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Controlled Striatal DOPA Production From a Gene Delivery System in a Rodent Model of Parkinson's Disease

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Conventional symptomatic treatment for Parkinson's disease (PD) with long-term L-3,4-dihydroxyphenylalanine (DOPA) is complicated with development of drug-induced side effects. *In vivo* viral vector-mediated gene expression encoding tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1) provides a drug delivery strategy of DOPA with distinct advantages over pharmacotherapy. Since the brain alterations made with current gene transfer techniques are irreversible, the therapeutic approaches taken to the clinic should preferably be controllable to match the needs of each individual during the course of their disease. We used a recently described tunable gene expression system based on the use of destabilized dihydrofolate reductase (DD) and generated a N-terminally coupled GCH1 enzyme (DD-GCH1) while the TH enzyme was constitutively expressed, packaged in adeno-associated viral (AAV) vectors. Expression of DD-GCH1 was regulated by the activating ligand trimethoprim (TMP) that crosses the blood–brain barrier. We show that the resulting intervention provides a TMP-dose-dependent regulation of DOPA synthesis that is closely linked to the magnitude of functional effects. Our data constitutes the first proof of principle for controlled reconstitution of dopamine capacity in the brain and suggests that such next-generation gene therapy strategies are now mature for preclinical development toward use in patients with PD.

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INTRODUCTION

Gene therapy strategies are emerging as possible future treatment alternatives for several disease indications including applications in the brain. Recent results in clinical trials in Parkinson's disease (PD) show that such interventions are essentially safe although robust efficacy indicators in placebo-controlled studies is at present lacking.[1–6] The animal efficacy data, *e.g.*, for enzyme replacement by gene therapy, suggests that this approach has high probability of success in the clinics as a restorative strategy, in particular for patients with an advanced disease state and those that display disabling side effects of oral L-3,4-dihydroxyphenylalanine (DOPA) pharmacotherapy. Troublesome side effects of long-term oral L-DOPA medication is thought to be a consequence of fluctuating levels of the drug and a progressive worsening of the disease.[7–10] Generation of DOPA in dopaminergic neurons is handled by tyrosine hydroxylase (TH; catalyzes the rate-limiting step in dopamine synthesis) in the presence of tetrahydrobiopterin (BH4).

We and others have shown that transduction of striatal neurons with transgenes encoding the TH and GTP cyclohydrolase 1 (GCH1; catalyzes the rate-limiting step in BH4 synthesis; for review see ref.

[11]) enzymes with the use of adeno-associated viral (AAV) vectors gives rise to continuous DOPA production in these cells.[12–14] As these neurons do not express the enzyme responsible for the conversion of DOPA to dopamine (DA), this final reaction takes place in cells containing the aromatic amino acid decarboxylase (AADC), namely the residual dopaminergic terminals or serotonergic cells.[15,16] Reconstitution of the synthetic pathway for production of DA in the striatum in turn provides the means for robust and near complete recovery from behavioral deficits seen in rodent models of PD.[12,13,17,18]

None of the strategies implemented in animal studies or in clinical trials for PD so far have a mechanism to control DA production from the transgenic enzymes delivered via the viral vectors in the brain, *i.e.*, they are irreversible and uncontrollable. It is widely acknowledged, however, that regulation of gene expression is an important component of long-term safety of the treatment and for the first time opens the possibility to truly personalize the treatment to the needs of the individual patients. To achieve this goal, in the present study, we used a tunable protein expression system[19] based on the recently described destabilized dihydrofolate reductase (DHFR) domain.[20,21] Briefly, DHFR and any protein of interest coupled to it (in this case an enzyme) are readily degraded in the cell in the absence of its ligand, trimethoprim (TMP). The addition of TMP stabilizes the protein complex, which in turn rescues the enzymatic activity leading to functional restoration in the transduced cells. Our goal here was to obtain the first proof-of-principle *in vivo* results in the rat model of PD that this protein regulation mechanism can be implemented as a gene therapy tool to control the synthesis of DOPA in the brain and secondly that the approach has robust efficacy in this model to justify scale-up to large animal experiments and later translated to clinical studies.

RESULTS

Reconstitution of DOPA and dopamine metabolites by the regulated gene expression system

The first part of the study (Experiment 1) was designed to validate the functionality of controlled DOPA synthesis in the striatum obtained by gene therapy incorporating a destabilized domain based on DHFR (DD) coupled to the GCH1 gene in combination with constitutively expressed TH (TH+DD-GCH1). For this purpose, we used online microdialysis (OMD) in anesthetized rats and determined (i) steady-state production of DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) under baseline conditions; (ii) total DOPA synthesis capacity after inhibition of AADC in the striatum of animals where stabilization of the GCH1 protein was induced with oral administration of TMP, thus permitting synthesis of BH4 that in turn activated the TH enzyme (**Table 1**).

In intact rats, DOPAC and HVA (*i.e.*, metabolites of newly synthesized DA) levels in the extracellular space were abundant (typically between 1 and 2 μmol/l), while DOPA concentrations were below reliable quantification limit of our system. In rats with complete 6-OHDA lesion of the ascending dopaminergic projections of the medial forebrain bundle, the DOPAC and HVA concentrations were reduced to 5.6–13.5 nmol/l, representing more than 99% depletion on the average. In lesioned animals that received the TH+GCH1 constitutively active vectors, both DOPAC and HVA levels were increased to values between 15 and 40% of intact controls. Notably, there was a readily detectable DOPA present in the extracellular space 167.9 ± 58.5 nmol/l. Similarly, in the group treated with TH+DD-GCH1 and maintained under TMP activation, the DOPA, DOPAC, and HVA levels were increased above the lesion baseline, although the DOPA concentrations appeared lower than the constitutively active group.

In order to explore the maximum capacity of the enzymatic machinery obtained from transgenic expression of the human TH enzyme, the protocol followed the initial baseline period with addition of NSD-1015 to block the AADC enzyme. Consequently, the levels of DOPAC and HVA declined while the DOPA accumulated in all vector-treated animals, suggesting that the newly synthesized DOPA was continuously converted to DA by the endogenously present residual AADC enzyme and contributed to generation of DOPAC and HVA measured in the samples obtained during the baseline phase of the OMD experiment. Notably, the results obtained with addition of TMP to the ringer solution used as the dialysate did not further increase the DOPA levels suggesting that the system was fully activated with the oral TMP given to the animals during the 2 weeks prior to the OMD experiment. Finally, we tested two oral TMP doses (0.5 and 2.0 mg/ml in drinking water) and found that both were effective in reaching a similar biochemical reconstitution under these experimental conditions.

After establishing that the efficacy of the controlled expression system in the steady state on long-term TMP administration was comparable to the constitutive expressing system, we turned our attention to the transition from a baseline state in the absence of TMP to the activated state upon introduction of the ligand. To be able to perform an OMD analysis in an analogous way, while anticipating a longer sampling period, we adapted the measurements to be carried out in awake and freely moving animals. Microdialysis samples were monitored continuously and we quantified levels of DOPAC, HVA and as a control also 5HIAA, the metabolite of serotonin (data from 1 hour baseline followed by 12 hours post-treatment sampling presented in **Figure 1a**). As in the first protocol performed in anesthetized rats, DOPAC and HVA levels in awake freely moving rats were severely depleted in the complete 6-OHDA lesioned group accounting for about 0.5% of values compared with intact controls. In rats treated with the AAV vector mix expressing the TH and GCH1 proteins constitutively (TH+GCH1 group), the DOPAC and HVA levels were about 30 and 80% of intact baseline, respectively. Notably, the TH+DD-GCH1 group (naive to TMP) had very low baseline levels of both DOPAC and HVA, corresponding to about 2-4% of normal values and slightly higher when compared with lesion controls. Addition of 20 μmol/l TMP in the microdialysis solution resulted in gradual accumulation of DOPAC and HVA. The levels of these metabolites measured at the 12-hour mark were comparable to the constitutive expression group, *i.e.*, reached to ~25 and 50% of normal values in intact animals as compared with about 70 and 60% in the TH+GCH1 group, respectively (**Figure 1a**). After 12 hours of TMP administration, six out of seven rats in the TH+DD-GCH1 group had lower HVA levels as compared with the constitutive TH+GCH1 and intact groups, while one reached to about 25% higher than the intact average value (**Figure 1b**).

