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Ο ρόλος της Ακτιβίνης-Α στην προστασία από τη φλεγμονή του ΚΝΣ και τη διάδοση της παθογένειας της νόσου του Πάρκινσον

Role of Activin-A in the protection from inflammation to the central nervous system and in the propagation of the pathology of Parkinson's disease

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Contents

Abstract (English Version)

Neuroinflammation is of pivotal importance in the pathogenesis and propagation of Parkinson's disease (PD). In the process of neuroinflammation, it seems that a-synuclein (AS) strongly implicates. In general, A-synuclein is a protein with an unidentified role but it has a significant role in the pathology of Parkinson's disease. Hence, the key point for controlling PD progression is the identification of novel immunosuppressive agents that can limit the levels of inflammation in the diseased brain. Our studies are focused on the delineation of the role of the cytokine activin-A in the pathogenesis of PD. Activin-A participates in a diversity of biological processes and contributes to the regulation of immune responses. Recent evidence revealed a neuroprotective role of this cytokine suggesting that activin-A could represent a key cytokine in neuroinflammation. We hypothesized that activin-A could also implicate in the pathogenesis of PD and indeed our experimental data suggest that *in vivo* administration of activin-A in a mouse model of PD in which we administered AS preformed fibrils stereotaxically in the striatum, leads to a reduction of AS pathological accumulations in the SNpc, protecting against alpha-synuclein (AS)-mediated brain pathology. Finally, activin-A reduces the level of iba^{+} cells in the SNpc.

Abstract (Greek Version)

Η νευροφλεγμονή είναι κεντρικής σημασίας στην παθογένεια της νόσου του Πάρκινσον. Στη διαδικασία της νευροφλεγμονής φαίνεται πως συμμετέχει και η α-συνουκλεΐνη, μια πρωτεΐνη με άγνωστο ρόλο ακόμα, αλλά εξαιρετικά σημαντική στην παθολογία της νόσου του Πάρκινσον. Έτσι, το σημείο κλειδί στον περιορισμό της προοδευτικής εξέλιξης της νόσου είναι η εξεύρεση ανοσοκατασταλτικών μορίων που θα οριοθετούν τα επίπεδα της φλεγμονής στο νοσούντα εγκέφαλο. Οι μελέτες μας στοχεύουν στην αποσαφήνιση του ρόλου της κυτταροκίνης ακτιβίνης-Α στην παθογένεια της νόσου του Πάρκινσον. Η ακτιβίνη-Α συμμετέχει σε μια πληθώρα βιολογικών διεργασιών και ρυθμίζει τις ανοσολογικές αποκρίσεις. Πρόσφατες μελέτες αποκάλυψαν έναν νευροπροστατευτικό ρόλο αυτής της κυτταροκίνης υποδηλώνοντας ότι η ακτιβίνη-Α μπορεί να αποτελεί μια κυτταροκίνη κλειδί στη νευροφλεγμονή. Υποθέσαμε ότι η ακτιβίνη-Α μπορεί να εμπλέκεται στην παθογένεια της νόσου του Πάρκινσον και πράγματι τα πειραματικά μας δεδομένα υποδηλώνουν ότι η *in vivo* χορήγηση της ακτιβίνης-Α σε πειραματικό μοντέλο της νόσου του Πάρκινσον στο οποίο χορηγήσαμε προσχηματισμένα ινίδια α-συνουκλεΐνης στερεοταξικά στο ραβδωτό σώμα, οδηγεί σε μείωση των AS-παθολογικών συσσωρεύσεων στη μέλανα ουσία, προστατεύοντας τους νευρώνες από AS-μεσολαβούμενη παθολογία. Τέλος, η ακτιβίνη-Α μειώνει τα επίπεδα των iba^{+} κυττάρων στη μέλανα ουσία.

Introduction

Parkinson's disease

'Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with propensity to bend the trunk forwards, and to pass from a walking to a running pace; the senses and intellects being uninjured.' That was the analysis that James Parkinson used to describe the definition of 'shaking palsy' on the 1817 monograph '*An Essay on the Shaking Palsy'* in which he studied six cases of patients **[1]**. Until then, this devastating disease was unclassified as a unique pathological disorder because of the inability to observe and finally connect an amount of different symptoms of long duration. In 1872, Jean-Martin Charcot classified bradykinesia as one of the most pivotal symptom of the disease and proposed to entitle the syndrome as Parkinson's disease (PD) **[2]**.

The main clinical features are bradykinesia, tremor, rigidity and gait instability. Also at the early stages of the disease, fatigue and stiffness could be symptoms but rarely are noticed. Loss of smell, coordination and even changes in writing are often symptoms that are not noticed by the patient, at least at the early years of the disease. As Parkinson's disease progresses, the patient's speech slows down to a monotonous phase, the face becomes completely expressionless and the ability to walk is lost due to unplasticity. The almost constant tremor does not allow the patient to sleep. The median age of the disease appearance is almost 60 years with a duration of 15 years after initial diagnosis **[32]**.

The basic pathologo-anatomical characteristics of Parkinson's disease are the neurodegeneration of dopaminergic neurons in the region of substantia nigra pars compacta (SNpc) of the midbrain and the formation of intraneuronal inclusions called as Lewy bodies. The main component of these inclusions is the protein α-synuclein in an abnormal and aggregated form. There are two types of Lewy bodies, the brainstem and the cortical type, depending on their

morphology. Cortical Lewy bodies are often correlated with dementia in Parkinson's disease **[3]**.

The triggering effect of Parkinson's disease in not yet known. There are evidence implicating environmental and genetic factors as triggering causes of the disease. Furthermore, because of the complexity of the disease, there are many theories attempting to delineate the starting key event of PD. These theories lean on results that will be presented below. Few of the environmental risk factors of PD studies showed that low exposure to pesticides and past head injuries could potentially increase the development of PD **[4]**. Also there is a positive correlation between smoking and the development of PD. It seems that smokers are about twice as likely not to develop PD comparatively to never smokers **[5, 6]**. Caffeine seems also to be 'protective' from development of PD **[7, 8, 9]**. A possible explanation could be that nicotine and caffeine promote the augmentation of the levels of dopamine in the striatum and at the same time lead to inhibition of the mitochondrial enzyme monoamine oxidase which catalyzes the oxidative deamination of amines like dopamine and serotonin. Oxidative stress is provoked by high activity level of monoamine oxidase. Genetic studies have shown some specific gene mutations that lead to rare familial forms of Parkinson's disease**.** A study has reported six pathogenic mutations in leucine rich repeat kinase 2 (LRRK-2), also known as dardarin (encoded by *park8* gene) **[10, 11]**. The mutation with the highest frequency was Gly2019Ser mutation and was linked to 1% of sporadic cases and to 4% of hereditary parkinsonism cases. Other studies have pointed out the significance of mutations, mainly loss of function mutations, of genes such as *parkin, DJ-1, PINK1* and *ATP13A2* that could lead to an early development of the disease. PINK1 and parkin are in the same mitochondrial cascade **[12, 13]**. Mutations in these genes could lead to an autosomal recessive development of Parkinson's disease **[14]**. PINK1 is a mitochondrial serine-threonine protein kinase responsible for controlling the degradation of a misfunctional mitochondria. In such a case, PINK1 accumulates at the membrane of the damaged mitochondria recruiting parkin, an E3 ubiquitin ligase, in order to degrade the mitochondria through autophagy. Gene mutations on these genes support the theory of mitochondrial damage as a triggering effect of PD development.

Another protein involved in the oxidative process is DJ-1 (*park7*). DJ-1 is a deglycase which targets and stabilizes the nrf2 anti-oxidant response, inhibiting the binding of keap1 to nrf2 **[15]**. Keap1 is the inhibitor of nrf2 anti-oxidant response. Abnormal intracellular a-synuclein processing by proteasomes and lysosomes could suggest another triggering event in the pathogenesis of PD. A great question about the role of a-synuclein in the function of proteasomes is whether a-synuclein binds to proteasomes inhibiting their activity or if degradation of aggregated forms of a-synuclein has an impact on other proteasomal protein processing. There are studies showing decreased proteasomal activity in the SNpc of PD patients **[16]**. Also, chaperone mediated autophagy (CMA), a lysosomal degradation pathway, seems to have a significant implication in PD pathology. Lysosome-associated membrane protein 2a (Lamp2a), also known as CD107b, is a lysosome receptor and there are evidence that overexpression of lamp2a reverses the loss of TH-positive neurons mediated from a-synuclein accumulation in the SN **[17]**. Braak proposed that PD initiates at the gastric autonomic plexus of Meissner and the olfactory neuronal terminals. The pathology spreading is gradual and is subdivided into six stages. The a-synuclein pathology propagates from the lower brainstem to SNpc reflecting the advanced stage of the disease **[18]**.

There are many studies empowering the presented theories and together with these evidence, neuroinflammation seems to becoming a new player in the understanding of PD pathology. There also many studies indicating a novel propagating mechanism of the a-synuclein protein that could explain the disease progression.

A-synuclein

A-synuclein is a protein that is found mostly in presynaptic terminals in the site where synaptic vesicles accumulate. In that evidence leans on the initial consideration of the a-synuclein involvement in the regulation of the release of the synaptic vesicles. There are studies suggesting a pivotal role in the

stabilization of the SNARE protein complex. SNARE complex is responsible for the priming stage before fusion pore opening between the cellular membrane of the neuronal terminal and the membrane of the synaptic vesicle **[19, 20, 21, 22]**. But in general, the role of a-synuclein is not yet elucidated.

A-synuclein is found in many different forms. A very characteristic process concerning a-synuclein conformation, is called aggregation. Under specific conditions a-synuclein accumulates forming oligomeric or fully fibrillar species. The level of aggregation is very significant for the toxic potential of a-synuclein although it is not clear which form of a-synuclein is supposed to be toxic **[23]**. There are evidence that a-synuclein proline mutations lead to enhanced speed of oligomerisation without fibrillisation which was more toxic **[24]**.

