Development of Methods for Measurement of the Integrity of Dopaminergic Pathways in a Rat Model of Parkinson's Disease[1]

Edina Torlaković
Department of Cognitive Science, Carleton University
edina_@scs.carleton.ca

Bruce Hutcheon
Department of Neuroscience, Carleton University
Bruce_Hutcheon@carleton.ca

Introduction

Parkinson's disease (PD) is a progressive neurological disorder manifested by difficulty initiating movement, muscular rigidity, resting tremor, and other postural abnormalities. It is associated with a loss of neurons in the midbrain that supply the forebrain with dopamine. The resulting chemical deficit in forebrain structures results in a disruption of integrated motor output.

Before a description of the experimental studies is given, an outline of the anatomy and basic operation of the motor circuits is needed.

The regions of the brain that participate in voluntary movements are the basal ganglia, motor nuclei of the brain stem, red nucleus, and cerebellum. The region of interest in the case of PD is the basal ganglia.

1. The basal ganglia consists of four subcortical nuclei (see Figure 1): striatum [ST] (a collective term for the caudate nucleus and putamen),
2. globus pallidus (consisting of external [GPe] and internal segments [GPi]),
3. subthalamic nucleus [STN], and
4. substantia nigra [SN] (consisting of the pars compacta [SNc] and pars reticulata [SNr]).

[1] Carleton University Cognitive Science Technical Report 2002-08
URL http://www.carleton.ca/iis/TechReports

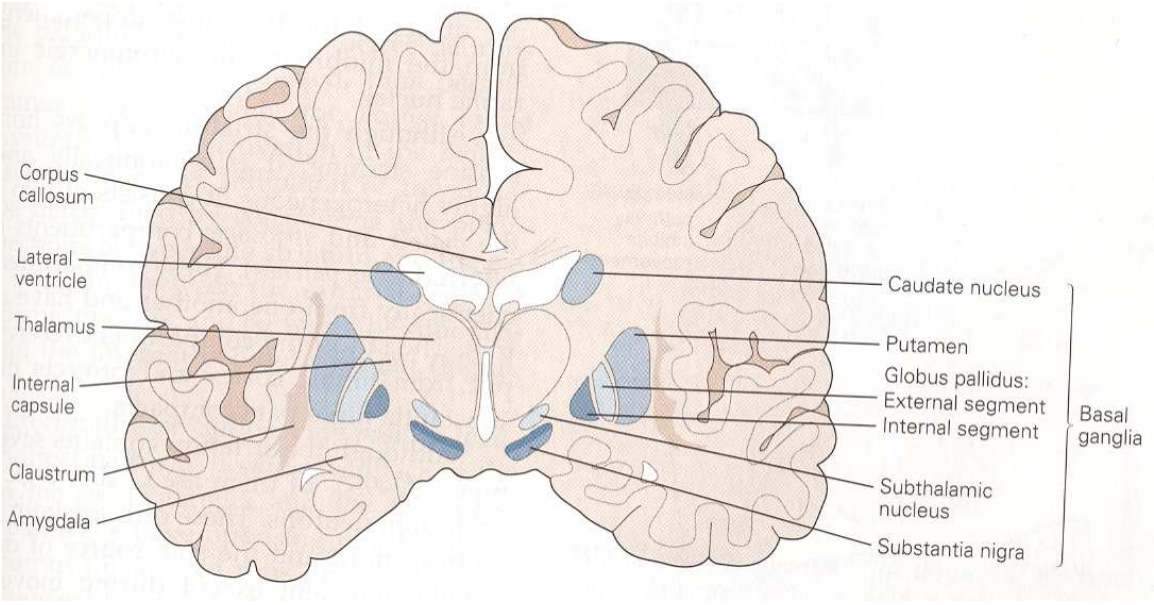


Figure 1: Coronal section of the basal ganglia (adapted from Nieuwenhuys et al. 1981)

These nuclei participate in a feedback loop which begins at the motor cortex, proceeds through the basal ganglia (with the striatum as the input centre), to the thalamus (the main conduit for sensory information reaching the brain), and back to the motor cortex. The loop runs in parallel to both descending motor commands from the cortex to the spinal cord and sensory information ascending to the cortex through the thalamus. Thus, it is in a position to coordinate sensory and motor information. When it becomes dysfunctional, as in PD, it is this coordination that is disrupted.

Two major pathways transmit information through the basal ganglia: the so-called direct and indirect pathways. The direct pathway projects from the striatum to the internal segment of the globus pallidus and substantia nigra pars reticulata which, together, form the output structures of the basal ganglia. In the indirect pathway, information leaving the striatum detours through the external segment of globus pallidus and subthalamic nucleus before proceeding to the output structures.

The function of these anatomical pathways is affected by the nature of the neurotransmitters they release and whether the released transmitter excites or inhibits other cells. In the basal ganglia, fast excitatory neurotransmission is accomplished through release of glutamate and fast inhibition is accomplished via release of gamma-amino butyric acid [GABA]. The direct pathway is purely inhibitory. The indirect pathway, while it has both excitatory and inhibitory elements (see Fig. 2) nevertheless functions as an excitatory circuit because of the way it is constructed. Specifically, the inhibitory connections from the striatum to the globus pallidus are directly followed by inhibitory connections to the subthalamic nucleus. This sets up an 'inhibition of inhibition' which is functionally similar to excitation (Steg et al.). Since the remainder of the indirect pathway, before it merges with the direct stream, contains mainly excitatory connections, the indirect pathway ends up with a net excitatory effect. The two pathways through the basal ganglia are thus functionally opposed.

Dopaminergic inputs to the basal ganglia apparently control the relative efficacy of these two opposing streams of information transfer. This balance determines whether the net relationship between the inputs and outputs of the basal ganglia will be inhibitory or excitatory. Finally since the basal ganglia themselves inhibit the thalamus and the thalamus excites the cortex, increased outflow from the basal ganglia result in inhibition of the cortex. Overall, then, excess activity in the direct pathway results in positive feedback in the motor loop back to the cortex. Conversely, increased activity in the indirect pathway results in negative feedback. A bias towards activity in the indirect pathway will down-regulate signals travelling around the feedback loop. In PD, such a bias occurs when the dopaminergic inputs to the basal ganglia are lost. The resulting down-regulation of information flow in these circuits is associated with the negative symptoms of Parkinson's disease.

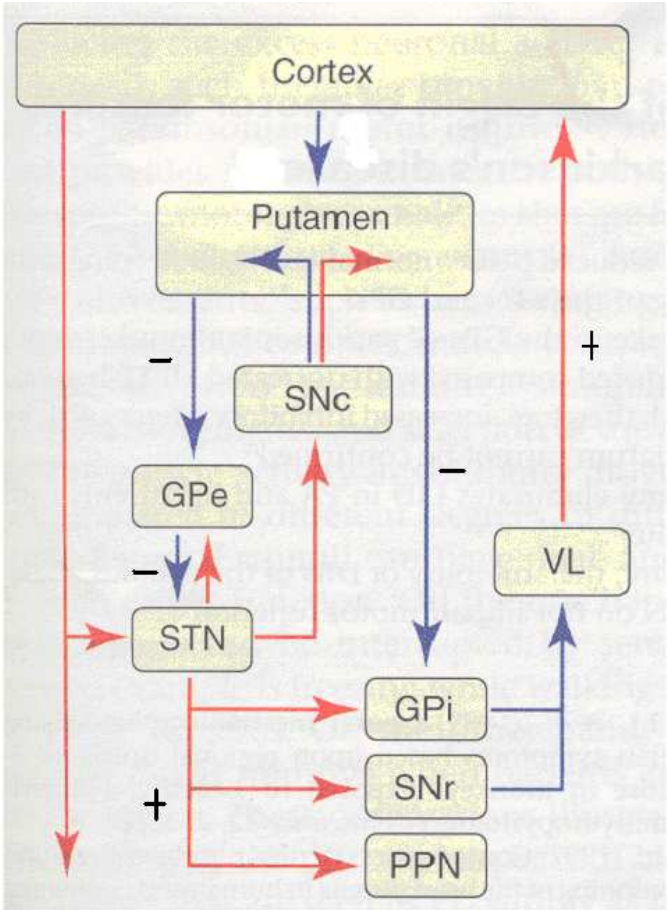


Figure 2: Schematic model of the basal ganglia; normal (Adapted from Obeso et al., 2000)

