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The Effect of Drugs Pramipexole, Citalopram and Reboxetine to Revert the Anxiety And Depression-Like Behaviors of Mice with Parkinson's Disease

Dr. Abdul Alim [1]*, Dr. Alessandra B. Oliva [1], Dr. G. Fisone [1]

1. Department of Neuroscience, Retzius väg 8,17177, Karolinska Institutet, Stockholm, Sweden.

Corresponding Author: Dr. Abdul Alim, Department of Neuroscience, Retzius väg 8,17177, Karolinska Institutet, Stockholm, Sweden.

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. It is characterized by motor symptoms, such as rigidity, resting tremor, and bradykinesia, mainly caused by the progressive death of midbrain dopaminergic neurons in the nigrostriatal cells. Accumulating evidence indicates that PD is also accompanied by a wide range (50% of PD patients) of non-motor symptoms (NMS) including sleep disturbances, and neuropsychiatric and cognitive deficits. These symptoms often appear in the early, pre-motor stage of the disease, when the dopaminergic degeneration is still partial. The pathophysiology of NMS in PD is still a matter of debate and, as a consequence, an efficient therapy has not yet been identified. For these reasons, the preclinical research on PD is nowadays particularly focused on the development of new animal models for the study of the biological correlates of these symptoms. To investigate the NMS of PD, we produced a mouse model characterized by partial depletion of the nigrostriatal dopaminergic system. For this purpose, we subjected C57BL/6J mice to a bilateral injection of 6-hydroxy dopamine into the dorsal striatum. The efficacy of the surgery was verified by western blotting quantification of the remaining tyrosine hydroxylase in the striatum of mice. Nonetheless, the lesioned and control mice were tested with behavioral tasks commonly used to evaluate depression and anxiety in mice. We showed that the lesion produces in mice an anxiety- and depression-like phenotype. Moreover, we found that these deficits were insensitive to L-DOPA, the most common drug used to treat motor symptoms in PD patients. Conversely, both anxiety and depression were reverted in our mouse model, by the administration of the selective dopaminergic D2 receptor agonist Pramipexole. Interestingly, we also found that Citalopram and Reboxetine, serotonin and noradrenaline transporter inhibitors respectively, fully restored the behavior of lesioned mice. Importantly, these data are in line with clinical observations that report a therapeutic effect of these drugs on anxiety and depression in PD patients.

Keywords: *Parkinson's disease, Non-motor symptoms, L-DOPA, Drugs, Anxiety and depression.*

Introduction

Parkinson's disease (PD) is one of the leading neurodegenerative diseases, described by the progressive loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta, ultimately causing different classical motor symptoms including rigidity, tremor, and bradykinesia (Braak et al., 2003). PD is an age-related disease that infrequently affects a person before the age of 50 years. More than 1.5% of the total global population older than 65 years received a diagnosis of PD (Meissner et al, 2011). In 1817, the disease was first described by James Parkinson. The non-motor NMS includes sleep disorders, bowel problems, and uncontrolled salivation. The incidence of a wide range of NMS affects almost 50% of PD patients with sleep disturbances, neuropsychiatric and cognitive deficits, and autonomic and sensory dysfunctions which are increasingly receiving more attention in recent years. These symptoms often appear in the early or pre-motor stage of the disease, when the DAergic degeneration is still partial (Chaudhuri et al., 2009).

After Alzheimer's disease, PD is the second most common neurodegenerative disease, and the pathophysiological mechanisms are still incomplete (Barone 2010). In some cases, positive family history has been related to a higher risk of PD, which leads to studies on the genetic factor and family background of the disease. Several experiments have indicated that some causative monogenetic mutations can explain about 10% of PD cases (De Lau et al, 2006). Moreover, mutation of Parkin gene seems to induce a severe loss of DAergic neurons in the absence of Lewy bodies, aggregates (5-25 μm in size) of normal, misfolded, and truncated proteins (Ferrer 2011). In addition to Parkin, a rare missense mutation of α-synuclein (encoded by SNCA gene) leads to autosomal dominant PD (Dawson 2000). Alpha-synuclein (α- syn) is a 140-amino acid protein, which is highly conserved among vertebrates and the major filamentous element of Lewy bodies and Lewy neuritis. Consequently, PD presents two distinct features such as clumps of the protein named Lewy bodies and dramatic death of DAergic neurons. Nevertheless, about 90% of PD cases can be rather related to non-genetic factors like different environmental toxins. The current idea of the deficit is that mitochondrial dysfunctions, oxidative stress, and protein mismatch, could be responsible for PD pathogenesis. Furthermore, neuroinflammation, glutamatergic excitotoxicity, lack of neurotropic factors, and environmental factors are also the reason for the additional risk in PD (Meissner et al. 2011).

The preclinical research has generated several animal models suitable for the study of motor and NMS of PD. Transgenic mice are easy to use once they are generated, if the mutation does not impair breeding or survival. Many different lines of mice have been generated in the last 10 years employing different promoters (PDGF- β, Thy-1, TH promoter, prion promoter, and many others) to overexpress

α-syn wild-type or mutated forms (two missense mutations found in α -syn gene in familial forms of autosomal PD). Tyrosine hydroxylase (TH) is an enzyme which is crucial for catalyzing the conversion of the amino acid L-tyrosine to L-3,4- dihydroxyphenylalanine (L-DOPA) and it is also a precursor of dopamine (DA). Dopamine β- hydroxylase (DBH) instead is the enzyme responsible for the conversion of DA to Noradrenalin (NE). Moreover, while both noradrenergic (NEergic) and DAergic cells express TH, only NEergic neurons express DBH and the two enzymes are usually used as a reference for DA and NE activity in the brain (Fernstrom and Fernstromi, 2007; Nestler et al., 2009).