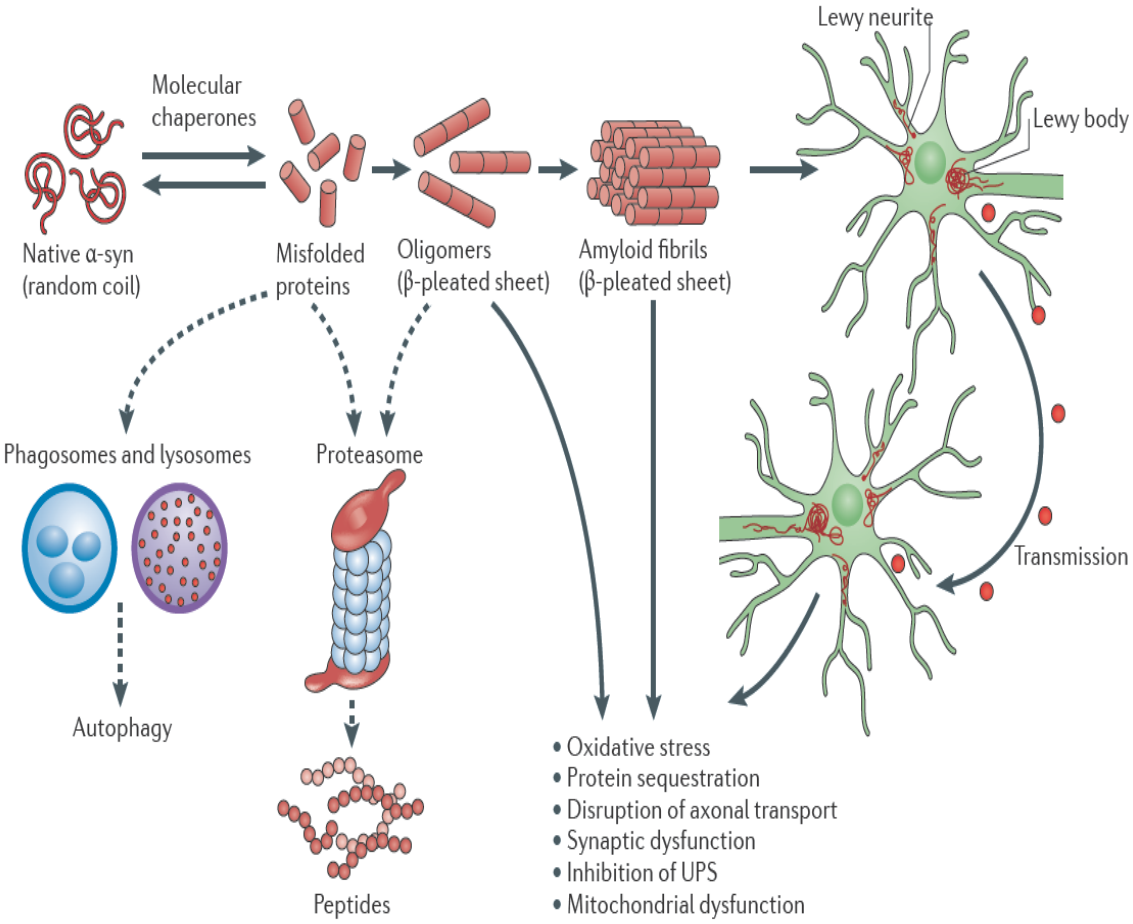


Figure 1. Propagation theory. Under unknown pathological conditions native a-synuclein starts to misfold, leading to the formation of pathogenic a-synuclein species like dimers, trimers and oligomers. Further aggregation will lead to the formation of more pathogenic structures like Lewy bodies and Lewy neurites. *Adapted from Irwin et al. Parkinson's disease dementia: convergence of α-synuclein, tau and amyloid-β pathologies. Nat Rev Neuro, 2013: 626–36.*

In contrast with that finding, there is another study in a rat viral model of a-synuclein nigral overexpression, where mutations which were promoting only oligomerisation and not fibrillisation led to more toxic results **[25].** A potential explanation about the selective neurodegeneration of dopaminergic neurons of SNpc could be that dopamine and its metabolites leads to an inhibitory effect to the conversion of protofibrils to fibrils **[26]**.

Another key point about a-synuclein is its secretory pathway and disease spreading. The propagation of a-synuclein relies on the fact that under pathological conditions that are not completely elucidated, a-synuclein undergoes misfolding states, forming a-synuclein pathogenic species. These pathogenic species must in some way propagate from cell to cell in order to spread the disease condition **[Figure 1.]**. There are many studies supporting a complete unconventional secretory pathway of a-synuclein through externalized vesicular structures that are very similar to exosomes **[Figure 2.]**. Detection of internalized a-synuclein into such vesicular structures and detection of oligomeric and monomeric a-synuclein species released in association with such vesicles in an a-synuclein expressing inducible neuroblastoma cell line **[27, 28]**. This vesicle-release could potentially suggest a communication mechanism between cells since these vesicles are able to transfer many cellular components to other cells but also in the extracellular matrix. A-synuclein seems that exploits this mechanism and propagate to other cells, spreading the PD pathology.

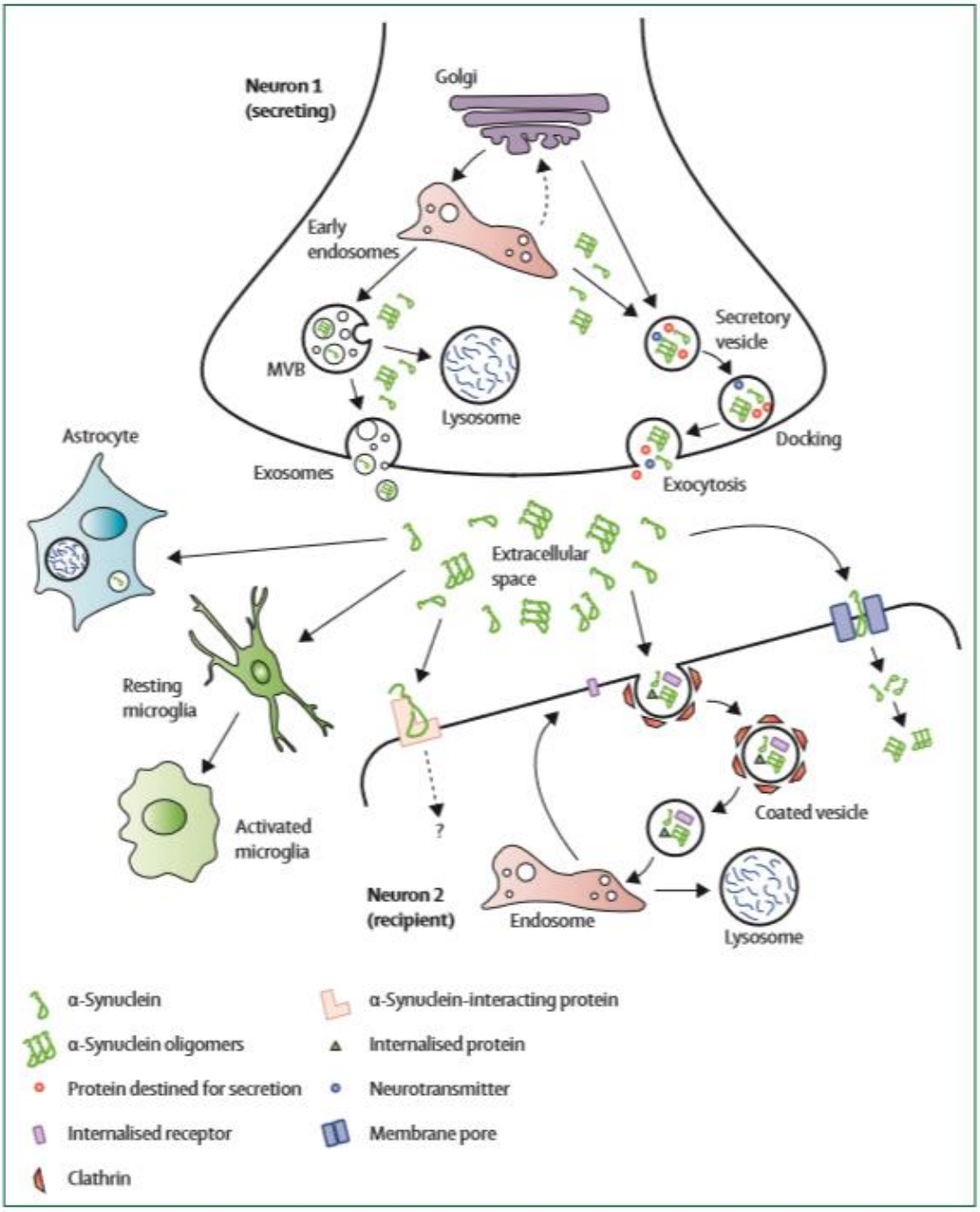


Figure 2. A-synuclein secretion and its cascade of effects. An unconventional secretory pathway of a-synuclein is presented. Cytoplasmic a-synuclein is internalized to multivesicular bodies (MVBs). There are two possibilities. Either MVBs will fuse to the lysosomes initiating the degradation process or will fuse with the cellular membrane of the presynaptic neuronal terminal to release exosomes to the extracellular matrix. Another secretory pathway could be via exocytosis of secretory vesicles that contain a-synuclein oligomers or monomers. Both types of a-synuclein secretion affects astrocytes, microglia and recipient neurons. *Adapted from Vekrellis K. et al. Pathological roles of a-synuclein in neurological disorders. Lancet Neurol, 2011:1015-25.*

In the context of neuroinflammation, a-synuclein seems to have a significant role. A-synuclein induces the activation of rat primary cultured microglia cells and the expression of specific metalloproteinases. Inhibition of these metalloproteinases led to diminished levels of NO and reactive oxygen species. Also suppression of TNF-α and IL-1β was observed **[29]**. A30P and A53T a-synuclein mutations led to greater levels of microglia activation comparatively to wild type protein **[30]**. Although a-synuclein seems that do not propagate to microglia cells, astrocytes internalize a-synuclein directly from neurons secreting pro-inflammatory cytokines and chemokines **[31]**.

Neuroinflammation in Parkinson's disease

During the last years, there are plenty of research studies implicating inflammation in the CNS as one of the major key players in the progression of Parkinson's disease. Neuronal cell death is a basic characteristic of this disease and apoptotic or/and necrotic phase neurons could trigger immune cells to activate and produce pro-inflammatory mediators like cytokines and chemokines. Neurons are extremely sensitive to such mediators **[33, 34, 35, 36]**. For example, dopaminergic neurons have diminished surviving ratio in exposure to cytokines like TNF-α and IFN-γ. Depending on the inflammatory mediator, many different neurotoxic dysfunction could be induced like oxidative stress, lysosome and proteasome dysregulation and abnormal mitochondria function. Also neuronal degeneration lead to a release of damage-associated molecular patterns (DAMPs), including heat-shock proteins, uric acid, chromatin, ATP, tau, and of course a-synuclein. DAMPs through pattern recognition receptors (PPRs) will induce an immune response mainly via TLRs. Permeability of blood brain barrier (BBB) is another significant element inducing neuroinflammation in the CNS through the increased infiltration levels of immune cells, mostly cells of the adaptive immunity. The increased influx of adaptive immune cells into the CNS could provoke neuronal injury which would lead to a more intense infiltration, creating a positive feedback loop of inflammation **[36]**. Other evidence suggesting the significant role of inflammation, is the increased levels of cytokines, mostly pro-inflammatory like

IL-6, IL-1β, TGF-β and IFN-γ as quantified post-mortem in the CSF of PD patients comparatively to same age healthy controls **[35, 37, 38, 39]**. Another important element is the observation of complement proteins in extraneuronal Lewy bodies post-mortem **[40]**. Microglia, T cells and B cells are implicated to the inflammatory pathway of PD, highlighting its complexity.

