



# Succinic semialdehyde dehydrogenase deficiency, a disorder of GABA metabolism: an update on pharmacological and enzyme-replacement therapeutic strategies

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## Abstract

We present an update to the status of research on succinic semialdehyde dehydrogenase (SSADH) deficiency (SSADHD), a rare disorder of GABA metabolism. This is an unusual disorder featuring the accumulation of both GABA and its neuromodulatory analog, gamma-hydroxybutyric acid (GHB), and recent studies have advanced the potential clinical application of NCS-382, a putative GHB receptor antagonist. Animal studies have provided proof-of-concept that enzyme replacement therapy could represent a long-term therapeutic option. The characterization of neuronal stem cells (NSCs) derived from *aldehyde dehydrogenase 5a1*<sup>-/-</sup> (*aldh5a1*<sup>-/-</sup>) mice, the murine model of SSADHD, has highlighted NSC utility as an in vitro system in which to study therapeutics and associated toxicological properties. Gene expression analyses have revealed that transcripts encoding GABA<sub>A</sub> receptors are down-regulated and may remain largely immature in *aldh5a1*<sup>-/-</sup> brain, characterized by excitatory as opposed to inhibitory outputs, the latter being the expected action in the mature central nervous system. This indicates that agents altering chloride channel activity may be therapeutically relevant in SSADHD. The most recent therapeutic prospects include mTOR (mechanistic target of rapamycin) inhibitors, drugs that have received attention with the elucidation of the effects of elevated GABA on autophagy. The outlook for novel therapeutic trials in SSADHD continues to improve.

**Keywords** GABA · Succinic semialdehyde dehydrogenase deficiency · Gamma-hydroxybutyric aciduria · mTOR · Torin 2 · Enzyme replacement therapy

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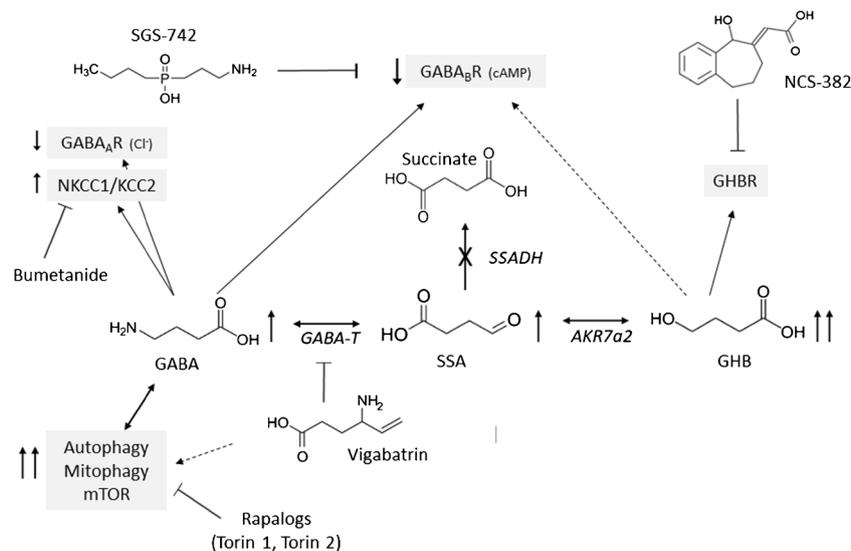
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## Introduction

Progress in understanding the pathophysiology and clinical features of succinic semialdehyde dehydrogenase deficiency (SSADHD) has continued since its description in the 1980s (Jakobs et al 1981; Gibson et al 1983). A major advance came in 1999 with the development of a murine knockout model, so-called aldehyde dehydrogenase 5a1 (*aldh5a1*<sup>-/-</sup>) mice (Hogema et al 2001). While the phenotype of this animal model is severe, with early lethality at 3 weeks of life, it has provided insights into GABAergic, glutamatergic, GHBergic, metabolic, oxidative stress, and other parameters otherwise unavailable (Gupta et al 2002; Cortez et al 2004; Buzzi et al 2006; Wu et al 2006; Jansen et al 2008; Pearl et al 2009; Vogel et al 2015, 2016, 2017a, b, c, d, e, f). Thus far, therapeutics that rescue this model from premature lethality include GABA<sub>B</sub> and GHB

receptor antagonists, the non-physiological amino acid taurine, the antiepileptic agent vigabatrin, the ketogenic diet, and rapalog agents such as Torin 2, the latter an mTOR inhibitor. SGS742, a GABA<sub>B</sub>R antagonist, is the subject of an ongoing clinical trial ([www.clinicaltrials.gov](http://www.clinicaltrials.gov); NCT02019667).

GABA, the major central inhibitory neurotransmitter (Schousboe and Waagepetersen 2007) and its related structural analog, gamma-hydroxybutyric acid (GHB), accumulate to supraphysiological levels in SSADHD (Malaspina et al 2016) (Fig. 1). The degree to which each contribute to pathophysiology remains unknown. However, emerging new roles for GABA exist beyond that of inhibitory neurotransmitter, including neuro-endocrine effects along the gut-brain axis, autophagy, circadian rhythms, and others (Kilb 2012; Lakhani et al 2014; Chellappa et al 2016; Mittal et al 2017). These roles provide novel opportunities to explore pathomechanisms in SSADHD. Here, we highlight new directions in SSADHD research over the last several years, and introduce pilot data supporting novel directions for research and preclinical drug development.



