**Background**

Parkinson’s disease (PD) is characterized by the loss of dopaminergic (DA-ergic) neurons in the substantia nigra pars compacta (SNpc). Among the most recent treatment strategies, in vivo astrocyte reprogramming is a promising therapy which is actively investigated. Da neurons could be generated through astrocyte reprogramming using transcription factors (TFs) or microRNAs (miRNAs). One-way miRNAs contribute to reprogramming is by targeting polypyrimidine tract binding (PTB) protein. PTB down-regulation induces neuronal PTB (nPTB) to express more neuronal gene (PTB/nPTB loop). Knowing that 70% of these neurons are gone by the time a patient is diagnosed, these therapies have to be thoroughly validated which require rigorous experiments to confirm that neurons derived though astrocytes reprogramming are functional DA neurons [1].

**The experiment**

Researchers [2] transduced mouse cortical astrocytes with a lentivirus expressing a small artificial segment of RNA that targets Ptbp1 (shPTB), the gene that encodes PTB. Next, they injected an adeno-associated virus (AAV) expressing shPTB in the subtantia nigra of a GFAP-Cre transgenic mouse in which DA neurons were ablated by 6-hydroxydopamine (6-OHDA), a dopamine analog toxic to DA neurons which results in the loss of substantia nigra neurons and depletion of striatal dopamine. 10~12 weeks after injection, they observed an increase in neurons and dopamine levels in the striatum were restored to 65% of control levels.

**Biological question**: design a battery of tests to evaluate the recovery of motor and non-motor functions of the mice impaired by the loss of DA neurons and which received AAV-shPTB therapy.

PD is characterized in mice by motor symptoms: balance, coordination, mobility or limb problems and it manifests as well by non-motor symptoms (NMS) such as emotional, cognitive deficits and aberrant social behaviors [3]. We will create three groups of mice 1) one group injected with AAV-shPTB 2) a group injected only with AAV-empty and 3) a group of wild-type (WT) mice and each group will have mice of different ages (3-,6- and 12-month-old). We will use the GFAP-Cre mouse expressing Cre recombinase from astrocyte GFAP promoter with a red fluorescent protein (RFP)-coding unit in the shPTB hairpin. After surgeries the mice will recover for 12 weeks and the three groups will be exposed to a sequence of tests alternating motor and non-motor assessments without exceeding two tests within a day to avoid exhaustion of the mice. We will subject each group of mice to the same two tests in separate environments. The two types of tests will be repeated for a week before moving the following week to the next two tests. After completion of all the tests, the animals will be deeply anesthetized and their brain removed surgically for immunohistochemistry (IHC) analysis. We will use IHC assay with primary antibodies to detect DA-ergic neurons, counting the total number of TH+ cells within the SNpc and compare this number between the injected and WT mice.

**Behavioral Assessment**

As a general rule, we will minimize light exposure and carefully clean the testing areas to remove any human or animal scents after each test. We will habituate the mice to be handled by the investigators, and will manipulate them gently.

We will seek to reduce variance between test results by stratifying our mice in groups of the same size, age, sex, genetic characteristics and we will reduce the noise in order to avoid to influence the mice behavior. We will then assess motor and non-motor recoveries among the three groups of mice. It is important to expose the mice to a battery of tests since it has been observed that mice can display impairments on a specific test but no other tests.

