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Gene therapy treatment for Parkinson's disease

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Genterapi

Parkinsons sjukdom är den näst vanligaste neurologiska sjukdomen efter Alzheimers sjukdom och idag lever ungefär 24000 personer med Parkinsons sjukdom i Sverige. Vanliga symptom är skakningar i vila, muskelstelhet samt försämrad rörelseförmåga. Symptomen uppkommer till följd av en sänkt koncentration av dopamin i hjärnan. Till en början är motorsymptomen lindriga men förvärras med tiden då fler dopamin producerande nervceller i hjärnan dör.

Det finns idag ingen behandling som stoppar sjukdomsförloppet utan dopamintillverkande nervceller forsätter att brytas ner och förstöras trots medicinering. Behandlingen fokuserar istället på att lindra sjukdomssymptom genom att på olika sätt återställa dopamin-nivån i hjärnan. Levodopa, förstaidet till dopamin, används för öka dopamin-nivån i hjärnan hos Parkinsonspatienter. Levodopa tas upp av hjärnan och omvandlats där till dopamin. Efter en tids behandling uppkommer ofta svåra bieffekter vilka tros uppstå på grund av en fluktuerande dopamin-nivå.

Genterapi är en potentiell och förhoppningsvis i framtiden möjlig behandling av Parkinsons sjukdom. Oftast används en djur modell för att testa ut behandlingsmetoder. I genterapi introduceras genmaterial i utvalda celler hos djuret för att komplettera eller reparera en gen. Överföringen av genmaterialet sker med hjälp av inaktiverade viruspartiklar. I denna studie introducerades genetiskt material för två enzymer involverade i produktionen av dopamin: tyrosin hydroxylas och GTP-cyklohydrolas. Reglering av enzymaktiviteten har möjlig gjorts att koppla en destabiliseringsdomän till ett av enzymen. Enzym kopplade till destabiliseringsdomänen bryts ner i frånvaro av en antibiotika som heter trimetoprim och enzymaktiviteten kunde på så sätt regleras genom tillsats av trimetoprim.

Resultaten visade att regleringen med hjälp av trimetoprim inte fungerade optimalt och detta tros bero på en för låg dos av trimetoprim i hjärnan. Ytterligare experiment måste därför göras för att optimera trimetoprim-dosen.

Abstract

Parkinson's disease (PD) is the most common neurodegenerative disorder after Alzheimer's disease and today more than 6 million people are living with PD world-wide (2018). PD is characterized by muscle stiffness, shaking at rest, and postural imbalance. The symptoms are caused by a decreased dopamine (DA) level in the brain due to a gradual loss of DA producing neurons.

The cause and trigger of the disease is unknown but is believed to be a combination of both environmental and genetic factors. No treatment that can cure or slow down the disease progression is available; there are on the other hand treatments for symptom relief. Gene therapy is one potential treatment option for symptoms relief in PD. In gene therapy, genetic material is introduced into cells in the brain using viral vectors with the goal of restoring DA production.

In this study gene material for two important enzymes in the dopamine production has been injected into the brain of rats using viral vectors. The two enzymes are Tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1). A destabilization domain was added to regulate the activity of the enzymes. All proteins coupled to the domain are degraded in the absence of trimethoprim (TMP), thus enabling regulation and fine tuning of the enzyme activity in the brain.

The dopamine and serotonin in the brain, as well as their precursors and metabolites were monitored using HPLC-ECD. The result showed a restored production in the non-regulated group and the GCH1 regulated group but not in the TH regulated ones. The TMP concentration in brain was monitored using HPLC-MS and found to be lower than expected. Further studies have to be done in order to optimize the conditions and fine tuning the regulation of the constructs.

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List of abbreviations

3-MT	3-Methoxytyramine
3-OMD	L-3-O-Methyl-DOPA
5HIAA	Indoleacetic acid
5HT	Serotonin
5HTP	5-Hydroxy-L-tryptophan
6-OHDA	6-hydroxydopamine
AADC	Aromatic amino acid decarboxylase
AAV	Adeno-associated viruses
BH4	5,6,7,8-tetrahydrobiopterin
COMT	Catechol-O-methyltransferas
CSF	Cerebrospinal fluid
DA	Dopamine
DBS	Deep brain stimulation
DD	Destabilization domain
DOPAC	3,4-dihydroxyphenylacetic acid
ESI	Electrospray ionization
GABA	Gamma-Aminobutyric acid
GCH1	GTP cyclohydrolase 1
GTP	Guanosine 5’-triphosphate
HVA	Homovanilic acid
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO	Monoamine oxidase
NA	Noradrenalin
OSA	1-Octanesulfonic acid
PBS	Phosphate buffered saline
PD	Parkinson’s disease
TAE buffer	Tris-acetate and EDTA buffer
TH	Tyrosine hydroxylase
THE buffer	Tris-HCL and EDTA buffer
TMP	Trimethoprim

Introduction

Parkinson's disease

Parkinson's disease (PD) was first described 1817 by James Parkinson and is the most common neurodegenerative disorder after Alzheimer's disease. Presently, more than 6 million people are living with PD world-wide (2018) (Kalia & Lang, 2015). The number of people with PD is expected to increase by more than 50 % by 2030 due to better living conditions and thereby an increased aging of the population (Kalia & Lang, 2015).

The cause and trigger of the disease is still unknown, but is believed to be a combination of environmental and genetic factors. Environmental factors found to be associated with an increased risk of developing PD are head injury, rural living, agricultural occupation, and well-water drinking (Noyce et al., 2012).The biggest risk factor for developing this idiopathic form (the cause is unknown) of the disease is age (Kalia & Lang, 2015). A hereditary form of PD exists but is relatively rare contributing to less than 10 % of all PD cases. This form of PD is caused by gene mutation and is passed from generation to generation (Beal & Thomas, 2007). A number of different mutations has been associated with PD since the first genetic factor was discovered 1997 (Funayama et al., 2002; Kitada et al., 1998; Polymeropoulos et al., 1997; Valente et al., 2004)

PD is characterized by rigidity (muscle stiffness), resting tremor (shaking at rest), bradykinesia (slowness in executing movements), akinesia (inability to initiate voluntary movement), and postural imbalance (Fahn, Libsch, & Cutler, 1971). Although PD is considered to be a movement disorder, non-motor symptoms as depression, anxiety, and sleeping problems are also a part of the disease (Borek, Amick, & Friedman, 2014). Today, no treatment that slows down the disease progression is available, nor is there any cure for the disease. There are on the other hand drugs for symptom relief of both motor and non-motor symptoms.

The human brain

The human brain consists of several regions that have different functionalities. The *cerebrum* fills most of the cranial cavity (the space within the skull) and is divided into two by a vertical slit. The two parts are called the left and the right *cerebral hemisphere* respectively. Other parts of the brain are *cerebellum* (the little brain) and the brainstem (Figure 1) (Brodal, 1992).

The interior of the *cerebral hemispheres* contains grey substance (groups of neurons with various tasks), including the *basal ganglia* (Figure 1 C). The *basal ganglia* contain a number of interconnected nuclei and are involved in the control of movements. The main components of the basal ganglia are the *caudate nucleus*, the *putamen*, and the *globus pallidus*. Other components often included are the *subthalamic nucleus* and *substantia nigra*. The *putamen* and the *caudate nucleus* are together called *striatum* (Brodal, 1992).

Striatum and *substantia nigra* are connected through neurons. The neurons have the ability to send nerve impulses over a long distance and consist of a cell body, an axon and dendrites. One neuron has several dendrites but only one axon. The axon is built to conduct the nerve impulse from one neuron to another. There is, however, no direct contact between two neurons and the transmission of a nerve impulse occurs instead by release and binding of signal molecules (neurotransmitters) (Brodal, 1992). Dopamin, serotonin, gamma-Aminobutyric acid (GABA), and glutamate are all examples of neurotransmitters found in the human brain (Purves et al., 2004).

