Enzyme Replacement Therapy for Succinic Semialdehyde Dehydrogenase Deficiency: Relevance in γ-Aminobutyric Acid Plasticity

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Abstract
Succinic semialdehyde dehydrogenase deficiency (SSADHD) is a rare inborn metabolic disorder caused by the functional impairment of SSADH (encoded by the ALDH5A1 gene), an enzyme essential for metabolism of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). In SSADHD, pathologic accumulation of GABA and its metabolite γ-hydroxybutyrate (GHB) results in broad spectrum encephalopathy including developmental delay, ataxia, seizures, and a heightened risk of sudden unexpected death in epilepsy (SUDEP). Proof-of-concept systemic SSADH restoration via enzyme replacement therapy increased survival of SSADH knockout mice, suggesting that SSADH restoration might be a viable intervention for SSADHD. However, before testing enzyme replacement therapy or gene therapy in patients, we must consider its safety and feasibility in the context of early brain development and unique SSADHD pathophysiology. Specifically, a profound use-dependent downregulation of GABA_A receptors in SSADHD indicates a risk that any sudden SSADH restoration might diminish GABAergic tone and provoke seizures. In addition, the tight developmental regulation of GABA circuit plasticity might limit the age window when SSADH restoration is accomplished safely. Moreover, given SSADH expressions are cell type–specific, targeted instead of global restoration might be necessary. We therefore describe 3 key parameters for the clinical readiness of SSADH restoration: (1) rate, (2) timing, and (3) cell type specificity. We aim to understand the impacts of specific SSADH restoration protocols on brain physiology, accelerating bench-to-bedside development of enzyme replacement therapy or gene therapy for SSADHD patients.

Keywords
epilepsy, genetics, inborn errors of metabolism, metabolism, mitochondrial disorder, neurodevelopment, seizures, status epilepticus, treatment

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GABAA receptor composition, intracellular trafficking, lateral mobility on neuronal surfaces, and synapse stability.19,20 SSADH fundamentally impact brain development. Brain plasticity is heightened during early critical periods of brain development.21,30 If successful SSADH restoration requires GABA circuit (i.e., receptor) auto regulation to accommodate a profound decline in GABA concentration, then such therapy might only be effective in younger patients. Conversely, in older patients who lack GABA circuit plasticity, SSADH restoration might be necessary to avoid sudden reversal of chloride homeostasis.16-18 In SSADHD, altered chloride homeostasis might lead to depolarizing GABAergic neurotransmission,22 which in turn might elicit host immune responses that often lead to reduced therapeutic efficacy or total resistance,28,29 limiting tests of sustained SSADH restoration. Second, functional activity of injected enzymes or viral-mediated transgene expression are uncontrollable in aldh5a1−/− mice. Unmanaged SSADH restoration in aldh5a1−/− mice leads to difficulty in evaluating therapeutic efficacy and dose-response relationship. Third, cell-specific SSADH restoration for therapeutic relevance is unachievable in aldh5a1−/− mice without the use of viral vectors with cell-specific promoters, but currently available viral tools do not achieve cell type-specificity necessary to differentiate various cell types (e.g., interneuron subtypes) relevant for SSADH expression.

To date, the aldh5a1−/− mouse is the only available SSADHD mouse model, which mimics a severe form of the disorder.27 However, there are several major limitations pertaining to the use of aldh5a1−/− mice in testing SSADH-restoring strategies such as enzyme replacement therapy. First, injected enzymes elicit host immune responses that often lead to reduced therapeutic efficacy or total resistance,22,28 limiting tests of sustained SSADH restoration. Second, functional activity of injected enzymes or viral-mediated transgene expression are uncontrollable in aldh5a1−/− mice. Unmanaged SSADH restoration in aldh5a1−/− mice leads to difficulty in evaluating therapeutic efficacy and dose-response relationship. Third, cell-specific SSADH restoration for therapeutic relevance is unachievable in aldh5a1−/− mice without the use of viral vectors with cell-specific promoters, but currently available viral tools do not achieve cell type-specificity necessary to differentiate various cell types (e.g., interneuron subtypes) relevant for SSADH expression.

Given that testing the full range of preclinical readiness of SSADH-restoring strategies requires sustained and regulated enzyme replacement paradigms, we proposed to develop a novel SSADHD mouse model that allows conditional aldh5a1 reactivation under precisely defined molecular control. In this novel mouse strain aldh5a1lox−/TA−STOP, the basal activity of aldh5a1 gene is disrupted, but is reconstituted upon Cre-mediated recombination (Figure 3). We will use this novel mouse genetic tool to address 3 key questions regarding safety and efficacy of SSADH restoration:

1. How rapidly can SSADH be restored without provoking seizures? If SSADH restoration leads to ambient GABA reduction, then a safe rate of enzyme restoration will be determined by the maximum rate at which GABA (particularly GABA\textsubscript{\textalpha}) receptors are upregulated. That is, we hypothesize that abrupt SSADH restoration will correspond to abrupt GABA decline without accompanying increase in GABA receptor expression—this may lead to seizures and brain injury. In contrast, gradual SSADH replacement should enable compensatory GABA receptor upregulation and (we predict) will be better tolerated. Using this novel mouse model, we will be able to test the safety and efficacy of a range of rates of enzyme restoration in SSADHD, and will explicitly address rate, rather than dose, as these pertain to gene therapy for epilepsy (Figure 4).

2. Given tight developmental regulation of GABAergic signaling, is SSADH restoration safe and effective across all ages? Or is safe and effective SSADH restoration restricted to specific developmental windows? GABA circuit plasticity is heightened during early critical periods of brain development.21,30 If successful SSADH restoration requires GABA circuit (i.e., receptor) auto regulation to accommodate a profound decline in GABA concentration, then such therapy might only be effective in younger patients. Conversely, in older patients who lack GABA circuit plasticity, SSADH...
restoration might be ineffective and unsafe. This too requires explicit preclinical testing (Figure 5).

3. Wild-type SSADH expression appears to be biased toward certain cell populations in the hippocampus and the cerebellum (Figure 6). Therefore, global SSADH restoration might risk adverse effects due to nonspecific reduction in GABAergic signaling. If this is true, then is limiting SSADH restoration to relevant brain regions safer and sufficient to rescue SSADHD? That is, we propose to test the safety and efficacy of regional and global SSADH restoration as a step toward identifying whether brain region–directed SSADH restoration (which may be safer than global SSADH restoration) is sufficient for SSADHD phenotype reversal.

**Materials and Methods**

**Institutional Assurance of Animal and Virus Use**

All animal treatment procedures and viral materials described in this study were covered by protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee at Boston Children’s Hospital.

**AAV Injection into C57Bl/6 Mice**

AAV-PHP.B: CAG-GFP (2.36 × 10^{13} genome copies/mL) was prepackaged and obtained from the Viral Core of Boston Children’s Hospital. AAV was suspended in sterile physiological saline and was administered into C57Bl/6 mice via intraperitoneal injection at postnatal day 10 (P10). Injections were performed once or across multiple days (refer to experimental paradigms outlined below).

**Immunofluorescence Staining**

Perfusion of cortical tissue and immunostaining procedures were performed as described previously. Under deep anesthesia, mice were perfused transcardially with ice-cold phosphate-buffered saline followed by 4% paraformaldehyde. Brain tissues were harvested, post-fixed in 4% paraformaldehyde, and cryopreserved in Tissue-Plus OCT Compound (Fisher Healthcare, Waltham, MA) for at least 24 hours.
Images were obtained at –20°C, washed briefly with phosphate-buffered saline, incubated with primary antibodies (see information below) overnight at 4°C, washed again, incubated with Alexa Fluor 594-conjugated secondary antibodies for 1 hour at room temperature, then mounted on glass slides. All perfusion, tissue fixation, and immunostaining procedures were carried out under the same conditions. Individual channels were acquired sequentially. Confocal images under low power (10× objective) and high power (40× objective) were acquired using the same batch of buffers to minimize variability between samples.

Antibodies

Different primary antibodies against specific interneuron subtypes are used in this study: calretinin, vasoactive intestinal polypeptide, and parvalbumin. These interneuron subtypes show differential expression with brain region specificity.

Image Acquisition

Immunostained brain sections were identified by fluorescence imaging under low power magnification (10× objective). Image acquisition was carried out using the FV10-ASW software (v2.1 C), with the following parameters: 20% laser output, ×1 gain control, laser intensity between 500 and 700, offset between 10% and 15% such that signals were within the linear range. Individual channels were acquired sequentially. Confocal images under low power (10× objective) and high power (40× objective) were acquired in selected brain regions. The amount of AAV-mediated transgene expression was quantified by confocal imaging, represented by GFP intensity in arbitrary units (arbitrary units).

The Novel Inducible SSADH Mouse Model

(Construction Work-in-Progress)

The endogenous aldh5a1 gene is located in chromosome 13 (GenBank). We use a 1-step mouse genome editing strategy termed Efficient Additions with Single-stranded DNA Inserts-CRISPR (Easi-CRISPR)37 to directly insert a lox-rtTA-STOP cassette via homology directed repair into a single-cell embryo (Figure 3A). In this mouse, aldh5a1lox-rtTA-STOP, endogenous SSADH expression is disrupted. To avoid alternative splicing leading to aldh5a1 gene read-through and basal SSADH expressions, we designed the lox-rtTA-STOP cassette to harbor a polypyrimidine tract directly upstream of the inserted splice acceptor site to ensure spliceosome and lariat formation.38 The additional SV40 polyadenylation signal further enables mRNA processing and stability. This inserted cassette ensures premature termination of the endogenous aldh5a1 gene, leading to its loss of function. Instead, this mouse expresses rtTA proteins driven by the endogenous aldh5a1 gene promoter upon the insertion of the lox-rtTA-STOP cassette (Figure 3B). Microinjection of CRISPR materials into single-cell embryos has been performed at Boston Children’s Hospital Mouse Gene Manipulation Core, and pups are being validated for gene insertion and integrity by Next-Generation DNA sequencing as of the preparation of this manuscript.

At basal condition, we anticipate that this mouse will phenocopy aldh5a1–/–, representing the severe form of the human SSADHD syndrome.27 When injected with adeno-associated virus which encodes Cre recombinase (AAV-Cre), the rtTA cassette will be removed via Cre-lox recombination, leading to reconstituted aldh5a1 gene activities under the control of its own promoter transcriptional elements and SSADH expression restored (Figure 3C). AAV-Cre will be injected at custom timing and dosage to test for therapeutic efficacy. The design of the aldh5a1lox-rtTA-STOP mouse allows versatile approaches to further study the impacts of conditional SSADH restoration. Alternatively, when bred to a mouse line expressing Cre-recombinase driven by a cell-specific promoter (e.g., Gad2-IRES-Cre mouse39), SSADH will be restored in selective cell types. This will give insight into whether cell targeted SSADH restoration might be viable therapeutic options.40-42

The aldh5a1lox-rtTA-STOP mouse will be further bred to a novel mouse line containing a tetracycline responsive element (TRE) driving a recombinant aldh5a1 gene, the TRE-aldh5a1 mouse (to be made separately), to allow reversible SSADH expression (Figure 7). The recombinant gene cassette TRE-aldh5a1 will be inserted in a previously characterized tightly regulated (TIGRE) genomic locus,44 such that SSADH expression is tightly controlled by the doxycycline (dox) level. This mouse system might be particularly useful when an adaptable pace of SSADH restoration is needed over the Cre-dependent
strategy (little control on AAV activities). This dox-mediated approach also allows reversible SSADH expression, so SSADH depletion can be studied systematically. Given the wide spectrum of clinical presentations among SSADHD patients traceable to their \textit{aldh5a1} mutations,\textsuperscript{44,45} this mouse model might offer an opportunity to study individual patient’s response to SSADH replacement. Importantly, these additional experiments are unachievable using \textit{aldh5a1}\textsuperscript{–/–} or \textit{aldh5a1\textsuperscript{lox-rtTA-STOP}} mouse in a Cre-dependent fashion alone (irreversible). Overall, this inducible SSADH mouse model allows controllable, reversible cell-targeted SSADH restoration, which is currently unachievable using the existing animal model.

**Statistics**

GFP intensity values from confocal imaging (represented by arbitrary units) were compared across experimental groups (i.e., across 1, 3, or 5 days of AAV injection) at 2 different postinjection time points (i.e., 7 or 14 days). One-way analysis of variance was used to compare across groups, followed by post hoc Bonferroni multiple comparison test for statistical significance. Data from 2 independent experiments were combined.

**Results**

**Rate-Dependent Transgene Expression in Brain via AAV-PHP.B Systemic Injections**

A proof-of-concept study was conducted to establish experimental paradigms for various rates of transgene expression via AAV vectors. Using an AAV construct that expresses GFP under constitutively active promoter (AAV-PHP.B: CAG-GFP, or AAV-GFP in short), we found that transgene expression is directly proportional to the rate of virus vector injection. Figure 8 summarizes results from a pilot study where identical viral loads were delivered at once or in 3-5 divided daily doses. We administered AAV-GFP via intraperitoneal injection in C57Bl/6 mice on postnatal day 10 (P10) and quantified AAV transduction efficiency by confocal imaging on perfused brain...
slices at 7 or 14 days postinjection (d.p.i.). Using this injection paradigm (Figure 8E), we observed widespread GFP expression in the brain, including the hippocampus and the cerebellum, which are relevant sites of robust SSADH expression. Importantly, our dosing strategies yielded >3-fold differential rates of gene expression in terms of GFP intensity at 7 d.p.i. (gradual = 20.53 ± 2.83 a.u.; moderate = 41.80 ± 7.48 a.u.; rapid = 66.03 ± 3.47 a.u.) but the cumulative GFP intensity at 14 d.p.i. was largely diminished to <1.4-fold across groups (gradual = 87.05 ± 8.47 a.u.; moderate = 107.2 ± 6.86; rapid = 119.7 ± 7.96) (Figure 8F).

AAV-PHP.B Transduces Interneuron Subtypes in the Hippocampus and the Cerebellum

To further characterize the cell identities of transduced cells on AAV-PHP.B intraperitoneal injections, we performed immunostaining on cryopreserved brain sections. Selected antibodies against cellular markers of different interneuron subtypes were used. Notably, we found that at 14 d.p.i., a majority of AAV-transduced GFP-expressing cells (~80%) in the hippocampus (CA1) are calretinin positive (Figure 9). In the cerebellum, however, GFP-expressing cells were vasoactive intestinal polypeptide-positive (~60%) or parvalbumin-positive (~40%).

Discussion

We describe our current efforts in developing enzyme-restoring strategies for SSADHD. From a brain developmental perspective, we consider the role that GABAergic signaling and its plasticity play in successful enzyme replacement therapy. We outline 3 critical parameters of SSADH restoration (i.e., rate, timing, and cell-specificity) that should be empirically established for the clinical readiness of enzyme replacement therapy in SSADHD.

Our work on the aldhsa1lox-rtTA-STOP mouse construction is a necessary first step to establish safety and efficacy parameters for SSADH restoration in clinical practice. In the future, functional SSADH restoration will be further developed to achieve cell type and subcellular precision. Nonetheless, several challenges remain: First, SSADH is a mitochondrial enzyme (Figure 1) with defined cell type expression profiles. A mitochondria-directed, cell-penetrating SSADH delivery strategy might be necessary to ensure functional SSADH restoration and to avoid off-target effects. Second, cell-specific SSADH delivery might be
achieved via characteristic extracellular environment of relevant cell types. Notably, the majority of mature parvalbumin-positive cells are enwrapped by perineuronal nets (PNN) recognizable by specific proteoglycan domains and sulphation patterns; enzymes or viral biomolecules packaging strategies might be designed to accommodate specific extracellular interactions to target relevant cell types. Third, peripheral SSADH restoration might be an alternative realistic treatment option. However, the impacts of peripheral SSADH restoration on patients' brain physiology and long-term effectiveness must be examined in great detail. This might be addressed by the additional use of specific Cre-expressing lines with the aldh5a1lox-rtTA-STOP mouse.

Our proposed mouse model, if fully constructed, will allow reversible SSADH expression via tetracycline (or doxycycline) dosing. We note that the potential leakiness of the tetracycline system is a reasonable technical concern. In our model design, we are relying on previous work, that is, the use of (1) an improved version of the rtTA transcription factor, (2) minimal Tet-responsive promoter elements, and (3) a genomic locus for transgene insertion with low basal transcriptional activities. As a precaution and part of the characterization work of our mouse model, we will measure and correlate baseline SSADH activities with its phenotypes. As control, we will include the total SSADH knock-out mouse (aldh5a1–/–) in our future studies.

We note that the aldh5a1–/– mouse model represents a very severe form of the disorder. Aldh5a1–/– mice exhibit spontaneous seizures around 2 weeks of postnatal age and premature lethality by 3 weeks of age. This is in contrast to the clinical circumstances of SSADHD, where a significant number of patients live through adulthood. Therefore, an improved mouse model that better recapitulates the clinical manifestations and severity of SSADHD is needed, which is in part the

![Figure 9. AAV-PHP.B intraperitoneally (IP) injected at P10 transduced various interneuron cell types in the mouse brain. Representative confocal micrographs of cryopreserved brain sections showing AAV-PHP.B-CAG-GFP transduced cells (top row in green) in the hippocampus and the cerebellum. Immunostaining was performed using various interneuron cellular markers (middle row in red). Arrow heads indicate GFP-expressing cells co-immunostained by respective interneuron cellular markers (bottom row). Selected identified GFP+ cells are shown in high magnification in insets. Scale bar: 50 μm. VIP, vasoactive intestinal polypeptide-expressing interneurons; PV, parvalbumin.](image)
motivation of this project. We believe that our inducible SSADHD mouse model will achieve this goal via the control of SSADHD expression on precise doxycycline dosing. Another advantage of using the Tet-on approach is that SSADH expression is reversible, allowing systematic investigation of risks on long-term decline of restoration efficacy (such as immune tolerance to ongoing enzyme replacement therapy).

Our proposed mouse model might be useful to study another aspect of SSADH, in which patients’ residual enzymatic activity is not recapitulated by the aldh5a1lox-rtTA-STOP model. We note that a relationship between the amount of residual activity and clinical phenotype has not been established. It remains to investigate whether there is a detectable range of enzymatic activity (perhaps corresponding to different variants of aldhl1 mutations) that might associate with the clinical spectrum of SSADH. Importantly, the fact that mutation carriers having half-normal enzymatic activities are asymptomatic suggests that there is a certain key threshold between 0% and 50% of normal SSADH activity permissive for symptomatic emergence. We anticipate that our Tet-on mouse model will enable the establishment of such delicate genotype-phenotype relationships, given that in our model SSADH expression can be dialed up or down precisely in a range controllable via doxycycline dosing.