**Fig. 1 GABA metabolism and intracellular interactions.** The site of the defect in patients with SSADHD is indicated by “X”. Abbreviations: GABA,  $\gamma$ -aminobutyric acid; GABA<sub>A</sub>R, ionotropic GABA<sub>A</sub> receptors; GABA<sub>B</sub>R, metabotropic GABA<sub>B</sub> receptors; GABA-T, GABA-transaminase; SSA, succinic semialdehyde; AKR7a2, aldo-keto reductase 7a2; GHB,  $\gamma$ -hydroxybutyric acid; cAMP, cyclic AMP; NKCC1, sodium potassium chloride cotransporter 1; KCC2, neuronal potassium chloride cotransporter 2. In SSADHD, GABA, SSA, and GHB accumulate ( $\uparrow$ ). Increased GABA and GHB activate GABA<sub>A</sub> and GABA<sub>B</sub> receptors and a putative GHB receptor (molecular nature unknown). However, a compensatory downregulation of GABA and GHB receptors ( $\downarrow$ ) has been reported in SSADHD suggesting excess GABA does not lead to increased inhibitory neurotransmission in vivo. NKCC1 and KCC2 control transmembrane chloride gradient and determine GABA<sub>A</sub> receptor

## NCS-382, a potential novel therapeutic for SSADHD

### Pharmacological and structural considerations

NCS-382 is a putative GHB receptor (GHB<sub>R</sub>) antagonist with a  $K_i$  14 times lower than that of GHB (Fig. 1; Maitre 1997; Vogensen et al 2013), and may be the only known antagonist of GHB<sub>R</sub>s (Bay et al 2014), whose molecular structure(s) remain undefined. NCS-382 was effective in rescuing *aldh5a1*<sup>-/-</sup> mice from premature lethality and in blocking the motor deficits induced by the GHB prodrug, gamma-butyrolactone (GBL; Ainslie et al 2016; Gupta et al 2002). NCS-382 exists as a racemic mixture (*hydroxyl*- carbon; Fig. 1). The *R*-isomer is twice as potent as the racemic mixture, and 13-fold more potent than the *S*- enantiomer (Castelli et al 2004). Prior to our initial studies described below, preclinical pharmacokinetic/toxicological analyses of NCS-382, mandatory considerations prior to clinical intervention, had not been reported.

directional transmembrane chloride flux. In experimental SSADHD, the expression ratio of NKCC1 and KCC2 (NKCC1/KCC2) is elevated, suggesting that activation of GABA<sub>A</sub> receptors causes chloride efflux from brain cells, membrane depolarization and activation of neurotransmission (see Fig. 3 for further details). NKCC1 inhibitors like bumetanide lower intracellular chloride concentration. In SSADHD, bumetanide may thus restore GABA inhibitory neurotransmission activity and efficiently suppress seizures. Finally, in SSADHD as well as in vigabatrin-treated animals, increased GABA activates the mTOR pathway with secondarily increased mitochondria number, oxidative stress, autophagy, and mitophagy. mTOR inhibitors, such as torin 1 and torin 2, improve GABA-induced, mTOR-pathway mediated intracellular defects and significantly prolong the lifespan of *aldh5a1*-deficient mice

## Pharmacokinetics and toxicology of NCS-382 and potential for drug-drug interactions

Limited earlier studies on the pharmacodynamic characteristics of NCS-382 were performed in baboon and pigeon, and were designed to exploit antagonist specificity for the GHR in order to interrogate the central effects of GHB (Quang et al 2002; Castelli et al 2003, 2004). We obtained detailed pharmacokinetic measures following i.p. administration of NCS-382 (100 to 500 mg/kg) in C57/B6 mice (Ainslie et al 2016). The plasma elimination  $t_{1/2}$  for NCS-382 ranged from 0.25–0.68 h in a dose-dependent fashion. Brain residence for NCS-382 was longer, with  $t_{1/2}$  ranging from 0.76–0.97 h, and decreasing with increasing dose. The brain-to-plasma ratio based on area under the concentration-time curves (AUCs) ranged from 0.72–1.8. The trend for decreased brain  $t_{1/2}$  with increasing dose may reflect saturation of central GHB binding sites, leaving more unbound NCS-382 for return to the systemic circulation. The fraction of total NCS-382 dose recovered in the urine was low (<4%), and undetectable in the feces (<150 ng/mg feces), suggesting metabolism as the primary route of elimination featuring glucuronidation (major product) and dehydrogenation (minor product) at the *hydroxyl*- moiety (Fig. 1). The intrinsic clearance of NCS-382 ( $Cl_{int}$ ) in the presence of NADPH and assessed by monitoring parent disappearance was 0.587 and 0.513 mL/min/mg protein in mouse and human liver microsomes (MLMs, HLMs), respectively. Calculated murine and human hepatic clearances were 5.2 and 1.2 L/h/kg body weight, respectively. The Michaelis constant ( $K_m$ ) for dehydrogenation was  $29.5 \pm 10$  and  $12.7 \pm 4.9$   $\mu$ M in mouse and human, respectively. Glucuronide formation was linear in both species up to 100  $\mu$ M. UGT2B7 (uridine 5'-diphosphoglucuronosyltransferase; UDP-glucuronosyltransferase 2B7) was suspected as the primary isoform of glucuronidating enzymes responsible for NCS-382 metabolism, based upon competition studies with the UGT2B7 inhibitor, diclofenac (Ainslie et al 2016). Co-administration of diclofenac (25 mg/kg) improved the efficacy of NCS-382 (300 mg/kg) to block the sedative and motor effects in animals treated with GBL. Plasma levels of glucuronides of NCS-382 and diclofenac decreased in combinatorial treatment relative to mice receiving either agent alone.

The identity of drug metabolizing enzymes active in the biotransformation(s) of NCS-382 also has not been reported, nor has the ability of NCS-382 to inhibit the typical enzymes, namely cytochrome P450s (CYP), involved in the metabolism of drugs. Accordingly, we employed HLMs and FDA-recommended probe substrates in reactions catalyzed by seven CYP isoforms (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) (Vogel et al 2017b). NCS-382 did not inhibit any of the tested enzymes at the highest tested dose (30  $\mu$ M). Further,

NCS-382 manifested minimal capacity to induce nuclear receptors involved in drug biotransformation and transport (aryl hydrocarbon, constitutive androstane, and pregnane X receptors) at supraphysiological doses (up to 500  $\mu$ M). Collectively, these findings indicate a low-risk for CYP P450-mediated drug-drug interactions.

HepG2 cells were subsequently used to examine the cellular toxicity of NCS-382 at up to 1 mM. Multiple biomarkers assessing cellular integrity, survival, and organelle function revealed little evidence for NCS-382 cytotoxicity (Vogel et al 2017b). Gene expression studies using NCS-382 in HepG2 cells revealed only a minor number of genes (of 370 tested) showing dysregulation (Table 1). Additionally, high-dose NCS-382 demonstrated only minimal pharmacotoxicity in neural stem cells (NSCs, or neuronal progenitor cells) derived from *aldh5a1*<sup>-/-</sup> mice (e.g., *aldh5a1*<sup>-/-</sup> NSCs) (Vogel et al 2017e). These cells were developed as an in vitro model of SSADHD, showing increased GHB content in culture medium, enhanced biomarkers of oxidative stress and increased mitochondrial number and highlighting the utility of NSCs as a useful preclinical screening tool for evaluating therapeutics for SSADHD (Vogel et al 2017f). In sum, although a number of additional studies will be needed, pilot pharmacokinetic/safety/toxicological evaluations support the potential for clinical application of NCS-382 in SSADHD.

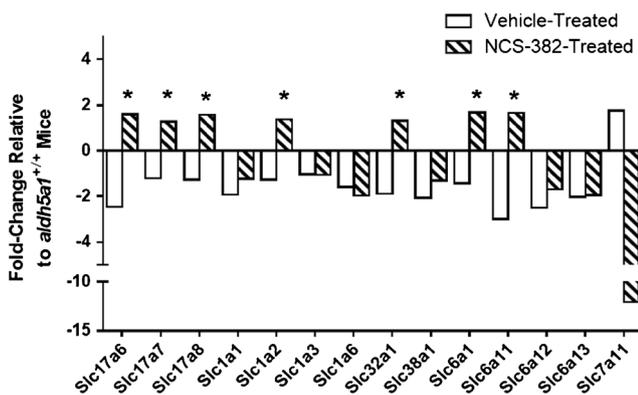
### Preclinical efficacy of NCS-382 in *aldh5a1*<sup>-/-</sup> mice

We subsequently turned attention to the transport of NCS-382 in vitro. Our objective here was to investigate the potential of NCS-382 to block uptake of GHB. We demonstrated for the first time using Madin-Darby canine kidney (MDCK) cells that NCS-382 is actively transported and capable of inhibiting GHB transport (Vogel et al 2017e). Following these in vitro assays with in vivo studies in *aldh5a1*<sup>-/-</sup> mice, we found the ratio of brain/liver GHB to be unaffected by chronic NCS-382 administration (300 mg/kg; 7 consecutive days), which appeared paradoxical. This finding suggests that potential future application of NCS-382 may only be modestly beneficial since brain GHB levels do not appear to be modified with chronic treatment. We examined cortical regions from the NCS-treated mice and evaluated the expression of a number of solute carriers involved in neurotransmitter transport. As shown in Fig. 2, we found essentially all of these transporters down-regulated in *aldh5a1*<sup>-/-</sup> cortex in the absence of treatment. NCS-382 normalized the aberrant expression of seven of these carriers, including both glutamate and GABA transporters, but had no effect on six and actually induced significant down-regulation of the glutamate-cystine antiporter. This finding is of interest

**Table 1** Genes altered >4-fold by NCS-382 (0.5 mM) in HepG2 cells

Gene	Gene product	Function	Gene grouping	Change
<i>CD36</i>	CD36 molecule	Involved in platelet activation, signaling and aggregation and metabolism	Steatosis	↓
<i>HTRA4</i>	High-temperature requirement factor A4	Degrades misfolded secretory proteins	ER Stress & unfolded protein response	↑
<i>SERPINA3</i>	Serpin family A member 3	plasma protease inhibitor; deficiency has been associated with liver disease	Phospholipidosis	↓
<i>SLC2A3</i>	Glucose transporter type 3, brain	Facilitative glucose transporter	Phospholipidosis	↓
<i>SLC51A</i>	OST-alpha	Transports the major species of bile acids	Cholestasis	↓
<i>TNFRSF1A</i>	Tumor necrosis factor receptor superfamily, member 1A	Activates NF-κB, mediates apoptosis, and functions as a regulator of inflammation	Apoptosis & necrosis	↑

in view of the significant depletion of glutathione in this animal model, the fact that glutathione is composed of glutamate, cysteine and glycine, and the earlier finding that glutamate/glutamine levels are abnormal in *aldh5a1*<sup>-/-</sup> brain (Gupta et al. 2004; Chowdhury et al. 2007). These results provide modest preclinical support for the use of NCS-382 in SSADHD. Additional in vivo studies are in progress in *aldh5a1*<sup>-/-</sup> mice using NCS-382, assessing lifespan, body weight, and neurobehavioral outcomes, and using both chronic and acute administration paradigms.



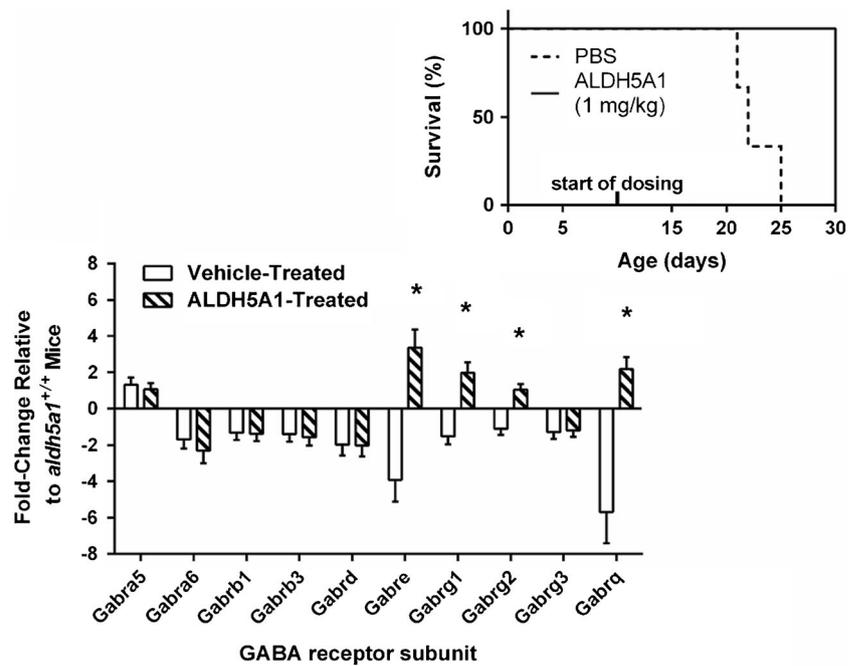
**Fig. 2** Cortical gene expression profile of solute carriers (Slc) in *aldh5a1*<sup>-/-</sup> mice following NCS-382 administration (7 days, q.i.d., 300 mg/kg). Relative levels are displayed, normalized to control *aldh5a1*<sup>-/-</sup> mice receiving vehicle. Functional role of carriers: \*17a6, \*17a7, \*17a8, vesicular glutamate (glu) transporter, glu cotransporter, and glu transporter 3, respectively; 1a1, \*1a2, 1a3, 1a4, excitatory amino acid transporters 3, 2, 1, and 4, respectively; \*32a1, GABA vesicular transporter; 38a1, Na<sup>+</sup>-coupled amino acid transporter 1 (glutamine transport); \*6a1, plasma membrane GABA transporter; \*6a11, 6a12, 6a13, Na<sup>+</sup>-dependent GABA presynaptic terminal reuptake, Na<sup>+</sup>/Cl<sup>-</sup>-dependent betaine, and Na<sup>+</sup>/Cl<sup>-</sup>-dependent GABA 2 transporters, respectively; 7a11, glutamate-cysteine antiporter. Asterisked genes demonstrated correction of expression following chronic NCS-382 administration. Values represent pooled data of biological triplicates (n = 3 animals each, NCS-382 and vehicle)

## Enzyme replacement therapy for SSADHD

As a therapeutic approach, enzyme replacement therapy (ERT) has gained prominence in the lysosomal storage disorders, although it should be feasible in organic acidemias (Darvish-Damavandi et al 2016). We examined the feasibility of ERT in *aldh5a1*<sup>-/-</sup> mice using a GST(glutathione)-tagged human *ALDH5A1* gene construct that overexpresses the GST-hSSADH fusion protein with accompanying ampicillin resistance in *E. Coli* (DNASU Plasmid Repository; Narayanan et al 2004). Crude extracts of *E. coli* were harvested that had been transfected and grown in standard LB broth supplemented with ampicillin at 37 °C overnight, pelleted and lysed with Pefabloc (protease inhibitor; Sigma-Aldrich, St. Louis, MO USA) and lysozyme. GST-hSSADH resident in crude lysates of *E. coli* was purified using Pierce™ GST spin purification kits, followed by dialysis and subsequent concentration using polyethersulfone (PES) columns. Resultant protein content was quantified using a standard BCA protein assay. The GST-tagged enzyme activity was cleaved with thrombin and the activity of *ALDH5A1* determined employing spectrofluorometry based on the NAD/NADH couple (Gibson et al 1991).

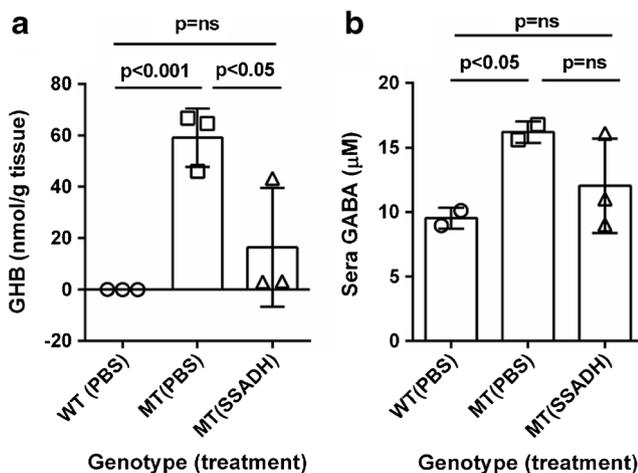
The feasibility of ERT using bacterially-produced *ALDH5A1* was subsequently evaluated in *aldh5a1*<sup>-/-</sup> mice. As endpoint, we employed rescue of this model from premature lethality (day of life (DOL) 21–23; endpoint, DOL 30, which is highly significant survival), the latter endpoint chosen in view of the limited availability of treatment protein. Purified *ALDH5A1* was administered (i.p., 1 mg/kg in PBS, q.d.) beginning at day of life 10. Median survival of vehicle-treated *aldh5a1*<sup>-/-</sup> mice was 22 days compared to 80% survival rate (4 of 5 mice) to 30 days with ERT treatment (Logrank; *p* = 0.04; Fig. 3 (inset)). Brain, liver, and sera were collected from surviving ERT-treated subjects harvested at DOL 30. The expression of GABA receptor genes was contrasted between ERT (DOL 30) and untreated (DOL 21)