The two regions of the basal ganglia crucially involved in PD are the substantia nigra pars compacta and the striatum. Neurons of the substantia nigra pars compacta normally manufacture the dopamine that is released in the striatum. In PD, dopaminergic neurons of the substantia nigra die thus depriving the striatum of dopaminergic innervation. This results in the imbalance between the direct and indirect pathways

described above. The symptoms of PD occur when 80% or more of dopamine in the striatum is lost (DeLong el al., 2000).

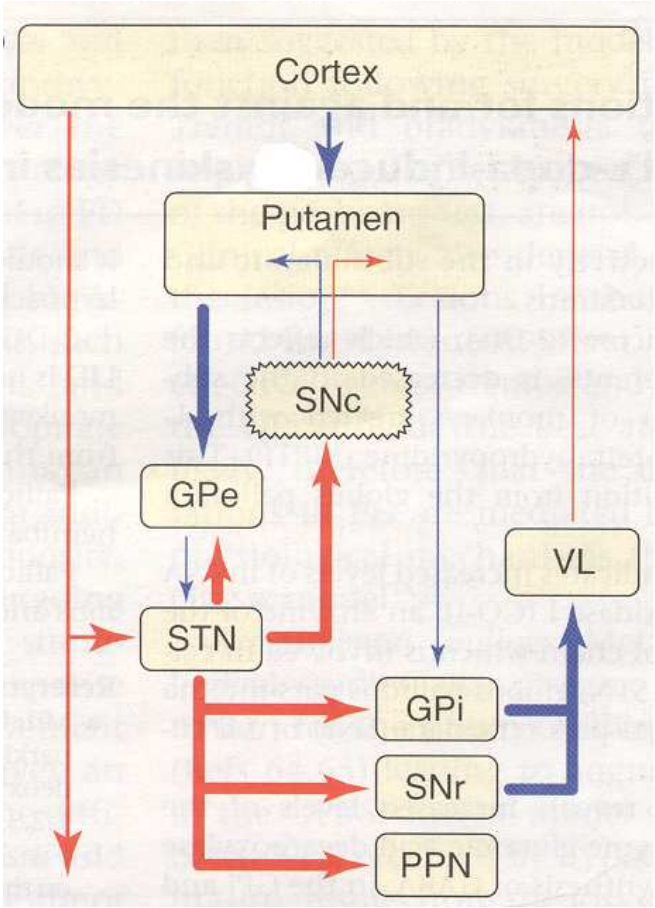


Figure 3: Schematic model of the basal ganglia; PD (Adapted from Obeso el al., 2000)

There are two types of treatment used for PD: surgical and pharmacological. Surgical treatment involves electrocoagulative lesioning (e.g. pallidomy, thalamotomy) or transplantation (eg, fetal nigral transplantation) of the parts of the brain responsible for motor control (Affifi et al., 1998). Even though this type of treatment has proven successful, it is limited by a number of ethical and cross-infection concerns (Zhang et al.). Pharmacological treatments are more commonly practiced. An example is replacement of dopamine using agents such as L-dopa which can be turned into dopamine inside the brain. Dopamine cannot be administered directly because it cannot cross blood-brain barriers. Research shows that L-dopa treatment reduces morbidity and mortality, however it only does so in the short term. Inevitably, the treatment becomes less effective and disease progresses. Therefore, the goal is to find a drug that will have a long-term positive effect.

Recent research (Steiner et al., 1997) shows that systematic injection of an immunophilin ligand, GPI-1046, causes regeneration of brain tissue and neuritic

branching in damaged neurons. In the case of Parkinson's disease, in which a population of dopaminergic neurons innervating the striatum is damaged, this should correspond to a regrowth, in the striatum, of dopaminergic terminals from surviving neurons.

Zhang et al. (2001) studied the influence of GPI-1046 in an animal model of PD (the same model as used in this study). In their study they used rats in which dopaminergic neurons had been lesioned by unilateral microinjection of 6-hydroxydopamine (6-OHDA) in the substantia nigra. The functional consequences of this lesion were a reduction in corticostriatal LTP and abnormal motor behaviour following injection of amphetamine sulfate. Their findings show that treatment with GPI-1046 significantly restored striatal LTP and reduced amphetamine-induced abnormalities. Therefore, they argue, this immunophilin ligand may be effective in treating PD. Previous studies (Steiner, 1997) had already shown that these effects are associated with a re-establishment of dopamine levels and dopaminergic fibres in the affected striatum. Apparently, in these cases, GPI-1046 replenished dopamine levels in the striatum through a process of regrowth of fibres from pre-existing neurons rather than by causing an increase in the number of dopaminergic neurons available for innervating the striatum.

To further investigate the efficacy of GPI-1046 in the rat model of Parkinson's disease we wished to refine the system used for quantifying striatal dopamine levels and the number of dopaminergic neurons in substantia nigra pars compacta. Eventually, this system will yield information on dopamine abundance in individual animals which will be combined with results from electrophysiological investigations in the same animals to yield an overall picture of the restoration of brain chemistry and neurological function resulting from GPI-1046 treatment. The quantitative methods developed here will also be useful in answering the question of where the new dopaminergic innervation of the striatum comes from. In particular, if the number of neurons in the substantia nigra pars compacta is significantly decreased by the lesion (and not increased by the administration of GPI-1046), does all the new dopamine found in striatum originate from the remaining neurons, or do dopaminergic processes from other areas innervate the striatum?

The remaining section of this report describes the part of the study centred on the development of a quantitative system to assess the effect of GPI-1046 on a PD animal model.

The study

The study is divided into 4 series. The first two rounds have been completed while Round 3 and Round 4 are still in progress. In this report findings based on the first two rounds, for which the analyses were done separately, are presented.

Animals

Adult male Long-Evans hooded rats were used for the study. Initially 24 animals were used in each round, however some of them were used for preliminary physiological experiments and some died following lesioning surgery. Therefore, the analysis for Round 1 is based on 15 animals, and of Round 2 on 20. The experimental design for both series is the same; the difference is only in the stereotaxic coordinates used for injection of neurotoxins into the brain, which were slightly changed for Round 2.

The model

The animals were lesioned by unilateral microinjection of a neurotoxin, 6-hydroxydopamine (6-OHDA), in the substantia nigra pars compacta (intracerebral administration). 6-OHDA affects dopaminergic neurons by acting as a substrate for the enzyme tyrosine hydroxylase which occurs in these cells as part of the metabolic machinery for manufacturing dopamine. The interaction between 6-OHDA and tyrosine hydroxylase creates cytotoxic products which destroy these cells. The intracerebral administration of 6-OHDA therefore produces a lesion and lowers the level of dopamine in substantia nigra pars compacta as well as in all the areas of the brain where it projects (predominantly in the striatum).

