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COMPREHENSIVE INVITED REVIEW

Succinic Semialdehyde Dehydrogenase: Biochemical–Molecular–Clinical Disease Mechanisms, Redox Regulation, and Functional Significance

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Abstract

Succinic semialdehyde dehydrogenase (SSADH; aldehyde dehydrogenase 5a1, ALDH5A1; E.C. 1.2.1.24; OMIM 610045, 271980) deficiency is a rare heritable disorder that disrupts the metabolism of the inhibitory neurotransmitter 4-aminobutyric acid (GABA). Identified in conjunction with increased urinary excretion of the GABA analog gamma-hydroxybutyric acid (GHB), numerous patients have been identified worldwide and the autosomal-recessive disorder has been modeled in mice. The phenotype is one of nonprogressive neurological dysfunction in which seizures may be prominently displayed. The murine model is a reasonable phenocopy of the human disorder, yet the severity of the seizure disorder in the mouse exceeds that observed in SSADH-deficient patients. Abnormalities in GABAergic and GHBergic neurotransmission, documented in patients and mice, form a component of disease pathophysiology, although numerous other disturbances (metabolite accumulations, myelin abnormalities, oxidant stress, neurosteroid depletion, altered bioenergetics, *etc*.) are also likely to be involved in developing the disease phenotype. Most recently, the demonstration of a redox control system in the SSADH protein active site has provided new insights into the regulation of SSADH by the cellular oxidation/reduction potential. The current review summarizes some 30 years of research on this protein and disease, addressing pathological mechanisms in human and mouse at the protein, metabolic, molecular, and whole-animal level. *Antioxid. Redox Signal.* 15, 691–718.

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I. Historical Perspectives and Background

A. Identification of succinic semialdehyde dehydrogenase deficiency

Jakobs and colleagues (87) were the first to report the increased excretion of gamma-hydroxybutyric acid (GHB) in humans. These investigators examined the urine of three developmentally delayed children with minimal language development, using combined gas chromatography–mass spectrometry methodology. The mass spectrometric analysis was a match to library data consistent with GHB. Jakobs and coworkers speculated that a block at the level of succinic semialdehyde dehydrogenase (SSADH) would lead to the accumulation of succinic semialdehyde (SSA), which would undergo conversion to GHB. Further support for this hypothesis came from the fact that these children were born to related parents (consanguineous marriages), strongly supportive of an autosomal-recessive inheritance pattern (Fig. 1). This hypothesis was eventually proven correct.

Under normal physiological conditions, SSADH (also aldehyde dehydrogenase 5a1, ALDH5A1; E.C. 1.2.1.24; chromosome 6p22) works in tandem with GABA (gamma-aminobutyric acid; also 4-aminobutyric acid) transaminase to convert the carbon backbone of GABA to succinic acid, the latter a source of energy within the tricarboxylic acid (TCA) cycle (Fig. 1). This pathway is important in brain mitochondria, effectively coupling the metabolism of the primary neurotransmitters glutamate and GABA to ATP generation *via* the pathway of oxidative phosphorylation. In further support of his hypothesis, Jakobs noted that GHB was a compound with unusual neuropharmacological activities (118, 197), and this observation was consistent with the neurological abnormalities observed in the patients. Although the role of GABA as a major central nervous system (CNS) inhibitory neurotransmitter is well established, the exact function of the GABA-analog GHB in CNS function (neuromodulator or neurotransmitter?) is still widely discussed and debated even today (197, 198), despite a growing literature focused on GHB research that approaches some 2000 PubMed citations and spans almost 50 years (75).

In the early 1980s, there was no evidence that SSADH would be expressed in tissues outside of the CNS. Early studies confirmed that SSADH activity could not be readily demonstrated in cultured fibroblasts, but a mixed polymorphonuclear population isolated from whole blood expressed measurable SSADH enzyme activity (57). Accordingly, an enzyme procedure and peripheral tissue source (whole blood) became available in which to test the hypothesis that SSADH enzyme activity was deficient in patients with gamma-hydroxybutyric aciduria. It was subsequently demonstrated that absence of SSADH enzyme activity was directly correlated with increased excretion of GHB in physiological fluids from patients (57–61, 67).

B. Human SSADH deficiency: early clinical, metabolic, and enzymatic findings

Between the 1980s and into the mid-1990s, research on SSADH deficiency primarily focused upon patient identification, improving methodology for enzyme and metabolite analyses, and working on purification of the protein from a mammalian source for eventual cDNA cloning (28, 31, 63–65, 138, 184). More fully described later in this review, SSADH deficiency leads to protean CNS findings, and remains a disease that is challenging to identify based solely upon clinical examination. Patients invariably display global delays in mental and motor development and expressive speech, which transitions to behavioral disabilities in adolescence and adulthood. Parental consanguinity has been noted often, a finding consistent with rare recessive disorders in which the causative alleles occur infrequently in the human population (67, 68, 70, 139, 140).

The original assay employed to quantify SSADH enzyme activity in white blood cells utilized a coupled system (conversion of radiolabeled GABA to succinate, basically coupling the activities of GABA-transaminase with SSADH), which lacked sensitivity for heterozygote detection or for potential prenatal diagnostic studies (83, 138). A significant methodological improvement in enzyme analysis came with the development and implementation of a sensitive fluorometric

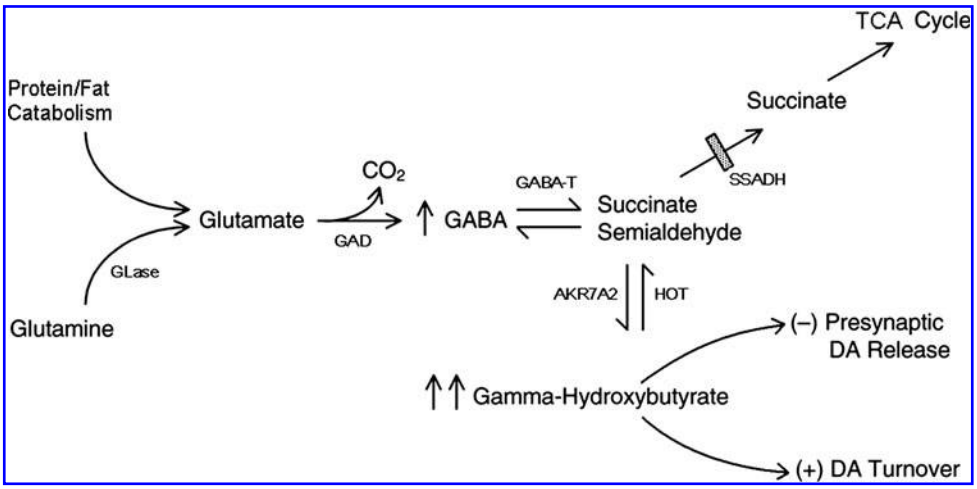

FIG. 1. Schematic diagram of GABA formation and catabolism in mammals. The block in heritable SSADH (ALDH5A1) deficiency is indicated by the cross-hatched box. Decarboxylation of glutamate (catalyzed by glutamic acid decarboxylase; GAD) produces GABA (arrow depicts its elevation in patients and null mice). Succinate semialdehyde is normally oxidized to succinate, thus moving the carbon skeleton of GABA into the TCA cycle for further metabolism. When blocked, accumulated SSA can be converted to gamma-hydroxybutyrate (double upward arrows indicating the significant increase in both mice and humans with ALDH5A1 deficiency). Gamma-hydroxybutyrate, at increased levels, inhibits presynaptic DA release and enhances DA turnover. AKR7A2, aldo-keto reductase 7A2; Aldh5a1, aldehyde dehydrogenase 5a1; DA, dopamine; GABA, gamma-aminobutyrate; GABA-T, GABA-transaminase; GHB, gamma-hydroxybutyric acid; GLase, glutaminase; HOT, hydroxyacid-oxoacid transhydrogenase (catalyzing the cofactor-independent conversion of GHB to succinic semialdehyde with coupled conversion of 2-oxoglutarate to D-2-hydroxyglutaric acid); SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase; TCA, tricarboxylic acid.

assay for SSADH, based upon NAD^+ reduction coupled to SSA oxidation (65, 89). Supplementing this enzyme assay with a highly sensitive stable-isotope dilution, gas chromatographic–mass spectrometric assay for GHB in physiological fluids (urine, blood, cerebrospinal fluid, and amniotic fluid) enabled the possibility of prenatal diagnosis for SSADH deficiency in families with an affected proband (64, 83, 88).

C. Pharmacology of GABA and GHB

SSADH deficiency is unique in light of the fact that it alters the levels and function of two neuromodulatory species, GABA and GHB. Quantitatively speaking, increased levels of GHB are much higher than those of GABA, yet it is probable that at least a component of the neuropathology of this disease is linked to GABA elevation. GABA is the major inhibitory neurotransmitter found in mammalian brain, where at least 1/3 of all synapses utilize it for neurophysiological inhibition. GABA is obtained from precursor L-glutamate by decarboxylation, catalyzed by glutamic acid decarboxylase (Fig. 1). GABA is packaged into vesicles in an energy-requiring process, released into the synaptic cleft and bound to receptors that induce inhibition of neurotransmission (20, 55, 122, 192). Removal of GABA from the synaptic cleft occurs *via* a number of GABA transporters (92, 114). With respect to neurotransmitter cycling between nerve-terminals and their surrounding astrocytes, the key biochemical intermediate is glutamine. In astrocytes, glutamate is converted to glutamine *via* glutamine synthase. Glutamine is then shuttled to nerve terminals and converted to glutamate *via* the action of L-glutaminase (Fig. 2), with the glutamate produced serving as precursor of GABA. GABA is ultimately recycled *via* metabolic sequences that produce intermediates employed for GABA reformation (Fig. 2). The key reactions are catalyzed by GABA transaminase and SSADH. Of interest, α-ketoglutarate from the tricarboxylic acid cycle is the nitrogen acceptor for the GABA-transaminase reaction, thereby producing a stoichiometric amount of glutamate for each molecule of GABA converted to succinic acid (the so-called GABA shunt).

Three GABA receptor classes have been identified (49) (Fig. 3). GABA(A) and GABA(C) receptors are ionotropic (*e.g.*, multisubunit pore channels that are involved in ion movement associated with fast inhibitory neurotransmission). From a pharmacological perspective, the GABA(A) receptor is remarkably diverse in structure, with $\sim$20 cloned subunits already identified (53, 127, 189). Of relevance for several neurodevelopmental disorders, activation of GABA(A) receptors leads to fast excitation in neurons in early development (128, 134, 155), which switches to fast inhibition in adulthood. On the other hand, the GABA(B) receptor is metabotropic, a heterodimer which functions *via* a G-protein system that is coupled to adenylate cyclase or directly to selected ion channels (13). As opposed to the GABA(A) receptor, the GABA(B) receptor induces slower inhibitory neurotransmission throughout development, including hyperpolarization of the membrane potential and depression of Ca^{2+} influx into neurons. However, GABA(B) receptors in glial cells stand out by promoting Ca^{2+} influx into these cell types (124), which might be important when GABA(B) receptor agonists accumulate in the extracellular fluids (such as in SSADH deficiency). An extensive discussion of GABA receptor neuropharmacology can be obtained from the primary literature (13, 33, 53, 137, 159).