Microglia are the tissue-resident macrophages of the CNS. The ontogeny of microglia was a great debate the past years but the recent discovery that microglia cells are originated from the yolk sac of the embryo mouse, completely remap the conventional ontogenic diagrams of the mononuclear phagocytic system **[41, 42, 43]**. Initially, the concept of the origin of all macrophages was that macrophages originated from hematopoietic stem cells (HSCs) that differentiate into myeloid progenitors and then to monocytes that circulate in the blood. When these monocytes enter a tissue, the environmental cues of this tissue, mostly cytokines and chemokines, will reprogram these monocytes into the tissue-resident macrophages. The study that revealed that microglia exist in embryonic developing mouse brain (E9.5), was a triggering key point in the microglia fate analysis **[44, 45, 46, 47]**. Also there are many studies indicating a differential gene expression pattern of many genes between microglia cells and other tissue-resident macrophages **[48, 49, 50]**. Nowadays, the previous conventional fate map needs to be renewed since the majority of microglia cells are originated from HSCs of the embryo yolk sac **[Figure 3.]**.

Microglia cells are key players in the regulation of neuroinflammation and there are evidence for microglia engagement in the pathology of PD. Microglia express pattern recognition receptors (PPRs) that are able to recognize DAMPs that are released from a damaged neuron. The components or molecules that are released due to neurodegeneration are recognized by TLRs, NLRs or other PPRs leading to the activation of microglia cells **[51]**. Microglia cells express TLR1 to TLR9 **[52]**. The activation of microglia promote a proinflammatory mediators secretion **[53, 54, 55]**. Also microglia activation can occur due to the loss of the CD200:CD200R inhibitory signal after neuronal damage **[53, 56]**.

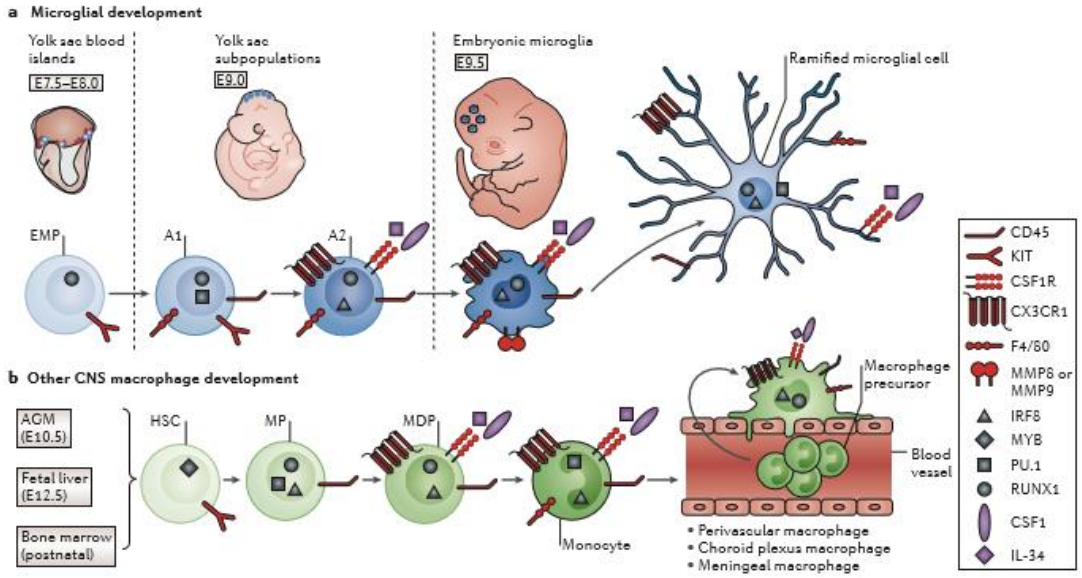


Figure 3. Differential ontogeny of microglia cells and other CNS macrophages. a| Microglia cells are derived from erythromyeloid progenitors (EMPs) at the embryonic day 7.5 – 8.0 in the yolk sac. At embryonic day E9.0, the ongoing-differentiation microglia cells upregulate the CD45 antigen, a stage usually referred to as A1 stage. The expression of myeloid markers such as F4/80, CX3C chemokine receptor 1 (CX3CR1) and colony-stimulating factor 1 receptor (CSF1R) marks the transition to A2 stage in which microglia cells start to migrate to the CNS. b| Other CNS macrophages originate from the definitive hematopoiesis that initiates at embryonic day E10.5, in the aorta-gonad-mesonephros (AGM) region, at the embryonic day E12.5 in the fetal liver and postnatally in the bone marrow. HSCs differentiate to monocytes that circulate throughout the blood and these monocytes reprogram to macrophages and/or dendritic cell progenitors (MDPs). IL-34, interleukin-34; IRF8, interferon regulatory factor 8; MMP, matrix metalloproteinase; MYB, transcriptional activator MYB; PU.1, transcription factor PU.1; RUNX1, runt-related transcription factor 1. *Adapted from Prinz M, Priller J.2014. Microglia and brain macrophages in the molecular age: From origin to neuropsychiatric disease. Nat Rev Neurosci 15: 300–312.*

In response to that type of activation, microglia cells can also produce besides cytokines and chemokines, reactive oxygen species and prostanoids that are supposed to have an immunoregulatory role **[57, 58, 59]**. Microglia activation lead to MHCII upregulation, responsible for the antigen presentation process to $CD4^+$ T cells. MHCII is highly expressed by activated microglia cells in PD patients while it is not detectable in healthy controls. Microglia are mostly found in the nigrostriatal tract **[60, 61, 62]**.

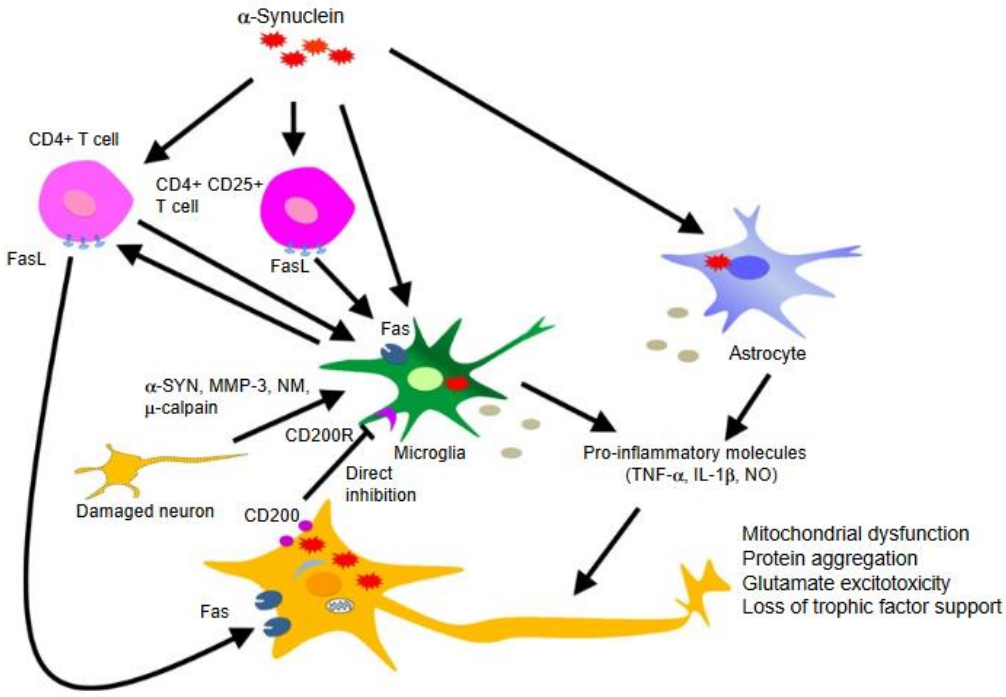


Figure 4. A potential overview of neuroinflammation. A-synuclein and DAMPS are released from degenerating neurons. This release stimulates microglia and astrocytes. Microglia cells are activated and secrete proinflammatory mediators. Also chemokines are secreted recruiting CD4+ T cells that interact with the Fas of neurons leading to its degeneration. Finally neurodegeneration interrupts the signal of CD200/CD200R activating microglia cells. *Adapted from Tufekci KU et al. Inflammation in Parkinson's disease. Adv Protein Chem Struct Biol. 2012;88:69-132.*

Besides the importance of microglia in the pathogenesis of PD, adaptive immunity seems also to implicate in the progression of the disease. There are studies indicating the role of T cells since infiltrating T cells were found in the SNpc of PD patients comparatively with healthy controls **[63, 64, 65]**. Also memory T cells population are increased in PD patients, diminishing the ratio of naïve T cells **[66]**. $CD45RO^+$ T cells are increased in PD patients indicating the presence of activated memory T cells **[67]**. About the levels of T regulatory cells in the periphery, there are some studies with conflicting results. Furthermore, $CD4^+CD25^+CD127^-$ Tregs cells show diminished suppressive capacity in PD patients than Tregs of healthy controls **[67]**. Immunization of T cells with nitrated a-synuclein led to IL-17 and TNF-α secretion after *ex vivo* restimulation. Adoptive transfer of these Th17-like polarized in wild type mice, increased the levels of neurodegeneration while *ex vivo* polarization to Th1 and

Th2 phenotype did not reveal alterations in the levels of neurodegeneration. Polarization to Tregs and adoptive transfer of these T cells led to diminished levels of neurodegeneration **[68]**.

Inflammation has a pivotal role in the pathogenesis of PD and that is why we need to identify a novel immunosuppressive factor able to dampen inflammatory responses.

Experimental models for Parkinson's disease

There are plenty of experimental animal models for PD. There are the toxin models with MPTP model and 6-OHDA model being the most conventional alongside the genetic models of a-synuclein, LRRK2, DJ-1 and PARKIN **[69]**. 6-hydroxydopamine (6-OHDA) or 2,4,5-trihydroxyphenethylamine is a neurotoxin leading to an almost complete neurodegeneration of the nigrostriatal tract **[70, 71]**. It is suggested to be a useful model in order to study the degeneration of the dopaminergic neurons therapeutically. But there are many disadvantages with 6-OHDA model. The neurotoxin effect is very intense (12h to 3 days after injection). Also 6-OHDA does not cross efficiently the BBB so systemic administration is not possible, only intrastriatal stereotaxic injection. Another important fact is that Lewy bodies are not formed. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin that targets also the nigrostriatal pathway. Lacking of formation of pathological inclusions of Lewy bodies is a great issue together with the inability to detect behavioral alterations except some distinct cases **[72, 73, 74]**. There are various a-synuclein transgenic mice with A53T mutation of a-synuclein being the most effective. The significant issue about a-synuclein transgenic mice is that although LB formation is detected, no great levels of dopaminergic neurodegeneration are detected **[75, 76, 77]**. In general, the animal experimental models of PD do not present great resemblance with the disease. A model that is used very often in order to study the disease progression and a-synuclein propagation, is the model with the intrastriatal stereotaxic a-synuclein injection, a model that we also used in our experiments.