Experimental design

One half of the animals were injected with 6-OHDA while for the other half normal saline (a salt solution) has been used. Saline should not have any effect, however it was administered in order to find out whether or not the mechanical injury caused by the injection has an effect. Both groups of animals were treated identically.

After two weeks all animals were injected with amphetamine. Amphetamine blocks the uptake of dopamine once it has been released leading to raised extra-cellular dopamine levels and stimulation of dopamine receptors in animals that have had 6-OHDA lesions. This build-up of dopamine in the striatum occurs only on the unlesioned side leading to an imbalance in motor activity.

In order to evaluate the effects of 6-OHDA, the rotational behaviour induced by amphetamine was observed. Since the basal ganglia control motor activity, animals with a 6-OHDA lesion on only one side of their brain tend to turn consistently to one side when they move. To assess this, the number of turns in each direction was recorded over 15 minute periods for all animals. The asymmetry in turning estimates the extent of lesion behaviourally.

The next step was to separate animals into four experimental groups. The 6-OHDA lesioned animals were divided into 2 groups and saline-injected animals into the other two groups. This was done randomly. The animals were identified according to whether they received a 6-OHDA injection (Yes and No groups).

Two weeks after the administration of 6-OHDA the animals were injected with either GPI-1046 (+ vehicle) or an inactive injection. A small amount of ethanol was included to dissolve the GPI compound and was also included in the control injection. The animals were injected once a day, subcutaneously. Therefore, the four experimental groups are identified as:

- Group 1: N-N (not lesioned with 6-OHDA, not treated with GPI-1024),
- Group 2: N-Y (not lesioned with 6-OHDA, treated with GPI-1024),
- Group 3: Y-N (lesioned with 6-OHDA, not treated with GPI-1024), and
- Group 4: Y-Y (lesioned with 6-OHDA, treated with GPI-1024).

After one week of treatment, the rotational behaviour was again observed. The statistical analysis of the data collected has not been completed; therefore the findings for this part of the study cannot be reported yet.

Let us now describe the fluorescence imunohistochemical analyses that were done for this study in more detail.

Fluorescence Imunohistochemical Analysis

The goal of this part of the study was to quantify dopamine levels in the different treatment groups listed above. This was done in two parts of the brain (striatum and substantia nigra). The strategy adopted was to determine dopamine levels indirectly by using an antibody stain directed to the synthetic enzyme tyrosine hydroxylase (the same enzyme that interacts with 6-OHDA to produce the lesion). The antibody was tagged with a fluorescent molecule to enable its visualization with a microscope. High fluorescence in a region indicates high levels of tyrosine hydroxylase enzyme and, presumably, high dopamine levels. It is important to mention that for this part of the study blind analysis were done (the individual was instructed about how the readings should be done, yet was not aware of the experimental design).

The equipment used for this part of the study is a Leica DM microscope with the OpenLab 3.03 software installed on a Macintosh PC.

Step one was to measure the level of dopamine in the ST by quantifying the fluorescent tyrosine hydroxylase (TH) signal. First the immunofluorescent image of the striatum of each animal in the operated and non-operated hemispheres was looked at. In each image the difference of the level of brightness between the striatum (see figure 4) and a nearby dopamine-negative region (called the “external capsule”) was measured. The dopamine-negative region served as measure of non-specific fluorescence in the sample and constituted the background signal. When this background is subtracted from the raw fluorescence levels in the striatum this yields the true level of specific tyrosine hydroxylase associated fluorescence in the image. The procedure for calculating fluorescent levels is given in point form below.

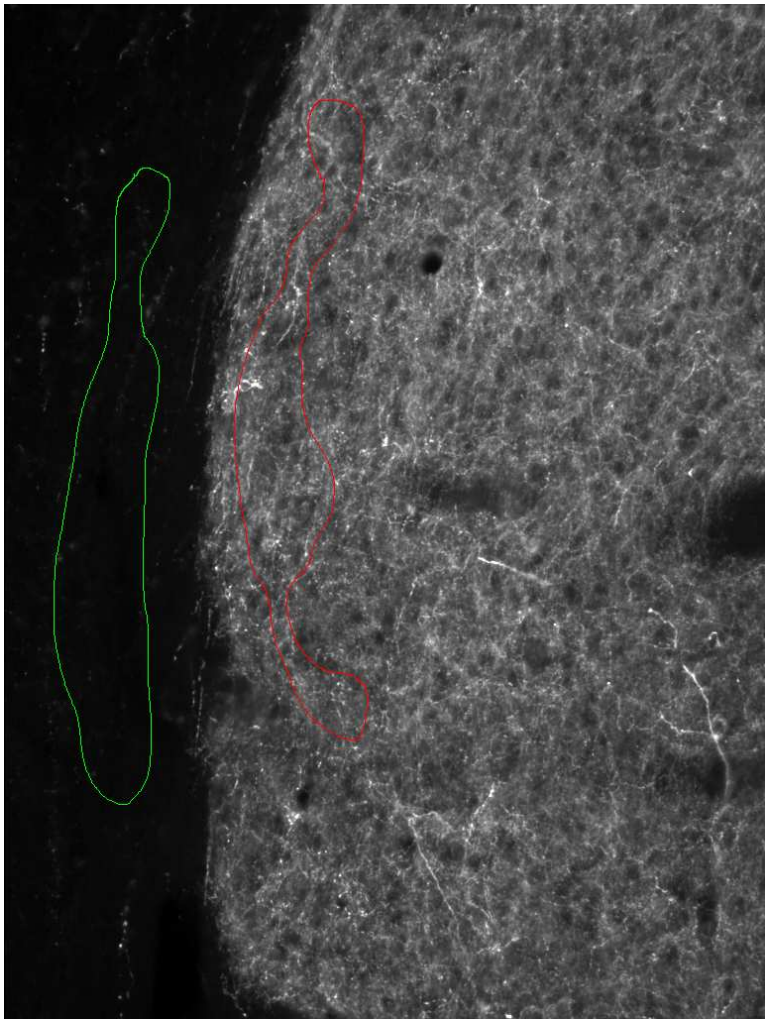


Figure 4: The background subtraction method – measuring the TH signal in the striatum

1. measure difference between TH signal in the ST and the external capsule on operated side:
2. TH signal operated – TH background operated = TH background-subtracted operated.
3. measure difference between TH signal in the ST and the external capsule on non operated side:
4. TH signal non-operated – TH background non-operated = TH background-subtracted non-operated.
5. form the DA_ratio = TH background subtracted operated / TH background subtracted non-operated

The DA ratio calculated in the last step expresses the relative abundance of tyrosine hydroxylase enzyme (and, by extension, dopamine levels) in the two hemispheres. This quantifies the extent of the lesion induced by 6-OHDA.

A separate measure of the severity of 6-OHDA lesions was generated by counting tyrosine-hydroxylase-containing neurons in the substantia nigra pars compacta. As mentioned earlier, this is the point of origin of the dense network of dopaminergic fibres found in the striatum. Unlike the dense forest of interwoven dopamine-containing processes in the striatum this region contains discrete dopamine-containing cells. Injection of 6-OHDA kills a subset of these neurons. The lesion created should therefore be visible as a difference, between the operated and non-operated sides, in the number of tyrosine hydroxylase positive cells.

The counting process used consists of looking through the eyepiece of the microscope and counting within predefined fields. The procedure is given below in point form similar to that listed for the quantification of striatal dopamine earlier.