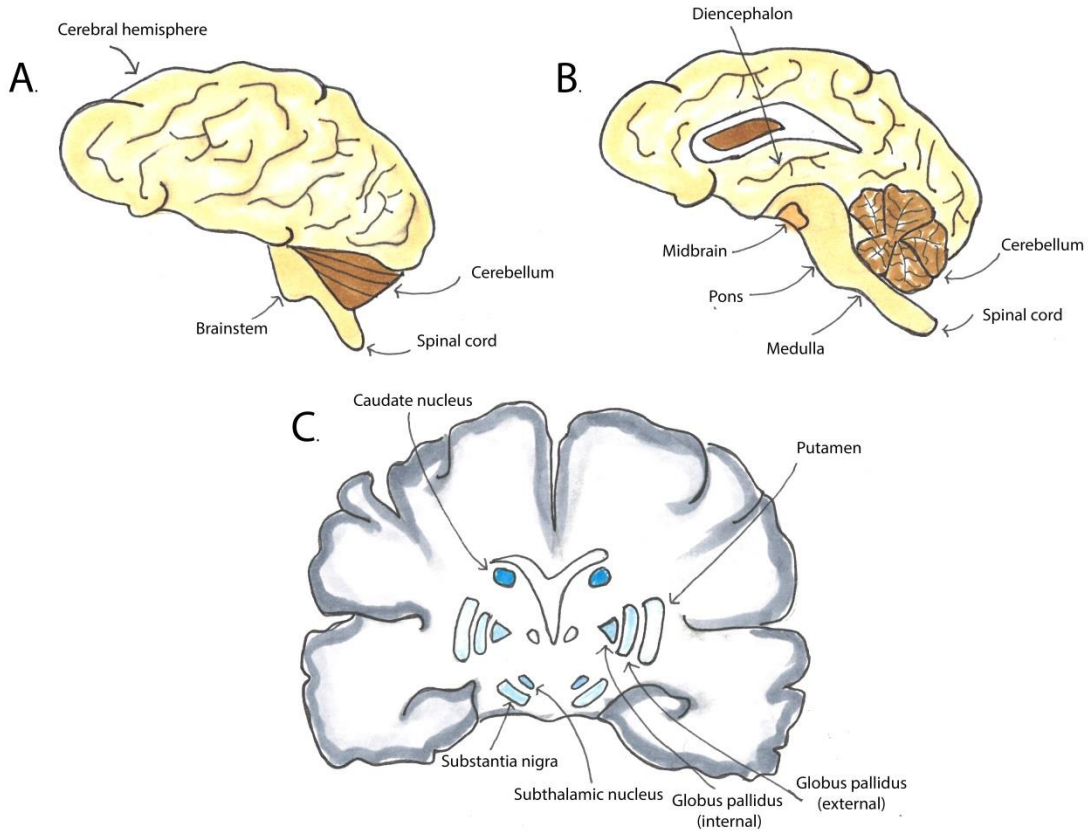

Figure 1 (A) The brain can be divided into *cerebellum*, the brainstem and the *cerebral hemispheres*. (B) Cross section of the brain: The brainstem consists of the *medulla*, the *pons*, and the midbrain. The *diencephalon* (where *thalamus* is included) and the *cerebral hemispheres* are included in the forebrain. (C) The main components of the *basal ganglia* are the *caudate nucleus*, the *putamen*, and the *globus pallidus*. Other components often included are the *subthalamic nucleus* and *substantia nigra*. *The putamen* and the *caudate nucleus* are together called *striatum*. (the picture is based on Brodal, 1992)

Patients with PD have a loss of dopaminergic neurons (dopamine producing neurons) in *substantia nigra*, the part of the brain connected through neurons to *striatum*. DA production in the *basal ganglia* (which includes both *substantia nigra* and *striatum*) is important for coordination of movements and a decreased dopamine level gives rise to the motor symptoms so characteristics for PD (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973; Fahn et al., 1971; Ma, Röyttä, Rinne, Collan, & Rinne, 1997) Interestingly, up to 60 % of the dopaminergic neurons in substantia nigra are degraded before development of symptoms making the disease hard to diagnose (Nyholm, 2007).

Dopamine production

DA production in the brain starts with the conversion of the amino acid tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by Tyrosine hydroxylase (TH). This is the rate limiting step in the production of DA and TH activity is dependent on the accessibility of its cofactor 5,6,7,8-tetrahydrobiopterin (BH4), as well as Fe(II) and oxygen (*A new era of catecholamines in the laboratory and clinic. [Elektronisk resurs]*, 2013). BH4 is generated from guanosine 5'-triphosphate (GTP) in a three step reaction involving the enzyme GTP cyclohydrolase 1 (GCH1) (Figure 2).

TH consists of two domains, a regulatory part located at the N-terminous of the enzyme and a catalytically part located at the C-terminous. The regulatory part controls enzyme stability and activity while the catalytically part binds the substrates (tyrosine and oxygen) and the cofactor (BH4). The enzyme activity is also regulated through feedback inhibition by DA. Phosphorylation of four serine residues (8, 19, 31, and 40) located in the regulatory part of the enzyme is important for regulation of enzyme activity (*A new era of catecholamines in the laboratory and clinic. [Elektronisk resurs]*, 2013).

L-DOPA is converted to DA by aromatic amino acid decarboxylase (AADC) and stored in vesicles by vesicular monoamine transporter 2 (Lohoff, 2010). DA is metabolised in the cell by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC), which is further converted to homovanilic acid (HVA) by catechol-O-methyltransferas (COMT) (Brooker, Widmaier, Graham, & Stiling, 2014) (Figure 2).

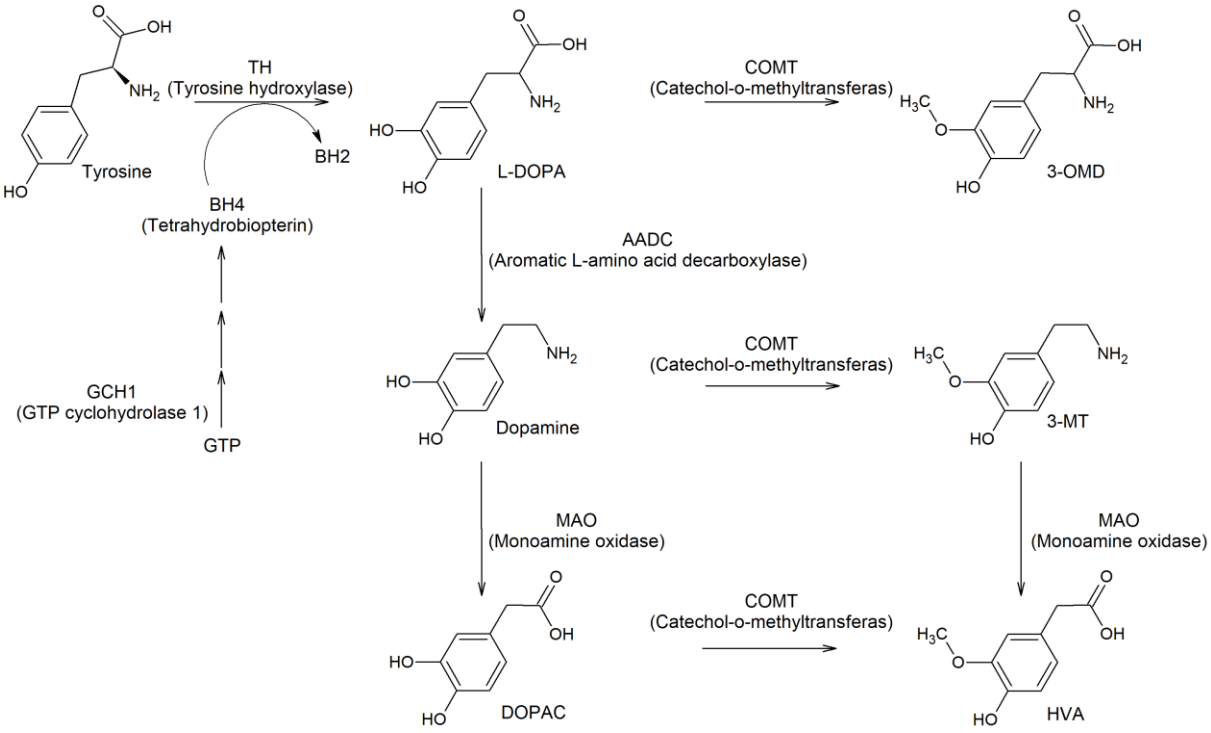

Figure 2 DA production and metabolism. DA production starts with the conversion of the amino acid tyrosine to L-DOPA. The reaction is the rate limiting step in the production of DA and is dependent on the enzyme TH. TH activity is dependent on its cofactor BH4 and iron. BH4 is produced in a three step reaction from GTP involving the enzyme GCH1. L-DOPA, the precursor to DA, is then converted to DA by AADC.

Treatment of Parkinson's disease

L-DOPA – motor-symptoms relief

L-DOPA is the precursor to DA and it has been used as a standard treatment for PD since the late 1960s (Cotzias, Papavasiliou, & Gellene, 1969). It has the ability to cross the blood-brain barrier and is converted to DA by dopaminergic neurons in *substantia nigra*. In early stages of PD, there are still a sufficient number of dopaminergic neurons left to allow a relatively normal DA release. As the disease progresses, more dopaminergic neurons are lost and other types of cells take up and convert L-DOPA. These cells can however not store DA and it leaks into the presynaptic area and is thereafter degraded. Therefore, L-DOPA treatments improve the motor symptoms during the early stages of the disease but the therapeutic window narrows as the disease progresses and more dopaminergic neurons are lost. (Nyholm, 2007)

Side effects such as dyskinesias normally occurs 6-7 years after started treatment (Evans et al., 2011). Dyskinesia is uncontrolled, involuntary movements and can affect the entire body or a body part such as the head, arm, or leg. It is thought to be caused by fluctuating levels of DA (Cenci, 2014)

L-DOPA is often taken in combination with drugs that prevents L-DOPA breakdown. There are drugs targeting COMT, the enzyme that converts L-DOPA to L-3-O-Methyl-DOPA (3-OMD). With inhibition of COMT more L-DOPA can be transferred to the brain and converted to DA (Brooks, Sagar, & Group, 2003).

Gene therapy

Gene therapy is a potential treatment option for symptoms relief in PD. Genetic material is introduced into cells in the brain with the goal of restoring DA production. The gene material codes for enzymes important in the DA production, and is delivered using viral vectors. Today, a number of viral vectors are used including viral vectors based on adenoviruses, adeno-associated viruses (AAV), and retroviruses. AAV vectors are most frequently used (Daya & Berns, 2008).

AAV is a virus with a linear single-stranded DNA genome belonging to the *parvoviridae* family. The virus is non-pathogenic in the absence of a helper virus (adenovirus or herpesvirus), making it suitable for gene therapy (Daya & Berns, 2008). The wildtype genome contains two open reading frames, both coding for important viral replication and capsid proteins. Inverted terminal repeats are located in both ends of the genome. These regions are important for replication and packing of the genome. To create a recombinant AAV vector suitable for gene therapy, the open reading frames are removed and replaced by a target gene. Genes from adenovirus together with important genes from the wild type AAV genome are essential for production of recombinant AAV vectors. These are expressed in trans (on a separate plasmid) and not included in the final AAV vector (Daya & Berns, 2008; Shin, Yue, & Duan, 2012).

