our intention was to clarify whether STEP is expressed by midbrain reactive glia or not. For this purpose, we performed an immunocytochemical analysis of STEP and GFAP, an astrocyte marker, in neuron-astrocyte midbrain co-cultures stimulated with MPP^+ (as mentioned above). The reactivity of astrocytes after a specific stimulus leads to an increase of GFAP expression (*Olanow & Tatton, 1999*). Immunocytochemistry suggest an increase of GFAP expression at 20 µM MPP^+ when compared to other concentrations (Figure 12). However, in the control (0 µM) some reactive astrocytes

were also observed. In what concerns STEP, we did not observe any notable changes in STEP expression after MPP^+ (Figure 12, second lane).

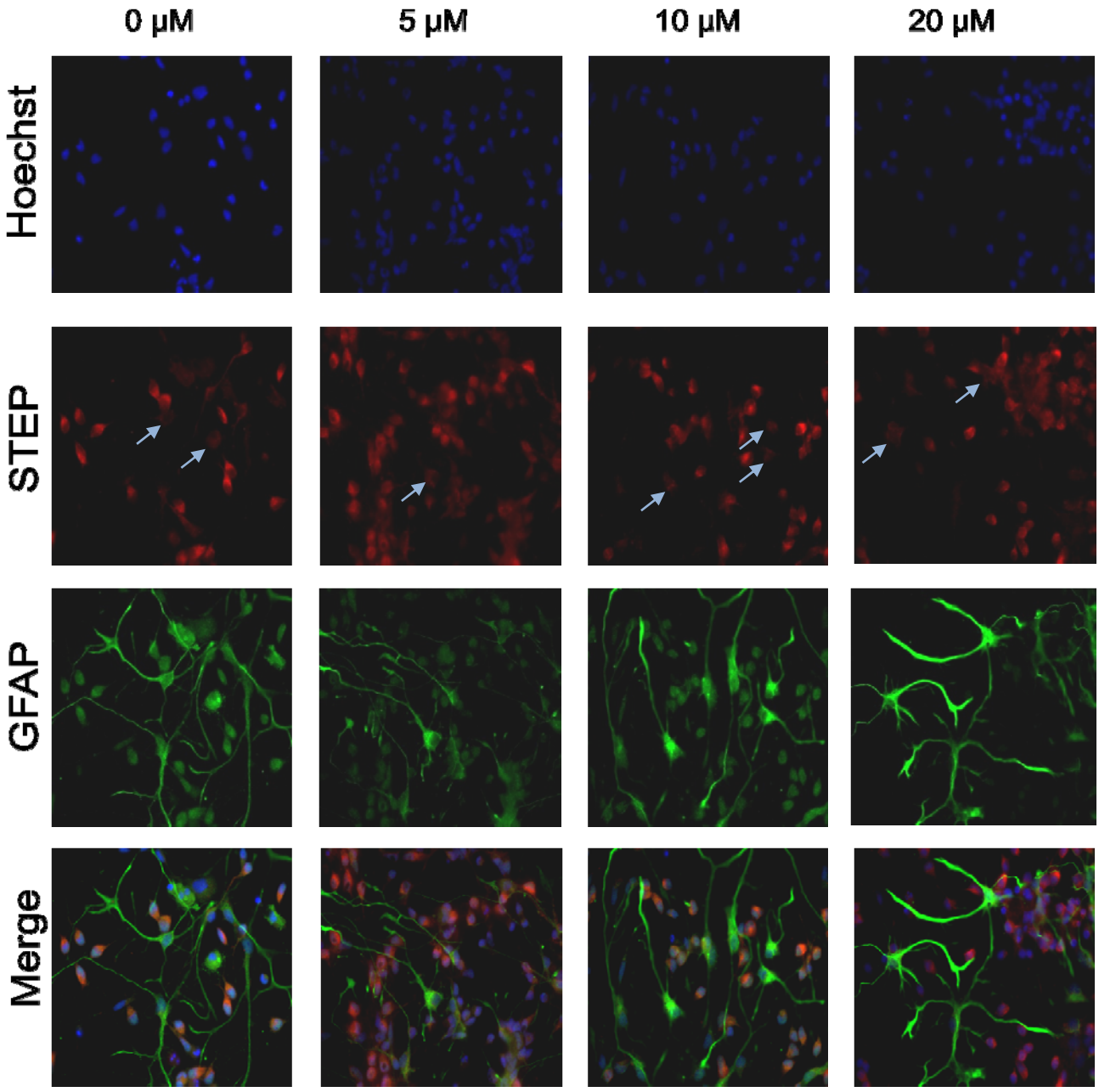


Figure 12. Immunocytochemical staining of STEP in midbrain astrocytes. Representative images of Immunocytochemical analysis of midbrain co-cultures stimulated with different concentrations of MPP^+ (0 µM, 5 µM, 10 µM and 20 µM). The analysis was performed with staining for nuclei (Hoechst, blue), STEP (red) and GFAP (green) proteins. The experiment was repeated in three independent cell preparations. Snaps were taken with the same exposure and at the same time. White arrows correspond to STEP staining in reactive astrocytes.

To further investigate how the levels of GFAP are modified by MPP^+ stimulus, we analyzed these same cultures by Western blot using α-tubulin as loading control (Figure 13). However, no significant differences were detected between control and other concentrations of MPP^+.