The most recent theory related to the etiology of anxiety symptoms in PD disputed that they are "reactive" and secondary to the psychosocial anxiety of the chronic disease and related disability. Nonetheless, there is increase evidence indicating that anxiety may be directly associated with the neurochemical changes in PD. Among others, NMS include olfactory dysfunction, rapid eye movement disorder (REM), neuropsychiatric problem and cognitive deficits (Schrag, 2004; Chaudhuri and Odin, 2010). However, all of these symptoms may precede motor impairment by decades (Claassen et al., 2010) and they usually enhance the intensity with disease progression, sometime leads to frank dementia in the late phase of PD (Cools et al., 2001; Goetz et al., 2008). Neuropsychiatric and mood-related disorders are common symptoms in PD patient (Cummings, 1992; Schrag, 2004) with a prevalence of around 20% (Reijnders et al., 2008), and apparent as apathy, depression, and anxiety. Eventually, these types of disorder strongly affect the quality of life, and negatively interfere with the diagnosis. The cognitive impairment in these patients affects executive functions, resulting in a phenotype resembling that of frontal lobe patients (Robbins and Arnsten, 2009), with insufficiency in attention (Ballard et al., 2002), planning, concept formation and working memory (Kehagia et al., 2010). In particular, the memory deterioration affects the both spatial (Levin et al., 1991) and non-spatial working memory domain (Matison et al., 1982), implied memory (Asselen et al., 2009), periodic memory and procedural learning (Dujardin K, et al., 2003), whereas the ability to form new periodic memories is preserved (Knowlton et al., 1996; Dubois & Pillon, 1997). Furthermore, the progression of the cognitive deficit into dementia, in PD patients, has been recommended to be predictable based on the prevalence of frontostriatal executive deficits, such as visuospatial and memory functions, with the latter associated with an increased risk (Ray and Strafella, 2012).

Depression is the most common psychiatric disorder of PD, and it affects almost 40-50% of PD patients. The causes of depression in PD patients are still matter of debate. However, different hypothesis has suggested that psychological, behavioral, and physiological factors all contribute to depression in PD (Simuui and Sethi 2008). Most of the studies have been explored the mechanisms underlying depression in PD patients by focusing on the DAergic, NEergic and Serotonergic (SEergic)

systems, which are identified to be affected in PD. The depression in a patient with PD might underlie due to the down regulation of monoamines particularly DA and NE. In fact, NE is thought to be associated with idiopathic depression like symptoms (Lambert et al., 2000). The first-choice treatment for depression in PD patients are the selective serotonin re-uptake inhibitors (SSRIs) (Simuui and Seth 2008; Taylor et al., 2009), which are preferred to other antidepressants due to the lack of anticholinergic side effects (Lemke 2008). On the other hand, the involvement in depression of dysfunctions related to the DAergic system has been largely discussed and the related theories represent the rationale for the use of DAergic drugs as antidepressants (Lemke 2008). According to the epidemiological study of psychiatric illness, anxiety affects 15.7 million people per year in the United State and 25-30% of PD patients (Dissanayaka et al., 2010; Gotez, 2010; Leentjens et al., 2011a). Anxiety in PD is defined, as a psychological reaction through the development of other symptoms, mainly motor disturbance during the progression of the disease. The relationship between clinical anxiety disorders and DAergic transmission are quite poorly understood (Millan, 2003). Social phobia is one of the most common characteristics in PD patients, which is associated with the sustained suppression of both DAergic transmission and the activity of DA receptors (Grant et al., 1998; Stein et al., 2002). Functional magnetic resonance imaging study has revealed that the involvement of a perturbation of DAergic input to the amygdala is related the abnormal emotional expression in PD patients (Benke et al., 1998; Tessitore et al., 2002). Moreover, several studies has pointed out that the onset of anxiety symptoms due to the emergence of motor impairments in PD patients (Bower et al., 2010; Wesskopf et al., 2003) and also in pre-clinical animal models of PD ((Branchi et al., 2008; Tadaiesky et al., 2008; Eskow Jaunarajs et al., 2010).

Pramipexole is a non-ergot DA receptor agonist, selective for the DA D2 receptors, which showed to improve both motor and non-motor symptoms in PD patients (Naoko et al., 2010). Citalopram is a selective serotonin reuptake inhibitor that reverts anxiety in rodents during behavioral tests such as elevated plus maze (EPM). Citalopram has been chosen rather than other anxiolytic drugs due to its low affinity against receptors of the DAergic as well as another major neurotransmitter system. Reboxetine is a potent, specific, and selective noradrenergic reuptake inhibitor (SNRI). Based on the pharmacological studies, Reboxetine is to be expected as a selective and potent tool for psychopharmacological research and the uses of this drug will help to clarify the function of norepinephrine in depression deficit (Hindmarch 1997; Versiani et al 1999).

L-DOPA is a metabolic precursor of the DA. In case of DA deficiency, as in PD, it is endogenously administered and easily crosses the Blood-brain barrier (BBB), whereas it is converted to DA by endogenous aromatic amino acid decarboxylase. In addition, it is stored in surviving nigrostriatal

terminals. Notably, most of the drug is decarboxylated to DA in the periphery, resulting in different side effects such as nausea, vomiting, hypotension and cardiac arrhythmias. Therefore, it is usually administered together with a peripheral dopa- decarboxylase inhibitor (DDCI) such as Carbidopa. Since, it is unable to cross the blood brain barrier; Carbidopa prevents the formation of dopamine peripherally while increasing the availability of L-DOPA in the CNS. Treatment with L-DOPA is nowadays the most effective therapy for PD. Whereas, the deficit of DAergic function in the basal ganglia is the principal cause of motor impairment in PD patient, the biological correlates of affective disorders are more difficult to assess (Chaudhuri et al., 2009; Dubois et al., 1997; Stern et al., 1985). Indeed, the appearance of these symptoms in PD patients correlates not only to the progressive depletion of DA, but also to the concomitant degeneration of NEergic, SEergic and cholinergic systems (Braak et al., 2003). Different hypotheses have been suggested to explain the affective disorders in early PD patients. At present, affective disorders in PD patients are treated with standard therapies, normally employed to handle depression and anxiety in non-PD patients (Blonder and Slevin 2011). These interventions have limited efficacy and do not take into account potential negative interactions with the concomitant administration of L-DOPA. Thus, the high frequency of NMS in PD and the lack of a definitive therapy, emphasize the need of new studies on the pathophysiological mechanisms of these disorders, to pave the way for the design of novel more effective treatments.