Activin-A

Activin-A is a well characterized cytokine involved in many biological processes. The importance of activin-A is mirrored by the fact that almost every cell type has the ability under specific conditions to synthesize and secrete activin-A. Although activin-A has been firstly purified as a gonadal protein inducing the secretion of follicle-stimulating hormone from the pituitary gland, there are plenty of studies at this moment indicating the involvement of activin-A in a plethora of processes. Embryogenesis, erythropoiesis, tissue repair, wound healing, neuronal survival, neurogenesis and stem cell maintenance and its fate determination are some of the most essential biological processes that activin-A participates. Member of the TGF-β superfamily that consists of more than 45 proteins, activin-A is a homodimeric protein with two βA subunits (βA βA) linked with a disulfide bond. In general, activins are found also in both homodimeric and heterodimeric forms depending on the combination of the subunits **[78]**. Thus, there are homodimeric activins-B, C and E (consisting of two βB, βC and βE subunits respectively), and the heterodimeric activin-AB (consisting of one βA and one βB subunit) **[79, 80]**. Activin-A has a molecular weight of 25kDa approximately. Also, every cell type of the innate and adaptive immunity is dictated by activin-A, highlighting this cytokine as a great immune regulator **[Figure 5.].**

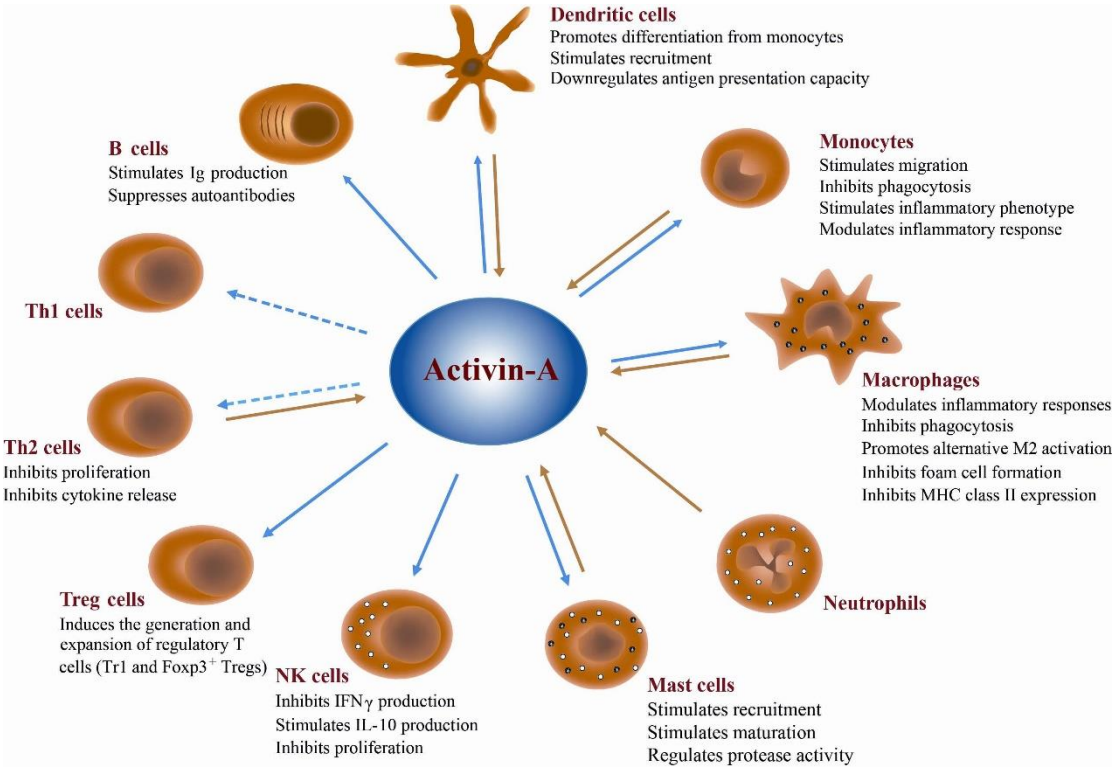


Figure 5. Activin-A effects on innate and adaptive immune responses. Monocytes, macrophages, dendritic cells, neutrophils, mast cells and Th2 lymphocytes (yellow arrows) are capable of producing Activin-A depending of the conditions. The role of activin-A on a diversity of biological processes is noted down on distinct immune cell subsets (blue arrows). The effect of activin-A in Th1 and Th2 cell responses is not clearly known (broken arrows). *Adapted from Activin-A: A New Piece in the Puzzle of Tolerance in Asthma, Tousa et al. 2014, Clinical Anti-Inflammatory & Anti-Allergy Drugs*

Signaling pathways of activin-A

As mentioned before, activin-A is a cytokine that participates in a diversity of biological processes. This pleiotrophy is imprinted by a highly complex signaling pathway. The starting point of this signaling cascade is the heterotetrameric receptor complex which is composed by two types of homodimeric activin receptors. Type I or ActRI homodimeric receptors containing activin-like kinase (ALK) receptor 2, 4 and 7 and type II or ActRII homodimeric receptors (ActRIIA and ActRIIB) **[79, 81]**. Structurally, type I and type II receptors are highly similar with a serine threonine/kinase domain intracellularly and a small cysteine-rich extracellular domain. The main difference between those two types of receptors

is that type I receptor contains also a juxtamembrane region necessary for the signal cascade **[81]**. It should be pointed out that the latent heterotetrameric complex receptor is not formed until the binding of activin-A to ActRII. This binding is the initial step for the linkage of ActRII to the inactive ActRI, leading to the formation of the heterotetrameric complex receptor. This linkage leads to the phosphorylation of the mothers against decapentaplegic (Smad) proteins which are the main regulators of the signaling cascade of activin-A and form the canonical signaling pathway. Eight Smad proteins have been identified and every one of them plays a specific role in the signaling cascade. Smad-1, Smad-2, Smad-3, Smad-5 and Smad-8 consist the group of the receptor-regulated Smad proteins while Smad-6 and Smad-7 represent the Inhibitory Smad (I-Smad) protein group. The moment the heterotetrameric receptor will be formed, the kinase domains of the receptor complex will commence to phosphorylate the group of the receptor-regulated Smad proteins in order to activate them. The phosphorylated Smad-2 and Smad-3 interact with Smad-4 (Co-Smad) leading to the formation of a protein complex **[82]**. Subsequently, this protein complex translocates to the nucleus, activating the transcription of specific genes **[Figure 6.]**. This nuclear translocation is achieved by a signal transduction by Smad-4 and CREB binding protein (CBP)/p300 and transforming growth interacting factor (TGIF) **[83]**. As mentioned before, the Smad proteins consist the canonical signaling pathway but there are evidence suggesting that activin-A can initiate also different signaling pathways like the mitogen activated protein kinase/extracellular signal regulated kinase (MAPK/ERK), c-Jun N-terminal kinase, JNK and p38 **[Figure 6.] [84]**. The involvement of activin-A in a diversity of biological processes makes mandatory the presence of a modulatory molecule. Follistatin (FS) is the main regulator of activin-A. FS binds potently to activin-A, neutralizing and inhibiting its action. However, FS has inhibitory effects not only on activin-A but also on several TGF-β superfamily cytokines. There are two isoforms of follistatin arising from alternative splicing, each one has a distinct role in the clearance of activin-A. The FS288 isoform interacts potently with the heparin sulfate proteoglycans and regulates the functional ability of activin-A while the FS315 isoform clears out activin-A from the circulation without binding to the proteoglycans **[85]**.

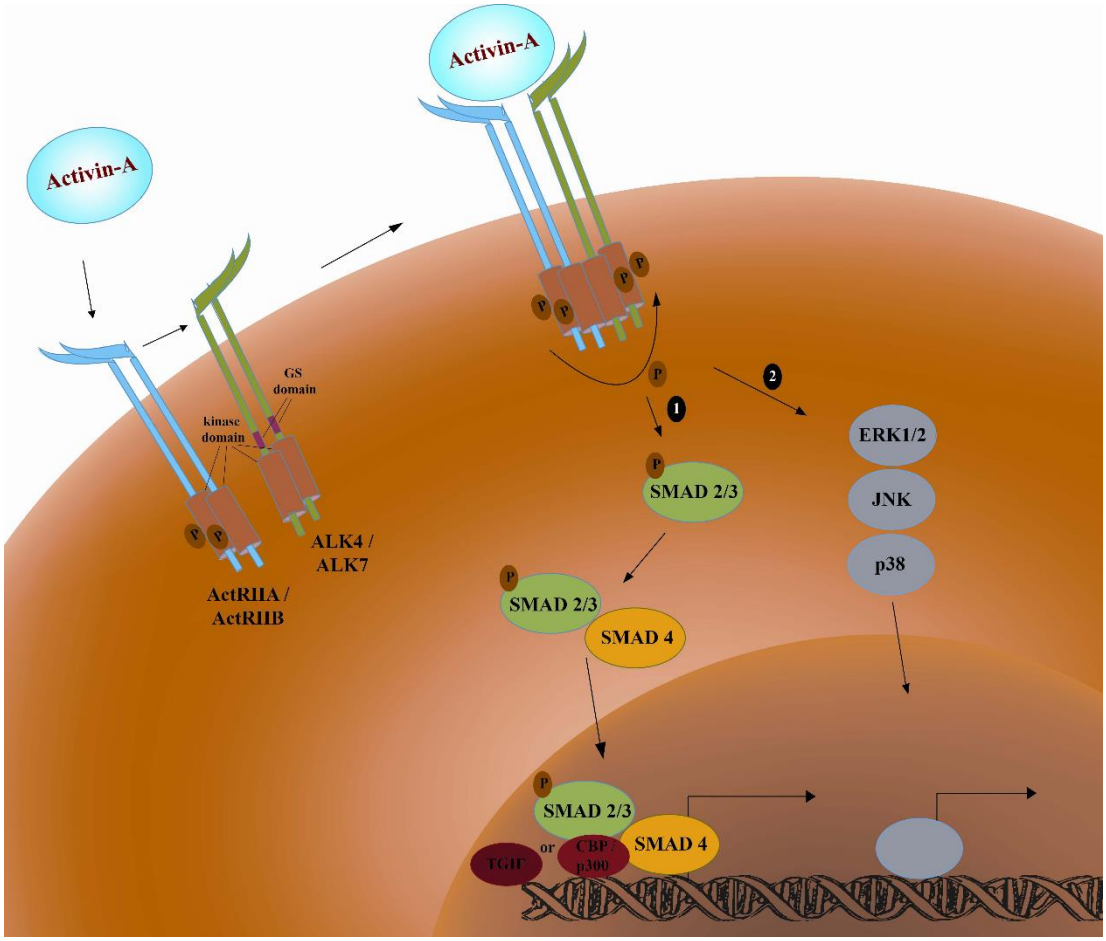


Figure 6. Signaling pathways of activin-A Ligation of activin-A to ActRII leads to the recruitment of the ActRI (ALK4/ALK7). The activation of the latent form of the receptor promotes the signal transduction through Smad2/3. pSmad2/3 interacts with Smad4 inducing its nuclear translocation. At the nucleus the Smad complex regulates the transcription of several target genes with co-operation of transcriptional factors such as CBP/p300 or TGIF). Furthermore, Activin-A activates the ERK, p38 or JNK signal pathway targeting the transcription of several genes. *Adapted from Activin-A: A New Piece in the Puzzle of Tolerance in Asthma, Tousa et al. 2014, Clinical Anti-Inflammatory & Anti-Allergy Drugs*

The Janus profile of activin-A

Activin-A is a cytokine produced by almost every cell type. But the complexity of the biology of this cytokine arises from the janus profile that activin-A has. A

great repertoire of functions through pro-inflammatory and anti-inflammatory effects exerted by activin-A **[Figure 7.].**

TNF-α and IL-1β production, two cytokines that indicate a pro-inflammatory response, are induced by activin-A together with the secretion of prostaglandin E2, thromboxane and iNOS by mouse bone-marrow macrophages **[86]**. Immature human and murine DCs are induced to secrete CXCL12 and CXCL14 chemokines after activin-A administration, promoting their migration *in vitro* **[88]**. Human monocytes are differentiated into Langerhans cells after activin-A stimulation and then migrate through IL-12p70 upregulation **[89]**. Mouse $CD8^+$ T cells release higher levels of antigen-driven IFN-γ after activin-A administration **[87]**. Also, mouse peritoneal macrophages after activin-A stimulation produce increased levels of nitric oxide (NO) and IL-1β and upregulates the CD14/CD68 expression inducing their phagocytic capacity **[90]**.