In 2008, the result from the first phase 1 clinical trial using AAV2-induced AADC expression in PD patients was published (Eberling et al., 2008). The method needs to be combined with exogenous L-DOPA supply as the enzyme converting tyrosine to L-DOPA (TH) is not available. The effect of gene therapy has shown to be long lasting in nonhuman primates. The primates were followed for 8 years post injection to confirm that the AADC transgene expression remained unaffected (Hadaczek et al., 2010).

Another approach is to completely reconstruct the dopamine production apparatus by introducing the most important enzymes involved in the conversion of tyrosine to DA (TH, GCH1, and AADC). TH converts the amino acid tyrosine to L-DOPA and TH activity is dependent on the accessibility of its cofactor BH4 which is generated from GTP in a three step reaction involving GCH1. L-DOPA produced by TH from tyrosine is further converted to DA by AADC (Figure 2). Delivery of all three genes in one AAV vector is not possible as they together are too large to fit in a single AAV capsule. This can be solved by using three individual vectors or by using a lentiviral vector (Azzouz et al., 2002; Cederfjäll, Sahin, Kirik, & Björklund, 2012; Shen et al., 2000). Another approach is to genetically modify the enzymes to fit into one single AAV vector.

In this study gene material for TH and GCH1 has been delivered using AAV5. AADC is more unspecific than TH and can be found in several types of neurons other than DA neurons (e.g. serotonergic neurons) and was therefore excluded (Arai, Karasawa, & Nagatsu, 1996; Hökfelt, Fuxe, & Goldstein, 1973). The TH was truncated at the N-terminal, where the activity and stability of the enzyme is regulated. By excluding AADC and delete the regulatory domain of TH the gene material could be delivered using one single AAV vector. This is an advantage compared to methods using three separate AAV vectors. Using one vector only will assure that all three genes are expressed in the same cell.

A destabilization domain (DD) was used to regulate the activity of TH and thereby the levels of L-DOPA produced. The DD is based on the protein dihydrofolate reductase from *E. coli* and engineered to be degraded in the absence of a stabilising ligand called trimethoprim (TMP). TMP's ability to cross the blood-brain barrier makes it suitable in regulation of enzymes involved in the nervous system (Iwamoto, Björklund, Lundberg, Kirik, & Wandless, 2010). All proteins coupled to the DD are degraded in the cell in the absence of TMP, enabling regulation of the L-DOPA level in the brain. However, addition of TMP stabilizes the protein and ensures enzyme activity. This enables fine tuning of the L-DOPA production, making it possible to enhance or shut down the production of L-DOPA.

Method

Chemicals

All chemical were of analytical/biological grade and bought from Sigma Aldrich, if nothing else stated.

Biological and gene material

Two animal studies were performed. The first study included thirty-six female adult Sprague-Dawley rats (Charles River, Germany) whereas the second study included sixty-two. Neuronal lesion of the animals' right side of the brain was performed by injecting 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle to simulate a PD brain. The left side was kept intact to serve as positive control. The animals in the first study were divided into three groups according to gene material injection (Table 1): (group 1, buffer) negative control by Injection of buffer instead of AAV vector; (group 2, TH + GCH1) Injection of two viral vectors, containing gene material for TH and GCH1 respectively; (group 3, DD(I)-tTH-GCH1) Injection of one viral vector, containing gene material for truncated TH coupled to both a DD(I) and GCH1.

In the second study, the animals were divided into six groups. To simplify the comparison of the result between the two studies, two of the groups (group 2 and group 3) were represented in both study number one and study number two. Study number two included two additional variants of the DD, this to see if the gene expression was disrupted by the present of the DD. The groups present only in study number two are as follow (Table 1): (group 4, DD(II)-tTH-GCH1) Injection of one viral vector containing gene material for truncated TH coupled to both DD(II) and GCH1; (group 5, DD(WT)-tTH-GCH1) Injection of one viral vector containing gene material for truncated TH coupled to both DD and GCH1. A wild-type variant of the DD that does not affect the protein stability was used ;(group 6, TH+ DD(I)-GCH1) Injection of two viral vector. One containing gene material for TH and the other one containing gene material for the DD(I) coupled to GCH1. Group 6 differs from the other in the aspect that the GCH1 activity not the TH activity was regulated using TMP. As for study one, a control group were injected with buffer instead of viral vector. All gene material was delivered using AAV-5 vectors. Behavioural tests were performed throughout the experiments, before and after lesion, after viral injection and after TMP treatment, to confirm lesion and to monitor potential recovery.

Table 1 The animals were divided into group according to gene material injection. A total of eleven animals were injected with buffer instead of viral vector and did served as negative controls. All gene material was delivered using one AAV vector with exception for group 2 and group 6 where two vectors were used for the delivery. All groups with a DD were further divided into two groups: one receiving TMP subcutaneously and one that did not.

Group	Treatment	Number of animals (study 1)	Number of animals (study 2)	Total number of animals	TMP
1	Buffer	8	3	11	No
2	TH+GCH1	10	4	14	No
3a	DD(I)-tTH-GCH1	9	1	10	No
3b	DD(I)-tTH-GCH1	9	1	10	Yes
4a	DD(II)-tTH-GCH1	-	10	10	No
4b	DD(II)-tTH-GCH1	-	10	10	Yes
5a	DD(WT)-tTH-GCH1	-	10	10	No
5b	DD(WT)-tTH-GCH1	-	10	10	Yes
6a	TH+DD(I)-GCH1	-	10	10	No
6b	TH+DD(I)-GCH1	-	9	9	Yes

In both studies, all animals in all groups expect the group1 and group 2 were further divided into two subgroups (Table 1): one given TMP and the other not. TMP (0.15 g/ml) was injected subcutaneous (under the skin) once weeks throughout the experiment starting from 17 week after AAV injection for study one and 20 weeks after AAV injection for study two. Plasma, serum, CSF, and *striatum* were collected 28 weeks after AAV injection in the first part of the study and 32 weeks after AAV injection in the second part of the study. TMP levels were also monitored by weekly harvest of plasma in the first part of the study.

HEK293 cells were transfected for overexpression of TH and used as positive control in the TH assay. The cells were transfected using 16.61 µg plasmid diluted in optiMEM reduced serum medium (Prod.no 3185-062, Thermo Fisher) into a total volume of 1611 µl. 49.83 µl TransIT-2020 (Prod.no MIR5400, Mirus) was added followed by 15 minutes incubation. The prepared solution was added to a 75 ml flask with HEK293 cells. The cells were harvest three days after transfection using PBS with 5 mM EDTA to dislodge the cells from the growing surface. After harvest, the cells were stored in -80^{o} C until further analysis.

PC12 cells were cultured to serve as positive control in the TH assays. The cells were thawed in water bath and added to a T175 flask together with 40 ml RPMI 1640 medium (prod.no 31870074, Thermo Fisher). Glutamine, horse serum and fetal bovine serum were added to the medium to give a final concentration of 2 mM glutamine, 10 % horse serum, and 5 % fetal bovine serum. The medium was changed every second day and the cells were harvest nine days after seeding using PBS with 5 mM EDTA to dislodge the cells from the growing surface. The cells were stored at -80^{o} C until further analysis.

Sample preparation

The striatum collected from the animals in the two studies were prepared by sonication. For the sonication, buffer was added to give a 1:5 tissue to buffer ratio. 150 µl Tris-acetate and EDTA buffer (TAE buffer) with pH 7.4 was added to *striatum* collected from the first study, whereas 150 µl Tris-HCl and EDTA buffer (THE buffer) with pH 6.8 was added to *striatum* collected from the second study. The final concentration of Tris in the buffer was 20 mM and the EDTA concentration was 0.5 mM. The acetic acid concentration was 10 mM in the TAE buffer. Both buffers contained phosphatase inhibitors (Prod.no04906837001, Roche) and protease inhibitors (Prod.no 11836153001, Roche). 1 tablet of Protease inhibitor and 1 tablet phosphatase inhibitor were added to 10 ml of buffer.