The primary biomarker of SSADH deficiency is GHB, whose elevated levels leads to the differential diagnosis and to further diagnostic enzyme and molecular studies. GHB is an endogenous component of animal brain which is derived from GABA, yet it is present at only a minor fraction of the concentration of GABA (about 1%, or from 1 to 4 μM) (43, 44), whereas GABA levels in total brain extract approach

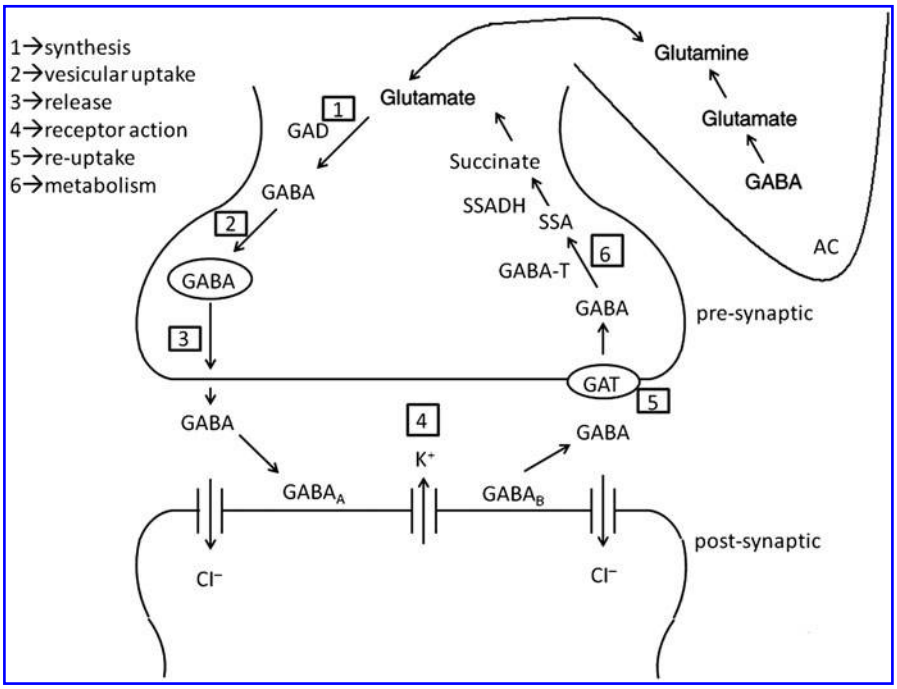

FIG. 2. Schematic diagram of the GABAergic synapse. The contribution of the astrocyte (AC) to GABA uptake and metabolism is depicted in the upper right quadrant of the figure. The pertinent processes in synaptic transmission are diagrammed as synthesis, vesicular formation, release into the synaptic cleft, receptor interaction, reuptake, and metabolism. Not all steps are shown. GABA(A), GABA(A) receptor; GABA(B), GABA(B) receptor; GABA-T, GABA-transaminase; GAD, glutamate decarboxylase (pyridoxine dependent); GAT, GABA transporter (many are known, see text). Sources of glutamate primarily include 2-oxoglutarate from the TCA cycle, as well as glutamine (producing glutamate by the action of L-glutaminase in the neuronal presynaptic terminal). As well, the nitrogen acceptor for the GABA-T reaction is 2-oxoglutarate, which yields one molecule of glutamate for each molecule of GABA catabolized through SSA. Note that glutamine represents the key shuttle form for glutamate and GABA between neuronal terminals and astrocytes.

millimolar levels (while n*M* in the extracellular fluids). When given exogenously, GHB readily crosses the blood–brain barrier and produces a number of unusual behavioral, electrophysiological, and biochemical outcomes, in addition to absence seizures (178, 179, 197, 198). Depending upon the dose administered, these effects include short-term amnesia and memory loss at low dose, seizure, or paradoxical sleep induction at moderate dosage, and stupor, coma, and potentially respiratory arrest at very high dosages (72, 75, 179). GHB was initially developed as a GABA-analog with potential anesthetic properties (and is still used in this mode today), but early testing in rat and primates led to movement abnormalities and disturbances of the electroencephalogram (EEG) (177). In the clinic, GHB has found utility as an anesthetic, for the treatment of drug dependence, in treating familial insomnia, as a tissue preservative and protectant for cardiac tissue, and more recently with very effective results in the treatment of cataplexy associated with narcolepsy (trade name, sodium oxybate, or Xyrem®) (121, 178, 197, 198). In the last 10–15 years, GHB has emerged as a recreational drug, especially in the rave party scene. The latter are generally all night parties where other illicit drugs may be consumed, accompanied by loud and repetitive music. GHB consumption has been described as producing a euphoric state with loss of inhibition and some respiratory depression, but instances of vomiting, agitation, seizures, and even death have been noted with its illicit consumption (131). There is now a well-described addiction/withdrawal syndrome associated with chronic GHB consumption, and access to this drug is facilitated by the fact that its progenitor (gamma-butyrolactone) is a widely used industrial solvent, which can be readily converted to GHB by treatment with NaOH.

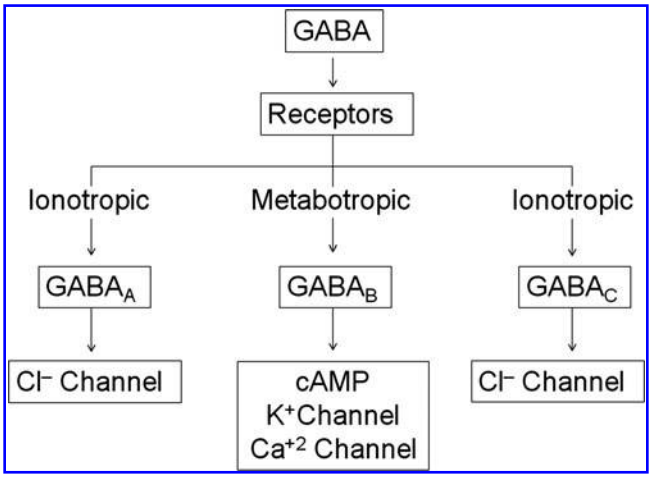

FIG. 3. Schematic diagram of receptor interactions for GABA. GABA(C) receptors are predominantly located in ocular tissue. Ca, calcium; cAMP, cyclic adenosine monophosphate; Cl, chloride; K, potassium.

Pharmacologically, GHB has a complex and interesting profile, and its full effects on brain tissues are not yet completely understood. However, it is well established that GHB acts *via* GABA(B) receptors with very low affinity (millimolar range) (11, 26, 119). In addition, GHB acts with mixed affinity (from nanomolar to low micromolar) at specific GHB receptors in CNS (111, 130). Wu and colleagues (199) have produced preliminary data to support the concept that GABA(B) and GHB receptors are functionally different, although both appear to be metabotropic systems. Many investigators believe that the intrinsic biological activities of GHB are me-

diated through the GHB receptors in brain; conversely, current thinking and literature indicates that a major component of the clinical and pharmacological effects of GHB, especially when administered in high-dose settings (abuse situations, addiction, and recreational usage) are most likely effected though interaction at the GABA(B) receptor (38, 96).

II. Molecular Genetics of SSADH Deficiency and Functional Polymorphisms of the *SSADH* Gene

A. SSADH protein characterization

A major objective in research on SSADH deficiency in the late 1980s and early 1990s was to demonstrate functional alterations of the *SSADH* gene as the underlying etiology of SSADH enzyme deficiency. This required cDNA cloning of the *SSADH* gene, and protein sequence data were unavailable. To generate degenerate oligonucleotide primers for potential cDNA isolation, purified SSADH protein was required for eventual protein digestion and amino acid sequence determination. Chambliss and Gibson (28) purified rat brain SSADH to apparent homogeneity and found relative molecular masses of 203,000 and 191,000 Da for rat and human, respectively. Denaturing electrophoretic analysis revealed a single subunit of molecular mass 54,000 Da for rat and 58,000 Da for human. For both proteins, K_m (Michaelis constant) values for SSA were in the 2–4 μM range, consistent with those values determined in other species (Table 1). The subunit structure of both enzymes was investigated in brain extracts and purified preparations by immunoblotting, using a polyclonal rabbit antiserum against the purified rat brain enzyme. For rat and human extracts, single bands were detected at 54,000 and 58,000 Da, comparable to findings in the purified preparations. Immunoblotting analyses in other species (guinea pig, hamster, mouse, and rabbit) revealed single subunits of 54,000–56,500 Da, suggesting that SSADH was composed of weight-identical subunits. The purified rat brain protein was subjected to Edman degradation for peptide generation, with subsequent development of degenerate oligonucleotide primer pools necessary for cDNA library screening.

Table 1. Kinetic Parameters of Succinic Semialdehyde Dehydrogenase Proteins from Different Organisms

Species (tissue)	*K_m for SSA (μM)*	*K_m for NAD^+ (μM)*	*V_{max} (U/mg)*	*References*
Human (brain)	1	40	68	25, 161
Rat (brain)	2–5	20–99	27–29	24, 29
Mouse	3	227	7	158
Monkey (brain)	3	—	—	3
Bovine (brain)	5.5	127	28	74
Bacteria	54	33	9	163, 180
Tick	3	43	0.7	158
Potato	5	31	6.5	166
Fly	5	91	1.7	157
Plant	15	130	23	21

1U = 1 μmol/min.
SSA, succinic semialdehyde.

B. Molecular genetics of SSADH deficiency–cDNA cloning and pathogenic mutations

Chambliss and coworkers (29) reported the isolation of three rat brain cDNA clones (3500, 1465, and 1135 bp) encoding *SSADH*. Composite clones encoding the processed SSADH protein predicted a polypeptide with 488 amino acids (molecular mass 52,000 Da). The cDNA clones were confirmed by expression analysis and protein sequence data from the purified rat brain *SSADH*. Two human liver *SSADH* cDNA clones of 1091 and 899 bp were also isolated, but isolation of complete cDNA clones was hampered by the high GC content of the 5-prime region of the human *SSADH* gene. Comparative analysis of human and rat brain SSADH proteins and genomic sequences revealed 83% and 91% identity in nucleotide and protein sequence, respectively. Northern blot analysis revealed two differentially expressed transcripts of ~2.0 and 6.0 kb in rat and human tissues, respectively. Southern blotting of the human gene revealed that both transcripts derived from a single copy gene >38kb (16, 29, 30).

Akaboshi and coworkers (2) presented an overview of ~30 mutations in the *SSADH* gene derived from patients with gamma-hydroxybutyric aciduria. These included missense, nonsense, gene deletions, and splicing errors, without a major mutation hotspot. Alterations of several well-conserved glycine residues, previously reported as critical for enzyme function in the aldehyde dehydrogenase superfamily, led to nearly complete ablation of enzyme activity (2, 191). Since the report by Akaboshi and colleagues, only sporadic reports of inherited mutations have been presented. Bekri and coworkers (10) described a mildly affected patient homozygous for a small deletion in exon 10, producing a significantly truncated polypeptide. Lemes and coworkers (108) documented SSADH deficiency in a patient of Spanish ancestry whose c.1226G>A allele was consistent with others previously reported from the Mediterranean region (2).

Additional disease-associated mutations were characterized in two unrelated probands with severe clinical features. For one, a splicing defect leading to removal of exon 7 resulted in the maintenance of the reading frame (17). In the second patient, the c.667T>C missense mutation resulted in a substitution of arginine for cysteine (Cys) (p.C223R) at position 223 of exon 4 (41); loss of the same Cys residue had previously been shown to dramatically decrease SSADH activity in an *in vitro* assay (2). Jung and colleagues (93) described a combined case of SSADH deficiency and WAGR (aniridia, hemihypertrophy, and Wilms' tumor) syndrome that was associated with a homozygous c.587G>A allele (p.G196D), altering a highly conserved glycine residue in the aldehyde dehydrogenase protein superfamily. Very recently, Iglesias Escalara and coworkers (51) reported a family with two probands in whom the patients presented primarily with axial hypotonia and fine motor deficits. Two previously identified alleles (c.278G>T; p.C93F and c621delC; p.S208fsX2) were identified in the probands.