There are also many studies indicating the anti-inflammatory role of activin-A. In RAW264.7 cell line, activin-A suppresses the secretion of IL-1β and NO and downregulates the CD14, CD68 and TLR4 expression after LPS stimulation *in vitro* **[91]***.* Furthermore, activin-A induces the differentiation of peripheral $CD4^+$ $CD25^-$ T cells to $Foxp3^+$ Treg cells *in vivo* **[92]**. Activin-A also inhibits the proliferation of NK cells and the expression of CD25 and T-bet and secretion of IFN-γ, CCL4, CCL3, CXCL8 and CXCL10 by NK cells *in vitro* **[93]**. LPS-activated mouse peritoneal macrophages after activin-A stimulation downregulate the levels of CD14 and MHCII expression, diminishing their phagocytic capacity **[94]**. Activin-A suppresses TLR4 expression on mouse peritoneal macrophages inhibiting LPS stimulation effects **[95]**. The levels of monocytic phagocytosis and IL-6-mediated murine B cell proliferation are decreased after activin-A administration *in vitro* **[96]***.* During polyclonal stimulation activin-A suppresses the proliferation of $CD4^+$ T cells **[97]**.

Activin-A has a pivotal role in the suppression of Th2 cellular responses. Th2 cell-mediated allergic responses are exacerbated upon *in vivo* neutralization of endogenously produced activin-A, uncovering its protective role in experimental asthma. Our group has previously shown that activin-A can induce a

$CD4^+Foxp3^-IL\text{-}10^+$ regulatory T cell population that suppress Th2 cell-mediated responses, highlighting a novel immunoregulatory role for activin-A **[98]**.

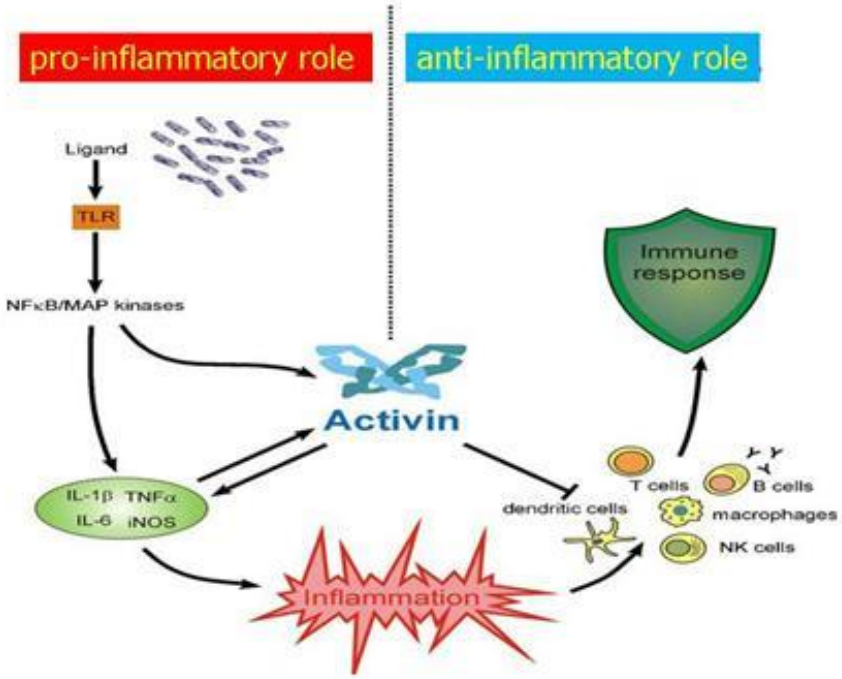


Figure 7. Dual role of activin-A in immune responses. Pro- and anti-inflammatory role of Activin-A depends on the cellular type, the cytokine milieu and the context of the response. *Adapted from Cytokine and Growth Factor Reviews 2009*

Activin-A and neuroprotection

Activin-A and its receptor is expressed in the brain and is highly upregulated in a variety of brain disorders like epilepsy, stroke and excitoxic injury **[99, 100]**. Activin-A is expressed by most of the CNS cell types including neurons, microglia and oligodendrocytes **[101, 102]**. M2 microglia derived activin-A promotes oligodendrocyte differentiation during remyelination **[102]**. In human CSF, activin-A was increased after severe traumatic brain injury and perinatal asphyxia and considered as a biomarker of brain injury in pre-term neonates **[103, 104, 105]**. In an animal model of acute excitotoxic brain injury, the neurotrophic effect of the basic fibroblast growth factor (bFGF) depends on the induction of the expression of activin-A **[106]**. Furthermore, activin-A exert

potent neuroprotective function attenuating cerebral damage in a model of stroke **[107].** Intrastratial infusion of activin-A attenuated the neuronal degeneration in an animal model of Huntington's disease **[108]**. Notably, activin-A inhibits serum deprivation and hydroxydopamine -induced death in SHSY5Y neuroblastoma cells and suppresses 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-driven DA neurotoxicity **[109]**. Activin-A reverses the activated state of microglia cells to ramified (inactivated microglia) after LPS stimulation. Also, in a model of kainic acid hippocampal neurodegeneration, activin-A promotes neurogenesis indirectly by limiting the levels of inflammation **[110]**. Activin-A enhances the phosphorylation and the activation of N-methyl-D-aspartate receptors (NMDA) in primary hippocampal neuronal cultures **[111]**. Finally, follistatin overexpressing mice showed diminished late phase of long-term potentiation (LTP) but after activin-A administration *in vivo,* LTP late phase was prolonged **[112]**.

Hypothesis and Aims

We hypothesize that **«*activin-A represents a key cytokine in PD that can suppress AS-associated neuroinflammation and protect against disease propagation*»**.

Aims:

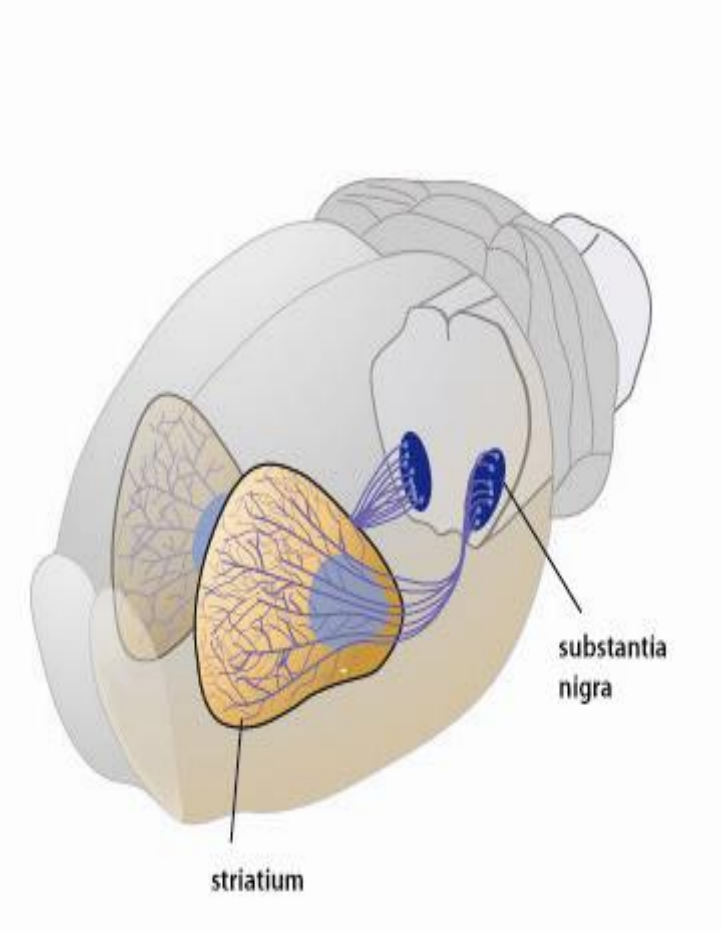
1. To delineate activin-A expression pattern in every cell type of the CNS.
2. To investigate the role of activin-A in the protection against AS pathological accumulations and the mechanism behind that potential protection.

Materials and Methods

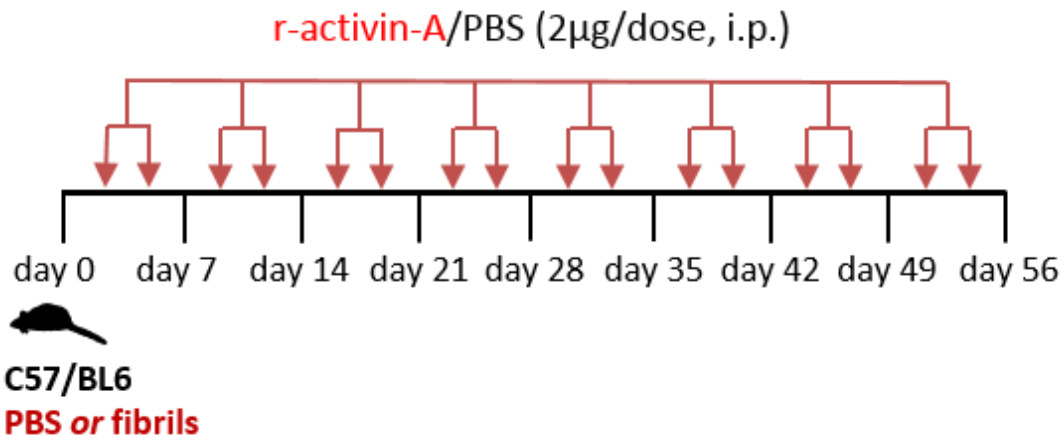
Intrastriatal stereotaxic injections of wt a-synuclein fibrils targeting the nigrostriatal pathway

C57/BL6 mice (2-4 month aged)

Injected material: 4,25µg of wt a-synuclein fibrils



Experimental approach



To determine the in vivo role of activin-A in the suppression of AS-induced pathology, we utilized our previously-established mouse model which consists of administration of human AS preformed fibrils (PFF) or control (PBS), in the striatum of C57BL-6 mice. In this model, phosphorylated-alphasynuclein^{+} accumulations are formed at 60 days post injection, at the SNpc (ipsilaterally) and in distinct cortical layers. Notably, at the contralateral site, in the cortex, pathological accumulations of phospho-alpha synuclein are also observed though at a lower extend, indicative of AS-induced transmission of disease pathology (unpublished observations). The effects of activin-A were investigated after administration of either recombinant activin-A (at 2µg/dose, intraperitoneally) or control (PBS), (twice/week for 8 weeks) into AS-injected mice (n = 3 mice/group). Subsequently, mice were sacrificed and distinct brain regions were obtained and utilized for immunohistochemical analysis to assess AS phosphorylation.