**Fig. 3 Enzyme replacement therapy (ERT) in experimental SSADHD. (insert) *Aldh5a1*<sup>-/-</sup> mouse survival-rate to day of life (DOL 30) as a function of enzyme replacement intervention.** Purified human ALDH5A1 was administered daily (1 mg/kg/day), beginning at DOL 10, via i.p. injection. Also shown is the expression of GABA-related genes following ERT in *aldh5a1*<sup>-/-</sup> mice (fold change relative to *aldh5a1*<sup>+/+</sup> mice; sagittal slices of 21 day old mice). Abbreviations: *Gabra5*, GABA<sub>A</sub> receptor subunit α5; *Gabra6*, α-6; *Gabrb1*, β-1; *Gabrb3*, β-3; *Gabrd*, δ; *Gabre*, ε; *Gabrg1*, γ-1; *Gabrg2*, γ-2; *Gabrg3*, γ-3; *Gabrq*, θ. Asterisked values represent directional correction of expression as a function of ERT



*aldh5a1*<sup>-/-</sup> mice, with data normalization to DOL 21 *aldh5a1*<sup>+/+</sup> mice. The expression of several GABA<sub>A</sub> receptor subunits (primarily gamma, epsilon, and theta) were significantly corrected in *aldh5a1*<sup>-/-</sup> mice with enzyme intervention (Fig. 3). Since we hypothesized that parenterally administered SSADH would lower metabolites (GHB, GABA) in blood and other tissues, these intermediates were quantified (Fig. 4) in sham and enzyme-treated subjects using LC/MS-MS (Gibson et al 1990; Kok et al 1993). Although the numbers

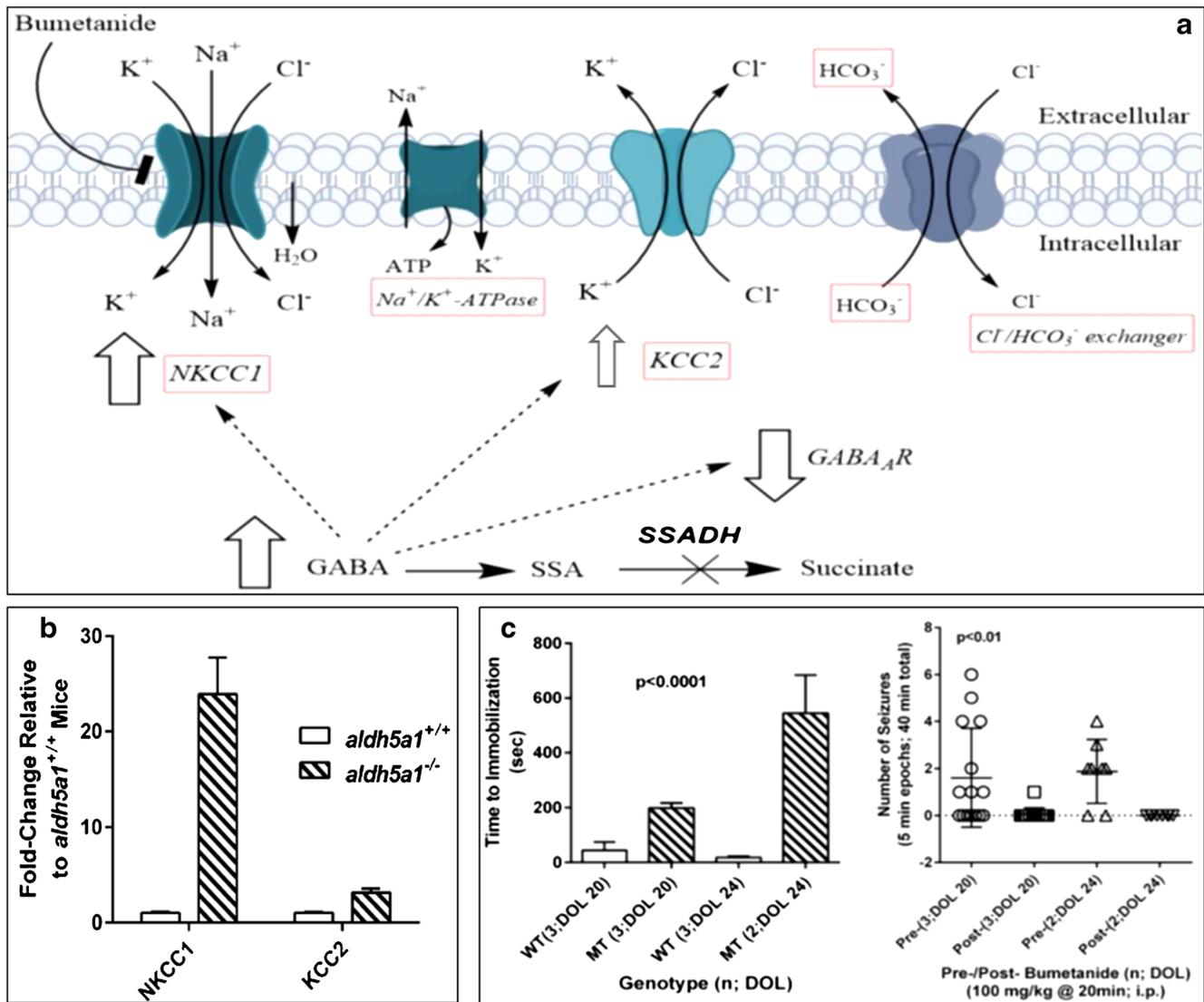
were low, we found a significant correction of GHB in the brain of enzyme treated animals, and a trend toward improved levels of GABA in blood. This promising trial was not sufficiently powered for biochemical measures, and more extensive evaluations are needed with larger n values. In particular, we need to assess the levels of SSADH activity in blood and organs, and investigate the potential of PEGylation to increase protein t<sub>1/2</sub> and reduce immunogenicity (Bell et al 2017).



**Fig. 4 Metabolic measures in animals treated with ERT.** The treatment scheme is described in the legend to Fig. 3. **a** Brain GHB content as a function of genotype (WT = wild-type, *aldh5a1*<sup>+/+</sup> mice; MT = mutant, *aldh5a1*<sup>-/-</sup> mice; PBS, phosphate-buffered saline; SSADH, recombinant SSADH). **b** GABA in sera. Data depicted as mean ± SD. Statistical analyses employed one-way ANOVA with post-hoc analysis (*t* test)

### The paradox of seizures in a SSADHD, a hyperGABAergic disorder

Chloride directional flux through the GABA<sub>A</sub> receptor in mammalian brain is regulated by the transmembrane chloride gradient which is itself controlled primarily by two transporters, the sodium-potassium-chloride symporter (NKCC1) and the potassium-chloride cotransporter (KCC2) (Fig. 5a; Kilb 2012). Activation of the GABA<sub>A</sub> receptor when the activity of NKCC1 is increased and intracellular chloride concentrations are elevated results in chloride efflux, plasma membrane depolarization and paradoxical neurotransmission activation. Such a situation is observed in the fetal brain but is reversed postnatally when GABA<sub>A</sub> receptor activation consistently leads to increased chloride cellular uptake, hyperpolarization and neurotransmission inhibition. We hypothesized that in SSADHD, postnatal seizures were caused by overexpression of NKCC1 and a continuing excitatory capacity of GABA<sub>A</sub> receptors after birth. If confirmed, this mechanism would explain the paradox of seizures in a hyperGABAergic condition (Vogel et al 2017c). Furthermore, it would also



**Fig. 5** Intracellular chloride homeostasis, GABAergic neurotransmission, and bumetanide in SSADHD (for abbreviations, see Fig. 1). **a** Schematic diagram of membrane ion transport and mechanism of action of bumetanide. **b** NKCC1 and KCC2 gene expression in the hypothalamus of  $aldh5a1^{+/+}$  ( $n=5$ ) and  $aldh5a1^{-/-}$  ( $n=5$ ) mice collected at postnatal day-of-life 20. Expression data were obtained using q-RT-PCR, validated pathway plates from Bio-Rad. **c** Time to sedation following acute dosing of 100 mg/kg bumetanide. Animals were evaluated at day of life (DOL) 20 and 24 for seizure events, and the time to immobilization (sedation) determined by visual recording using Noldus technology (see text). Bumetanide was administered intraperitoneally to the subject mice after 20 min. of initial observational

recording, followed by an additional 20 min of recording. Total number of seizures was quantified in 5 min blocks of the total 40 min. recording period. Bumetanide was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) and aseptically dissolved in DMSO vehicle. Vehicle-treated subjects did not show sedation.  $Aldh5a1^{-/-}$  mice (mutant; MT) were significantly more resistant to the sedative effects of bumetanide as compared to  $aldh5a1^{+/+}$  (wild-type; WT) mice, the latter showing almost instantaneous immobilization. During the active period in  $aldh5a1^{-/-}$  mice prior to immobilization (~3–8 min.), no seizure activity was noted. Additionally, this resistance increased significantly with age in  $aldh5a1^{-/-}$  mice ( $t$  test,  $p < 0.05$ ). Abbreviations employed: n, number of animals studied; DOL, day of life; sec, seconds; min, minutes

suggest that inhibition of NKCC1 might have positive therapeutic effects in SSADHD. In support of these hypotheses, we found that NKCC1 was highly overexpressed in  $aldh5a1^{-/-}$  brain (Fig. 5b). The expression of KCC2, which transports chloride ions out of the cell, was also increased but significantly less than that of NKCC1 (Fig. 5b). Hence, the NKCC1 to KCC2 ratio was significantly increased, suggesting that

intracellular chloride concentrations might be increased in the  $aldh5a1^{-/-}$  brain, favoring a depolarizing and excitatory activity as the predominant role for GABA<sub>A</sub> receptors. This remains to be quantitatively evaluated employing neurophysiological assessments in vitro via patch-clamp methodology, or perhaps in vivo using two-photon measurement with chloride ion probes.

The potential therapeutic role of NKCC1 inhibition in SSADHD was examined next. In this context, we postulated that there would be resistance to the sedative activity of NKCC1 inhibitors in SSADHD, because of the increased expression of NKCC1. We treated *aldh5a1<sup>+/+</sup>* and *aldh5a1<sup>-/-</sup>* mice with acute i.p. doses of bumetanide (25 and 100 mg/kg body weight) with video recording of seizure activity as well as assessment of the time to immobilization. Bumetanide is a known inhibitor of both NKCC1 and 2 originally approved for edema but with demonstrated antiepileptic efficacy in several neurological/epileptic disorders despite poor brain penetration (Levy and Bellou 2013; Oliveros et al 2011; Rahmanzadeh et al 2017; Cleary et al 2013). We employed Pinnacle technology (<https://www.pinnacle.com>) for recording of animal behavior in conjunction with a rubric developed by Noldus technology to evaluate seizure activity (<http://www.noldus.com/animal-behavior-research>). Our design was to quantify generalized tonic-clonic seizures in 5 min epochs for 40 min in continuum. At the 20 min mark, a single intraperitoneal administration of bumetanide (25 or 100 mg/kg) was given and the animals returned to the open field setting. A dose of 25 mg/kg bumetanide did not induce immobilization during the 40 min recording period for either *aldh5a1<sup>+/+</sup>* or *aldh5a1<sup>-/-</sup>* mice. Conversely, 100 mg/kg resulted in rapid induction of immobilization (Fig. 5c). Of interest, however, was the observation that *aldh5a1<sup>-/-</sup>* were significantly more resistant to the effect of bumetanide, at both DOL 20 and 24, with this resistance about threefold greater in the older *aldh5a1<sup>-/-</sup>* mice. In addition, no seizure activity was observed in the mutant mice in the time period between bumetanide injection and the onset of immobilization, an approximate period of 3–8 min. As well, the lower dose of bumetanide (25 mg/kg) also reduced seizure activity in *aldh5a1<sup>-/-</sup>* mice without sedation (data not shown). These preliminary data suggest the resistance of the *aldh5a1<sup>-/-</sup>* mice to the sedative effect of bumetanide is secondary to increased NKCC1 activity and disruption in the chloride gradient. This suggests that manipulation of NKCC1 and restoration of intracellular chloride homeostasis represents a potentially attractive therapeutic target in SSADHD.

## GABA and mTOR: treatment strategies and pathophysiological insights in SSADHD

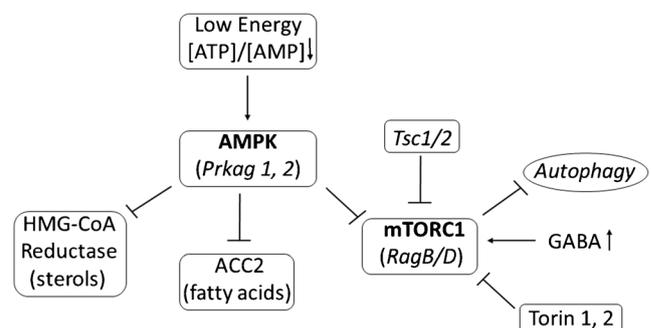
### Inhibition of mTOR, a therapeutic target in SSADHD

Increased mitochondrial numbers (including both the size and total number of organelles) were first documented in *aldh5a1<sup>-/-</sup>* mice in pyramidal hippocampal neurons (Nylen et al 2008). Subsequently, Lakhani et al (2014) demonstrated that increased GABA levels in *S. cerevisiae* led to activation of mTOR (molecular target of rapamycin), manifesting as

elevated mitochondrial numbers and enhanced oxidative stress. This same group of investigators further documented that mitochondrial numbers in brain and liver derived from *aldh5a1<sup>-/-</sup>* mice were increased and associated with enhanced oxidant stress, all of which could be normalized by the mTOR inhibitor, rapamycin.

These studies were the genesis of further preclinical interventional studies in *aldh5a1<sup>-/-</sup>* mice with mTOR inhibitors (rapamycin, temsirolimus), dual mTORC1/2 and PI3K inhibitors, as well as mTOR-independent autophagy inducing drugs (Vogel et al 2016; Fig. 6). Lifespan extension (rescue from premature lethality) for *aldh5a1<sup>-/-</sup>* mice was observed across a number of mTOR-specific and dual inhibitors, and findings with the dual inhibitor XL765 and Torin2 were striking. XL765 induced a modest weight improvement over 35 days until sacrifice at DOL 50 in *aldh5a1<sup>-/-</sup>* mice (Vogel et al 2016). Conversely, mTOR-independent inducers of autophagy were without benefit in mitigating premature lethality. These data suggest that induction of autophagy through mTOR-independent mechanisms was insufficient for rescue, suggesting that other functions associated with mTOR may be involved in the clinical efficacy of mTOR blockade in *aldh5a1<sup>-/-</sup>* mice.

Studies with Torin 2 administration in *aldh5a1<sup>-/-</sup>* mice, beginning at DOL10 under an escalating dose paradigm, were evaluated for seizures using a three channel recording electrocorticography (Pinnacle Technology; <https://www.pinnacle.com/eeg-emg-systems.html>) and electrode-implanted *aldh5a1<sup>+/+</sup>* and *aldh5a1<sup>-/-</sup>* mice. EEG was scored offline using semi-automated detection (Neuroscore, Data Sciences International, St. Paul, MN) for seizure frequency (total



**Fig. 6** Simplified schematic diagram of the roles of AMPK and mTORC1 in the regulation of autophagy. Both adenosine monophosphate-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) have key roles in the regulation of intracellular energetics, growth, and survival. *Prkag1* and *Prkag2* (protein kinase cAMP-activate  $\gamma$  subunits 1 and 2) represent components of the active AMPK trimeric structure. Conversely, *RagB* and *D* (ras-related GTP-binding proteins B and D) are involved in the regulation of mTORC1 function. *Tsc1/2* (tuberous sclerosis proteins 1 and 2) are negative regulators of mTORC1. Abbreviations: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; ACC, acetyl-CoA carboxylase; ATP, adenosine triphosphate; AMP, adenosine monophosphate

number of seizures), and total ictal time (cumulative time spent in seizures); where a seizure event was defined as a run of continuous spikes  $\geq 3$  s in duration on the EEG. Automatically detected events were verified by visual inspection (Dhamne et al 2017). Our prediction was that Torin 2 administration would lead to improvement in both parameters. We found that Torin 2 was ineffective at reducing seizure frequency in *aldh5a1*<sup>-/-</sup> mice (Table 2), and unexpectedly found that it significantly extended the total ictal time (Table 2). It is of interest that Torin 2 corrected (up-regulated) a number of GABA(A)ergic receptor subunits (Vogel et al 2016). If these receptors remain immature, consistent with our hypothesis for bumetanide (see above), then their subsequent up-regulation could conceivably exacerbate depolarization, and thus enhance cumulative epileptic outburst duration, as we observed (Table 2). On the other hand, others have provided evidence that mTOR inhibitors prevent the sprouting of mossy fibers, a form of synaptic reorganization which occurs in epilepsy and can lead to the formation of recurring excitatory circuits (Dudek and Shao 2004). Which mechanism explains enhanced seizure activity with Torin 2 remains under investigation.