C. Variation in the SSADH gene

Malaspina and coworkers (120) noted additional heterogeneity in *SSADH* transcripts for the 3′ untranslated (UTR)

and promoter regions of the gene. Functional characterization of the 3′-UTR revealed the presence of two polyadenylation signals whose alternative use was responsible for production of the two mRNAs of 1.8 and 5.2 kb, respectively (previously estimated by Chambliss and coworkers (29) as ~2 and ~6 kb). mRNA turnover studies provided no evidence that the different 3′-UTR of the transcripts was associated with alteration of stability or decreased efficiency of translation. Although currently unknown, it remains possible that differential properties of these two transcripts could be restricted to specific tissues and/or active under specific physiological conditions. For example, different *cis*-acting UTR elements might be present in the two mRNAs and result in specific localization within subcellular compartments, especially with respect to neuronal and non-neuronal mitochondria (16). Based upon what we have recently learned concerning the redox characteristics of the SSADH protein (see below) (97), it would be of great interest to determine if the levels of these transcripts are differentially regulated in response to the oxidation–reduction status of the cell.

Additional variation in the *SSADH* gene was documented *via* single-nucleotide polymorphisms detected in the 0.8 kb sequence upstream of the ATG (methionine [usually referenced as the methionine start codon in translation]) start site, characterized by the presence of multiple promoter elements (16). Lorenz and coworkers (113) identified seven variable positions in this region in three groups of patients with idiopathic generalized epilepsy and photosensitivity and controls ($n = 3$ each) of Germanic descent. Further resequencing of the 0.8 kb region in 24 individuals representing broad geographical origins revealed polymorphism at nine positions, of which eight had been reported in the single nucleotide polymorphism DataBank (113, 120). Significant linkage disequilibrium was detected within/between this region and the coding sequence, resulting in limited promoter-coding arrangements. Reconstruction of haplotypes revealed six possible arrangements for the eight variable promoter sites. Constructs in which these sequences had been cloned upstream of the luciferase gene were assayed to evaluate functional effects. Two variant positions (rs4646828 and rs2744575) contributed significantly to the level of *SSADH* transcription, and confirmed a positive regulatory element between −184 and −136 bp upstream of the *SSADH* start codon (16)

D. Functional polymorphisms in the SSADH *gene and evolutionary considerations*

SSADH activity determined in cultured cells or freshly isolated tissues shows substantial variation (65). For example, the range of control values in extracts of cultured human lymphoblasts is 0.7–4.6 nmol/min/mg protein (mean ± SD, 2.44 ± 1.04, coefficient of variation 43%). Molecular characterization of random individuals revealed eight missense and one same-sense variants (16), and four attained frequencies consistent with polymorphism (*e.g.*, c.106G>C (p.G36R), c.538C>T (p.H180Y), c.545C>T (p.P182L), and c.709G>T (p.A273S)). All variants, except c.709, targeted nonconserved residues, and the functional consequence of these alleles was evaluated *via* expression analysis in mammalian cells. There was a wide variation of SSADH enzyme activities associated with each amino acid substitution, and significant activity reduction was observed for the P182L protein (16). Moreover, *in vitro* studies indicated that haplotypes with multiple amino acid alterations resulted in a cumulative reduction of enzyme activity (2). Identification of individuals heterozygous for several polymorphisms led to a study of the compound genotypes, to understand the haplotype phase(s) of these alleles.

Haplotype analyses of five coding variants revealed only 5 of 32 expected outcomes, in addition to linkage disequilibrium between c.538T and both c.106C and c.545T alleles (17). This suggests that a variety of enzymes with potentially varying levels of catalytic activity may function in human. Genotyping individuals from diverse populations and all continents revealed an interesting geographical pattern for the distribution of c.538 and c.545 variants (17, 109). These studies confirmed only a limited number of haplotypes, with the c.538C>T polymorphism most common, and the c.538C allele demonstrating a range of frequency (0.50–1.0), as well as the lowest frequency in Africans and the highest in Chinese populations. A high frequency of the c.538T/c.545T haplotype was observed in all Asian populations. This unusual pattern of human diversity suggests evolutionary constraint on the SSADH protein, which was characterized in primate coding sequences through evaluation of nonsynonymous/synonymous substitution rates. Of 25 nonsynonymous substitutions in primate sequences, none altered any of five amino acid motifs critical for enzyme activity, indicative of strong evolutionary constraint (120).

Conversely, a higher nonsynonymous/synonymous substitution ratio was displayed in the human lineage, suggesting increased substitutions along the evolutionary line. Further, the variable coding positions in human could be assessed for the ancestral state using interspecific analyses. For all but one polymorphism, the most common human allele was represented by the ancestral state, which is shared with primates. Conversely, the c.538C allele is later-derived, with high frequencies in the majority of human populations, and replacing the ancestral allele. The c.538C allele leads to replacement of a tyrosine conserved in primates and rodents, with a histidine in human that is not observed in other eukaryotes. These findings indicate the possibility of recent positive selection in the human lineage, perhaps associated with the advantage conferred by the maximal enzyme activity related to the c.538C allele (17).

III. Tissue and Subcellular Localization of SSADH and Alternative Roles

A. Localization of SSADH in neural and nonneural tissues

Despite its importance in the catabolism of GABA, there have been limited investigations on the subcellular localization of SSADH in mammalian brain. This question is becoming of greater significance with the expanding use and consumption of GHB both clinically and nonclinically. SSADH is a mitochondrial matrix protein (as is GABA-transaminase); conversely, the enzyme converting SSA to GHB, aldo-keto reductase 7a2 (AKR7A2; so-called SSA reductase), is located in the cytosol. Accordingly, this suggests that there is either a pool of SSA in both mitochondria and cytosol, or that a transport protein (or proteins) affects translocation of SSA across the mitochondrial membrane. Such a protein has hitherto not been identified.

Several reports have identified SSADH activity in mammalian brain (4, 28, 94, 151, 156, 161). SSADH activity has also been reported in other tissues, including the myenteric plexus of rat small intestine (102), bovine adrenal medulla and platelets (74), monkey retina (152), adult human liver (42), human fetal kidney and liver (31), and rat liver, heart, kidney, and spleen (32). Many of these studies included only an enzyme assay, and potentially a number of aldehyde dehydrogenases can act on SSA as substrate, thus making a definitive assessment of the tissue distribution of this enzyme difficult. Conversely, several SSADH proteins purified from a number of species reveal very low K_m values for SSA (Table 1), indicating that in all likelihood they represent specific SSADH proteins. Bernocchi and colleagues (12) identified SSADH in cerebellar Purkinje neurons and liver hepatocytes using immunohistochemical methodology. However, for Purkinje cells, staining associated with SSADH activity was localized on the mitochondria (especially on the outer membrane), although a very slight extramitochondrial SSADH staining was observed. A similar distribution pattern was observed in hepatocytes. Chambliss and colleagues (32) examined the non-neural distribution of SSADH by combining enzyme analyses with immunohistochemistry using a polyclonal antisera. These investigators presented clear evidence for SSADH protein expression in brain, liver, pituitary, heart, ovary, and kidney. The presence of SSADH has so far not been identified in mammalian pancreas, ova, oviduct, testis, or sympathetic ganglia (185).

The precise localization of SSADH in non-neural tissues is of great interest, since recent data show that GABA may modulate not only endocrine function of the gastrointestinal system (18, 196), but may also affect pathogenic T lymphocyte proliferation (15). Therefore, intracellular enzymes (such as SSADH) that regulate extracellular GABA concentrations might be linked to the regulation of a large variety of functions in cells and tissues in both neural and non-neural organs. Such general functions may include brain signaling, metabolic state, and immune function.

Subcellular localization studies of SSADH in mammalian brain were undertaken in the 1970s and early 1980s (104, 160, 194), with a particular emphasis on synaptosomal and non-synaptosomal mitochondrial localization. Those studies provide evidence for isoforms of SSADH that may exist in brain neuronal and non-neuronal mitochondria. Nonetheless, detailed immunohistochemical localization in discrete brain regions and multiple subcellular compartments has not been presented. To begin characterizing the brain region and cell-specific expression and function of SSADH, immunohistochemical studies of SSADH expression in the adult mouse brain are currently underway(Delenclos and Jensen, unpublished). Emerging data show that SSADH is widely expressed throughout most brain regions, although a particularly strong expression is observed in the primary and secondary motor cortex, the amygdala, and the basal ganglia. Ongoing experiments will address the colocalization of SSADH with markers of neuronal subtypes in the forebrain and midbrain, including glutamic acid decarboxylase (67,000 Da species), which specifically labels cell bodies of GABAergic interneurons, or parvalbumin, which labels sets of fast-spiking interneurons with high metabolic demand (56). As well, the colocalization of SSADH and AKR7A2 in brain is a highly interesting topic that is being investigated.

B. Role of SSADH in 4-hydroxynonenal metabolism

4-hydroxy-trans-2-nonenal (4-HNE) is one of several α,β-unsaturated aldehydes implicated as inducers of oxidant stress in a number of neurodegenerative disorders, including Alzheimer's disease (129, 150). Murphy and coworkers employed competition, inhibition, and immunoblotting studies to demonstrate that SSADH, in rat brain, was the major aldehyde dehydrogenase responsible for 4-HNE disposition, but only a minor contributor to its metabolism in liver. This raises the possibility that 4-HNE may accumulate in neural tissue of SSADH-deficient patients and might contribute to pathophysiology (144–146).

IV. The Clinical Phenotype of SSADH Deficiency: Pathophysiology and Pharmacological Concepts

A. Clinical phenotype and pharmacologic considerations

Human SSADH deficiency typically has certain clinical features, characterized by a relatively nonprogressive encephalopathy presenting with hypotonia and delayed acquisition of motor and language developmental milestones in the first 2 years of life. Virtually constant characteristics identifiable in early childhood are intellectual disability with profound expressive language deficits, hypotonia, and ataxia but preserved independent ambulation, and hyporeflexia on neurological examination. Psychiatric symptoms may be the most disabling and are manifest by hyperkineticism, inattention, and sometimes aggression in early childhood, and anxiety and obsessive-compulsive disorder in adolescence and adulthood (98, 141). The disorder does not usually display the intermittent or episodic course of other organic acidemias and metabolic encephalopathies, although the disease in some patients is diagnosed after having unanticipated difficulty recovering from otherwise ordinary childhood illnesses, probably related to previously unidentified hypotonia. Additionally, a subgroup of about 10% of patients has a degenerative course characterized by regression and prominent extrapyramidal manifestations (143). These manifestations include chorea, myoclonus, and dystonia. In this group, the disease in the patients was diagnosed earlier, often with severe manifestations in the first months of life.

A clinical database using systematic questionnaires of 68 patients indicates that developmental delay is a universal presentation. Common clinical features include intellectual disability, behavior problems, and motor dysfunction (Tables 2 and 3). To address the long-term outlook, we reported on 33 patients (52% males) over 10 years of age (98). The mean age of

Table 2. Clinical Features of Succinic Semialdehyde Dehydrogenase Deficiency

	Number	*Percentage*
Developmental delay	67	98.5
Mental retardation	65	95.6
Hypotonia	57	83.8
Behavior problems	47	69.1
Seizures	34	50.0
Ataxia	50	73.5

$n = 68$.

Table 3. Neuropsychiatric Disturbances in Patients with Succinic Semialdehyde Dehydrogenase Deficiency

	Number	*Percentage*
Inattention	31	72.1
Sleep disturbances	28	65.1
Hyperactivity	24	55.8
Anxiety	17	39.5
OCD	16	37.2
Aggression	9	20.9
Hallucinations	5	11.6
Pervasive developmental delay/Autism	5	11.6

$n = 43$.
OCD, obsessive compulsive disorder.

this patient cohort is 17.1 ± 6.4 years (range 10.1–39.6 years). The mean age when symptoms first appeared was 11 months (range 0–44 months) and the mean age at diagnosis was 6.6 years (range 0–25 years).

