Exploration of Variability of Arkypallidal and Prototypical Projections to Dopamine 1 and Dopamine 2 Populations of the Putamen

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July 13, 2016

Abstract

The human brain is one of the world's greatest mysteries, the process of mapping its interactions have only just begun. The *basal ganglia*, which is the medial section of the human brain, is believed to have a crucial role in our ability to voluntarily move. This study investigates the internal connections of the basal ganglia; more specifically the monosynaptic connections between the *putamen* and the *external segment of globus pallidus*. The connections of prototypic and arkypallidal neurons (located in the external segment of globus pallidus) to both dopamine 1 and dopamine 2 receptor neurons of the putamen are explored by using retrograding viral tracing and immunofluorescence staining. The results of this study show the location of neurons in the external segment of globus pallidus projecting to the either dopamine 1 or dopamine 2 receptor neurons. The results also show the preferential target neurons of both arkypallidal and prototypic neurons.

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List of Abbreviations

CP_u	Putamen
Cre	Cre recombinase
Cy	Cyanine
D1	Dopamine 1
D2	Dopamine 2
G	Rabies Glycoprotein
GFP	Green Fluorescent Protein
GP	Globus Pallidus
GP_e	External segment of Globus Pallidus
GP_i	Internal segment of Globus Pallidus
PBS	Phosphate-buffered saline
RVdG	Rabies-glycoprotein-deleted rabies virus
STN	Subthalamic Nucleus
TBS-T	Triss-buffered saline containing 0,03% Trikon

1 Introduction

Patients suffering from neuropsychiatric disorders, such as depression, schizophrenia, autism, Parkinson's disease and bipolar disorders, are today treated with psychotherapeutic drugs developed from findings brought to light before 1960 in conjunction with clinical observation. Researchers are currently working on mapping the neural pathways for future use in medical purposes. If the human brain were successfully mapped, many side effects of current treatments could be avoided. Since there are both ethical and practical difficulties with examining the human brain, animal models are frequently used, most commonly used are transgenic mice. [1]

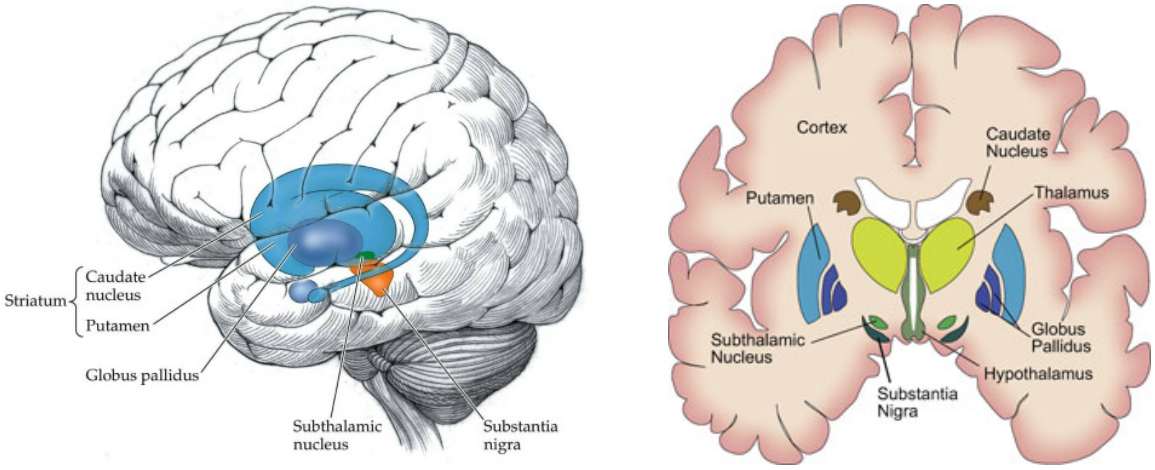
1.1 The Basal Ganglia

The human brain contains neurons, glial cells and blood vessels and is divided into several sections [2]. The basal ganglia, part of the cerebrum and located under the cerebral cortex, has a central role in the voluntary movement and, contradictory to other parts of the motor system, does not directly interact with the spinal cord [2]. Research has lead to an overall knowledge about the basal ganglia circuits and due to its relevance for future treatments of movement disorders, the motor circuit is the most recently studied, a work we will continue [3].

1.1.1 Anatomy of the Basal Ganglia

The basal ganglia consists of numerous linked subcortical nuclei that receive input from the cerebral cortex and thalamus. The nuclei send the output through the thalamus back to the cortex and directly to the brain stem. The four functionally distinct groups important for the motor circuit are: the striatum, the globus pallidus (GP), the substantia nigra and the subthalamic nucleus, see (Figure 1a). [2]

The striatum is divided into two regions: the caudate nucleus and the putamen (CP_u), see Figure 1b. The striatum is thought to be the primary input station for information from the cerebral cortex, thalamus and the brain stem. Further on, the signals arriving in the striatum proceed to reach the globus pallidus and the substantia nigra. These two sections of the basal ganglia are considered to be the output nuclei and inhibit their target area in the thalamus and brain stem. The inhibitory outputs are thought to be controlled by two different neural pathways: the direct and the indirect pathway. [2]



(a) (b)

