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**Design of a single AAV vector for co-expression of TH and GCH1  
to establish continuous DOPA synthesis in a rat model of  
Parkinson's disease**

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**Running title:** Single AAV vector-mediated DOPA delivery

**Abbreviations:** 5-HT: serotonin, 6-OHDA: 6-hydroxydopamine, AADC: aromatic L-amino acid decarboxylase, AAV: adeno-associated virus, AP: anteroposterior, BH4: 5,6,7,8-tetrahydro-L-biopterin, Ctx: cortex, DA: dopamine, DAB: 3,3'-diaminobenzidine, DOPA: 3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenylacetic acid, DV: dorsoventral, gc: genome copies, GCH1: GTP cyclohydrolase-1, GP: globus pallidus, HPLC: high performance liquid chromatography, HVA: homovanillic acid, ITR: inverted terminal repeat, MFB: medial forebrain bundle, ML: mediolateral, NAc: nucleus accumbens, PD: Parkinson's disease, PFA: paraformaldehyde, SNpc: substantia nigra pars compacta, SNpr: substantia nigra pars reticulata, Str: striatum, Syn-1: synapsin 1, TH: tyrosine hydroxylase, Tu: olfactory tubercle, VTA: ventral tegmental area.

## Abstract

Pre-clinical efficacy of continuous delivery of 3,4-dihydroxyphenylalanine (DOPA) with adeno-associated viral (AAV) vectors has recently been documented in animal models of Parkinson's disease. So far, all studies have utilized a mix of two monocistronic vectors expressing either of the two genes, tyrosine hydroxylase and GTP cyclohydrolase 1, needed for DOPA production. Here we present a novel vector design that enables efficient DOPA production from a single AAV vector in rats with complete unilateral dopamine lesions. Functional efficacy was assessed with drug-induced and spontaneous motor behavioral tests where vector-treated animals showed near complete and stable recovery within one month. Recovery of motor function was associated with restoration of extracellular dopamine levels as assessed by online microdialysis. Histological analysis showed robust transgene expression not only in the striatum but also in overlying cortical areas. In globus pallidus we noted loss of NeuN staining, which might be due to different sensitivity in neuronal populations to transgene expression. Taken together, we present a single AAV vector design that result in efficient DOPA production and wide-spread transduction. This is a favorable starting point for continued translation toward a therapeutic application, although future studies need to carefully review target region, vector spread and dilution with this approach.



## Introduction

The current standard treatment for Parkinson's disease (PD) is based on restoration of striatal dopamine (DA) neurotransmission by peripheral administration of the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) <sup>1,2</sup>. Although L-DOPA is very effective in the early phase of the disease, the majority of PD patients start experiencing adverse effects as the disease progresses. The most prominent are L-DOPA-induced involuntary movements (dyskinesias) that are thought to, at least partly, develop due to the intermittent administration of the drug <sup>3</sup>. Clinical observations suggest that these dyskinesias can be alleviated by constant administration of L-DOPA via either the intravenous or the duodenal route <sup>4,5</sup>.

Continuous administration of DOPA can also be achieved by viral vector-mediated delivery of the genes encoding the enzymes necessary for its production - namely tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1). GCH1 is a key enzyme in the synthesis of the cofactor 5,6,7,8-tetrahydro-L-biopterin (BH4). Together, TH and GCH1 are the rate-limiting enzymes in the production of DOPA from the dietary amino acid tyrosine <sup>6</sup>. DOPA is then converted into DA by the aromatic L-amino acid decarboxylase (AADC). By intrastriatal delivery of TH and GCH1 a local and continuous production of DOPA can be achieved, and further decarboxylation presumably occurs in spared serotonergic or dopaminergic neurons <sup>7</sup>. This therapeutic strategy has the potential to alleviate motor complications in PD without induction of dyskinesias or other adverse events induced through off-target DA receptor stimulation. While other vector types have allowed multi-cistronic expression cassettes

containing DA synthesizing enzymes, until now, all studies on the continuous DOPA delivery approach have utilized two separate adeno-associated viral (AAV) vector constructs<sup>8-12</sup>. In this study we have designed and validated a single AAV vector including both TH and GCH1 (Fig 1). There are several advantages with a single-vector design. Transduction will assure that both transgenes are co-expressed in the same cells. This is favorable from a clinical translational perspective as it ensures that all transduced cells that drive the transcription of the vector also can synthesize DOPA. Furthermore, by avoiding a mix of two vector constructs, the reproducibility between production batches should also be simplified. We have previously shown that when the two transgenes are delivered from a dual vector injection, at a GCH1:TH ratio of 1:5, optimal DOPA production is achieved<sup>13</sup>. To achieve a favorable stoichiometric relationship in the new single-vector design, we used a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), down-stream the TH gene. The WPRE sequence functions on the mRNA level (i.e., solely on the expression cassette for the TH gene) through enhancement of mRNA trafficking to the ribosome and increased translation.

In this study, we validated the single AAV vector construct expressing both TH and GCH1 in the complete 6-OHDA lesioned rat PD model with behavioral and biochemical readouts. Therapeutic efficacy was evaluated with several behavioral tests to study functional recovery in simple and complex motor performance and learning tasks. Enzyme function and DA synthesis was studied with online microdialysis, post-mortem biochemistry and histology of transgene expression.

## Results

### **Long-term assessment of functional motor recovery**

The therapeutic efficacy of the TH-GCH1 single-vector construct was assessed with a series of behavioral tests. Amphetamine- and apomorphine-induced rotation tests and the cylinder test were performed pre-AAV treatment to validate the 6-OHDA lesion and to balance the experimental groups - the AAV-TH-GCH1 vector treatment group (TH-GCH1) and a lesion control group (Les-Sham). After AAV treatment, the asymmetrical forepaw-use in the cylinder test was restored after 5 weeks in the TH-GCH1 group, and maintained at the 12- and 14-week time points (Fig. 3a). Further, the animals were re-assessed with amphetamine- and apomorphine-induction at 10 and 14 weeks, where the TH-GCH1 treatment resulted in a 50 % decrease in rotational bias in both tests (Fig. 3d,e). At baseline (post-lesion), the majority of animals displayed a slight ipsiversive spontaneous rotational bias. However, at the beginning of the fourth week, animals in the TH-GCH1 group instead started showing indications of contraversive rotational bias. Therefore, all animals' drug-naïve rotational behavior was quantified at week 5, 10 and 14 (Fig 3f). At week 5, the animals in the TH-GCH1 group rotated close to 60 turns toward the side of injection during the evaluation time, compared to the Les-Sham group that rotated slightly toward the side contralateral to the lesion. At 10 weeks this difference was decreased and at the last assessment there was no significant difference between the groups.

Three behavioral tests, in which the animals were not pre-trained, were used to increase the support for the functional efficacy of the TH-GCH1 vector. Four weeks after AAV treatment, the animals were trained in the stepping test to assess forelimb akinesia by quantifying the ability to do adjusting side steps. Animals in the Les-Sham group displayed severe impairment in the forepaw contralateral to the lesion (Fig. 3b). Their performance did not improve over the 14 weeks of assessment. In the TH-GCH1 group, however, the animals showed equal performance on the left paw (contralateral to the lesion) as the right paw. This recovery was maintained throughout the experiment.