The aim of this study was to focus on the validation of a PD mouse model of affective disorders and the identification of biochemical mechanisms behind anxiety and depression- like symptoms. To achieve this aim, mice were subjected to partial degradation of the nigrostriatal DAergic pathway through bilateral injection of 6-OHDA into the dorsal striatum. Interestingly, this type of lesion produced a loss of DAergic and NEergic neurons, which are known to be affected in the early stages of PD. Furthermore, the lesioned and control mice were tested with the standard behavioral tests to assess depression and anxiety-like behaviors. These studies are followed by the analysis of specific pharmacological interventions aimed at reducing or abolishing depression- and anxiety-like behaviors caused by 6-OHDA lesion. Receptor agonists and neurotransmitter transporters inhibitors were systemically administered to lesioned mice and the behavioral performance was evaluated by depression and anxiety tests.

Materials and Methods

Animals

Sixty male (C57BL/6J strains), 8 weeks old mice (Taconic, Tornbjerg, Denmark) weighting 25-30 g at the beginning of the experiments, were used for this study. Mice were housed in standard cages in groups of a maximum of five/cage in a temperature-controlled room (23±1°C) under a light-dark cycle with free access to food and water. All of the experiments were carried out during the light phase, in accordance with the guidelines of the Research Ethics Committee of Karolinska Institutet, Swedish Animal Welfare Agency, and European Communities Council Directive 86/609/EEC. Mice were mainly divided into two groups, called "lesion" and "sham". The lesion group (n= 40) received 6-OHDA injection into the dorsal striatum and the sham or control mice (n= 20) received vehicle. After the surgery, mice were left to recover for three weeks before starting the behavioral analysis.

Drugs

6-OHDA, supplied from Sigma-Aldrich (St. Louis, Missouri), was dissolved immediately before use, in 0.9% saline (NaCl) and 0.02 % ascorbic acid with the concentration of 4µg/µl. The neurotoxin effective drug 6-OHDA was injected bilaterally into the dorsal striatum with a volume of 1 µl per injection. In addition, a group of control mice were injected with the same volume of vehicle (0.9% saline and 0.02 % ascorbic acid). Lesioned mice were treated with drugs such as L-DOPA, 20 mg/kg (Sigma Aldrich, Sweden); Pramipexole dihydrochloride 0.6 mg/kg (TOCRIS bioscience, Bristol, UK); Reboxetine mesylate 20 mg/kg (abcam Biochemicals®, Cambridge, UK) and Citalopram 20 mg/kg (abcam Biochemicals®, Cambridge, UK). These drugs were injected 30 minutes before the experiments.

6-OHDA lesioning

All mice were anesthetized with a mixture of Hypnorm Solution (VetaPharma, Leeds, UK), Midazolam 5 mg/mL (Hameln Pharmaceutals GmbH, Hameln, Germany) and water (1:1:2) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA). Firstly, the skull of the mouse was exposed and one hole per side was drilled with a burr. A bilateral injection of 1 µl of 6-OHDA (4µg/µl) was made into the dorsal striatum, according to the following coordinates (mm): antero-posterior (AP) + 0.6; medio-lateral (ML) ± 2.2 and dorso-ventral (DV) -3.2 (Franklin and

Paxinos, 1997). Control mice, called Sham, were similarly injected with the same volume of vehicle instead of 6-OHDA.

Behavioral task

Elevated Plus Maze Test

EPM test is an important behavioral test to measure anxiety-like behavior in mice (Belzung and Griebel, 2001). The apparatus of this experiment is a maze composed of four black plastic arms, organized like a cross, and located 55cm above the ground. Two arms, opposite to each other, are enclosed by lateral walls (50cm x 6cm x 40cm) named closed arms, whereas the other two arms are without walls (50cm x 6cm x 0.75cm) called open arms; the middle of the cross is a small, squared area (6cm x 6cm) named center. A 60w lamp is located above the apparatus. The mice were placed into the center to perform the test, facing one of the two open arms, and let explore the maze for 5min. The behavior of each mouse was recorded with a digital handy-cam (SONY, HDR-CX115E). Afterwards, movies were analyzed, and the time (in seconds) spent by each mouse in the arms and in the center of the apparatus was measured by an experiment blind to the groups.

Tail Suspension Test

Tail Suspension is a very common behavioral test for the measurement of depression-like behavior in mice (Yan et al., 2010). The animals were suspended by the tail on a metal rod, installed 30cm above a table, with an adhesive tape and left in this position for 6 min. The behavior of each mouse was recorded by a digital handy-cam (SONY, HDR-CX115E). Due to the uncomfortable position, mice spontaneously move in order to recover their normal vertical position. Thus, during this test the immobility time is considered to be an index of hopelessness and depression. The immobility time (in seconds) over the total 6min, was then examined by the researcher. Immobility time is defined as the time when the animals are hanging passively and when they are completely motionless.

Forced Swim Test

The Forced Swim test is an important behavior test to measure the depression-like behavior of animals. According to this test animals were singularly put into a glass cylinder, 25cm in high and 13cm in diameter filled up to 15cm with tap water at a temperature of 25°C and let swim for 10min. At the end of the test the mouse was removed from the cylinder, gently dried and placed in its home cage under

the red light (50w), for 10min, to keep it warm. The swimming behavior of each mouse was recorded by a digital handy-cam (SONY, HDR-CX115E). The total immobility time (in seconds) of each mouse during the test was then measured by a researcher though watching the videos, and considered as a measure of depression-like behavior. Since there is no way to escape from the aversive situation of being in a cylinder filled with water, the more depressed the mouse is the less effort it makes to swim. Immobility is defined as floating behavior of the mouse with only the minimal movements to keep the head above the water surface.