Figure 1: Structure of the basal ganglia. (a) The four bigger sections of the basal ganglia; the striatum, the globus pallidus, the subthalamic nucleus and the substantia nigra.[1](b) Cross-section of the human brain showing; the caudate nucleus and the putamen (striatum), globus pallidus (internal and external segment), substantia nigra and subthalamic nucleus. The figure also illustrates the cortex, thalamus and the hypothalamus. [2]

[1]Nostalgos. Neural Circuits of Fear and Anxiety towards the Extinction of Anxiety, Phobias and Psychological Traumas [Online]. [cited 2016-07-09] Available from: `http://www.nostalgos.com/8.html`

[2]We All Have Unique Brains. Basal Ganglia [Online] [cited 2016-07-12] Available from: `http://www.weallhaveuniquebrains.com/brain_anatomy/basal_ganglia/`

1.1.2 The Motor Circuit

The striatum receives input from the cortex and the substantia nigra [4]. The output neurons of the striatum have either dopamine 1 (D1) or dopamine 2 (D2) receptors which bind to dopamine, a common neurotransmitter [2]. These receptors generate the direct and indirect pathway [2], found in Figure 2. D2 receptors activate the indirect pathway whereas the direct pathway is activated by binding to D1 receptors [2].

The direct pathway proceeds from the striatum, more specifically from the caudate nucleus and the putamen, when inhibitory projections reach the inhibitory neurons in the internal section of the globus pallidus (GP_i). The GP_i then projects to the thalamus. In the indirect pathway inhibitory neurons in the external section of globus pallidus (GP_e) are activated by signals proceeding from inhibitory neurons in the striatum (caudate and putamen). The neurons of the GP_e project to the subthalamic nucleus. The subthalamic nucleus also receives strong excitatory signals from the cortex. All signals picked up in the subthalamic nucleus then proceed to the GP_i. The activating drive of the subthalamic nucleus then works to resist the disinhibitory response of the direct pathway. In corresponding ways the indirect pathway regulates the direct pathway effect. [4]

The motor circuit (the circuit between the basal ganglia and the thalamus) is provided with both positive and negative feedback. The positive feedback comes from the direct pathway whereas the negative feedback is received from the indirect pathway. By stimulation of the direct pathway, the thalamus is disinhibited and increases its activity. The indirect pathway works the other way around and decreases the thalamocortical activity. This results in an activation of the direct pathway which promotes movement, whereas an inhibition of movement is accomplished by stimulation of the indirect pathway. [2]

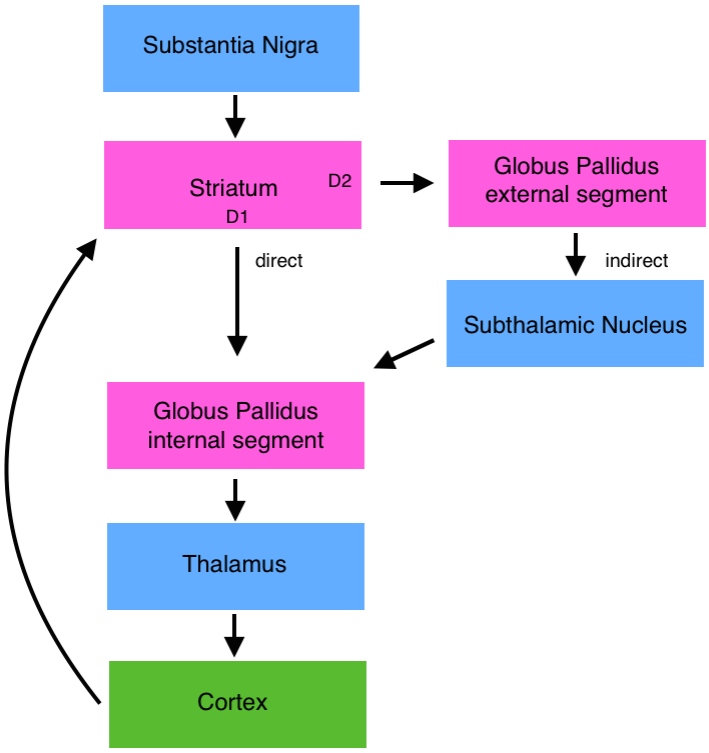


Figure 2: The direct and indirect pathway of the basal ganglia. The direct pathway (activated by dopamine binding to D1 receptors) proceeds from the striatum to the thalamus via the GP_i. The indirect pathway is activated when dopamine binds to the D2 receptors and proceeds from the striatum to the GP_i via the GP_e and the subthalamic nucleus before reaching the thalamus. The striatum receives input from the cortex and the substantia nigra.

