Impairment of Neurogenesis in the Olfactory Bulb of Transgenic Mice Overexpressing Human Wildtype Alpha Synuclein Under the Thy-1 Promoter

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that impairs motor function. Patients with PD display motor symptoms including bradykinesia, akinesia, rigidity, resting tremor, and postural instability. In addition, recent studies have found early non-motor symptoms in patients with PD including olfactory impairments, gastrointestinal dysfunction, sleep disturbances, anxiety and depression. The genetically engineered alpha-synuclein overexpressing mice (ASO) under the Thy-1 promoter have high levels of alpha-synuclein and demonstrate distinctive proteinase K-resistant alpha-synuclein inclusions throughout the brain including the thalamus, basal ganglia, cortex and olfactory bulb (Rockenstein et al., 2002). These mice demonstrate early and progressive motor impairments without the loss of nigrostriatal dopaminergic neurons at nine months of age (Fleming et al., 2004). The ASO mice under the Thy-1 promoter demonstrate olfactory impairments in olfactory tests and thus may present an early model of Parkinson’s disease (Fleming et al., 2005).

It has been documented that Parkinson’s patients have impairments outside of the nigrostriatal pathway. One area that can be affected is the olfactory senses. The olfactory neurons are renewed throughout life: the neurons originate from the subventricular zone (SVZ) and then migrate along the rostral migratory stream (RMS), which are surrounded by olfactory ensheathing glia, and eventually reach the olfactory bulb (Gritti et al., 2002). Recent studies have shown that the ASO mice under the PDGFβ promoter have impairments in neurogenesis of the olfactory bulb and hippocampus, presenting a mouse model of PD with impairments outside the of the nigrostriatal pathway (Winner et al., 2004). The goal of the project outlined in this proposal is to investigate whether animals exhibiting an accumulation of alpha-synuclein in the olfactory bulb demonstrate impairments in the olfactory senses. This study can provide a better understanding of early symptoms of Parkinson’s disease. It will also provide better criteria for testing and diagnosing the earlier stages of Parkinson’s disease and may allow for early therapeutic interventions.

A. Specific Aims:

This project aims to gain an understanding of the relationship between alpha-synuclein accumulation in the olfactory bulb and olfactory impairments. The main hypothesis is that alpha-synuclein accumulation in olfactory bulb causes the olfactory impairments. The alpha-synuclein accumulation in the olfactory bulb may cause a disruption in neurogenesis and cause the olfactory impairments.

Hypothesis 1: To test whether mice overexpression of alpha-synuclein under the Thy-1 promoter exhibit an impairment of neurogenesis in the olfactory bulb.

Hypothesis 2: To test in the alpha-synuclein over-expressing mice under the Thy-1 promoter, whether olfactory impairments are correlated with a perturbation in the neurogenesis of the olfactory bulb.

Hypothesis 1 will be tested by quantifying the number of bromodeoxyuridine (BrdU) positive cells in the olfactory bulb of the ASO and wild type (WT) littermate controls using the techniques of immunohistochemistry and stereology. In addition, hypothesis 1 will be addressed by the quantification of the following double-label immunohistochemical experiments: BrdU and Tunnel, BrdU and alpha-synuclein, BrdU
and Neu-N, BrdU and tyrosine hydroxylase (TH). Hypothesis 2 will be addressed by testing olfactory impairments with olfactory tests including the block test, habituation-dishabituation test and by correlating the quantification of the BrdU-positive cells and α-synuclein accumulation in the ASO mice compared to WT.

B. Background and Significance

Parkinson’s disease (PD) is a neurological disorder characterized by the degeneration of dopaminergic neurons in the nigrostriatal pathway. The accumulation of alpha-synuclein in the brain and peripheral tissues results in the formation of Lewy bodies, a hallmark of PD. (Braak et al., 2004) Patients often suffer a combination of motor symptoms including resting tremor, rigidity, bradykinesia and postural instability. By the time patients are diagnosed with PD, 80% of their dopaminergic neurons in the substantia nigra pars compacta have degenerated causing devastating effects on their motor function (DeKosky et al., 2003).