Behavioral recovery in TH+DD-GCH1-treated rats in response to oral administration of TMP

In Experiment 2, we assessed long-term motor recovery in lesioned animals treated with active vectors using the stepping and corridor tests and compared them to lesion and intact controls (see **Figure 2** for experimental design). Intact naive rats (n = 11; black squares in **Figure 3**) perform on average 9–11 steps in the forehand direction and 11–13 steps in the backhand direction in the stepping test (**Figure 3a**,**b**). The normal performance in the corridor test is equal number of nose pokes in lids (retrievals) positioned on the left and right side in the corridor (**Figure 3c**). The 6-OHDA-lesioned rats (n = 9; open squares in **Figure 3**) used in this study have a severe impairment in all three parameters; *i.e.*, make few or no adjusting steps and have a near complete bias to taking pellets from the lid on the right side, ipsilateral to the lesion. Treatment with TH+GCH1 (n = 10;

black circles in **Figure 3**) resulted in near complete recovery in the stepping test already 6 weeks post-AAV and this effect was maintained throughout the rest of the study (**Figure 3a**,**b**). In the corridor test, animals in this group showed an initial overcompensation at the 6-week assessment point where they almost exclusively made left retrievals (**Figure 3c**). This effect was decreased at the following assessment time points but the animals continued to display an approximate 75% left side bias for the remaining time of the study until 33 weeks post-AAV injection. Animals that received the TH+DD-GCH1 (n = 19) vector showed little to no recovery during the baseline assessment at 6, 9, and 12 weeks (open circles in **Figure 3**). During this assessment period, we observed that this group behaved similar to lesion controls and their own baseline measurements prior to vector treatment suggesting that in the absence of TMP, *i.e.*, when GCH1 enzyme is destabilized and presumably BH4 levels are low. This was in contrast to the constitutively active TH+GCH1 group that showed clear signs of functional changes in both behavioral tests. This follow-up period of 3 months allowed us to demonstrate that the DD-regulated delivery system was tightly controlled and could be maintained without any functional consequences to the animals over long-term.

Fifteen weeks post-AAV, half of the animals in this group (n = 10; gray circles in **Figure 3**) started receiving oral TMP emulsion in their drinking water while the remaining rats (n = 9; open circles in the TMP dose escalation phase) continued receiving drinking water without TMP. Three concentrations of TMP were administered to the animals in escalating doses between 0.5, 1, and 2 mg/ml, each lasting 6 weeks. Based on this concentration and the amount of water consumed per day, the TMP dose per rat was estimated to be 22.8 ± 2.8, 40.9 ± 6.2 and 87.1 ± 14.6 mg/kg/ day, respectively. Behavioral assessment was performed at 3 and 6 weeks with each TMP dose. During the 0.5 mg/ml TMP administration phase, TH+DD-GCH1 group showed partial recovery already at the first assessment point at 3 weeks, in contrast to animals that received the same vector but no TMP or lesion controls (Mann-Whitney U-comparison, $P < 0.001$ in both tests in all three test parameters). When the animals received a TMP dose of 1 and 2 mg/ml, the therapeutic effect in the stepping test increased gradually to a magnitude comparable to the TH+GCH1 group. Notably, at the second assessment of the 1 mg/ml TMP dose, an overcompensation effect was seen in the corridor test (~90% retrievals on the left, previously neglected side) similar to the observations in the TH+GCH1 group at 6 weeks after transduction (**Figure 3c**). This effect was transient and was not observed in the following test 3 weeks later, despite the fact that the dose of TMP was increased to 2 mg/ml.

Oral TMP administration stabilizes DHFR-coupled GCH1 and rescues TH expression

After the last behavioral assessment, brain tissue from animals used in this experiment was processed for histological analysis. The expression of the GCH1 transgene was documented using an antibody specific to the human protein (with no cross reactivity to the rodent species; see lack of staining in the untreated side, **Figure 4a**). As expected, this analysis showed that there was a robust expression in both the constitutively active TH+GCH1 group (**Figure 4b**,**j**) and the TH+DD-GCH1 group receiving the TMP ligand (**Figure 4d**,**k**), while those animals that were maintained without TMP showed minimal or no immunoreactivity to GCH1 protein (**Figure 4c**,**i**). Limited or no GCH1 transduction was observed in cortex and corpus callosum (**Figure 4j**,**k**). Using an antibody directed to the DHFR protein, we were able to confirm that the GCH1 immunoreactivity in the TH+DD-GCH1 group was accompanied with a matching staining for the DD fusion protein (**Figure 4h**) and that the same antibody stained only few cells in the group that did not receive TMP (**Figure 4g**).

With respect to the expression of the TH enzyme, 6-OHDA lesion removes the endogenous dopaminergic fiber terminals in the striatum essentially completely (compare **Figure 5a** with **Figure 5f**), which can also be appreciated as loss of pre-terminal axon projections seen transiting the globus pallidus (GP) (compare **Figure 6a** with **Figure 6f**). Expression of the human TH transgene from the AAV vector introduces a new TH enzyme pool within the striatal neurons (**Figure 5k**) as well as the GP (**Figure 6k**). Interestingly in the TH+DD-GCH1 group that did not receive TMP, the absence of GCH1 immunoreactivity was associated with low level of TH expression observed only in a few cells (**Figure 5p** and **Figure 6p**). This was in contrast to the animals in the TH+DD-GCH1 group that received TMP, which showed similarly robust TH expression that were seen in animals in the constitutively active TH+GCH1 group (compare panels k and u in **Figures 5** and **6**). To quantify how the transduction volume differed in the treatment groups and how TMP affected the expression of the TH protein, we compared the transduction volume with the total striatal volume in TH stained specimens. The transduction in the TH+GCH1 group corresponded to 63.6% of the total striatal volume, whereas the animals that received the regulated vector combination had only 37.2% in the absence of oral TMP ($P < 0.001$ compared with TH-GCH1), whereas addition of TMP increased this measure to 50.5%.

Finally, we assessed any potential toxic effects of transgene expression by staining serial sections from all groups of animals with either CV to see all cellular profiles, NeuN to assess the total neuronal profiles, or Iba1 and ED1 antibodies for evidence of microglial activation. The panel of antibodies and analysis performed here was chosen based on a similar work we have done recently in another vector system associated with toxicity problems.[22] CV and NeuN stained specimens at the level of striatum and GP (second and third columns in **Figures 5** and **6**) showed no evidence of cell loss or perivascular hypercellularity and no apparent alterations were seen in NeuN-positive profiles either. In addition, when quantifying NeuN-positive neuronal profiles in both the striatum (**Figure 7a**) and GP (**Figure 7b**), no difference was found between any of the groups. No clear alterations in microglial morphologies were noted in the Iba1-stained specimens and abundance of ED1 profiles were only minimally increased suggesting that the activation of the neuroinflammatory processes were of low grade (compare groups within column 4 and 5 in **Figures 5** and **6**).

DISCUSSION

Gene regulation systems have been developed utilizing, *e.g.*, rapamycin dimerization, lac operator-repressor, different steroids receptors (ecdysone and mifepristone), RNA interference, and aptamers (see ref. [23] for a recent review). The most studied and characterized regulatory system is the one that is built on the tetracycline (tet) responsive promoters.[24,25] This system uses a fusion protein composed of the *Escherichia coli* tet-repressor protein and the HSV-1 VP-16 transcription factor, that is constitutively expressed, and initiates transcription when bound to a transgenic promoter containing a tet-operon. In the presence of its inhibitory ligand tetracycline, or an analogue, *e.g.*, doxycycline, the interaction of the fusion protein and the promoter sequence is inhibited (thus termed *tet-off* system). Many alterations of this system have been developed including versions where administration of the ligand instead promotes transcription (*tet-on* system).[25]

Both *tet-on* and *tet-off* systems have been used in a number of gene therapy applications *in vivo* including those in the brain (see ref. [26] for review). In particular, tet-dependent regulation of TH, AADC, and GDNF has been reported in rodent models of PD.[27–30] However, to our knowledge, this system has not been developed toward use in humans, most likely due to the concern that the

expression of the HSV-1 protein in the constructs is potentially immunogenic and since the majority of the adult population have circulating antibodies against this virus.[31] Secondly, the requirements for dosing for effective gene regulation in the brain may be unfavorable as tetracycline derivatives have low blood brain barrier penetration capabilities.[32] In addition, ligand- induced toxicity can be a potential problem and has been suggested for the doxycycline, at least in cell culture experiments.[33] Notably, despite the relatively long preclinical characterization period over two decades, there is still limited information available on the efficacy of the tet-operant controlled gene expression systems in nonhuman primate models of any brain disease.