The amount of dopamine released determines the balance between the direct and indirect pathway signals. A release of dopamine in the striatum increases the motion of signals along the direct pathway which is initially activated by the binding of dopamine and D1 receptors. Dopamine released in the striatum also acts on D2 receptors, connected to the indirect pathway, and decreases efficiency along the indirect pathway. In case of a lower dopamine concentration in striatum, the indirect pathway works more efficiently than the direct pathway. [3]

1.1.3 The External Segment of Globus Pallidus

Although the direct and indirect pathway model hypothesizes that the GP_e receives input from the striatum and sends downstream signals to the subthalamic nucleus, some re-

searchers believe that the pathway communication through the GP_e is even more complex [5]. It has also been predicted that a deeper understanding of the GP_e neurons heterogenity would be crucial to comprehend both the basal ganglias function and dysfunction [5]. Research has indicated that the GP_e consists of two types of neurons; *arkypallidal* and *prototypic neurons* [6]. The arkypallidal neurons are thought to only project input to the striatum whereas the prototypic neurons are believed to sporadically innervates the striatum but also consistently send downstream signals in the basal ganglia circuit [5]. The prototypic neurons express, among others, the transcription factor Nkx2-1 while arkypallidal neurons express the FoxP2 transcription factor [6]. This genetic difference could be important when differentiating the two GP_e neurons. The majority of the prototypic neurons also express the protein parvalbumin (PV) [5].

1.2 Background to Method

1.2.1 Retrograding Viral Neural Tracing with Deactivated Rabies Virus and Cre-expressing Mice Lines

Around 2007 a method using a rabies virus where rabies glycoprotein (G) has been deleted (RVdG) was introduced and has been widely used since. This method has enabled researchers to identify presynaptic partners to specific neurons regardless of the distance between two neurons. Except from the modified rabies virus, transgenic animal models need to be used. [7]

The natural habitat of an intact rabies virus includes spreading though numerous synaptic steps. For scientific purposes, the rabies virus has been modified to only spread monosynapticly. This is achieved by eliminating the gene for G in the virus genome. The deletion of G also prevents the organism from being infected by rabies since the virus becomes monosynapticly restricted. [7]

A genome sequence for green fluorescent protein (GFP) needs to be inserted into the rabies virus genome for researchers to be able to mark the infected cells. For the GFP to be expressed in the infected cells, it is essential that the target cells have a genome that codes for the enzyme Cre recombinase (Cre). Therefore mice lines that are genetically manipulated to express Cre have been developed. [7]

To enable the rabies virus to bind to the Cre-expressing target neurons, a helper virus is injected into the mouse brain. The helper virus will bind to Cre positive cells and transfer their genome so that Cre positive cells exclusively express an avian-specific retroviral receptor (TVA) and G. TVA is the receptor that makes it possible for the rabies virus to bind to the cells and the function of G is for the rabies virus to recognize which cells to bind to. When the helper virus, as seen in step 1 (Figure 3), is injected to the area of interest, this results in the expression of TVA and G in the Cre-expressing cells (marked with red and green in step 2, Figure 3). Later the rabies virus is injected in the same location (see step 3 Figure 3). The rabies virus will infect the starter cells labeled with TVA and G and its genome will be replicated in the starter neurons. The Cre will bind to the genome sequence and cleave off the sequence coding for GFP, this resulting in the production of rabies viruses with G on their membrane. This G + RVdG virus will spread trans-synapticly to input cells which will then express GFP (seen as green in step 4 Figure 3). The G + RVdG virus goes back one synapse, which means it is a retrograding virus. Both the starter neuron and the input neurons will be marked with GFP (green in Figure 3) whereas Cre negative neurons and neurons which are not directly presynpatic to the starter neurons will be unlabeled. [7]

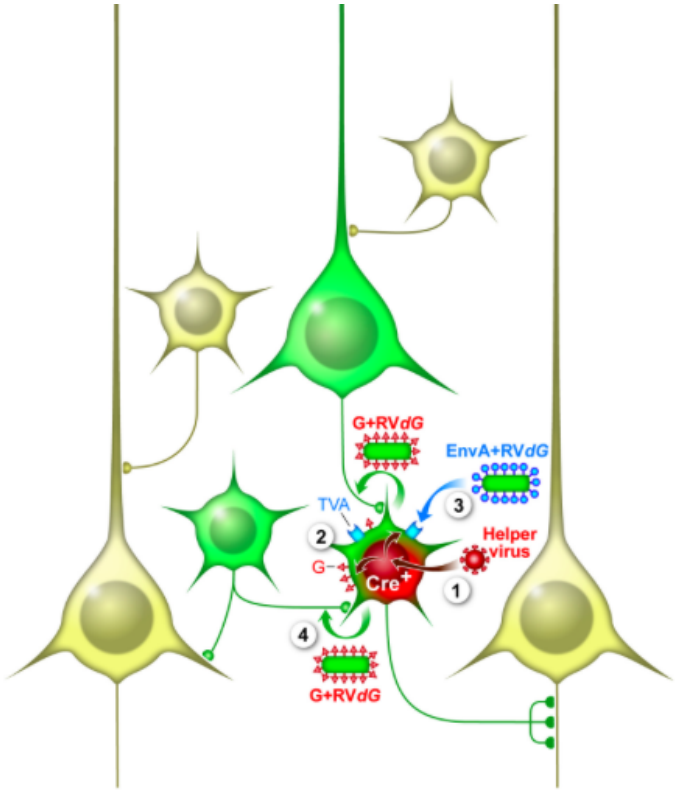


Figure 3: (1) infection of helper virus, (2) expression of TVA and G in Cre-expressing neuron, (3) infection of the rabies virus (4) monosynaptic spread of the G + RVdG virus.[3]

1.2.2 Antibodies and Immunofluorescence Staining

Antibodies are Y-shaped proteins and can be divided into primary and secondary antibodies [8]. The primary antibodies are developed to target specific target genes or proteins whereas the secondary antibodies are meant to bind to the primary antibodies [9]. The primary antibodies used in laboratories are produced in different host species [9]. Commonly used are rabbits, goats, chickens and guinea pigs [9]. Whether or not a secondary antibody can bind to a specific primary antibody depends on the host of the primary antibody [9]. For example, a secondary anti-rabbit antibody can only bind to a primary antibody produced in a rabbit [9]. This mechanism is utilized in a laboratory technique to target genes and proteins called immunofluorescence staining [10].