Recent studies have shown that prior to the motor deficits typically indicative of PD, a number of other symptoms develop, including olfactory impairments, gastrointestinal dysfunction, sleep disturbances, anxiety and depression. The staging of pathology in sporadic PD demonstrates that lesions in the olfactory bulb and cortex develop in the early stages of PD, whereas the nigrostriatal cell loss occurs in the later stages of PD (Braak et al., 2004). This provides strong evidence for the value of studying olfaction in PD. Transgenic mice that overexpress alpha-synuclein (ASO) under the Thy-1 promoter demonstrate high levels of alpha-synuclein inclusions throughout the brain including the olfactory bulb, thalamus, basal ganglia and cortex (Rockenstein et al., 2002). Male ASO mice at 3-4 months under the Thy-1 promoter demonstrate significant (p< 0.01) olfactory impairments with olfactory tests such as the novel olfaction capacity test (figure 1) and the habituation/dishabituation test (figure 2) (Fleming et al., 2005). The mechanism of the olfactory impairments in the PD is not well understood.

The olfactory bulb has neurogenesis throughout life. The olfactory neurons originate from the subventricular zone (SVZ) and then migrate along the rostral migratory stream (RMS), surrounded by olfactory ensheathing glia, eventually reaching the olfactory bulb (Gritti et al., 2002). Renewal of olfactory neurons is essential for proper olfactory detection and discrimination. It has been documented that ASO mice under the PDGFß promoter have impairments in neurogenesis of the olfactory bulb (Winner et al., 2004). The ASO mice under the Thy-1 promoter exhibit numerous alpha-synuclein inclusions in the olfactory bulb and display demonstrable olfaction impairments. (Rockenstein et al., 2002; Fleming et al., 2005). It is critical to test whether alpha synuclein aggregates cause a disruption of neurogenesis in the olfactory bulb in the ASO mice. This would allow for a better understanding of the early symptoms of PD and thereby provide for possible early detection and early intervention in humans.
C. Preliminary Data

Figure 1: At 3 months, WT and ASO were tested for olfactory capacity with the block test (Spinetta et al., 2005). On trial 7, the mice were exposed to blocks 1, 2, 3 and 5 (novel block from another animal’s cage) and video taped for 30 seconds. The time the animal spent in olfactory investigation was measured. WT (n=16) compared to ASO (n=7) spent significant (∆p < 0.005) more time in olfactory investigation of block 5. In addition, WT spent significant (*p < 0.001) more time in olfactory investigation with block 5 compared to blocks 1, 2 and 3.

D. Experimental Design

This is a two-part study to evaluate the olfactory impairments in the ASO mice. First, the animals will be tested using olfactory tests such as the block test and habituation-dishabituation test. The second part of the study will analyze the olfactory bulb for neurogenesis, neuronal survival, neuronal differentiation and for the accumulation of alpha-synuclein in ASO mice compared to WT mice.

Animals

Human alpha-synuclein overexpressing transgenic mice under the Thy-1 promoter (ASO) and wild type (WT) litter mate controls will be tested (Rockenstein et al., 2002). There will be two time groups for olfaction testing. Forty male mice will be tested for the olfactory function; ASO (n=10) and WT controls (n=10) at 3-4 months of age and ASO (n=10) and (WT) controls (n=10) at 9 months of age.
Olfacton Tests

Olfaction Capacity Test / Block Test  Animals are individually housed with five wooden blocks (2.5 cm$^3$) numbered 1-5 placed in each animal’s cage for at least one week prior to testing. On the day of the testing, animals habituate to the room for one hour of low light. During this time, all five blocks and some bedding are removed from each cage and placed in a plastic bag labeled with the animal’s identification. At the time of testing blocks 1-4 from the animal’s cage are placed in the middle of the cage. The mouse is then videotaped for 30 seconds. The test is repeated for a total of six exposures to blocks 1-4. On the seventh trial, block 4 is replaced with block 5 from another mouse’s cage; thus blocks 1, 2, and 3 from its own cage and the novel block 5 are placed in the animal’s cage. If olfaction function is intact, the mouse should detect the novel odor and therefore spend more time sniffing the novel block. The animals are recorded on a video camera for 30 seconds and the time spent in olfactory investigation is measured and calculated by a rater blind to genotype (Spinetta et al., 2005).