After addition of buffer, the brain tissue was sonicated on ice 10 times 3 seconds using a sonicator of the model VC50 (Sonics and Materials inc). The amplitude was set to 60. The crude extract was separated into four tubes: one for HPLC-ECD analysis, one for HPLC-MS analysis, one for determination of the total protein concentration and one for TH activity measurements and stored at -80°C until further analysis (Figure 3

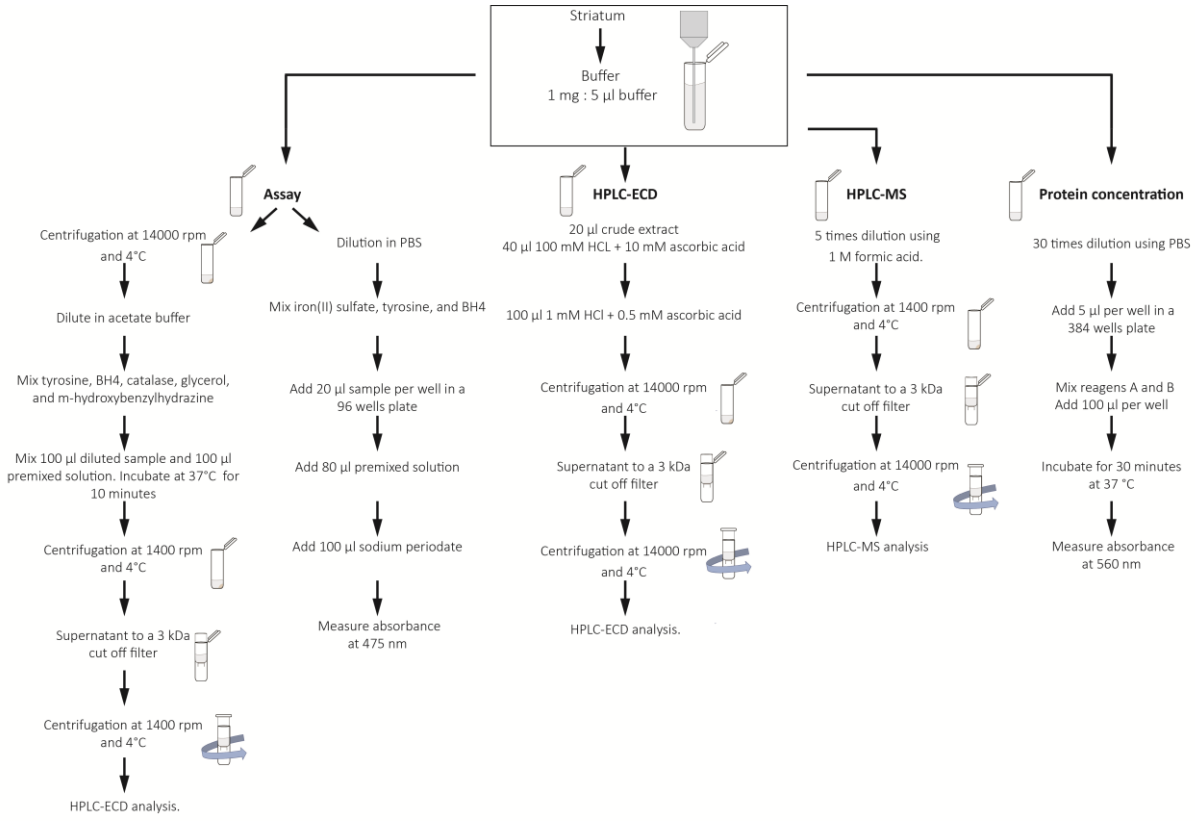

Figure 3 The *striatum* collected from the animals in the two studies were homogenized by sonication.5 µl buffer was added for every mg *striatum*, giving a 1: 5 *striatum* buffer ratio. The sonicated *striatum* was aliquoted into different tubes for HPLC-ECD analysis, HPLC-MS analysis, TH activity measurements, and total protein determination.

For the HPLC-ECD analysis, 20 µl crude extract was transferred to a new tube containing 40 µl 100 mM HCL + 10 mM ascorbic acid, giving a 3 times dilution. The samples were incubated 20 minutes on ice followed by addition of 100 µl 1 mM HCl + 0.5 mM ascorbic acid giving a final 8 times dilution of the crude extract. The samples were centrifuged 10 minutes at 1400 rpm and 4°C. The supernatant

was transferred to a 3 kDa cut off filter (Prod.no 82031-346, VWR) followed by 45 minutes centrifugation at 14000 rpm and 4°C. 30 µl of the filtered sample were transferred to vials for HPLC-ECD analysis.

The TMP levels in plasma, serum, CFS and *striatum* were analysed using HPLC-MS. The samples were thawed on ice followed by addition of 1 M formic acid to give a 2 times dilution for serum and plasma, and a 5 times dilution for *striatum*. After dilution, the *striatum*, plasma and serum were centrifuged 10 minutes at 14000 rpm and 4°C before addition of the supernatant to a 3 kDa cut off filter. The samples were centrifuged 45 minutes at 14000 rpm and 4°C. The CSF was not diluted and therefore directly transferred to a 3 kDa cut off filter. After the filtration, 25 µl was added to vials for HPLC-MS analysis.

HEK 293 cells and PC12 cells were prepared the same way as *striatum* by addition of 150 µl 0.5x TAE buffer (pH 7.4) followed by sonication 10 times 3 seconds on ice using sonicator VC50. The amplitude was set to 60.

Total protein concentration

The total protein concentration was measured using a BCA kit (prod.no:23225, Thermo Scentific) with the following modifications of the standard protocol: 5 µl diluted crude extract or protein standard was added per well to a clear 384 wells polystyrene plate with flat bottom wells followed by addition of 100 µl working reagent. The crude extract was diluted 30 times in PBS in order to fit into the range of the calibration curve (Figure 3

The reaction was incubated 37° C for 30 minutes and thereafter the absorbance was measured at 560 nm using EnVision 2104 multilabel Reader (Perkin Elmer).

Quantification of monoamines in striatum using HPLC-ECD

A total of 10 analytes were quantified in both left and right *striatum* including L-DOPA and DA as well as metabolites of DA and L-DOPA (Figure 2 Noradrenalin (NA) was also monitored to ensure that the correct region had been collected and to ensure that L-DOPA was accurately identified as NA and DA has similar retention times.

L-DOPA is converted to DA by AADC (Figure 2 AADC, in contrast to TH, is found in several neurons other than dopaminergic neurons including serotonergic neurons. In PD patients receiving Levodopa treatment it has been shown that L-DOPA is converted to DA using AADC from serotonergic cells when no dopaminergic cells are present (Arai et al., 1996). The HPLC-ECD analysis included monitoring of three monoamines in the serotonin pathway to make sure that it was not affected in a negative way by the gene therapy.

HPLC is a liquid based chromatographic method used for separation and identification of compounds in a sample. The analytes in the sample are separated based on interactions with the stationary phase (the column) and the mobile phase (used to migrate the sample through the system). The separation can be achieved by difference in size, charge, hydrophobicity and hydrophilicity. In reversed phase chromatography, the stationary phase is hydrophobic and the mobile phase is a mixture of polar solvents (methanol, acetonitrile, water). The analytes can also be separated using ion-pairing, where an iron-paring reagent is added to the mobile phase. The iron-paring reagent has a hydrophobic region making interaction with the stationary phase possible and an ionic part making

interaction with charged analytes possible. Analytes interacts differently with the ion-pair reagent depending on charge and size. The mobile phase composition can be isocratic (same throughout the analysis) or a gradient (change throughout the analysis).

The Ultimate 3000 (Thermo Fischer) HPLC system used for this analysis was equipped with a biocompatible isocratic pump, 0.250 ml/min flow rate, 25 µl of sample was injected and a reversed phase C18 column with a particle size of 2.5 µm and 2.1x150 mm dimensions (Prod.no 186006739, Waters) was used for analyte separation. The monoamines were detected by an ECDRS electro chemical detector using an amperometic detector cell with glassy-carbon electrode set to 750 mV. The mobile phase consisted of 50 mM phosphate buffer (pH 2.5) 1-Octanesulfonic acid (OSA), methanol, and acetonitrile. The concentrations of OSA, methanol, and acetonitrile were varied to achieve good separation of all analytes (Table 2). Not all mobile phases gave a separation of all ten monoamines due to matrix interference and peak overlap. The mobile phase was therefore optimized during the study. An external calibration curve was constructed by analysing a master mix of all ten individual analytes at increasing concentrations. All data was evaluated in Chromeleon 7.2 using the peak area to calculate the concentration of each monoamine and the concentration was finally normalised against the total protein concentration of *striatum*.

Table 2 Different mobile phases used. Not all mobile phases used gave a separation of all ten monoamines du to matrix interference.

Mobile phase	Used for samples from	Problem with mobile phase
300 mg/l OSA and 6.75 % acetonitrile	Study one	No quantification of HVA
330 mg/l OSA, 5.4 % acetonitrile and 2 % methanol	Study one and study two	No quantification of DOPAC
280 mg/l OSA and 6.25 % acetonitrile	Study two	-

Quantification of TMP using HPLC-MS

The TMP concentration in plasma, CSF and *striatum* was relatively quantified using HPLC-MS. The HPLC instrument used was a 1269 system (Agilent), 1 µl sample was injected and a reversed phase C18 column with a particle size of 3.5 µM and 2.1x50 mm dimensions (Prod.no: 186005255, waters) was used for separation. A mobile phase with triple component gradient was used (Table 3 and Table 4). MS analysis using quadrupole time-of-flight (qTOF) with electrospray ionization (ESI) interface followed the separation.

The retention time of TMP was determined by spiking in TMP in the sample matrix and MS-MS data was collected to control that TMP and nothing else was measured. The fragmentation pattern collected was compared to the fragmentation pattern of pure TMP. 5-10 samples from animals without TMP treatment were pooled to serve as negative control. The pooled samples were prepared as described previously (see sample preparation) and spiked with TMP to give a final concentration of 0.148, 0.077, and 0.039 mg/ml TMP. This data was used for relative quantification of TMP in the samples. The analysis was randomized, meaning that the analysis was not organized after the six groups to avoid introducing any analysis related bias in the data.

Table 3 Composition of the mobile phase used for separation.

Name	Composition
A	0.1 % formic acid and 0.1 % ammonia formate
B	95 % ACN in 0.1 % formic acid and ammonia formate
C	95 % MeOH in 0.1 % formic acid and ammonia formate

Table 4 A mobile phase with triple component gradient was used for separation.

