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Selective vulnerability in dopaminergic neurons in Parkinson's Disease

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Abstract

The onset of the typical symptoms of Parkinson's Disease (PD) is correlated with the selective loss of dopaminergic neurons located within the substantia nigra pars compacta (SNpc) in the midbrain. These neurons project their axonal terminals to the striatum, and the depletion of dopamine causes typical symptoms such as resting tremor, bradykinesia, and rigidity.

Previous studies have highlighted the presence of more vulnerable and more resilient dopaminergic neurons. Understanding the molecular basis of this differential vulnerability is essential for identifying the onset of the pathology during the prodromal phase, when dopaminergic neurons have not yet undergone cell death but already present functional alterations. Identifying early signals at this stage is crucial for the development of effective neuroprotective strategies.

The objective of this thesis is to analyze the expression of specific markers, such as Tyrosine Hydroxylase (TH), Calbindin, and ALDH1A1, in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), using the conditional knockout mouse model of the *Ndufs2* gene, coding for a catalytic subunit that is essential for the functionality of MCI (mitochondrial complex I), which presents a phenotype that mimics the same peculiarities of the neurodegenerative state characteristic of Parkinson's Disease.

Through immunofluorescence and microscopy techniques, a spatial mapping of the midbrain was realised to localize these markers and compare their expression levels in wild-type (WT) and pathological conditions (PD). The investigation aims to determine whether the expression of these biomarkers is correlated with the progressive loss of dopaminergic neurons or if their presence may confer an intrinsic neuroprotective advantage. In particular, an overall neuronal loss of 51% of dopaminergic neurons (identified by TH expression) was observed. This degeneration mainly affected the subpopulation expressing ALDH1A1, confirming their greater vulnerability; in contrast, neurons characterised by calbindin expression were found to be significantly more resistant to the neurodegenerative process.

Introduction

Parkinson's Disease

Parkinson's disease is the second-most common neurodegenerative disorder (after Alzheimer), that affects 2–3% of the population ≥65 years of age (Poewe et al., 2017).

The condition owes its name to James Parkinson, who first described its symptoms in 1817 in *"An essay on the shaking palsy" (Parkinson, 1817)*: tremor at rest, alterations in locomotion and postural reflexes, as well as speech (Parkinson, 2002).

The symptomatology of this pathology is heterogeneous, and is characterised by motor and non-motor symptoms.

The principal motor symptoms are:

- tremors at rest,
- slowness of movement (bradykinesia) and difficulty in initiating purposeful movements,
- akinesia (lack of movement),
- hypokinesia (reduced movement),
- stiffness of limbs and neck,
- reduced facial expressions.

Non-motor symptoms:

- cognitive deficits,
- language deficits,
- depression,
- insomnia.

The cause of PD is still unknown, but it appears to result from a complicated interplay of genetic and environmental factors affecting numerous fundamental cellular processes (Wirdefeldt et al., 2011).

The clinical symptoms are a consequence of the degeneration of dopaminergic neurons in the SNc (substantia nigra pars compacta) and the depletion of dopamine in the striatum with the consequent loss of modulation of the basal ganglia circuits that are fundamental in the regulation of movements (Surmeier et al., 2017).

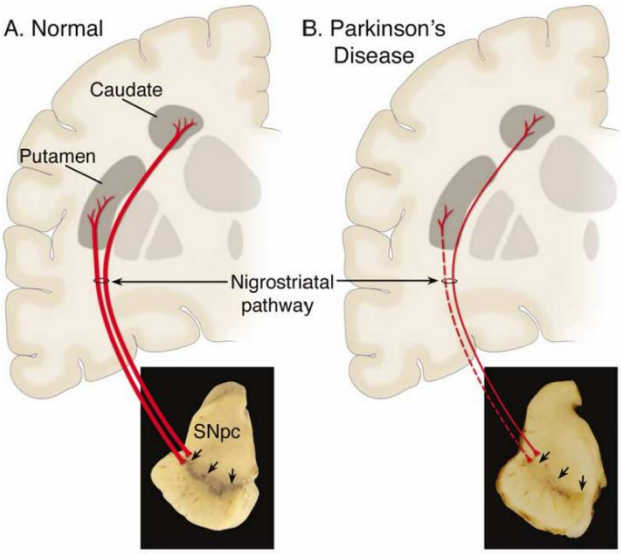


Figure 1 Schematic representation of the nigrostriatal pathway and a cross-section of the midbrain. Adapted from (Dauer & Przedborski, 2003). The image shows the degeneration of neurons in the substantia nigra pars compacta (SNpc) and the consequent reduction in innervation of the striatum (putamen and caudate).

When there is a loss of dopamine in the nigrostriatal pathway, various symptoms occur in the somatodendritic region. First the axons of dopaminergic neurons, located in the striatum, are involved. The motor phenotype is not yet manifested at this stage. Subsequently, the soma of dopaminergic neurons, located in the substantia nigra pars compacta, is involved. The characteristic motor phenotype of PD manifests itself at this stage. The phase prior to diagnosis is called prodromal and manifests with non-motor symptoms.

The motor symptoms of PD occur when the loss of dopaminergic neurons in the SNc is about 50/60%, and the resulting loss of dopamine release in the striatum is 60/70%.

The challenge, therefore, is to be able to diagnose PD before the onset of symptoms, and thus the loss of a significant percentage of dopaminergic neurons.

To this end, it is necessary to understand which characteristics give the SNc neurons the vulnerability typical of PD and which type of neurons are most affected by the disease.

Diagnostic criteria

It is possible to identify the fundamental neuropathological hallmarks of the disease, which underpin the clinical manifestations and the diagnostic framework:

- Progressive loss of dopaminergic neurons of the SNc, which innervate the basal ganglia;
- An anatomopathological hallmark is the presence of Lewy bodies (LB): protein aggregates that contribute to dysfunction and neuronal death;
- Dopamine (DA) deficiency in striatum and SNc, as the striatum receives dopaminergic projections from SNc (region most affected by PD).

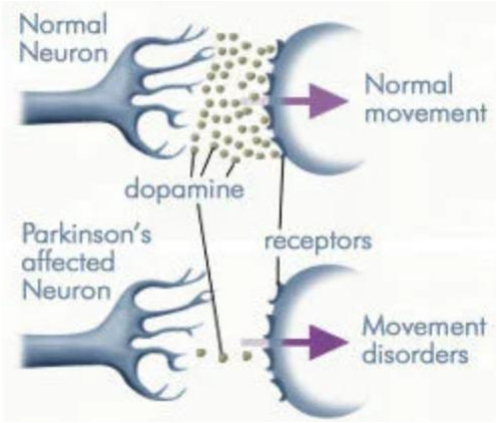


Figure 2 Dopamine levels in a normal vs. Parkinson's affected neuron from (Bazazeh et al., 2016)

We can also distinguish PD into idiopathic, spontaneous (95%), and 5/10% familiar, monogenic, linked to mutation of disease-associated genes. To date, there is no known cure for the PD.

One possible treatment involves the administration of LEVODOPA, but it is necessary to progressively increase the dose in order to continue to have a response over time, and in addition there are many side effects due to continued administration (Poewe et al., 2017).

Another possible approach involves stimulation of the brain, but not all patients can undergo this type of treatment, it does not always work, and the results are not always good. Therefore, both approaches have disadvantages and none is conclusive.

Nigrostriatal pathway

A pivotal point in the study of PD is the nuclei of the basal ganglia, which constitute fundamental circuitry for the performance of important motor functions such as planning, execution of movements and motor learning (procedural memory).

The striatum, composed mostly of projection neurons called MSNs (medium spiny neurons), in addition to receiving axonal projections from the cerebral cortex and thalamus, is innervated by the dopaminergic axons of the SNc and VTA (ventral tegmental area).

In particular, the projections of the axons of the SNc innervate mainly the striatum at the dorsal level, while the axons of the VTA project mainly to the ventral region of the striatum and at the level of the cortex (Haber et al., 2000).

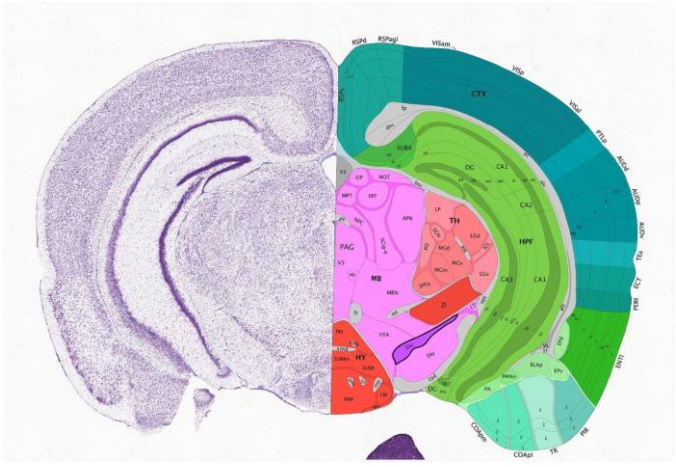


Figure 3 Localisation of dopaminergic nuclei in mouse's midbrain from Allen Brain Atlas

PD's models

The heterogeneity of PD makes it difficult to find a precise transposition of the patients' characteristic pathological phenotype.

Rodents are commonly used as the animal model because of the ease of care in laboratory environment and availability of transgenic mouse strain as well as established protocols. Specifically, ***rodents are used to model PD because the nigrostriatal dopaminergic degeneration directly correlates with motor deficits observed in these animals****. The behavioral phenotype of rodent unilateral lesions can be examined through a series of tests including measuring movement, grip, or strength of the front paws* (Wirdefeldt et al., 2011).