Nearly half of patients develop epilepsy, usually with generalized seizures of the tonic-clonic and atypical absence types (Table 4) (144). Electroencephalographic findings include background slowing, epileptiform abnormalities (usually generalized and sometimes multifocal), and, occasionally, photosensitivity and electrographic status epilepticus of sleep (Table 5). Heterozygosity for SSADH deficiency has been reported in one family with a parent and sibling having generalized spike-wave discharges, photosensitivity, and absence and myoclonic seizures (39).

Sleep disorders are commonly reported (144). The majority of patients manifest excessive daytime somnolence, and ~20% have disorders of initiating or maintaining sleep. A study of a single patient having two nights of polysomnography demonstrated prolonged stage rapid eye movement (REM sleep) onset and, on the second consecutive night, excessive EEG background slowing after a generalized seizure during stage 4 sleep (6). We have studied 10 patients with overnight polysomnography and daytime multiple sleep latency testing and have reported prolonged REM latency (mean 272 ± 89 min *vs.* controls 90 min), and reduced stage REM percentage (mean 8.9%, range 0.3%–13.8% *vs.* controls 20%–29%) (147). Half of patients showed a decrease in daytime mean sleep latency on MSLT, indicating excessive daytime somnolesence. Thus, there appears to be a reduction in REM sleep in SSADH deficiency. Animal models have demonstrated that hyperGABAergic states, for example, *via* inhibition of GABA transaminase using vigabatrin, are associated with reduction of REM sleep and prolongation of the transition phase between sleep stages nonrapid eye movement (*NREM* sleep) and REM (169).

Table 4. Seizure Activity in Patients with Succinic Semialdehyde Dehydrogenase Deficiency

	Number	*Percentage*
Generalized tonic clonic	18	52.9
Absence	17	50.0
Myoclonic	8	23.5
Unspecified	3	8.8
Other (apparent life threatening event, atonic partial, febrile)	16	47.1

$n = 34$.

Table 5. Electroencephalogram Findings in Patients with Succinic Semialdehyde Dehydrogenase Deficiency

	Number	*Percentage*
Normal electroencephalogram	17	42.5
Abnormal electroencephalogram	23	57.5
Background abnormal/slowing	12	30.0
Spike discharges	11	27.5
Electrical status epilepticus during sleep	1	2.5
Photosensitivity	3	7.5

$n = 40$.

Recently, the first postmortem examination of SSADH deficiency was presented. The diagnosis had been made retrospectively in a 19-year-old deceased girl after the confirmed diagnosis in her living sister. Her living sister had been followed with a history of developmental delay and borderline cognition (IQ = 70), until presenting with seizures and a subsequent diagnosis of SSADH deficiency. The decedent presented with developmental delay, seizure onset at age 13 years, and SUDEP (sudden unexpected death in epilepsy) at age 19 after having experienced escalating convulsive seizure activity. A postmortem evaluation was performed with the assigned diagnosis of epilepsy of unknown etiology. A pathogenic homozygous mutation of Aldh5A1 (p.Gly409Asp) was detected in the living sister, and subsequently confirmed in the decedent and both parents (heterozygotes). The major neuropathological finding was striking discoloration of the globus pallidi, along with leptomeningeal congestion. On microscopic examination, there was hyperemia and granular perivascular calcification of the globus pallidus and superior colliculus, consistent with chronic excitotoxic injury, without significant neuronal loss or gliosis of *Cornu Ammonis* 1 region of the hippocampus, the area that would have been considered most vulnerable to epileptic or hypoxic injury (99). These postmortem findings further suggest the globus pallidus as an anatomical region prone to damage in SSADH deficiency, as described for additional patients below.

B. Radiographic findings

Cerebral magnetic resonance imaging (MRI) demonstrates a dentatopallidoluysian pattern with T2-weighted hyperintensity and corresponding T1-weighted hypointensity involving the cerebellar dentate nuclei, globus pallidi, and subthalamic nuclei in a typically symmetrical distribution (Fig. 4). Similar signal abnormalities have also been identified in the subcortical white matter and brainstem, particularly substantia nigra (144). Other findings include delayed myelination, cerebral atrophy, and cerebellar atrophy (Fig. 5) (201, 203). Although the pallidal hyperintensity is the most consistent imaging finding and is usually homogeneous and equally affects the internal and external portions, variants

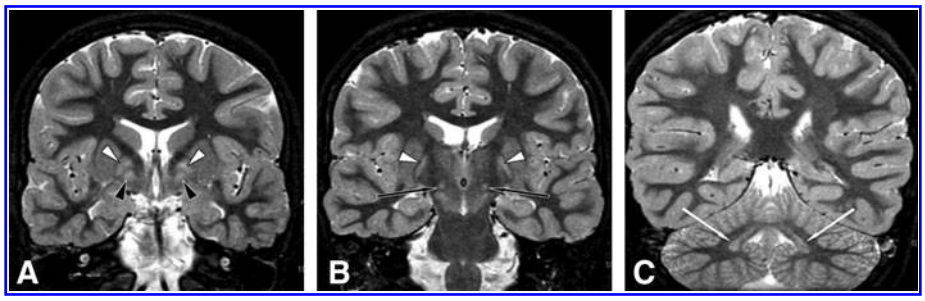

FIG. 4. T-2 weighted MRI demonstrates characteristic symmetrical dentatopallidoluysian pattern of SSADH deficiency. (A) *Arrowheads* show globus pallidus interna (black) and externa (white). **(B)** *Long arrows* show subthalamic nucleus. **(C)** *Long white arrows* show dentate nucleus. MRI, magnetic resonance imaging.

occur. Of seven patients studied in a recent clinical protocol, five had the typical dentatopallidoluysian pattern described above. One patient had asymmetric involvement of the globus pallidus that has proven to be stable over 7 years, with minimal abnormality on the right but marked increased T2-weighted and decreased T1-weighted signal on the left accompanied by expansion of the left globus pallidus and bilateral ventriculomegaly (Fig. 6). In the oldest patient studied (age 27 years), the pallidal signal abnormality was subtle, but associated with clear volume loss and commensurate ex vacuo dilatation of the third ventricle, without abnormalities of the subthalamic or dentate nuclei. Magnetic resonance spectroscopy that is edited for small molecules has shown elevated levels of GABA and related compounds (including GHB and homocarnosine) in patients but not obligate heterozygotes (52, 144). Fluoro-deoxyglucose positron emission tomography studies have shown decreased cerebellar glucose metabolism in patients with cerebellar atrophy demonstrated on structural MRI (5, 140).

Cerebrospinal fluid has been studied for GABA concentrations and related metabolites (70). There is a 2- to 4-fold increase in GABA and 30-fold increase of GHB in cerebrospinal fluid (CSF) of SSADH-deficient patients (139). There are also elevated levels of homocarnosine and a trend toward low glutamine levels, implicating dysfunction of the neuronal-glial shuttle wherein glutamine is synthesized only in astroglia with subsequent glutamate and GABA formation in neurons.

C. Neurophysiology and transcranial magnetic stimulation

The common feature of generalized epileptic seizures in the murine and human conditions led to initiation of noninvasive imaging and electrophysiologic studies to address GABAergic cortical activity. The pathophysiology of absence seizures underscores the importance of altered GABA(A) and GABA(B) mechanisms. Typical absence seizures present as staring episodes associated with three-per-second, generalized spike-wave discharges on EEG. Atypical absence seizures have relatively similar semiology but are associated with a slower spike-wave frequency (1.5–2.5 Hz) and EEG background slowing in the setting of a symptomatic neurodevelopmental disorder. Absence seizures are caused by alteration of the thalamocortical circuitry involving thalamic relay neurons, thalamic reticular neurons, and cortical pyramidal neurons. Thalamic relay neurons activate cortical pyramidal neurons in either a tonic mode causing wakefulness or REM sleep, or a burst mode that uses T-type calcium channels to produce non-REM sleep. The thalamic reticular neurons hyperpolarize the thalamic relay neurons through GABA(B) receptors and cause burst firing during wakefulness, which

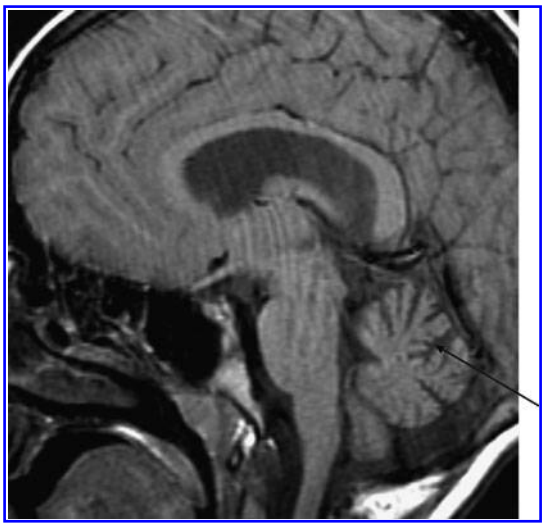

FIG. 5. Mid-sagittal MRI in SSADH deficiency showing cerebellar vermian atrophy (highlighted by the *black arrow*).

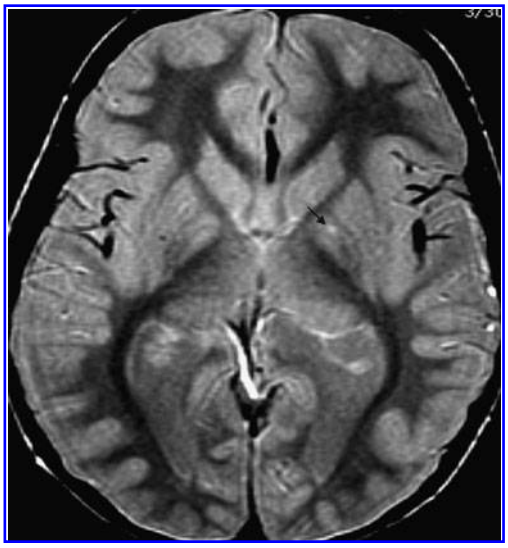

FIG. 6. MRI reveals the variant pattern of speckled, asymmetric pallidal signal hyperintensity (highlighted by *dark arrow*) in a 7-year-old boy with SSADH deficiency.

may lead to absence seizures. The thalamic reticular neurons are in turn inhibited by neighboring reticular neurons through activation of GABA(A) receptors. Therefore, both GABA(A) and GABA(B) receptors are involved in absence epilepsy, with the former mediating the inhibitory postsynaptic potentials regulating thalamocortical behavior and the latter synchronizing thalamocortical circuitry.

GABA(B)-mediated activity is responsible for physiologic de-inactivation of T-type calcium channels that lead to the spike discharge in absence seizures. Hence, excessive GABA(B) activity may be predicted to exacerbate absence seizures, as seen with administration of vigabatrin, raising overall GABA levels, or more specifically baclofen, a GABA(B) receptor agonist. Further, decreased GABAergic activity could potentially be associated with a transition from absence to generalized convulsive seizures later in life.

Positron emission tomography (PET) with [11C]-flumazenil, a benzodiazepine receptor antagonist, was used to study GABA(A) receptor activity in SSADH-deficient patients. Flumazenil PET was obtained with coregistration to MRI in 7 SSADH deficiency patients, 10 unaffected parents (obligate heterozygotes), and 8 healthy adult controls. Significant reductions in flumazenil binding were noted in all regions of interest (basal ganglia, amygdala, hippocampus, and cerebellar vermis, frontal, parietal, and occipital cortices) in patients ($p < 0.001$), with no significant differences between controls and parents (146). There was no gender effect. Given that previous flumazenil PET studies have shown that binding is higher in healthy children than in adults, these results support the presence of decreased GABA(A)-benzodiazepine binding site availability in SSADH deficiency, consistent with use-dependent downregulation of GABA receptors as demonstrated in the animal model (vide infra).