The suitability of controlled gene therapy depends not only on the characteristics of the specific construct used but also the ligand required for regulation of the transgene expression. Ideally, the ligand used to control the functional effects of the transgenic proteins should be an Food and Drug Administration–approved molecule, readily cross the blood brain barrier and activate expression in a dose range that does not induce any unwanted effects. Furthermore, it is important that the ligand is potent enough to induce high expression for optimal therapeutic benefit in the dose range it can be applied over long-term. The ligand used with the DD based on DHFR, TMP, is a well-known and routinely pre- scribed antibiotic usually in combination with sulfamethoxazole. TMP has a well-documented safety profile and can be prescribed over extended periods for prophylaxis against urinary tract infections at a dose of 100 mg/day over 4–6 months and for treatment of acute urinary tract infections at 300–400 mg/day typically given over 3–4 days. Only when the dose is raised to 8 g as single-dose administration can mild intoxication has been observed. In the present study, we approximated that the 0.5–2.0 mg/ml TMP emulsion would correspond to about 20–80 mg/kg based on the amount of liquid rats drank daily. Even at a TMP dose of 40 mg/ kg, the animals showed complete recovery in the behavioral tests without decreased water consumption or body weight. Thus, the TMP dose needed for therapeutic efficacy in this model is well below the safe doses prescribed to humans for chronic use.

In a study by Sando *et al*.[34] that utilized the DD regulation concept with Cre recombinase, the investigators observed that the concentration of TMP, dissolved in DMSO and saline, peaked 10 minutes after i.p. injection in the mouse brain and was below detection limit after 30 minutes. Although the administration route used in this experiment was different, the clearance of TMP from the brain is presumably comparable to the present study and would argue that effects after 30 minutes are associated with the half-life of stabilized GCH1 and the corresponding BH4 synthesis. Thus, the turn ON of this regulation system can be rapid, as seen in the microdialysis experiment in awake rats, whereas the turn OFF can take longer time and will depend not only on the half-life of GCH1 but the corresponding rate of depletion of newly synthesized BH4, the decrease of TH enzyme activity and the corresponding decrease of DA, DOPA, and metabolites. Thus, the analysis of turn OFF kinetics of this regulation system requires long-duration experiments that take all these parameters into account and should preferably be carried out in nonhuman primates for better predictability of the situation in humans.

In the motor assessment part of the study, we found that animals in the TH+DD-GCH1 group that were given 0.5 mg/ ml TMP in their drinking water showed significant recovery in spontaneous behavior tests. There was a significant increase in the behavioral response when these animals were subjected to 1.0 mg/ml TMP. No further improvements were seen when going to 2.0 mg/ml dose, although the recovery in animals had reached close to the behavioral performance in intact controls in the stepping test and was already overcompensated in the corridor test, thus creating the ceiling

effect in this model. In future studies, it would be interesting to define the lowest effective dose of TMP for this gene therapy strategy for a defined level of transduction efficiency and also explore in further detail the time effect. Here for practical reasons, we designed the experiment as a dose-escalation study and defined each dose administration duration to be 6 weeks. It is possible that longer treatment periods with 0.5 mg/ ml (or an even lower dose) might be sufficient to obtain complete recovery in this model.

A number of early stage clinical trials have been performed in testing the safety and efficacy of viral vector-mediated gene delivery in PD patients.[1–6] None so far have employed a mechanism to directly control the therapeutic efficacy of the approach. Only one of the clinical trials, aiming at increasing the decarboxylation capacity in the striatum by expression of AADC in striatal neurons, anticipated an indirect control mechanism by requiring peripheral administration of L-DOPA to result in production of DA from the newly acquired AADC pool. However, the patients treated with the AAV2 vectors encoding for the AADC gene reported no functional benefit by reduction of UPDRS scores when on peripheral L-DOPA treatment, while some changes were noted when the patients were scored in off state.[5,6] Thus, at present, there is not a clear demonstration of regulation of transgene expression in the clinical setting in that scenario. It should be noted here also that the two studies above were both open labeled safety studies and functional end-points were only secondary measures reported.

Side effects associated with L-DOPA pharmacotherapy are linked to fluctuations in plasma levels of the drug.[35] As the disease progresses, the need for higher doses of L-DOPA in combination with higher frequency of administration become necessary for maintenance of on-time during the day. Continuous DOPA delivery with gene therapy could solve this issue and avoid off target effects of oral L-DOPA therapy by delivery of the drug only in the intended target areas of the brain. In addition, the use of controlled gene expression systems would have many advantages over constitutive expression in the clinical setting. The properties of a tunable gene therapy that makes it superior to conventional unregulated approaches can readily be exemplified in the case of PD. First, cohorts of PD patients represent a heterogeneous group in terms of disease severity and corresponding dopaminergic depletion. Secondly and in further support for the use of controlled gene delivery systems, PD is a progressive disorder, in which the patients' requirements for optimal therapeutic efficacy change over the course of the disease. Thus, a single time interventional treatment that cannot be terminated or modified would pose concerns in the selection of the dose for each patient. A regulated system, on the other hand, would allow post-vector injection tailoring of the treatment efficacy based on each patient's specific needs. Thus, both in clinical development phase and during its use as a marketed drug, the capability of regulation for fine-tuning and individualizing potency of the treatment would be highly desirable. In the absence of such a control mechanism, even if the vector dose is carefully titrated and matched to the needs of a patient at the time of intervention, the selected dose will fall out of the range for optimal treatment benefits in the long-term. If, on the other hand, the dose selected for the same patient were adjusted to meet the needs over a longer period of time, the initial response might lead to adverse effects; making adjustments to initially "over-dose" the patients are unlikely to be feasible in the clinical setting.

MATERIALS AND METHODS

Subjects. One hundred fifty-five female Sprague-Dawley rats (Charles River, Schweinfurt, Germany) weighing 225–250 g were used in this study. Animals were housed two to three per cage under a 12-hour

light/12-hour dark cycle with free access to food and water except during assessment with corridor test (as described below). All experimental procedures were approved by the Ethical Committee for use of Laboratory Animals in the Lund-Malmö region.

Experimental design. The study was conducted in two separate experiments. Thirty-six animals were used in Experiment 1, where four groups of rats were subjected to an *in vivo* OMD using one of two protocols as described below. The second experiment was designed to assess the long-term behavioral effects of the regulated gene expression system tested in this study and included a total of 38 animals (selected from a total of 89 rats with 6-OHDA lesions) with validated severe and stable motor behavioral impairments and 11 intact control rats. The selection criteria used for inclusion in the second study was >6 net turns/min ipsilateral to the lesion side after challenge with amphetamine (2.5 mg/kg), <5% left retrievals in the corridor test, and no left forehand adjusting steps. Collectively, these three measures mark animals with severe impairments induced by dopamine depletion.

Surgical procedures. Anesthesia was induced by fentanyl citrate (Fentanyl, Apoteksbolaget, Sweden) and medetomidine hydrochloride (Dormitor, Apoteksbolaget, Sweden) injected i.p. at doses of 6 ml/kg (300 and 0.3 mg/kg, respectively). Animals were placed in a stereotactic frame (Stoelting, Wood Dale, IL) and intracerebral injections were made with a Hamilton syringe (Hamilton, Bonaduz, Switzerland) fitted with a glass capillary. The antero-posterior (AP) and mediolateral (ML) coordinates were calculated from bregma and the dorsoventral (DV) coordinates from the dural surface, according to the atlas of Watson and Paxinos.[36]

6-OHDA lesions. Fourteen micrograms free base 6-OHDA (Sigma-Aldrich AB, Sweden) were dissolved in ascorbate-saline (0.02 %), resulting in a concentration of 3.5 µg/µl, and injected into the right medial forebrain bundle using the following coordinates: AP: -4.4 mm; ML: -1.2 mm, and DV: -7.8 mm with the tooth bar set to -2.4 mm. The injection speed was constant at a speed of 1 µl/minute and the needle was kept in place for 3 minutes before it was slowly retracted.

