Rationale for model development

Parkinson’s disease (PD) is a clinical condition characterized by progressive and extensive loss or degeneration of dopaminergic neurons in substantia nigra pars compacta. This will lead in final stage of natural evolution to massive depletion of dopamine in striatum (caudate and putamen) and loss of connection between those two dopaminergic structures. After Alzheimer disease, Parkinson’s disease is considered the second most common neurodegenerative disorder that affects about 1-3% of the elderly population.

Etiology of Parkinson disease is complex and remains, unfortunately, insufficiently elucidated. The pathophysiology of PD is most likely a consequence of an unknown relationship and interrelation between genetic factors (PARK 1-11 genes) and environmental factors (heavy metal, pesticides and fungicides) which induce a cascade of molecular events in neuronal and dopaminergic pathways. On the other hand, these alterations will produce protein aggregation, which results in formation of cytoplasmic inclusions in dopaminergic neurons (Lewy bodies), a pathological marker of Parkinson’s disease. The L-Dopa still remains the standard therapy of treatment of PD. If that approach has good results in first years
of therapy, studies and observations have concluded that after 5-7 year this therapy loses its effectiveness.

We needed an animal model of Parkinson’s disease in order to try to elucidate the underlying mechanisms and for the development of new potential pharmacological and non-pharmacological therapy. Small animals, mice and rats, represent the most common animals used in PD modeling. Most of these animal models have been developed using a range of neurotoxins. In the last year, the use of genetic models has been possible, thanks to genomic manipulations. Both models, toxic and transgenic, have their own advantages and limitations and choosing what model is reliable for experiments depends on the specific hypothesis that is being pursued.

Steps in model development/implementation

A wide range of pharmacological agents or neurotoxins (pesticides and fungicides) is capable to induce alterations in dopaminergic structures, alterations that are similar with those observed in human PD. This model represents the oldest and remains the most used model in experimental studies focusing on PD. Although numerous toxic agents are available for developing PD in rodents, these are classified mainly by administration route (systemic or local) and the species involved. The toxins that are mainly used in present are 6-hydroxydopamine (6-OHDA), 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, paraquat, reserpine, methamphetamine, and 3-nitrotyrosine.
Experimental models in rodents

We implemented a model based on local administration of 6-hydroxydopamine (6-OHDA). Its use as a neurotoxin to induce PD is one of the oldest and most common models in the last 50 years, because its capability to produce acute and severe nigrostriatal degeneration. This is the most functionally described model and much of the information on the anatomy, behavioral, biochemical, and physiological effects of dopamine depletion and nigral cell loss was derived from this model.

6-OHDA is a structural analog of dopamine that is highly electroactive and oxidizes to form reactive oxygen species. When the toxin is administered systemically it will affect only terminal sympathetic nerves in the peripheral nervous system, due to its inability to cross the blood-brain barrier. This is why 6-OHDA must be administered in the specific area in central nervous system, in order to produce selective neurodegeneration. In the central nervous system 6-OHDA can be administered either intracerebroventricular or in the specific interested nuclei. Both methods require knowledge and stereotaxic equipment. To specifically target the nigrostriatal dopaminergic pathways, 6-OHDA can be stereotactically administered in substantia nigra, nigrostriatal tract or in striatum compound by caudate and putamen nuclei. Once administered intracerebrally, 6-OHDA is efficiently taken up by monoaminergic neurons that have a membrane transport mechanism for catecholamines (dopamine or noradrenalin), which accounts for the high specificity of its actions. After injection in substantia nigra or nigrostriatal tract, 6-OHDA causes anterograde degeneration of nigrostriatal system. In the other situation when 6-OHDA is administered in striatum, the toxin produces a slow retrograde degeneration of nigrostriatal system over a period of weeks. In this case is a progressive
model of nigrostriatal degeneration, which is more similar to the gradual evolution of the neurodegenerative process of human PD. Overall, in both situations, dopaminergic terminal depletion starts within 12 h after injection. To further develop PD in a rat model, researchers need advanced knowledge of central nervous system anatomy and performant stereotaxic equipment (Figure 1). In our laboratory, we have chosen to obtain an animal model of PD by selective administration of 6-OHDA in the striatum of Wistar rats, respectively in the caudat putamen nucleus (Cpu). Using this method, we obtained a retrograde degeneration of dopamine neurons bodies within 12h after injection, an effect lasting more than 2 weeks.
Experimental models in rodents