Pharmacological models

Pharmacological models are based on the administration of neurotoxins that produce neurodegeneration of dopaminergic neurons, simulating the PD phenotype.

The principal pharmacological models are:

6-Hydroxydopamine (6-OHDA)

The first model was generated by administering 6-OHDA. The administration is by intracerebral injection, because it doesn't penetrate the blood-brain barrier. Once in the cell, 6-OHDA causes the production of reactive oxygen species responsible for mitochondrial dysfunction.

The administration of 6-OHDA in different brain regions causes a distinctive pattern of neuronal degeneration. For example, injection of 6-OHDA into the striatum will first damage the axonal terminals in the striatum, followed by degeneration of dopaminergic neurons in the substantia nigra (SN). In contrast, injection of 6-OHDA into the SN leads to massive destruction of dopaminergic neurons. Therefore, the latter approach results in relatively severe symptoms (Perese et al., 1989).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP is a neurotoxin accidentally discovered to have caused parkinsonism-related symptoms without the presence of LB in young people who had taken it as a synthetic heroin.

Being a lipophilic molecule, it is able to cross the blood-brain barrier. After systemic administration, MPTP is oxidised to MPP+, a toxic metabolite that is taken up by dopaminergic neurons due to its similarity to dopamine (Martí et al., 2017). MPP+ inhibits complex I of the respiratory chain, resulting in a decrease in ATP levels in the striatum and SN and neuronal death (Kim et al., 2015; Sayre et al., 1989). This induces the progressive loss of DA neurons in the SN and consequently a decrease in dopamine levels in the striatum (Bezard et al., 1999).

Rotenone

Rotenone is a natural substance used as an insecticide. Its lipophilic nature makes it able to penetrate through the blood-brain barrier and inhibit the mitochondrial complex I.

In particular, chronic administration of low doses of rotenone produces selective cell degeneration in the nigrostriatal region (Inden et al., 2011).

Key symptoms of PD, such as motor deficits, catecholamine depletion and loss of dopaminergic neurons, can be observed in animal models.

Initially, mainly pharmacological models were used. The problem is that the methods used to obtain these models are very aggressive, killing dopaminergic neurons. As a result, the phenotype obtained is the late stage phenotype.

What is useful, however, is to be able to follow neuronal degeneration progressively, from early to late stages, to better understand which neurons are involved and how they are

involved. This kind of understanding was possible with the introduction of transgenic mice, the manipulation of which makes it possible to eliminate or overexpress PD-related genes.

Genetic models

The complex I of the mitochondrial respiratory chain plays an important role in the onset of PD: the inhibition of this complex is responsible for the death of dopaminergic neurons. These genetic animal models include:

Ndufs4

About ten years ago, with the aim of examining the effects of the loss of complex I activity on the function and survival of dopaminergic neurons, the Ndufs4 model was generated by deletion of this subunit (van de Wal et al., 2022).

In mice generated by conditional knockout of the Ndufs4 subunit (Ndufs4 KO) there was no loss of dopaminergic neurons in the SN or motor deficits typical of PD (Kim et al., 2015). But the absence of a phenotypic pattern related to PD is not due to the fact that mitochondria do not play a critical role in PD: in fact, Nfdus4 is an accessory subunit. This means that even with the deletion of this subunit, complex I activity still continues, thanks to the ability of neurons to compensate for the partial destruction involving mitochondrial oxidative phosphorylation (OXPHOS).

For this reason, the model Nfufs2 was generated.

Ndufs2

In this model, the deletion in dopaminergic neurons of the *Ndufs2* gene, coding for a catalytic subunit that is essential for the functionality of MCI (mitochondrial complex I), makes it possible to observe a phenotype that can be associated with PD (González-Rodríguez et al., 2021).

Ndufs2 was ablated specifically in dopaminergic neurons by selective breeding of mice expressing Cre under the control of the dopamine transporter (DAT) promoter with mice containing a floxed allele of the *Ndufs2* gene.

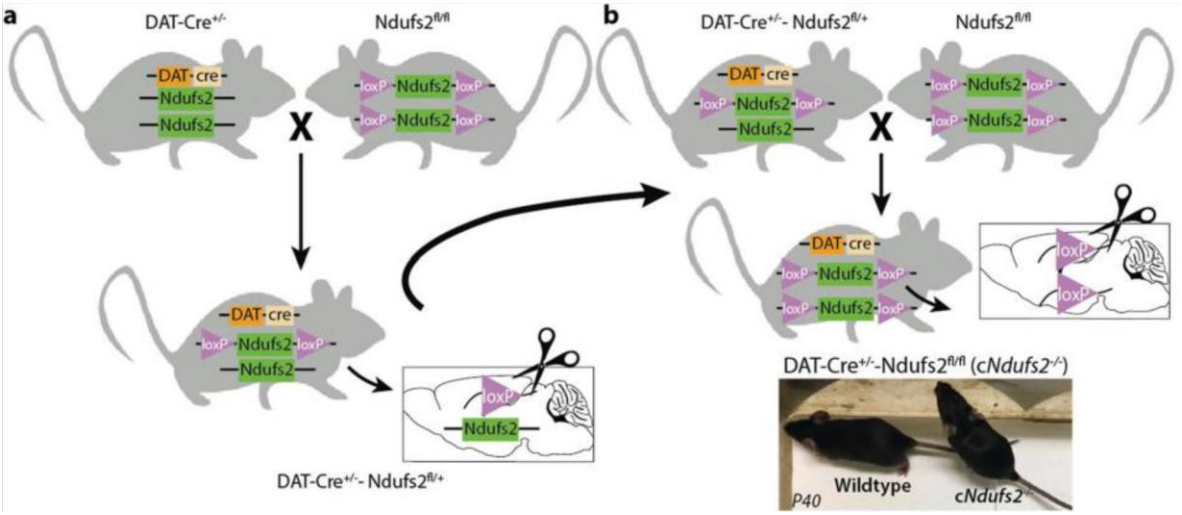


Figure 4 Deletion of Ndufs2 gene in mice from ***(González-Rodríguez et al., 2021)***

These conditional *Ndufs2*-knockout mice displayed progressive, axon-first, levodopa-responsive parkinsonism, resembling that seen in humans.

Progressive neuronal loss was demonstrated by immunostaining with **tyrosine hydroxylase (TH)**. The link between this marker and the survival of dopaminergic neurons stems from the fact that tyrosine hydroxylase is the enzyme that catalyses the conversion of L-tyrosine

into L-DOPA and, consequently, dopamine. For this reason, it allows us to track the progressive dopamine neuron loss in Parkinson's Disease.

It was observed that, on day P30, TH levels were downregulated in the striatum, but at this time point there were no significant changes in the SN or the ventral tegmental area (VTA). In contrast, on day P60, TH expression in the SN was approximately half that of wild-type controls, and dopamine release had decreased by approximately 75%.

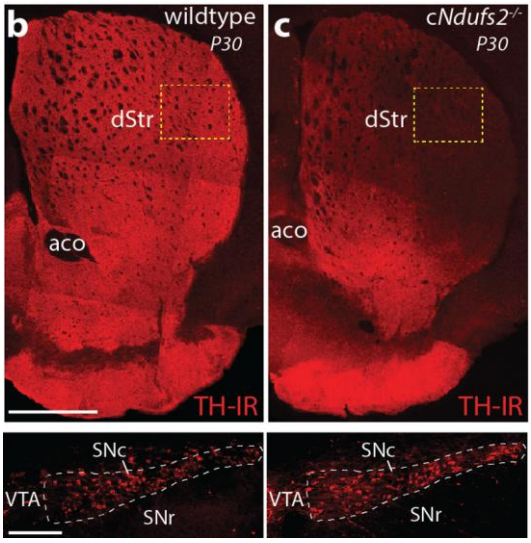


Figure 5 Representative images showing a significant reduction in TH staining in the dorsal striatum but not in the SNc in P30 wild-type and c*Ndufs2*$^{-/-}$ mice, from ***(González-Rodríguez et al., 2021)***

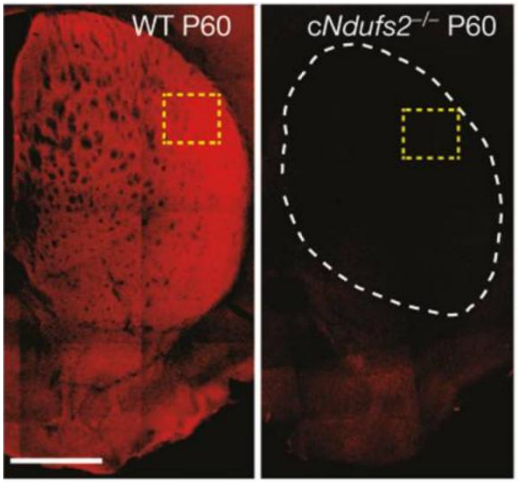


Figure 6 Representative images showing TH immunostaining in the striatum in wild-type and c*Ndufs2*$^{-/-}$ mice at P60, from ***(González-Rodríguez et al., 2021)***

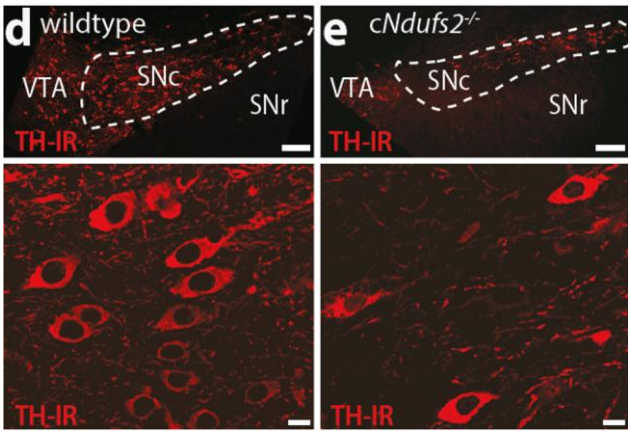


Figure 7 Representative images showing a significant reduction in TH staining in the SNc of c*Ndufs2*$^{-/-}$ mice (e) compared with wild-type mice (d) at P60 from ***(González-Rodríguez et al., 2021)***

In contrast to other models of PD characterised by rapid dopamine depletion, in *cNdufs2*$^{-/-}$ mice it is possible to trace the progression of the disease and to evaluate the correlation between the decrease in dopamine release and the onset of symptoms.