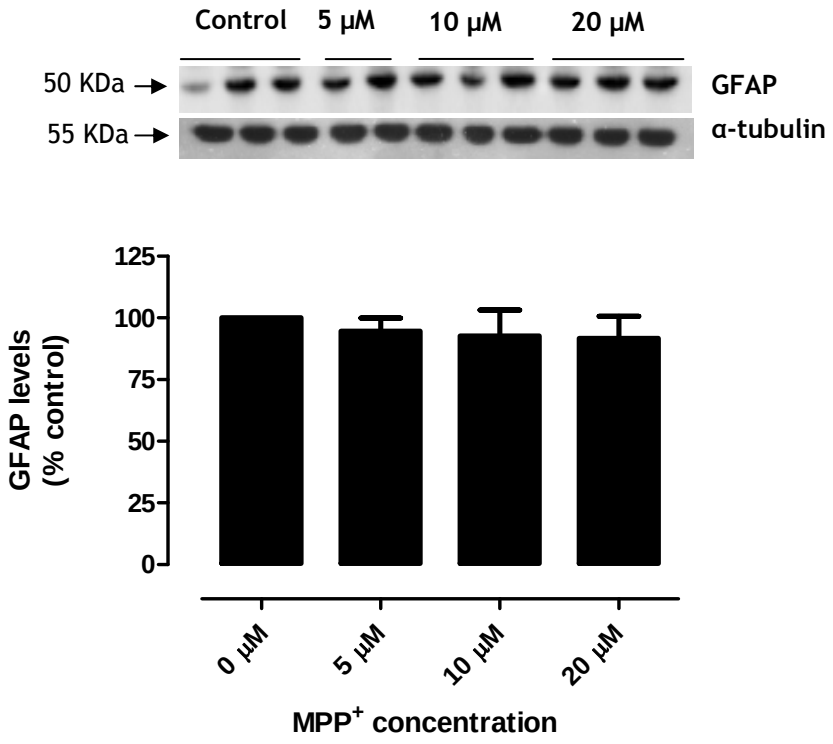


Figure 13. GFAP levels in midbrain cultures were not affected by MPP^+ exposure. Western blotting analysis of MPP^+ stimulated-cultures (n=4). Representative Western blot probed for GFAP (MW: 50KDa) and α-tubulin (MW: 55kDa) proteins. Optical densitometry analysis revealed that there were no significant differences in the GFAP expression when comparing control and MPP^+. Each GFAP lane was normalized according to α-tubulin band (used as a loading control). Results are expressed as GFAP/ α-tubulin ratio. Values are expressed as mean ± SEM ($P>0.05$; One-Way ANOVA followed by Dunnett's Test).

4.2.2. *IN VIVO* ASSAY – MPTP - INDUCED LESION

After accessing data regarding how MPP^+ induces neurodegeneration of dopaminergic neurons in primary midbrain cultures, we next studied the effect of the dopaminergic lesion in a more complex model, a mouse MPTP model. For this purpose mice were injected with saline (control group) or MPTP (30mg/kg/day) for 5 consecutive days. We tried to unravel if it was observed any alteration in the *SN* TH^+-neurons after MPTP- induced lesion and establish if there was any relationship between the dopaminergic lesion and STEP expression. We started by determining the efficiency of the lesion of this procedure.

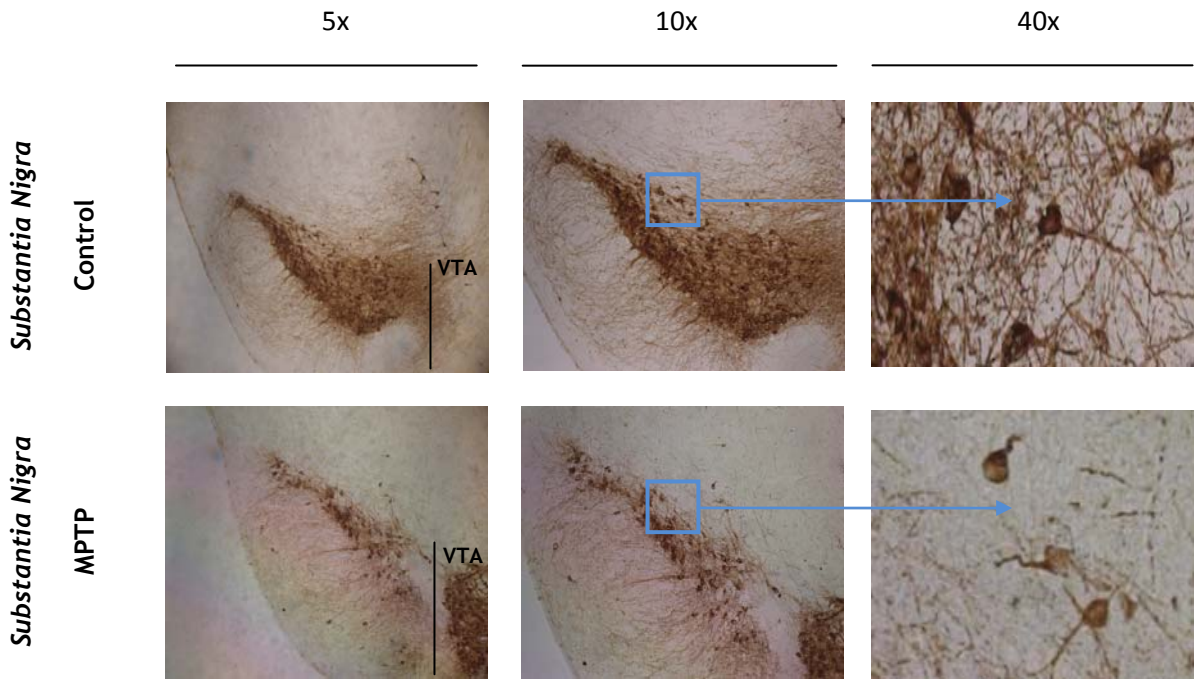
4.2.2.1. Extension of MPTP - induced lesion

To investigate the extension of lesion produced by MPTP, we performed an immunohistochemical staining for TH in brain sections of C57BL/6 mice. Not all mice within MPTP-treated group presented Parkinsonian motor abnormalities, such as tremor or rigidity.

The number of TH^+-cells in the *SN* of slices from control and MPTP-treated animals was determined by immunohistochemical procedure (Figure 14A). It was also possible to observe a decrease in the striatal-TH fibers in slices from MPTP exposed animals (Figure 14B).

Immunohistochemistry and quantitative analysis of TH^+-neurons showed that MPTP induced a reduction of 26% in the number of TH^+-cells (Figure 15).

A.



B.