The equipment is composed by a set of screws capable for smooth movements in 3 directions: anterior-posterior (X axis), right-left (Y-axis) and up-down (Z-axis). Each screw is being driven by an electrical motor, which is controlled by a specific software (Stereo Drive). In this way it is possible to make smooth movements in the direction of 3 axes with an accuracy of 50 µm. Dental drills are attached to the stereotaxic system for better stability and accuracy.

After anesthesia with a mixture containing ketamine and xylazine, the rat weighting 250-300 g is carefully positioned in the stereotaxic equipment. The head of the rat was gently positioned by means of a rat nose clamp, supplemented by rat ear bars placed lightly in the external auditory meatus to locate the interaural line (Figure 2).

![Fig.2. Skull positioned in the stereotaxic device with bregma and lambda sutures in the same horizontal plane](image)

It is important to notice that ear bars must be inserted with only a few grams of pressure or else the bones of the external
auditory meatus or the skull will be crushed with a severe negative impact on vital prognostic. In other cases, if the ear bars are inserted with too much pressure, the animal will develop breathing difficulties followed by death. The position of the head was adjusted so that the skull surface at bregma and lambda are in the same horizontal plane.

After positioning the rat in the stereotaxic device, the scalp was incised and the skull was leveled in order to easily identify the lambda and bregma. Lambda was defined as the point of intersection of the best-fit lines passing through the sagittal suture and the left and right portion of the lambdoid suture. Bregma was defined as the point of intersection of the best-fit lines passing through the sagittal suture and the left and right portion of the bregmatic suture. To establish the stereotaxic position of brain structures, reference needle tracks were made perpendicular to the horizontal and coronal planes at predetermined distances from bregma and the interaural line. In this experiment bregma was used as a reference point in order to establish necessary coordinates for caudat putamen nucleus (CPu as shown in Figure 3).
Thus, the following coordinates were used:

- X axes: 1.44 mm from bregma
- Y axes: 2.75 mm from sagittal suture
- Z axes: 5 mm.

Unilateral injection into putamen nucleus was made by a mixture of 6-OHDA, ascorbic acid and sodium chloride in a total volume of 5µL at a rate of 1 µL/min; injection was made with a Hamilton syringe connected to a stereotaxic brain cannula. Note that ascorbic acid was used to prevent or reduce damage on noradrenalinergic neurons, which are more sensitive to 6-OHDA than dopaminergic neurons. After the administration is finished, it is important that the stereotaxic cannula should be gently removed from the brain. A successful intervention must be free of superficial bleeding or intracranial
hemorrhage, situation often associated with death of the animal. At the end, the hole in the skull surface must be covered with an inert substance (dental cement in our case) and the skin is sutured.

In the first phase, the injection with 6-OHDA is commonly carried out unilaterally with the contralateral hemisphere serving as control. There is also the added benefit of lower mortality rate as compared with bilateral injections in the same intervention. In our laboratory, second injections was made seven days after full recovery and only after the animals have developed specific symptoms of unilateral nigrostriatal cell loss. These unilateral lesions caused by 6-OHDA induce typical stereotypes in response to drugs that are able to stimulate striatal dopamine receptors, either directly, such as apomorphine, or indirectly, such as amphetamine, which prompts the release of dopamine from striatal terminals of the intact hemisphere. In the first case, animals will rotate contralaterally towards the site of injection, whereas, in the other case, they will rotate ipsilaterally.