Western Blotting

In order to verify the success of the 6-OHDA injection in terms of amount of DA depletion, at the end of the behavioral test, all mice were sacrificed by decapitation and the left and right striatum and hippocampus were rapidly dissected out on an ice-cold surface. The tissue samples were then sonicated in 750µl (striatum) and 500µl (hippocampus) 1% sodium dodecyl sulfate (SDS) and boiled for 10min to solubilize the proteins. According to the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA) aliquoted 5µl per homogenate were used for the protein quantification for each sample. The samples were then processed by Western Blotting, to determine the amount of TH. Western Blotting involves three main steps: the gel electrophoresis, the protein transfer and the protein detection.

At the gel electrophoresis, the proteins are mainly separated through a 10% polyacrylamide gel by an electrical field according to their molecular weight and electrical charges. Basically, the smaller and highly charged molecules run faster through the gel than the bigger, less charged ones. During the transfer phase, the proteins migrate from the gel into the PVDF membrane (GE healthcare, Little Chalfont, UK) through an electrical field, due to make them accessible for the antibody staining. When the proteins are transferred to the membrane, it is required to block the non-specific binding sites in order to prevent the high background and to get a clear specific protein signal. Lastly, the protein staining is the step based on an amplification chain where the specific primary mouse anti-TH antibody (1:3000, Chemicon International, Massachusetts, USA) binds its target region and a horseradish peroxidase- conjugated secondary anti-mouse antibody (1:30000), made in the specie of the primary, recognizes and binds the protein-primary antibody. Finally, the way followed the results in a specific binding that is visualized by using a substrate for the conjugated enzyme, the ECL solution, (Pierce, Rockford, IL, USA). The chemiluminescent signal, in turn, is detectable by a film, which then display the stained proteins as bands. According to the protocol, the membranes were incubated with PBS-Tween and 5% milk-blocking solution for 50 min. Next, the membranes were washed three times for

10 minutes with PBS-Tween and stained overnight with a primary antibody mouse anti-TH (1:3000, Chemicon international, Massachusetts, USA). Subsequently, the antibody solution was rinsed and the membranes were incubated for 2h with a horseradish peroxidase-conjugated secondary anti-mouse. The membranes were then incubated 5min with the ECL solution (Pierce, Rockford, IL, USA) and the protein signal was detected by a high performance chemiluminenescence film (GE Healthcare Little Chalfont, UK). Quantification was done by Quantity One software Odyssey (LI-COR Biosciences, Cambridge, UK) and the level of each protein was expressed as percentage of control. If the Western Blotting showed that the surgery of lesioned mice was not successful, then that animals were not considered for the analysis of the behavioral test battery.

Statistical Analysis

All behavioral data were analyzed with one or two-way analysis of variance (ANOVA), and post hoc comparisons between the groups were made with the Fisher's post hoc test. Student't test with equal variances was used to analyze experiments with two groups.

Results

1 6-OHDA lesion induces an anxiety- and depression-like phenotype in mice

To evaluate the effect of the 6-OHDA lesion on anxiety and depression in our mouse, lesioned and sham mice have been tested by the EPM test (for anxiety) and the Forced Swim test (for depression). EPM is based on the physiological aversion of mice to open spaces. In fact, our control mice clearly spent more time in the closed arm than in the open one, during the 5 min test. However, this difference was much bigger in the lesioned mice. Notably, lesion and control mice spent a similar amount of time in the central part of the apparatus (Fig. 1, panel A). The effect of the lesion on the time spent in the different arms of the apparatus has been statistically analyzed by a 2-way ANOVA and showed a significant interaction "arm" x "group" interaction [$F(2,80)=13.109$, $p<0.0001$]. The following Fisher posthoc comparison confirmed the difference between the two groups in the time spent in both the open and closed arms (± SEM. ** $p< 0.01$).

Similarly, when the animals have been tested in the Forced Swim test, we found a significant difference in the two groups, indicating that the lesion actually induces a depression-like behavior. In fact, lesioned mice totalized a bigger amount of immobility time, compared to sham mice, during the 10 min test (Fig. 1, panel B).