1. counting the number of tyrosine hydroxylase positive cells in the region of the substantia nigra pars compacta on the lesion side = SN count operated
2. counting the number of tyrosine hydroxylase positive cells in the region of the substantia nigra pars compacta in the non-lesioned side = SN count non-operated
3. form the C_ratio = SN count operated /SN count non-operated

The last step of our analysis was to plot, for each animal, the DA ratio in the striatum vs the C_ratio in the substantia nigra. This forms the core of our assay. In animals with no lesion both ratios should, on average, be equal to 1. In lesioned animals that are *not* treated with GPI-1046, both ratios should be reduced in a correlated fashion. In lesioned animals treated with GPI-1046, however, the DA_ratio in the striatum should be restored back in the direction of the unlesioned animals (i.e., back towards a ratio of 1) whereas the C_ratio in the substantia nigra will remain unaltered. Thus, when the data from animals in all the different groups are plotted on the DA_ratio vs C_ratio graph, all points except for the lesioned and GPI-106 treated rats (the Y-Y group, see above) should fall along the same line. This will be the mark that GPI-1046 has been effective in causing resprouting of dopaminergic fibres in the striatum.

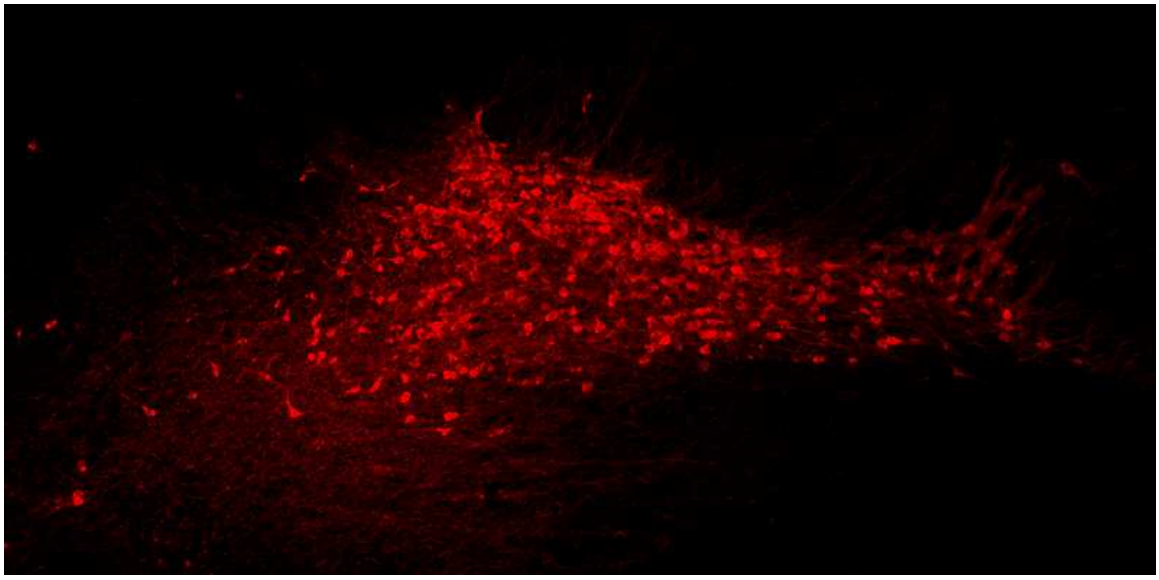


Figure 5: CY3 – identification of dopaminergic neurons in substantia nigra pars compacta

By merging the two images of substantia nigra parc compacta taken under different light wavelengths, we were able to identify the co-localized dopaminergic neurons that projected to the striatum (see figure 6).

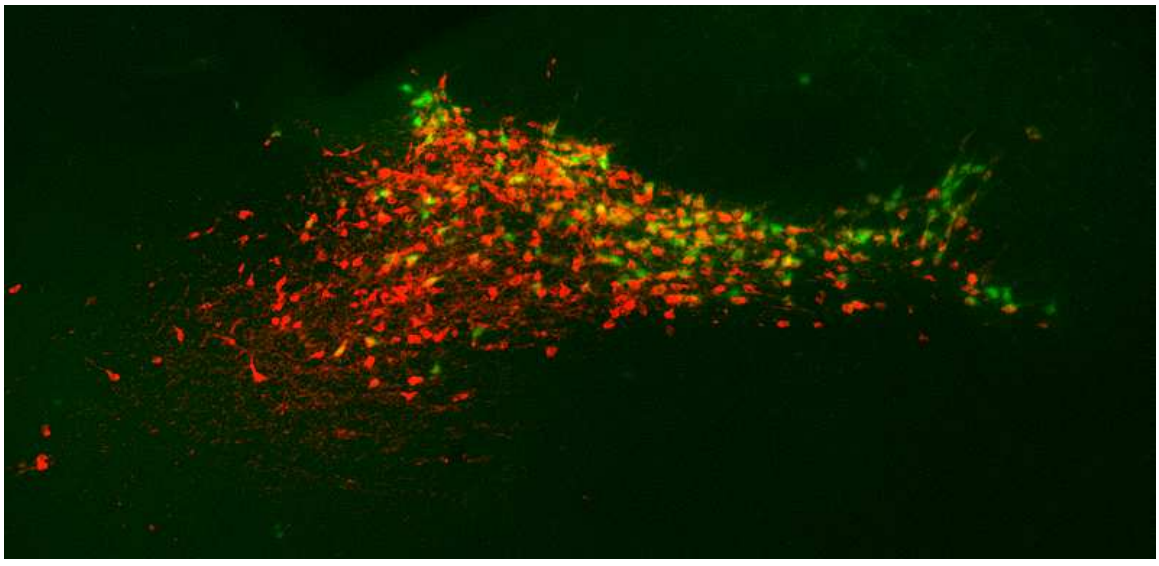


Figure 6: Merged CY3 and fluorogold images – identification of the co-localized dopaminergic neurons

The images taken under the light wavelength that identifies CY3 were also used in order to find the ratio of the number of cells in the substantia nigra pars compacta of both sides of the brain for each animal. By using the immunofluorescent images of substantia nigra pars compacta, a region of substantia nigra pars compacta of non-lesined

and lesioned side, of approximately the same size, was looked at and the number of cells was counted in each (see figures 7, 8, and 9).