It was observed that at P30, in the so-called **prodromal phase**, when only the striatal tissue is affected, it is possible to restore dopamine release through systemic treatment with levodopa (6 mg/kg) and prevent the pathophysiology associated with Parkinson's disease.

At P60, in the **symptomatic phase**, when the substantia nigra and the ventral tegmental area are also affected, it is not possible to restore dopamine release or physiological functions; the disease spreads and the typical disorders related to walking and movement appear.

This acquired awareness makes it all the more important to identify the prodromal phase and take action before the progression of the disease renders the symptoms irreversible.

Vulnerability of SNc neurons in PD

The SNc has an important neuronal vulnerability in PD resulting in the degeneration of about 80/90% of its neurons (in contrast, in VTA about 30/40% of neurons are lost).

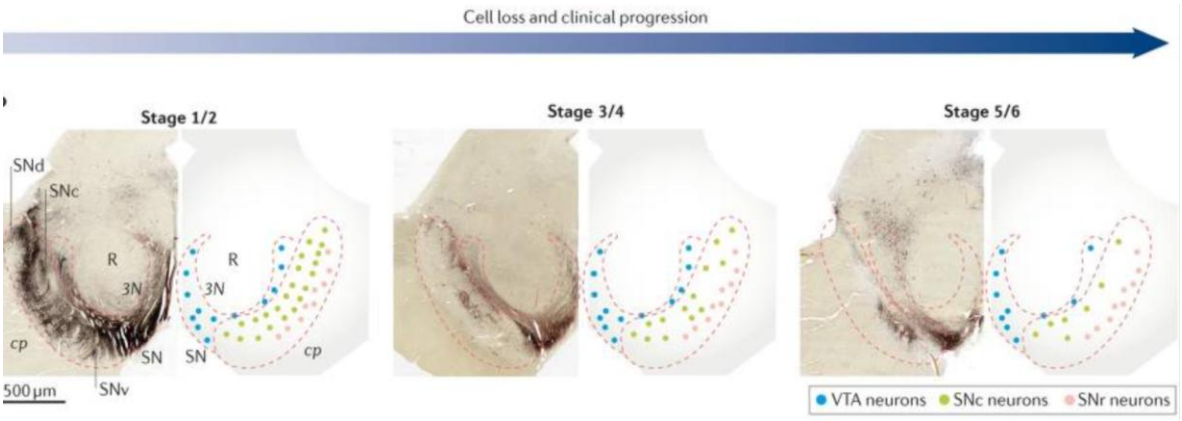


Figure 8: **Staging of neurodegeneration in clinical Parkinson disease** taken from **(Surmeier et al., 2017)**

Transverse sections of the midbrain, the normal distribution of tyrosine hydroylase-immunopositive dopaminergic neurons is shown in the left panels, and the pattern is schematized in the right panels. Heavily pigmented neurons of the substantia nigra pars compacta (SNc) are depicted in green; less pigmented neurons of the ventral tegmental area (VTA) are depicted in blue; neurons of the SN pars reticulata (SNr) are depicted in pink. The initial loss of ventral-tier SNc observed in patients with stage 4 cPD is depicted in the middle panel, with greater cell loss observed over time at later stages, as indicated in the right panel.

This condition is favoured by the unique physiology of dopaminergic neurons, which are characterised by intracellular calcium oscillations controlled by the opening of the voltage-dependent Cav1 calcium channels located on the plasma membrane. In fact, these neurons function as autonomous pacemakers, generating large action potentials (spikes) even in the absence of excitatory synaptic input. This activity triggers the activation of Cav1 voltage dependent calcium channels, belonging to the L-type (long-lasting) family, which open during membrane depolarization and exhibit a slow inactivation rate, thereby allowing a prolonged influx of Ca^{2+} into the cytoplasm. This cytoplasmic calcium can interact with other proteins due to the intrinsic low level of calcium buffer proteins, such as calbindin.

These oscillations maintain energy balance because the mitochondrial uptake of Ca^{2+} promote oxidative phosphorylation (OXPHOS) to meet the neuron's high ATP demand.

However, when these channels are overexpressed, a condition of chronic oxidative stress at the mitochondrial level arises in the axons of DA neurons of the SNc. The result is an increased production of ROS (reactive oxygen species), RNS (reactive nitrogen species) etc., leading to mitochondrial damage, senescence and selective neuronal death.

Another physiological feature peculiar to dopaminergic neurons that could explain the increased vulnerability is the numerous axonal branches that increase neurotransmitter (DA) release, and consequently the neuron's own energy demand.

Another factor that could contribute to the selective vulnerability of dopaminergic neurons in the SNc is the presence of neuromelanin, a pigment that gives the SNc its characteristic black colour, which has the function of capturing iron. Neuromelanin accumulates with age, resulting in iron accumulation that contributes to reactive oxygen species and oxidative stress.

Thanks to research conducted by Poulin and colleagues (Poulin et al., 2020), it has been possible to classify the dopaminergic neurons of the midbrain in different subpopulations, and this is crucial for understanding the molecular cascades underlying selective vulnerability in Parkinson's disease.

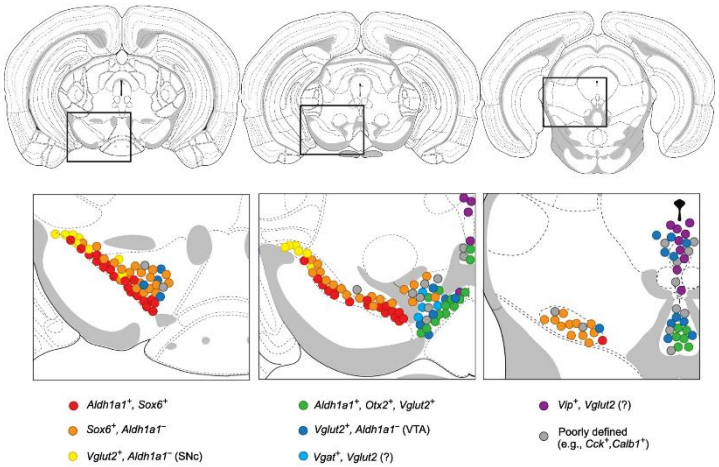


Figure 9 Differential expression of various markers in different dopaminergic neurons of the midbrain in mice from ***(Poulin et al., 2020)***

In particular, the identification of molecularly distinct neuronal populations requires the measurement of multiple markers in individual cells, focusing on two anatomical groups in the midbrain: SNc and VTA. This research highlights several key markers:

- Sox6 represents a hallmark of the most vulnerable dopaminergic population;
- Aldh1a1 identifies ventral SN neurons, which are among the first to degenerate in Parkinson's Dissease;
- Otx2 is a transcription factor that defines neurons in VTA projecting to the limbic system and cortex;
- Calb is associated with a higher degree of resilience durind PD's neurodegeneration.

Research aims

The key point of my research work is to investigate the functional differences between VTA and SN neurons, in order to understand why neurons localised in the VTA can survive a lot and neurons localised in the SN die, despite the fact that they are both dopaminergic neurons.

Several factors may be involved: morphology, physiological and bioenergetic phenotype, or genetic profile of this neurons.

I will focus my work on the analysis of the genetic profile to know also if the difference between SN and VTA is something posterior at the disease, or maybe something intrinsic of these dopaminergic neurons, by comparing samples of midbrain sections from wild-type and knockout mice.

In particular, I will analyse by immunostaining the expression of markers involved in PD, as TH, Calbindin and ALDH1A1.

Immunostaining

To evaluate dopaminergic cell loss, free-floating sections of mice's midbrain were processed for immunostaining against Tyrosine Hydroxylase (TH), Calbindin and ALDH1A1. This technique allows to identify and localize dopaminergic neurons that express a specific protein, using two antibodies: a primary antibody directed against the target antigen, and a secondary antibody conjugated to a fluorochrome, which recognizes the constant region (Fc) of the primary antibody, binds to it, and emits a fluorescence that can be detected through microscopy.

TH (Tyrosine Hydroxylase)

As mentioned previously, TH is the enzyme that catalyses the conversion of L-tyrosine into L-DOPA. For this reason, it is considered the hallmark of dopaminergic neurons and will be

essential in immunostaining experiments to identify dopaminergic neurons in the SNpc (Substantia Nigra pars compacta) and VTA (Ventral Tegmental Area).

Calbindin

Calbindin acts as a calcium buffer by binding the ion, regulating intracellular calcium levels and protecting the cell from neurotoxicity. This action is crucial in the dopaminergic neurons of the midbrain, as they are autonomously active and exhibit pacemaker activity that keeps the calcium channels (Cav1) open for longer, allowing calcium to move into the cellular compartment and exposing the cells to higher levels of oxidative stress (López González del Rey, 2022). Calbindin binds and sequestrates the calcium present in the cytoplasm, increasing the resistance of dopaminergic neurons.