AAV vector injections. Vector preparations of TH+GCH1 or TH+DD-GCH1 were injected at two sites in the striatum with two deposits along each tract. In total, 5-µl vector was injected per animal, distributed by 1.5 µl in the ventral and 1.0 µl in the dorsal deposit in each site. A pulled glass capillary (outer diameter 60–80 µm) was mounted on a Hamilton syringe with a 22-gauge needle to the minimize tissue damage and improve accuracy. The injection coordinates were: (i) AP: +1.0 mm; ML: -2.8 mm and DV: -4.5, -3.5 mm and (ii) AP: 0.0 mm; ML: -4.0 mm and DV: -5.0, -4.0 mm with the tooth bar set to -2.4 mm. The injection speed was kept constant at 0.4 µl/minute and the needle was kept in place for 1 minute after the ventral and 3 minutes after the dorsal deposit. Animals in the intact and lesion control groups underwent sham surgery by drilling a burr hole at the corresponding position in the skull but without penetrating the dura.

AAV vectors. The viral vectors used in this study were AAV serotype 5 with ITR sequences from serotype 2, and all transgenes were driven by the chicken beta actin (CBA) promoter, which includes a rabbit-gamma globulin intron and a cytomegalovirus (CMV) enhancer, and terminated with an early SV40 poly-A sequence. The two transgenes were human TH and GCH1. Regulation of GCH1 expression was achieved by coupling a destabilizing domain (DD) derived from *E. coli* dihydrofolate reductase (DHFR) to the N-terminal side of the protein (DD-GCH1). Generation of the controllable DHFR domains has been described in detail earlier.[20]

Two vector combinations were used in this study; TH and GCH1 constitutively expressed (group denoted TH+GCH1) and constitutive expression of TH combined with regulated GCH1 (DD-GCH1) (group denoted TH+DD-GCH1). Both were prepared in Dulbecco's phosphate- buffered saline (DPBS) mixed at 5:1 ratio of TH over GCH1 or the DD- GCH1. The final titer of the vector used in the TH+GCH1 and TH+DD- GCH1 groups were 1.9E+14 gc/ml (resulting in 9.5E11 gc injected) and 1.8E+14 gc/ml (resulting in 9.0E11 gc injected), respectively.

AAV vectors were produced in HEK-293 cells grown in tissue culture flasks for adherent cells (BD Falcon) to about 60–80% confluence. Transfection was achieved with the calcium-phosphate method and included equimolar amounts of transfer and helper plasmid DNA (pDP5 encoding for the AAV5 capsid proteins). The cells were incubated for 3 days before harvesting with PBS-EDTA. They were then centrifuged (1,000 × g for 5 minutes at 4 °C), resuspended with lysis buffer (50 mmol/l Tris, 150 mmol/l NaCl, pH 8.5) and lysed by freeze-thawing cycles with dry ice/ethanol baths. The lysate was treated with benzonase (Sigma-Aldrich AB, Sweden) and then purified by centrifugation to remove cellular debris (4,500 × g for 20 minutes at 4 °C) followed by ultracentrifugation (1.5 hours at 350,000 × g at 18 °C) in a discontinuous iodixanol gradient[37] and then by ion-exchange chromatography using an Acrodisc Mustang Q membrane device (Pall Life Sciences, Port Washington, NY). Briefly, the Mustang Q membranes were preconditioned according to the manufacturer's instructions with a final wash with a low salt buffer (20 mmol/l Tris, 15 mmol/l NaCl, pH 8.0). The virus suspension was diluted threefold in the same low salt buffer, before initiating the purification. Addition of the virus to the membranes was followed by a wash with the same low salt buffer. The virus was eluted from the membranes using a high salt buffer (20 mmol/l Tris, 250 mmol/l NaCl, pH 8.0). The virus suspension was then buffer exchanged ~100-fold by adding DPBS buffer (Life Technologies, Waltham, MA) and concentrated with a centrifugation filter device (Millipore Amicon Ultra 100kDa MWCO) at 2,000 × g and 18 °C. Dilutions of viruses were done using the same DPBS buffer. The titers of the vector preparations were determined with TaqMan quantitative PCR using primers targeting the ITR sequence promoter.[38]

Oral TMP administration. Nine of 19 TH+DD-GCH1-treated animals in behavioral part of the experiment (Experiment 2) received oral TMP suspen- sion (Meda AB, Solna, Sweden) in their drinking water 15 weeks post-AAV injection. TMP was administered in three different doses in 6-week intervals—starting concentration 0.5 mg/ml, 1.0 mg/ml at 21 weeks, and finally 2.0 mg/ml at 28 weeks (**Figure 1**). Drinking behavior was closely monitored in a subset of animals, which enabled approximation of the corresponding dose, 22.8 ± 2.8, 40.9 ± 6.2, and 87.1 ± 14.6 mg TMP/kg/day, respectively.

Behavioral tests. Amphetamine-induced rotation test was used as an initial screen to exclude animals with incomplete dopaminergic lesion and was performed 5 weeks after 6-OHDA surgeries. Animals received injections of D-amphetamine sulphate (2.5 mg/kg, i.p., Apoteksbolaget, Sweden) and their full left and right body turns were quantified over 90 minutes using automated rotometer bowls (AccuScan Instruments, Columbus, OH). The cut-off value for net ipsilateral rotational asymmetry score was 6 full body turns/minutes.

Corridor test was first described by Dowd *et al*.,[39] and measures lateralized sensory neglect. Briefly, the rat was placed in the end of a corridor (150 × 7 × 23 cm) with 10 adjacent pairs of cups filled with five sugar pellets evenly distanced along the floor of the corridor. Animals were allowed to explore the corridor freely. An investigator blinded to the group identity directly quantified retrievals; defined as each time the rat poked its nose into a unique cup, regardless of if it ate any pellets. Revisits in the same cup were not scored unless retrieval was made from another cup in between. All rats were tested until 20 retrievals were made or the test duration exceeded 5 minutes. Before testing, all rats were placed in an empty corridor for 5 minutes to reduce novelty of the environment. The rats were food restricted the day prior and during the 2–3 days of testing. Results were calculated as an average of the contralateral retrievals (left) and presented as percentage of total retrievals.

Stepping test, developed by Schallert *et al*.[40] and modified by Olsson *et al*. [41] was employed in this study. In brief, a blinded investigator assessed forelimb use by holding the rat with two hands only allowing one forepaw to touch the table surface. The investigator then moved the rat sideways over a defined distance of 90 cm with a constant speed over 4–5 seconds and scored the amount of steps in both forehand and backhand direction for each forelimb. Each direction was scored twice on each testing day and the average score was calculated over 3 days.

OMD procedure. Two microdialysis protocols were employed in separate groups of animals. In total, 36 animals were used for this part of the experiment. The first protocol was performed in four groups of TMP-naive animals, namely the TH+DD-GCH1 (n = 7), TH+GCH1 (n = 4), Les-Sham (n = 4), and intact controls (n = 4) groups.