***Striatum* Control** ***Striatum* MPTP**

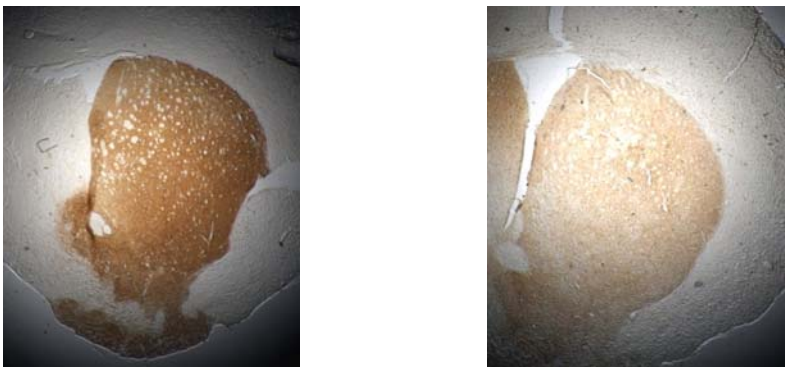


Figure 14. Decreased TH-immunoreactivity in the *SN* and striatum of MPTP-treated mice. (A, B) TH immunohistochemistry in the *SN* and in the striatum of brains from MPTP mice (MPTP, n=3) and control mice (Control, n=3). Sections were counterstained with DAB. (A) Representative sections of the *SN* from control and MPTP's brains stained for TH. A notable decreased in the number of TH^+-cells could be observed when compared MPTP mice to control mice. The TH^+-neurons were counted only in the *SN* and not in the ventral tegmental area. (B) Representative sections of the striatum from control and MPTP's brains also stained for TH. A striatal-TH fibers loss could be observed when compared the striatum of a control-group with the striatum of a MPTP-group. The immunohistochemical analysis was performed in three animals of each group (control and MPTP), with at least six sections per animal.

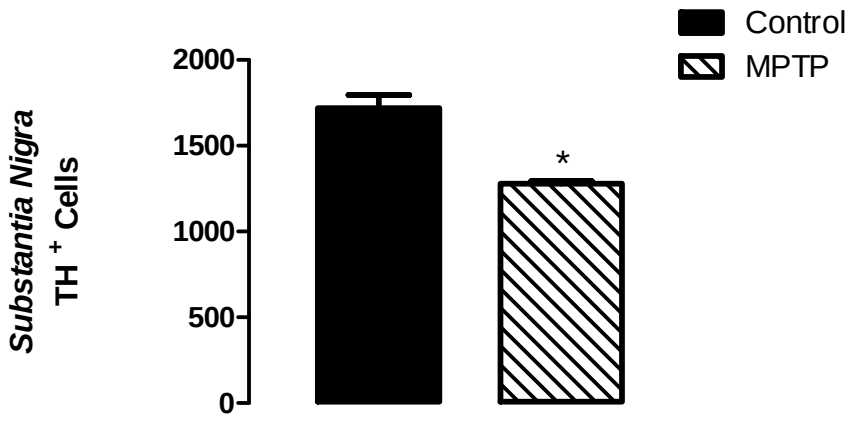


Figure 15. *SN* TH^+-neurons decreased after MPTP-induced lesion. Quantitative analysis of TH-immunoreactivity revealed a significant reduction in the number of TH^+-neurons in the *SN* of MPTP-treated mice when compared to the same region of control animals (26% of reduction). Values are expressed in total TH^+-neurons ± SEM. *P<0.05 (Student's t-test).

We also analyzed the extension of dopaminergic lesion by determining the TH levels by Western blot (Figure 16).

Although we did not obtain significant changes in TH levels (in relation to ß-actin) after MPTP lesion for any region analyzed, a decrease tendency was observed. This difference is more notable in the *SN*, however no statistically significant differences were detected.

A.