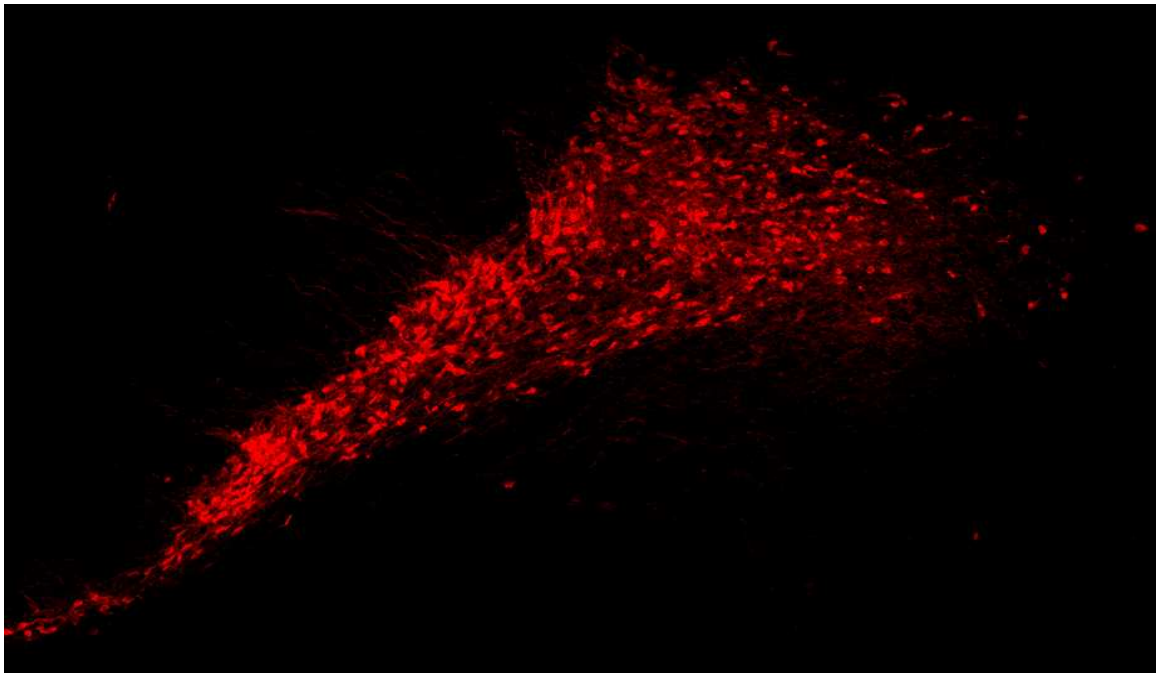


Figure 7: CY3 – substantia nigra pars compacta non-lesioned side

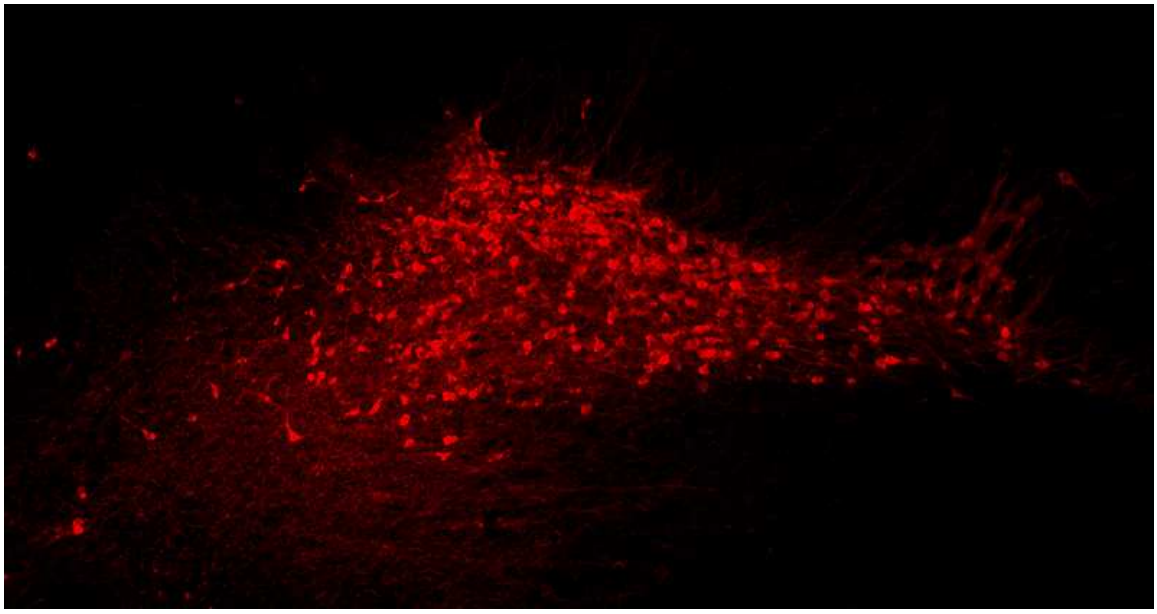


Figure 8: CY3 – substantia nigra pars compacta lesioned side

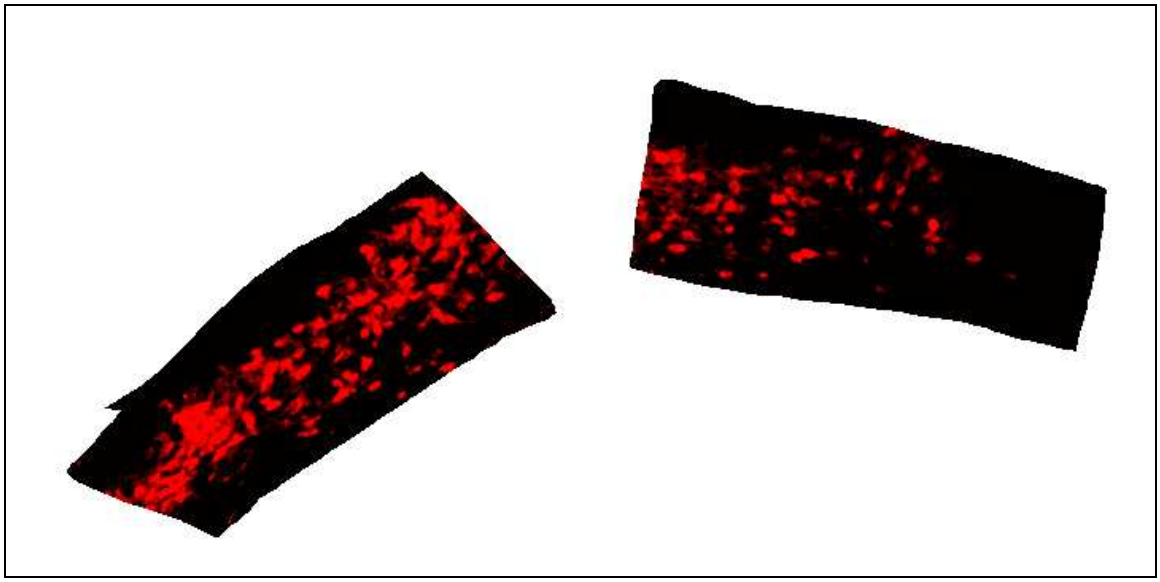


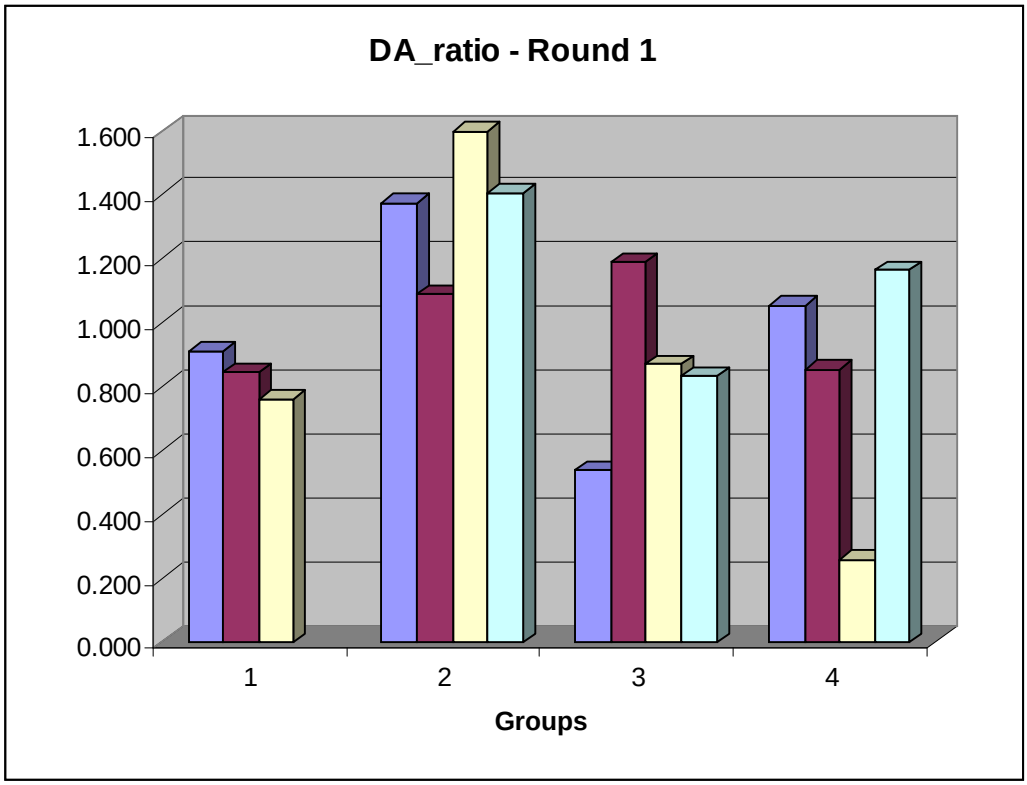
Figure 9: CY3 – Cell count

Analysis of dopamine abundance in the striatum and substantia nigra

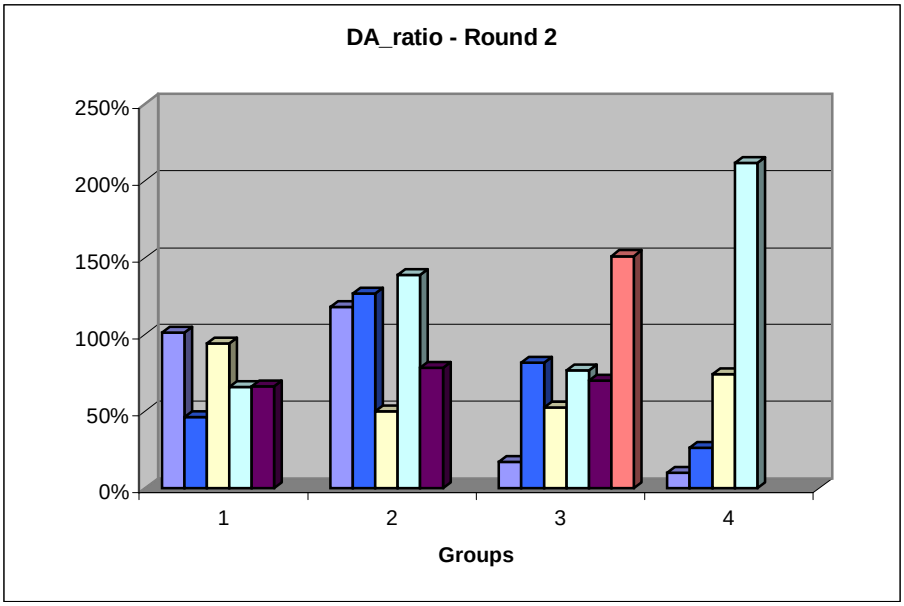
As mentioned earlier in the report, the data collected in Round 1 and 2 was analyzed separately. ANOVA procedures were used to compare the dopamine levels of the four experimental groups. We also compared the groups according to the number of