Journal of Child Neurology
1-10

DOI: 10.1177/0883073821993000
journals.sagepub.com/home/jcn
SAGE

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

Henry Hing Cheong Lee, PhD[1], Phillip L. Pearl, MD[2], and Alexander Rotenberg, MD, PhD[1,2]

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. Our work focuses on the construction of a novel SSADHD mouse model that allows "on-demand" SSADH restoration for the systematic investigation of these key parameters. 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

Received November 10, 2020. Received revised January 4, 2021. Accepted for publication January 12, 2021.

Succinic semialdehyde dehydrogenase deficiency (SSADHD) is a rare autosomal recessive metabolic disorder (prevalence: ~200 documented cases worldwide) caused by loss-of-function mutations in the aldehyde dehydrogenase 5 family member A1 (*ALDH5A1*) gene.[1,2] *ALDH5A1* encodes SSADH, which is essential for metabolic conversion of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Figure 1). In the absence of SSADH, GABA and its metabolite γ-hydroxybutyrate (GHB) accumulate to pathologic levels in the brain, resulting in nonprogressive broad-spectrum encephalopathy.[3-6] Paradoxically, despite profound increase in extrasynaptic GABA, patients with SSADHD experience frequent seizures and significant risk of sudden unexpected death in epilepsy (SUDEP) in a hyper-GABAergic state.[7] This likely results from use-dependent compensatory downregulation of $GABA_A$ and (to a certain extent) $GABA_B$ receptors.[8,9] To date, treatment for SSADHD is symptomatic.[10-13] A therapy that addresses the underlying enzyme deficiency in SSADHD is absent.

Proof-of-concept experimental enzyme replacement therapy[14] or liver-directed adenoviral *aldh5a1* gene transfer[15] increases survival of *aldh5a1*$^{-/-}$ mice. This raises realistic prospects for clinical SSADH-restoring therapies. However, the

[1] FM Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA, USA
[2] Department of Neurology, Boston Children's Hospital, Boston, MA, USA

Corresponding Author:
Henry Hing Cheong Lee, MD, FM Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA 02115-5724, USA.
Email: hingcheong.lee@childrens.harvard.edu

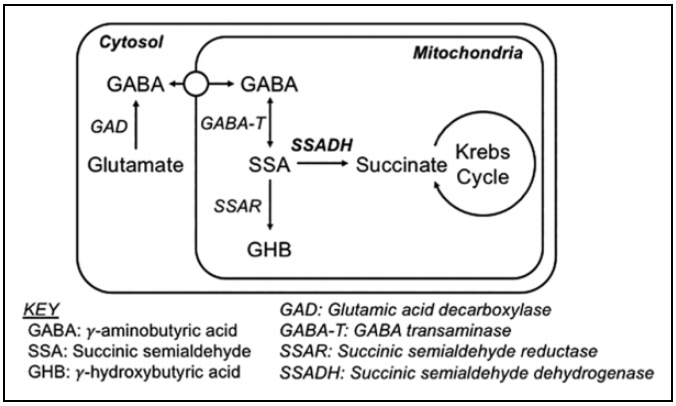

Figure 1. γ-Aminobutyric acid (GABA) metabolic pathway. Cytosolic glutamate is converted by glutamic acid decarboxylase (GAD) to form GABA, which is subsequently translocated into the mitochondria, where GABA is reversibly converted by GABA transaminase (GABA-T) to succinic semialdehyde (SSA). SSA is converted either by SSA reductase (SSAR) to γ-hydroxybutyric acid (GHB), or by SSA dehydrogenase (SSADH) to succinate, which then enters the Krebs cycle. In the absence of SSADH (i.e., SSADHD), GABA and GHB are accumulated to pathologic levels.

profound reductions in GABA catabolism and altered signaling in SSADHD fundamentally impact brain development. Brain plasticity and the status of GABAergic functions might play a key role in determining the outcomes of such SSADH-restoring strategies. Postsynaptic GABAergic responses undergo an early developmental switch from excitation to inhibition mediated by tight regulation of chloride homeostasis.[16-18] In SSADHD, altered chloride homeostasis might lead to depolarizing GABAergic neurotransmission.[14] It is not known how SSADH restoration might impact neuronal chloride transport, but certain plasticity mechanisms might be necessary to avoid sudden reversal of chloride homeostasis and overexcitation. Neuronal activities dynamically modulate $GABA_A$ receptor composition, intracellular trafficking, lateral mobility on neuronal surfaces, and synapse stability.[19,20] SSADH restoration might lead to further reduction of GABA-mediated signaling in a setting of reduced $GABA_A$ receptor availability, resulting in seizures. Adaptive changes (i.e., plasticity) in GABA receptors must be in place to accommodate loss of ambient GABA and loss of inhibitory tone, and avoid provoking seizures (Figure 2). More broadly, GABA circuit maturation triggers critical period plasticity in the cortex.[21-24] Knockdown of critical factors for GABA circuit maturation delays critical period onset across brain regions.[23-25] It is unclear how critical period timing is affected in SSADHD, and whether such plasticity might represent an opportunistic window for successful therapy later in life. This is particularly relevant for adult SSADHD patients, whose critical period might have closed prematurely because of predominant GABA accumulation in early life. Subsequent lack of $GABA_AR$ upregulation on SSADH restoration might render enzyme replacement therapy ineffective. In this situation, adjunctive therapy targeted to reopen critical period plasticity[26] might become a realistic consideration along with enzyme replacement therapy.

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,[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.

Given that testing the full range of preclinical readiness of SSADH-restoring strategies requires sustained and regulated enzyme restoration 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 *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$, 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 $GABA_A$) 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

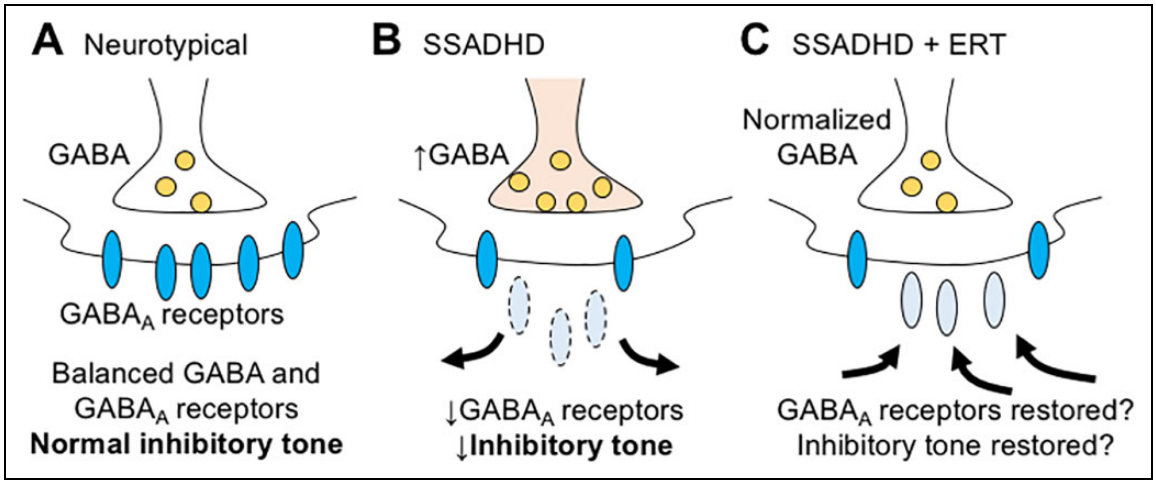

Figure 2. Use-dependent compensatory $GABA_A$ receptor expression underlies seizures in SSADHD and potential enzyme replacement therapy (ERT) response. (A) Under neurotypical situations, balanced levels of GABA and $GABA_A$ receptors result in normal inhibitory tone. (B) In SSADHD, pathologic accumulation of GABA leads to use-dependent reduction of $GABA_A$ receptors. Despite a hyper-GABAergic condition, the overall inhibitory tone is sufficiently impaired, resulting in seizures in SSADHD patients. (C) ERT in SSADHD normalizes (reduces) GABA levels in a setting of reduced $GABA_A$ receptors. Successful ERT outcomes depends on plastic restoration of functional $GABA_A$ receptors and inhibitory tone.

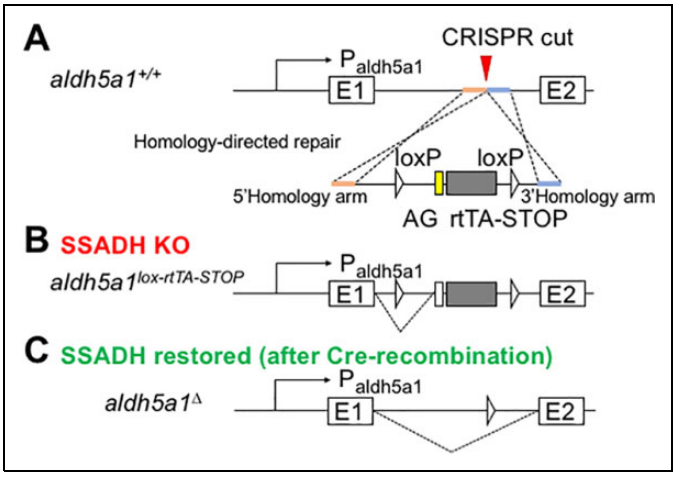

Figure 3. Construction of the *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ mouse. (A) The endogenous *aldh5a1* gene is disrupted by CRISPR/Cas9-mediated homology directed repair in its first intron with the insertion of a gene cassette containing a splice acceptor (AG) and the rtTA-STOP sequence flanked by 2 loxP sites. (B) At baseline, *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ mice are SSADH-null as a result of the disrupted *aldh5a1* gene. Instead, rtTA expression is driven by endogenous *aldh5a1* promoter activities (to combine with a second mouse, *TRE-aldh5a1*, for doxycycline-mediated rescue strategy; see Figure 7 for details). (C) Upon Cre-recombination, *aldh5a1* is reconstituted for re-expression (*aldh5a1*$^{\Delta}$).

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[31,32] (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.[33] 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

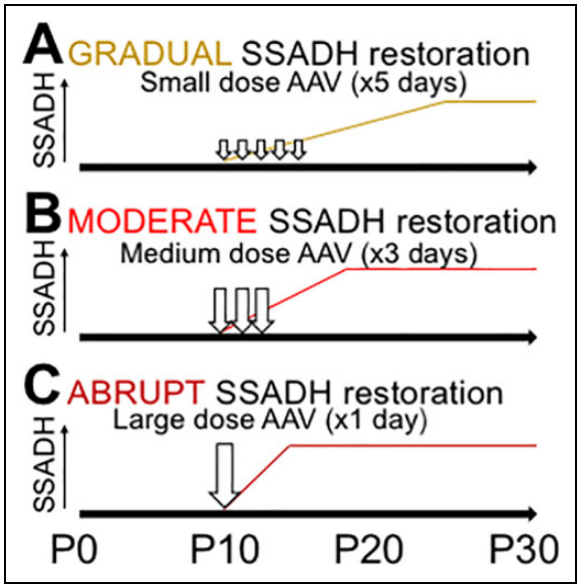

Figure 4. Experimental paradigms studying the impacts of rate of SSADH restoration in mice. Same total amount of AAV will be injected across different time spans (1, 3, or 5 days) to represent (A) gradual, (B) moderate, and (C) abrupt SSADH restoration.

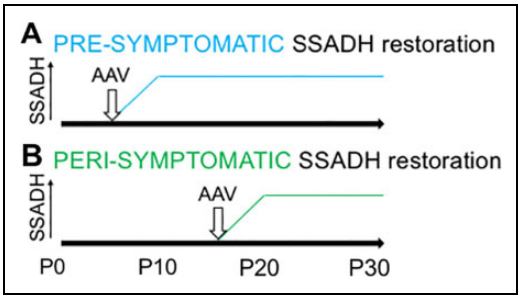

Figure 5. Experimental paradigms studying the impacts of age of SSADH restoration in mice. Same total amount of AAV will be injected at different ages (P5 or P15) to represent (A) presymptomatic and (B) perisymptomatic SSADH restoration.

before sectioning. Free-floating cryosections covering the hippocampus and the cerebellum (sagittal, 30 µm, midline ± 1.2-1.7 mm)[34-36] 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 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 (a.u.).

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, $aldh5a1^{lox\text{-}rtTA\text{-}STOP}$, 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* 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 contrasting timing and dosage to test for therapeutic efficacy. The design of the $aldh5a1^{lox\text{-}rtTA\text{-}STOP}$ 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* mouse[39]), 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 $aldh5a1^{lox\text{-}rtTA\text{-}STOP}$ 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,[43] 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

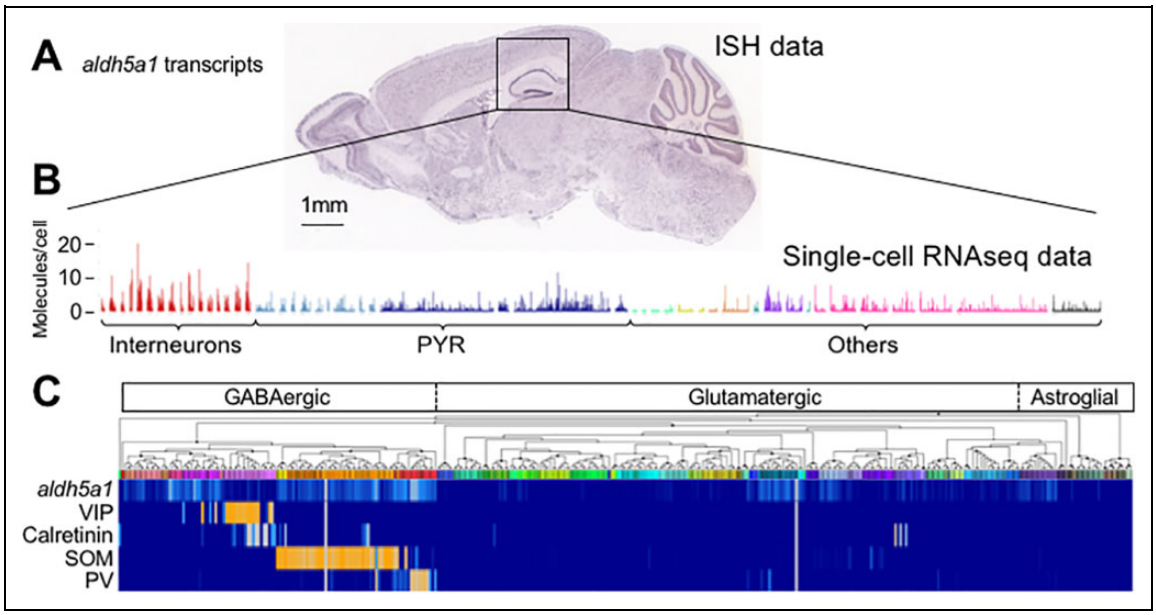

Figure 6. Transcript expressions of SSADH in the mouse brain. (A) In situ hybridization (ISH) data of *aldh5a1* transcripts in adult (P56) C57Bl/6 J mouse brain. Credit: Allen Brain Institute online database (http://mouse.brain-map.org/). Note the brainwide expression of *aldh5a1*, and its enhanced expression in the hippocampus and the cerebellum. (B) Single-cell RNAseq data of *aldh5a1* in mouse cortex and hippocampus. Credit: The Linnarsson lab (http://linnarssonlab.org/cortex/).[32] Cell types are classified as interneurons and pyramidal cells (PYR). (C) Single-cell RNAseq data of *aldh5a1* in the mouse whole cortex and the hippocampus. Credit: Allen Brain Institute online database (https://celltypes.brain-map.org/rnaseq/mouse_ctx-hip_10x). Single-cell RNAseq data in (B) and (C) suggested that *aldh5a1* expression is biased toward GABAergic interneurons. Molecular markers of interneuron subclass are included for comparison. PV, parvalbumin; SOM, somatostatin; VIP, vasoactive intestinal polypeptide.

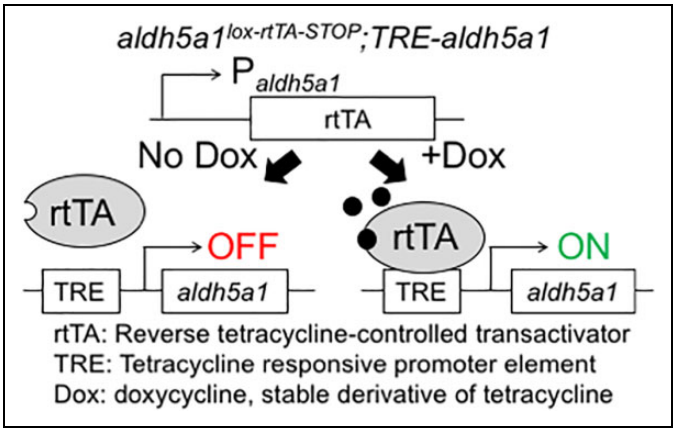

Figure 7. Conceptual design of a reversible SSADH mouse model. Breeding *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ and *TRE-aldh5a1* mice allows reversible expression of recombinant *aldh5a1* in the presence of doxycycline (Dox) tightly driven by a Tet-responsive element (TRE).

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 *aldh5a1* mutations,[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 *aldh5a1*$^{-/-}$ or *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ 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

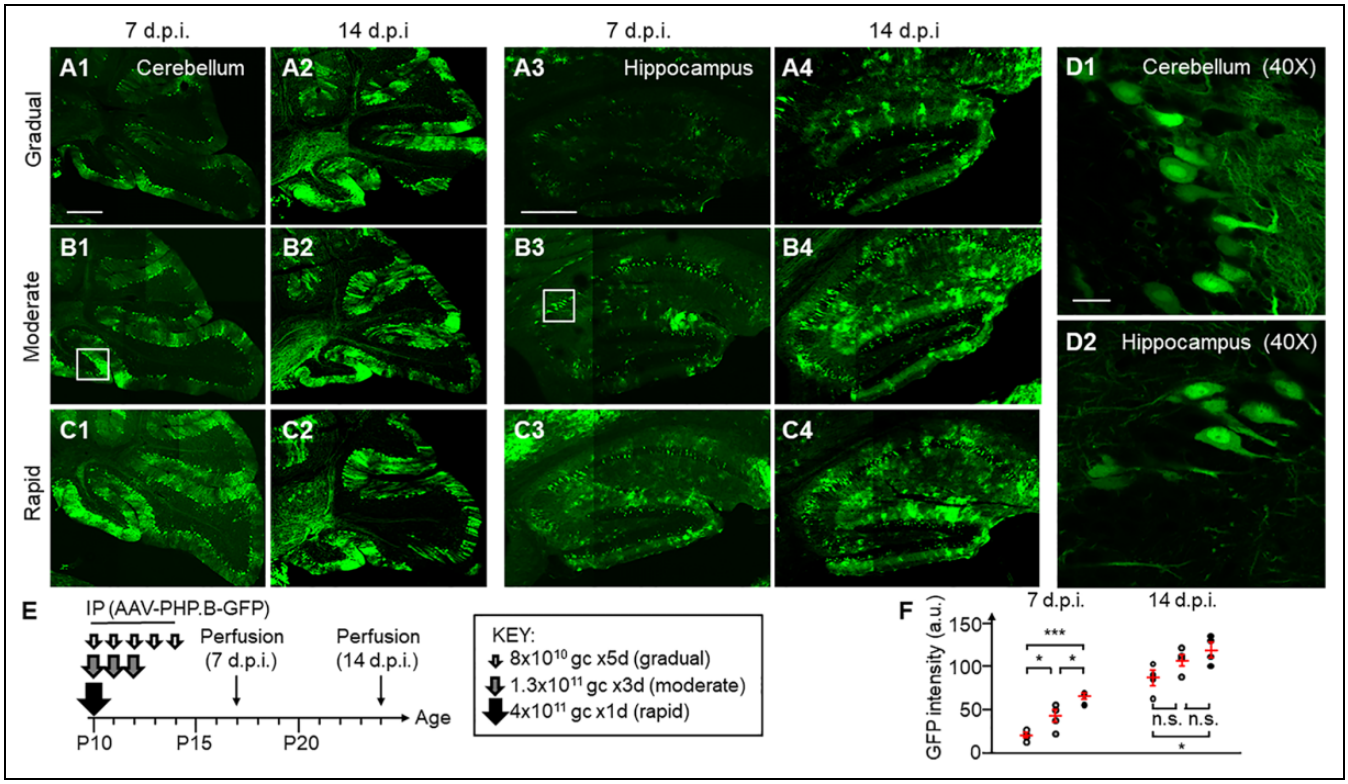

Figure 8. Rate-dependent GFP expression via AAV-PHP.B injected across various time spans. Representative confocal micrographs showing the cerebellum (A1-2, B1-2, and C1-2) and the hippocampus (A3-4, B3-4, and C3-4) at 7 and 14 days postinjection (d.p.i.), in low magnification (10X), using escalating doses of AAV-PHP.B to mimic gradual (A), moderate (B), and rapid (C) transgene expression across various time spans (E). High magnification (40×) of individual neurons in selected brain regions in B are shown in D. Quantification is presented in F. Scale bars: 500 μm (A1, A3), 20 μm (D1). Note rate-dependent expression at 7 d.p.i across these 3 dosing schedules (L to R: gradual, moderate, and rapid), but these changes are largely diminished at 14 d.p.i. N=4 (per dosing schedule and postinjection time point) from 2 independent experiments. Statistical analysis: One-way analysis of variance followed by Bonferroni multiple comparison test. *$P < .05$, ***$P < .001$. n.s., not significant.

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 *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ 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.[31,42] A mitochondria-directed, cell-penetrating SSADH delivery strategy[46] might be necessary to ensure functional SSADH restoration and to avoid off-target effects. Second, cell-specific SSADH delivery might be

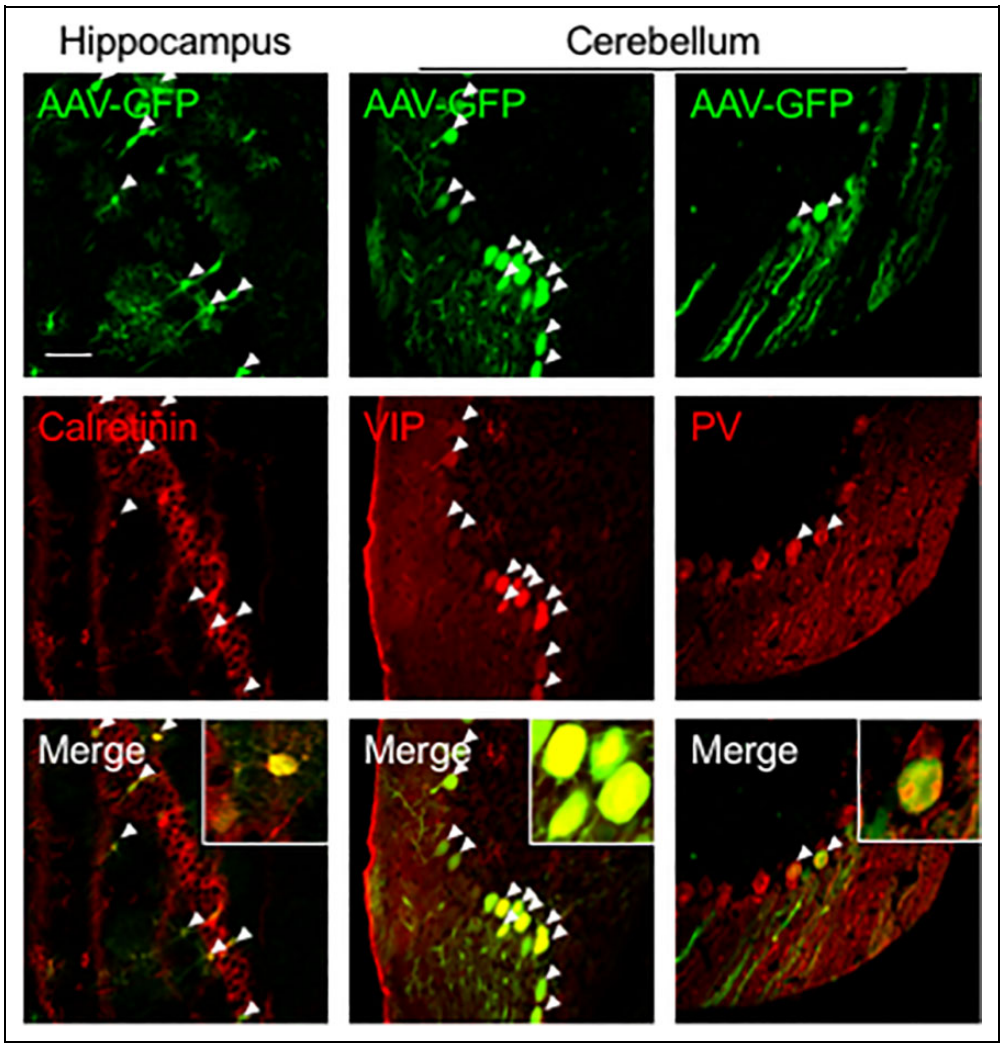

Figure 9. AAV-PHP.B intraperitoneally (IP) injected at P10 transduced various interneuron cell types in the mouse brain. Representative confocal micrographs of cryopreserved brain sections showing AAV-PHP.B-CAG-GFP transduced cells (top row in green) in the hippocampus and the cerebellum. Immunostaining 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.

achieved via characteristic extracellular environment of relevant cell types.[47] Notably, the majority of mature parvalbumin-positive cells are enwrapped by perineuronal nets (PNN) recognizable by specific proteoglycan domains and sulphation patterns[24,25,48]; 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.[14,15] 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 *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ 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,[49] (2) minimal Tet-responsive promoter elements,[50] and (3) a genomic locus for transgene insertion with low basal transcriptional activities.[43] 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.[27] This is in contrast to the clinical circumstances of SSADHD, where a significant number of patients live through adulthood.[51] Therefore, an improved mouse model that better recapitulates the clinical manifestations and severity of SSADHD is needed, which is in part the

motivation of this project. We believe that our inducible SSADHD mouse model will achieve this goal via the control of SSADH 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 SSADHD, in which patients' residual enzymatic activity is not recapitulated by the *aldh5a1*$^{-/-}$ 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 *aldh5a1* mutations) that might associate with the clinical spectrum of SSADHD. 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 approach to achieve rate-dependent transgene expression. We used AAV-PHP.B, a recently developed capsid pseudotype with superb brain penetrance 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 *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ mouse using an AAV-PHP.B expressing Cre-recombinase (i.e., pAAV-CAG-Cre-WPRE-hGH, characterized in our previous studies[54]), 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 *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ mouse) will be a powerful genetic tool to model functional enzyme restoration in SSADHD.

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 *aldh5a1*$^{-/-}$ 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 *aldh5a1* 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.

Acknowledgments

We thank the Intellectual and Development Disabilities Research Center (IDDRC) at Boston Children's Hospital (CHB IDDRC U54HD090255) for research support infrastructures, including the Viral Core (Dr. Yiming Zhang, PI: Dr Zhigang He) for providing AAV constructs, the Animal Behavioral & Physiology Core (directed by A.R.) for viral injection procedure room, and the Mouse Gene Manipulation Core (Dr. Mantu Bhaumik, PI: Dr Clifford Woolf) for embryo microinjection with CRISPR-Cas9 materials for the *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ mouse construction. We also thank MGH DNA core for Next-Generation Sequencing service, Dr Gerald Marsischky for consultation on the *aldh5a1*$^{lox\text{-}rtTA\text{-}STOP}$ CRISPR knock-in strategy, and Dr Mike Gibson for comments on this manuscript. The authors declare no conflict of interest in this study.

Author Contributions

H.H.C.L., P.L.P. and A.R. conceived the central hypothesis and study design of this project. H.H.C.L., P.L.P. and A.R. received funding for this project. H.H.C.L. performed experiments, analyzed data and prepared figures. H.H.C.L., P.L.P. and A.R. discussed and interpreted data. H.H.C.L., P.L.P. and A.R. wrote and revised this paper.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article. This work is supported by a research grant from the SSADH Association (to H.H.C.L, P.L.P. and A.R.).

ORCID iDs

Henry Hing Cheong Lee https://orcid.org/0000-0003-4975-7330
Phillip L. Pearl https://orcid.org/0000-0002-6373-1068

References

1. Gibson KM, Sweetman L, Nyhan WL, et al. Succinic semialdehyde dehydrogenase deficiency: an inborn error of gamma-aminobutyric acid metabolism. *Clin Chim Acta*. 1983;133:33-42.
2. Pearl PL, Parviz M, Vogel K, Schreiber J, Theodore WH, Gibson KM. Inherited disorders of gamma-aminobutyric acid metabolism and advances in ALDH5A1 mutation identification. *Dev Med Child Neurol*. 2015;57:611-617.
3. Jakobs C, Bojasch M, Mönch E, Rating D, Siemes H, Hanefeld F. Urinary excretion of gamma-hydroxybutyric acid in a patient with neurological abnormalities. The probability of a new inborn error of metabolism. *Clin Chim Acta*. 1981;111:169-178.
4. Pearl PL, Wiwattanadittakul N, Roullet JB, Gibson KM. Succinic Semialdehyde Dehydrogenase Deficiency. *GeneReviews((R))*. Initially posted May 5, 2004; updated April 28, 2016. Seattle, WA: University of Washington.
5. Malaspina P, Roullet JB, Pearl PL, Ainslie GR, Vogel KR, Gibson KM. Succinic semialdehyde dehydrogenase deficiency (SSADHD): pathophysiological complexity and multifactorial trait associations in a rare monogenic disorder of GABA metabolism. *Neurochem Int*. 2016;99:72-84.
6. Jansen EE, Vogel KR, Salomons GS, Pearl PL, Roullet JB, Gibson KM. Correlation of blood biomarkers with age informs pathomechanisms in succinic semialdehyde dehydrogenase deficiency (SSADHD), a disorder of GABA metabolism. *J Inherit Metab Dis*. 2016;39:795-800.
7. Pearl PL, Shukla L, Theodore WH, Jakobs C, Gibson KM. Epilepsy in succinic semialdehyde dehydrogenase deficiency, a disorder of GABA metabolism. *Brain Dev*. 2011;33:796-805.
8. Pearl PL, Gibson KM, Quezado Z, et al. Decreased GABA-A binding on FMZ-PET in succinic semialdehyde dehydrogenase deficiency. *Neurology*. 2009;73:423-429.
9. Parviz M, Vogel K, Gibson KM, Pearl PL. Disorders of GABA metabolism: SSADH and GABA-transaminase deficiencies. *J Pediatr Epilepsy*. 2014;3:217-227.
10. Pearl PL, Schreiber J, Theodore WH, et al. Taurine trial in succinic semialdehyde dehydrogenase deficiency and elevated CNS GABA. *Neurology*. 2014;82:940-944.
11. Pearl PL. Succinic semialdehyde dehydrogenase deficiency: lessons from mice and men. *J Inherit Metab Dis*. 2009;32:343-352.
12. Vogel KR, Pearl PL, Theodore WH, McCarter RC, Jakobs C, Gibson KM. Thirty years beyond discovery—clinical trials in succinic semialdehyde dehydrogenase deficiency, a disorder of GABA metabolism. *J Inherit Metab Dis*. 2013;36:401-410.
13. Gropman A. Vigabatrin and newer interventions in succinic semialdehyde dehydrogenase deficiency. *Ann Neurol*. 2003;54(suppl 6):S66-S72.
14. Vogel KR, Ainslie GR, Walters DC, et al. Succinic semialdehyde dehydrogenase deficiency, a disorder of GABA metabolism: an update on pharmacological and enzyme-replacement therapeutic strategies. *J Inherit Metab Dis*. 2018;41:699-708.
15. Gupta M, Jansen EEW, Senephansiri H, et al. Liver-directed adenoviral gene transfer in murine succinate semialdehyde dehydrogenase deficiency. *Mol Ther*. 2004;9:527-539.
16. Ganguly K, Schinder AF, Wong ST, Poo M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell*. 2001;105:521-532.
17. Rivera C, Voipio J, Payne JA, et al. The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature*. 1999;397(6716):251-255.
18. Lee HH, Deeb TZ, Walker JA, Davies PA, Moss SJ. NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents. *Nat Neurosci*. 2011;14:736-743.
19. Jacob TC, Moss SJ, Jurd R. GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci*. 2008;9:331-343.
20. Luscher B, Fuchs T, Kilpatrick CL. GABAA receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron*. 2011;70:385-409.
21. Hensch TK. Critical period plasticity in local cortical circuits. *Nat Rev Neurosci*. 2005;6:877-888.
22. Reh RK, Dias BG, Nelson CA 3rd , et al. Critical period regulation across multiple timescales. *Proc Natl Acad Sci U S A*. 2020;117:23242-23251.
23. Huang ZJ, Kirkwood A, Pizzorusso T, et al. BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell*. 1999;98(6):739-755.