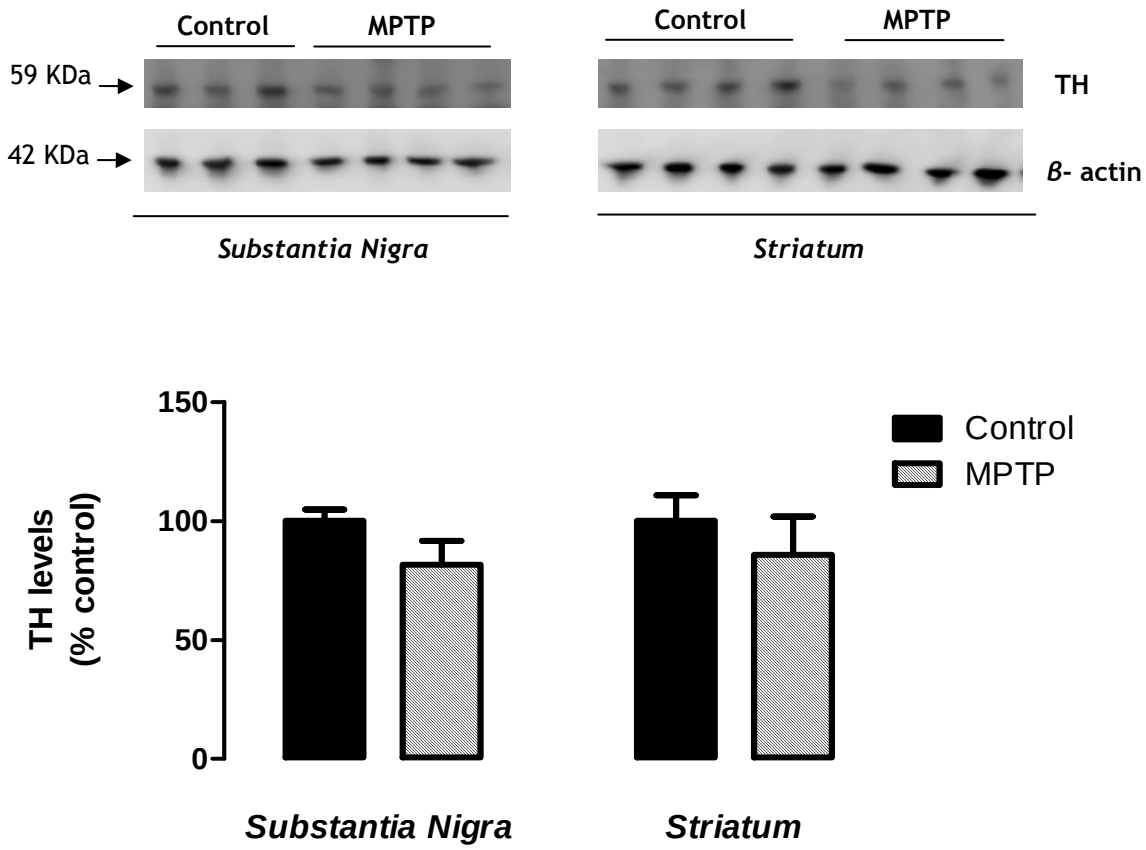


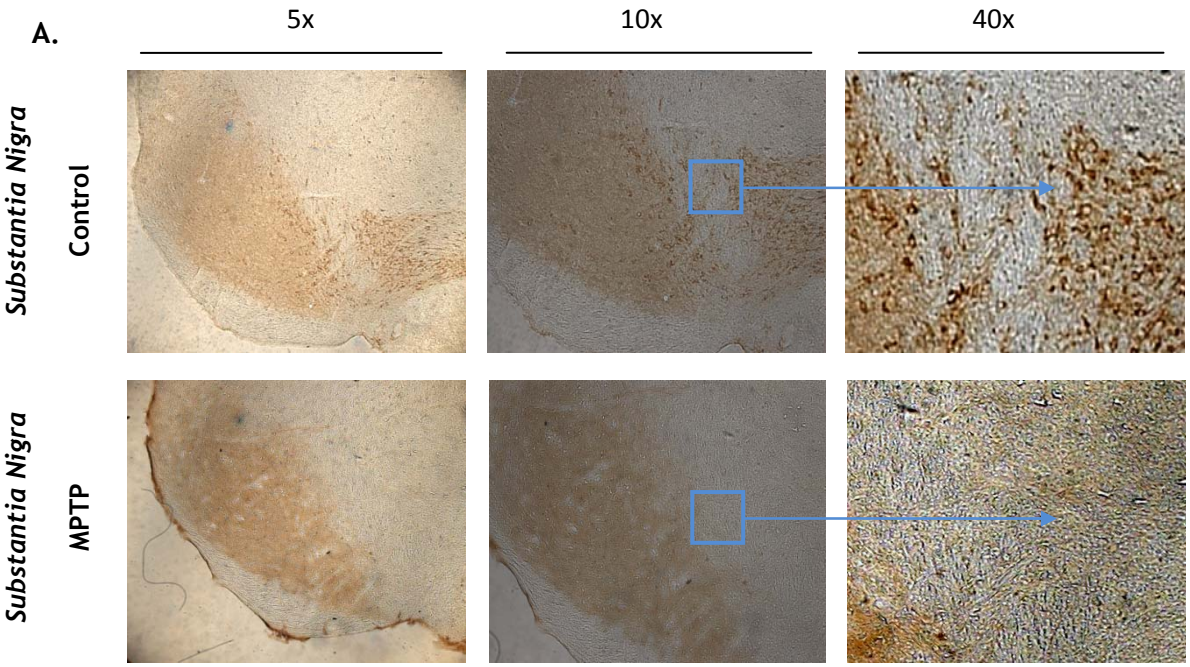
Figure 16. Extension of MPTP - induced lesion. Western blotting analysis of *SN* and striatum sections of control (n=14) and MPTP (n=13) mice. Representative Western blot probed for mouse Tyrosine-Hydroxylase (MW: 59KDa) and ß-actin (MW: 42KDa) proteins. Optical densitometry analysis revealed a decrease in the TH levels in the *SN* from MPTP mice as compared to the control, however, without significant differences. No significant differences in TH levels were also observed in the striatum between both groups. Each TH lane was normalized according to the ß-actin band. Results were expressed as TH/ ß-actin ratio. Values are expressed as mean ± SEM (P>0.05; One-Way ANOVA followed by Bonferroni's Multiple Comparision Test).

4.2.2.2. Is STEP expression influenced by MPTP-lesion?

To investigate if there is a relationship between dopaminergic lesion and STEP expression we carried out an immunohistochemical staining against STEP in brain sections (*SN* and striatum) of C57BL/6 mice. Although STEP staining in the *SN* was extremely scarce, we observed STEP expression in some cell bodies in lateral area of *SN*, of control mice (Figure 17A, upper panel). However, we didn't distinguish any staining against STEP in other *SN*-cell bodies analyzed (control and MPTP groups) (Figure 17A, lower panel).

Even though STEP was not found extensively in the *SN* of brains from control or MPTP mice, it is possible to observe STEP expression in the striatum of both groups (Figure 17B); nevertheless, we could confirm that immunorecativity wase very weak.

A Western blot analysis suggests that the levels of STEP increased in *SN* of MPTP-group as compared to control-group (Figure 18; left panel). Nevertheless, the STEP levels in the striatum were unaltered (Figure 18; right panel). Densitometric quantification, using α-tubulin as loading control, confirmed that STEP levels were significantly increased in *SN* of MPTP-group in relation to the control-group and no significantly different in the striatum of both groups (Figure 18).