The corridor test was used to study the sensorimotor aspect of the lesion, which was performed as a single assessment at 25 weeks post-AAV treatment. In this test, the read-out variable is the animals' lateralized response selection by nose-pokes in small equally spaced containers filled with sugar pellets along a corridor. Animals in the Les-Sham group showed a strong bias toward retrieving pellets only on the right side (ipsilateral to the lesion) (Fig 3c left), with only single retrievals on the animals left side ( $1.3 \pm 1.0$  % of total retrievals). Again the animals in the TH-GCH1 group displayed a completely symmetrical response (left retrievals  $49.6 \pm 4.6$  % of total) (Fig. 3c right).

At 21-24 weeks post-injection of the TH-GCH1 vector, the animals were trained and tested in the staircase version of the paw-reaching test, utilizing a wide platform (35 mm) for maximal stringency of the test. In the test setup on day 1 through 15, both sides of the staircase box were baited with 40 sugar pellets. Day 16-18, only the left staircase was filled, presenting the animals with a forced choice (FC-L) and day 19-21 the opposite staircase was utilized (FC-R).

The animals continuously improved in the number of taken and eaten pellets over day 1-6 (Phase I, learning phase) using both the ipsilateral and contralateral paw (Fig. 4a,b and d,e). The rate of learning in both groups was evaluated by regression coefficient comparison over phase I. This confirmed that the two groups improved at the same rate in number of eaten pellets on the ipsilateral paw (Fig. 4b), whereas the animals in the TH-GCH1 group improved at a significantly higher rate on the contralateral paw (Fig. 4e) ( $1.49 \pm 0.63$  more pellets eaten per day  $p=0.020$ ). Similarly, the animals in the treatment group improved in the number of pellets taken at a higher rate using both paws (Fig. 4a,e) ( $3.44 \pm 1.31$  and  $2.38 \pm 1.02$  more pellets taken per day for right and left paw respectively, as compared to the Les-Sham group,  $p=0.01$  and  $p=0.021$  respectively). Day 7-10, the animals still displayed high variability in their performance in the test and thus, this was considered a calibration phase (Phase II). Day 11-15 the animals displayed very little variation (Phase III, Stable phase). This was validated by three-way ANOVA that confirmed no significant time effect over days 11-15. During the stable phase, the animals in the TH-GCH1 group managed to pick up significantly more pellets on the side contralateral to the lesion, compared to the animals in the Les-Sham group (Fig. 4g). This was coupled with a significantly greater success in the number of pellets eaten on that side (Fig. 4h), resulting in a decreased error rate (Fig. 4f, i), possibly at the expense of a slightly increased error rate with the intact paw (Fig. 4c, i).

## **Efficient BH4 and DOPA synthesis from the single TH-GCH1 AAV vector**

For quantitative analysis of the TH-GCH1 gene expression and enzyme function, a subgroup of the animals (n=6 per group) was sacrificed 28 weeks post-AAV treatment for biochemical analysis. Three regions of the forebrain were analyzed in each hemisphere - the dorsal striatum, ventral striatum and the frontal cortex. Together, these fractions constituted the whole forebrain and thus the total forebrain concentration could be calculated (Fig. 5). As reported previously<sup>13-15</sup>, the complete 6-OHDA lesion induced an almost 90 % decrease in dorsal striatal BH4 levels ( $0.16 \pm 0.02$  pmol/mg compared to  $1.4 \pm 0.009$  pmol/mg) (Fig. 5a). The same pattern was observed in the ventral striatum and frontal cortex, although to a lesser extent. The striatal TH-GCH1 injection caused a significant increase in BH4 levels both in the ipsilateral and the contralateral hemisphere. More than eight-fold levels compared to intact levels were observed in the ipsilateral dorsal striatum ( $11.4 \pm 1.5$  pmol/mg) and supra-physiological levels were also detected in the ventral striatum and the frontal cortex (Fig. 5a). The striatal TH-GCH1 over-expression also led to increased DOPA synthesis. The animals were sacrificed without pre-treatment of a centrally acting AADC enzyme inhibitor such as NSD-1015. Therefore, the DOPA levels observed reflect either a saturation of the AADC enzyme activity or accumulation in sites that do not contain AADC enzyme activity. Whereas the DOPA levels in the intact and lesioned rat brain remained close to the detection limit, the levels in the dorsal striatum ipsilateral to the injection of animals that received the TH-GCH1 vector approached 15 pmol/mg ( $14.8 \pm 1.9$  pmol/mg) (Fig. 5b). Interestingly, whereas

the transduction resulted in increased BH4 levels in the intact hemisphere, there was no detectable increase in DOPA levels in these regions except for the ventral striatum. This further supports our interpretation that accumulation of DOPA is a result of saturation of the AADC enzyme in the denervated forebrain regions and that in the projection region, the contralateral hemisphere, the AADC enzyme is not saturated.

In the rat striatum after a complete 6-OHDA lesion there is no neuronal population that retains the ability to efficiently recover DA from the extracellular space and therefore prevent degradation. While the TH-GCH1 gene delivery reconstituted only 9 % of intact striatal DA levels ( $5.0 \pm 1.2$  pmol/mg compared to  $56.3 \pm 3.6$  pmol/mg) (Fig. 5d), the DOPAC and HVA levels were reconstituted to 50 % and 200 % respectively (Fig. 5e,f). In this study, we also evaluated the effect of the total AADC enzyme activity in the three regions, as the activity of this endogenous enzyme is a key component of the DOPA delivery strategy. In the dorsal striatum, the lesion induced around 85 % decrease in AADC enzyme activity (Fig. 5c). In the TH-GCH1 group, decarboxylation activity was decreased around 50 % in ventral striatum whereas it was unaltered in frontal cortex.

### **Striatal monoamine levels after AAV-mediated TH-GCH1 treatment**

Forty-weeks after AAV-treatment, assessment of striatal monoamines was performed with online microdialysis in a subset of animals (TH-GCH1 n=4, Les-Sham n=3, and intact controls n=3. These animals were not followed with behavioral tests). Samples were collected in 15 min time bins in anaesthetized animals and sampling started after 60 min equilibration following the

intrastratial probe placement. Sampling consisted of three baselines samples, a 15 min KCl challenge then followed by four washout samples (Fig. 6a,b). Baseline levels of DA in the intact rat striatum were  $3.3\pm 0.8$  nM, which was significantly decreased by the 6-OHDA lesion. The TH-GCH1 treated animals had higher baseline levels of DA than intact controls ( $10.5\pm 3.8$  nM), however, had much lower release when challenged with KCl ( $16.0\pm 7.5$  nM compared with  $176.1\pm 21.5$  nM). Animals in the Les-Sham group showed slightly lower release of serotonin (5-HT) when challenged with KCl than intact controls (Fig. 6b). Interestingly, animals in the TH-GCH1 group released substantially lower amounts of 5-HT in response to KCl compared to the Les-Sham group. Extracellular levels of DOPA and DOPAC followed the same pattern as the total levels quantified post-mortem with high extracellular DOPA levels and partially reconstituted DOPAC (Fig. 6c,d). Extracellular HVA levels were however not reconstituted to the same extent (Fig. 6e).