* **Y-Maze**: tests by computing *spontaneous alternations*, e.g., a mouse entering a different arm of the maze in each of 3 consecutive arm entries, the spatial working memory of the rodent. After calibrating the data/video acquisition system, we will place gently the mouse in the center of maze, step away and start the recording of the experiment. Then we will proceed to data analysis.
* **Novel Object Recognition [4]**: we will stick firmly two identical objects in a test area, at equal distance to the edges of the area, leaving enough room for the mice to move freely. 2-3 h after the sample phase, we will assess exploratory behavior directed toward a familiar and novel object and compute the *object bias score* which is the ratio between time of exploring an object over the total exploring both objects. Similarly, we will compute the *novelty preference* and *discrimination indices*. We will change the interval between the sample and test phases from minutes, to test short term memory (STM), to hours (Intermediate Term Memory) or days (Long Term Memory).
* **Corticospinal Function Evaluation**: we will remove the mouse from its cage and suspend it by the tail for 5-10s, and give a score which reflects whether the mouse exhibits limb clasping. We could supplement this test in observing how the mouse hang/grip to a wire.
* **Challenging Beam Traversal Procedure [5]**: this test will evaluate fine motor coordination and sensorimotor skills.
* **Cylinder Test** **[6]**: after placing the mouse in a cylinder, we will count paws lifts against the cylinder wall. This test indicates the level of the forelimb motor function and motor performance.
* **Open Field Test [6]:** we will measure movement path, speed of movement, total distance travelled. This test assays general locomotion activity.
* **Rotorod [3]:** An accelerating rotating roller tests motor coordination, ataxia, attention and stress.
* **Kinematic Gait Analysis [7]:** Bradykinesia is a key symptom of PD, and manifests in gait impairment. While mice are running on a treadmill with adjustable angle and speed, posture and gait dynamic data are collected to assess balance and coordination.

**Emotional and Cognitive Assessment**

In PD patients, non-motor symptoms (NMS) include: cognitive impairment, sleep disorders, gastrointestinal and sensory dysfunction and depression. To some extent, rodents can express similar symptoms. We need to use them to evaluate such symptoms.

* **Elevated Plus Maze [3]**: the maze comprises two open arms and two closed arms, animals are recorded exploring these arms, and percentage of time spent in the open arm is an index of anxiety-like behavior.
* **Forced Swim Test [3]**: mice are placed in a cylinder filled water. Depressive-like state is defined as an increase in time of immobility and decrease in latency to immobility.
* **Sucrose Preference Test [3]**: We will deprive the mice of food and water before exposing them to a bottle containing water and the other one filled with a sucrose solution. Reduction of sucrose preference is indicative of anhedonia thus depression-like behavior.
* **Morris Water Maze [3]**: this test will evaluate the ability to learn the position of a platform and test spatial reference memory and assess cognitive flexibility.

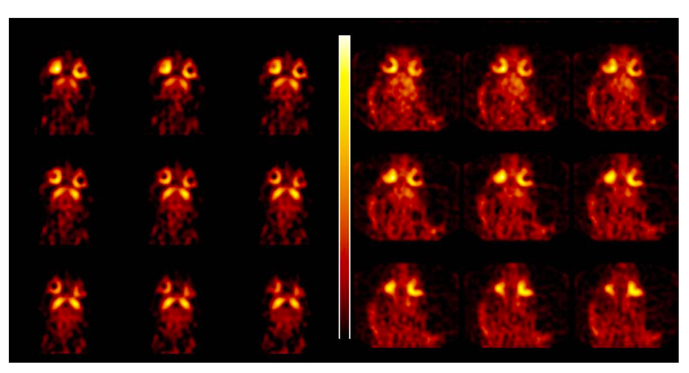
To perform the next assessments, mice will be deeply anesthetized and brain slices will be prepared.

**Electrophysiological, Transcriptomic and Morphologic Single Neuron Assessment**

We will follow the Patch-seq technique (Path-clamp recording technique) and compare electrophysiological, gene expression and molecular properties of a DA reprogrammed neuron and an endogenous DA neuron [8].

**DAT-SPECT Assessment**

Dopamine Transporter (DAT) SPECT is highly accurate to detect the loss of striatal binding and nigrostriatal cells in early PD cases [9]. We will use micro-SPECT with binding of SPECT tracers to DA D2/3 receptors to evaluate striatal DA binding. We will also check integrity of dopaminergic nerve terminals and the number of dopaminergic cells in the substantia nigra and investigate if there is any correlation between number of TH+ cells in the SN and the striatal DA concentration [10][11] for the different groups of mice.



Example of DAT-SPECT showing loss of striatal binding

on the MPT-lesioned mouse on the right vs. control mouse

on the right.

In conclusion, multiple factors need to be considered in evaluating the effects of astrocyte reprogramming on the CNS functions and the tests described here decrease uncertainty when contemplating applying this strategy to patients with PD. More rigorous tests are needed to evaluate long-term impact of reprogrammed neurons on the brain and overall patient condition.

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