All animals were surgically implanted with a probe guide, which was cemented to the skull 2 days prior to the actual sampling. This was achieved with two screws fastened to the skull without penetrating the dura and drilling at the position of the vector injections, *i.e.*, AP: +0.5 mm; ML: –3.7 mm and DV: –1.7mm with the tooth bar set to –2.4 mm. The DV coordinate was calculated; so, the membrane of the probe was positioned in the center of the transduction. A tether screw was then placed on the positioned to later hold the tether and then dental cement was added to fixate all components to the skull bone. The animal was given analgesia after the surgery and allowed to recover for at least 2 days before the experiment. At the day of the experiment, the animal was briefly sedated with isofluorane to facilitate removal of the guide dummy inside the probe guide, the sampling probe was inserted and then the tether was attached to the screw. The rat was placed in the testing cylinder where it had free access to food and water throughout the experiment. Following an equilibration period of 90 minutes, baseline samples were collected over 60 minutes (five samples at 12-minute intervals). The ringer solution of artificial CSF was changed to ringer containing 2E-5 M TMP lactate salt (Sigma-Aldrich AB, Sweden) so that the next sample became the first time bin when TMP was infused to the brain via reverse MD. This approach resulted in a precise measure of the time of initiation and controlled exposer of TMP over several hours following this time point. The animal was allowed to freely move in the test chamber for an additional 12 hours and the dialysates were instantly injected and analyzed with a HPLC coupled to the outlet of the OMD system while the samples were collected every 12 minutes. The microdialysates were then analyzed by HPLC with the ALEXYS monoamine analyzer system (Antec Leyden, The Netherlands) consisting of a DECADE II detector and VT-3 electrochemical flow cell. DA and metabolites were detected with a mobile phase consisting of 50 mmol/l citric acid, 8 mmol/l NaCl, 0.05 mmol/l EDTA, 15% methanol, 700 mg/l 1-octanesulfonic acid sodium salt, at pH 3.15, with 1 mm × 50 mm column with 3-mm particle size (ALF-105) at a flow rate of 90 ml/minutes. The quantification limit of the system was ~2 nmol/l. Peak identification and quantification was conducted using the Clarity chromatographic software package (DataApex, Prague, Czech Republic).

The second microdialysis experiment was performed in anaesthetized animals was performed in TH+DD-GCH1-treated animals that received oral TMP administration in their drinking water at least 2 weeks prior sampling; either 0.5 mg/ml (n = 4) or 2 mg/ml (n = 5) (same TMP emulsion that was administered to the animals followed with behavior tests). In addition, this experiment also included animals from the TH+GCH1 (n = 3), Les-Sham (n = 2), and intact control (n = 3) groups. As described above, probe placement was calculated to position the membrane of the probe in the center of the transduction area in striatum, which corresponded to the coordinates: AP: +0.5 mm; ML: –3.7 mm and DV: –5.7mm with the tooth bar set to –2.4 mm. After 90 minutes equilibration, baseline samples were collected before 1E-5 M NSD- 1015 (Sigma-Aldrich, St Louis, MO) was administered via the probe in the ringer solution for 2 hours. This was then followed by a 2-hour administration of 2E-5 M TMP lactate salt in addition to 1E-5 M NSD-1015 in the ringer solution. Samples were analyzed readily as described for the first microdialysis experiment. After the last sample was collected, the animal was terminated and brain tissue was taken for histology.

Histological processing. After the last behavioral assessment point all animals were anaesthetized by an injection of 1.2 ml sodium pentobarbital (i.p., Apoteksbolaget, Sweden) and then transcardially perfused with 50 ml room temperature saline followed by 250 ml ice-cold 4% paraformaldehyde (PFA) in 0.1 mol/l phosphate buffer adjusted to pH 7.4, at a 50 ml/minute rate. The brains were then dissected and postfixated in 4% PFA for 24 hours before cryoprotection in 25% sucrose for 24–48 hours. The fixed brains were cut in coronal orientation at a thickness of 35 μm on a semiautomated freezing microtome (Microm HM 450) and

collected in 8 and 6 (striatum and substantia nigra, respectively) series and stored in antifreeze solution (0.5 mol/l sodium phosphate buffer, 30% glycerol, and 30% ethylene glycol) at –20 °C until further processing. Immunohistochemistry was performed using antibodies raised against TH (P40101-0, rabbit IgG 1:2,000 Pel-Freez, Rogers, AR), GCH1 (MCA3138Z, mouse IgG 1:2,000 AbD Serotec, Oxford, UK), AADC (AB1569, rabbit IgG, 1:500, Millipore, Billerica, MA), NeuN (MAB377, mouse IgG 1:500, Millipore), IBA1 (019-19741, rabbit IgG 1:1,000, Wako, Richmond, VA), ED1 (MCA341-R, mouse IgG 1:200, Serotec), and DHFR (custom made, rabbit IgG, 1:50,000). Incubation with biotinylated secondary antibodies (BA1000, goat anti-rabbit 1:200 and BA2001, horse anti-mouse 1:200, Vector Laboratories, Burlingame, CA) was followed by a second 1-hour incubation with avidin-biotin peroxidase solution (ABC Elite, Vector Laboratories). The staining was visualized using 3,3′-diaminobenzidine in 0.01% H_2O_2.

Stereological analysis. NeuN-positive cells in striatum and GP were quantified with design-based stereology to rule out any toxic effects as a consequence of vector transduction. In this study, we utilized the areal counting frame originally described by Gundersen *et al.* [42] with a Nikon Eclipse 80i light microscope connected to the NewCast Module in VIS software (*Visiopharm A/S*, Horsholm, Denmark). Delineation was performed unilaterally in 2× magnification in the entire rostrocaudal axis, approximately between bregma 2.28 mm and –2.76 mm and bregma –0.24 mm and –2.76 mm for striatum and GP respectively.[36] Every 16th section was quantified for striatal counts, resulting in seven sections per brain and for GP every 8th or 16th section was quantified (4–7 sections/brain). Quantification was performed with a 60× Plan-Apo oil lens (NA = 1.4) by a researcher blinded to the group identity. The optical fractionator principle was then used to estimate the total number of objects present in the target nuclei.[43] Transduction volume was determined in the same as delineation with 2× magnification of TH stained specimens.

Statistical analysis. Statistical comparisons were conducted with the SPSS statistical package version 21 (SPSS, Chicago, IL). In **Figure 3**, nonparametric statistics were performed with Mann-Whitney *U*-test between group comparisons and Friedman test followed by Wilcoxon signed-rank test for the effect of TMP dose. One-way analysis of variance followed with Tukey's honest significant difference was performed on comparisons of transduction volumes and in **Figure 7**.

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FIGURE LEGENDS

Figure 1 Striatal 3,4-dihydroxyphenylacetic acid, homovanillic acid (HVA), and 5-hydroxyindoleacetic acid metabolites measured in awake-freely moving animals with online microdialysis (Experiment 1). All animals were trimethoprim (TMP)-naive at the start of the experiment. After the baseline sampling was done over 1 hour duration (five samples every 12 minutes), 20 μmol/l TMP-lactate (dissolved in ringer solution) was administered via the probe using reverse microdialysis principle and maintained throughout the rest of the experiment. Panel **a** shows 1-hour average data from baseline (BL) and 6- and 12-hours after start of TMP infusion in the TH+DD-GCH1 (n = 7), TH+GCH1 (n = 3), Les-Sham (n = 2), and intact control (n = 4) groups (values in brackets indicate the standard deviation). HVA levels from animals in the TH+DD-GCH1 group (n = 7) are plotted individually (black lines in panel **b**), while the group averages of intact and lesion controls as well as the constitutively active TH+GCH1 treatment group are presented for reference (gray lines). Data traces pres- ent in panel **b** are floating point averages of two data points from con- secutive samples plotted over a 13-hour period of observation.

Figure 2 Experimental design for the long-term behavioral assessment study (Experiment 2). Eighty-nine Sprague Dawley rats received 6-hydroxydopamine injections in the medial forebrain bundle and were then screened and pre-scored with amphetamine induced rotations, corridor test, and stepping test. Rats were then selected based on the severity of the lesion-induced behavioral impairments and allocated into two vector treatment groups and one lesion control group. In addition, 11 rats were included in the study as intact control group. Ten weeks after the lesion, nine animals were injected with a mixture of two vectors encoding for constitutively active TH and GCH1 genes (depicted as TH+GCH1 group), while another 19 were injected with a vector mix in which TH expression was combined with a regulated GCH1 expression (depicted as TH+DD-GCH1). All rats were then followed with repeated behavioral assessment with corridor and stepping test over a 33-week period. At 15 weeks post-AAV injection, 10 out of 19 animals in the TH+DD-GCH1 group received trimethoprim (TMP) in their drinking water. TMP was administered in three concentra- tions—0.5, 1.0, and 2.0 mg/ml—for 6 weeks each in a dose-escalation study design. After 33 weeks, all animals were terminated and brain tissue taken for histological processing.

Figure 3 Motor behavioral assessment of the animals was performed using the stepping (a,b) and corridor (c) test paradigms over a 33-week follow-up period. The stepping test results are presented in the forehand (**a**) and the backhand direction (**b**). Three measurements were conducted post-AAV at 6, 9, and 12 weeks before the introduction of trimethoprim (TMP) in drinking water at three doses from 0.5 mg/ml to 1 and 2 mg/ml every 6 weeks. During this period, 2 tests were performed with 3 weeks intervals. Note that during the post-AAV assessment period, the TH+DD-GCH1 group (open circles) was an average of 19 animals; while in the TMP dose-escalation phase of the study 9 animals were followed without TMP (open circles) and 10 were followed with TMP (gray circles). Mann-Whitney U-tests were used to study the effect of TMP by comparing averages of the TH+DD-GCH1 and Les-Sham or intact control groups before and during TMP administration. Friedman test followed by Wilcoxon signed-rank test was used to study the effect of TMP dose in the TH+DD-GCH1 group and in corridor test all TMP doses were significantly different from pre TMP administration and between doses ($P < 0.001$) except between 1.0 and 2.0 mg/ml. *TH+DD-GCH1 group significantly different from Les-Sham ($P < 0.001$). Error bars represent standard error of the mean.

Figure 4 Regulation of transgenic GTP cyclohydrolase 1 (GCH1) fused with destabilized dihydrofolate reductase (DHFR) (depicted as DD-GCH1). Animals treated with constitutively expressed TH+GCH1 showed robust expression of transgenic human GCH1 in the striatum (**b**). Note that this antibody does not cross-react with rodent GCH1 and thus no immunoreactivity is observed in intact animals (**a**). In the absence of trimethoprim (TMP), TH+DD-GCH1-treated rats have very faint or no GCH1 or DHFR immunoreactivity (**c,g**), whereas in animals receiving TMP the immunoreactivity of GCH1, as well as DHFR, is increased to readily detectable levels (**d,h**). At 2 mg/ml dose level (as shown in these panels) GCH1 expression is similar to constitutive expression group (compare **b** and **d**). Low power images of coronal brain sections from animals that received either TH+DD-GCH1 (with TMP in **k** and without in **i**) or TH+GCH1 (**j**). Scale bar in panel **h** represents 100 μm in panels **a–h** and 2.3 mm in **i–k**.

Figure 5 High-magnification images obtained from the striatum. Transduction area stained using tyrosine hydroxylase immunoreactivity (TH; **a,f,k,p,u**), cresyl violet (CV; **b,g,l,q,v**), NeuN (**c,h,m,r,w**), and glial markers Iba1 (**d,i,n,s,x**) and ED1 (**e,j,o,t,y**; equivalent to human CD68) in columns from left to right. Each experimental group is represented in rows. Scale bar in y represents 100 μm and applies to all panels.

Figure 6 High-magnification images obtained from the globus pallidus. Stained using tyrosine hydroxylase immunoreactivity (TH; **a,f,k,p,u**), cresyl violet (CV; **b,g,l,q,v**), NeuN (**c,h,m,r,w**), and glial markers Iba1 (**d,i,n,s,x**) and ED1 (**e,j,o,t,y**; equivalent to human CD68) in columns from left to right. Each experimental group is represented in rows. Scale bar in y represents 100 μm and applies to all panels.

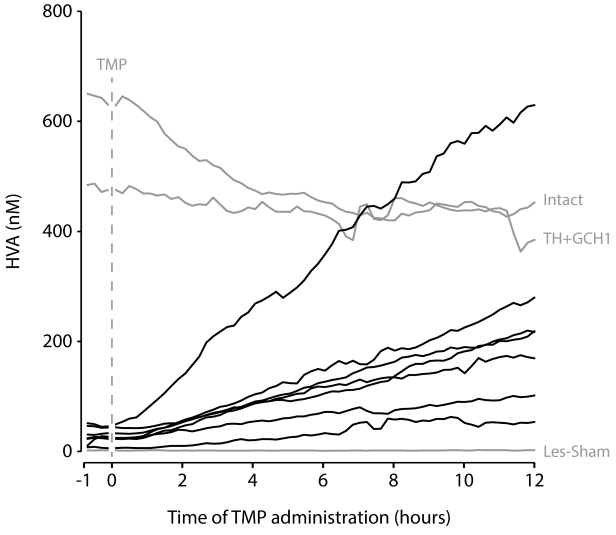
Figure 7 Stereological quantification of NeuN-positive neuronal profiles in striatum (a) and globus pallidus (b). Number of cells in both striatum and globus pallidus were not significantly affected by the 6-OHDA lesion (Les-Sham) when compared to intact animals (open bars). Expressing tyrosine hydroxylase constitutively in combination with either GCH1 or DD-GCH1 did not cause any reduction of these cells in either nuclei. Error bars represent standard error of the mean.

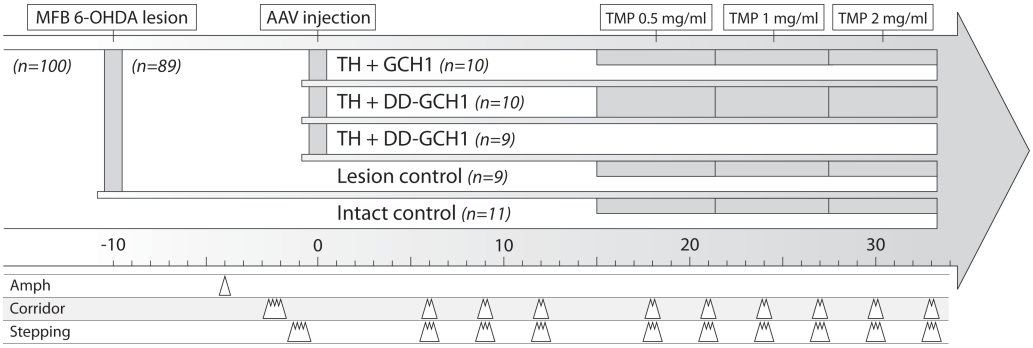
A

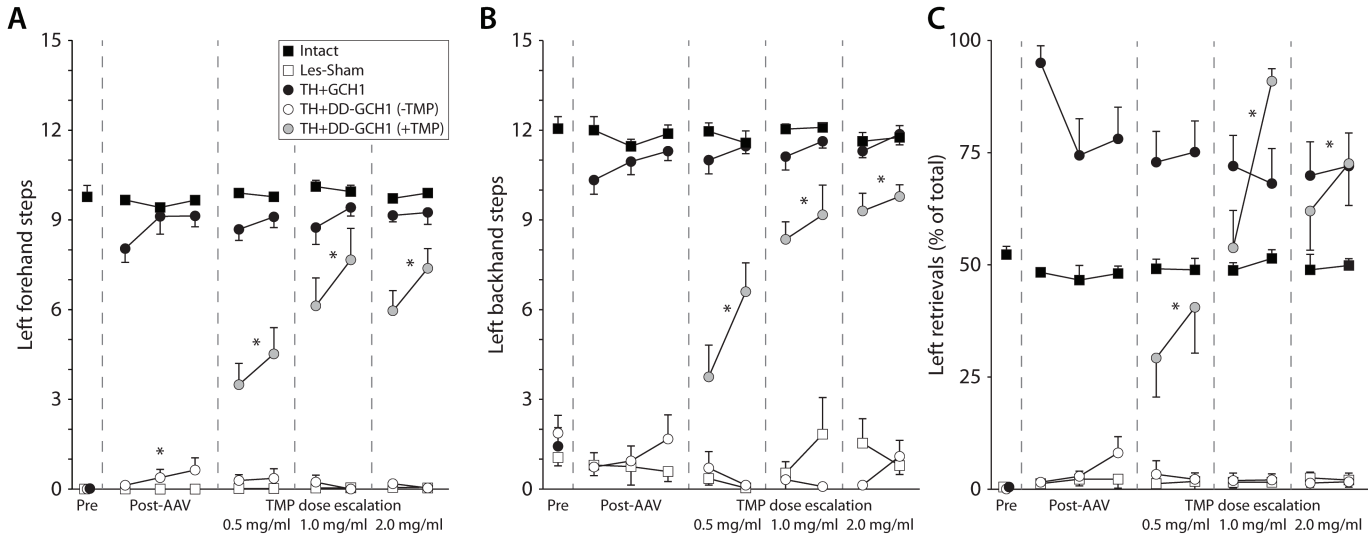
	Intact			Les-Sham		
	BL	TMP-6h	TMP-12h	BL	TMP-6h	TMP-12h
DOPAC	**891.4** (290.0)	**697.0** (241.5)	**663.5** (332.6)	**3.0** (2.6)	**4.0** (1.1)	**2.6** (1.7)
HVA	**640.8** (145.3)	**456.7** (133.4)	**449.7** (141.6)	**2.0** (0.8)	**1.7** (1.0)	**1.9** (0.9)
5-HIAA	**236.9** (36.6)	**180.8** (17.6)	**196.5** (65.5)	**318.2** (50.1)	**279.9** (94.9)	**332.4** (65.6)