### **Widespread transgene expression in the rodent striatum**

Immunohistochemistry was used to qualitatively assess transgene expression. The injection of 6-OHDA into the medial forebrain bundle in the right hemisphere resulted in a complete loss of the neurons located in the ipsilateral substantia nigra pars compacta and the nuclei they innervate - the striatum and substantia nigra pars reticulata (Fig. 7a,d). The 6-OHDA injection also depleted part of the ventral tegmental area that projects to the nucleus accumbens and olfactory tubercle. The contralateral side remained visually unaffected. The two-site injection of the TH-GCH1 vector induced a strong ectopic TH expression in the dorsal, central and caudal striatum (Fig. 7b), but also in the cortex and globus



pallidus. The GCH1 staining was overlapping with TH but with less intensity (Fig. 7c). The transduction of striatal projection neurons resulted in an anterograde transport of both TH and GCH1 to the substantia nigra pars reticulata (Fig. 7e for TH and GCH1 not shown). A clear reduction in NeuN positive cells could be observed in the ipsilateral globus pallidus in vector-treated animals compared with the contralateral side (Fig. 7f,g), which was coupled with increased microglial activation, assessed with Iba1 staining (data not shown).

## Discussion

The use of a single-vector for co-expression of the TH and GCH1 genes for continuous DOPA delivery has been hampered by the limited packaging capacity of AAV vectors. The yield of the recombinant vector is optimal around the size of the wild-type AAV genome (4.7 kb). If the recombinant genome significantly exceeds this size, the production titers and *in vivo* efficacy become compromised. The vector construct tested here uses a somewhat uncommon approach (Fig 1). We decided to create two independent expression cassettes where each transgene is expressed from its own promoter. This approach is different from the multicistronic constructs utilized in other studies and gives an important benefit of increased expression predictability<sup>16</sup>. In addition, this is the first clinical candidate vector for enzyme replacement in PD not to utilize a modified form of the cytomegalovirus promoter<sup>17-20</sup>, but instead an unmodified promoter of human origin. The small synapsin-1 (Syn-1) promoter we used here is preferentially expressed in neurons<sup>21</sup>. Furthermore, adding the WPRE enhancer element in the TH expression cassette, we could amplify the expression of this gene over the GCH1 gene. Presence of the WPRE sequence has been reported to enhance expression in the order of 5-10 fold<sup>22</sup>, bringing it very close to the ratio we have previously determined to be optimal for this approach<sup>13</sup>. As we have found AAV5 to be our serotype preference for this application, both in rats and non-human primates<sup>12,23</sup>, we produced this vector also as that serotype.

We have previously shown that full motor recovery with the continuous DOPA delivery approach could take up to 12 weeks post-AAV treatment<sup>12</sup>. We

attributed this to modulation of the down-stream neuronal populations might be required for the symptomatic relief. In this study, we show that this modulation is not required for full recovery in a number of behavioral tests. We did, however, observe emergence of an contraversive rotation bias at 4 weeks post-AAV in the TH-GCH1 group, suggesting a transient over-activation. This is around the time when the expression of the vector reaches maximal level <sup>24</sup>. This effect declined over time suggesting that the post-synaptic modulation and normalization of the basal ganglia circuitry is a slow process, but one that is necessary for normal function to be recognized. Taken together with previous data, this suggests that the post-synaptic plasticity can work to adjust to both a sub-normal and supra-normal synaptic DA concentration, as long as it remains stable <sup>12</sup>. However, the data from this study alone cannot exclude the alternative explanation that there is a slight down-regulation of the transgene expression over time.

The magnitude of normalization in the drug-induced rotation was less than that observed in spontaneous motor behavioral tests. The reduction to about 50 % of baseline apomorphine-induced rotation, in the MFB 6-OHDA lesion model, appear to be somewhat of a floor effect with the AAV-mediated DOPA delivery, as we and others have observed this magnitude with vastly different efficacy of the vector <sup>8,25</sup> and unpublished observations. However, this appears to be a poor predictor of both protection against L-DOPA induced dyskinesias and recovery in spontaneous motor behavioral tests <sup>12</sup>. The mechanism behind this deserve further investigation.

The study design also included the staircase test to study the effects of continuous DOPA delivery on motor learning. This showed that TH-GCH1 treated animals were quicker to learn how to pickup and eat sugar pellets using the paw affected from the lesion as compared with the Les-Sham group. After the initial 10 days of testing the animals in the treatment group managed to pickup ten more pellets (a 50 % increase) using the affected paw and also ate almost twice as many compared to the Les-Sham group. Motor learning and its dependence on DA has been recently shown in an elegant study in Pitx3 deficient “aphakia” mice where motor learning, independent on motor function, was restored through long-term peripheral L-DOPA administration <sup>26</sup>. Our data suggest that a similar effect is achieved through the AAV-mediated DOPA delivery and that this may contribute to the significant therapeutic potential of the treatment.

The robust behavioral recovery indicates a very efficient DOPA synthesis. To evaluate the resulting DOPA and DA synthesis capacity and turnover, we measured monoamine content both in the extracellular space *in vivo* and the total tissue content post-mortem. Striatal monoamine measurements with online microdialysis showed reconstituted levels of baseline DA in the extracellular space. In a previous study by Mandel et al., microdialysis was conducted under similar circumstances but with a two-vector mix of AAV2 vectors with significantly lower transduction and expression efficacy. In this study, extracellular baseline DA levels were below detection limit <sup>11</sup>. Here we show that the DA levels not only could be restored but that even supra-physiological levels could be achieved. This suggests that even though treatment with TH and GCH1 only reconstitutes 9 % of striatal DA levels in tissue homogenates, compared

with intact controls, extracellular levels are restored. However, the ability to release through KCl infusion is still limited confirming that storage machinery is not restored with this treatment. This may also explain the less than complete reversal of amphetamine-induced rotation in these animals. However, the reduced 5-HT release suggest that at least a fraction of the *de novo* synthesized DA is co-stored in the releasable vesicle pool in serotonergic neurons. The reduced KCl-induced release of 5-HT is approximately 1.5 nM. This would correspond to less than 1 % of the releasable pool of intact DA innervation and a change of less than 10 % from the transduced DA baseline. This is very similar to what has been observed after peripheral L-DOPA administration in the 6-OHDA lesioned rat <sup>27</sup>.