Time (min)	A (%)	B (%)	C (%)
3.00	40.0	30.0	30.0
3.10	0.0	25.0	75.0
4.00	0.0	25.0	75.0
4.10	95.0	0.0	5.0
8.00	95.0	0.0	5.0

The data was evaluated in Mass Hunter and the TMP concentration was calculated from the area of the peaks. The concentration in *striatum* was normalised against the total protein concentration of *striatum*. The *striatum* and plasma was normalised against the measured concentration of TMP in the negative control.

Activity assay

It is important to be able to monitor the TH activity in the *striatum* and up till this point it has in our department been done using radioactive reagents to monitor the enzyme activity. The aim of this part of the study was to develop a non-radioactive assay for TH activity measurements to reduce cost and improve lab safety.

Monitoring tyrosine hydroxylase activity in striatum using spectrophotometry

The assay is based on the monitoring of a chromophore produced in the reaction between L-DOPA and sodium periodate. The absorbance at 475 nm gives a measure of the amount of chromophore produced giving an indirect measure of the amount of L-DOPA in the sample.

The TH activity assay was performed as described by Vermeer (Vermeer, Higgins, Roman, & Doorn, 2013) with modification of the sodium periodate concentration used. A reaction mixture containing BH4, iron(II) sulfate, and tyrosine was premixed. Different concentrations of the components in the reaction mixture were tested in order to find the optimal assay conditions. 80 µl of the premixed solution, 20 µl of sample diluted in PBS, and 100 µl sodium periodate (1800 µM) was added to a 96 well clear polystyrene plate with flat bottom wells. The reaction was incubated at 37°C and the absorbance was measured using EnVision 2104 multilabel Reader (Perkin Elmer) once every minute during 10 minutes.

Monitoring tyrosine hydroxylase activity in striatum using HPLC-ECD

The TH activity assay was performed as described by Hooper (Hooper et al., 1997) with following modifications. The sample was centrifuged followed by dilution of the supernatant in 0.05 M acetate buffer (pH 6.0). A reaction mix was prepared containing tyrosine (substrate), m-hydroxybenzylhydrazine (AADC inhibitor), BH4, catalase, and glycerol (Table 5). 100 µl of diluted sample was added to 100 µl premixed reaction mix followed by incubation in heat block at 37 °C for

10 minutes. The reaction was terminated using 100 µl 100 mM HCl + 5 mM ascorbic acid followed by 10 minutes centrifugation at 14000 rpm and 4 °C. The supernatant was transferred to a 3 kDa cut off filter and centrifuged 30 minutes at 14000 rpm and 4 °C. The L-DOPA concentration was measured using HPLC-ECD (for HPLC-ECD set up, see quantification of monoamines in striatum using HPLC-ECD).

Table 5 Reaction mix components and concentrations for activity measurements of TH in *striatum* using HPLC-ECD.

Chemical	Concentration
Tyrosine	200 µM
m-hydroxybenzylhydrazine	1.25 mM
BH4	1.0 mM
Catalase	19.5E3 units/ml
Glycerol	200 mM

PC12 cells were prepared the same way as *striatum* to serve as positive control. Two negative controls were prepared. The first one contained reaction mix but no sample (*striatum* or PC12 cells). The second negative control did include both reaction mix and sample. However, the incubation step was excluded and the reaction was instead immediately terminated.

Result and discussion

DA production in striatum

The aim of this study was to restore and regulate the L-DOPA production in *striatum* through gene therapy using AAV vector injections. All constructs have been tested *in vitro* before used for *in vivo* studies.

It has been shown in PD patients receiving L-DOPA treatment that the serotonergic neurons convert L-DOPA to DA (Arai et al., 1996; Hollister, Breese, & Mueller, 1979; Navailles, Bioulac, Gross, & De Deurwaerdère, 2010; Tanaka et al., 1999). Genetic material for AADC (the last enzyme in both the serotonergic and dopaminergic production pathway) was therefore not included in the AAV vectors used in this study and L-DOPA was expected to be converted to DA using serotonergic neurons present in the brain. The HPLC-ECD data shows that the serotonin pathway was not affected in a negative way as no significant difference in 5-HT, 5-HIAA, and 5-HTP levels in the striatum was detected for any of the groups between the left and the right side of the brain (control vs treated side).

DA, 5-HT, NA and their metabolites and precursors were quantified using HPLC-ECD. The peak area was used for calculations of the concentration. The average was calculated for each monoamine in each group (for all groups, see table 1). Animals with no detectable levels of a monoamine were not included in the average of that monoamine. The DA amount in *striatum*, for both the positive control side (left side) and the right side is shown in Figure 4 For group 1 (the negative control), the DA concentration in the right side was below the detection limit and for group 2 (the unregulated positive control) the DA concentration was clearly detectable. The DA-level was similar in all left sides (naïve side). This result confirms that the lesion of the right side of the brain was successful and that the naïve control side was not affected by the lesion nor the gene therapy.

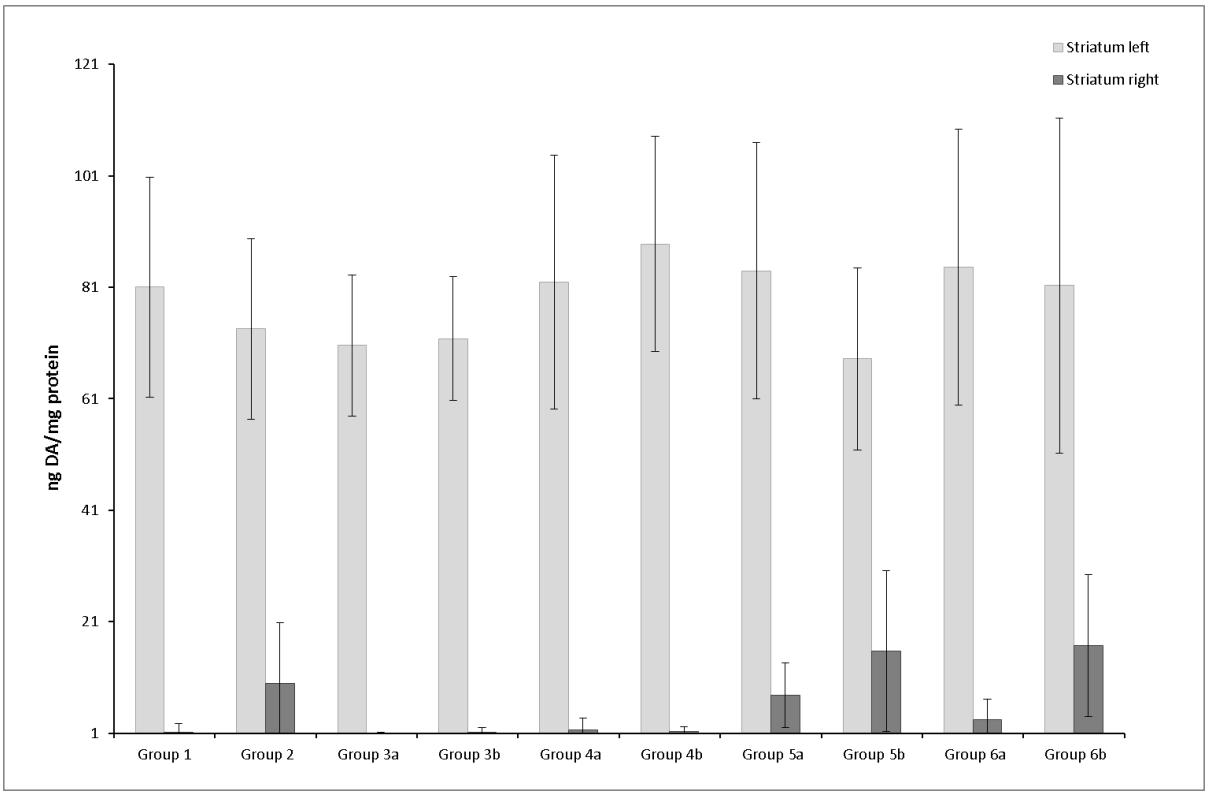

Figure 4 DA amount in *striatum* for both left (control side) and right side of the brain. The result indicates that the lesion using 6-OHDA was successful as the DA level of the right side in the buffer group (group 1) is significantly lower than for the left side (the control side).

The TMP concentration in CSF, serum and *striatum* was measured using HPLC-MS and found to be very low in both CSF and *striatum* (Figure 5 thus no upregulation of the L-DOPA production was expected for any of the regulated groups. All HPLC-MS data was collected from plasma, CSF, and *striatum* harvested from animals in study number one. The TMP was administered the same way with the same dose in the second part of this study and thus TMP concentrations were assumed to be the same for all animals included in this study.