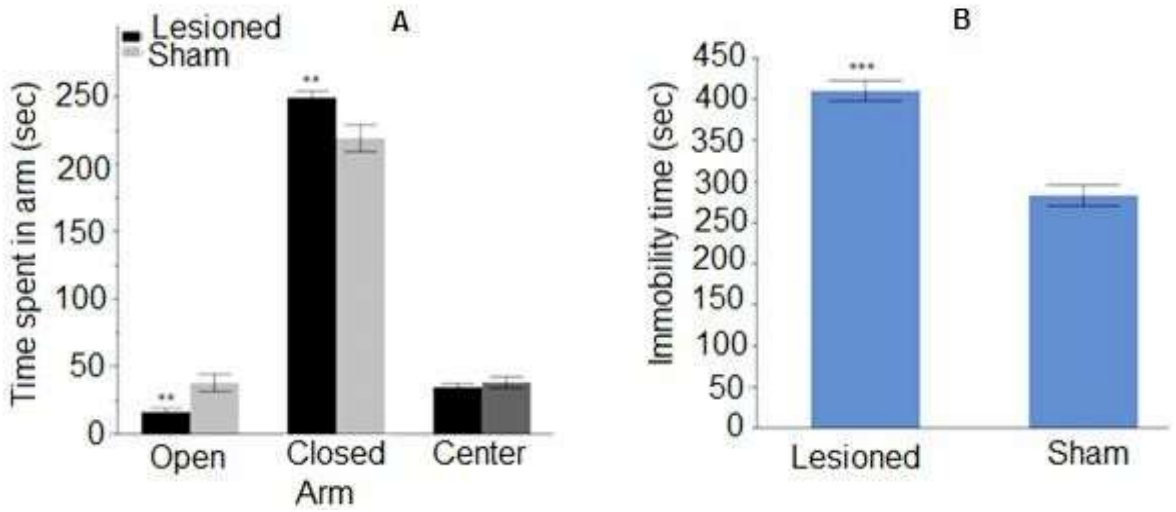

Figure 1. Anxiety- and depression-like phenotype in 6-OHDA-lesioned mice. A) Elevated Plus maze test performed in sham (n=12) and lesioned (n=13) mice. The graph shows the time (measured in sec) spent by the animals in the center as well as in the open and closed arms of the apparatus, during 5 min test. B) Forced Swim test in sham (n=12) and lesioned (n=13) mice. The graph shows the immobility time (measured in sec) totalized by the animals, during a 10 min test. Data are expressed as mean ± SEM. ** $p< 0.01$, *** $p< 0.0001$ vs sham mice, same arm.

In addition, our study could also show the Tail Suspension test which represents a significant effect of the lesion on the total immobility time spent by the animals during 6 min test [$F(1,24) = 1.737$; $p <0.05$], analyzed by one-way ANOVA. The lesioned mice showed higher immobility time compared to sham mice (Fig. 2). This data indicates that 6-OHDA- lesioned mice have a slight depression-like behavior.

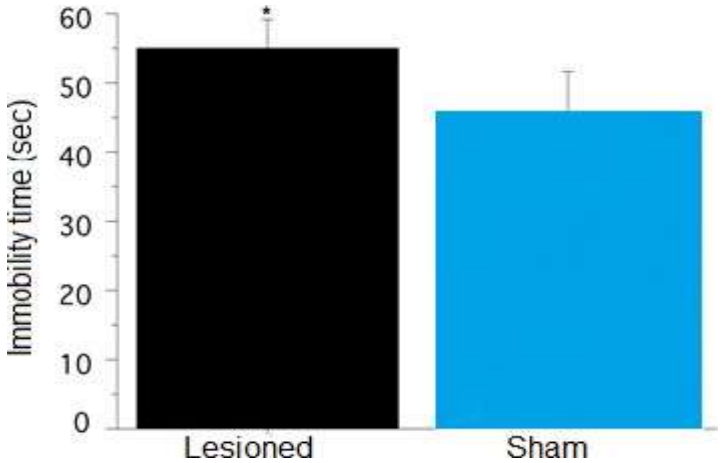

Figure 2. Tail Suspension Test. The analysis of the Tail Suspension test revealed a significant effect of the lesion on the total immobility time (measured in sec) spent by the animals during 6 min test [$F(1,24) = 1.737$; $p <0.05$], analyzed by one-way ANOVA. This data indicates that 6-OHDA- lesioned mice have a slight depression-like behavior.

All together these data demonstrate that our mouse model effectively mimics the psychiatric, mood-related disorders present in PD patients.

Pramipexole, but not L-DOPA, reverts anxiety- and depression-like behavior in lesioned mice

At first, we wanted to test the efficacy of the goal standard therapy for PD, L-DOPA, to revert the anxious and depressed phenotype of our lesioned mice. We treated the lesioned mice with this drug and submitted them to Forced Swim test. We found that L-DOPA was not able to revert the symptom (Fig. 3, panel B). Since L-DOPA is the precursor of DA, it binds both D1 and D2 DA receptor in the striatum. For the other group of lesioned mice, we also analyzed the effect of the selective D2 receptor agonist Pramipexole through the same behavioral task. Interestingly, we found that this drug was able to reduce the depression in our lesioned mice (± SEM. ** $p < 0.01$ vs sham, ## $p < 0.01$ vs lesioned mice treated with saline).

Thus, we submitted sham and lesioned mice to the EPM. We confirmed that the lesioned mice show an anxious behavior, and we also found that this pathological phenotype is reverted by the administration of Pramipexole, but not by the administration of L-DOPA (Fig. 3 panel A). In fact, as shown in Fig. 3 panel A, the amount of time spent in the open arm is always less, compared to the time spent in the closed arm, in all groups. However, Pramipexole-treated mice were similar to the sham, whereas there was no difference between saline-treated and L- DOPA-treated lesioned mice (mean ± SEM).