[3]Callaway E, Luo L. Monosynaptic Circuit Tracing with Glycoprotein-Deleted Rabies Viruses. The Journal of Neuroscience. 2015;35(24): 8979-8985

There are two types of immunofluorescence staining; direct and indirect [10]. The method used in this study will involve the indirect immunofluorescence, which means that a primary antibody is added to the target cells and later on a secondary antibody conjugated to a fluorescent dye is added [10]. Cyanine dyes (Cy) are commonly used fluorescent dyes that exists in several versions, among others Cy3 and Cy5 [11].

1.3 Aim of Study

The aim of this study is to explore the variability of arkypallidal and prototypic neurons in the GP_e and investigate their projections to D1 (direct pathway) and D2 (indirect pathway) populations in the CP_u. This could be essential for development of future psychotherapeutic drugs and treatments.

2 Method

The neural pathways of the brain were studied by using Cre-expressing mice lines whose brains had been injected with a helper virus and modified rabies virus. Fragmentation of the mice brains was done, thereafter immunostaining and an optical microscope were used for analysis.

2.1 Stereotactic Viral Injection

Two mice from different mice lines (Adora2a-cre and D1-cre) were used for the injection of helper virus and rabies virus. In the Adora2a-cre mice line the Cre had been expressed in cells which had the D2 receptor and in the D1-cre mice line, Cre was instead expressed in cells containing the D1 receptor. Both lines were of the laboratory mouse type C57BL/6. A surgery was performed where helper virus (TVA-V5-2A-RG) was injected in the putamen (CP_u) of both mice, see Figure 4. One week after the helper virus injection, rabies virus (Rabies-EGFP) was injected into both mice in the same area. The mice brains were then removed and perfused with 4% paraformaldehyde (PFA), to keep the tissue alive.

To prepare the mice brains for further use they were placed in holders, then filled with liquid agaros gel. The holders were placed in a refrigerator for one hour for the agaros gel to solidify.

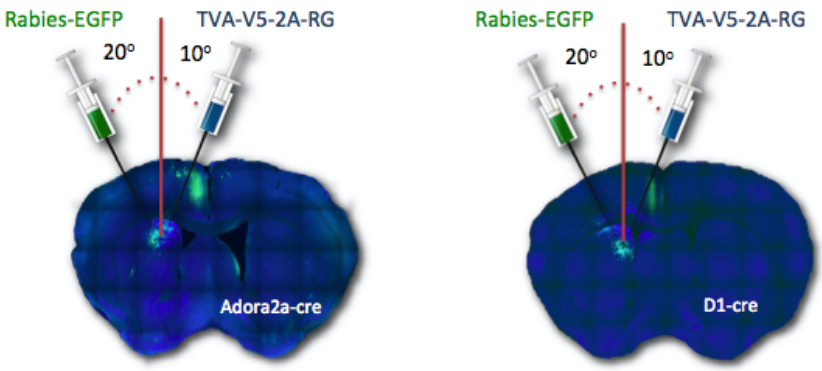


Figure 4: Helper virus (TVA-V5-2A-RG) injection and rabies virus (Rabies-EGFP) injection in the two mice (One mouse from the D1-cre mice line and one from the Adora2a-cre mice line). The picture displays which angles were used for the injection. Rabies-EGFP were injected with an 20° angle and the TVA-V5-2A-RG with an 10° angle, to avoid contamination.

2.2 Fragmentation of Mice Brain Using Vibratome

A vibratome (Leica VT1200 S) was used for the fragmentation of the mice brains and the setting for fragment thickness was set to 60 µm. The mice brains situated in agaros gel were attached to a specimen disc using super glue. After each section was cut it was placed in a marked tissue culture plate containing Phosphate-buffered saline (PBS). This course of events was repeated until the whole brain had been fragmented. Thereafter the marked tissue culture plate containing the brain fragments was placed in a cold room for future usage.

The brain fragements were placed in Triss-buffered saline containing 0,03 % Trikon (TBS-T) for ten minutes. The TBS-T affects the cell membrane's permeability by making it more unstable. This enables antibodies to more easily pass through the membrane, and

was important to prepare for future usage.

2.3 Immunofluorescence Staining

To differentiate the prototypic and the arkypallidal neurons in the GP_e, immunostaining was performed which utilizes the cells' ability to express different transcription factors and proteins. The fragments were placed in Donkey-serum to block interfering antibodies before the immunostaining. Three primary antibody solutions were prepared (listed in Table 1). One third of the fragments of D1-cre mice line brain were placed in each solution. The same procedure was repeated with the fragments from the Adora2a-cre mice line and the brain tissue fragments were left in the solutions over night.