One principal requirement a the successful outcome of the continuous DOPA delivery as a clinical therapy is the availability of sufficient decarboxylase activity at the site of transduction <sup>28</sup>. Indeed we find that close to 90 % of the AADC activity in the dorsal striatum appear to reside in DA terminals and is efficiently depleted with the 6-OHDA lesion. We have previously shown that a considerable amount of the residual AADC activity resides in 5-HT terminals in the striatum and that these are indeed important for the symptomatic relief of the therapy <sup>12</sup>. This raises the important consideration of how the availability of AADC is in the human, Parkinsonian brain. While previous studies have shown that the threshold for symptomatic relief of L-DOPA does not increase significantly with disease progression <sup>29</sup>, supporting the concept of a continuous DOPA delivery strategy, other studies have tried to assess the AADC function in the diseased brain. In these studies, the AADC levels appear to be significantly

reduced although technical challenges have rendered them highly variable <sup>30,31</sup>. More robust findings have been made in MPTP lesioned macaques where the reduction in AADC activity is suggested to limit the L-DOPA pharmacotherapy <sup>32</sup>. What implications this may have on the clinical potential for this therapy requires further investigations.

The finding of neuronal loss in the globus pallidus raise questions regarding potential toxicity due to high expression of the transgenes and their products. It has been shown *in vitro* that high levels of DA and DOPA can cause oxidative stress through formation of reactive oxygen species <sup>33</sup>. Similarly, Chen and colleagues have shown that ectopic accumulation of DA in striatal projection neurons can cause rapid degeneration in a transgenic mouse model <sup>34</sup>. Therefore, it is not unreasonable to expect that a very efficient DOPA synthesis *in vivo* at high enough titers may generate similar effect. Further studies are required to elucidate the vulnerability of transduced neurons and to determine the therapeutic window of the treatment and to exclude the pallidal cell loss contribution to symptomatic relief .

Taken together, the results from this study show the possibility to create a single AAV vector containing both TH and GCH1, that results in very efficient DOPA synthesis, superior to what we have achieved with two separate vector constructs of the same serotype. Furthermore, this study shows that an enzyme replacement strategy can result in complete recovery in a number of motor tests already 4 weeks after initiation of the therapy but that the post-synaptic adjustment to a new equilibrium may take longer time. This opens up for further developments of this very promising treatment alternative for PD.

## Materials & Methods

### Subjects

Forty-four female Sprague-Dawley rats weighing 225-250 g were purchased from Charles River (Germany) and were housed 2-3 per cage with free access to food (except during the staircase and corridor tests, see below) and water under a 12 h light/12 h dark cycle. All experimental procedures performed in this study were approved by the Ethical Committee for use of Laboratory Animals in the Lund-Malmö region.

### Surgical procedures

All surgical procedures were conducted under anesthesia induced by an i.p. injection of a 20:1 mixture of Fentanyl and medetomidine (Dormitor, Apoteksbolaget, Sweden) in approximately 6 ml/kg. The stereotactic injections were conducted with a Hamilton syringe with the animal mounted in a stereotactic frame (Stoelting, Wood Dale, IL). The anteroposterior (AP) and mediolateral (ML) coordinates were calculated from bregma and the dorsoventral (DV) coordinates from the dural surface <sup>35</sup>.

*6-OHDA lesions.* The neurotoxin 6-OHDA (Sigma-Aldrich AB, Sweden) was injected unilaterally into the right medial forebrain bundle to lesion the ascending DA projections <sup>36</sup>. Fourteen µg free base 6-OHDA was administered in ascorbate-saline (0.02 %) at a concentration of 3.5 µg/µl into one deposit along the fiber bundle to ensure a specific lesion of the DA fibers, sparing the serotonin

projection system<sup>37</sup>. The injection was conducted at the following coordinate: AP: -4.4 mm; ML: -1.2 mm and DV: -7.8 mm with the tooth bar set to -2.4 mm with a 26-gauge needle attached to the Hamilton syringe. The toxin was injected at a speed of 1  $\mu$ l/min and the needle was kept in place for an additional 3 min before it was slowly retracted from the brain parenchyma.

*AAV vector injections.* The animals in the TH-GCH1 treatment group received intrastriatal injections of 5  $\mu$ l AAV vector in ringer lactate suspension. The injections were performed at two sites with two deposits in each, 1.5  $\mu$ l in the more ventral and 1  $\mu$ l in the more dorsal, delivered with a pulled glass capillary (outer diameter 60-80  $\mu$ m) mounted on a 22-gauge needle attached to the Hamilton syringe. The injection coordinates were: (1) AP: +1.0 mm; ML: -2.6 mm and DV: -4.5, -3.5 mm and (2) AP: 0.0 mm; ML: -3.2 mm and DV: -5.0, -4.0 mm with the tooth bar set to -2.4 mm. The injection rate was 0.4  $\mu$ l/min and the needle was kept in place for 1 min after the first and 3 min after the second deposit, before it was slowly retracted.

### **AAV vector design**

The viral vectors utilized in this study were pseudotyped AAV2/5 vectors, where the transgene of interest is flanked by inverted terminal repeats of the AAV2 packaged in an AAV5 capsid (for reasons of simplicity the vectors were referred to as AAV elsewhere in the paper). For this experiment, a novel plasmid was generated, expressing both transgenes, TH and GCH1, under the human Synapsin-1 (Syn-1) promoter (Figure 1). The cDNA for the human GCH1 gene was followed by a late SV40 poly-A sequence that then preceded the second Syn-



1 promoter controlling the expression of the human TH-1 cDNA. To achieve a superior expression of the TH gene over GCH1, the trafficking of the TH mRNA was improved by the addition of a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). This vector contains the wild-type WPRE with the open reading frame for a fragment of a potential oncogene, the X protein. However, this can be removed without compromising the function <sup>38</sup>. The full sequence was terminated by an early SV40 poly-A sequence. The AAV vectors were produced with a double-transfection method with the appropriate transfer plasmid and the helper plasmid containing the essential adenoviral packaging genes, as described previously <sup>39</sup>. They were thereafter purified by iodixanol step gradients and Sepharose Q column chromatography <sup>40</sup>. The purified viral vector suspension was titered with TaqMan quantitative PCR <sup>41</sup>, but with primers and probe targeted toward the WPRE sequence. The final titer of the injected AAV vector suspension was 9.7E13 gc/ml.

## **Behavioral tests**

*Drug-induced rotation* tests were performed by measuring left and right full body turns with automated rotometer bowls (AccuScan Instruments Inc., Columbus, Ohio), after an injection of either D-amphetamine sulfate (2.5 mg/kg, i.p., Apoteksbolaget, Sweden) or apomorphine-HCl (0.05 mg/kg, s.c., Sigma-Aldrich AB, Sweden) and quantified continuously for 90 and 40 min, respectively. Rotational asymmetry scores are presented as net full body turns/min, either as ipsiversive (clockwise, i.e., toward the lesion) or contraversive (counter clockwise) rotations. The drug-naïve spontaneous rotation test was performed with the same setup with the exception of the drug challenge.

*Cylinder test* assessing exploratory forelimb use asymmetry was performed as described by <sup>42</sup> with minor modifications from <sup>43</sup>. Each rat was placed in a 20 cm in diameter glass cylinder and exploratory behavior was recorded with a digital video camera. Two perpendicular mirrors were placed behind the glass cylinder, which allowed the complete surface of the cylinder to be clearly visible on the recording. The animal was left in the cylinder until at least 20 forelimb touches on the cylinder wall were observed. Forelimb placement and use on the cylinder wall was scored off-line with frame-by-frame analysis by an observer blinded to the group identity of the animals. The paw used during each of the 20 contacts with the cylinder walls during rearing was scored and presented as left forepaw touches as a percentage of the total number of touches.