Immunofluorescence

To determine the expression of activin-A, alpha synuclein (phospho S129), iba-1, neuronal class III β-tubulin (tuj-1), tyrosine hydroxylase and p-smad2/3 in the cortex, the striatum and the SNpc, we performed immunofluorescence

experiments with 35μm cryostat free floating sections from the brain. Firstly, we washed 3 times the sections with PBS 1x for 15 minutes each time. Citrate buffer was used for antigen retrieval at 80 °C in waterbath for 30 minutes. PBS 1x washes 3 times for 15 minutes each time. Blocking step with 5% Donkey Serum and 0.1% TritonX in PBS 1x for 60 minutes. Primary antibodies with 2% Donkey Serum in PBS 1x for ~44hours at 4 °C. Anti-activin-A (RnD AF338) was used in a final concentration of 10 μg/ml, anti-alpha synuclein (phospho S129) antibody (Abcam 51253) was used in a final dilution of 1/2000, anti-iba 1 (Wako 019-19741) was used in a final concentration of 1,25 μg/ml , neuronal class III β-tubulin (tuj-1, Covance MMS-435P) was used in a final concentration of 0,66 μg/ml, anti-tyrosine hydroxylase antibody (TH, Millipore AB152) was used in a final dilution of 1/2000, p-smad2/3 (SantaCruz sc-11769) was used in a final concentration of 0,66 μg/ml. PBS 1x washes 3 times for 15 minutes each time. Secondary antibodies with 2% Donkey Serum for 90 minutes at RT. PBS 1x washes 2 times for 15 minutes each time. To-pro-3 (ThermoFischer Scientific T3605) was used in a final dilution of 1/1000 for 8 minutes. PBS 1x washes 2 times for 15 minutes each time. Fluoromount-G (eBioscience 00-4958) was used to mount the sections to superfrost plus microscope slides (Thermo Scientific).

Statistical Analysis

For statistical analysis, we used Graph Pad Prism Version 5 Software (Graph Pad Software Inc, San Diego, CA). We applied Unpaired Student's *t* test and Mann-Whitney ή Wilcoxon tests depending on the nature of the experiments. All of the results are presented as mean ± SEM. Differences with *p* value less that 0.05 are supposed to be statistical significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Validation of experimental model

As stated before, the experimental model we used relies on the propagation of AS fibrils into SNpc from the striatum of C57BL-6 mice that initially were injected. Striatum consist of many terminals of dopaminergic neurons of the SNpc. The AS preformed fibrils were injected in the right striatum of the brain (ipsilaterally) and after 60 days post injection, these AS fibrils propagated to SNpc, forming these AS roundy pathological accumulations that seem like rings. In order to validate that our experimental model works correctly, we performed single stainings with p-a-synuclein antibody in SNpc brain sections. Our results showed that these AS pathological accumulations are formed 60 post injection only in the injected side of the brain (ipsilaterally). In the non-injected side of the brain (contralaterally), no AS preformed fibrils or AS pathological accumulations were formed 60 days post injection **[Figure 8.].**

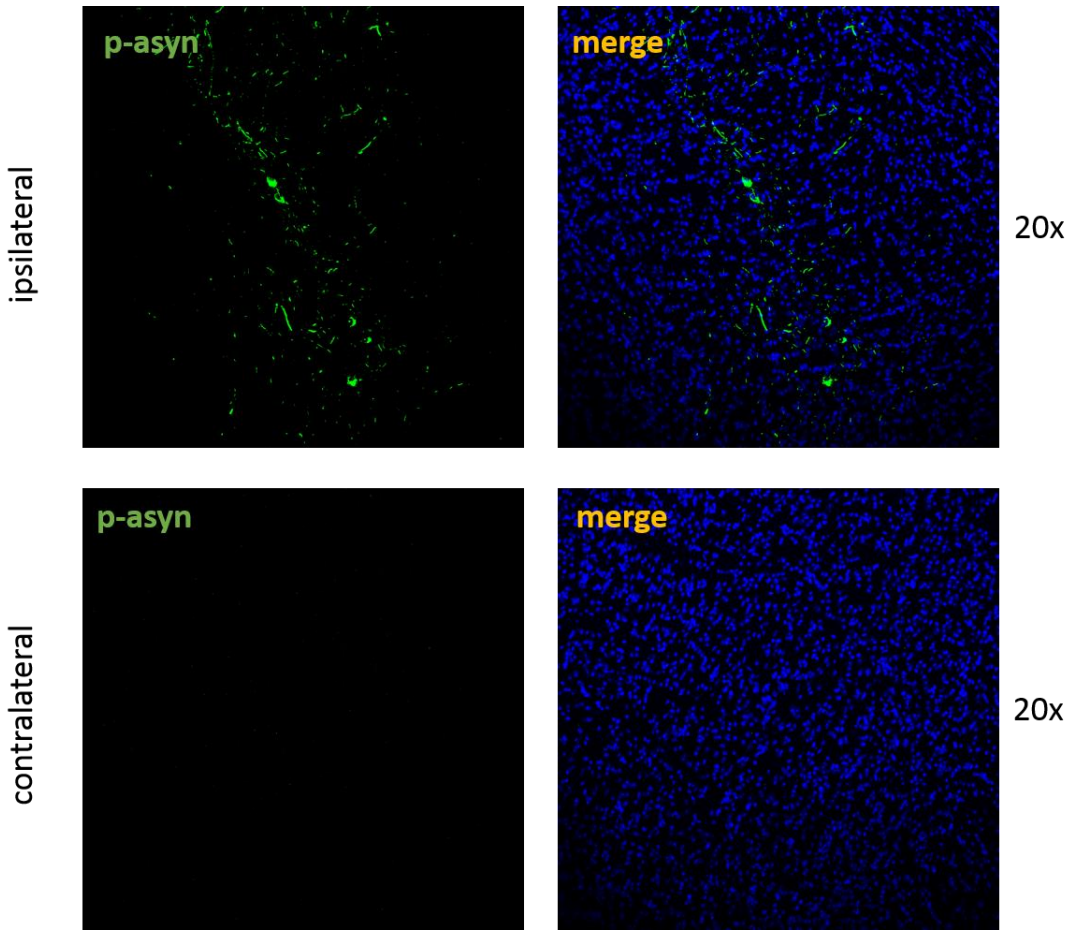


Figure 8. Validation of experimental mouse model. Administration of human AS preformed fibrils (PFF) in the striatum of C57BL-6 mice. Phosphorylated-alphasynuclein^{+} accumulations are formed at 60 days post injection at the SNpc in the injected side (ipsilaterally) and not in the non-injected side (contralaterally).

Activin-A is not expressed by iba^{+} cells

In order to delineate the activin-A expression pattern, we started with the 'immune' cells of the brain, the microglia cells. We performed double-staining immunofluorescence experiments which revealed that Iba^{+} cells do not express activin-A in the cortex, the striatum and the SNpc of AS preformed fibrils injected mice and of wt mice injected with PBS (data not shown). Iba-1 is a calcium-binding protein that is expressed by macrophages, microglia and monocytes. Furthermore, we performed immunofluorescence experiments in order to find out if ALK4, an activin-A receptor, is expressed by iba^{+} cells in the SNpc. Our results revealed that ALK4 was partially expressed by iba^{+} cells of AS preformed fibrils injected mice, ipsilaterally and contralaterally (data not shown) **[Figure 9.].**

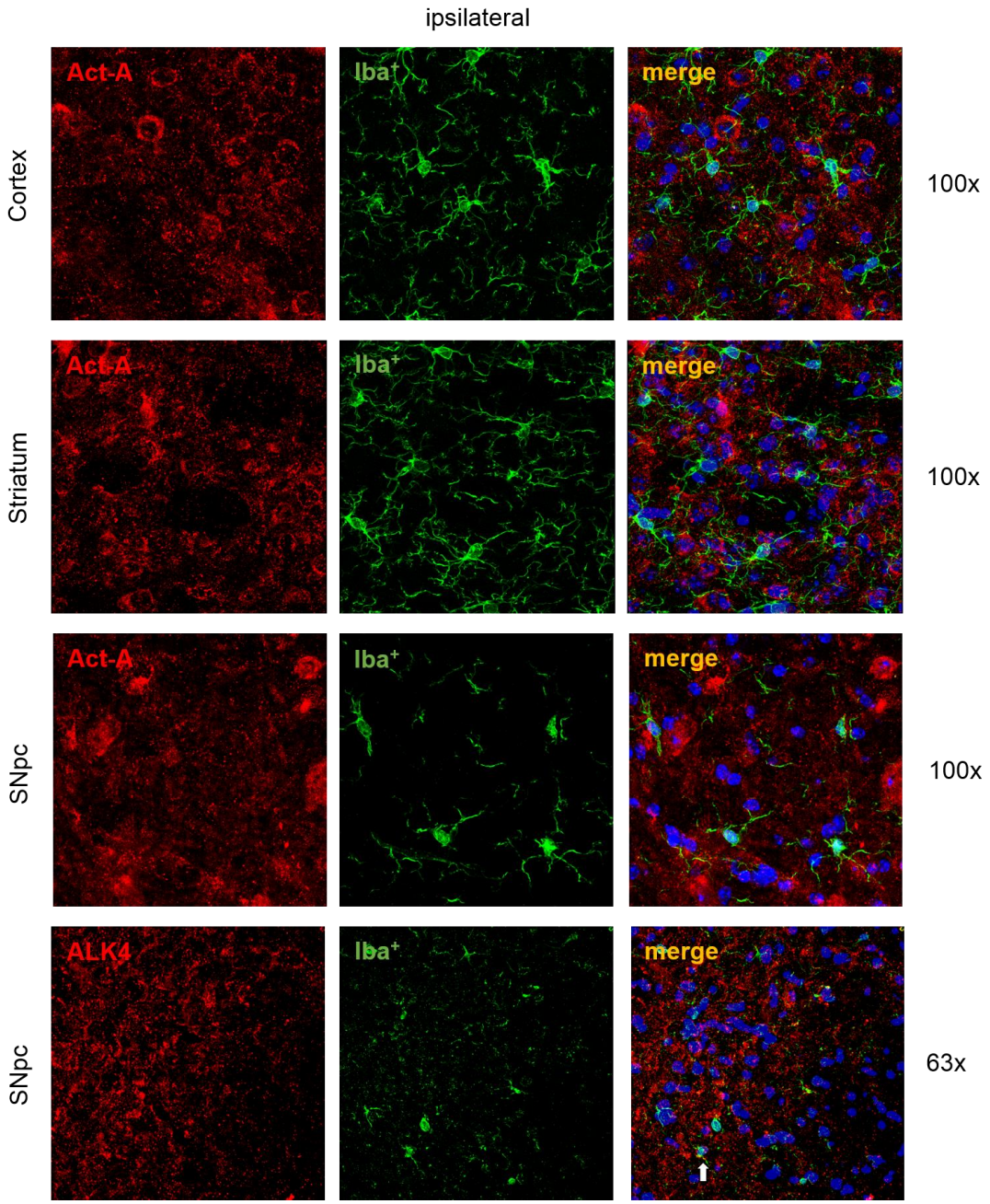


Figure 9. Brain cryosections of mice injected with AS fibrils (PFF) were stained with antibody against activin-A and ALK4, the receptor of activin-A (red) and antibody against iba$^+$ cells (green) and analyzed by confocal microscopy. In the cortex, the striatum and the SNpc, no activin-A expression was detected by iba$^+$ cells. In the SNpc, ALK4 was partially expressed by iba$^+$ cells. Nuclei are stained blue with DAPI. Arrows indicate positively stained cells for ALK4 and iba$^+$ antibodies. Data are representative from *n* = 2 mice.

Activin-A is predominantly expressed by neurons

The next step for the delineation of the activin-A expression pattern was to investigate whether neuronal cells express activin-A. We performed double-

staining immunofluorescence experiments which revealed that tuj-1^{+} cells predominalty express activin-A. Neuronal class III β-tubulin (tuj-1) is a structural protein for microtubule stability and is expressed by neurons of the CNS and PNS. Our data revealed that tuj-1^{+} predominantly express activin-A in the cortex, the striatum and the SNpc of AS preformed fibrils injected mice **[Figure 10.]** and of wt mice injected with PBS (data not shown).

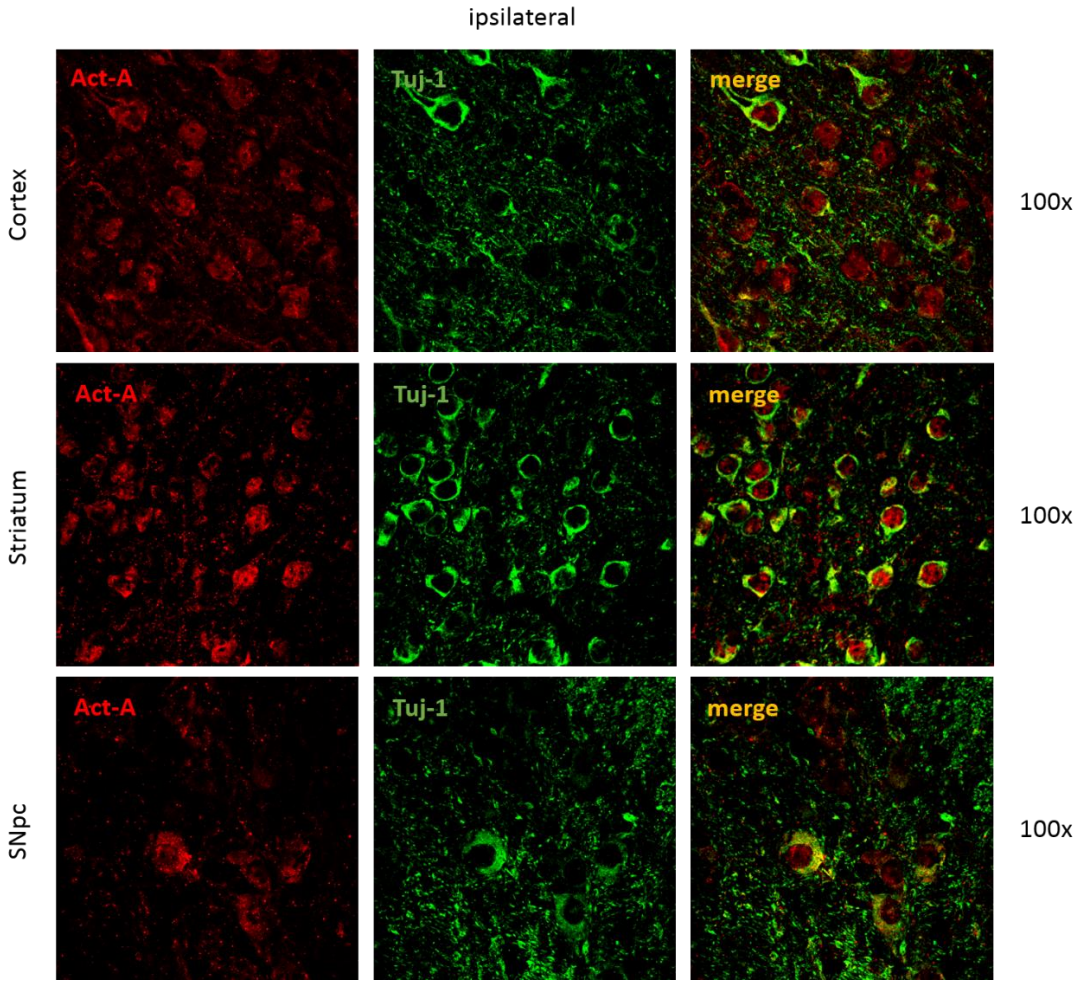


Figure 10. Brain cryosections of mice injected with AS fibrils (PFF) were stained with antibody against neuronal class III β-tubulin (tuj-1 as green) and antibody against activin-A cells (red) and then analyzed by confocal microscopy. In the cortex, the striatum and the SNpc, tuj-1^{+} cells predominantly expressed activin-A. Data are representative from *n* = 3 mice.

Activin-A, ALK4 and p-smad2/3 are expressed by dopaminergic neurons in the SNpc

In the previous figure we showed that activin-A is predominantly expressed by neuronal cells. The main pathology of PD leans on the neurodegeneration of

dopaminergic neurons and since we showed that activin-A is expressed by neurons in the SNpc, we decided to check out if dopaminergic neurons were expressing activin-A. Indeed, our results showed that dopaminergic neurons of the SNpc of AS preformed fibrils injected mice predominantly expressed activin-A **[Figure 11A.]**. In order to uncover the mechanism by which activin-A reduced the AS pathological accumulations as it will presented below **[Figure 12.]**, we performed immunofluorescence experiments with ALK4 and p-smad2/3 antibodies together with tyrosine hydroxylase staining. ALK4 is a type I activin-A receptor and p-smad2/3 is a pivotal signaling molecule in the canonical signaling pathway of activin-A. Our data revealed that dopaminergic neurons of the SNpc of AS preformed fibrils injected mice expressed ALK4 and predominantly expressed p-smad2/3 **[Figure 11B, 11C.].** No differences were observed between ipsilateral and contralateral sides (data not shown).

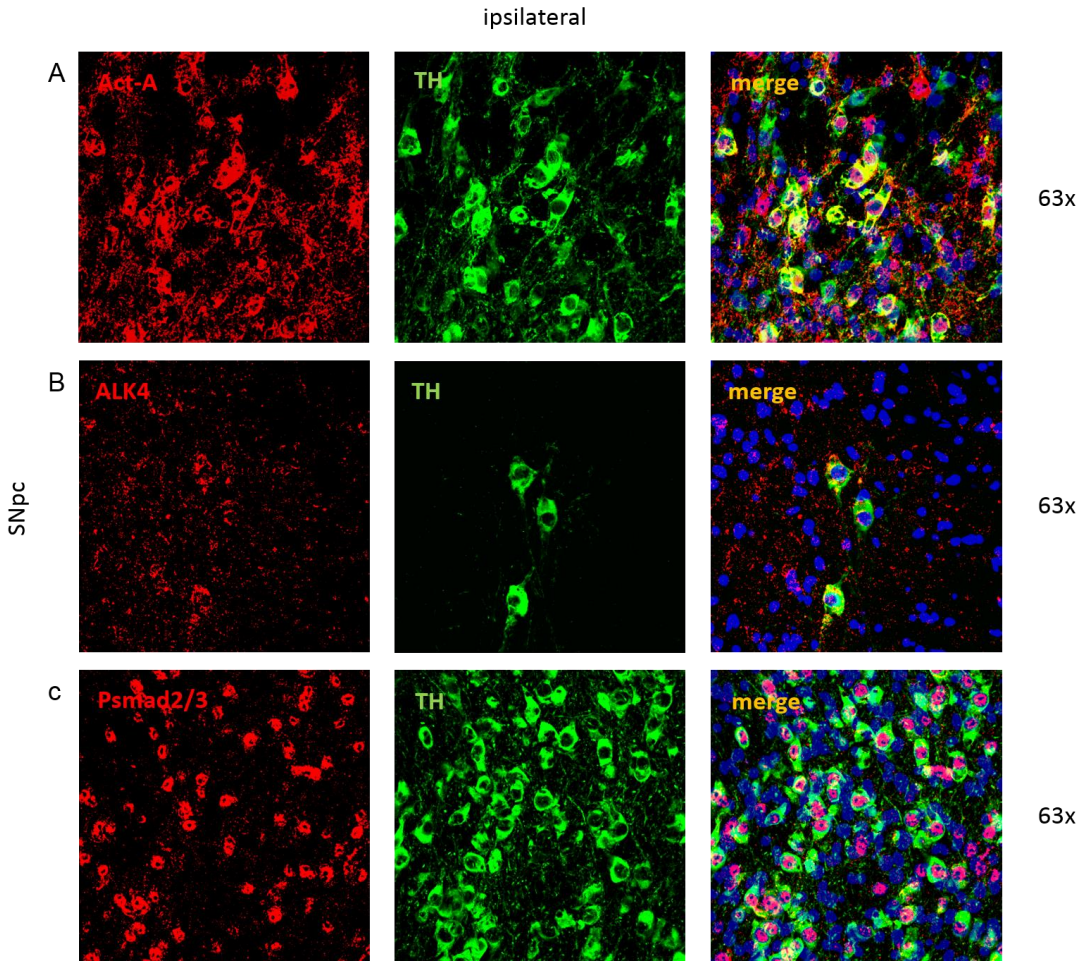


Figure 11. Brain SNpc cryosections of mice injected with AS fibrils (PFF) were stained with antibody against tyrosine hydroxylase (green) and antibody against activin-A, ALK4 and p-smad2/3 (red) and then analyzed by confocal microscopy. **A.** TH^+ cells predominantly expressed activin-A. **B.** TH^+ cells expressed ALK4 **C.** TH^+ cells mainly expressed p-smad2/3. Nuclei are stained blue with DAPI. Data are representative from *n* = 3 mice.