Table 1: Preparation of primary antibody solutions. The different primary antibodies added were; *c-a-V5*: chicken anti-V5, *r-a-Nkx2.1*: rabbit anti-Nkx2.1, *r-a-foxp2*: rabbit anti-foxp2 and *gp-a-PV*: guinea pig anti-PV

Solution	TBS-T	c-anti-V5	r-a-Nkx2.1	r-a-foxp2	gp-a-PV
1	500 µL	1 µL	-	-	-
2	1000 µL	-	1 µL	-	1 µL
3	1000 µL	-	-	1 µL	1 µL

After lying in antibody solutions over night the brain fragments were washed in TBS-T for ten minutes to eliminate unattached primary antibodies. This washing step was performed three times. Afterwards the fragments were placed in solutions containing secondary antibodies with Cy for two hours. The markers used were Cy3-rabbit, Cy3-guinea pig, Cy5-rabbit, Cy5-guinea pig and Cy5-chicken.

Later, the fragments from the D1-cre mice line were placed in containers with different secondary-antibody-solutions based on which primary-antibody-solution they had first been placed in; Solution 1, 2 or 3 (Table 2).

Table 2: Different secondary antibodies were added to various D1-cre fragments based on which primary antibody solutions they were first placed in.

D1-cre fragments from:	**Markers on added secondary antibodies:**
Solution 1	Cy5-chicken
Solution 2	Cy3-rabbit, Cy5-guinea pig
Solution 3	Cy3-rabbit, Cy5-guinea pig

The fragments from the Adora2a-cre mice line were placed in containers with different secondary-antibody-solutions based on which primary-antibody-solution they had first been placed in; Solution 1, 2 or 3 (Table 3).

Table 3: Different secondary antibodies were added to various Adora2a-cre fragments based on which primary antibody solutions they were first placed in.

Andora2a-cre fragments from:	**Markers on added secondary antibodies:**
Solution 1	Cy5-chicken
Solution 2	Cy3-guinea pig, Cy5-rabbit
Solution 3	Cy3-guinea pig, Cy5-rabbit

TBS-T washing steps were repeated twice, thereafter all brain tissue fragments were washed four times in PBS to remove TBS-T and placed in a cold room.

2.4 Sample Mounting

To be able to observe the results, the mice brain fragments were placed on microscope slides. One brain tissue fragment at a time, starting with the most anterior fragment and proceeding posteriorly, was taken from the tissue culture plate and first placed in a PBS-filled petri dish to be fixed on a microscope slide by using glycerol and a cover slip. When all brain fragments had been placed on microscope slides, the slides were stored cold for future operation.

2.5 Epifluorescence Microscope

The microscope slides were removed from the cold room and placed in an epifluorescence microscope (Leica Fluorescence Microscope). Photos were then taken where different markers were represented by different colours (green, red and blue) depending on which filter were used.

3 Results

The following results represent pictures taken after injection of Rabies-EGFP and TVA-V5-2A-RG in one mouse from the D1-cre and one from the Adora2a-cre mice line. The pictures taken of the D1-cre mouse brain indicates connections between GP_e neurons projecting to D1-neurons in the CP_u. The connections investigated in pictures taken of the Adora2a-cre mouse brain are those between GP_e neurons and neurons in the CP_u with D2-receptors. The D1 receptors are connected to the direct pathway while the D2 receptors are connected to the indirect pathway.

Control pictures of the starter cells of the injection in CP_u can be seen in Figure 5. In Figure 6 the location of the CP_u-projecting neurons in the GP_e are shown. Figure 7 and 8 identify the CP_u-projecting neurons as prototypic or arkypallidal. Different colours are used to identify the markers for the transcription factors Nkx2-1 and Foxp2 and the PV protein. Lastly, two diagrams summarizing the pictures in Figure 7 and 8, are shown in Figure 9 and 10.

3.1 Retrograde Labeling of Starter Neurons in Putamen

See Figure 5, for photos taken at the area of injection (the CP_u) to be able to evaluate the results. The blue colour shows Cre-expressing neurons in the CP_u which have been infected by TVA-V5-2A-RG. The green fluorescence in the pictures shows the CP_u-oriented Cre-expressing neurons that through Rabies-EGFP infection have become GFP positive.