*Stepping test* was initially described by <sup>44</sup>, and the modified side-stepping version <sup>45</sup> was performed in the this study. Forelimb akinesia was assessed by an investigator blinded to the animals group identity by holding the rat with two hands only allowing one forepaw to reach the table surface. The animal was then moved sideways over a defined distance of 90 cm at a constant pace over 4-5 sec. The investigator scored the numbers of adjusting side steps in both forehand and backhand direction twice, and the average was calculated. The animals were trained for 6 days during the third week post-AAV injection and then scored by taking the average of their performance during day 7-9, when their daily variability had minimized. During the remaining time points at 10-, 12- and 14-weeks post-AAV injection, the animals only needed habituation for one or two days and were then scored during three consecutive days.

*Staircase test* was used to assess the skilled forelimb reaching and grasping abilities by with a modified version of the original test design described by <sup>46</sup> according to <sup>47</sup>. Briefly, sugar pellets (TestDiet, Richmond, VA) were placed on steps of a double staircase divided by a wide central platform (35 mm), all enclosed in a small Plexiglas box (285 x 60 x 90 mm). The animals were kept on a restricted food intake (6-8 g/day) during the test period with food only administrated after the daily test session. The staircase test was performed as a single session 21 weeks post-AAV injections. The animals were trained in the staircase test for 21 consecutive days (15 min/day) with 10 sugar pellets placed on each 2-5 step on both sides (total 40 pellets/side), where day 1 was defined as the first day the rats started retrieving sugar pellets. The data from day 11-15 was used as a stable performance score. At day 16-18 and 19-21, sugar pellets were only placed on the left and right shelf, respectively, challenging the rats with a forced choice. Skilled forelimb test results were expressed as the number of pellets eaten (taken - missed) on the contralateral (left) side.

*Corridor test* was performed 25 weeks post-AAV injections, as previously described <sup>48</sup> to study lateralized sensorimotor response selection. Briefly, the rats were placed in the end of a corridor (1500 x 70 x 230 mm) with 10 adjacent pairs of lids, evenly distributed along the floor. Each lid was filled with approx. 5 sugar pellets. Retrieval was defined as each time the rat poked its nose into a unique cup, regardless of if it ate any pellets. Revisits without interleaving other retrievals were not scored. Each rat was tested until 20 retrievals were made or a maximum time of 5 minutes elapsed. The rats were food restricted prior and throughout testing, as described above for the staircase test, and habituated for

10 minutes for 2 days in the corridor, with sugar pellets scattered along the floor. To reduce exploratory behavior the rats were placed in an empty corridor before scoring. The rats were then scored for 4 days and the results were presented as an average of the last 2 days of the number of contralateral retrievals (left) as a percentage of total retrievals.

### **Histological analysis**

Four animals from both the TH-GCH1 and Les-Sham groups were anaesthetized by an i.p. injection of 1.2 ml sodium pentobarbital (Apoteksbolaget, Sweden) and then transcardially perfused with 50 ml room tempered saline followed by 250 ml ice-cold 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer adjusted to pH 7.4, at a 50 ml/min rate. The brain was then dissected and post-fixated in 4 % PFA for 2 hours before cryoprotection in 25 % sucrose for approximately 24-48 hours. The fixed brains were cut into 35  $\mu$ m coronal sections on a semi-automated freezing microtome (Microm HM 450) and collected in 6 series and stored in anti-freeze solution (0.5 M sodium phosphate buffer, 30 % glycerol and 30 % ethylene glycol) at -20 °C until further processing. Immunohistochemistry was performed with antibodies raised against TH (P40101-0, rabbit IgG 1:2000 Pel-Freez, Rogers, AR), NeuN (MAB377, mouse IgG 1:500, Millipore, Billerica, MA) and IBA1 (019-19741, rabbit IgG 1:1000, Wako, Richmond, VA). Antibodies against human GCH1 (used at 1:5000) was provided by R. Mandel and was raised in rabbits with a synthetic 12 amino acid (GFPERDPPRPGP, aa 21-32) peptide conjugated to a KLH antigen, as described in <sup>11</sup>. The staining was visualized with biotinylated secondary antibodies (BA1000, goat anti-rabbit and BA2001, horse

anti-mouse, Vector Laboratories, Burlingame, CA) followed by a 1-hour incubation with avidin-biotin peroxidase solution (ABC Elite, Vector Laboratories, Burlingame, CA) developed by 3, 3'-diaminobenzidine in 0.01 % H<sub>2</sub>O<sub>2</sub> for color reaction.

### **Biochemical analysis**

Six rats from both the TH-GCH1 and Les-Sham groups were killed by decapitation, where after the brain was removed and sliced in the coronal axis into two parts with a brain mold. The striatal tissue from each hemisphere of the anterior part was then rapidly dissected and frozen individually on dry ice and stored at -80 °C until further analysis. The caudal part containing the midbrain-hindbrain regions was post-fixed in 4 % PFA for 24 hours at 4 °C and then kept in 25 % sucrose for 24 hours and later processed for histological validation of the 6-OHDA lesion, as described above. The dissected brain tissue was homogenized and prepared with a modified version of a previously described protocol, which enables detection of monoamines and BH4 by high performance liquid chromatography (HPLC), *in vitro* enzyme activity and western blot analysis from the same sample <sup>49</sup>. Briefly, the tissue was homogenized on ice in Tris-acetate buffer (5 µl/mg, 20 mM, pH 6.1) with an ultrasonic disintegrator. Eighty µl of the homogenate was then pipetted into ice-cold 0.8 mM perchloric acid for HPLC measurements. The remaining homogenate was centrifuged (15 min at 4600 x *g* at 4 °C) where after the supernatant was frozen until analyzed in the AADC enzyme activity assay.

*HPLC analysis of monoamines and BH4.* The tissue homogenate in perchloric acid was incubated on ice for at least 20 min before centrifugation for 15 min at 4600 x *g* at 4 °C. The supernatant was filtered through 96-well 0.45 µm PVDF filter plate by centrifugation for an additional 5 min at 4600 x *g*. Thereafter the sample was diluted 1:4 in Milli-Q filtered de-ionized water and stored in -80 °C until analysis. The tissue extracts were then analyzed by HPLC-EC in three separate measurements for (1) DA and 5-HT; (2) DOPA; and (3) BH4. For each measurement, 25 µl of each sample was injected by a cooled autosampler (Spark Holland Midas) into an electrochemical detector (ESA Coulochem III) coupled to a guard cell (ESA 5020) and a glass-carbon electrode analytical cell (ESA 5011). For DA, 5-HT and DOPA detection a reverse phase C18 column (3 µm ReproSil-pur, 4.6 mm Ø, 150 mm length, Chrompack) was used for compound separation, whereas for BH4 detection, this was replaced with another reverse phase C18 column (5 µm ReproSil-pur, 4.6 mm Ø, 250 mm length, Chrompack) preceded by a C8 column (5 µm ReproSil 80, 4.6 mm Ø, 33 mm length, Chrompack).