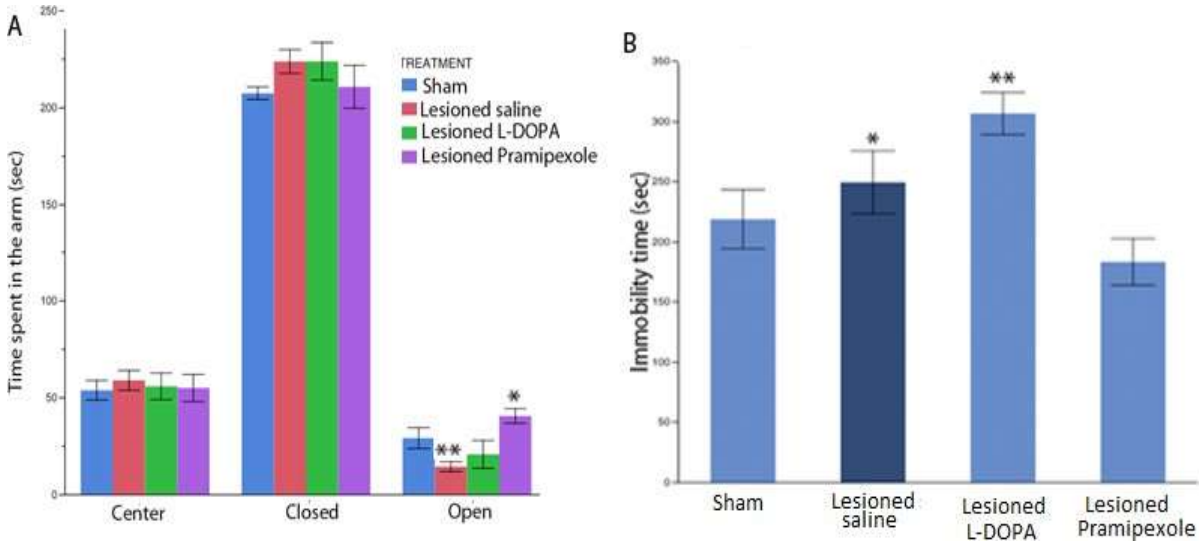

Figure 3. Pramipexole reverts anxiety- and depression-like behavior, but L-DOPA does not revert these deficits in lesioned mice.

A) Elevated Plus maze test performed in sham (n=8) and lesioned (Les) mice treated with saline (n=6), 0.6 mg/kg Pramipexole (n=7) and 10 mg/kg L-DOPA (n=5). The graph shows the time (measured in sec) spent by the animals in the center as well as in the open and closed arms of the apparatus, during a 5 min test. Data are expressed as mean ± SEM. * p< 0.05, ** p< 0.01 vs sham mice.

B) Forced Swim test in sham (n=8) and lesioned (Les) mice treated with saline (n=6), 0.6 mg/kg Pramipexole (n=7) and 10 mg/kg L-DOPA (n=5). The graph shows the immobility time (measured in sec) totalized by the animals, during a 10 min test. For both Elevated Plus maze and Forsed Swim test, the mice received the drug injection 30 min before the experiment. Data are expressed as mean ± SEM. * p< 0.05, ** p < 0.01 vs sham.

Anxiety and depression-like behaviors are reverted by the administration of Reboxetine and Citalopram

To investigate the involvement of noradrenaline and serotonin neurotransmission systems in the anxiety- and depression-like phenotype found in lesioned mice, we verified the ability of both NET and SERT inhibitors (Reboxetine and Citalopram, respectively) to revert the deficits. Thus, we subjected the sham and lesioned mice to the EPM. We found that 6-OHDA lesion induces an anxiety-like behavior in our mice, and this pathological phenotype is reverted by the administration of Reboxetine (20 mg/kg), as well as Citalopram (20 mg/kg) shown in Fig. 4 as for anxiety in panel A and for depression in panel B. Importantly, we also found that when lesioned mice were treated with Reboxetine and Citalopram, their behavior was similar to control mice (Fig. 4, panel B).

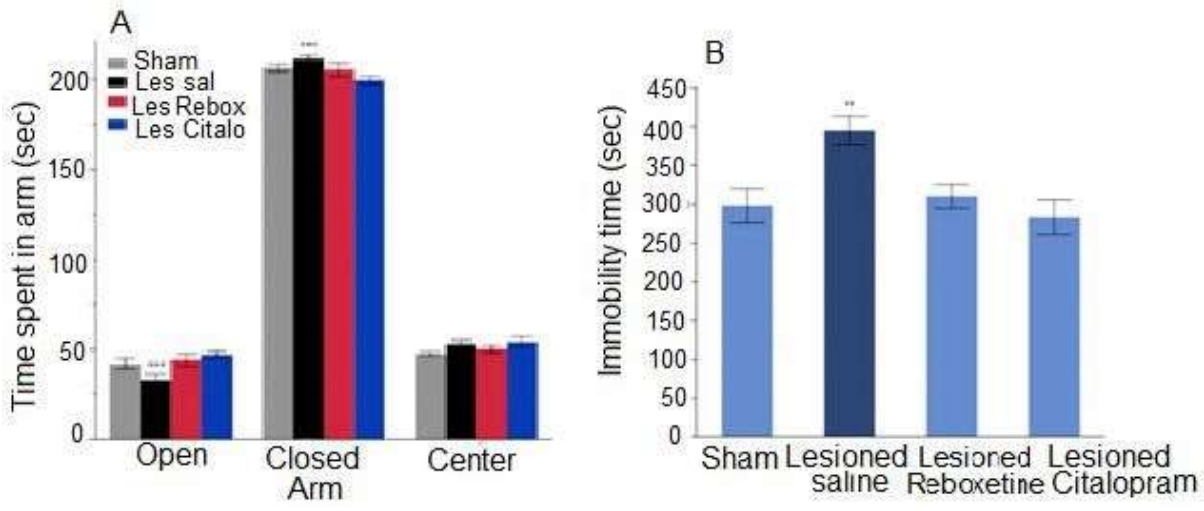

Figure 4. Reboxetine and Citalopram revert anxiety- and depression-like behavior in lesioned mice. A) Elevated Plus maze test performed in sham (n=10) and lesioned (Les) mice treated with saline (n=6), 20 mg/kg Reboxetine (n=7) and 20 mg/kg Citalopram (n=9). The graph shows the time (measured in sec) spent by the animals in the center as well as in the open and closed arms of the apparatus, during a 5 min test. Data are expressed as mean ± SEM. *** p< 0.0001 vs sham mice, same arm. B) Forced Swim test in sham (n=10)

and lesioned (Les) mice treated with saline (n=7), 20 mg/kg Reboxetine (n=7) and 20 mg/kg Citalopram (n=7). The graph shows the immobility time (measured in sec) totalized by the animals, during a 10 min test. For both Elevated Plus maze and Forsed Swim test, the mice received the drug injection 30 min before the experiment. Data are expressed as mean ± SEM. ** $p < 0.01$ vs sham.