24. Lee HHC, Bernard C, Ye Z, et al. Genetic Otx2 mis-localization delays critical period plasticity across brain regions. *Mol Psychiatry*. 2017;22:680-688.
25. Miyata S, Komatsu Y, Yoshimura Y, Taya C, Kitagawa H. Persistent cortical plasticity by upregulation of chondroitin 6-sulfation. *Nat Neurosci*. 2012;15:414-422, S411-S412.
26. Gervain J, Vines BW, Chen LM, et al. Valproate reopens critical-period learning of absolute pitch. *Front Syst Neurosci*. 2013;7:102.
27. Hogema BM, Gupta M, Senephansiri H, et al. Pharmacologic rescue of lethal seizures in mice deficient in succinate semialdehyde dehydrogenase. *Nat Genet*. 2001;29:212-216.
28. Desai AK, Li C, Rosenberg AS, Kishnani PS. Immunological challenges and approaches to immunomodulation in Pompe disease: a literature review. *Ann Transl Med*. 2019;7:285.
29. Shirley JL, de Jong YP, Terhorst C, Herzog RW. Immune responses to viral gene therapy vectors. *Mol Ther*. 2020;28:709-722.
30. Le Magueresse C, Monyer H. GABAergic interneurons shape the functional maturation of the cortex. *Neuron*. 2013;77:388-405.
31. Blasi P, Boyl PP, Ledda M, et al. Structure of human succinic semialdehyde dehydrogenase gene: identification of promoter region and alternatively processed isoforms. *Mol Genet Metab*. 2002;76:348-362.
32. Zeisel A, Muñoz-Manchado AB, Codeluppi S, et al. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science*. 2015;347:1138-1142.
33. Hsieh TH, Lee HHC, Hameed MQ, Pascual-Leone A, Hensch TK, Rotenberg A. Trajectory of parvalbumin cell impairment and loss of cortical inhibition in traumatic brain injury. *Cereb Cortex*. 2017;27:5509-5524.
34. Paxinos G, Franklin KBJ. *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates*. 4th ed. Amsterdam: Elsevier/AP; 2013.
35. Devito LM, Kanter BR, Eichenbaum H. The hippocampus contributes to memory expression during transitive inference in mice. *Hippocampus*. 2010;20:208-217.
36. Kaemmerer WF, Reddy RG, Warlick CA, Hartung SD, Mclvor RS, Low WC. In vivo transduction of cerebellar Purkinje cells using adeno-associated virus vectors. *Mol Ther*. 2000;2:446-457.
37. Quadros RM, Miura H, Harms DW, et al. Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. *Genome Biol*. 2017;18:92.
38. Coolidge CJ, Seely RJ, Patton JG. Functional analysis of the polypyrimidine tract in pre-mRNA splicing. *Nucleic Acids Res*. 1997;25:888-896.
39. Taniguchi H, He M, Wu P, et al. A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron*. 2011;71:995-1013.
40. Concolino D, Deodato F, Parini R. Enzyme replacement therapy: efficacy and limitations. *Ital J Pediatr*. 2018;44:120.
41. Toscano MG, Romero Z, Muñoz P, Cobo M, Benabdellah K, Martin F. Physiological and tissue-specific vectors for treatment of inherited diseases. *Gene Ther*. 2011;18:117-127.
42. Didiasova M, Banning A, Brennenstuhl H, et al. Succinic semialdehyde dehydrogenase deficiency: an update. *Cells*. 2020;9:477.
43. Zeng H, Horie K, Madisen L, et al. An inducible and reversible mouse genetic rescue system. *PLoS Genet*. 2008;4:e1000069.
44. Akaboshi S, Hogema BM, Novelletto A, et al. Mutational spectrum of the succinate semialdehyde dehydrogenase (ALDH5A1) gene and functional analysis of 27 novel disease-causing mutations in patients with SSADH deficiency. *Hum Mutat*. 2003;22:442-450.
45. Akiyama T, Osaka H, Shimbo H, et al. SSADH deficiency possibly associated with enzyme activity-reducing SNPs. *Brain Dev*. 2016;38:871-874.
46. Collard R, Majtan T, Park I, Kraus JP. Import of TAT-conjugated propionyl coenzyme A carboxylase using models of propionic acidemia. *Mol Cell Biol*. 2018;38:e00491-17.
47. Lam D, Enright HA, Cadena J, et al. Tissue-specific extracellular matrix accelerates the formation of neural networks and communities in a neuron-glia co-culture on a multi-electrode array. *Sci Rep*. 2019;9:4159.
48. Beurdeley M, Spatazza J, Lee HHC, et al. Otx2 binding to perineuronal nets persistently regulates plasticity in the mature visual cortex. *J Neurosci*. 2012;32:9429-9437.
49. Faedo A, Laporta A, Segnali A, et al. Differentiation of human telencephalic progenitor cells into MSNs by inducible expression of Gsx2 and Ebf1. *Proc Natl Acad Sci U S A*. 2017; 114:E1234-E1242.
50. Loew R, Heinz N, Hampf M, Bujard H, Gossen M. Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol*. 2010;10:81.
51. Lapalme-Remis S, Lewis EC, Meulemeester CD, et al. Natural history of succinic semialdehyde dehydrogenase deficiency through adulthood. *Neurology*. 2015;85:861-865.
52. Deverman BE, Pravdo PL, Simpson BP, et al. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat Biotechnol*. 2016;34:204-209.
53. Hordeaux J, Wang Q, Katz N, Buza EL, Bell P, Wilson JM. The neurotropic properties of AAV-PHP.B are limited to C57BL/6J mice. *Mol Ther*. 2018;26:664-668.
54. Bei F, Lee HHC, Liu X, et al. Restoration of visual function by enhancing conduction in regenerated axons. *Cell*. 2016;164: 219-232.
55. Hordeaux J, Yuan Y, Clark PM, et al. The GPI-linked protein LY6A drives AAV-PHP.B transport across the blood-brain barrier. *Mol Ther*. 2019;27:912-921.
56. Huang Q, Chan KY, Tobey IG, et al. Delivering genes across the blood-brain barrier: LY6A, a novel cellular receptor for AAV-PHP.B capsids. *PLoS One*. 2019;14:e0225206.
57. Matsuzaki Y, Konno A, Mochizuki R, et al. Intravenous administration of the adeno-associated virus-PHP.B capsid fails to upregulate transduction efficiency in the marmoset brain. *Neurosci Lett*. 2018;665:182-188.
58. Naso MF, Tomkowicz B, Perry WL 3rd, Strohl WR. Adeno-associated virus (AAV) as a vector for gene therapy. *BioDrugs*. 2017;31:317-334.
59. Lundstrom K. Viral vectors in gene therapy. *Diseases*. 2018;6.
60. Liu Y, Hegarty S, Winter C, Wang F, He Z. Viral vectors for neuronal cell type-specific visualization and manipulations. *Curr Opin Neurobiol*. 2020;63:67-76.
61. High-dose AAV gene therapy deaths. *Nat Biotechnol*. 2020; 38:910.