The mobile phase for DA and 5-HT detection contained 60 mM sodium acetate, 90 µM Na<sub>2</sub>-EDTA, 460 µM 1-octanesulfonic acid in 9 % methanol, where pH was adjusted to 4.2. For DOPA detection it contained 100 mM NaH<sub>2</sub>PO<sub>4</sub> adjusted to pH 3.0 with H<sub>3</sub>PO<sub>4</sub>, 90 µM Na<sub>2</sub>-EDTA, 2.8 mM 1-octanesulfonic acid in 10 % methanol. The mobile phase used for BH4 detection, on the other hand, was modified from a previously described EC BH4 detection protocol<sup>50</sup> and composed of 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA, 160 µM DTE in 5 % methanol, pH 5.2. The mobile phases were delivered at a flow rate of 500 µl/min for catecholamines and 1 ml/min for BH4. Peak identification and

quantification was performed with the Clarity chromatographic software package (DataApex, Prague, Czech Republic).

*AADC enzyme activity assay.* To study *in vitro* AADC enzyme activity a modified protocol from <sup>51</sup> was used. Briefly, 5  $\mu$ l of tissue supernatant was mixed with 95  $\mu$ l of assay buffer solution (0.01 mM pyridoxal phosphate, 0.17 mM ascorbic acid, 0.1 mM pargyline, 1 mM 2 $\beta$ -mercaptoethanol, and 0.1 mM EDTA in 50 mM sodium phosphate buffer, adjusted to pH 7.2). Samples were run in triplicates where one sample received assay buffer and the two other received assay buffer including 0.25 mM L-DOPA. The samples were incubated at 37 °C for 20 min before the reaction was halted by addition of 150  $\mu$ l of ice-cold 0.1 M perchloric acid. After centrifugation (15 min at 4600 x *g* at 4 °C), the supernatant was transferred to a 96-well 0.45  $\mu$ m PVDF filter plate (Whatman, Florham Park, NJ) and the filtrate was diluted in 450  $\mu$ l ice-cold 0.1 M perchloric acid before analysis of DA content with HPLC, as described below. There after, the measurement from the sample not receiving L-DOPA was subtracted from the L-DOPA receiving samples. The average of the two background subtracted values constituted the final dependent variable.

*Online microdialysis.* Microdialysis was used to quantify extracellular levels of DA, DOPA, 5-HT and metabolites in baseline conditions and in response to KCl. Rats from the TH-GCH1 (n=4), Les-Sham (n=3), and intact control (n=3) groups were anesthetized with 1-2 % isoflurane mixed with O<sub>2</sub> and N<sub>2</sub> and placed in a stereotaxic frame. The microdialysis probes were made of polyethersulfone, with a membrane length of 3 mm, a 0.5 mm outer diameter, and had a 6 kD cut-off value (Agnthos Microdialysis, Stockholm, Sweden). The probes were inserted

into the striatum with the help of a probe holder and positioned at AP: +0.5 mm, ML: -3.7 mm relative to bregma and DV: -5.7 mm from the dural surface. The tooth bar was set to -2.4 mm. These coordinates were chosen to position the membrane of the probe in the center of the transduction area in the striatum. The probes were connected to a syringe infusion pump (Model 100; CMA Microdialysis, Solna, Sweden) via polyethylene tubing and perfused with normal ringer solution containing 145 mM NaCl, 3 mM KCl and 1.3 mM CaCl<sub>2</sub>, pH 6.4, at a constant rate of 0.6 µl/min. To rescue easily oxidized compounds, e.g., 5-HT, an antioxidant solution containing 1 M HAc, 0.27 mM EDTA, 33 mM L-cysteine, and 5 mM ascorbic acid<sup>52</sup>, was mixed with the dialysate after the outlet of the probe. The dialysate was directly analyzed on Alexys online monoamine analyzer microdialysis system (Antec Leyden, The Netherlands) consisting of a DECADE II electrochemical detector and VT-3 electrochemical flow cell. The outlet of the microdialysis probe was connected to a 14-port external valve that can direct the dialysate into two separate flow paths. Two different mobile phases, optimized for the detection of the respective metabolites, were used in each of the two flow paths. The first mobile phase (50 mM phosphoric acid, 8 mM NaCl, 0.1 mM EDTA, 12.5 % methanol, 500 mg/l octane sulphate; pH 6.0) was used for the detection of DA and 5-HT ran through a 1 mm x 50 mm column with 3 mm particle size (ALF-105) at a flow rate of 75 ml/min. The second mobile phase (50 mM phosphoric acid, 50 mM citric acid, 8 mM NaCl, 0.1 mM EDTA, 10 % methanol, 600 mg/l octane sulphate; pH 3.2) was used for the detection of DOPA, DOPAC and HVA, which passed through a 1 mm x 150 mm column with 3 mm particle size (ALF-115) at a flow rate of 100 ml/min. The dialysate samples were transferred via 5 µl loops simultaneously into each flow path and analyzed by the



online HPLC in 15 min time bins. One hour of equilibration was followed by analysis of 3 baseline samples before the dialysate was changed to a modified ringer lactate solution containing KCl (51 mM NaCl, 100 mM KCl and 1.3 mM CaCl<sub>2</sub>) for 15 min in order to stimulate the readily releasable pool of DA and then switched back to the normal ringer lactate solution. Dialysate samples were analyzed for an additional 60 min following the KCl challenge. After collecting the last baseline sample the probe was withdrawn and the animal was perfused, as described in above, and probe placement was confirmed with histology.

### **Statistical analysis**

All statistics were conducted with the SPSS statistical package (SPSS Inc., Chicago, IL). Repeated measures ANOVA for data in figure 3 was performed with the general linear model. This was followed by a one-way ANOVA at each time-point with Tukey's HSD *post hoc* test. Two-way ANOVA tests performed on data presented in figure 4 and 6 were analyzed with the univariate general linear model. In figure 4, *post hoc* side comparisons were conducted with Bonferroni's-corrected T-tests and group comparisons on the treated side with one-way ANOVA followed by Tukey's HSD. In cases where the Levene test for unequal variance was significant, Tukey's HSD was replaced with Dunnett's T3 test. One-way ANOVA was performed at for each region individually in figure 5, with *post hoc* comparison with Tukey's HSD or Dunnett's T3 test. In figure 6, *post hoc* time comparisons were conducted with Bonferroni's-corrected T-tests and group comparisons with one-way ANOVA followed by Tukey's HSD or Dunnett's T3 test.

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## Figure Legends

**Figure 1.** AAV5 Single-vector design. A novel AAV vector construct was generated to express both tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1) from a single-vector genome. To achieve an increased expression of TH over the GCH1 gene, a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was added downstream the TH gene. Both genes were driven by the human Synapsin 1 promoter (SYN-1) and trafficking was enhanced with a SV40 polyadenylation sequence (pA). The complete gene sequence was inserted between inverted terminal repeats (ITR) from an AAV serotype 2, which was packaged in an AAV serotype 5 capsid.