The 2-way ANOVA analysis showed a significant "arm" x "group" interaction [$F(6,95)=5.697$, $p=0.0001$]. The following Fisher post-hoc comparison confirmed the difference between the two groups in the time spent in both the open and closed arms. Similarly, when the animals have been tested in the Forced Swim test, we found a similar therapeutic effect exerted by Reboxetine, as well as by Citalopram, on the depression-like behavior showed by lesioned mice. The 1-way ANOVA revealed a significant treatment effect [$F(3,29)=5.863$, $p=0.0034$], and the following Fisher post-hoc indicated that in fact lesioned mice totalized a bigger immobility time compared to the sham mice, and that Reboxetine- and Citalopram-treated mice have a lower immobility time compared to lesioned mice treated with saline.

Reduction of Tyrosine Hydroxylase showed the successful lesion of PD rodents

By using Western Blotting we found that the reduction of TH following 6-OHDA lesion was about 60% in the striatum compared to sham mice (Fig.5). Mice with a reduction of TH less than 60% were excluded from the behavioral analysis.

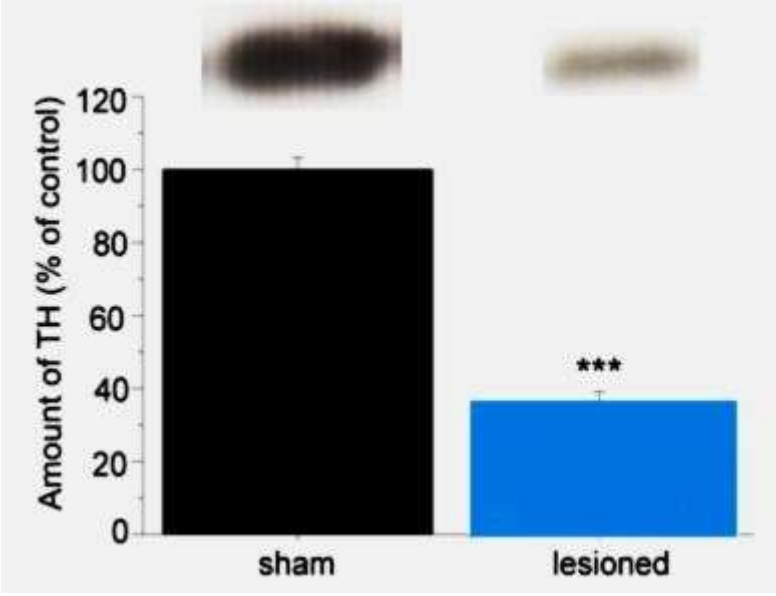

Figure 5. Quantification of TH in the striatum of sham and lesioned mice. The Western Blotting analysis showed that the remaining TH level was about 40% in the striatum of lesioned mice, compared to control mice. Data are shown as mean ± SEM. *** $p < 0.01$ vs sham.

All together these results demonstrate that the anxiety- and depression-like phenotype induced by the 6-OHDA lesion in our mouse model, can be reverted by the administration of a NET (Reboxetine) and a SERT (Citalopram) inhibitors. Importantly, this data is in line with clinical observations that report a therapeutic effect of these drugs on anxiety and depression in PD patients.

Discussion

PD is a neurodegenerative disorder typically defined by the progressive death of midbrain DAergic neurons projecting to the basal ganglia and by the emergence of motor symptoms such as rigidity, tremor, and bradykinesia (Braak et al., 2003; Jankovic 2008). Several studies have demonstrated that PD is also accompanied by a wide range of NMS, appearing in the early stages or even pre-motor phase of the disease and affecting a large proportion of patients (50%) (Aarsland et al., 2004; Chaudhuri and Schapira, 2009). NMS include different types of deficits such as cognitive, autonomic, and sensory dysfunctions as well as sleep disturbance. However, NMS also include neuropsychiatric disorders, like depression and anxiety (Aarsland et al., 2004; Chaudhuri and Schapira 2009), that have been for a long time under- reported and under-recognized as PD symptoms. In this study, we firstly demonstrated that the bilateral injection of 6-OHDA into the striatum of mice induced a DA reduction of around 60% in the striatum. Importantly, we showed that the partial DA depletion we obtained in our model leads to anxiety-like behavior, as demonstrated by the mice performance in the Elevated Plus maze. Notably, these data are consistent with other studies (Leonibus et al., 2007; and Tadaiesky et al., 2008). Furthermore, they closely mimic the pathophysiologal signs occurring at the early stage of the disease in PD patients.

Besides, the anxiety-like behavior, we also proved that the 60% DA depletion in the striatum induces a depression-like behavior in our CB57L/6J mice model, similarly to what has been reported by Tadaiesky et al. (2008). By using Tail Suspension test and the Forced Swim test we found that our lesioned mice have a depression-like phenotype. At the end of the behavior experiments, the animals were sacrificed, and the amount of TH left in the lesioned striatum was quantified by western blotting. This analysis confirmed that the procedure we used efficiently induced a partial (around 60%) bilateral depletion of DA in the striatum of our mice.

It has been shown that in PD patients not only the striatal DAergic system is affected but also other brain structures such as the anterior olfactory structures, amygdale, raphe nuclei, locus coeruleus, hippocampus, autonomic nervous system, cerebral cortex are involved. In particular, the severity of anxiety and depression seems to correlate with functional abnormalities in the amygdale, which

receives NEergic and DAergic innervation that are both reduced in human PD (Remy et al. (2005). Furthermore, it has been demonstrated that the serotonin system is affected in PD, and stimulation of this system reduces or diminishes several NMS (Barone 2010). All together our results demonstrate that the anxiety- and depression-like phenotype induced by the 6-OHDA lesion in our mouse model, can be reverted by the administration of NET (Reboxetine), SERT (Citalopram) inhibitors and DA D2 receptor agonist (Pramipexole). The main result of this study is that our mouse model of CB57L/6J with a partial DA lesion develops an early stage of PD with underlying NMS such as anxiety- and depression-like behavior.