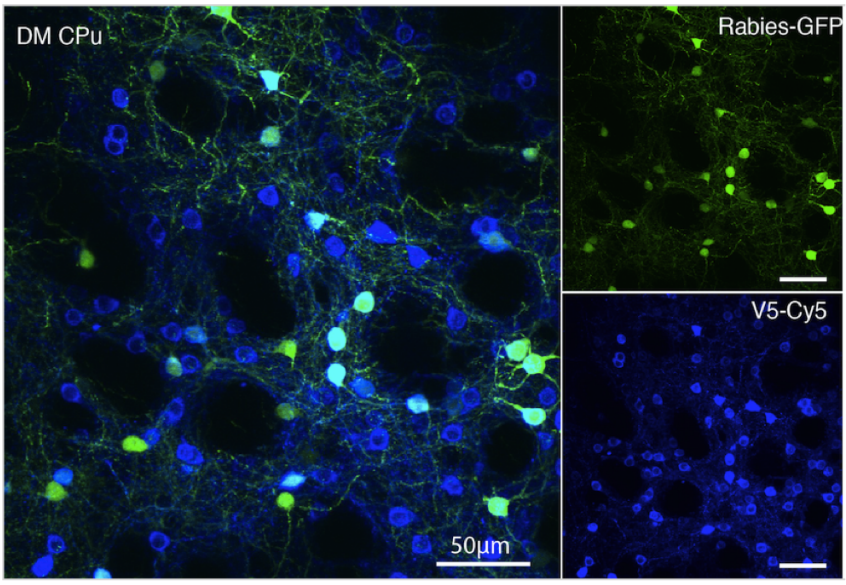
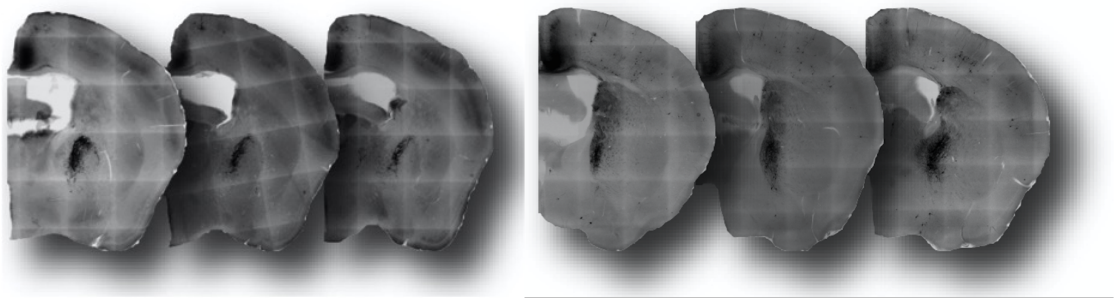


Figure 5: Retrograde labeling of starter neurons in the CP_u. Blue fluorescence representing TVA-V5-2A-RG infected neurons and green fluorescence representing Rabies-EGFP infected neurons.

3.2 Topography of the Putamen-projecting Neurons in the External Segment of Globus Pallidus

Photos of brain fragements where the target area of neurons projecting signals to the CP_u can be seen, in both the Adora2a-cre mouse (Figure 6a) and the D1-cre mouse (Figure 6b). The three photos taken from each mouse brain proceeds from an anterior fragment to more posterior fragments of the brain.

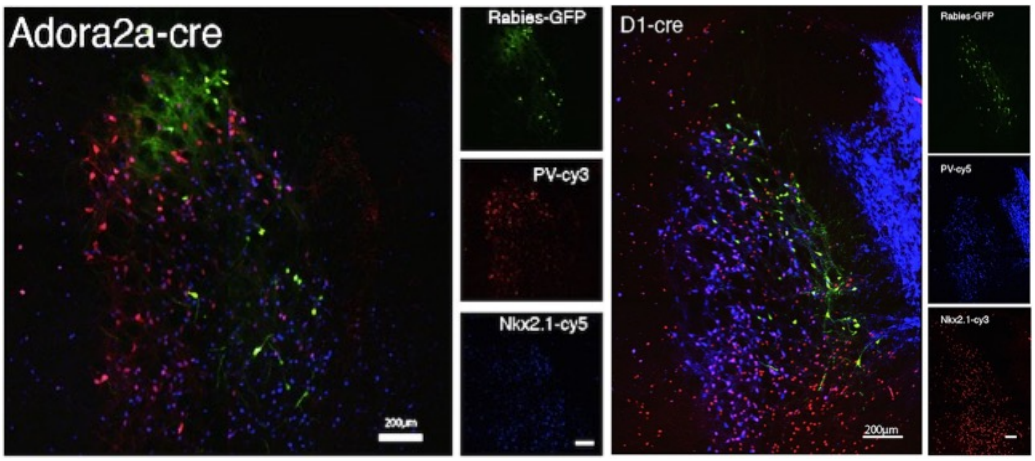


(a) Adora2a-cre (b) D1-cre

Figure 6: Pictures taken of CP_u-projecting neurons in the two mice line brains (Adora2a-cre and D1-cre). The pictures proceed from the most anterior fragment of the brain (right) to more posterior parts (right).

3.3 Immunofluorescences Staining to Identify Prototypic and Arky-pallidal Neurons in the External Segment of Globus Pallidus