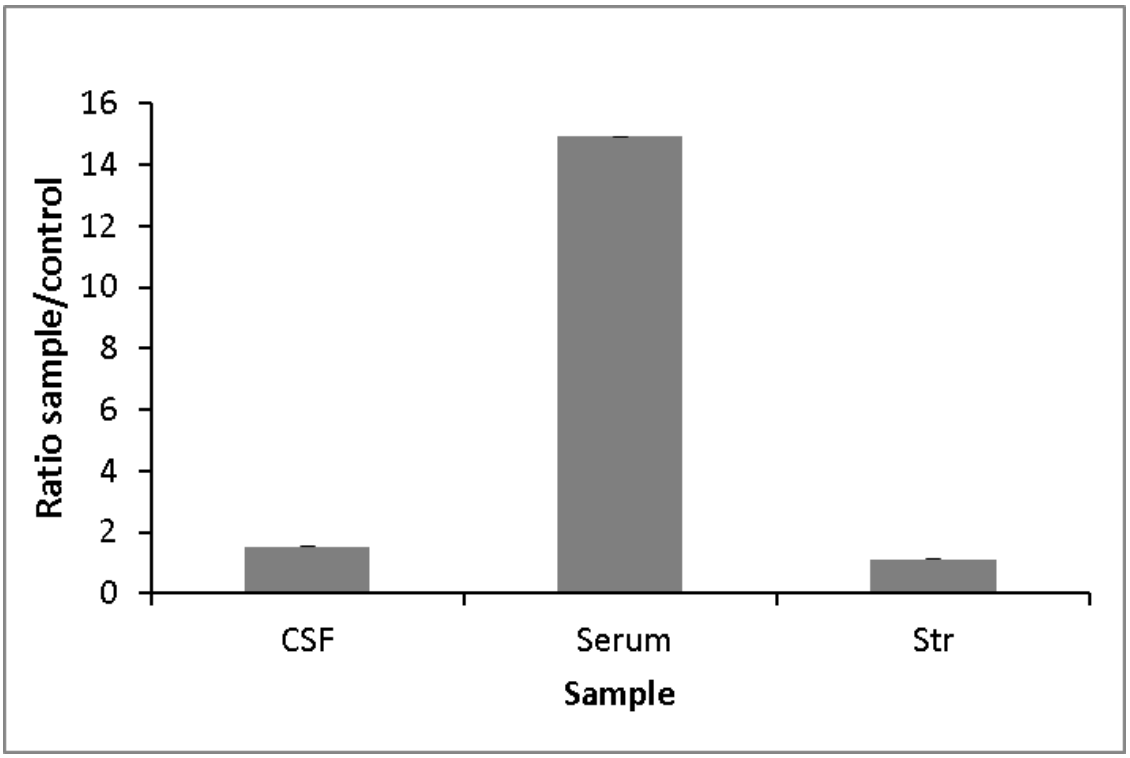

Figure 5 TMP concentration in CSF, serum, and *striatum*. The highest concentration of TMP was measured in serum. This can explain the low L-DOPA amount for the animals in the groups were TH is regulated (group 3 and group 4).

L-DOPA data shows a restored L-DOPA production in group 2 (the non-regulated group), group 5a, group 5b, and group 6b (Figure 6 Group 3 and 4 have similar L-DOPA levels as group 1 (the negative control group) even when given TMP. Except for the DD, there is no difference in the constructs used for group 3, 4, and 5. In group 5 a wild type DD is coupled to TH, giving no regulation using TMP and L-DOPA data shows that the construct for group 5 works. The results from the *in vitro* test show that the regulation works when a sufficient amount of TMP is accessible in the cells. From this, the conclusion that the low TMP concentration in *striatum* affects the regulation is drawn and further studies has to be done to optimize the TMP dose and administration.

The results indicate an up-regulation of group 6 despite the low TMP concentration in the brain. This can be explained by the results from an earlier study concluding that a 5:1 ratio between TH and GCH1 is optimal (Björklund et al., 2009). Group 6 has a non-regulated TH and a regulated GCH1 and only a minor up-regulation of GCH1 due to low TMP presence might give a significant improvement of TH activity. Thus a low amount of active GCH1 can still give a restored L-DOPA production whereas this is not the case for regulation of TH.

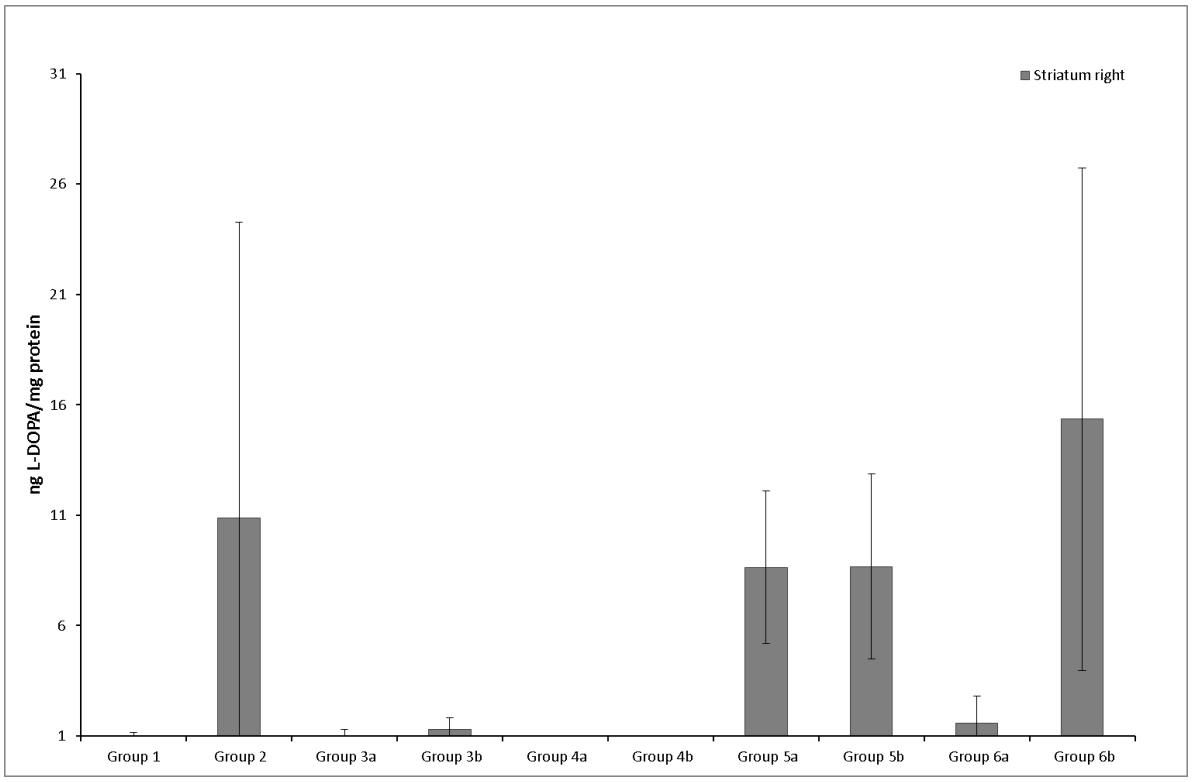

Figure 6 L-DOPA amount in right *striatum* for the six different groups. The data shows a restored L-DOPA production in non-regulated group (group 2), as well as group 5a, 5b, and 6b.

Metabolites

The mobile phase was optimized during the analysis due to peak overlap from matrix interference (Figure 7 All samples were analysed twice and the results were compared. Mobile phase 1 and mobile phase 2 were used for analysis of the samples from study one whereas mobile phase 2 and mobile phase 3 were used for the samples from study two (Table 2). All metabolites were quantified using the same mobile phase conditions (mobile phase B) except for DOPAC which was analysed using mobile phase 1 and mobile phase 3. The limited amount of sample made a third rerun of the samples from the first part of the study impossible and DOPAC was therefore quantified in different mobile phase condition for the first and second part.

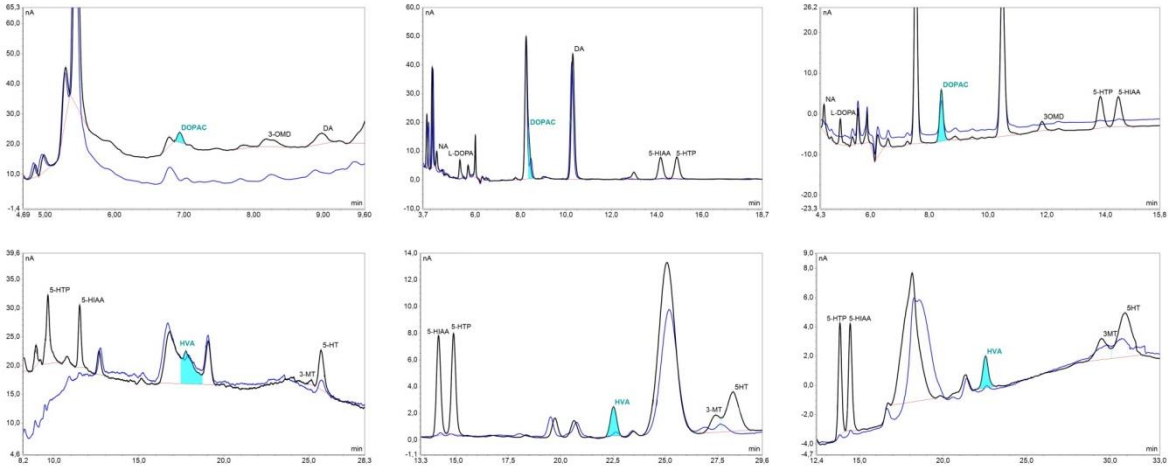

Figure 7 (A) A peak overlap matrix interference was observed for HVA using mobile phase 1. The blue line is the sample matrix whereas the black line is the same sample matrix spiked with HVA and DOPAC. (B) A peak overlap matrix interference was observed for DOPA using mobile phase 2. The blue line is the sample matrix whereas the black line is the sample matrix spiked with HVA and DOPAC. No matrix interference was observed for HVA. (C) No peak overlap matrix interference was observed when using mobile phase 2. The blue line is the sample matrix whereas the black line is the sample matrix spiked with HVA and DOPAC.