dopaminergic cells found in the substantia nigra pars compacta. For the relationship between the level of dopamine in the striatum and number of dopaminergic cells in the substantia nigra pars compacta MANOVA procedures (with two dependent variables) were used.

DA_ratio

Our data show that there was no significant difference between the dopamine ratio of the four experimental groups in Round 1 ($F_{3,4}$=3.481, p>.05; see Graph 1); and Round 2 ($F_{4,6}$= .331, p>.05; see Graph 2).



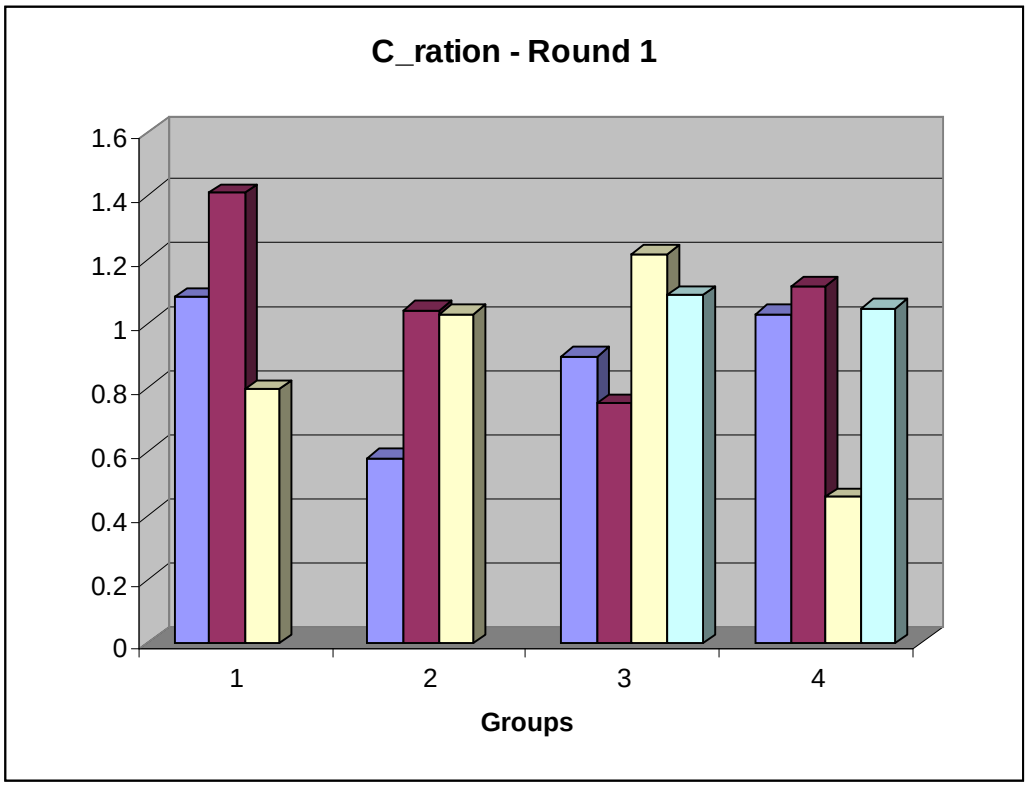
Graph 1: Dopamine ratio of each animal in Round 1. Data are grouped by treatment as explained above: Group 1, N-N; group 2, N-Y; group 3 Y-N; group 4, Y-Y.