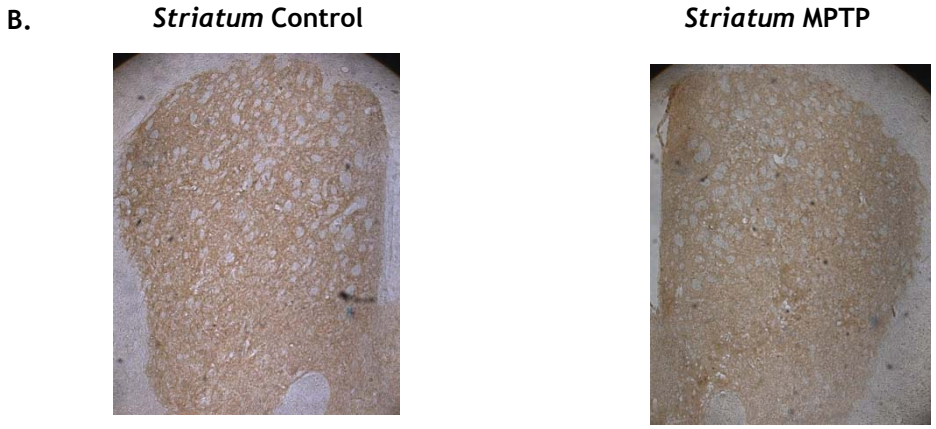


Figure 17. Immunostaining against STEP in *SN* and striatum of C57BL/6 mice. Mice were subjected to i.p. injections of saline or MPTP (30mg/Kg/day) for 5 consecutive days. (A) STEP immunoreactivity was observed in some *SN*-cell bodies from control-group. (B) STEP immunohistochemical analysis of the striatum of both groups revealed a STEP expression but without significantly differences between the control-group and MPTP-group. The immunohistochemical analysis was performed in three animals of each group (control and MPTP), with at least six sections per animal.

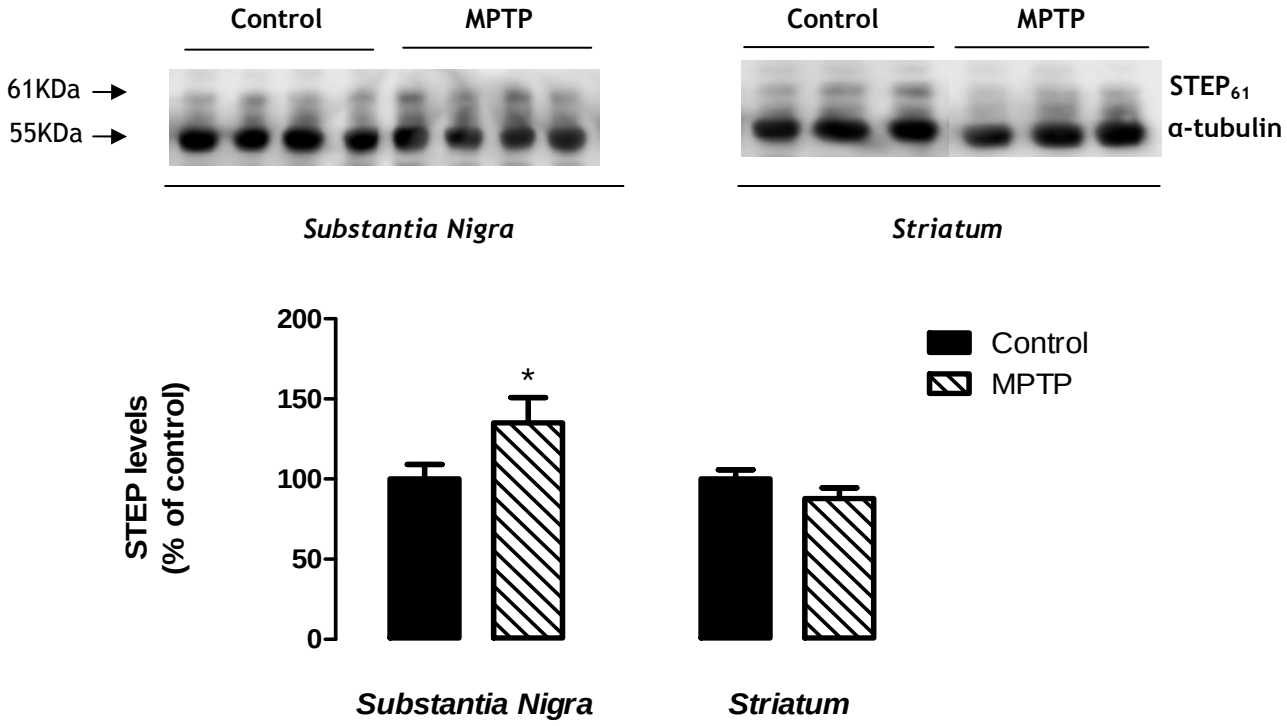


Figure 18. Increased STEP levels in *SN* after MPTP induced-lesion. Western blotting analysis of *SN* and striatum sections of control (n=14) and MPTP (n=13) mice. Representative Western blot probed for STEP (MW: 61KDa) and α-tubulin (MW: 55KDa) proteins in both regions. Each STEP lane was normalized according to the α-tubulin band used as a loading control. Results were expressed as STEP/ α-tubulin ratio. Values are expressed as mean ± SEM. **P*<0.05 (One-Way ANOVA followed by Bonferroni's Multiple Comparision Test).

4.2.1.3. How do Astrocytes respond to the lesion induced by MPTP?

Following the data obtained in the *in vitro* assay, and in order to investigate how the reactivity of astrocytes can be modified by dopaminergic lesion, we also analyzed the lysates from *SN* and striatum of both groups used for *in vivo* assay by Western blot.

To clarify if in the experimental conditions used in this work the MPTP exposure induced an increase of astrocytes reactivity, *SN* and striatal extracts from both control and MPTP-treated mice were subjected to Western blot analysis for GFAP. Densitometric quantification using ß-actin as loading control showed that levels of GFAP are increased in the *SN* of MPTP mice when compared to control mice (Figure 19). However, no significant differences in the GFAP levels were detected in striatum of both groups. If we made a parallelism with the results observed *in vivo*, for STEP expression, in the *SN* (Figure 18), we observe that may exist a correlation between the increase in the GFAP levels – as a marker of reactivity of astrocytes – and the STEP levels.