To noninvasively study neurophysiologic parameters and allow for a quantitative approach to measuring excitatory *versus* inhibitory factors, transcranial magnetic stimulation (TMS) was utilized with both single and paired pulse stimuli. Eight families (parents and affected probands) were studied with determination of standard TMS parameters: resting motor threshold, motor evoked potential recruitment, short and long interval intracortical inhibition, cortical silent period, and intracortical facilitation (154). There is a loss of long interval intracortical inhibition in patients compared to heterozygous and control groups ($p < 0.0001$), and the cortical silent period is significantly shorter in SSADH-deficient patients compared to parents and volunteers ($p < 0.01$). These indicators of decreased inhibitory factors are consistent with impaired GABA(B) receptor cortical activity. This supports use-dependent downregulation of GABA(B) receptors in the human condition, consistent with analogous activity in the animal model (vide infra).

D. Treatment strategies and challenges

The treatment for SSADH deficiency remains problematic and no consistently successful therapy has emerged (76, 144). Treatment is generally symptomatic and targeted. Options include anxiolytic agents or selective serotonin reuptake inhibitors and related medications for obsessive-compulsive disorder. Appropriate antiepileptics are chosen for generalized epilepsy other than avoidance of valproate due to its ability to inhibit any residual SSADH enzymatic activity (175). Vigabatrin, an irreversible inhibitor of GABA-transaminase, is a logical choice because it will inhibit the conversion of GABA to gamma-hydroxybutyrate, a putative pathogen in this condition. A video-manuscript report of two adolescent brothers demonstrates exercise-induced paroxysmal dyskinesias, manifest by a prominently lurching gait, which showed some improvement after vigabatrin therapy (110). Vigabatrin, however, has not been a consistently helpful therapeutic for these patients, and there have been many reports of lack of effect or, worse, worsening of symptoms ranging from seizure control to alertness. Further, there have been concerns regarding vigabatrin, resulting thus far in absence of U.S. Food and Drug Administration approval, initially because of intramyelinic edema and white matter vacuolation in rats and dogs (22, 148, 153). In clinical trials, 30% of patients treated with vigabatrin for epilepsy report visual field defects after 1 year of treatment (103, 181, 190). As this deficit begins with peripheral visual field constriction, it would be particularly difficult for patients with a neurodevelopmental disorder such as SSADH deficiency to be alert to the early signs of visual toxicity. Utilization of electroretinography and ocular Doppler ultrasonography technology for assessment of optic nerve fiber layer thickness remains preliminary at this time for such applications. MRI signal changes, particularly prominent in the GABA-rich thalamus and basal ganglia, in infants treated with relatively high doses of vigabatrin pose additional concerns regarding vigabatrin use in SSADH deficiency (145, 146). While vigabatrin will lead to at least transient decreases in CSF GHB levels (50), there may be a deleterious effect related to attendant increases in CSF (and brain) GABA levels (142).

E. Status of therapeutic trials

Both human and animal investigations have pointed to several specific therapeutic trials. Taurine, an amino sulfonic acid sold as a dietary supplement, has osmoregulatory, neuromodulatory, and tropic roles, but its exact mechanism of action is unknown. The SSADH mutant mouse study showed a survival rate of 55.6% at day 20 after intraperitoneal injection of taurine [250 mg/kg (77)]. Oral taurine at a 5000 mg/kg dosage improved survival to 46.0% ($p < 0.01$). Taurine is associated with an observed safe level in humans at 3 g/day, and higher dosages have been tested without significant adverse effects. In a single case report, taurine was reported to improve gait, coordination, and energy of a 2½-year-old boy with SSADH deficiency (165). The patient was given 4 g/day ($\sim$200 mg/kg) over 1 year. Higher doses were associated with insomnia. At 9 months teachers reported improved behavior, peer interactions, increased level of activity, and coordination. At 12 months, the boy's MRI was interpreted as improved. No correlation was found between improved behavior and urine GHB levels. The case was neither controlled or blinded, but has led to planning of further clinical trials.

As mentioned earlier, preliminary animal work in the SSADH mutant model has suggested benefit from treatment with SGS 742, a GABA(B) receptor antagonist. Since our earlier studies had shown promise with a similar compound, CGP-35348 (77, 84) (Fig. 7), we undertook examination of SGS-742 in SSADH-deficient mice, especially since this compound could be used in patients. Early reports on the intervention in humans with SGS-742 showed cognitive

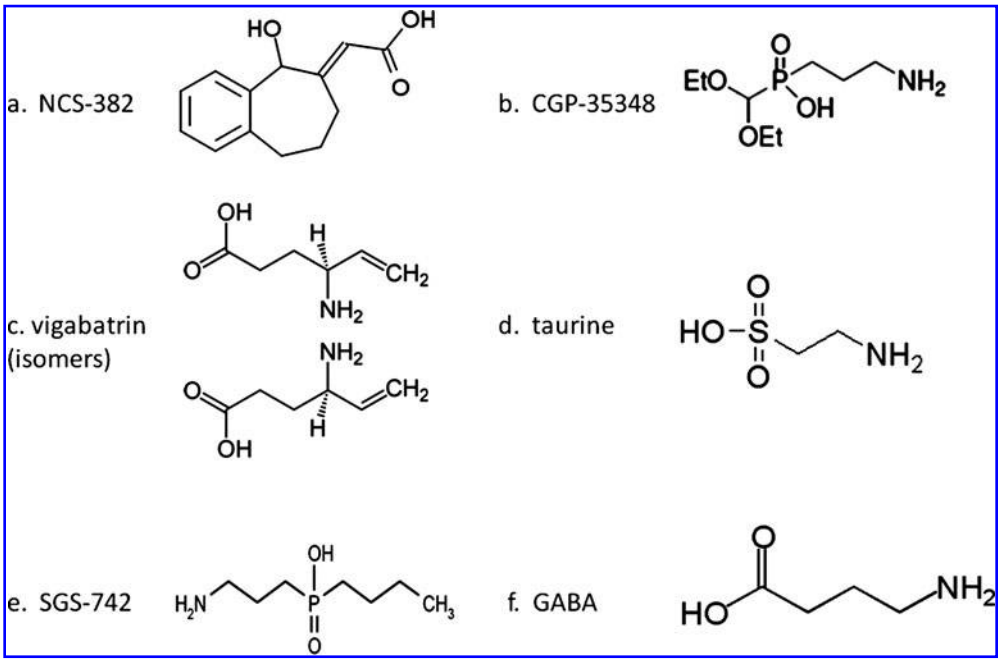

FIG. 7. Compounds employed therapeutically in Aldh5a1-deficient mice (and occasionally in human patients) with demonstrated efficacy. Full IUPAC names include (a) NCS-382 = (2E)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7]annulen-6-ylidene) ethanoic acid; (b) CGP-35348 = (3-Aminopropyl) (diethoxymethyl)phosphinic acid; (c) Vigabatrin = (*RS*)-4-aminohex-5-enoic acid; (d) Taurine = 2-aminoethanesulfonic acid; and (e) SGS-742 (also CGP-36742, see Ciba-Geigy Pharmaceuticals) = 3-aminopropyl-*n*-butyl phosphinic acid. For comparison, GABA is shown in the bottom right corner (f, 4-aminobutyric acid). Note that b–e have structural correlations with the GABA backbone, although both SGS-742 and CGP-35348 are phosphinic acid derivatives. Similarly, NCS-382 has structural characteristics corresponding to GHB on the top portion of the heptane ring (more closely related to 4-hydroxycrotonic acid). Figure adapted from structures available on Wikipedia (www.wikipedia.org).

enhancement such as improved attention, reaction time, visual information processing, and working memory in mice, rats, and monkeys. This agent has been compared with topiramate on electrocorticography (ECoG) using the mutant mouse as the study subject. Topiramate is a polymechanistic antiepileptic, with properties including enhancement of GABAergic effects, attenuation of voltage-gated Na^+ currents, and inhibitory actions on kainate and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (105). ECoG recordings ($n = 4$) were taken from frontal and parietal cortex bilaterally after electrode implantation at day-of-life 18 (P18). The drugs were administered intraperitoneally under continuous ECoG monitoring at P19 with topiramate (3, 4.5, and 6 mg/kg) and SGS-742 (30, 100 mg/kg). SGS-742 showed a dramatic dose-dependent improvement in epileptiform activity on ECoG, whereas topiramate was ineffective. As ECoG is state dependent, the doses of SGS-742 and topiramate utilized were chosen by experimentally determining the dose below the threshold for sleep.

The minimum level of SGS-742 found to be effective in the ECoG studies was then used for assessment of spike-wave discharges and survival. The cumulative spike-wave duration effects of SGS-742 at different concentrations have been characterized (145). At baseline (no drug administered), aldehyde dehydrogenase 5a1 (Aldh5a1)$^{-/-}$ (*e.g.*, SSADH-deficient) mice displayed significantly higher spike-wave discharge duration than wild-type (Aldh5a1$^{+/+}$) littermates ($p < 0.05$). SGS742 significantly improved the spike-wave duration in dose-dependent fashion and controlled absence seizures. These data support earlier data using CGP 35348 (37) and verify a prominent GABA(B) component associated with the absence seizures in Aldh5a1$^{-/-}$ mice. SGS742 significantly reduced spike-wave duration in both mutant SSADH-deficient and wild-type mice who received gamma-butyrolactone, a precursor of GHB.

In a Phase II double-blind, placebo-controlled clinical trial of SGS-742 in 110 patients with mild cognitive impairment, oral administration of 1800 mg divided into three daily dosages for 8 weeks significantly improved attention, reaction time, visual information processing, and working memory (54). Future plans are to administer this compound to patients with SSADH deficiency and utilize accompanying TMS measurements as a biomarker of GABA(B)-mediated neurotransmission.

V. Murine Model of SSADH Deficiency

To better characterize the pathophysiology of SSADH deficiency and to begin to explore effective treatment interventions, a murine model was generated using standard gene-targeting in embryonic stem cells (84). At approximately postnatal day 16–22 SSADH-deficient (Aldh5a1$^{-/-}$) mice displayed ataxia and loss of motor control, and developed generalized seizures leading to rapid death by the fourth week of life. Metabolic measurements revealed increased amounts of GHB and total GABA in urine, brain, and liver homogenates, and there was significant gliosis in the hippocampus of affected animals. In an attempt to rescue the lethal seizure phenotype, therapeutic intervention with phenobarbital or phenytoin was ineffective at prolonging the lives of affected mice; conversely, intervention with vigabatrin (gamma-vinylGABA; Sabril^R, an irreversible inhibitor of GABA-transaminase) or the GABA(B) receptor antagonist CGP 35348 prevented tonic-clonic convulsions and significantly enhanced survival of the mutant mice (Fig. 7). Since neurologic deterioration coincided with weaning, the