Metabolites levels for the regulated groups were expected to be similar to metabolites levels in the non-regulated group when given TMP and similar to the buffer group when not given TMP. The reasoning behind this is as follow: the negative control group (group 1) has no DA production and therefore no DA metabolites whereas an increased level of DA metabolites indicates a restored or partly restored L-DOPA production. 3OMD was monitored in order to be able to confirm that the produced L-DOPA was converted to DA and not degraded to 3-OMD (see Table 6 for all monoamines included in the HPLC-ECD analysis, for a schematic over the dopamine system see Figure 2

Table 6 Monoamines included in the HPLC-ECD analysis. At the top of the table are monoamines included in the dopamine system are listed. In the bottom of the table are compounds included in the 5-HT system listed. The L-DOPA and DA levels as well as its metabolites were measured in both left and right *striatum*. Three monoamines in the serotonin pathway were also included in the analysis to make sure that the serotonergic neurons were not affected in a negative way by the gene therapy.

Dopamine system		
Dopamine	DA	
3,4-Dihydroxy-L-phenylalanine	L-DOPA	DA precursor
L-3-O-Methyl-DOPA	3-OMD	L-DOPA metabolite
3,4 dihydroxyphenylacetic acid	DOPAC	DA metabolite
Homovanilic acid	HVA	DA metabolite
3-Methoxytyramine	3-MT	DA metabolite
Serotonin system		
Serotonin	5-HT	
5-Hydroxy-L tryptophan	5-HTP	5-HT precursor
indole acetic acid	5-HIAA	5-HT metabolite

For 3-OMD (the L-DOPA metabolite), DOPAC and HVA, the result shows a restored DA production in animals in group 5a, group 5b and group 6b as the DOPAC and HVA levels are similar to the 3-OMD, DOPAC and HVA levels in group2 (the non-regulated group)(Figure 8).

The result also shows a successful regulation in group 6 as Group 6b has a higher 3-OMD, HVA, and DOPAC levels than 6a (Figure 8 No difference in 3-OMD, HVA, and DOPAC levels was detected between animals given TMP and not given TMP in group 5 (DD-WT). All other groups had 3OMD, HVA, and DOPAC levels similar to group 1 (the negative control group) or group 6a and are considered as not measurable regulated. A low 3OMD level in these groups confirms that L-DOPA was not produced and degraded into 3OMD instead of converted to DA.

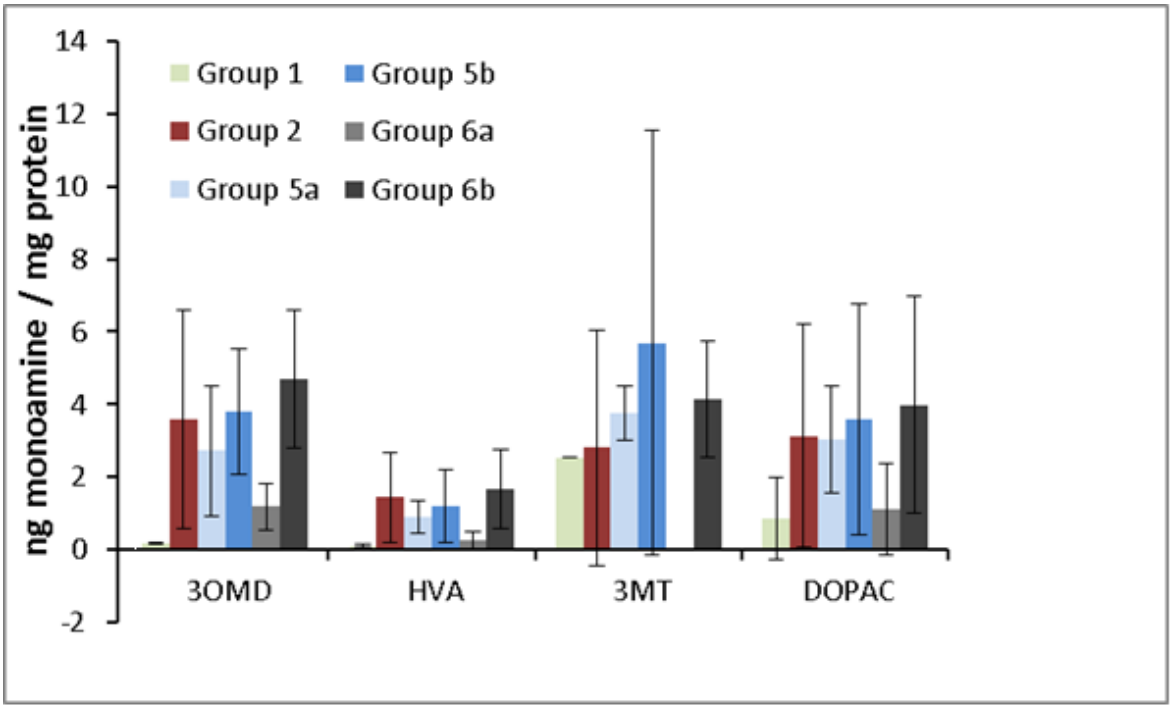

Figure 8 Striatal concentration levels of the metabolites included in the L-DOPA and DA production.

The 3MT level in the negative control group was similar to the 3MT level in group 2, group 5a, group 5b, and group 6b. The result was not expected as the 3 MT level in the control group (group 1) was expected to be significantly lower than for the regulated groups when given TMP. Also no difference can be seen between the groups given TMP and not given TMP except for group 6 where no 3MT was detected for the group not receiving TMP (group 6a).

Tyrosine hydroxylase activity

Monitoring Tyrosine hydroxylase activity in striatum using spectrophotometry

Optimal sodium periodate concentration

Different concentrations of sodium periodate were tested in order to find the optimal sodium periodate concentration. The absorbance at 475 nm increased with increasing sodium periodate concentration up to 2400 µM sodium periodate. The absorbance at 475 nm was constant at

concentrations higher than 2400 µM. From this, the optimal sodium periodate concentration was determined to 1800 µM (Figure 9 Using optimal sodium periodate concentration increases the sensitivity of the method and the L-DOPA concentration can therefore be determined more accurately (Figure 10

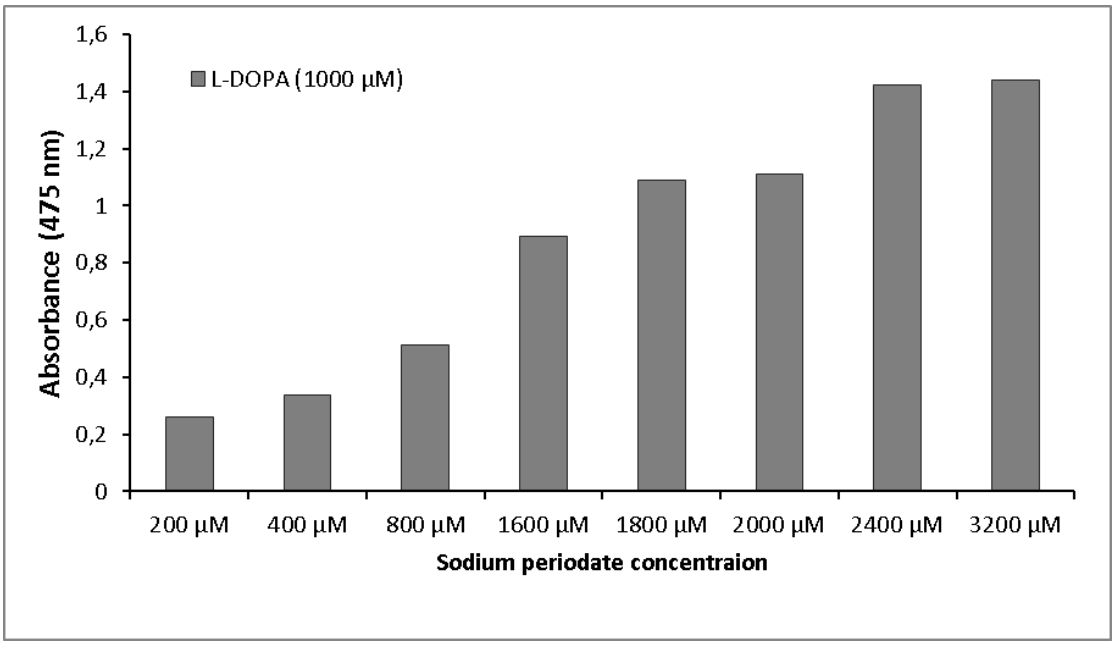

Figure 9 Different sodium periodate concentrations were tested in order to find the optimal concentration. The optimal sodium periodate concentration was determined to 1800 µM as it is shown to give a high signal at 475 nm but are still not saturated. The L-DOPA concentration used was 1000 µM for all sodium periodate concentrations.