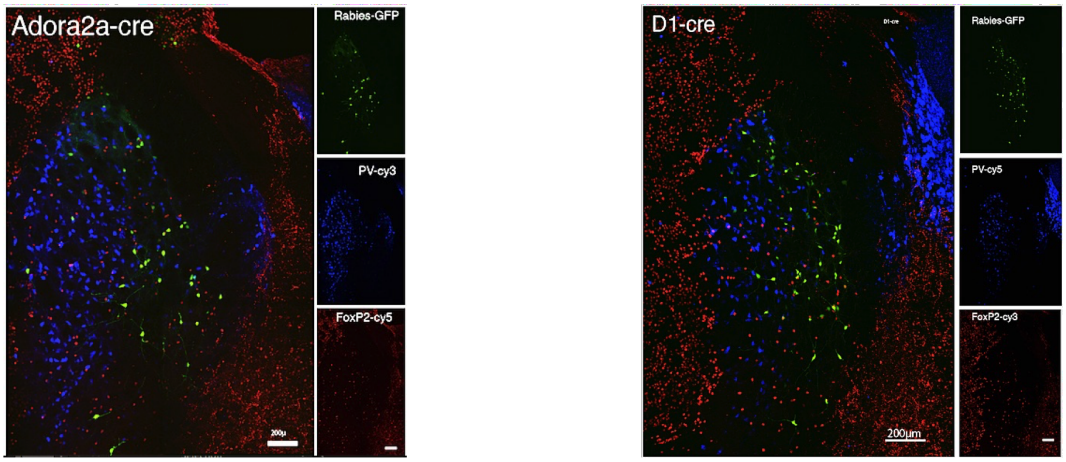
Figure 7 and 8 represent pictures taken of GP_e in both mice lines that projects to CP_u, where markers representing FoxP2, Nkx2-1, PV and GFP can be seen.



(a) GFP-expressing neurons (green), PV-expressing neurons (red) and Nkx2-1 marked neurons (blue) in Adora2a-cre mice line GP_e.

(b) GFP (green) and PV-expressing (blue) neurons and Nkx2-1 marked neurons (red) in D1-cre mice line GP_e.

Figure 7: Pictures taken from the GP_e in both mice lines investigating the occurrence of GFP, PV and Nkx2-1.



(a) GFP-expressing neurons (green), PV-expressing neurons (blue) and Nkx2-1 marked neurons (red) in Adora2a-cre mice line GP_e.

(b) GFP-expressing neurons (green), PV-expressing neurons (blue) and Nkx2-1 marked neurons (red) in D1-cre mice line GP_e.

Figure 8: Pictures taken from the GP_e in both mice lines investigating the occurrence of GFP, PV and FoxP2

Figure 9 summarizes the results of Figure 7a and 8a into a bar diagram where the percentage of all GFP positive cells of the GP_e which project to D2-neurons (Adora2a) are shown. The result differentiates prototypic and arkypallidal neurons.

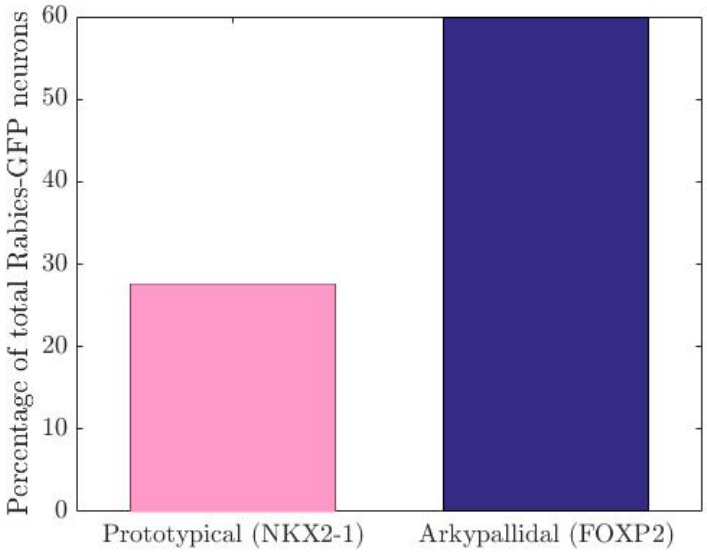


Figure 9: The percentage of all GFP-expressing neurons in GP_e which are prototypic and project to D2-neurons (Adora2a) of the CP_u.the pink bar represents prototypic (Nkx2-1-marked) neurons and the purple bar represents arkypallidal (FoxP2-marked) neurons. The pink has a value of 27.5% and the purple bar a value of 60%.

Figure 10 summarizes the results of Figure 7b and 8b into a bar diagram where the percentage of all GFP positive cells of the GP_e which project to D1-neurons are shown. The result differentiates prototypic (pink) and arkypallidal (purple) neurons.

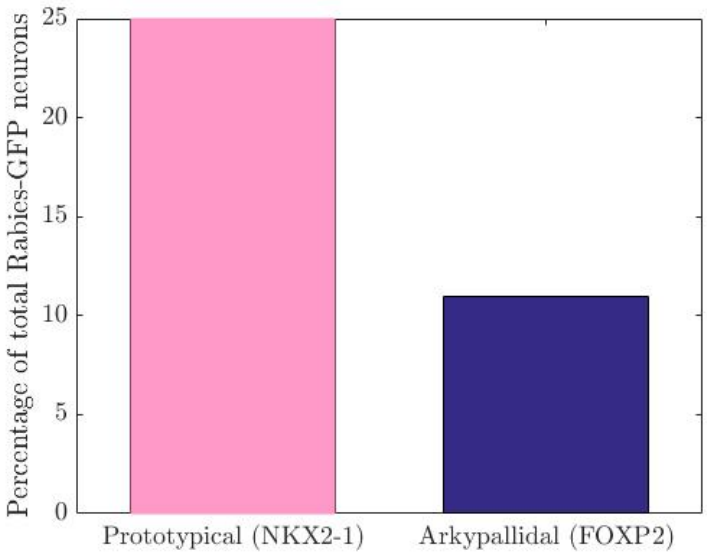


Figure 10: The percentage of all GFP-expressing neurons in GP_e which are arkypallidal and project to D1-neurons of the CP_u. The pink bar (prototypic) shows a value of 25% whereas the purple bar (arkypallidal) shows 11%.