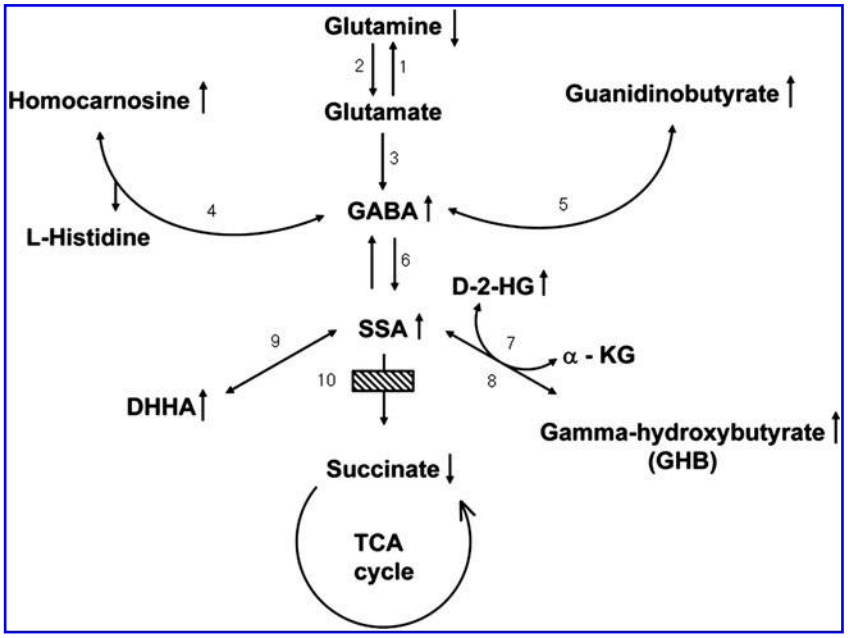

FIG. 8. Biochemical perturbations identified in patients and knockout mice with inherited Aldh5a1 deficiency. The site of the block in human Aldh5a1-deficient patients and in knockout mice is indicated by the cross-hatched box. Arrows depict the qualitative level of abnormality (downward, decreased; upward, increased). α-KG, α-ketoglutarate; D-2-HG, D-2-hydroxyglutarate; DHHA, 4,5-dihydroxyhexanoic acid; GABA, 4-aminobutyrate. Numbered enzymes include 1, glutamine synthase; 2, glutaminase; 3, glutamic acid decarboxylase; 4, carnosinase (not completely characterized); 5, L-arginine:glycine amidinotransferase (likely, but not proven); 6, GABA-transaminase; 7, HOT, hydroxyacid-oxoacid transhydrogenase (catalyzing the cofactor-independent conversion of GHB to SSA with coupled conversion of 2-oxoglutarate to D-2-hydroxyglutaric acid); 8, AKR7A2, aldo-keto reductase 7A2; 9, pyruvate dehydrogenase reaction (most likely, not examined in any detail); 10, SSA dehydrogenase.

presence of a protective compound in breast milk was hypothesized to be involved. Accordingly, treatment of mutant mice with the amino acid taurine, very high in mammalian breast milk, also resulted in significant rescue of the lethal murine phenotype. Metabolically, the SSADH-deficient mouse model has been a representative phenocopy of the human disease, yet neurologically it seems to represent the extreme end of the severity scale, since only about half of human patients have an epileptic disorder (145, 146). This may reflect the observation that the mutation in the mouse is a null allele, whereas in patients most mutations may still yield some residual enzyme activity. Nonetheless, this animal model has provided a wealth of important insight into pathomechanisms in SSADH deficiency.

A. Metabolic findings in the murine model of SSADH deficiency

As noted above, the knockout mouse model is a viable phenocopy of the human disease, at least with respect to GHB and GABA (69, 71, 78, 80, 90, 145) (Fig. 8). Estimates of GHB levels in the brain, both globally and regionally, are on the order of 200–250 μM, with levels in corresponding wild-type mice of 1–4 μM. Although these levels are quite elevated, they are not sufficient to activate GABA(B) receptors, for which the IC_{50} for GHB is in the millimolar range (179). Nonetheless, intracellular concentrations of GHB could be significantly larger than those measured above, and could not be estimated without *in vivo* microdialysis or sensitive spectroscopy methods. GABA levels in null mouse brain are consistently threefold increased above normal, very consistent with levels measured in cerebrospinal fluid of patients (69, 70, 72, 90). While these increases are not comparable to that of GHB, during embryonic development, and most likely for the first 1–2 weeks of life, GABA is excitatory in the developing rodent brain, primarily at the GABA(A) receptor (82, 173), and this hyperGABAergic state in the mouse model may be a contributory factor to the evolution of seizures.

A striking anomaly in the murine model is a significant depletion of glutamine in brain that accompanies the elevation of GABA (69). This was observed in whole brain homogenates, as well as in extracts from different brain regions (including frontal and parietal cortices, hippocampus and cerebellum). Glutamine is a major storage form and intermediate for both GABA and glutamate, and the metabolism of this species is well-regulated through localization of specific synthetic/degradative enzymes in various neural intracellular compartments (193). For example, glutamine synthase is localized specifically to astrocytes, whereas the glutamate forming enzyme, glutaminase (converting glutamine to glutamate), is regionalized to the neuronal terminals (71). In this way, glutamine serves as a shuttle form of glutamate and GABA between different cell types in neural tissue. To unravel the potential causes of glutamine deficiency, Chowdhury and colleagues (35) examined *in vivo* oxidation of stable-isotope-labeled glucose and acetate in the brain of SSADH-deficient mice. These substrates were chosen based upon their tissue specificity for metabolism, in which glucose is oxidized by both neurons and glial cells, whereas acetate is almost strictly oxidized within the glia. Chowdhury and colleagues found that although there were surprisingly few effects on GABA formation in the murine model, both glutamate and glutamine formation were markedly decreased in the brain of mutant mice, suggesting that glutamine was preferentially utilized to maintain glutamate levels (35).

Several other metabolic abnormalities have been identified in SSADH-deficient mice. Schultze and colleagues (170) noted that treatment of epileptic patients with vigabatrin led to the accumulation of guanidinobutyrate in physiological samples (primarily CSF). Since vigabatrin leads to elevated GABA (see

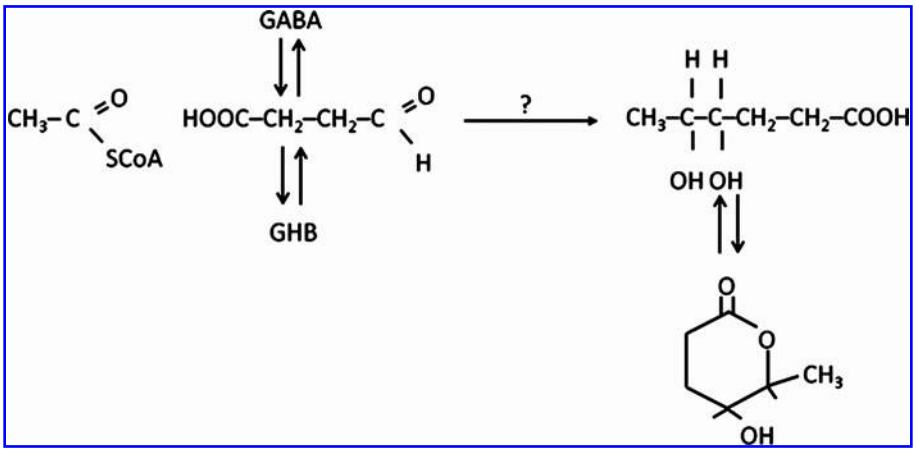

FIG. 9. Hypothetical formation of 4,5-dihydroxyhexanoic acid from SSA and a putative 2-carbon activated species, such as acetyl-CoA (shown). Lactonization of 4,5-dihydroxyhexanoic acid can readily occur in aqueous solution. GABA, 4-aminobutyrate; SCoA, coenzyme A.

above), Schultze and colleagues hypothesized that increased GABA was reacting with arginine *via* the AGAT (arginine:glycine amidinotransferase) reaction to form guanidinobutyrate in lieu of guanidinoacetate. Tissues derived from SSADH-deficient mice revealed increased levels of guanidinobutyrate, which also suggested a potential depletion of arginine (91), which could conceivably have ramifications for the nitric oxide system. Elevated levels of homocarnosine were also detected in brain tissue of mutant mice, and this was confirmed in the brain (MR spectroscopy) of human patients (134). Homocarnosine is the dipeptide form of GABA (L-histidine; GABA), and is found both in brain and skeletal muscle with various roles and properties (48).

Two additional metabolites, detected at increased levels, have been identified in the brains of SSADH-deficient mice. One of these is D-2-hydroxyglutarate (Fig. 8), a species detected at elevated levels in another heritable neurological disorder, D-2-hydroxyglutaric aciduria (101). D-2-hydroxyglutarate is believed to arise from the action of D-2-hydroxyglutarate transhydrogenase, an NAD^+-independent enzyme that converts GHB to SSA with stoichiometric generation of D-2-hydroxyglutarate from 2-oxoglutarate (95). A compound that thus far appears pathognomonic for human SSADH deficiency, 4,5-dihydroxyhexanoic acid (DHHA), is also elevated in tissues and fluids derived from SSADH deficient mice (Fig. 9). This unique compound is predicted to arise from the condensation of succinate semialdehyde with a two carbon species most likely derived from the pyruvate dehydrogenase reaction, as first postulated by Brown and colleagues (19). If this is the case, major bioenergetic perturbations may occur if SSA accumulates to an appreciable extent and interferes with the pyruvate dehydrogenase (or other) reactions.

B. Disturbances of myelin in SSADH-deficient mouse brain

Donarum and colleagues (45) undertook expression profiling of SSADH-deficient brain regions to examine GABAergic and glutamatergic components that were predicted to be altered. Unexpectedly, these investigators reported a significant downregulation for genes associated with myelin biogenesis and compaction, predominantly in hippocampus and cortex. The key proteins downregulated were myelin basic protein, myelin-associated glycoprotein, myelin-associated oligodendrocytic basic protein, and proteolipid protein (45). The expression data were confirmed by a number of alternative protocols, including immunohistochemistry, Western-blotting, and quantitative reverse transcription–polymerase chain reaction methods. As well, the levels of ethanolamine and choline plasmalogens, all core myelin components, were quantified and shown to be decreased, as was the level of myelin evaluated by toluidine staining and the thickness of the myelin sheath characterized by electron microscopy.

The expression profiling studies were extended by comprehensive examination of brain phospholipid fractions in mutant mice, including quantitation of fatty acids in individual phospholipid subclasses and estimation of hexose-ceramide. SSADH-deficient mice demonstrated a 20% reduction in the ethanolamine glycerophospholipid content as compared to wild-type littermates, whereas other brain phospholipids (choline glycerophospholipid, phosphatidylserine, and phosphatidylinositol) were not affected. Analysis of individual fatty acids in these fractions revealed consistent alterations in n-3 fatty acids, primarily increased 22:6n-3 levels (docosahexaenoic acid [DHA]). These results for DHA are somewhat paradoxical, since a highly oxidative environment (expected in the SSADH-deficient mouse) would more likely favor a reduction in DHA, especially in neurons where DHA is enriched in the synaptic membrane. Finally, in the phosphatidyl serine fraction there were marked increases in the proportions of polyunsaturated fatty acids with corresponding decreases of monounsaturated fatty acids (7).

Several potential explanations may help to explain the myelin abnormalities in these mice. Gupta and colleagues (78) presented data that the neuroactive steroids progesterone and allopregnanolone were decreased in the brains of SSADH-deficient mice. GABA synthesis may be activated by neurosteroids, especially allopregnanolone (116, 117), and GABA(B) receptors (downregulated in SSADH-deficient mice—see below) can influence the levels of myelin-associated proteins (115). Accordingly, the myelin effects observed in the mutant mice are likely a culmination of many processes, involving GABA levels, GABAergic receptors and neuroactive steroid levels (Fig. 10).