Habituation / Dishabituation Test  Similarly to the block test, animals are individually housed. An unscented tissue cartridge is placed into each animal’s cage for one week prior to testing in order for animals to habituate directly to the stimulus. On the day of testing, animals are transferred into the testing room and allowed to adjust to the low light for one hour. During testing the animals receive a total of seven exposure trials. For each of the first six trials animals are exposed to the same scent (banana) to habituate to that scent. On the seventh trial, animals are exposed to a novel scented cartridge (lemon). The animals are recorded on a video camera for 30 seconds and the time spent in olfactory investigation is measured and calculated by a rater blind to genotype (Bielsky et al., 2004). Tissue cartridges (Omnisette tissue cartridge, Fisher Scientific) are loaded with a cotton ball injected with the various odors. Every other day, paired scents are tested in the following order: banana and lemon, coconut and anise, pine and cinnamon, lemon and lime, lemon and orange, and lime and orange.

Bromodeoxyuridine (BrdU) Injections
After the olfaction test, the two groups of mice at 4 and 9 months receive five injections of BrdU every eight hours over a forty-hour period (Gotts et al., 2002, Winner et al., 2004). BrdU labels newly-born cells by labeling DNA synthesis in the S phase of the cell cycle (Bauer et al., 2005). The animals are then sacrificed one month after BrdU injections to study neurogenesis differentiation in the olfactory bulb.

Immunohistochemistry

Neurogenesis and cell survival
Double immunohistochemistry of BrdU with Tunnel (a cell death marker) effectively permits identification of those newly-born cells in the olfactory bulb that do not survive. The number of BrdU positive cells and the number of BrdU and Tunnel double labeled cells are stereologically quantified. This addresses the important question of how many newly born cells survive in the olfactory bulb of ASO as compared to WT so that actual genesis of the olfactory bulb is quantified.
**Neurogenesis and alpha-synuclein accumulation**

A double immunohistochemical label of BrdU and alpha-synuclein, which labels alpha-synuclein aggregates will be stereologically quantified in the olfactory bulb of the ASO and WT mice. This will address whether alpha-synuclein aggregates disrupt neurogenesis in the olfactory bulb.

**Neurogenesis and neuronal differentiation**

Double immunohistochemical label of the BrdU and Neu-N, which is a neuronal marker, would determine the number of BrdU positive cells that turn into neurons. A stereological analysis of the double labeled Neu-N and BrdU positive cells will be compared in olfactory bulb of the ASO to WT mice.

Double immunohistochemical label of the BrdU and tyrosine hydroxylase (TH), which labels dopaminergic cells would determine the number of BrdU-positive cells that turn into dopaminergic neurons. A stereological analysis of the double-labeled TH and BrdU positive cells in the olfactory bulb will be compared in the ASO to WT mice.

**Stereology**

The unbiased stereology will be performed with the use of the Stereoinvestigator software (Microbright Field Inc.). An optical dissector will be designed to allow for a systematic, random counting procedure and the volume will be determined by tracing the region of interest. Every 5th section (200 µm interval) of the left hemisphere of each animal will be processed for immunohistochemistry. The granule cell layer of the olfactory bulb labeled with BrdU, NeuN and Tunnel positive cells will be counted with a counting frame of 60µm x 60µm and a 200µm x 200µm counting grid. The TH-positive cells will be counted in the glomerular layer of the olfactory bulb with a 20µm x 20µm counting frame and a 100µm x 100µm counting grid (Winner et al., 2004).

**Limitations**

Other brain regions such as the olfactory cortex and frontal cortex could be perturbed, causing the olfactory impairments. Thus, it would be helpful to quantify of the neurons in the olfactory cortex and in frontal cortex to determine whether those regions are involved in the olfactory impairments. Another possible explanation could be there is a disruption in the cellular functionality. The olfactory impairments could be due to a disruption in the connectivity of the neurons in the olfactory bulb. In this case, it would be important to test the functional connectivity of the cellular layers in the olfactory bulb and test whether the projections to the olfactory cortex are intact. In addition, the olfactory cortex could be altered and may cause the olfactory impairments. Thus, the quantification of the neurons in the olfactory cortex would be another area to examine to establish the regions involved in the olfactory impairments.

**Future Directions**

This study will provide evidence for early staging of PD. This study has relevant clinical application for detecting the early stages of PD, which provides patients with the opportunity to use early therapeutic interventions. In addition, it would be important to study the migration of newly born cells from the SVZ to the olfactory bulb and to analyze the pathway for possible mechanisms of interference. Also, it is valuable to test ASO mice for the additional early non-motor symptoms including gastrointestinal dysfunction, sleep disturbances, anxiety and depression. This could allow for the development of better criteria to test for the early symptoms in Parkinson’s disease.
E. References:


