here will advance efforts toward clinical trial, it is important that preclinical studies are repeated to obtain long-term readouts, up to a year, of efficacy and to evaluate any evidence of toxicity arising from possible expression of hDAT in nondopaminergic neurons. Additional challenges relating to human translation are worthy of discussion; for such precision medicine approaches to be effective, there is a need for accurate neurosurgical targeting, which requires considerable expertise and optimal vector dosing to maximize efficacy and avoid neurotoxicity. A clear understanding of the disease is also necessary to ensure that the most suitable patients are put forward for trial, potentially determined by genotype, age, and disease stage for optimum therapeutic time window. One potential modification to improve safety and efficacy could be to use a dopaminergic neuron-specific promoter, although the difficulty in identifying a translatable dopaminergic neuron-specific promoter is widely acknowledged in the field. Studies of truncated human TH promoters have shown low to high promoter strength with variable specificity (*36*). These TH promoters have not been used clinically and are unlikely to improve our vector efficacy, safety, and translatability. Moreover, the current gene therapy trials for related disorders such as AADC deficiency (NCT01395641 and NCT01973543) used the AAV2 capsid with a ubiquitous promoter, combined with stereotactic delivery to successfully target specific brain regions such as the striatum and midbrain with marked patient benefit (*39*–*42*). Our use of hSyn promoter improves neuronal selectivity in comparison, and the efficacy and safety achieved through our vector design and delivery method clearly supports future translation of our approach toward a clinical trial of AAV gene therapy for patients with DTDS.

MATERIALS AND METHODS

Study design

The aim of this study was to (i) understand the mechanisms underpinning DTDS in an in vitro patient-derived mDA model and (ii) develop a gene therapy strategy to rescue disease in vitro and in vivo.

For the generation of a patient-derived neuronal model, we obtained fibroblasts from patients and reprogrammed them into iPSCs. To rule out the effect of genetic background, we generated an isogenic control iPSC line with correction of the disease-associated homozygous *SLC6A3* mutation. A tritiated DA uptake assay and HPLC were performed to confirm DAT dysfunction in mature derived mDA neurons. We then investigated cellular disease mechanisms by immunoblotting, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and immunofluorescence analysis, identifying disease-specific dysregulation of DA metabolites and neurodegenerative processes. A cell viability assay was performed on the mDA cultures to investigate inflammatory response and an enzyme-linked immunosorbent assay on patient CSF to investigate proinflammatory cytokine release. We then developed a lentiviral-based gene therapy delivery approach and analyzed DAT activity and markers of neurodegeneration after gene transfer.

For reproducibility and reliability of our dataset, we have used standardized protocols and performed blinded analyses, except in Figs. 3D and 5D and fig. S2B. The number of replications of each independent experiment is reported in the respective figure legends. Single experiments, which failed for technical reasons, have been selectively discarded from the analysis. Tritiated DA uptake assays of untreated (Fig. 1A) and pifithrin-μ–treated (Fig. 5A) lines have been performed simultaneously to avoid technical variability, and as such, they partially share the same dataset for untreated samples.

The in vivo studies were designed to test the hypothesis that AAV-mediated gene therapy would restore DAT function in the knockout mouse model of DTDS. We evaluated the efficacy of neonatal AAV9-mediated gene transfer with endpoints of survival, locomotor behavior, and neurotransmitter analysis. We assessed toxicity related to dosage and off-target expression and then delivered gene therapy to adult knockout mice with AAV2 capsid by stereotactic injection with the same endpoints. The number of biological replicates varied between studies ($n = 5$ to 17) and is indicated in the figure legends. Animals were randomly assigned to the vector treatment group. Assessment of outcomes was blinded through labeling without treatment information on behavioral analysis videos, tissue samples for biodistribution, and pathological analysis.

Statistical analysis

Statistical analysis tailored to each experiment was performed using GraphPad Prism version 8. For the statistical analysis of iPSC-derived data, when dual comparisons were required, two-tailed Student's *t* test was applied, whereas for multiple comparisons, one-way analysis of variance (ANOVA) was performed. In vivo experimental design and sample sizes were designed using NC3Rs guidance and power calculation. For most analyses of animal experiments, one-way or two-way ANOVA was performed with either Bonferroni or Tukey's multiple comparison test. Percentage of foot faults was converted by log transformation before ANOVA. For neuronal firing, Kruskal-Wallis distribution and χ^2 tests were applied.

SUPPLEMENTARY MATERIALS

stm.sciencemag.org/cgi/content/full/13/594/eaaw1564/DC1

Methods

Fig. S1. Generation of control, patient, and isogenic human iPSC lines.

Fig. S2. Differentiation of control and patient iPSC lines into mDA precursors.

Fig. S3. Differentiation of control and patient neural progenitors into mature, electrically active mDA neurons.

Fig. S4. Day 65 DAT gene and protein expression profiles for control and patient lines.

Fig. S5. Day 65 gene expression profiles for key enzymes involved in dopamine metabolism in control and patient lines.

Fig. S6. Day 65 quantification of mDA neurons in control and patient lines.

Fig. S7. Day 65 immunofluorescence for cleaved caspase-3 (cCASP3) in control and patient lines.

Fig. S8. Day 65 immunofluorescence for GFAP in control and patient lines.

Fig. S9. Therapeutic approaches for DTDS with pifithrin-μ and lentiviral gene transfer in the mDA neuronal model.

Fig. S10. In vivo AAV9 hSyn GFP marker gene study.

Fig. S11. Electrophysiological properties of medium spiny neurons after neonatal AAV9 hSLC6A3 gene therapy.

Fig. S12. AAV9.hSLC6A3 intracerebroventricular gene transfer at higher dosage.

Fig. S13. AAV2.hSLC6A3 in vitro transduction of knockout primary neurons.

Fig. S14. AAV2.hSLC6A3 stereotactic gene delivery to SN.

Table S1. Primers for CRISPR correction of *SLC6A3* variant c.1184C>T.

Table S2. List of antibodies.

Table S3. Primer sequences.

Table S4. Primer sequences for generation of vector expression cassette, viral vector titration, and qRT-PCR.

Movie S1. Untreated hyperlocomotor DAT knockout at P21.

Movie S2. Untreated DAT knockout showing parkinsonism at P35.

Movie S3. Open field DAT knockout treated with AAV9.hSLC6A3.

Movie S4. Open field DAT knockout treated with high dosage AAV9.hSLC6A3.

Movie S5. Open field DAT knockout treated with neat AAV2.hSLC6A3 compared with controls.

Movie S6. Open field dosage response of DAT knockout treated with AAV2.SLC6A3 2×10^{10} (neat), 2×10^{9} (1:10), and 2×10^{8} (1:100) dosages.

Data file S1. Raw data (provided as separate Excel file).

References (*43*–*52*)

View/request a protocol for this paper from *Bio-protocol*.