ALDH1A1 (Aldehyde dehydrogenase 1 family, member A1)

ALDH1A1 is an aldehyde dehydrogenase that catalyses the oxidation of DOPAL (a cytotoxic side-product of dopamine catabolism, highly reactive) into DOPAC, thereby protecting dopaminergic neurons from neurotoxicity.

Materials and methods

Processing of mouse brains

Perfusion

The mice were anaesthetised with Thiobarbital and perfused via a peristaltic pump through the ventricle. Initially (for 1-2 minutes), transcardial perfusion was carried out using buffered saline solution (PBS) for approximately two minutes, in order to ensure complete removal of the blood component. Once the outflow was clear, tissue fixation was carried out by infusion of 4% paraformaldehyde (PFA) for approximately 5 minutes.

At this point, the brain is dissected and placed in PFA solution for 1-2 hours.

Preparation of 4% paraformaldehyde (PFA):

Reagents:

- PFA, 20g
- PBS 10x, 50mL
- Distilled water
- HCl 37%

Procedure: the solution was prepared by dissolving 20 g of PFA in 250 ml of preheated distilled water. The beaker was placed on a magnetic stirrer and NaOH crystals were added to aid dissolution and clarify the solution. 50 ml of 10× PBS was added. The solution was left to cool and then brought to a final volume of 500 ml with distilled water, and the pH was titled to 7.4 using HCl.

Store in the fridge at 4°C.

Cryoprotection

After fixation with PFA, the brain is extremely rigid but still contains water. For this reason, to prevent the formation of ice crystals during freezing – which would destroy the morphology of the neurons – the brain must be subjected to a cryoprotection process using a 30% sucrose solution. The hypertonic solution will draw water out of the tissue, and the sucrose will enter the cells, lowering the freezing point and increasing the density of the brain until it sinks (overnight).

Preparation of the anti-freeze solution:

Reagents:

- PBS 10x, 100mL
- Sucrose, 300g
- Ethylene Glycol, 300mL
- PVP-40 (Polyvinylpyrrolidone 40kDa), 10g
- NaCl, 9g
- Distilled water until a final volume to 1L

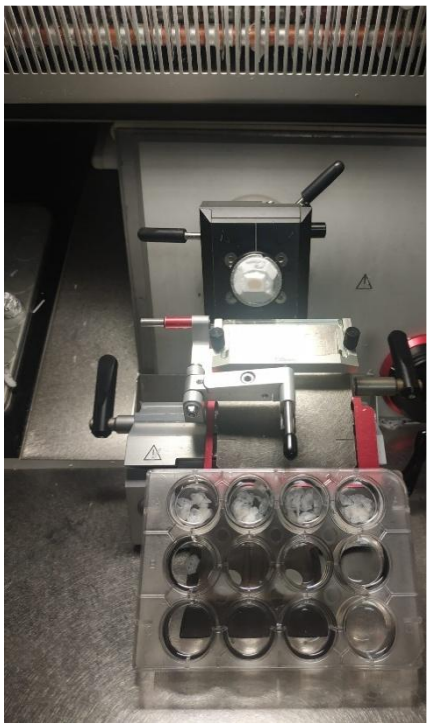
Store in the fridge at 4°C.

Inclusion in OCT

Once the brain has been cryoprotected, the excess solution is removed and the sample is embedded in OCT, taking particular care to ensure the correct orientation of the sample. OCT is a hydro-soluble polymer mixture that solidifies rapidly at low temperatures and provides structural support during cryostat sectioning.

The sample is rapidly cooled using dry ice and then transferred to -80°C to guarantee uniform solidification, permitting brain to freeze progressively.

Cryostat



The brain sections were cut using a cryostat: this is a microtome enclosed in a refrigerated chamber. During cutting, the temperature of the machine was maintained at -21°C (TM = -21°C) and the temperature of the sample was maintained at -19°C (TC = -19°C).

The cryostat allows the sample frozen in OCT to be cut into extremely thin sections.

Specifically, in the initial experiments the section thickness was 50 µm; subsequently, the protocol was optimised using 25 µm sections.

The support on which the sample is secured is fixed to a mechanical arm that moves vertically, moving forward by a preset thickness towards the fixed, sharp steel blade, against which the tissue slides. A glass slide is positioned on the blade to prevent the cut section from curling up. As the cut is made, the section slides between the blade and the glass slide and is collected using a small brush or a syringe needle and transferred to well plates containing PBS 1x.

Immunostaining

Immunostaining was performed on **free-floating sections** maintained under continuous orbital agitation during all incubation and washing steps to ensure optimal antibody penetration and uniform labeling.

Initial experiments were focused on the validation of single immunostaining protocols for TH, Calbindin, and ALDH1A1. This phase was essential to determine the optimal antibody concentrations and to assess the labeling efficiency of each marker independently.

To achieve high-quality double and triple labeling, an extensive optimization process was carried out. Several parameters were tested, including:

- Section thickness optimized during the initial stages of the study: we compared 50µm and 25µm thick sections; the latter were chosen for the final protocol as they provided superior antibody penetration and enhanced imaging resolution, while ensuring the physical integrity of the tissue during free-floating procedures;
- Host species: Evaluation of primary antibodies raised in mouse, rabbit, and donkey to avoid cross-reactivity;
- Antigen Retrieval: Comparison of different pretreatment methods, such as heat-induced epitope retrieval (HIER) using sodium citrate buffer an HCl 2N;
- Testing of various blocking buffers to minimize background noise and non-specific binding.

Subsequently, double staining (TH+/Calb+ and TH+/ALDH1A1+) was performed to verify the fluorescence overlap and to characterize the sub-populations of dopaminergic neurons.

Once the optimal conditions were established, a standardized triple staining protocol (TH+Calb+ALDH1A1) was finalized. This definitive procedure was applied to all experimental samples, including both Wild-Type (WT) and Knock-Out (KO) groups, to ensure consistency and reproducibility across the study.

Calbindin Immunostaining

Sample: 453, substantia nigra floating sections, 50µm.

- 6 washes in PBS 1x (2 minutes) with shaking to remove the Anti-freeze solution;
- M.O.M. ("mouse on mouse") pre-incubation: *the calbindin is a mouse IgG and when using a mouse primary antibody on mouse tissue , the risk is that the secondary antibody can't distinguish between the mouse primary antibody (Calbindin) and native immunoglobulins in mouse's tissues. M.O.M. technique can eliminate this background problems*.

  1,25mL of PBS.T 1x + 1 drop of M.O.M (incubation for 1 hour with shaking);
- Primary Antibody: **Calbindin**

  Four wells were prepared to evaluate calbindin immunostaining efficiency across a range of antibody dilutions:

  1. Negative control (Blocking Buffer only)
  2. Calb 1:10.000
  3. Calb 1: 2500
  4. Calb 1:500

  Incubation overnight at 4°C.

  N.B. The **Blocking Buffer solution(BB)** is used to prevent non-specific binding and reduce background noise.
  Preparation of BB (Vf: 10mL):
  1. 0,5mL of BSA
  2. 1mL of FBS
  3. 8,5mL of PBS.T 1x
- 3 washes in PBS.T 1x (5 minutes with shaking);
- Secondary Antibody: **Anti-mouse IgG Alexa flùor 568 1:1000**

  in each well: 1µL of Alexa flùor 568 in 1mL of BB

  Incubation for 1 hour.
- 3 washes in PBS.T 1x (5 minutes with shaking);
- 1 wash in PBS 1x + **DAPI** (1:5000);

- 1 wash in PBS 1x;
- Mounting of the sections on slides with fluorogel.

ALDH1A1 Immunostaining

Sample: 453, substantia nigra floating sections, 50µm.

- 6 washes in PBS 1x (2 minutes) with shaking to remove the Anti-freeze solution;
- M.O.M. pre-incubation

  1,25mL of PBS.T 1x + 1 drop of M.O.M (incubation for 1 hour with shaking);
- Primary Antibody: **ALDH1A1**

  Four wells were prepared to evaluate calbindin immunostaining efficiency across a range of antibody dilutions:

  1. Negative control (Blocking Buffer only)
  2. ALDH1A1 1:5000
  3. ALDH1A1 1:2500
  4. ALDH1A1 1:250

  Incubation overnight at 4°C.

- 3 washes in PBS.T 1x (5 minutes with shaking);
- Secondary Antibody: **Anti-rabbit IgG Alexa flùor 568 1:1000**

  in each well: 1µL of Alexa flùor 568 in 1mL of BB

  Incubation for 1 hour.
- 3 washes in PBS.T 1x (5 minutes with shaking);
- 1 wash in PBS 1x + DAPI (1:5000);
- 1 wash in PBS 1x;
- Mounting of the sections on slides with fluorogel.

αTH Immunostaining

Sample: 455, substantia nigra floating sections, 50µm.

- 6 washes in PBS 1x (2 minutes) with shaking to remove the Anti-freeze solution;
- M.O.M. pre-incubation

  1,25mL of PBS.T 1x + 1 drop of M.O.M (incubation for 1 hour with shaking);
- Primary Antibody: **TH 1:2500**

  0,4µL of Ab in 1mL of BB

  Incubation overnight at 4°C.
- 3 washes in PBS.T 1x (5 minutes with shaking);
- Secondary Antibody: **Anti-rabbit IgG Alexa flùor 488 1:1000**

  in each well: 1µL of Alexa flùor 488 in 1mL of BB

  Incubation for 1 hour.
- 3 washes in PBS.T 1x (5 minutes with shaking);
- 1 wash in PBS 1x + DAPI (1:5000);
- 1 wash in PBS 1x;
- Mounting of the sections on slides with fluorogel.