Troubleshooting

Although this approach to obtain a reliable model of Parkinson’s disease is simple in theory and relatively easy to understand, developing and ensuring of a real model is not an easy task.

A major requirement, that cannot be underestimated, is that the rat weight should be matched with the one specified in the atlas used to establish the coordinates. If that is not done correctly, there is a high probability to fail in targeting the
proposed structures of the central nervous system, considering the small volume of the brain. On the other hand, the animal should be gently positioned in the stereotaxic device as mentioned above, but in the same time is should be done in a secure fashion in order to avoid any movements during the procedure. Driling holes in the skull can induce lateral movements and shift the brain from its intended position.

The administration of 6-OHDA should be done slowly, otherwise there is risk of cerebral edema, intracranial hypertension and dead. Controlled volume injection is mandatory and we advise for an automatic system for the procedure. Another issue to consider is that after the second injection with 6-OHDA in the contralateral putamen nucleus, due to the motor specific manifestations of PD, the animals will not be able to feed and drink properly. Researcher dealing with animals with induced PD will have to allocate a lot of time caring for animals. Otherwise successful models risk side effects that will influence further evaluations.

Even if this model is widely used, there is a possibility of systemic administration of different neurotoxins in order to induce Parkinson’s disease in rodents and we believe that researchers ought to be aware when choosing accordingly

**MPTP model**

The first observations on neurotoxic effects of MPTP came in 1980s from Langston. He observed in young drug users clinical symptoms similar to those observed in Parkinson’s disease. These manifestations occurred after intravenous administration of a street preparation containing MPTP. After peripheral administration MPTP crosses the blood-brain
Experimental models in rodents

barrier and is transformed in astrocytes by monoamine oxidase-B into 1-methyl-4-phenylpyridinium ion (MPP+) which is the MPTP- active metabolite. After that, the MPP+ is taken up by dopamine transporter and selectively delivered to dopaminergic neurons from substantia nigra pars compacta. In this stage, an important role is played by MPP+ and its high affinity for dopamine transporter. In neurons of substantia nigra pars compacta, MPP+ inhibit complex I activity of mitochondria resulting in an oxidative stress.

For reasons that are not yet known, rats are very resistant to MPTP. On the other hand, rats are responsive to MPP+ only if administered specifically into substantia nigra pars compacta. In mice, MPTP is administered usually intraperitoneally. A major disadvantage of this model is the lack of Lewy bodies in dopaminergic neurons. Another limitation is the difficulty to maintain rodents with bilateral lesions, because the restricted capacity to feed and drink, due to Parkinson’s disease manifestations.

Rotenone model

Rotenone is a flavonoid found in the roots and stems of several plants, cataloged as an environmental toxin, a pesticide and a potential inhibitor of complex I. Betarbet et al. described this model first time as an alternative for MPTP model. The model is developed by chronic intravenous infusion of rotenone to rats. It easily crosses the brain-blood barrier due to its high lipophilic capacity. Unlike MPP+, rotenone does not depend on the dopamine transporter to enter in dopaminergic neurons. Once in the cell, rotenone inhibits mitochondrial complex I, leading to massive formation of reactive oxygen species. Thus it
inhibits proteasome activity, causing degeneration of nigrostriatal pathways and formation of Lewy bodies cytoplasmic inclusions, containing ubiquitin and α-sinuclein positive material in nigrostriatal cells. The rotenone model mimics some of the most important mechanisms and pathophysiological pathways assumed in human Parkinson’s disease. However, an important limitation of this model is that it has large variability in the individual response to toxin. Thereby, it is almost impossible to produce homogeneous alterations in animals and this makes the rotenone model unreliable, as a perfect model should be reproducible and standardized. Another issue is represented by high mortality due to high toxicity of rotenone on liver, heart, kidneys, and gastrointestinal tract.

References

4. Cenci MA, 6-OH Dopamine Rat Model, Encyclopedia of Movement Disorders, 2010, Pages 3-5