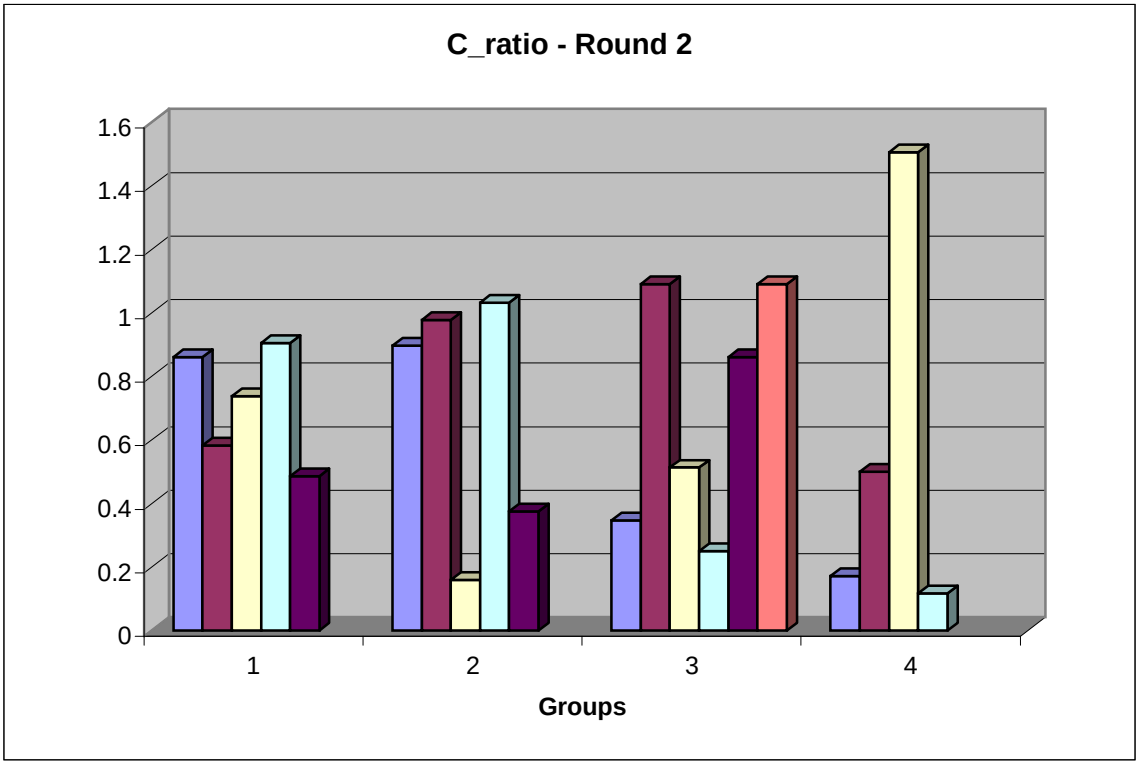
Graph 2: Dopamine ratio of each animal in Round 2. Data are grouping as in Graph 1.

C_ratio

There is also no significant difference between the ratio of dopaminergic cells in the substantia nigra between the four experimental groups in Round 1 ($F_{3,4}$=.94, p<.961; see Graph 3) and Round 2 ($F_{4,6}$=.100, p<.956; see Graph 4).



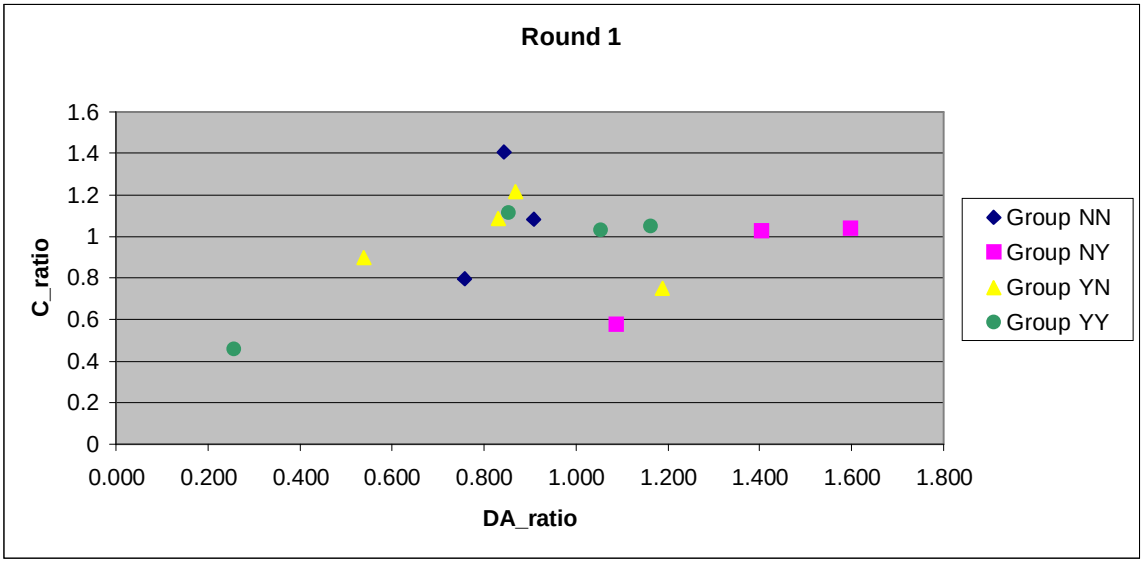
Graph 3: C_ratio of each animal in Round 1. Data are grouped as in Graph 1.



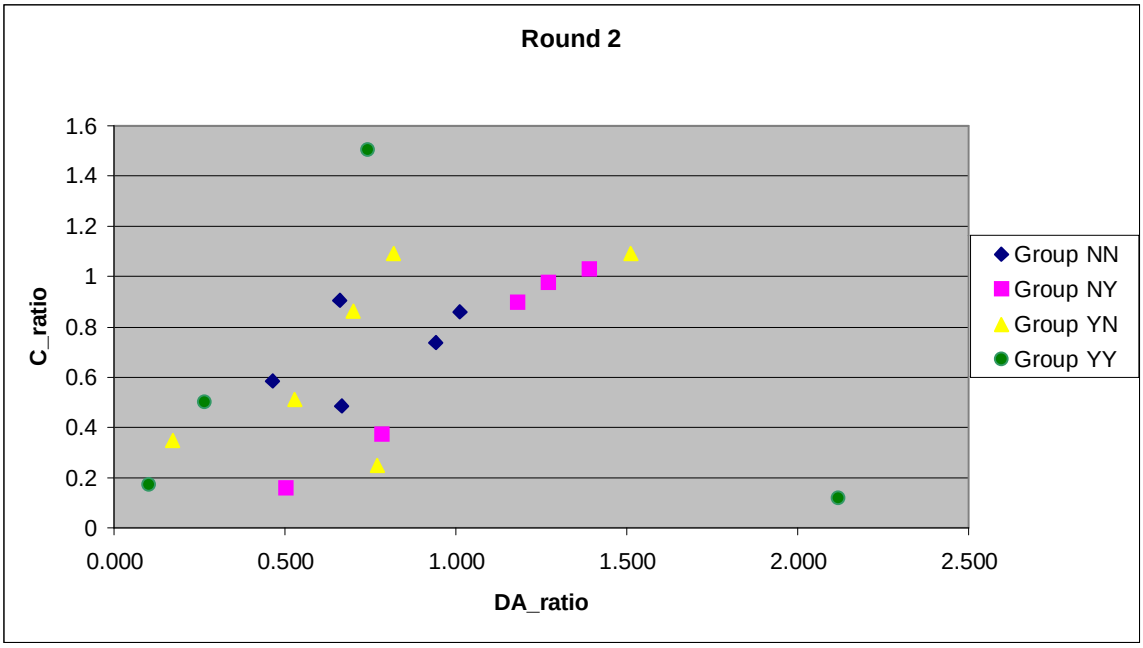
Graph 4 : C_ratio of each animal in round 2. Data are grouped as in Graph 1.

Plots of C_ratio vs DA ratio are shown below for rounds 1 and 2. These show that the two measures of lesion severity are correlated. Data points for the different experimental groups are coded by colour. The most potentially interesting group is composed of those animals in which a lesion has been produced and the animal then treated with GPI-1040 (green points in Figures ? and ?). As explained earlier, we expect these to form a distinctive grouping below and to the right of the rest of the data (i.e., low C_ratio and high DA_ratio) in such plots. This was not seen.

Statistically, our data show no significant difference between the four experimental groups in Round 1 ($F_{3,4}$=.331, p>.05); (see Graph 5) and Round 2 ($F_{4,6}$=.331 p>.05) (see Graph 6).