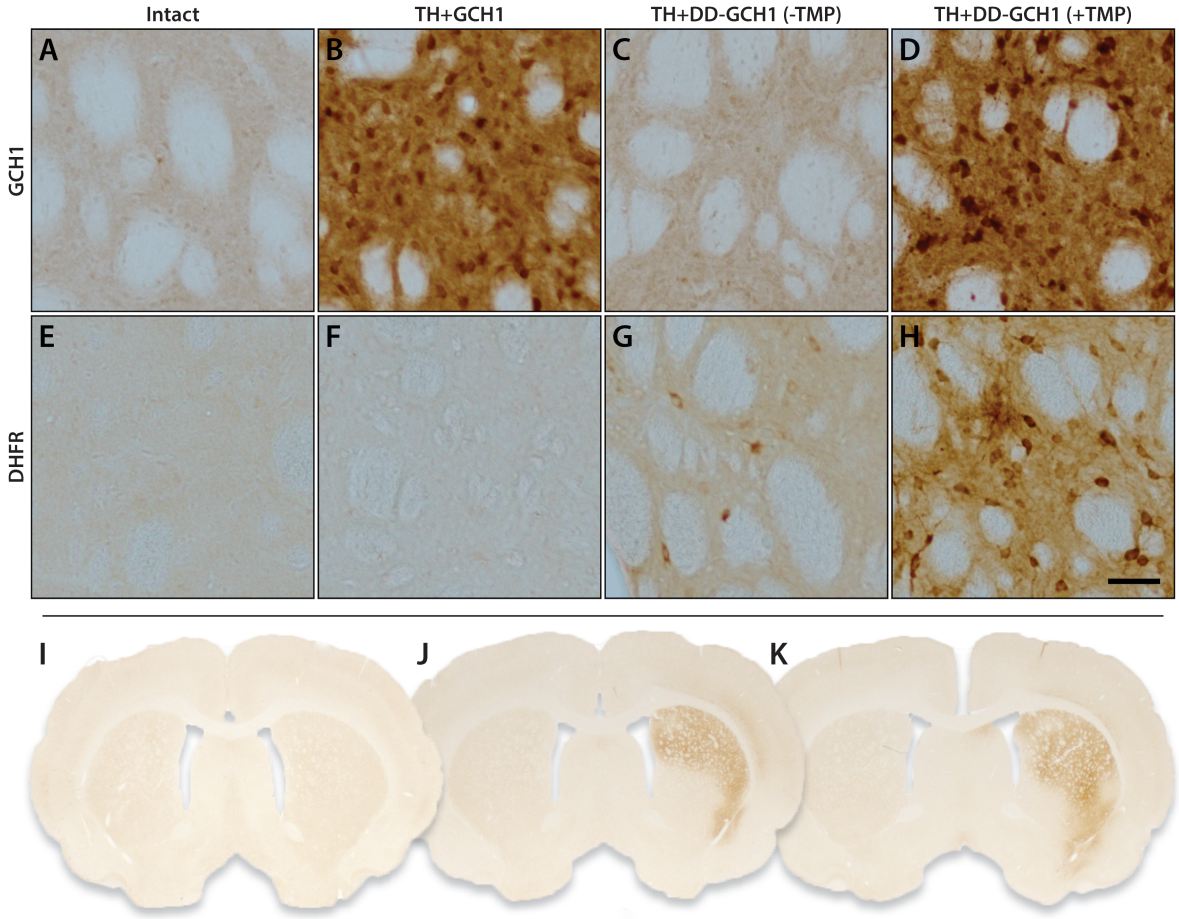
	TH+GCH1			TH+DD-GCH1		
	BL	TMP-6h	TMP-12h	BL	TMP-6h	TMP-12h
DOPAC	**283.4** (317.1)	**278.6** (269.6)	**233.9** (187.7)	**15.0** (12.8)	**101.8** (109.2)	**176.0** (171.8)
HVA	**507.9** (414.4)	**476.5** (418.7)	**376.3** (254.1)	**28.6** (15.3)	**135.5** (113.8)	**236.2** (182.8)
5-HIAA	**252.7** (70.2)	**231.6** (53.2)	**244.3** (88.5)	**292.0** (58.3)	**233.9** (63.8)	**271.6** (50.9)