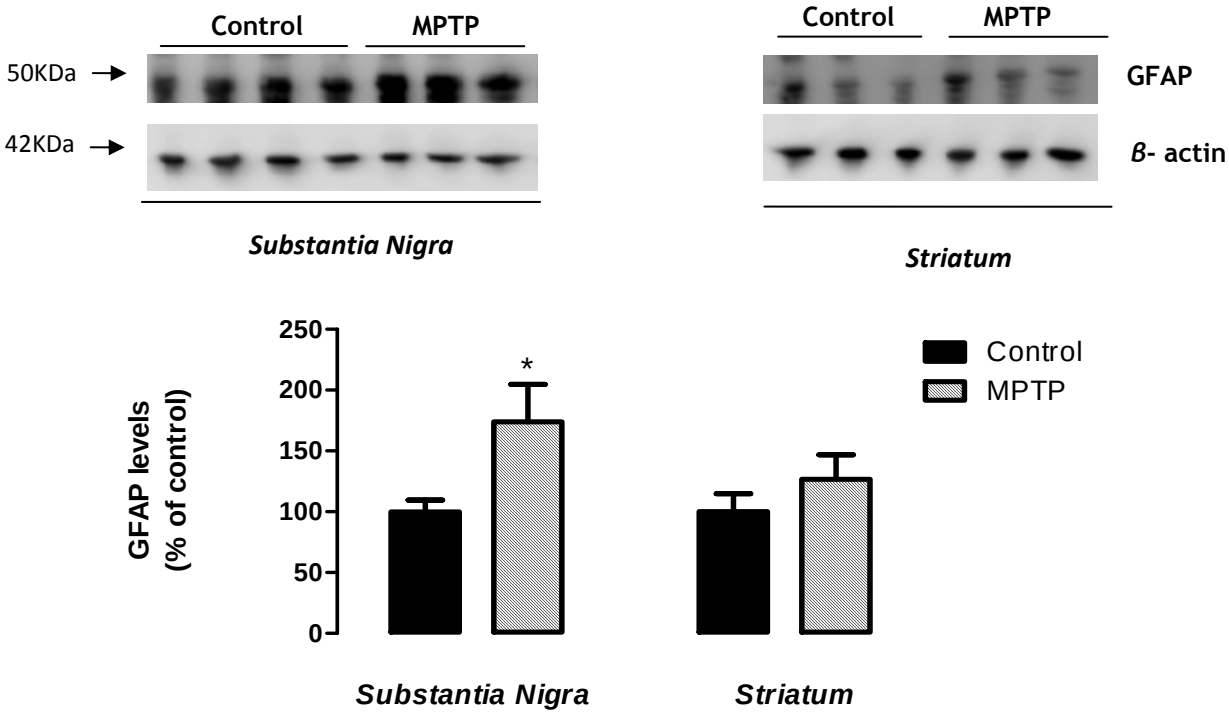


Figure 19. GFAP levels are increased in *SN* after MPTP-lesion induced. Western blotting analysis of *SN* and striatum sections of control (n=14) and MPTP (n=13) mice. Representative Western blot probed for GFAP (MW: 50KDa) and ß-actin (MW: 42KDa) proteins. Optical densitometry analysis revealed an increase in the GFAP levels in the *SN* from MPTP mice as compared to the control. No significant differences in GFAP levels were detected in the striatum between both groups. Each GFAP lane was normalized according to the ß-actin band. Results were expressed as GFAP/ ß-actin ratio. Values are expressed as mean ± SEM. **P*<0.05 (One-Way ANOVA followed by Bonferroni's Multilple Comparision Test).

Chapter 5

DISCUSSION

Considerable information regarding the biology of STEP has accumulated since its discovery (*Lombroso et al., 1991*). STEP was the first member of the family of nonreceptor tyrosine phosphatase to show specific expression within the CNS (*Boulanger et al., 1995*).

In this study we provide evidence of a relationship between Parkinson's disease and STEP, a protein that was already associated with neurodegenerative diseases, such as Alzheimer's disease (*Snyder et al. 2005; Kurup et al., 2010*), Huntington's disease (*Saavedra et al., 2011*), Fragile X Syndrome (*Venkitaramani & Lombroso, 2009; Goebel-Goody & Lombroso, 2012*). Although STEP has been linked to these neurodegenerative diseases, there is no information on the expression of STEP in Parkinson's disease. For this reason we propose to investigate the role of this phosphatase in the nigrostriatal pathway. Here, we further investigated if changes in dopaminergic signaling – as a result of a selective dopaminergic lesion in Parkinson's disease models, *in vitro* and *in vivo* - influence the expression of STEP in the nigrostriatal pathway.

In the first part of this study, we characterized the expression of $STEP_{61}$ in the dopaminergic neurons from ventral midbrain. STEP, as the name indicates, is highly enriched within the striatum in comparison to other brain regions (*Lombroso et al., 1993*), however, it is also expressed in the ventral midbrain (*Kim et al., 2008*). For this purpose, we decided to observe how STEP expression varies along age in both *SN* and striatum of C57BL/6 mice.

Interestingly, we observed a dynamic change in STEP expression, showing relatively stronger expression in ventral midbrain and in striatum in early development, however the expression profile in both areas are different. The highest expression of STEP occurs in *SN* in the first week of age that is followed by a significant decrease in STEP levels in the first two weeks and maintained levels in adult age. Surprisingly, in aged animals there is a considerable increase in STEP levels. In the striatum, contrary to the *SN*, the STEP levels are constant in the first four weeks, and slowly decreasing during adult age. A significant increase in STEP levels in aged animals was also observed. The results obtained for the first couple of months are in accordance with results obtained by Kim et al., (2008), which observed that STEP expression was restricted to mouse midbrain during the embryonic stage and decreasing in midbrain during adult stages. Our results show an unexpected increase in *SN* and striatum in aged animals (60 weeks-old) that, to our knowledge, was never reported. We can hypothesize that the increase, at this developmental stage can be related to a compensation mechanism where we can speculate that the increase in STEP levels would occur to annul the possible effects of the age and its consequences, such as, the cellular death. Since age is considered a

risk factor to neurodegenerative diseases, increased STEP levels in aged animals may also indicate a link between STEP and aging associated neurodegenerative diseases.

STEP is present in high levels in medium spiny neurons of the striatum, the dopaminoceptive neurons, where it is regulated by dopamine receptors (*Paul et al., 2000*). Besides the strong expression of STEP in this region - the target of dopaminergic neurons from the *substantia nigra* - we have observed that STEP is also expressed by midbrain dopaminergic neurons.