Graph 5: DA_ratio/C_ratio – Round 1



Graph 6: DA_ratio/C_ratio – Round 2

Studies using fluorescent retrograde tracers

As mentioned earlier, one of the questions addressed in this study is the site of origin of the increased dopamine found in the ST following treatment with GPI-1046: are the newly sprouted dopaminergic fibres exclusively from neurons in the substantia nigra that survived the lesioning. To answer this we made use of a retrograde neuronal tracer

known as “fluorogold”. This stain is used to identify populations of neurons that innervate a target region since, once injected into a brain region, it penetrates local fibres and travels backwards along the axon to the cell body.

Fluorogold fluoresces at a different set of wavelengths than the stain we used to mark tyrosine hydroxylase. By using appropriate sets of filters both fluorescent signals can be viewed in the same preparation. Dopaminergic neurons that project to the striatum are identified by merging the two views and noting in which cells the markers coexist (see figure 6). Normally, such neurons only exist in the substantia nigra pars compacta. Following lesioning and GPI-1046 treatment, we searched for such cells in other areas hoping to find regions that have been encouraged to send new dopaminergic projections to the striatum.

Results using retrograde tracer

The fluorogold results for both Round 1 and Round 2 show that the dopamine in the striatum does not come from the ventral tegmental area or contralateral unlesioned substantia nigra. (see figure 6). Almost all neurons with colocalized markers for tyrosine hydroxylase and fluorogold were located in the substantia nigra on the side of 6-OHDA (or, in the case of controls, saline) injection. Neurons bearing colocalized markers in the ventral tegmentum, retrorubal field, or contralateral substantia nigra were either absent (most animals) or exceedingly rare and no different from controls.

Discussion

In this study, by using the retrograde tracer fluorogold, we found that that in lesioned animals injected with GPI 1046 the dopaminergic re-innervation of the striatum is not through growth of branches from the dopaminergic neurons of the ventral tegmentum or from neurons in the contralateral (unlesioned) substantia nigra

However, at this point, our data do not completely support the effect of GPI-1024 on the PD animal model.

Concerning the level of dopamine in the ST, the expectations were the following:

a. Group 3, lesioned with 6-OHDA and not treated with GPI-1064, should be significantly different from the other three experimental groups.
b. The control group (Group 1), and the treated group (Group 4) should not differ if the drug has an effect.
c. Group 2, non-lesioned but treated, should not differ from Group 2 and Group 4, since the drug should not have an effect on healthy tissue.

Our findings do not support the first point, yet points b and c are supported.

In relation to the number or dopaminergic cells in the SNc, the expectations were the following:

a. Groups 1 and 2 should not significantly differ since neither one of these groups had a 6-OHDA lesion.
b. Groups 2 and 3 should not significantly differ since both of these groups were lesioned with 6-OHDA.
c. Groups 1 and 2 should significantly differ from Groups 3 and 4 (non-lesioned vs. lesioned groups).

Our finding support points a and b, however the most important point in this case, point c is not supported.

The MANOVA analysis of the DA_ratio and C_ratio data shows no significant difference between the the experimental groups. The expectations were the following:

- **a.** Groups 1 and 2 should not significantly differ, in other words the points on the graphs that represent animals from these two groups should cluster together. Their dopamine level as well as their cell ratio should be along the same lines.
- **b.** Group 3 should show low dopamine and small number of cells, since this groups is lesioned and non-treated.
- **c.** Group 4 should have number of cells similar to those of Group 3, however the level of dopamine should not significantly differ from the same of Groups 1 and 2.

The data of the Round 1 were far from what we had expected. The effect of GPI should have been to disrupt the straight-line relationship by increasing DA_ratio while leaving C_ratio constant. This was not seen. However, the data of Round 2 show better relationship between the two. The correlation between the DA_ratio and C_ratio is strong (see Graph 6) This means that, in any individual animal, when there is an imbalance between the number of dopaminergic neurons in the substantia nigra there is a corresponding imbalance in the TH signal in the striatum. Thus, the quantification techniques that we have developed are working well.

In general, our findings based on DA_ratio and C_ratio analysis separately, suggests that perhaps the drug does have an effect on a PD animal model. Nevertheless, such a claim cannot be made just yet. As mentioned earlier in this report, the study is still in progress, and in the first two series we experimented with the extent of the lesion as well as with its coordinates. Another possibility is that the injections in the striatum produce a mechanical lesion there in which case the immunophilin ligand GPI-1046 would have an effect independent from any lesion in the substantia nigra. The fluorogold injection to the striatum will be avoided in Round 3 and if necessary in Round 4. Another possible reason that the studies done so far had low statistical power is that they involved too few subjects, and thus the ability to detect an effect was limited. Perhaps if this number of animals was bigger, the expected difference between the groups could be demonstrated. Further research is needed and we expect an improvement in the series of experiments to follow.

However, it is important to note that we have developed a reliable and fairly accurate quantitative system for assessing the effects of GPI-046 on a PD animal model. The quantitative techniques we constructed and applied are exceptional for measuring the correlation between DA_ratio and C_ratio in individual animals. They accurately show that when there is imbalance between the number of dopaminergic cells in the substantial nigra, there is a corresponding imbalance in the TH signal in the striatum. Therefore, the quantification techniques used are working quite well.

References:

Afifi AK, Hoesen GV, Bechara A, and Rodnitzky R. 1998. Functional Anatomy of Basal Ganglia .Retrieved from www on March 12, 2002

[http://biology.about.com/gi/dynamic/offsite.htm?site=http://www.hitl.washington .edu/publications/prothero/node52.html]

DeLong, MR. 2000. *The Basal Ganglia*. In: ER. Kandel, JH Schwarts, and TM Jessel (eds.). *Principles of Neural Science.* 4th ed. New York McGraw-Hill.

Korczyn, AD. *Parkinson's Disease*. Retrieved from www on March 12, 2002 [http://www.acnp.org/g4/GN401000142/Default.htm]

Nieuwenhuys R, Voogd J, and Huijzen C. 1981. *The Human Central Nervous System: A Synopsis and Atlas*. 2nd ed. Berlin: Springer.

Obeso JA, Rodriguez-Oroz MC, Rodriguez M, Lanciego JL, Artieda J, Gonzalo N, and Olanow W. 2000. *Pathophysiology of the Basal Ganglia in Parkinson's Disease*. Trends in Neuroscience. Vol. 23, No. 10[268] (Suppl.). pp. S8-S19.

Schwarting RKW and JP Huston. *The Unilateral 6-Hydorxydopamine Lesion Model in Behavioural Brain Research. Analysis of Furnctional Deficits, Recovery and Treatments*. Progress in Neurobiology, Vol. 50, pp 275-331. 1996.

Steg G and Johnels B. Year. *Physiological Mechanisms and Assessment of Motor Disorders in Parkinson' s Disease*Advances in Neurology, Vol. 60:XX

Steiner JP, Hamilton GS, Ross DT, Valentine HL, Guo H, Connolly MA, Sauer H, Nowotnik AC, Suzdak PD, 1997. *Neurotrophic Immunophilin Ligands Stimulate Structural and Functional Recovery in Neurodegenerative Animal Models*. Proc Natl Acad Sci USA 94: 2019—2024.

Zhang C, Steiner JP, Hamilton GS, Hicks TP, and Poulter MO. *Regeneration of Dopaminergic Function in 6-Hydorxydopamine-Lessioned Rats by Neuroimmunophilin Ligand Treatment*. J. Neurosci. 2001, Vol. 21.