Antigen Retrieval

Sodium Citrate Buffer

(10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

Reagents:

- Tri-sodium citrate (dihydrate), 2.94 g
- Distilled water, 1000mL
- HCl 1N
- 0.05% Tween 20, 0,5mL

Procedure:

Mix tri-sodium citrate and distilled water to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0,5mL of Tween 20 and mix well.

Store this solution at room temperature for 3 months or at 4°C for longer storage.

Antigen Retrieval with Sodium Citrate Buffer:

- Pre-heat Sodium Citrate Buffer with microwave until temperature reaches 95-100°C (waiting for bubbles);
- pour on a 6-well plate and move the floating sections to the well;
- leave 5 min and repeat twice;
- Last incubation 15-20min until temperature drops to RT
- Repeat twice and after the third incubation allow the slides to cool down for 20-30min;
- PBS washes 3 x 5 min at RT;
- Blocking overnight at 4ºC with Blocking buffer (PBS, Triton X100 0.3 %, Normal Donkey Serum 5%).

Following antigen retrieval, non-specific binding was blocked by incubating the sections overnight at 4°C in a blocking solution containing 5% Normal Donkey Serum (NDS) and

0.3% Triton X-100 in PBS. The use of Triton X-100 ensured adequate tissue permeabilization to prepare the sample for successive incubation with the antibodies, while NDS was employed to minimize background noise.

HCl 2N

HCl unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies.

In fact, PFA forms cross-links between proteins, which masks the antigenic sites in the tissues giving weak or false negative staining for immunohistochemical detection of certain proteins.

Procedure: incubation of the samples in 2N HCl for approximately 10–20 minutes, followed by washing with PBS 1x.

Calbindin + αTH Immunostaining

Samples: C57J4,C57J5,C57J6, substantia nigra floating sections, 25µm.

- 6 washes in PBS 1x (5 minutes) with shaking to remove the Anti-freeze solution;
- M.O.M. pre-incubation

  1,25mL of PBS.T 1x + 1 drop of M.O.M (incubation for 1 hour with shaking);
- Primary Antibodies:
  - Calbindin: **Mouse monoclonal anti-Calbindin 1:5000**
  - TH: **Rabbit Polyclonal anti-TH 1:2500**

  0,6µL of Cb + 1,2 µL of TH in 3mL of BB (1mL for each sample)

  Incubation overnight at 4°C.
- 3 washes in PBS.T 1x (10 minutes with shaking);
- Secondary Antibodies:
  - **Goat Anti-Mouse IgG Alexa flùor 568 1:1000**
  - **Goat Anti-Rabbit IgG Alexa flùor 488 1:1000**

  in each well: 1µL of Alexa flùor 568+1µL of Alexa flùor 488 in 1mL of BB

  Incubation for 2 hours RT.
- 3 washes in PBS.T 1x (10 minutes with shaking);
- 1 wash in PBS 1x + DAPI (1:5000) 10 minutes with shaking ;
- 1 wash in PBS 1x 10 minutes with shaking;
- Mounting of the sections on slides with fluorogel.

ALDH1A1+αTH Immunostaining

Samples: C57J4,C57J5,C57J6, substantia nigra floating sections, 25µm.

- 6 washes in PBS 1x (2 minutes) with shaking to remove the Anti-freeze solution;
- Antigen Retrieval with Sodium Citrate pH=6
  Boil Na + Citrate solution with a microwave, pour on a 6-well plate and move the floating sections to the well. Leave 5 min and repeat twice.
  Last incubation 15-20min until temperature drops to RT.
- 3 washes in PBS 1x (5 minutes)
- Blocking overnight at 4°C with Blockin Buffer (PBS 1x, Triton X100 0,3%,Normal Donkey Serum 5%)
- Primary Antibodies:
  - ALDH1A1: **Goat Polyclonal anti-ALDH1A1 1:2500**
  - TH: **Rabbit Polyclonal anti-TH 1:2500**

  1,2µL of ALDH1A1 + 1,2 µL of TH in 3mL of BB (1mL for each sample)

  Incubation overnight at 4°C.

- 4 washes in PBS 0,05% Tween-20 (5 minutes with shaking);
- Secondary Antibodies:
  - **Donkey Anti-Goat IgG Alexa flùor 488 1:1000**
  - **Donkey Anti-Rabbit IgG Alexa flùor 568 1:1000**

  in each well: 1µL of Alexa flùor 488+1µL of Alexa flùor 568 in 1mL of BB
  Incubation for 2 hours RT.
- 3 washes in PBS 0,05% Tween-20 (5 minutes with shaking);
- 1 wash in PBS 1x + DAPI (1:5000) 5 minutes with shaking ;
- 1 wash in PBS 1x 10 minutes with shaking;
- Mounting of the sections on slides with fluorogel.

Triple staining: αTH + Calbindin + ALDH1A1

List of samples of substantia nigra floating sections kept at -20ºC in antifreeze solution after the cut with the cryostat obtaining 25µm sections.

Table 1 List of samples processed for triple immunostaining (TH/Calb/ALDH1A1), including genotype, age at sacrifice

	Sample	Age at sacrifice
WT	Ndufs2/NDI1-TH-IRES-CRE-D **446**	59
WT	Ndufs2/NDI1-TH-IRES-CRE-D **512**	59
WT	Ndufs2/NDI1-TH-IRES-CRE-D **516**	59
KO	Ndufs2/TH-IRES-CRE-D **538**	57
KO	Ndufs2/TH-IRES-CRE-D **549**	57
KO	Ndufs2/TH-IRES-CRE-D **562**	58

List of reagents:

- VECTOR® M.O.M.™Immunodetection Kit
- Mouse monoclonal anti-Calbindin D-28k (Swant®)
- Goat polyclonal anti-ALDH1A1 (R&D Systems)
- Rabbit polyclonal anti-TH (Novus)
- Donkey anti-mouse Alexa flùor 488 (Invitrogen)
- Donkey anti-goat Alexa flùor 633 (Invitrogen)
- Donkey anti-rabbit Alexa flùor 568 (Invitrogen)

Triple staining protocol:

- 6 washes in PBS 1x (5 minutes) with shaking to remove the Anti-freeze solution;
- M.O.M. pre-incubation

  1,25mL of PBS.T 1x + 1 drop of M.O.M (incubation for 1 hour with shaking);
- Primary Antibodies:
  - Calbindin: **Mouse monoclonal anti-Calbindin 1:1000**
  - TH: **Rabbit Polyclonal anti-TH 1:2500**
  - ALDH1A1: **Goat Polyclonal anti-ALDH1A1 1:2500**
- In each well: 1µL of Cb + 0,4 µL of ALDH1A1 + 0,4 µL of TH in 1mL of BB (PBS 1x, Triton X100 0,3%,Normal Donkey Serum 5%)

  Incubation overnight at 4°C.
- 3 washes in PBS 0,05% Tween-20 (5 minutes with shaking);
- Secondary Antibodies:
  - **Donkey anti-mouse Alexa flùor 488 1:1000**
  - **Donkey anti-rabbit Alexa flùor 568 1:1000**
  - **Donkey anti-goat Alexa flùor 633 1:1000**

  in each well: 1µL of Alexa flùor 488+1µL of Alexa flùor 568+1µL of Alexa flùor 633 in 1mL of BB

  Incubation for 2 hours RT.
- 3 washes in PBS 0,05% Tween-20 (5 minutes with shaking);
- 1 wash in PBS 1x + DAPI (1:5000) 10 minutes with shaking ;
- 1 wash in PBS 1x 10 minutes;

Mounting of the sections on slides with fluorogel.

Results

Initial results were obtained through **double immunofluorescence staining** to assess the protein expression patterns of Calbindin and ALDH1A1 in dopaminergic (TH+) neurons. Fluorescence microscopy analysis revealed distinct subpopulations of neurons: those expressing both markers (TH+CB or TH+ALDH1A1**)** and those characterized by mutually exclusive expression.

Expression of TH and Calbindin in the midbrain

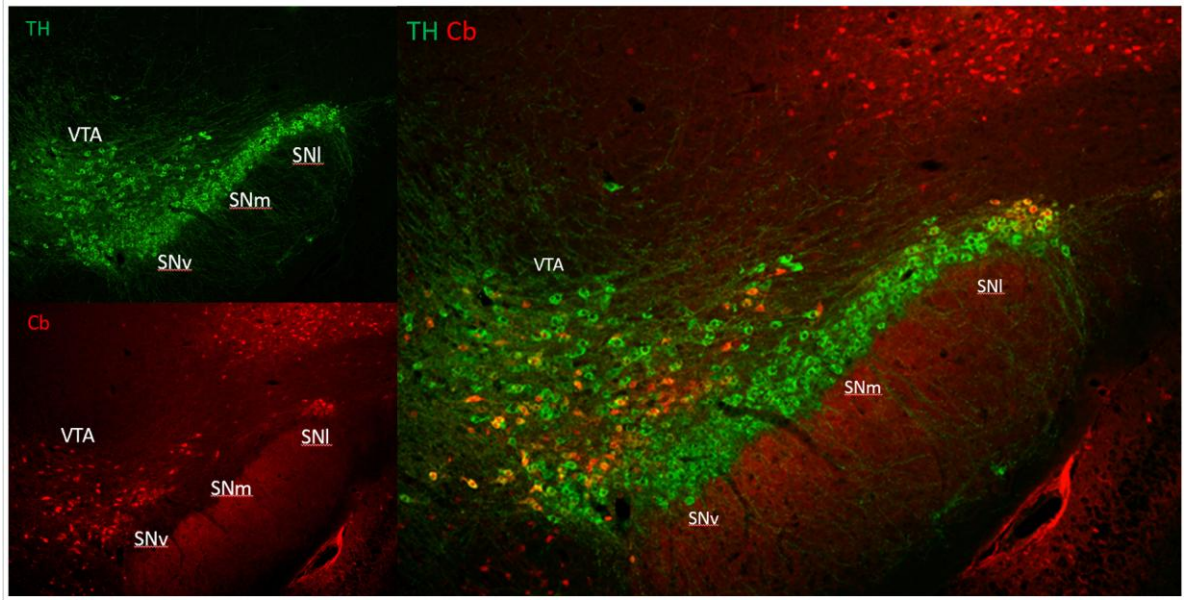


Figure 9 Expression of TH (green fluorescence) and calbindin (red fluorescence) in midbrain (on the left). Merge TH+Cb (on the right).