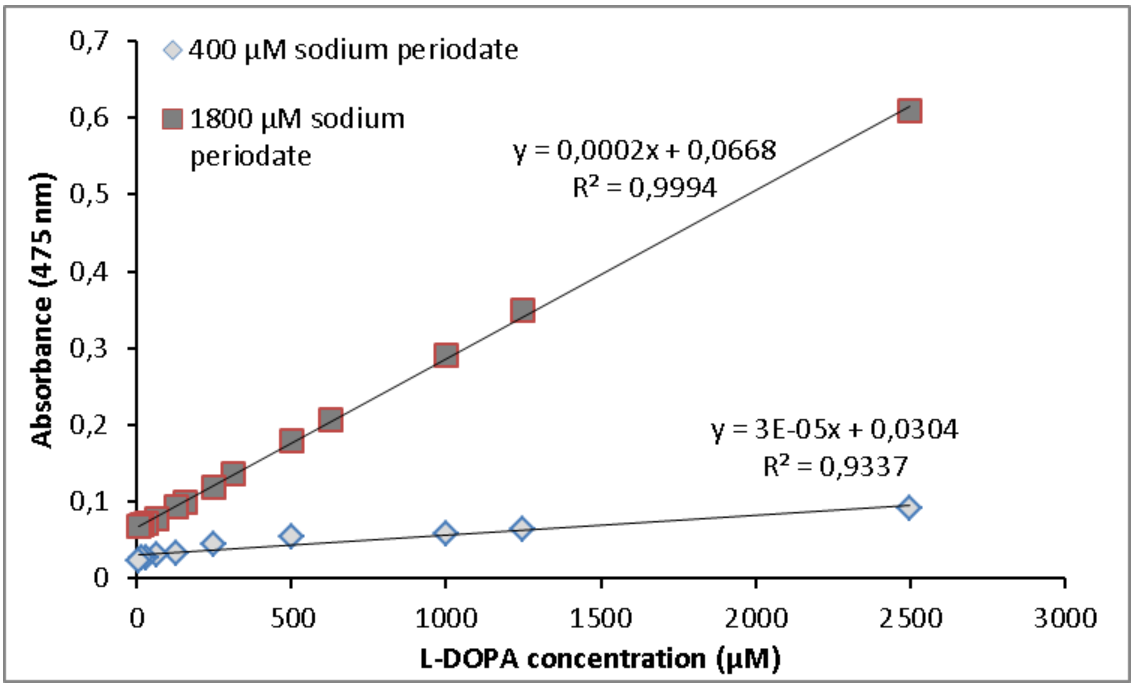

Figure 10 Calibration curve using 1800 µM and 400 µM sodium periodate. Using an optimal sodium periodate concentration (1800 µM) increases the sensitivity of the method.

TH activity in striatum and HEK293 cells

The TH activity was measured in naïve *striatum*, HEK293 cells overexpressing TH, and naïve HEK293 cells using a concentration of 400 µM sodium periodate. No activity could be measured in any of the samples. The TH activity in naïve *striatum* was also measured using 1800 µM sodium periodate as the sensitivity of the standard curve did improve by using 1800 µM sodium periodate instead of 400 µM (Figure 10 However, no activity could be measured using 1800 µM sodium periodate. A slight signal decrease was observed for the 5 times diluted samples and is believed to be caused by a matrix effect quenching the signal (Figure 11 The iron(II) concentration was 100 fold higher in all measurements using 1800 µM sodium periodate compared to the measurements using 400 µM sodium periodate. A standard curve using the lower concentration of iron(II) sulfate but the higher concentration of sodium periodate was made to prove that the iron concentration did not affect the signal.

All naïve *striatum* (left sides) tested in the TH activity assay were known to have properly working dopamine production (Figure 4 and therefore a sufficient amount of active TH. The assay has been reported to work for the measure of TH activity in mouse brain (Wang, Sung, & Chung, 2017). They did however not measure the TH activity in naïve brain but instead in mouse brain were the TH activity had been enhanced. The conclusion that the method is not sensitive enough for naïve brain is drawn.

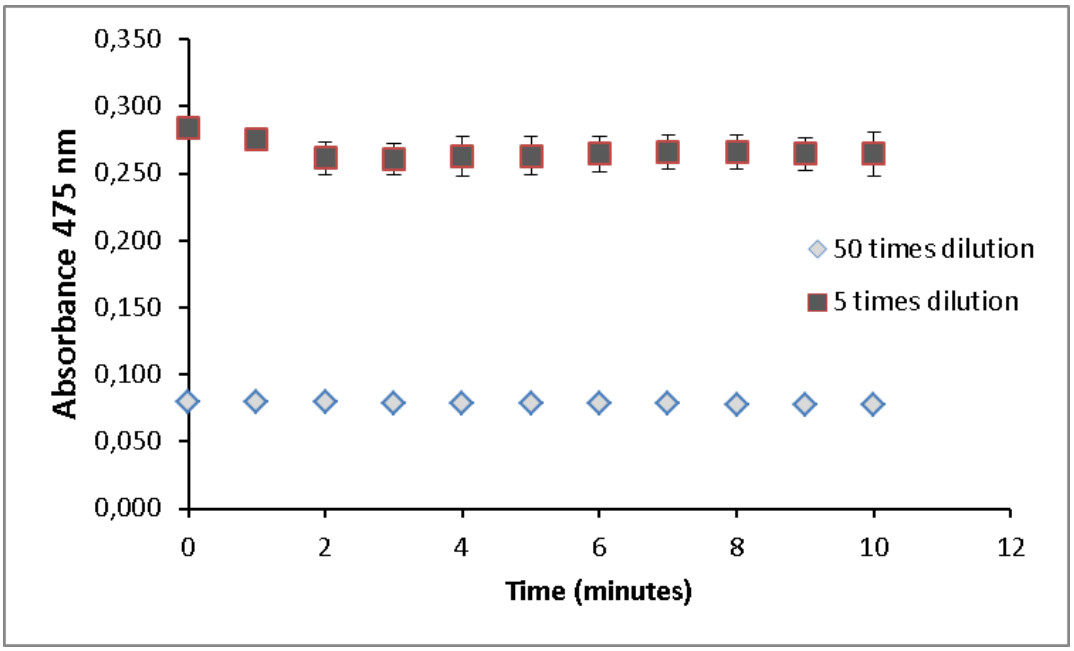

Figure 11 The absorbance at 475 nm was measured once every minute during ten minutes. The plate was incubated at 37°C between the measurements. Two different dilutions were tested: 5 times and 50 times dilution of naïve *striatum*. The periodate concentration was 1800 µM.

Matrix effect

To test if the non-detectable TH activity was caused by a matrix effect quenching the signal L-DOPA was added to the full assay reaction mix including diluted *striatum* or HEK293 cells, reaction buffer, and 400 or 1800 µM sodium periodate. No increase was seen for sample diluted 5 times whereas a significant signal increase was observed when adding L-DOPA to 50 times diluted naïve *striatum.* The signal increase was however larger in the blank (PBS) than in 50 times diluted naïve *striatum* (Figure 12 The same trend was observed for HEK293 cells. The result indicates that something in the sample quenches the signal at high sample concentrations and at higher sample dilutions the activity is too low to be detectable.

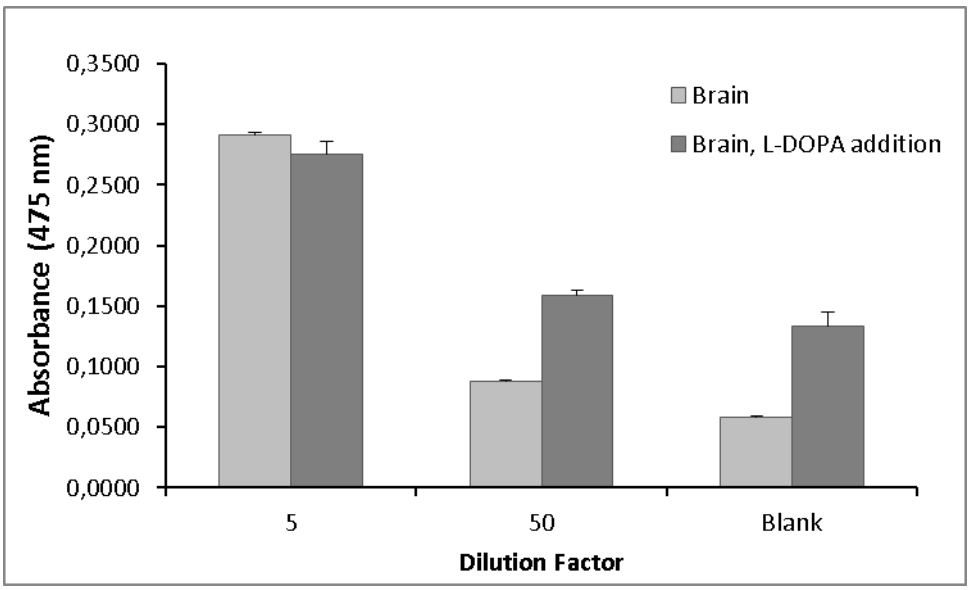

Figure 12 Increase or decrease in signal when 1 µl of 5 mM L-DOPA was added to the well. The absorbance at 475 nm was measured before and after addition of L-DOPA. The sodium periodate concentration was 1800 µM.

Monitoring Tyrosine hydroxylase activity in striatum using HPLC-ECD

A decrease of the signal was observed after injection of samples containing the full reaction mix. All components in the reaction mix was therefore analysed separately and the result showed that the signal decrease occurred only when injecting m-hydroxybenzylhydrazine (AADC inhibitor) or the full reaction mix. Cleaning the electrode gave a full signal recovery and the conclusion from this is that m-hydroxybenzylhydrazine affects the electrode negatively. To solve this problem, another detection method can be used or alternatively find another AADC inhibitor that does not foul the electrode. Further studies have to be done to be able to tell if this analysis method is useful in the monitoring of TH activity in *striatum*.

Conclusion

The result shows a restored L-DOPA production in the non-regulated group (group 2) as well as the GCH1 regulated group (group 6). No effect of the L-DOPA production was seen in the TH regulated groups. This might be due to the low TMP concentration in *striatum*. Further studies have to be done to optimize the TMP dose and administration.

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