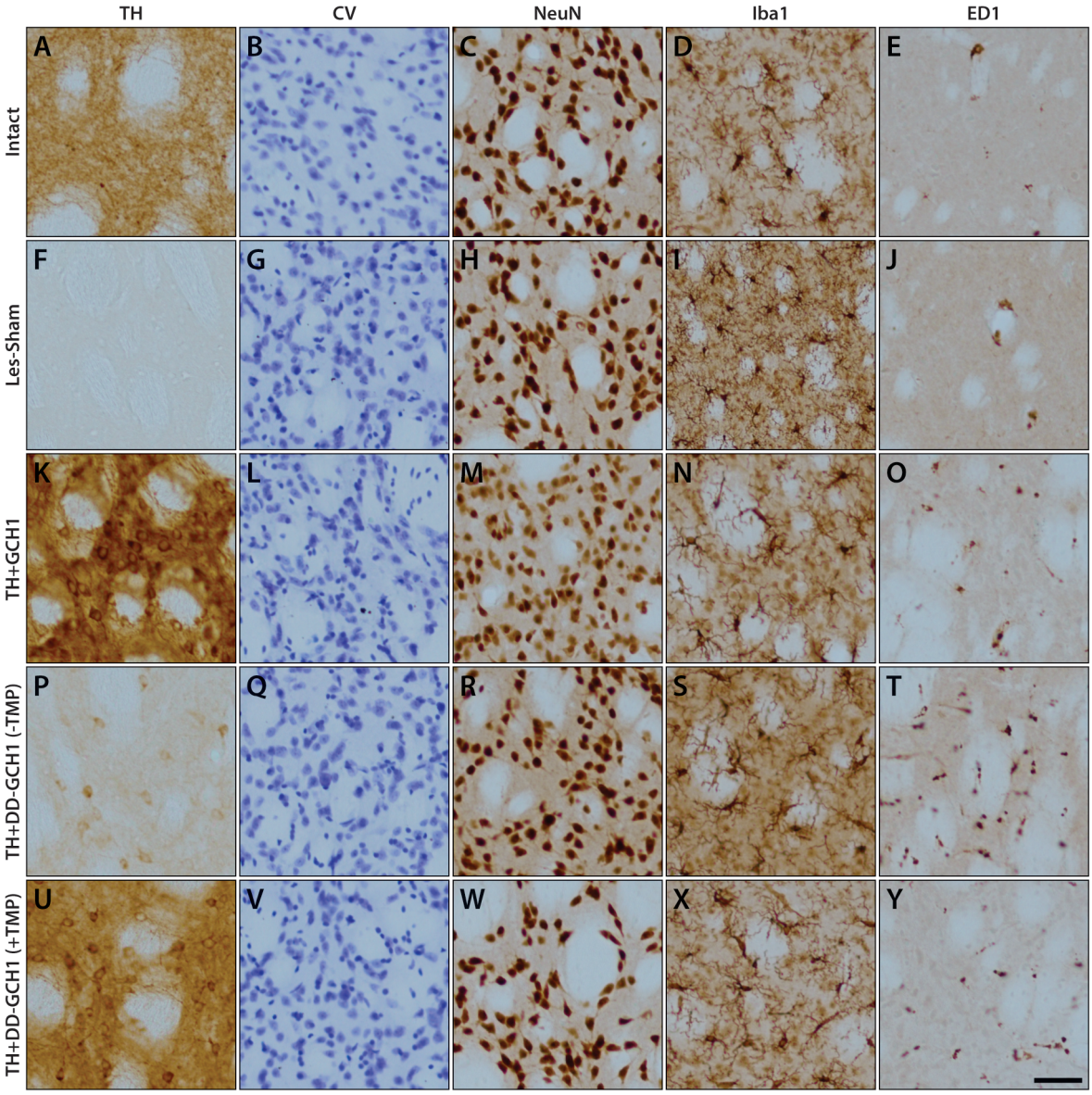
B

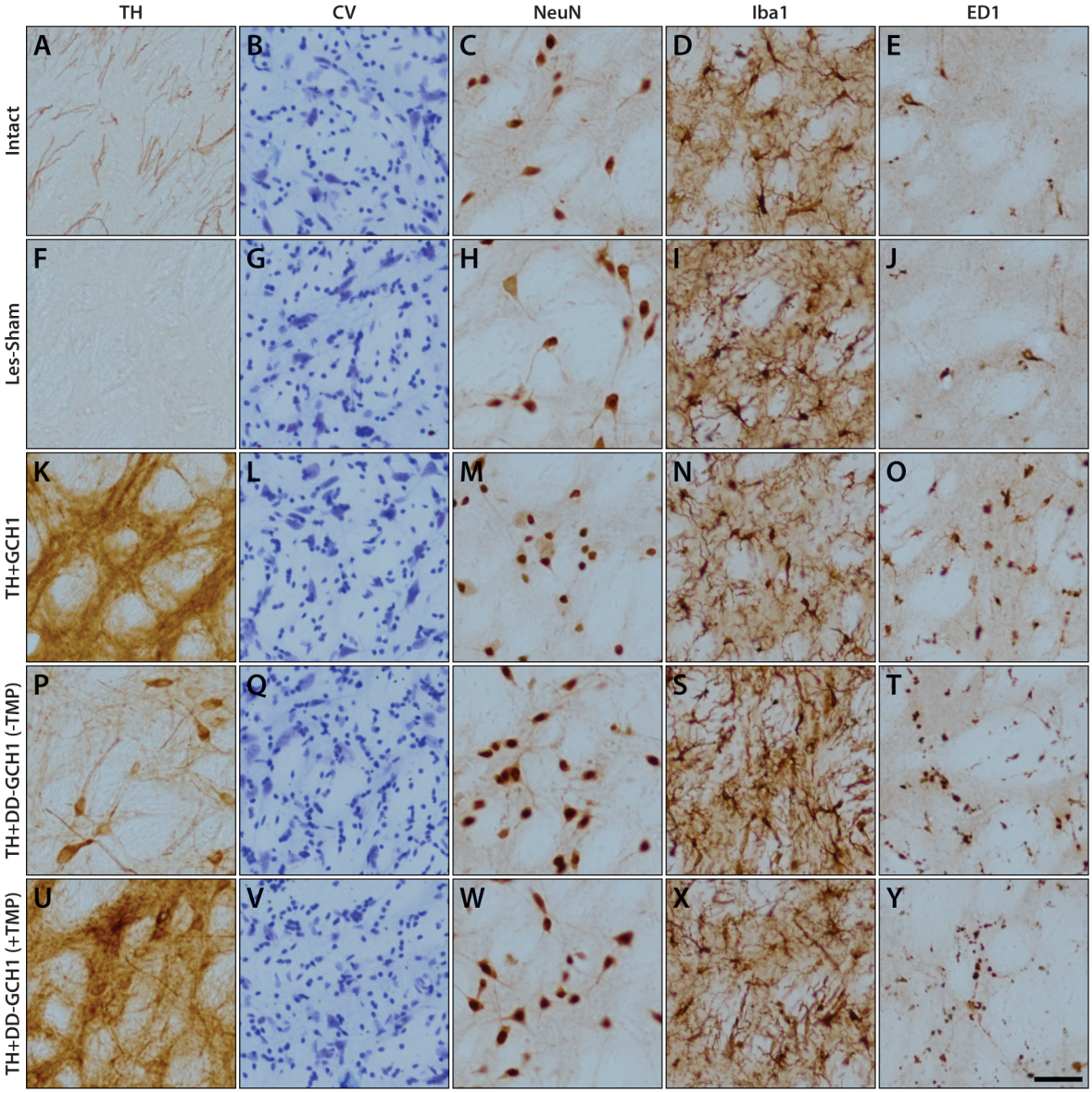



MFB 6-OHDA lesion
AAV injection
TMP 0.5 mg/ml
TMP 1 mg/ml
TMP 2 mg/ml
(n=100)
(n=89)
TH + GCH1 (n=10)
TH + DD-GCH1 (n=10)
TH + DD-GCH1 (n=9)
Lesion control (n=9)
Intact control (n=11)
-10
0
10
20
30
Amph
Corridor
Stepping


A
B
C
Intact
Les-Sham
TH+GCH1
TH+DD-GCH1 (-TMP)
TH+DD-GCH1 (+TMP)
Left forehand steps
Left backhand steps
Left retrievals (% of total)
Pre
Post-AAV
TMP dose escalation
0.5 mg/ml
1.0 mg/ml
2.0 mg/ml


Intact
TH+GCH1
TH+DD-GCH1 (-TMP)
TH+DD-GCH1 (+TMP)
GCH1
DHFR
A
B
C
D
E
F
G
H
I
J
K


TH
CV
NeuN
Iba1
ED1
Intact
Les-Sham
TH+GCH1
TH+DD-GCH1 (-TMP)
TH+DD-GCH1 (+TMP)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y


TH
CV
NeuN
Iba1
ED1
Intact
Les-Sham
TH+GCH1
TH+DD-GCH1 (-TMP)
TH+DD-GCH1 (+TMP)
A
B
C
D
E
F
G
H
I
J
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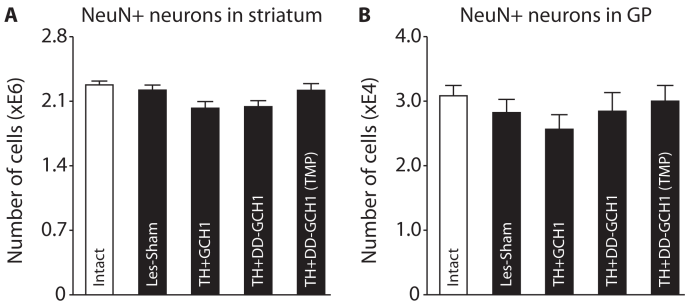
X
Y


A
NeuN+ neurons in striatum
Number of cells (xE6)
2.8
2.1
1.4
0.7
0
Intact
Les-Sham
TH+GCH1
TH+DD-GCH1
TH+DD-GCH1 (TMP)
B
NeuN+ neurons in GP
Number of cells (xE4)
4.0
3.0
2.0
1.0
0
Intact
Les-Sham
TH+GCH1
TH+DD-GCH1
TH+DD-GCH1 (TMP)

	DOPA						DOPAC						HVA					
	BL		+NSD		+NSD +TMP		BL		+NSD		+NSD +TMP		BL		+NSD		+NSD +TMP	
	AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD	AVE	SD
Intact	0.0	0.0	170.8	81.9	184.7	45.4	1950.5	796.7	1268.3	415.5	897.0	406.0	1162.7	692.0	1259.0	572.5	1098.5	508.7
Les-Sham	0.7	0.7	2.7	3.0	20.3	1.6	5.6	4.9	4.5	3.9	1.0	2.2	13.5	13.0	11.8	12.1	8.6	8.3
TH+GCH1	167.9	58.5	234.6	100.9	257.7	107.0	320.5	82.4	108.9	43.9	79.3	16.2	471.8	47.8	220.5	49.0	151.6	14.1
TH+DD-GCH1 (0.5 mg/ml)	91.6	72.4	121.3	91.2	120.7	88.9	302.6	238.8	121.4	115.7	90.4	78.0	468.6	269.1	228.0	184.9	153.6	115.6
TH+DD-GCH1 (2.0 mg/ml)	86.4	49.7	125.6	74.7	117.1	66.0	676.1	534.8	260.6	175.4	202.0	127.3	466.2	283.5	238.6	170.6	164.2	122.8