SNl: lateral substantia nigra; SNm: medial substantia nigra; SNv: ventral substantia nigra; VTA: ventral tegmental area

As can be seen in these pictures, it's possible to discriminate cells with only TH binding (green fluorescence), cells with only calbindin binding (red fuorescence), and cells with co-localisation.

It is possible to conclude that both the antibodies used show specificity towards the substantia nigra and VTA.

Expression of TH and ALDH1A1 in the midbrain

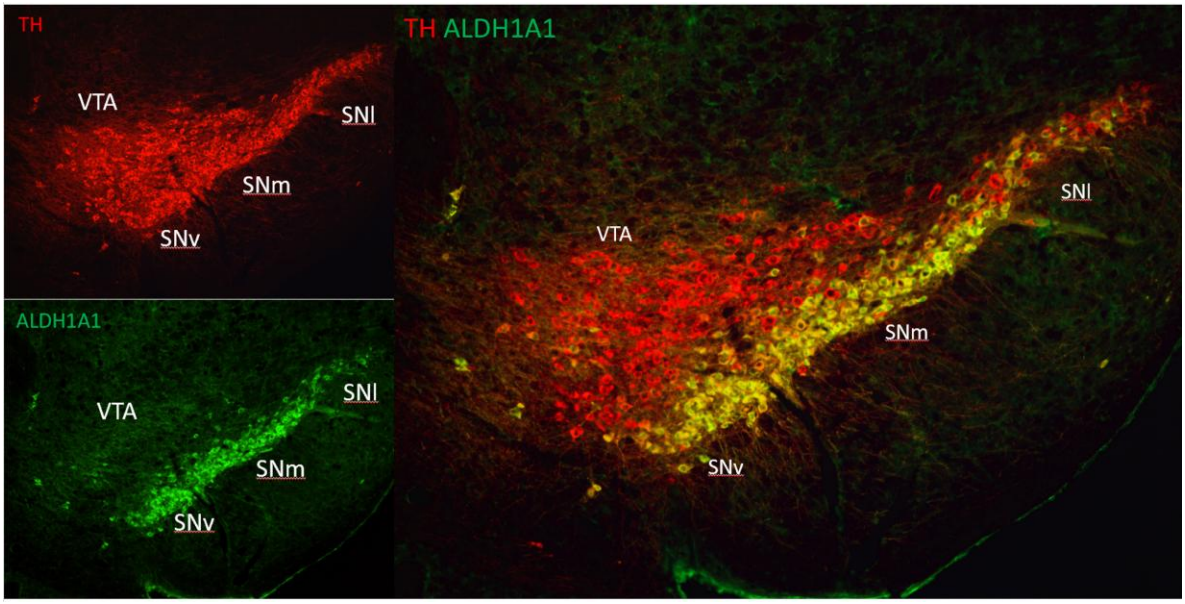


Figure 10 Expression of TH (red fluorescence) and ALDH1A1 (green fluorescence) in midbrain (on the left). Merge TH+Cb (on the right).

SNl: lateral substantia nigra; SNm: medial substantia nigra; SNv: ventral substantia nigra; VTA: ventral tegmental area

Similarly, this second double-labeling experiment highlights the possibility of discriminating between distinct dopaminergic subpopulations. Specifically, we identified neurons labeled exclusively by TH (marker of choice for identifying dopamine-producing neurons) characterized by red fluorescence, those positive only for ALDH1A1 (green fluorescence), and a subset of neurons exhibiting double-staining (co-localization) with and overlapping of the fluorescence.

Characterizing these distinct subpopulations is crucial for the comparative analysis, as it allows to evaluate whether the loss of a specific marker in **KO samples** selectively affects specific neuronal subtypes, such as the **double-positive (TH+/ALDH1A1+)** or **double-positive (TH+/Cb)** neurons, compared to **WT controls**.

Furthermore, the preliminary double-staining assays served as a fundamental tool for **anatomical mapping**. By analyzing the characteristic morphology of the **Substantia Nigra (SN)** across different sections, we were able to precisely **orient the cryostat-cut samples** along the rostro-caudal axis, accurately identifying the anatomical boundaries (start and end) of the region of interest.

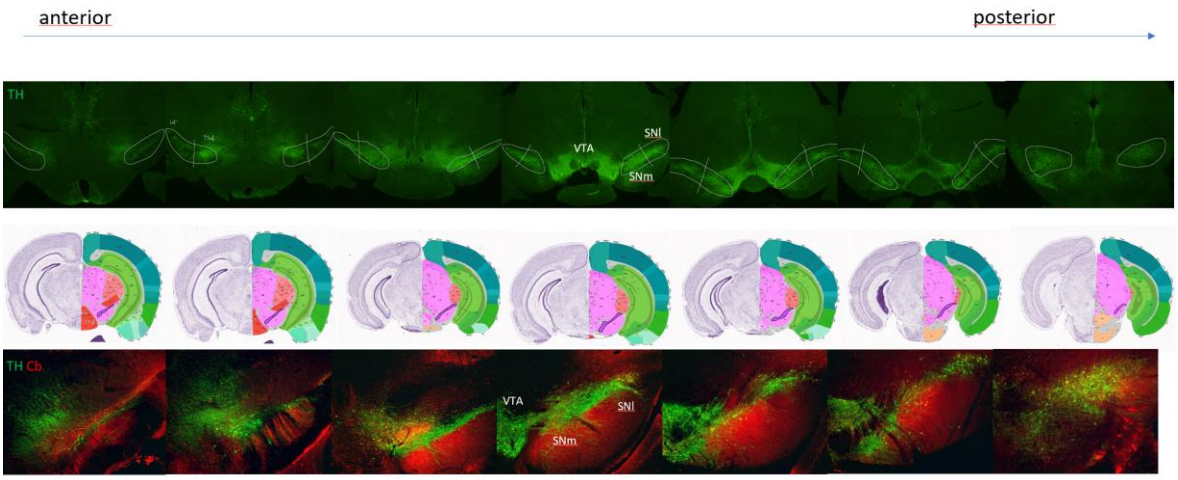


Figure 11 Anatomical progression of substantia nigra

In Figure 13, is characterized the anatomical progression of the Substantia Nigra (SN) along the **antero-posterior axis**. In the upper row, the overall distribution of dopaminergic neurons is visualized via **TH immunofluorescence (green)**. These experimental observations were cross-referenced with the **Paxinos Mouse Brain Atlas** (middle row) to precisely define the anatomical boundaries of the SN at each level. Finally, high-magnification images (bottom row) focus on the right hemisphere, demonstrating the co-localization of **TH and Calbindin (CB)** and revealing how these subpopulations are organized within the distinct tiers of the SN.

Building upon the optimized protocols and anatomical mapping previously described, we performed a triple immunofluorescence staining to simultaneously detect Tyrosine Hydroxylase (TH), Calbindin (CB), and ALDH1A1 across all experimental groups of sections. This approach allowed for a comprehensive visualization of the neurochemical diversity within the dopaminergic population: the differential expression of these markers within TH+ neurons is correlated with a higher level of vulnerability or resilience to neurodegeneration in KO samples. To translate these qualitative observations into quantitative data, high-resolution images were processed using Adobe Photoshop. A cell counting procedure was employed to determine the number of each neuronal subpopulation and the related distribution. This quantification is fundamental to identifying significant differences between WT and KO samples, providing the statistical basis for our final conclusions regarding.

Triple-stained sections were imaged using a Leica THUNDER Imager. This system utilizes Computational Clearing technology to eliminate out-of-focus blur (background) in real-time, providing high-resolution images comparable to confocal microscopy but with the speed of widefield acquisition. This advanced imaging approach was essential for the accurate spatial discrimination of TH, Calbindin, and ALDH1A1 signals within the 25µm thick sections, ensuring reliable cell counting and co-localization analysis.

Thanks to the THUNDER algorithm, has been removed the so-called 'haze' (background blur), allowing to clearly distinguish individual dopaminergic cell bodies even in densely populated tissue areas, thereby facilitating manual cell counting in Photoshop.