Parkinson's disease is characterized by the progressive degeneration of dopaminergic neurons from the *substantia nigra* that project to the striatum (*Nussbaum & Ellis, 2003; Jankovic, 2008*) resulting in reduced striatal levels of dopamine, a neurotransmitter that is known to regulate STEP activity (*Paul et al., 2000*). For this reason, we tried to unravel if through a Parkinson's disease mouse model, we might be able to observe any alterations on the expression of STEP in the regions that are mainly affected in this neurodegenerative disease (*Prensa et al., 2009; Lim & Tan, 2007; Jankovic, 2008*).

Therefore, we decided to perform this investigation in the MPTP mouse model of PD to explore the response of STEP to a specific dopaminergic injury. We chose to use the MPTP model as it is so far recognized as the best experimental model of sporadic PD, replicating most of the biochemical and pathological features seen in the clinical condition (*Przedborski et al., 2001; Dauer & Przedborski, 2003*). To make a parallelism with the MPTP mouse model, *in vivo*, we also used a primary culture of midbrain cell stimulated by MPP^+ - the active metabolite of MPTP.

Specific dopaminergic lesion was attained in both the cellular and *in vivo* model, as shown by the significative reduction in the number of TH^+ cells in cells or animals exposed to MPP^+ or MPTP. There are other studies that demonstrate MPP^+-induced neurotoxicity and consequently the reduction of TH^+-neurons (*Akaneya et al., 1995; Tian et al., 2007*). We obtained the same results in the *in vivo* assay, which means that MPTP induced a significant decrease in the number of dopaminergic neurons. Our observations are in accordance with He et al. (2004), who observe a significant reduction in the TH^+-neurons in an age-dependent manner after the same MPTP lesion regimen (30mg/Kg/day, for 5 consecutive days).

In order to confirm the lesion induced by MPP^+ or MPTP, *in vitro* and *in vivo*, a Western blot analysis of TH levels was performed. Tyrosine hydroxylase is an enzyme important in the biosynthesis of dopamine and is often considered a marker of nigrostriatal neuron sprouting (*Bezard et al., 2000; Rothblat et al., 2001; Jakowec et al., 2004*).

In cultures exposed to MPP^+ 10 µM we observed a significant increase of TH levels. This can be explained by a compensation mechanism since it is accepted that dopamine regulates both the expression and activity of TH. Reduced dopamine levels caused by MPP^+ exposure may lead to an increase of TH expression as a way to compensate the lack of dopaminergic

neurotransmission. *In vivo*, after MPTP administration, we could observe a slight reduction in the TH levels mainly in the *SN*; in the striatum there were no significant differences. Nevertheless, the reduction of TH levels is consistent with the neuropathology aggravation with the progression of the disease (*Nass & Przedborsky, 2008*). Despite using another MPTP administration regimen, Petzinger et al., (2006), observed that TH protein (when analyzed by Western blot) in the MPTP-treated mice was significantly lower compared to saline control, but these results are demonstrated only in the striatum.

The different results obtained between *in vivo* and *in vitro* models models can be explained by the fact that the direct application of the toxin to the cell cultures (*in vitro*) can induce more drastic effects than the administration of MPTP *in vivo*, and also because the animal possess alternative mechanisms of avoiding the lesion that are absent in the cell culture..

Once established the effects of MPP^+ and MPTP in the both models, we investigated the possible alterations in the STEP expression and how this phosphatase respond to changes in dopaminergic signaling. The expression of STEP in areas of the basal ganglia targeted by nigral dopaminergic neurons (*Braithwaite et al, 2006)* neurons suggested that STEP may be regulated by dopaminergic signaling. Moreover, in mice STEP siRNA knockdown significantly reduced the number of midbrain tyrosine hydroxylase positive cells (*Kim et al., 2008*).

It was demonstrated that STEP and TH co-localize in the *SN* of postnatal 8-day-old mice (*Kim et al., 2008*). In the present study we investigated if STEP was present in dopaminergic neurons in midbrain neurons-astrocytes co-culture. By using Immunocytochemistry, we observed that most TH neurons express STEP. Furthermore, we observed alterations, although not remarkable, in STEP expression when the cultures were submitted to a MPP^+ stimulus, suggesting that STEP can be susceptible to this specific stimulus. To confirm these results, a Western blot analysis was performed. We could then observe an increase of STEP expression at concentration of 10 μM. As a matter of fact, the changes in STEP expression observed with this MPP^+ concentration was in accordance with the reduction of TH neurons observed for the same concentration in the same cultures.

We could find the same results in the *in vivo* assay through the Western blot analysis that demonstrate that STEP levels were significantly increased in *SN* after MPTP-induced lesion. Nevertheless, STEP levels in the striatum seem to be unaltered. This increase, *in vivo*, may result from the lack of dopamine stimulation of medium spiny neurons in the phosphorylation of STEP via activation of D1R and PKA (*Paul et al., 2000; Paul et al., 2003*). In case of a reduction of dopamine, such as in PD, this neurotransmitter cannot promote adenylciclase activation, leading to the decrease of cAMP levels. Therefore, a lower activation of PKA pathway could be observed, leading to a decrease of phosphorylation levels of STEP, thereby activating STEP (see Figure 3, for a normal situation in presence of dopamine).

We observed the presence of STEP in some cell bodies in lateral area of *SN* from control mice but no stain for the same region of MPTP-treated mice. On the other hand, we confirmed STEP immunostaining in the striatum, nevertheless without significant differences, such as in the Western blot analysis. This can be explained by the fact that the lesion regimen was not enough to induce significant alterations in STEP levels in this region.

Our results regarding STEP immunostaining are in accordance with findings showed in Lombroso et al., (1993), which indicated that *SN*, a nucleus composed mainly of dopaminergic neurons, contains only a weakly stained subset of STEP-immunoreactivity cell-bodies. The same group demonstrates that the caudate-putamen contained many STEP-immunoreactivity cell bodies.

We cannot exclude that in the weak STEP staining can be related to a bad performance of the anti-STEP antibody in Immunocytochemistry or Immunohistochemistry since there are no published data concerning the use of this antibody with these techniques.