C. Neurophysiology and GABAergic abnormalities in SSADH-deficient mice

GHB induces absence-like seizures in rodents (179) and absence seizures have been reported to occur in children with SSADH deficiency (145). Sequential electrocorticographic and video ECoG recordings from implanted electrodes were

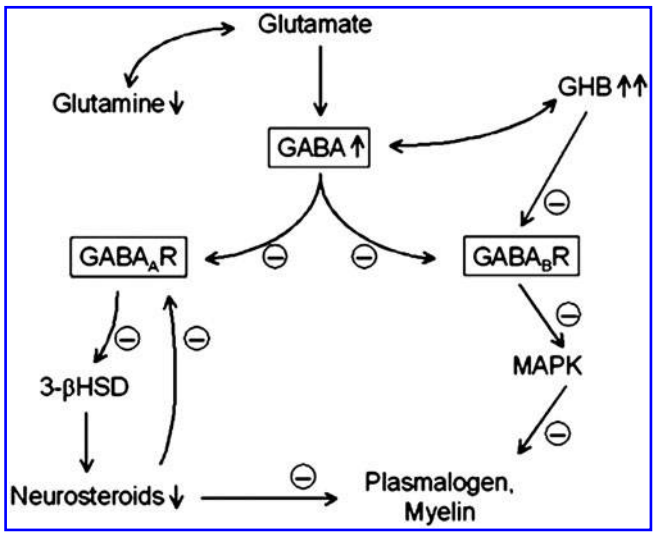

FIG. 10. Schematic of potential interactions resulting in myelin abnormalities in SSADH-deficient mice. Circled dashes represent regions of downregulation, whereas upward or downward pointing arrows indicate metabolite concentrations in mutant mice. Increased GABA/GHB result in use-dependent effects on GABA receptors and altered signaling *via* the mitogen-activated protein kinase pathway. These changes, coupled with decreases in brain neuroactive steroids in SSADH-deficient mice (78), may provide an explanation for low plasmalogen and myelin levels in mutant mice. 3βHSD, 3β-hydroxysteroid dehydrogenase; GABA, 4-aminobutyrate; GABA(A)R and GABA(B)R, GABA receptors; MAPK, mitogen-activated protein kinase. Figure adapted from Donarum *et al.* (45).

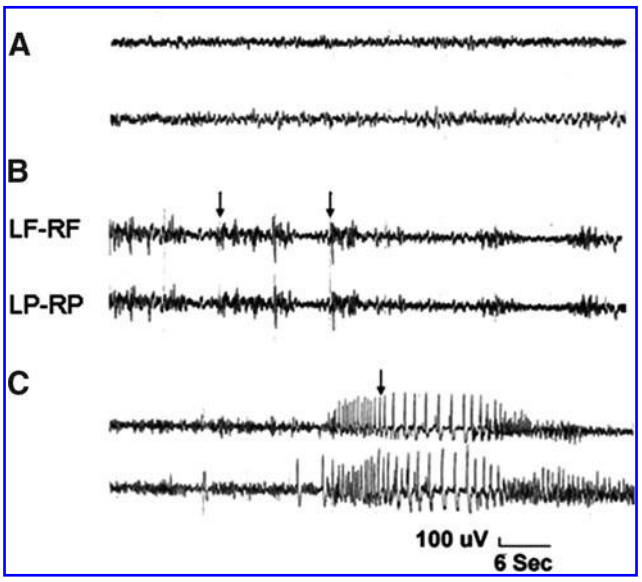

FIG. 11. Representative murine electrocorticograms obtained from implated electrodes. (A) Baseline ECoG recorded in a 16-day-old wild-type control mouse indicating an uneventful baseline with 35–50 μV and 5–7 Hz oscillations. **(B)** ECoG of 16-day-old SSADH-deficient mouse revealing 250–300 μV, 5–7 Hz spike-and-wave discharge lasting 3–6 s on average (*arrows*). **(C)** ECoG recording in 20-day-old mutant mouse revealed a transition from absence to a sustained rhythmic onset of generalized 600 μV at 5 Hz followed by 1.5–2 Hz spike-and-wave discharge associated with tonic-clonic seizures (*arrow*). ECoG, electrocorticogram; LF, left frontal; LP, left parietal; RF, right frontal; RP, right parietal leads, identical for A and C also. Figure adapted from Cortez *et al.* (37).

performed on SSADH-deficient mice from postnatal day (P) 10–21. These recordings revealed recurrent absence-like seizures during the second week of life and evolved into generalized convulsions late in the third week of life that were associated with an explosive onset of status epilepticus that was lethal (Fig. 11). The seizures in SSADH-deficient mice were consistent with typical rodent absence seizures with 7 Hz spike-and-wave discharge recorded from thalamocortical circuitry, the onset/offset of which was linked with ictal behavior characterized by facial myoclonus and immobility (37). Absence seizures were abolished by ethosuximide and the GABA(B) receptor antagonist CGP 35348. More recently, Stewart and colleagues (183) have demonstrated that generalized tonic-clonic seizures in SSADH-deficient mice show evidence of a circadian distribution, an observation that may have treatment ramifications for SSADH-deficient patients with seizures.

Because the SSADH-deficient mice manifest very high brain GABA and GHB levels, a systematic analysis of both GABA(A) and GABA(B) receptors was undertaken. In physiological concentrations, GHB acts at the GHB receptor, but in high concentrations such as those observed in the brains of patients, GHB is thought to be a direct agonist at the GABA(B) receptor. Accordingly, the possibility that both GHB receptor and GABA(B) receptor-mediated activity were altered in SSADH-deficient animals was comprehensively examined using both neurophysiology and biochemical/molecular biology-based techniques. There is significantly decreased binding of the specific GABA(B) receptor antagonist, CGP-54626A, in mutant mice by 2 weeks of life, particularly in hippocampus. As well, GABA(B) receptor-mediated synaptic potentials are decreased in SSADH-deficient animals. Strikingly, there is no detectable abnormality detected in GHB receptor structure or function in mutant animals (23, 123).

Alterations of the GABA(A) receptor were also observed in parallel studies. A selective decrease in binding of [(35)S]tert-butylbicyclophosphorothionate (TBPS; a ligand with selectivity for the chloride channel pore of the GABA(A) receptor) was observed in SSADH-deficient mice, which increased until reaching a low-point that correlated with the onset of generalized convulsive activity (200). Corresponding to these binding abnormalities was a downregulation of the β2 subunit, a reduction in GABA(A) receptor-mediated inhibitory postsynaptic currents (IPSCs), and augmented postsynaptic population spikes recorded from hippocampal slices. These data revealed a time-dependent progression and dysfunction associated with GABA(A) receptor activity that correlated with the profile of seizure transition (100, 145, 146, 162, 200).

To further extend the examination of these receptor anomalies, patch-clamp electrophysiology has recently been pursued to examine regional neuropathology. In cortical layer 2/3 pyramidal cells of SSADH-deficient brain, spontaneous inhibitory postsynaptic currents (IPSCs), reflecting activity of GABAergic synaptic contacts, and IPSCs evoked by electrical single-axon stimulation, were shown to be within normal

limits for SSADH-deficient mice (47). In contrast, tonic inhibition mediated by extrasynaptic GABA(A) receptors was increased, suggesting increased extracellular GABA levels and indicating that (350 μm thick) brain slice preparations preserve the pathophysiological environment surrounding cortical neurons (47). Dosa *et al.* (46) extended these studies by examining the effect of gene dosage and residual SSADH enzyme function (*e.g.*, heterozygotes [+/−] and mutant [−/−] mice) on single-cell hippocampal electrophysiology. These investigators documented that tonic extrasynaptic GABA(A) receptor-mediated currents increased exponentially with decreased enzyme function in heterozygous and mutant mice, whereas phasic synaptic GABA(A) receptor currents were unaltered in dentate gyrus granule cells. Similarly, tonic GABA(A) receptor-mediated currents increased nonlinearly in dentate gyrus interneurons of mutant animals, whereas phasic GABAergic neurotransmission is normal. These studies, exploring single-cell neuropathology in SSADH deficiency, suggest global disruption of cortical networks in SSADH-deficient mice, affecting both excitatory and inhibitory neurons (46, 47). Overall, the data suggest that extracellular ambient GABA concentrations increase exponentially with decreased SSADH enzyme function (164).

D. Evidence of oxidative damage and stress in murine SSADH deficiency

Several lines of evidence indicate that oxidant stress contributes to pathophysiology in both human and murine SSADH deficiency. As noted above, MRI for a number of patients (145, 146) has revealed hyperintense signals in the globi pallidi bilaterally. It is generally accepted that this reflects a degree of cytotoxic edema linked to metabolic toxicity (100, 145). MR imaging has not been reported in the SSADH-deficient mouse model. As described further below, the SSADH enzyme appears to be sensitive to the redox environment surrounding the protein, and the data we have obtained biochemically are consistent with these findings.

Latini and colleagues (107) extensively characterized the tissue antioxidant defenses and lipid peroxidation mechanisms in various cerebral structures (cortex, cerebellum, thalamus, and hippocampus) and in the liver of SSADH-deficient mice. The parameters analyzed were total radical-trapping antioxidant potential and glutathione (GSH) levels, the activities of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase, as well as thiobarbituric acid-reactive substances. These investigators observed that tissue nonenzymatic antioxidant defenses were significantly reduced in SSADH-deficient animals, particularly in liver (decreased total radical-trapping antioxidant potential and GSH) and in the cerebral cortex (decreased GSH), as compared to the wild-type littermate control mice. Further, superoxide dismutase activity is significantly increased in the liver and cerebellum, whereas the activity of catalase is significantly higher in the thalamus. Glutathione peroxidase activity is significantly diminished in the hippocampus. Finally, lipid peroxidation (thiobarbituric acid-reactive substance levels) is markedly increased in the liver and cerebral cortex, reflecting a high lipid oxidative damage in these tissues.

Any number of metabolic abnormalities could contribute to these findings, including accumulation of reactive SSA, 4-HNE accumulation (a substrate for SSADH; see above), and DHHA accumulation. Nonetheless, the data of Latini *et al.* (107) suggest that the pathophysiology of SSADH deficiency includes a component of oxidative stress or damage, consistent with limited findings in humans and with evidence of astrogliosis (84) in the murine knockout model.

Sauer and colleagues (167) extended these studies by looking at discrete mitochondrial processes in the SSADH-deficient mouse model. These investigators hypothesized that since GABA degradation is coupled to TCA cycle function in mammalian CNS (*via* the so-called GABA shunt, employing succinate and α-ketoglutarate), there might be dysfunction not only of global mitochondrial activity, but also of specific TCA cycle activities, leading to additional neuropathology. To test this hypothesis, cerebral activities of TCA cycle and respiratory chain enzymes, as well as the glutathione content in brain of SSADH-deficient mice, were characterized. Consistent with the report of Latini and coworkers (107), Sauer *et al.* found a significantly decreased glutathione content (hippocampus, cortex) and decreased activities of complexes I-IV primarily in the hippocampus, all consistent with increased oxidative stress and mitochondrial dysfunction. Conversely, specific activities of TCA cycle and respiratory chain enzymes were not affected by GABA, GHB, SSA, or DHHA (up to 1 mmol/L). Thus, despite evidence for both hippocampal and cortical oxidative damage in SSADH-deficient mouse brain, Sauer and colleagues (167) were unable to provide evidence that selected metabolites accumulating in SSADH-deficient mouse brain could directly induce selected derangements of energy metabolism *in vitro*.

E. Mechanistic considerations for oxidant stress in SSADH deficiency

A number of observations suggest that oxidant stress is a contributor to pathophysiology in both human and murine SSADH deficiency. Some of these include (i) hyperintense signals in the globi pallidi of patients bilaterally (likely cytotoxicity due to metabolite accumulation); (ii) depleted glutathione levels in SSADH-deficient mouse tissues; (iii) alterations of oxidative phosphorylation in the mouse model; (iv) perturbations of the Krebs cycle associated with 4,5-dihydroxyhexanoic acid and D-2-hydroxyglutarate accumulation (altering pyruvate and 2-oxoglutarate levels); (v) direct demonstration of altered biomarkers of oxidant stress in liver and brain of SSADH-deficient mice (107); and (vi) data indicating that mitochondrial SSADH is the principle catalyst for further metabolism of the lipid peroxidation product 4-HNE in brain. These are just a sampling of the data indicating oxidative damage in this disorder.

Historically, pathology associated with SSADH deficiency has been thought to be mitigated primarily through GABA and GHB. This is a reasonable hypothesis, since GABA is excitatory in the developing rodent brain (and elevated in SSADH-deficient embryo tissues) (82, 90), and since Sgaravatti and colleagues (174) demonstrated that direct administration of GHB in the rodent results in altered biomarkers of oxidant stress. However, less attention is focused on the potential toxicity of the species that interlinks GABA with GHB, SSA, which is significantly elevated in SSADH-deficient mouse brain when measured with accurate isotope dilution methodology (73).