Expression of TH, Calbindin and ALDH1A1 in WT/KO midbrain

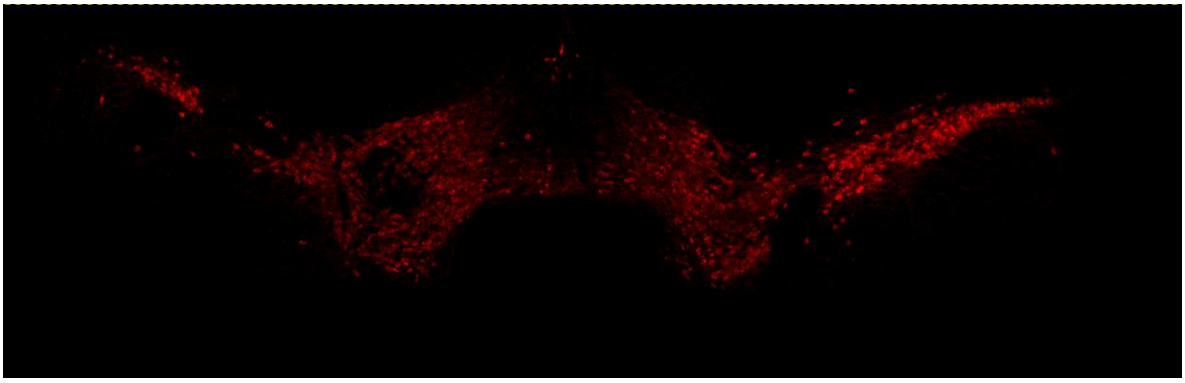


Figure 12 TH immunostaining in WT sample

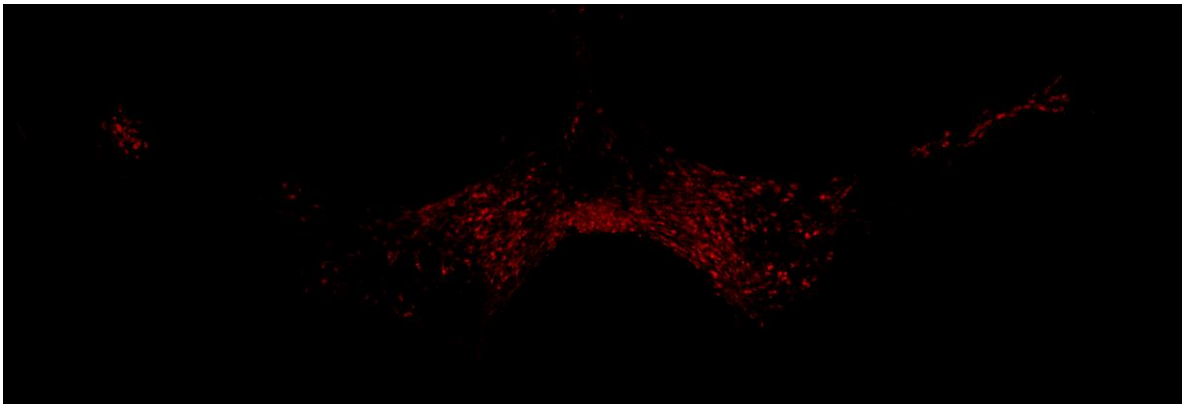
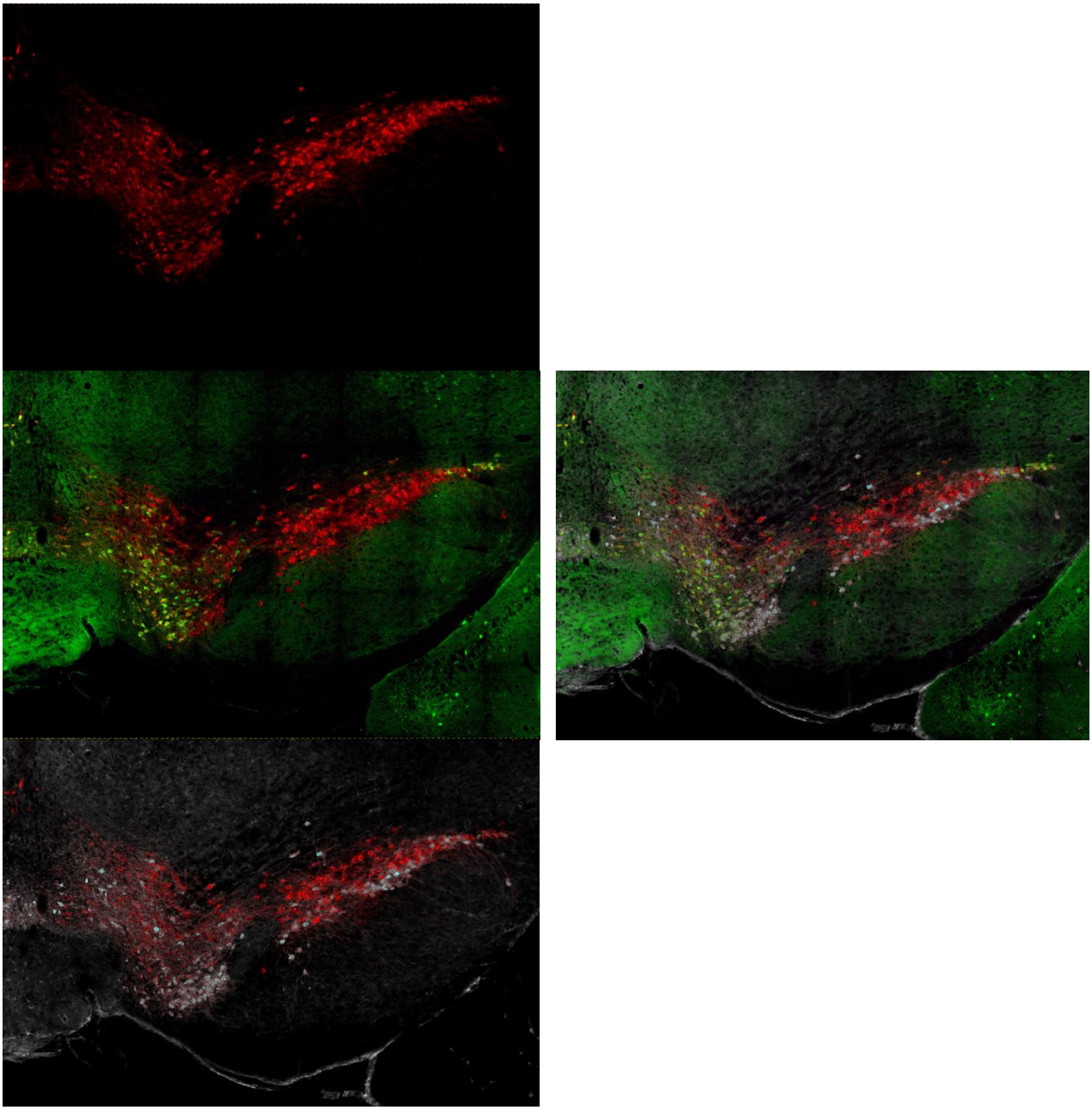
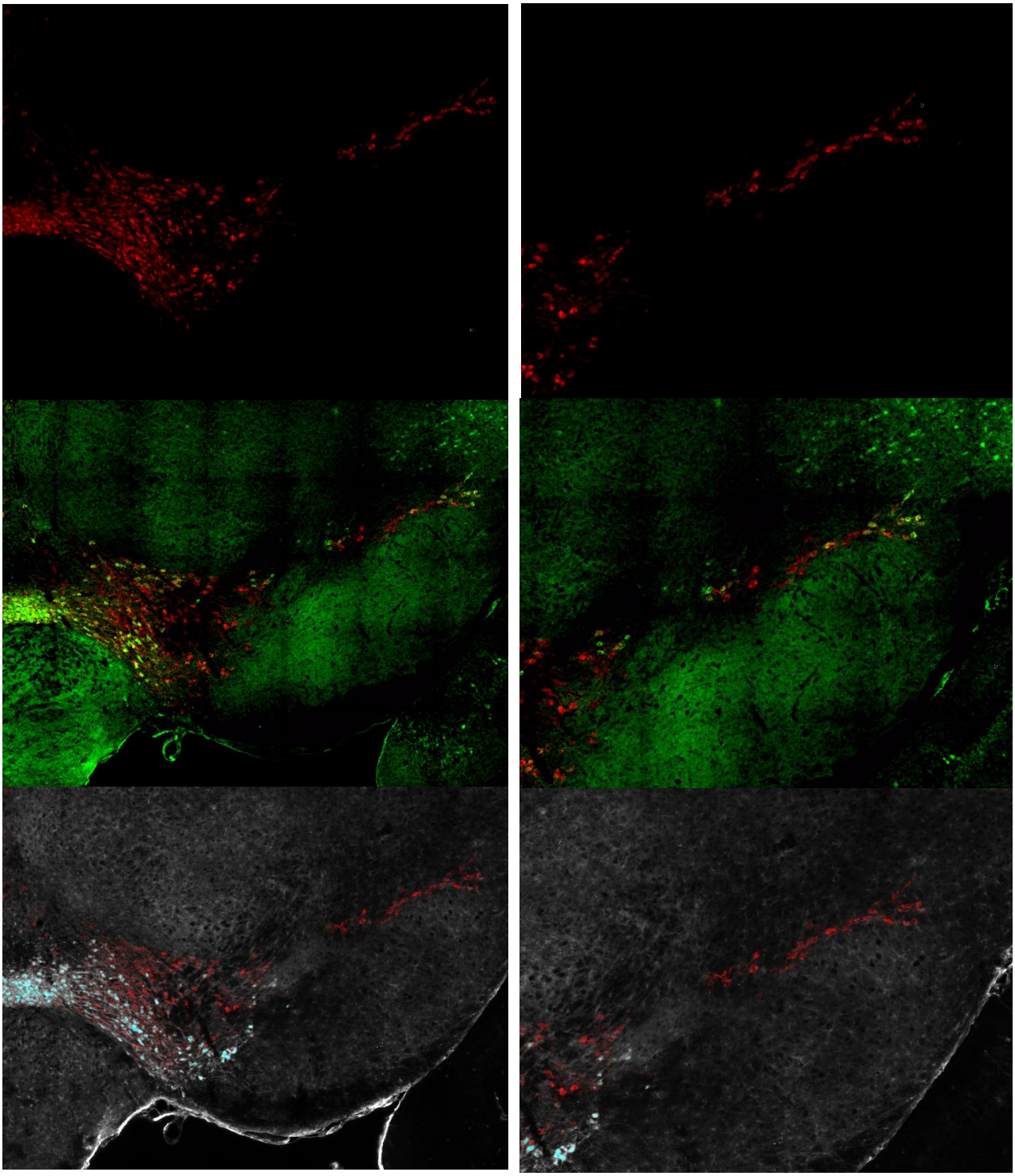


Figure 13 TH Immunostaining in KO sample

The comparison between figure 12 and figure 13 reveals a striking difference in **TH staining** (red fluorescence) between **WT and KO samples**. Given that Tyrosine Hydroxylase (TH) is the definitive marker for **dopaminergic neurons**, this marked reduction directly reflects the significant **loss of dopamine** and the extensive neurodegeneration occurring in the ***Ndufs2*-deficient** model.