## Future directions

### Dissecting the mTOR role using vigabatrin-treated *aldh5a1*<sup>+/+</sup> and *aldh5a1*<sup>-/-</sup> mice

Vigabatrin (VGB)-treated *aldh5a1*<sup>+/+</sup> mice represent a drug-induced form of GABA-transaminase deficiency, since VGB irreversibly inhibits this first enzyme of GABA catabolism (Fig. 1). Employing VGB, significantly elevated GABA in the CNS can be achieved at relatively low daily doses (~10 mg/kg), or via chronic subcutaneous delivery with calibrated osmotic minipumps (Vogel et al 2016, 2017b). A cardinal difference between these “models” resides in the absence of elevated GHB in VGB-treated *aldh5a1*<sup>+/+</sup> mice,

which enables us to begin to more clearly isolate the role of GABA and its effect on mTOR. We have begun exploring this aspect employing gene expression in these different models.

mTOR coordinates numerous intracellular bioenergetic cues that serve to control growth and catabolism (i.e., translation, autophagy) (Fig. 6). A number of gene expression changes correlated between the two animal models. *Prkag1* was down-regulated in brain of both models (Vogel et al 2017b, c). *Prkag1* is a gamma regulatory subunit of the heterotrimeric AMP-activated protein kinase (AMPK), which also contains an alpha catalytic subunit and a non-catalytic beta subunit (Fig. 6). AMPK is an important energy-sensing enzyme that monitors cellular energy status. In response to cellular metabolic stresses, AMPK is activated, and thus phosphorylates and inactivates acetyl-CoA carboxylase (ACC) and 3-hydroxy 3-methylglutaryl-CoA reductase (HMGCR), key enzymes involved in regulating de novo biosynthesis of fatty acid and cholesterol. AMPK acts via direct phosphorylation of metabolic enzymes, and acts longer-term via phosphorylation of transcription regulators. During low energy conditions AMPK is a critical negative effector of mTOR activation.

*Prkag2* was also down-regulated in *aldh5a1*<sup>-/-</sup> brain (and downregulated in VGB-treated mouse eye). Decreased expression of *Prkag1* and *2* was normalized by the mTOR inhibitor Torin 1, and correction of AMPK's activator (down-regulated in *aldh5a1*<sup>-/-</sup> mice), *Stk11*, was normalized by Torin2. As well, *Tsc1* and *2*, signaling systems downstream of AMPK, also exhibited lower expression which was normalized by Torin 1 and Torin 2 in *aldh5a1*<sup>-/-</sup> mouse brain. Rag GTPases activate mTOR through its amino acid sensing pathway, and *RagB/RagD* expression was upregulated in *aldh5a1*<sup>-/-</sup> brain (*RagB* was upregulated in VGB-treated eye tissue, while *RagD* was upregulated in VGB-treated brain tissue). Together, these findings strongly suggest that correction of AMPK is involved in the pro-survival effects of Torin drugs in *aldh5a1*<sup>-/-</sup> mice. Understanding the potential clinical utility of mTOR inhibitors to treat disorders of GABA metabolism will continue to be a central theme in our laboratory.

**Table 2** Effect of Torin 2 on seizure frequency and total ictal time in *aldh5a1*<sup>+/+</sup> and *aldh5a1*<sup>-/-</sup> mice

	Vehicle	Torin 2 (10 mg/kg)	<i>t</i> test (Veh. vs. Torin)
Seizure frequency (avg/h)			
<i>aldh5a1</i> <sup>+/+</sup>	5.4 ± 0.8	0.50 ± 0.59	<i>p</i> = 0.12
<i>aldh5a1</i> <sup>-/-</sup>	3.2 ± 0.8	9.6 ± 1.3	<i>p</i> = 0.11
<i>t</i> test	<i>p</i> = 0.41	<i>p</i> = 0.23	
Total ictal time (seconds) (avg/h)			
<i>aldh5a1</i> <sup>+/+</sup>	22 ± 1.7	2.1 ± 1.2	<i>p</i> = 0.15
<i>aldh5a1</i> <sup>-/-</sup>	19 ± 2.0	282 ± 8.0	<i>p</i> = 0.03
<i>t</i> test	<i>p</i> = 0.90	<i>p</i> = 0.30	

Data depicted as mean ± SEM. Seizure events included spike-trains, probable myoclonic as well as absence and tonic-clonic seizures

Given the multisystem dysfunction in SSADHD, it is likely that a combination of therapies will be required to leverage incremental improvements in the phenotype. A logical starting point would be VGB, if the issue of ocular toxicity could be overcome. Indeed, with regard to this, inhibitors of mTOR might be added to mitigate the effects of additionally increased GABA associated with VGB. An antioxidant agent would also be of value, given the evidence for oxidative stress in this disorder (Gupta et al 2002). As shown in the current report, ERT may have therapeutic benefit, which could be combined with genetic manipulations in the future, such as CRISPRCas9 approaches. Nonetheless, our short-term goals remain the development of targeted therapy for SSADHD.

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### Compliance with ethical standards

**Conflict of interest** K.Vogel, G. Ainslie, J. Rouillet, K. Gibson, D. Walters, A. McConnell, A. Rotenberg, and S. Dahmne declare that they have no conflict of interest

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