Activin-A reduces the formation of AS pathological accumulations

To determine the role of activin-A in the progression of the pathology of PD, we utilized our experimental model that was analyzed above. We administered activin-A (2μg/dose) or PBS in human AS preformed fibrils injected mice (PFF) for 60 days, two times per week. To analyse the role of activin-A, we performed immunofluorescence experiments in which we double-stained the brain SNpc cryosections with antibody against tyrosine hydroxylase in order to stain the dopaminergic neurons and with antibody against phospho-asynuclein in order to stain the AS fibrils and the AS pathological accumulations. Our results indicated a reduction in the AS pathological accumulations in the AS preformed fibrils injected mice in which we administered activin-A comparatively to AS preformed fibrils injected mice in which we administered PBS **[Figure 12.]**. In order to quantify the level of reduction, we measured the AS pathological accumulations to the number of tyrosine hydroxylase positive cells per mice. Our data suggest a neuroprotective role of activin-A which reduces the levels of AS pathological accumulations that lead to neurodegeneration of the dopanimegic neurons in the SNpc. At this point, it should be sounded that in the contralateral side of both mice group, no AS pathological accumulations were observed (data not shown). The results are representative from *n*=2 mice/group.

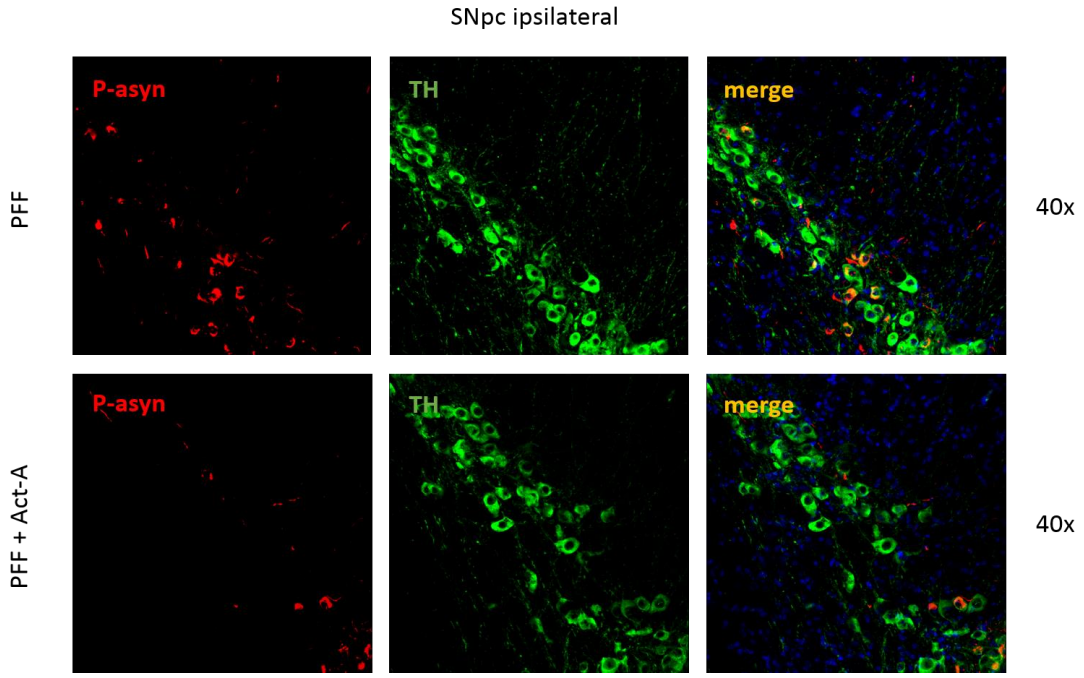


Figure 12. Brain SNpc cryosections of mice injected with AS fibrils (PFF) after PBS or activin-A administration were stained with antibodies against tyrosine hydroxylase (green) and phospho-asynuclein (red) and analyzed by confocal microscopy. Activin-A administration reduced the levels of AS pathological accumulations on dopaminergic neurons of the SNpc. Nuclei are stained blue with DAPI. The results are representative from *n*=3 mice/group.

In vivo activin-A administration reduces iba$^+$ cells in the SNpc

As stated before, we utilized an experimental model in which we injected AS preformed fibrils in the striatum of C57BL-6 mice. Then we administered *in vivo* activin-A or PBS, two times per week for 60 days. In order to uncover the mechanism by which activin-A leads to reduced levels of AS pathological accumulations, we performed immunofluorescence experiments with antibodies against tyrosine hydroxylase and iba-1. Tyrosine hydroxylase staining was performed in order to mark the whole region of the SNpc. After whole SNpc region was marked with the help of tyrosine hydroxylase staining, we counted all the iba$^+$ cells into the marked region. Our results showed that the levels of iba$^+$ cells were slighty reduced in both ipsilateral and contralateral sides of the AS preformed fibrils injected mice in which we administered activin-

A *in vivo* comparatively to the AS preformed fibrils injected mice in which we administered PBS **[Figure 13.]**.

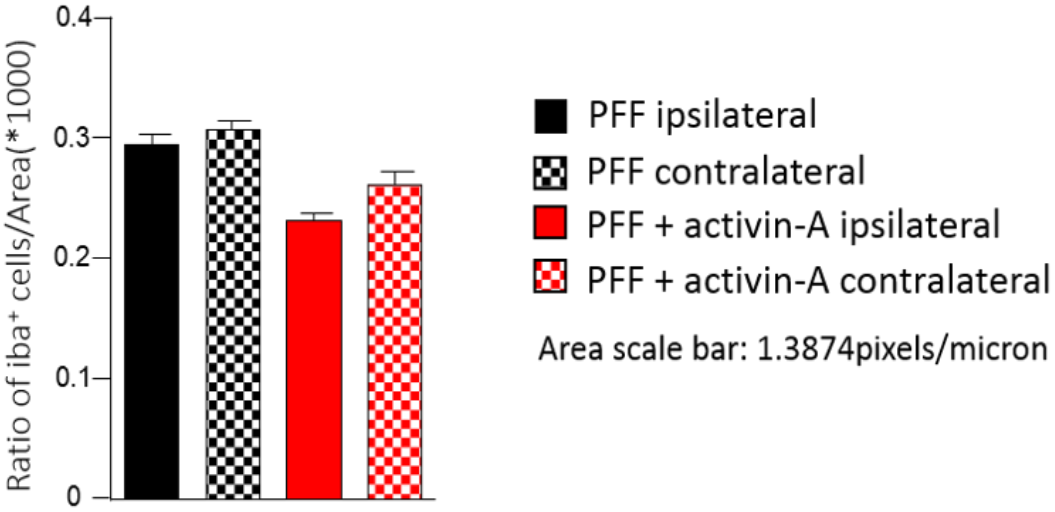


Figure 13. Wt mice injected with AS fibrils and treated with PBS or activin-A *in vivo*. In the SNpc we observed a reduction of Iba^+ cells in both ipsilateral and contralateral sides of mice injected with AS fibrils and treated with activin-A *in vivo.* Data are pooled from *n*=3 mice/group.

Discussion

The experimental model we utilized with the AS preformed fibrils injections in the striatum of C57BL-6 mice is a very useful mouse model in order to investigate the mechanisms of a-synuclein propagation and also new ways of targeted inhibition of the pathology transmission. In our case, we established our model in which AS pathological accumulations are only formed in the SNpc ipsilaterally. Although there are cases in which we observed AS fibrils in the contralateral cortex, no AS pathological accumulations were formed in the SNpc contralaterally at the timepoint we were investigating. These roundy AS pathological accumulations are formed 60 days post injection. This is also a very significant reason why this model is better in order to delineate the propagation process than 6-OHDA model in which more than 60% of dopaminergic neurons degenerate almost immediately.

In the CNS, iba^+ cells represent mostly microglia cells and low rates of infiltrating macrophages and monocytes. In our model, iba^+ cells do not express activin-A in the cortex, the striatum and the SNpc. There are other studies revealing that M2 microglia/macrophages cells secrete activin-A, significant for the remyelination process **[102]**. So an important step is to delineate the microglia phenotype. In PD, the level of immune response is low so it may explain the differences in the pattern of activin-A expression. Flow cytometric analysis of isolated primary microglia cells from mice with our experimental approach could elucidate the microglia phenotype in order to find out the role of microglia. Investigating a potential pro-inflammatory or anti-inflammatory profile and which cytokines and chemokines specifically express is of pivotal significance. The partial expression of ALK4 by iba^+ could be explained by the fact that the stained cells contain different cellular types like infiltrated macrophages and monocytes. So it is very essential to identify new experimental approaches to distinguish the different cell types of iba^+ cells.

The activin-A expression from almost all neuronal cells seems to indicate that activin-A has a pivotal role in the neuronal function. Also ALK4 and psmad2/3 implicate in the neuronal cell cycle. Activin-A, ALK4 and psmad2/3 expression by dopaminergic neurons were presented for the first time suggesting a

potential role in the disease progression. The next step is to analyse the activin-A expression by astrocytes that also have a key role in PD pathology. Astrocytes can internalise AS fibrils so activin-A could reveal a differential expression pattern after internalisation **[31]**.

The administration of activin-A in AS preformed fibrils injected mice led to a significant reduction of the formation of AS pathological accumulations in the SNpc. Our results indicate a novel neuroprotective function of activin-A. Uncovering the mechanism behind this neuroprotection is the next step. Activin-A is a cytokine involved in a variety of biological processes and also there are many studies indicating that this cytokine can implicate in the function of almost every cellular type. It is known that PD patients have an increased infiltration of T cells into the SN so a future experiment could be the analysis of T cells from the cervical lymph nodes in order to uncover the $CD4^+$ and $CD8^+$ T cells balance and also the Th subsets balance and the inflammatory mediators secreted. Furthermore, we could also analyse T cells population in order to find out if activin-A induces an immunoregulatory T cellular population that potentially could inhibit the formation of AS pathological accumulations. There are little evidence about the role of Treg cells in PD. A previous study showed that $CD4^+$ $CD25^+$ $CD127^-$ regulatory T cells from PD patients exhibited low suppressive capacity comparatively to healthy controls **[67]**. Recently a research group has found the receptor of a-synuclein preformed fibrils, lymphocyte-activation gene 3 (LAG3) expressed by neurons also besides T cells expression. No detectable levels of LAG3 were found in astrocytes and microglia cells **[113]**. A future experiment in order to delineate the mechanism of this significant reduction could be through confocal analysis of the potential downregulation of LAG3 expression by neurons of activin-A -treated AS fibrils injected mice comparatively to control AS fibrils injected mice. Furthermore, another important experiment would be the Ca^{+2} levels measurements before and after activin-A administration. Ca^{+2} has an essential role in the secretion of exosome-like vesicles containing a-synuclein monomers and oligomers **[114]**. Activin-A administration could affect the levels of Ca^{+2} inhibiting the propagation and formation of these AS pathological accumulations.

Finally, the slight reduction of iba^{+} cells probably is due to the inhibition of the infiltration of macrophages and monocytes to the CNS. Activin-A may lead to a reduction of these AS pathological accumulations and the iba^{+} cells attenuation may be the result of this reduction. Microglia and macrophages are activated after neuronal death through TLRs and NLRs. Since activin-A can reduce AS pathological accumulations then apoptotic neuronal cells do not release DAMPs in order to activate microglia and infiltrate immune cells like macrophage and monocytes.

In summary, our results revealed for the first time activin-A expression by dopaminergic neurons in the SNpc and highlight a novel neuroprotective role of this cytokine on PD pathogenesis. These findings may pave the way for the design of new experimental and therapeutic approaches in PD.

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