We also describe preliminary data using an AAV to achieve rate-dependent transgene expression. We used AAV-PHP.B, a recently developed capsid pseudotype with superb brain penetration and neurotropic properties.52,53 We established a systemic injection paradigm in young mice that allows robust brain-targeted expression, which is in agreement with previous AAV-PHP.B characterization using other intravenous injection approaches.52 To our knowledge, our results are the first to indicate rate-dependent brain transgene expression after intraperitoneal virus vector injection paradigm using AAV-PHP.B in developing mice. We further postulated that when this delivery paradigm is performed on the aldhl5a1lox-rtTA-STOP mouse using an AAV-PHP.B expressing Cre-recombinase (i.e., pAAV-CAG-Cre-WPRE-hGH, characterized in our previous studies54), we would be able to systematically study the impacts of different SSADH restoration rates on phenotype reversal.

Our pilot data further suggested that GABAergic neurons in the hippocampus and the cerebellum are targetable using our experimental injection (i.e., intraperitoneal injection at young age) different from those previously reported.52 Given the relevance of predominant SSADH expression in the hippocampus and the cerebellum GABAergic cells, we believe this AAV injection paradigm (when combined with the aldhl5a1lox-rtTA-STOP mouse) will be a powerful genetic tool to model functional enzyme restoration in SSADH.

We recognize that there are limitations regarding the choice of AAV-PHP.B in this study. Notably, AAV-PHP.B has been shown to demonstrate superb BBB penetration and neurotropism limited to only certain mouse strains (eg, C57BL/6 J), because of the selective availability of the lymphocyte antigen complex 6a (ly6a) upon intravenous infusion.55,56 In our proof-of-concept study, our main goal was to demonstrate that we can achieve rate-specific transgene expression via a temporally differential AAV injection paradigm from a young age (e.g., postnatal age day 10) in which systemic intraperitoneal injection is preferred over repeated direct cortical injections (Figure 8E). As above, investigation of transgene expression via this administration paradigm has never been documented. The more commonly used AAV serotypes (e.g., AAV9) are not ideal here because of their rather low transduction efficiency in the central nervous system via systemic injection, which will likely lead to uninterpretable results. In addition, our proposed mouse model is constructed in a C57BL/6 J background (to allow direct phenotypic comparison with the existing aldhl5a1lox-rtTA-STOP mouse model). Therefore, we chose AAV-PHP.B in this proof-of-concept study. Importantly, our experiments successfully demonstrate that in principle, rate-dependent AAV-mediated transgene expression can be achieved via systemic delivery, enabling the systematic investigation of SSADH restoration at different rates using our novel mouse model. This will be a critical first step to develop dosing schedules in relevant SSADH restoration therapies, including gene therapy and enzyme replacement therapy. More broadly, our findings might shed light in formulating similar restoration strategies for other genetic metabolic diseases, where pathologic compensatory changes caused by genetic mutations are often in place.

In terms of developing AAV-PHP.B for human gene therapy, we note that there are concerns including its acute toxicity at high viral dosage and low transduction efficiency as indicated in nonhuman primate studies.53,57 Therefore, AAV-PHP.B might not be an ideal candidate for human clinical trials of SSADH gene therapy. Instead, we propose that at least 3 key parameters must be established before such clinical trials, that is, (1) rate of restoration, (2) age of treatment, and (3) cell targets. We aim to use our novel SSADHD mouse model to generate relevant data that will guide the selection of appropriate viral vector candidates for eventual clinical trials. For example, if the rate of restoration cannot exceed a certain threshold (otherwise causing seizures and brain injury), viral vectors that allow repeated administration of smaller doses with low immunogenicity will be necessary.58,59 If restoration should be targeted to specific cell types, viral vectors that allow the incorporation of cell-specific regulatory promoter elements should be considered.60 Moreover, reports indicate that liver-directed gene transfer might be effective.15 Therefore, provided that toxicity is tolerable in patients,61 peripherally targeted SSADH restoration might be an alternative viable option.

Concluding Remarks

We introduce a novel genetic mouse model of SSADHD, which allows “on-demand” activation of the aldhl5a1 gene to fine-tune SSADH restoration. We anticipate that this novel mouse model allows systematic investigation of SSADH restoration parameters for preclinical readiness of enzyme replacement therapy and gene therapy in SSADHD.
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Author Contributions


Declaration of Conflicting Interests

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