The images illustrate the experimental markers in a representative **Wild-Type (WT) brain section**. From left to right: the top panel displays the TH immunostaining, the middle panel shows the merged signal for TH and Calbindin, and the bottom panel presents the merge for TH and ALDH1A1. Finally, the panel on the right provides a triple-merge, highlighting the physiological co-expression of all three markers within the dopaminergic population.



The images illustrate immunofluorescence analysis of dopaminergic subpopulations in ***Ndufs2*-KO mice**, highlighting the neurodegenerative phenotype.

The panels are organised into two columns. From top to bottom, the staining pattern is consistent for both columns: the upper panels show TH immunostaining (red); the middle panels display the merge of TH and Calbindin (red and green); and the bottom panels present the merge of TH and ALDH1A1 (red and white).

The right column presents high-magnification images of the same sections, offering a clearer view of the differential neuronal survival in KO mice. These detailed panels highlight how the majority of the remaining dopaminergic neurons belong to the Calbindin-expressing subpopulation (middle right). In stark contrast, the bottom-right image clearly demonstrates the near-total absence of ALDH1A1-expressing neurons, visually confirming the extreme vulnerability of this specific subset to *Ndufs2* deletion.

Quantification

Quantification was performed through manual counting of dopaminergic neurons, leveraging the high-resolution images acquired via the Leica THUNDER system.

The workflow involved the management of distinct layers corresponding to the individual fluorescence signals for each specific antibody (TH, Calbindin, and ALDH1A1). By selectively overlaying these layers, it was possible to precisely isolate and identify the various neuronal subpopulations. This approach enabled the discrimination between neurons expressing a single specific antibody and those characterized by the co-expression of two or three markers, ensuring that each cell was correctly classified within the analyzed WT and KO groups.

Table 2 Quantification of dopaminergic subpopulations in WT and KO SNpc

Genotype	Total TH+	TH+ CB+	TH+ ALDH1A1+	TH+ CB+ ALDH1A1+
WT	3361	706	2924	205
KO	1626	668	688	90
Loss (%)	-51,6%	-5,4%	-76,5%	-56,1%

The quantitative analysis is performed on three WT and three KO samples, as detailed in Table 1. The analysis was primarily focused on the SNpc (Substantia Nigra pars compacta), as qualitative evaluation of triple-stained sections did not reveal any substantial loss of dopaminergic neurons within the VTA in the KO group.

A first examination of the two experimental groups (WT and KO) separately revealed a high degree of intra-group homogeneity regarding the expression of the different markers. In the WT group the proportion of neurons expressing TH+/ALDH1A1+ amounted to approximately 40,1% of the total dopaminergic population. In parallel, the TH+/CB+ subpopulation represented approximately 10% of the total TH+ neurons. Finally, a small amount of triple positive neurons (TH+/CB+/ALDH1A1+) accounts for approximately 3% of total TH+ population.

The low variability observed across these three samples confirms that the identity and spatial distribution of dopaminergic neurons are conserved among individual animals of the same genotype. This uniformity is statistically supported by the low Standard Error of the Mean (SEM) recorded for each parameter, confirming minimal individual variation among the samples of the same experimental cohort.

Similarly, this homogeneity is maintained in the KO group, despite the massive reorganization of the cellular landscape.

The comparison between WT and KO groups of samples reveals a massive loss of total dopaminergic (TH+) neurons within the SNpc of KO mice, showing a 51% reduction compared to WT controls (from 3361 to 1626 cells). This finding confirms that the deletion of the *Ndufs2* gene, a fundamental subunit of mitochondrial Complex I, exerts a critical impact on neuronal survival, validating this system as a high-fidelity model of Parkinson's Disease.

However, neurodegeneration does not affect the entire dopaminergic population uniformly, highlighting a marked selective vulnerability among different subpopulations.

The most significant result concerns the TH+/ALDH1A1+ neurons, which undergo a dramatic 76% collapse (from 2924 to 688 cells). This suggests that ALDH1A1 expression identifies a neuronal subgroup extremely susceptible to the bioenergetic deficit and oxidative stress induced by Complex I dysfunction.

In stark contrast, TH+/CB+ neurons exhibit a striking relative resilience, with a minimal loss of approximately 5% (from 706 to 668 cells). Such stability indicates that Calbindin may act as a protective factor or identify a population with distinct metabolic requirements, rendering it intrinsically resistant to *Ndufs2* deficiency.

Finally, the impact on 'triple-positive' (TH+/CB+/ALDH1A1+) neurons, which show a 56% reduction (from 205 to 90 cells), suggests that the fragility associated with the ALDH1A1+ phenotype or the severity of the mitochondrial insult outweighs the potential protective effect of Calbindin in this specific subpopulation.

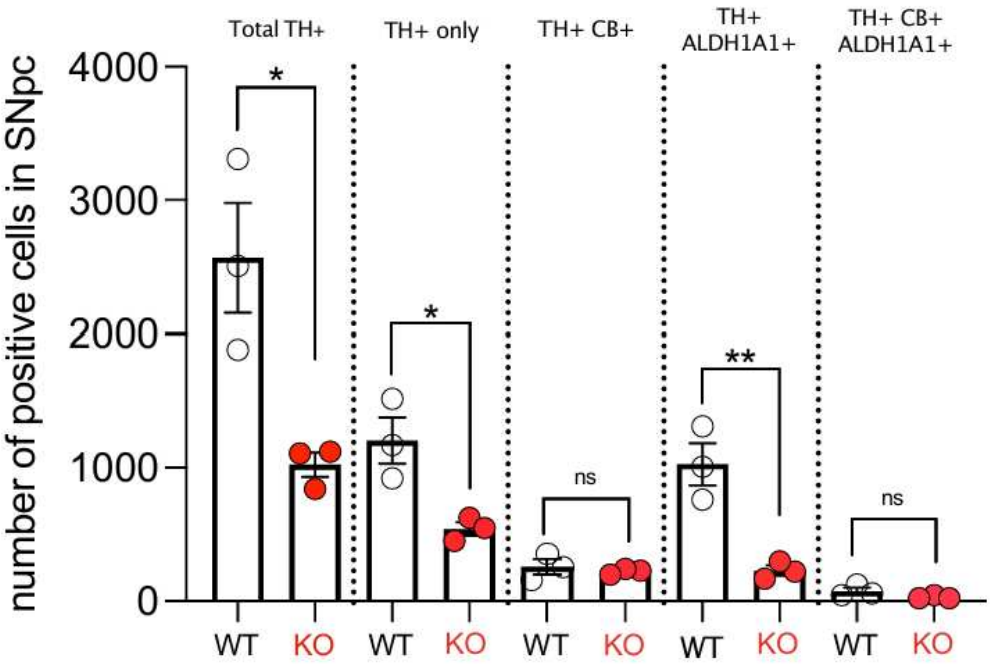


Figure 14 Quantitative analysis of dopaminergic subpopulations in the SNpc of WT and KO mice.

The scatter bar graph illustrates the number of positive cells for each neuronal marker combination across the two experimental groups (WT and KO).

Each point represents an individual animal (3WT in white and 3KO in red), with the bar height indicating the mean value and the error bars representing the Standard Error of the Mean (SEM).

Statistical comparisons between WT and KO groups are performed using an unpaired t-test (using GraphPad Prism software). A significant reduction is observed in Total TH+ cells (*$p < 0.05$) and in the TH+ only subpopulation ($p < 0.05$). Notably, the TH+/ALDH1A1+ subset shows the most dramatic decline (**$p < 0.01$), while no significant difference (ns) was found in the TH+/CB+ and TH+/CB+/ALDH1A1+ populations.

These results demonstrate a selective vulnerability of ALDH1A1-expressing dopaminergic neurons in the KO model. Interestingly, the TH+/CB+ subpopulation showed no significant (ns) reduction in the KO group: this lack of statistical significance highlights the selective resilience of calbindin-positive neurons to neurodegeneration affecting dopaminergic neurons in Parkinson's disease.

Conclusions and future perspectives

In conclusion, this study provides direct evidence of the selective vulnerability of dopaminergic neurons in the Ndufs2-KO mouse model. The deletion of this gene, by mimicking the mitochondrial Complex I deficiency typical of Parkinson's Disease, induced a significant neuronal loss (51%), which nonetheless almost entirely spared Calbindin-expressing cells. The most relevant finding is the collapse of the ALDH1A1+ subpopulation, identifying this marker as the primary target of mitochondrial dysfunction.

Future perspectives of this work may aim to investigate the molecular mechanisms underlying the resilience of CB+ neurons.

Understanding why these cells survive despite mitochondrial impairment could pave the way for new neuroprotective strategies. Furthermore, it would be of great interest to evaluate whether pharmacological interventions aimed at enhancing mitochondrial activity or reducing oxidative stress can specifically prevent the loss of ALDH1A1+ neurons, translating these findings toward potential therapeutic approaches for Parkinson's patients.

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