**Figure 2.** Experimental design. Twenty-seven rats with complete lesions of the nigrostriatal dopamine pathway, displaying > 6 ipsiversive full-body turns/min toward the lesioned side in the amphetamine-induced rotation test, were included in the study. Animals were balanced into two groups (TH-GCH1 n=10 and Les-Sham n=10) based on their performance in the cylinder test and in amphetamine- and apomorphine-induced rotations. Six weeks after the 6-OHDA lesion, the animals in the TH-GCH1 group received intrastriatal AAV injections and the animals in the Les-Sham group received sham surgery. The animals were then followed with a battery of behavioral tests for 6 months, some of which the animals had been pre-trained in and others were the treatment effect on motor function, sensorimotor integration as well as motor learning was studied. The animals' rotational behavior was studied at 10- and 14-weeks post-AAV injection with amphetamine- and apomorphine-induced rotation tests. At 5-, 10- and 14-



weeks post-AAV, the animals were scored in the same rotometer equipment without pharmacological challenge to assess spontaneous rotational behavior. The cylinder test was repeated 5, 12 and 14 weeks post-AAV. To study the treatment effect on motor learning, sensorimotor integration as well as motor function, training in the stepping, staircase and corridor tests was initiated once the therapeutic effect of the treatment was well manifested in the TH-GCH1 group. The stepping test was performed at 4-, 10-, 12- and 14-weeks post-AAV injection, the staircase test was performed as a single test session over 21 days between 21-24 weeks and the corridor test at 25 weeks post-AAV injection. Twenty-eight weeks post-AAV injection, animals from each group were sacrificed for either immunohistochemical analysis (n=4) or biochemical analysis (n=6). A subset of animals (TH-GCH1 n=4, Les-Sham n=3, and intact controls n=3) were analyzed with online microdialysis 40 weeks post-AAV. This enabled real time quantification of extracellular DA, DOPA, 5-HT and metabolites in baseline conditions and when challenged with KCl.

**Figure 3.** Recovery of motor function. Over a 34-week period, the animals were tested in a battery of behavioral tests. The animals were pre-scored in the cylinder test (a), amphetamine- (d) and apomorphine-induced rotation tests (e). After the AAV treatment, the animals in the TH-GCH1 group recovered to full paw use symmetry in the cylinder test within 5 weeks, which was maintained up to 14-weeks post-AAV (a). The stepping test was initiated 4 weeks post-AAV treatment without prior training (b). Also in this test, the animals in the TH-GCH1 group recovered to intact performance already at the first assessment point, which was maintained at the remaining time points at 10-, 12-, and 14-

weeks post-AAV treatment. Recovery in the drug-induced rotation tests was less pronounced but was still significant at 14-weeks post-AAV treatment (d, e). The rotational bias in the drug-naïve spontaneous rotation test was decreased in the TH-GCH1 group at 10- and 14-week time points compared with the first assessment at 5 weeks (f). Robust recovery was also seen in the corridor test at 25 weeks post-AAV, where TH-GCH1 treated animals performed equal amount of right and left retrievals, in contrast to the Les-Sham group. Statistics: Repeated Measures ANOVA a. Time  $F_{(3,54)}=3.44$   $p<0.05$ ; Time  $\times$  Group  $F_{(3,54)}=17.13$   $p<0.001$ ; b. Time  $F_{(3,54)}=2.45$   $p=0.074$ ; Time  $\times$  Group  $F_{(3,54)}=3.07$   $p<0.05$ ; d. Time  $F_{(2,36)}=0.87$   $p=0.43$ ; Time  $\times$  Group  $F_{(2,36)}=10.44$   $p<0.001$ ; e. Time  $F_{(2,36)}=9.97$   $p<0.001$ ; Time  $\times$  Group  $F_{(2,36)}=9.00$   $p<0.001$ ; f. Time  $F_{(2,36)}=4.82$   $p<0.05$ ; Time  $\times$  Group  $F_{(2,36)}=6.15$   $p<0.05$ ; Bonferroni corrected independent t-tests at each time-point.

**Figure 4.** Motor learning and fine motor function after AAV vector treatment. At 21-24 weeks post-AAV treatment, the animals were trained and analyzed in the staircase test utilizing a wide platform (35 mm) for maximal stringency of the test. In the test setup on day 1 through 15, both sides of the staircase box were baited with 40 sugar pellets. Day 16-18, only the left staircase was filled, presenting the animals with a forced choice (FC-L) and day 19-21 the opposite staircase was utilized (FC-R). The animals continuously improved in the number of taken and eaten pellets over day 1-6 (Phase I, learning phase) with both the ipsilateral and contralateral paw (a, b and d, e). Day 7-10, the animals still displayed high variability in their performance in the test and thus, this was considered an equilibration phase (Phase II). Day 11-15 the animals displayed

very little variation (Phase III, Stable phase). This was validated by three-way ANOVA that confirmed no significant time effect over days 11-15. During the stable phase, the animals in the TH-GCH1 group managed to pickup significantly more pellets on the side contralateral to the lesion, compared to the Les-Sham animals (g). This was coupled with a significantly greater success in the number of eaten pellets on that side (h), resulting in a decreased error rate (i). Statistics: Three-way ANOVA for day 11-15 (Time effect: Taken  $F_{(4,160)}=0.11$ ,  $p=0.98$ ; Eaten  $F_{(4,160)}=0.12$ ,  $p=0.97$ ; Error rate  $F_{(4,160)}=0.08$ ,  $p=0.99$ ); Two-way ANOVA over the Stable phase (III) Taken (Group  $F_{(1,176)}=43.19$ ,  $p<0.001$ ; Side  $F_{(1,176)}=277.09$ ,  $p<0.001$ ; Group  $\times$  Side  $F_{(1,176)}=42.00$ ,  $p<0.001$ ); Eaten (Group  $F_{(1,176)}=4.06$ ,  $p=0.045$ ; Side  $F_{(1,176)}=335.95$ ,  $p<0.001$ ; Group  $\times$  Side  $F_{(1,176)}=51.48$ ,  $p<0.001$ ) and Error rate (Group  $F_{(1,176)}=0.71$ ,  $p=0.4$ ; Side  $F_{(1,176)}=124.06$ ,  $p<0.001$ ; Group  $\times$  Side  $F_{(1,176)}=29.56$ ,  $p<0.001$ ). The Two-way ANOVA was followed by Bonferroni corrected post hoc comparisons with paired and independent t-tests. \* = significantly different from respective intact side, § = significantly different from lesioned side of Les-Sham.