Despite the pathology of PD is not yet fully understood, recently it has been suggested that astrocytes might play an important role in disease initiation and progression (*Maragakis & Rothstein, 2006)*. In response to neuronal damage, astrocytes can enter into a state called reactive astrogliosis, which is characterized by hypertrophy and increased expression of glial fibrillary acidic protein (*Greenstein & Greenstein, 2000; Wu et al., 2002*). In order to understand if MPP^+ or MPTP stimulus in a cellular or mice model can produce alterations in reactivity of astrocytes, we analyzed the GFAP levels in both models.

Although we have seen an increase of GFAP immunocytochemical staining, we concluded that there were no differences between the control and the stimulated cells, when quantifying GFAP levels by Western blot. This can be explained by the fact that mechanical manipulation associated with preparation of midbrain cultures can induce a reactivity state in astrocytes that leads to high GFAP levels in control conditions. However, it was described that higher concentrations of MPP^+ are thought to induce microglial and astroglial activation, resulting in the release of inflammatory cytokines and chemokines from the cells (*Yokoyama et al., 2008*). In fact, *in vivo*, an increase in GFAP labeling, compatible with the activation of astrocytes could only be observed in the *SN*. In the striatum of MPTP-treated mice no significant changes were observed.

Our main intention was to observe whether STEP could be present in reactive astrocytes after a specific stimulus, such as MPP^+. *In vitro*, we couldn't, nevertheless, observe alterations in STEP expression. Despite Hasegawa et al., (2000), could demonstrate the presence of STEP in reactive astrocytes after transient forebrain ischemia, in this specific activity, after a MPP^+ stimulus, we observed no changes in STEP expression. *In vivo*, we could observe that there is an increase in GFAP levels, which could indicate that astrocytic reactivity, in the *SN* may contribute to increased STEP levels observed for the same area in the same conditions. These

results, when using a PD mouse model, demonstrate that astrocytic reactivity can be an important factor in the STEP expression. Although, there will be the need to proceed with further studies to prove this relationship.

Finally, and as mentioned above, increased STEP protein levels could also be found in other neurodegenerative diseases, such as AD. Recent findings demonstrate the increase of STEP levels either in cellular, mice or human models of AD (*Snyder et al., 2005; Zhang et al., 2010; Kurup et al., 2010*). Above all, their findings are based in the genetic reduction of STEP levels that reverses cognitive and cellular deficits in AD mice, indicating STEP as a potential target of AD therapeutic approaches.

As we cannot base up in any other studies which could demonstrate a relation between PD and STEP, further studies are indeed necessary to understand the regulation of this phosphatase in PD, which is expected to contribute to find new strategic therapeutics to either suppress or retard this devastating neurodegenerative disease.

Chapter 6

CONCLUSIONS AND FUTURE PERSPECTIVES

Since its discovery, a considerable attention was paid to the role of STEP in synaptic plasticity and neurodegenerative diseases. STEP dephosphorylates and inactivates signaling enzymes, including ERK1/2, p38, and Fyn. Moreover, dephosphorylation of NMDARs by STEP promotes their endocytosis. For these reasons, STEP opposes LTP and facilitates LTD. The molecular mechanisms of STEP expression and activity are demonstrating to be a complex issue that maintains an appropriate balance of STEP function in the CNS. It is evident from recent studies that disrupting any of these mechanisms can alter the balance of STEP activity to either make it more or less active.

Despite the strong expression of STEP in the striatum, in this research we investigated STEP expression also in *SN* over the time, which seems to be altered in an age-dependent manner. Therefore, understanding the factors that interfere with STEP expression and age can be a step forward to reach a new and unexplored field in the therapeutics of neurodegenerative diseases. In our project, we hypothesized that changes in dopaminergic signaling, resultant from a selective dopaminergic lesion in Parkinson's disease models, can influence the expression of STEP in the nigrostriatal pathway.

In this activity we collected evidence for a significant correlation between STEP and Parkinson's disease. STEP may play a role in the pathogenesis of this neurodegenerative disease due to its significant increase after we used a stimulus which leads to dopaminergic neuronal death, either *in vitro* or *in vivo*. However, it remains unclear how this interactive connection occurs. Further studies are necessary to understand the regulation of this effect, which are expected to contribute to find new strategic therapeutics to suppress or delay the PD.

In order to strengthen our results, it would be appropriate to continue with other testing besides the observation of STEP expression in the *SN* and in the striatum, in the PD, as already referred.

To better understand how dopaminergic lesion affects STEP expression, it would be also pertinent:

- to evaluate dopaminergic lesion by quantification of the dopamine levels by electrochemical HPLC;

- to evaluate the effect of MPTP on the activity (phosphorylation levels) of STEP and in order to determine if the putative effects are related to the changes in the STEP regulated signal transduction pathways, to observe the phosphorylated and non-phosphorylated forms of STEP substrates, such as key signaling proteins that include the MAP kinases ERK1/2 and p38, as well as the tyrosine kinase Fyn;

- to determine if STEP levels affect the dopaminergic lesion induced by MPTP using STEP heterozygous and KO mice;

- to determine the regulation of STEP expression by dopamine antagonists applied selectively to the dopaminergic terminal or to the soma using postnatal cultures prepared in microfluidic chambers;

To clarify if STEP is expressed by midbrain reactive glia and if its expression depends upon glial activation we propose:

- to study the expression of STEP in neuron-astrocyte midbrain co-cultures and in midbrain microglia cultures, stimulated by TNF-alpha and lipopolysaccharide, respectively.

Since STEP has a critical role in synaptic plasticity we also want to understand how this specific protein can also be a target for dopaminergic transmission and how it can be modified. For this purpose we also pretend:

- to determine if STEP expression in the midbrain is regulated by neurotrophic factors relevant for the protection of DA neurons like brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) - a well-known neuroprotective molecule for dopaminergic neurons;

Taken together, we expect that this body of work validates STEP as a candidate for drug discovery in an effort to find STEP inhibitors as potential therapeutic agents for the treatment of PD.

Chapter 7

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