4 Discussion

The main results of this study (summarized in Figure 9 and 10) show that the prototypic neurons represent 25% of all GP_e neurons projecting to D1 or D2 (Adora2a) neurons of the CP_u. This means that approximately 25% of the GP_e-input to CP_u is prototypic. The prototypic CP_u-projecting neurons in the GP_e show no preferential innervation for neither D1 nor D2 striatal neurons. Figures 9 and 10 also indicate that 60% of all CP_u proceeding neurons in the GP_e are arkypallidal neurons which project to D2-neurons of the CP_u, whereas only 11% of all CP_u-projecting neurons in the GP_e are D1-projecting arkypallidal neurons. The arkypallidal neurons seem to preferentially innervate the D2-neurons of the CP_u.

The results are reliable because of successful injection of TVA-V5-2A-RG and Rabies-EGFP. This is shown in Figure 5 which displays which Cre-expressing neurons that have been infected by TVA-V5-2A-RG (blue) as well as which neurons that have been infected by the Rabies-EGFP (green). Since the TVA-V5-2A-RG enables the Rabies-EGFP to infect the neuron, a neuron can not be marked by green fluorescence if it is not marked by blue fluorescence. Analyzing Figure 5, a majority of the neurons marked by blue fluorescence are also marked by green fluorescence which indicates that the injection has been successful and almost all Cre-expressing neurons have been infected by the rabies virus.

According to the topography, see Figure 6a, most GP_e neurons projecting to D2-neurons (indirect pathway) in the CP_u are situated in the borders of the GP_e. This differs from the D1-neuron CP_u-projecting neurons (direct pathway) which are fixed in both the borders and the nucleus of the GP_e, see Figure 6b. It can also be seen in Figure 6b that the density of D1-projecting GP_e neurons increases in the posterior parts of the GP_e whereas the density of D2-projecting neurons is larger in the anterior parts of the GP_e.

4.1 Source of Errors

Since both the primary antibodies which bind to FoxP2 and Nkx2-1 expressing neurons are produced in the same host species (rabbit) it is not possible to compare the occurrence of FoxP2 and Nkx2-1 expressing neurons in the same fragment. This is because secondary rabbit-antibodies will bind to both, which makes us unable to differentiate the two types of neurons. Therefore PV was used as a replacement for prototypic (Nkx2-1) neurons when comparing the two since a majority of the prototypic neurons express PV. Since all prototypic neurons do not express PV, this contributes to uncertainty in the results when summarizing the pictures into bar diagrams.

4.2 Conclusion

This study shows that prototypic neurons located in the GP_e show no preferential innervation for neither D1 (direct pathway) nor D2 (indirect pathway) CP_u-neurons. The prototypic GP_e-neurons preferentially innervate both D1 and D2 neurons and represent in total 25% of all CP_u-projecting neurons of the striatum.

It is shown in this study that arkypallidal neurons of the GP_e innervate both D1 and D2 neurons of the CP_u, with a preferential innervation for D2-neurons since 60% of all CP_u-projecting neurons are D2-projecting arkypallidal neurons and only 11% of all CP_u-projecting neurons are arkypallidal and D1-projecting.

The results have shown differences in function, location and activity between prototypic and arkypallidal neurons, although further research is necessary to enable researchers to draw more trust worthy conclusions based on this study. If we new the function, location and activity of prototypic and arkypallidal neurons for sure, that information could be used in the development of future medical treatments.

4.3 Future Research

Since this study is based on only two mice from different mice lines (D1-cre and Adora2a-cre) this study needs to be performed on a higher quantity of mice for the results to be of bigger scientific signification. Finding more specific transcription factors for prototypic and arkypallidal neurons could be a further improvement of the method, since this could enable anti-FoxP2 and anit-Nkx2-1 to be present in the same solution which would eliminate the use of PV and lead to more reliable results. Since all neurons of the GP_e marked with GFP (CP_u-projecting) do not overlap with the markers for neither PV, Nkx2-1 nor FoxP2 (see Figure 7 and 8) this suggests that all CP_u-projecting neurons do not belong to either the prototypic or the arkypallidal type. Further research may provide a bigger understanding for the GP_e neural heterogeneity and enable identification of new types of CP_u-projecting neurons.

Acknowledgments

I would like to thank my mentor at the department for Neuroscience at Karolinska Instiute, Associate professor Konstantinos Meletis, for his support throughout my research process. I would also like to express my appreciation for PhD student Rania Tzorzi and Johanna Stergiadou for answering all my questions and providing help when needed. I have also appreciated having my laboratory partner, Jenny Angelin, by my side during this project. Jenny has contributed with her valuable enthusiasm and knowledge and I would like to thank her for sharing my ambition for this project. I would also like to thank Rays partners Tekniska Museet and Teknikföretagen their contributions. Last but not least I would like to thank the organizer group and all participants of Rays-for Excellence 2016 for making this summer one to remember.

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