**Figure 5.** Post-mortem tissue analysis after AAV-treatment. A sub-group (n=6) of the animals in each group were decapitated for retrieval of fresh brain tissue for biochemical analysis. HPLC-EC measurements of BH4 (a), monoamines (b, d, e, f) and in vitro AADC enzyme activity in dissected brain tissue subdivided in three fractions, dorsal striatum, ventral striatum (nucleus accumbens, ventral pallidum and the olfactory tubercle) and the frontal cortex. From these fractions, a measurement of total forebrain concentration was calculated. The expression of the transgene GCH1 was evaluated by measuring BH4 levels, which were

elevated in vector-treated animals ipsilateral to the AAV treatment compared with controls (a). The DOPA measurements gave indirect read-out of a functional vector-construct and the animals in the TH-GCH1 group showed a large increase of DOPA in the ipsilateral hemisphere compared with Les-Sham and intact controls (b). The *in vitro* AADC enzyme activity was severely reduced on the ipsilateral side in both groups, which was most profound in the dorsal striatum (c). Partial reconstitution of dopamine (DA) levels was observed in all regions of the hemisphere ipsilateral to the AAV injection (d), which was coupled with elevated levels of both DOPAC (e) and HVA (f), especially notable in the cortical fraction. Statistics: One-way ANOVA: BH4 (Dorsal striatum  $F_{(3,23)}=58.02$ ; Ventral forebrain  $F_{(3,23)}=30.03$ ; Frontal cortex  $F_{(3,23)}=551.19$ ; Total forebrain  $F_{(3,23)}=262.66$ , all with  $p<0.05$ ); DOPA (Dorsal striatum  $F_{(3,23)}=81.14$ ; Ventral forebrain  $F_{(3,23)}=12.42$ ; Frontal cortex  $F_{(3,23)}=100.13$ ; Total forebrain  $F_{(3,23)}=125.37$ , all with  $p<0.05$ ); AADC enzyme activity assay (Dorsal striatum  $F_{(3,23)}=20.11$ ; Ventral forebrain  $F_{(3,23)}=11.09$ ; Frontal cortex  $F_{(3,23)}=3.43$ ; Total forebrain  $F_{(3,23)}=22.91$ , all with  $p<0.05$ ); DA (Dorsal striatum  $F_{(3,23)}=91.76$ ; Ventral forebrain  $F_{(3,23)}=22.56$ ; Frontal cortex  $F_{(3,23)}=21.21$ ; Total forebrain  $F_{(3,23)}=76.68$ , all with  $p<0.05$ ); DOPAC (Dorsal striatum  $F_{(3,23)}=27.92$ ; Ventral forebrain  $F_{(3,23)}=14.16$ ; Frontal cortex  $F_{(3,23)}=180.77$ ; Total forebrain  $F_{(3,23)}=107.41$ , all with  $p<0.05$ ); HVA (Dorsal striatum  $F_{(3,23)}=92.01$ ; Ventral forebrain  $F_{(3,23)}=14.61$ ; Frontal cortex  $F_{(3,23)}=159.06$ ; Total forebrain  $F_{(3,23)}=40.87$ , all with  $p<0.05$ ). One-way ANOVA was followed by *post hoc* comparison with Tukey's in cases where Levene's test was significant ( $p<0.05$ ), otherwise with Dunnett's T3 test. o = significantly different from Les-Sham contralateral side, \* = significantly different from Les-Sham ipsilateral side, + =

significantly different from TH-GCH1 contralateral side.

**Figure 6.** Online microdialysis measurements in AAV-treated animals. Forty weeks post-AAV treatment, animals not followed with behavioral tests (TH-GCH1 n=4, Les-Sham n=3, and intact controls n=3) were studied with an online microdialysis setup. Dialysate was sampled in 15 min time bins and analyzed with HPLC-EC instantly. After probe placement an equilibration period of 60 min was allowed before 3 baseline samples were collected. During the collection of the last baseline the ringer solution was switched to a modified version containing high concentration KCl to stimulate the readily releasable pool of DA and 5-HT. Following the 15 min KCl challenge normal ringer solution was again administered and the sampling continued for 60 min. Animals treated with TH-GCH1 showed significantly higher baseline levels of DA compared with Les-Sham (a), however there was a clear lack of readily releasable stores compared with intact controls. In the 5-HT measurements, the baseline levels were similar between the groups but the TH-GCH1 group showed a decreased release in response to KCl compared with both Les-Sham and intact controls (b). Since baseline 5-HT levels were above detection limit, but under the quantified level (>2 nM), the results are presented as % of baseline. The measurements of DOPA (c), DOPAC (d), and HVA (e) are calculated as area under the curve (AUC) for both baseline and in response to KCl. The DOPA levels were greatly increased in the TH-GCH1 group compared with Les-Sham and intact controls (c). For the DA metabolites DOPAC and HVA, the TH-GCH1 group showed partial reconstitution compared with intact controls. Statistics: Panel (a) TH-GCH1 differed from Les-Sham in baseline DA measurements ( $F_{(2,9)}=5.81$ ,  $p<0.05$ ). One-way ANOVA

followed by Tukey's *post hoc* test. Panel (c-e) Two-way ANOVA: DOPA (Group  $F_{(2,14)}=32.06$ ; Time  $F_{(1,14)}=0.26$ ; Group  $\times$  Time  $F_{(2,14)}=0.42$ , all with  $p<0.05$ ); DOPAC (Group  $F_{(2,14)}=93.00$ ,  $p<0.05$ ); HVA (Group  $F_{(2,14)}=13.00$ ; Time  $F_{(1,14)}=11.21$ ; Group  $\times$  Time  $F_{(2,14)}=20.34$ , all with  $p<0.05$ ). Significant group effects were evaluated further with one-way ANOVA followed by *post hoc* comparison with Tukey's HSD in cases where Levene's test was significant ( $p<0.05$ ), otherwise with Dunnett's T3 test. DOPA baseline  $F_{(2,9)}=10.05$ ,  $p<0.05$ ; KCl  $F_{(2,9)}=11.46$ ,  $p<0.05$ ; DOPAC baseline  $F_{(2,9)}=40.03$ ,  $p<0.05$ ; KCl  $F_{(2,9)}=34.76$ ,  $p<0.05$ ; HVA baseline  $F_{(2,9)}=9.84$ ,  $p<0.05$ . Significant time effect was evaluated further with Bonferroni corrected independent T-test. o = significantly different from intact, \* = significantly different from Les-Sham, + = significantly different from respective contralateral side.

**Figure 7.** Transgene expression visualized with immunohistochemistry. Twenty-five weeks post-AAV treatment, animals from both the TH-GCH1 (n=4) and Les-Sham group (n=4) were deeply anaesthetized with pentobarbital, transcardially perfused, brains readily dissected and then stored in PFA for 2 hours. After overnight incubation in sucrose the brains were then cut in 35  $\mu$ m coronal sections in 6 series. The 6-OHDA injection in the right MFB resulted in a complete lesion of dopaminergic terminals in the striatum (a) and in the substantia nigra (SN) (d). Animals in the TH-GCH1 group showed expression of both transgenes, TH (b) and GCH1 (c), in the striatum but also in the SN (e for TH and GCH1 not shown). Injection of AAV rendered extrastriatal transduction in e.g., the cortex (Ctx) and globus pallidus (GP). NeuN stainings displayed a loss of GP neurons in the TH-GCH1 group (g) compared with the Les-Sham group (f) on

the ipsilateral side. CC: corpus callosum, NAc: nucleus accumbens, Str: Striatum, Tu: olfactory tubercle. Scale bars in c and e represent 1 mm in panels a,b,c and d,e respectively. Scale bar in g represent 100  $\mu\text{m}$  in panel f and g.