The mouse model of PD based on the striatal injection of 6-OHDA has been already demonstrated to be a good tool for the research on PD, even though it is classically applied to the study of motor symptoms. Bilateral injection of 6-OHDA into the striatum causes only partial depletion of striatal DAergic neurons instead of total depletion as in unilaterally injected models (Branchi et al 2010), leading to an animal model mimicking an early stage of PD. The main advantage of this is the absence of motor deficits that would represent an inconvenient variable in the analysis of mice non-motor behaviour. Moreover, striatal injection of 6-OHDA led to a fast damage of DAergic terminals in the striatum, and by a retrograde pattern it affects the projecting areas killing the DA neurons in the substantia nigra (Tadaiesky et al., 2008). Finally, the DA damage induced by 6-OHDA injection is not reversible and it can be easily used for long-term studies. However, whereas the motor symptoms of PD are known to be dependent on the only DA degeneration in the midbrain, the etiopathology of NMS is still unknown. In this regard it has been suggested that cognitive and neuropsychiatric deficits in PD are linked not only to the progressive degeneration of the dopaminergic nigro-striatal pathway. Actually, clinical and preclinical studies suggest that these symptoms might be only partially dependent from the death of DAergic neurons in the substantia nigra, but rather be linked to the consecutive involvement of other brain regions, such as hippocampus, prefrontal cortex and amigdala. Moreover, increasing evidence suggests that, in PD patients, NMS results from the disruption of the complex interaction between the DAergic, SEergic and NEergic transmitter systems, rather than from the only degeneration of DA neurons (Aarsland et al., 2011).

In line with previous description, our data totally support the hypothesis of the involvement of not only DAergic, but also NEergic and serotonergic systems in the anxiety and depression like phenotype of our PD mice. In fact, Pramipexole, a DAergic agonist, as well as Citalopram and Reboxetine, serotonin and noradrenaline agonist respectively, showed an antidepressant and anxiolytic effect in our model. In particular, Pramipexole has stronger affinity and selectivity to DA D2 and D3 receptors than the other ergot agonists (as compared to D1 and D2 receptor agonist). Thus, we were wondering about the

effect of a drug with a selective effect on a specific DAergic receptor. Antidepressant Reboxetine is a potent, specific and selective noradrenergic reuptake inhibitor. Citalopram is a selective serononin reuptake inhibitor which can modulate the anxiolytic effects on rodents during behavioral task such as Forced Swim Test (Belmaker and Agam, 2008). Citalopram has been chosen rather than other anxiolytic drugs due to its low affinity against receptors of the DAergic as well as other major neurotransmitter system. Our data demonstrated that all these drugs are able to revert the depression and anxiety like behaviors of lesioned mice, when tested in the Forced Swim test and the Elevated Place maze. Thus, we seen in Fig. 6 panel A, the amount of time spent in the open arm in less, compared to the time spent in the closed arm, in all groups. However, in lesioned mice this difference is bigger and they spent even more time in the closed arm, compared to sham mice.

Nowadays, the first choice of antidepressant in PD is considered the selective serotonin reuptake inhibitors and it is the most frequently prescribed first-line antidepressants (Simuui and Seth 2008; Taylor et al., 2009). However, the SSRIs are preferred due to their tolerable and lack of anticholinergic side effects as compared to other antidepressants (Lemke 2008). Recent studies highlighted the importance of DAergic mechanism for depression in PD patients and giving rise to the rationale for treating depression like deficits with the DA agonists (Lemke 2008). In line, with a heterogeneous neurobiological substrate of the disease, the motor symptoms are successfully treated with DA replacement therapies, such as L- DOPA administration but NMS including anxiety and depression like behaviors are not responding to this drug (Kulisevsky et al., 2000). However, L-DOPA has the property to bind both D1 and D2 receptor. This study has shown that the generic activation of dopamine agonists (induced by L-DOPA) is unable to revert anxiety-and depression like behaviors of PD rodents. Differently, a selective D2 receptor agonist like Pramipexole does revert these deficits.

DA depletion, nigral dopamine cell loss, and neurobehavioral disorders have been successfully accomplished using this model, but it does not produce or induce Lewy-like inclusions or those have been seen in PD. Additionally, it is hypothesized that L-DOPA itself can induce an increase in endogenous 6-OHDA levels that ultimately leads to Lewy body formation in DAergic neurons (Borah 2012). Conversely, there are few agents those are clinically under in a line that are currently able to slightly revert or suppress both motor and non-motor manifestations of the disorders, but there is no specific treatment still now for NMS in PD patients. Therefore, NMS are mainly handled with the therapies commonly used in NMS in PD patients, by taking into consideration the co-administration of antidepressant drugs to these same patients and a suitable specific treatment is still missing.

Conclusion

In conclusion, we validated a mouse model of PD for the study of NMS. This model is based on the bilateral injection of 6-OHDA directly into the striatum. This procedure produces a partial depletion of DA of around 60% in the striatum. We showed that such DA degeneration causes in our model depression and anxiety-like phenotype, as measured by the Tail Suspension, Forced Swim Test and the Elevated Place Maze. Interestingly, these symptoms were insensitive to treatment with L-DOPA, the goal standard treatment for PD motor symptoms. Conversely, a selective DA D2 receptor agonist reverted both depression and anxiety in our lesioned mice. Similarly, Reboxetine and Citalopram, noradrenalin and serotonin transporter inhibitors respectively, shared the ability to revert depression and anxiety in our lesioned mice. Notably, this data is in line with clinical observations that report a therapeutic effect of these drugs on anxiety and depression in PD patients. Certainly, more studies are needed to further investigate the mechanisms of the different behavior alterations found in this study. A single drug is able to revert motor symptoms as well as anxiety and depression in PD patients is certainly needed. For this reason, a deep understanding of the biochemical and molecular correlates of these deficits is a fundamental step for the identification of new target suitable for a definitive treatment of the disorders.

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