Aldehydes, such as SSA, comprise the major end-product of oxidation/peroxidation of lipids. Aldehydes are unsaturated, highly reactive species that can form adducts with a number of biomacromolecules, including proteins, lipoproteins, nucleic acids, and many others (36, 112, 133, 168, 188). Very high levels of aldehyde adducts are detected in a number of complex disorders, such as cardiovascular disease and in disorders featuring neurodegeneration (Parkinson's and Alzheimer's diseases) (36, 149, 150), whereas lower concentrations of the free aldehydes have the capacity to alter intracellular signaling and gene regulation (27). What intracellular reactions and pathways will affect the level of SSA? First, the redox status of the cell will determine the homeostatic relationship between GHB and SSA, and this impacts a component of the regulation of the SSADH protein outlined below (whereby a disulfide bridge prohibits entry of SSA into the active site of the protein). Second, the reaction catalyzed by D-2-hydroxyglutarate transhydrogenase generates a molecule of SSA for each molecule of D-2-hydroxyglutarate produced (95). Although generally with a K_{eq} (equilibrium constant) close to unity, GABA-transaminase will regulate the level of SSA produced, but enhanced release of GABA through a number of mechanisms (see below) may result in increased SSA levels.

F. Treatment approaches in murine SSADH deficiency

Early lethality in the SSADH-deficient mouse model provides both challenges and opportunities for the study of this animal. In one respect, long-term evaluation is hampered due to early lethality; conversely, early demise provides a useful investigative vehicle by which the utility of both pharmacologic and nonpharmacologic treatment interventions can be assessed (*e.g.*, extended survival). As noted above, several treatment strategies have proven efficacious, including taurine, CGP-35348, vigabatrin, and NCS-382, at moderate to high dosage, in the murine model (77, 84) (Fig. 7). Unfortunately, NCS-382 and CGP-35348 are not FDA-approved medications (although ongoing preclinical animal studies, coupled to toxicology data, may eventually support an orphan drug application). Vigabatrin is the most commonly used intervention in SSADH deficiency, and there has been mixed efficacy with this intervention (62, 66, 70, 71, 100). The logic of intervening with vigabatrin, in an already hyperGABAergic disease, also remains questionable.

Based upon the refractory seizures in SSADH-deficient mice, and the fact that pups who stayed with dams beyond the weaning period showed better survival than those weaned at earlier periods (perhaps related to ingestion of maternal milk, a significant source of fat), Nylen and colleagues (135) examined the feasibility of the ketogenic diet (KD) on SSADH-deficient mice. The KD is used primarily in those children whose epilepsy is refractory to conventional pharmacotherapy. Using a 4:1 ratio of fat to combined carbohydrate and protein KD, Nylen and colleagues contrasted the phenotype, *in vivo* and *in vitro* electrophysiology and [^{35}S]TBPS binding (see above; GABA(A) receptor associated) in SSADH-deficient mice. The KD prolonged the lifespan of mutant mice by >300% with normalization of ataxia, weight gain, and ECoG compared to mutants fed a control diet. SSADH-deficient mice (normal diet) showed significantly reduced mIPSC (miniature inhibitory postsynaptic current) frequency in *Cornu Ammonis* 1 hippocampal neurons as well as significantly decreased [^{35}S]TBPS binding in all brain areas examined. In KD fed mutants, mIPSC activity normalized and [^{35}S]TBPS binding was restored in the cortex and hippocampus. These investigators further showed that KD application to mutant mice enhanced the number of mitochondria in hippocampus and the ATP levels in mitochondria derived from this region, as compared to mutant mice receiving normal rodent chow (136). Accordingly, Nylen and colleagues suggested that the KD appears to normalize the perturbations seen in SSADH-deficient mice, and suggested that the KD could effectively restore GABAergic inhibition.

With respect to therapeutic concepts for any heritable (genetic) disorder, the long-term goal remains to achieve a more permanent (long-term) correction, which generally employs tissue transplantation and/or gene therapy. Chambliss and coworkers (32) demonstrated that hepatic tissue expresses 2/3 the level of SSADH protein and enzyme activity as compared to brain. In view of this observation, Gupta and colleagues (79) evaluated rescue of the SSADH-deficient model with liver-directed gene therapy using the E1-deleted adenoviral vector AD:pAD-Rous Sarcoma virus-human SSADH. The working hypotheses in this study were the following: (i) liver expresses considerable SSADH activity and therefore represents a major source of GHB generation; (ii) correction of liver enzyme deficiency will reduce GHB load both peripherally and in the CNS; and (iii) SSADH expression in hepatic tissue would improve survival. SSADH-deficient mice were treated under two protocols: (i) intraperitoneal injection of 10^8–10^{11} viral particles by day 10 of life or (ii) retro-orbital injection of 10^{11} viral particles at day 13 of life. Intravenous administration was prohibited by the small size and fragility of the mice. Maximal survival (39%; $P < 0.001$) was achieved with intraperitoneal administration (10^8 particles) at day 10; intraperitoneal (10^{10} and 10^{11} particles) and retro-orbital administration (10^{11} particles) yielded lower survival of 11–25% ($P < 0.02$). Under both protocols, the maximal hepatic SSADH enzyme activity was ~20% of wild-type liver activity (retro-orbital > intraperitoneal). At various time points postinjection, intraperitoneal-treated animals (10^8 viral particles) demonstrated upward of an 80% reduction in liver GHB concentration, with little impact on brain or serum GHB levels except at 48–72 h posttreatment (~50% reduction for both tissues). Accordingly, Gupta and colleagues harvested retro-orbitally treated animals at 72 h and observed significant reductions for GHB in liver, kidney, serum, and brain extracts. Histochemical analysis of liver from retro-orbitally treated mutants demonstrated substantial SSADH staining, but with variability both within tissues and between animals. These studies provided important proof of principle that liver-mediated gene therapy has efficacy in treating SSADH deficiency, and that hepatic tissue contributes significantly to the pool of GHB within the CNS. On the other hand, the feasibility of liver-directed gene intervention in human SSADH deficiency remains to be debated, since this is primarily a neurological disorder, and enzyme deficiency will still be present in neural tissue. A schematic diagram of the current pathophysiological mechanisms associated with heritable SSADH deficiency is shown in Figure 12.

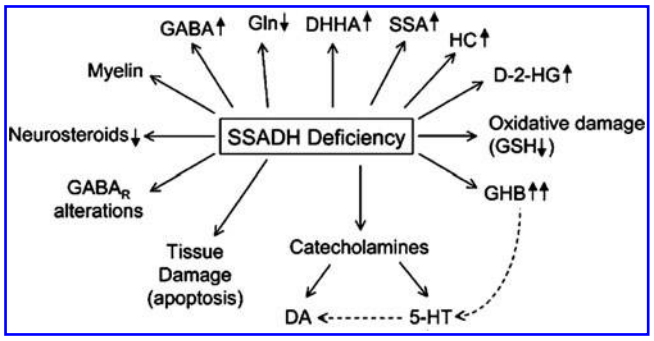

FIG. 12. Documented and hypothesized pathophysiological alterations in heritable SSADH deficiency. *Arrows* indicate the direction and magnitude of metabolic disturbances. *Dashed arrows* indicate proposed and/or hypothesized disturbances. D-2-HG, D-2-hydroxyglutarate; 5-HT, 5-hydroxytryptamine (serotonin); GABA, gamma-aminobutyric acid; $GABA_R$, GABA receptors; Gln, glutamine; GSH, glutathione; HC, homocarnosine.

VI. Crystal Structure of SSADH and Novel Regulatory Mechanisms

A. Enzymatic properties and production of SSADH protein

Human SSADH, encoded by the *ALDH5A1* gene and showing high amino acid homology with aldehyde dehydrogenases, is a member of the aldehyde dehydrogenase (ALDH) superfamily (81, 187). ALDHs utilize substrates with aliphatic tails, whereas SSADH uses SSA as a substrate with a hydrophilic carboxyl tail, which is most likely the explanation for why SSADH demonstrates a very high substrate specificity compared with other ALDHs. In the case of Drosophila melanogaster SSADH, the K_ms for substrate SSA and NAD^+ are 4.7 and 90.9 μM, respectively (157) (see also Table 1). Considering the crucial role of SSADH in the regulation of GABA homeostasis in the mammalian brain, and the high substrate specificity of the protein, Kim *et al.* (97) hypothesized that the 3D structural information of the protein would be important in providing detailed catalytic and regulatory mechanisms of this enzyme.

Human SSADH protein was generated from amino acid residues 48–535 (97). The N-terminal signal sequence (mitochondrial targeting) was removed to determine the 3D structure of the folded region of the protein. Data on the crystallized protein were collected at the 6C1(MXII) beamline of the Pohang Accelerator Laboratory using a QUANTUM 210 CCD detector (San Diego, CA). The crystals belonged to the space group F432 and had unit cell dimensions of $a = b = c = 265.706$. Information on three-dimensional structures and protein data bank codes for human SSADH are displayed in Table 6.

Table 6. 3D Structures and Protein Data Bank Codes of Human Succinic Semialdehyde Dehydrogenase

pdb code	*Type of protein*	*Form of protein*	*Resolution (Å)*
2w8n	Wild-type	Oxidized form	2.0
2w8o	Wild-type	Oxidized form	3.4
2w8p	C340A mutant	—	2.3
2w8q	C340A mutant	SSA bound form	2.4
2w8r	C340A mutant	SSA bound form	2.4

pdb, protein data bank (www.pdb.org/pdb/home).

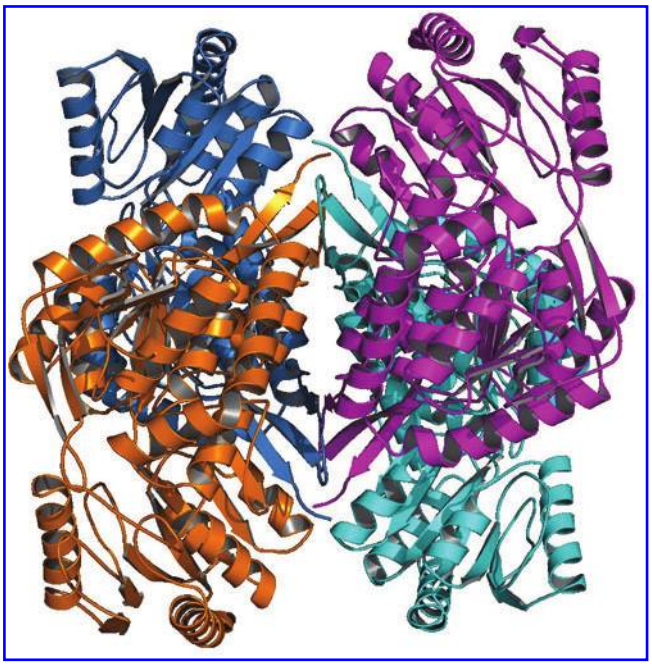

FIG. 13. Crystal structure of human SSADH. Shown is the tetrameric structure of human SSADH. SSADH functions as a tetramer as for other aldehyde dehydrogenases. The tetrameric structure is shown as a ribbon diagram showing one dimer in light blue and orange and the other dimer in magenta and cyan. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

B. Crystal structure of the oxidized form of human SSADH

The overall structure of SSADH shares the general fold of ALDH class 1 and 2 proteins (126, 182), and forms a tetrameric structure that can be generated by F432 crystallographic symmetry (Fig. 13). The monomeric SSADH protein is comprised of three domains: an N-terminal NAD-binding domain (residues 48–173, 196–307 and 509–524), a catalytic domain (residues 308–508), and an oligomerization domain (residues 174–195 and 525–535) (Fig. 14). The NAD-binding and catalytic domains are representative of α/β structures, whereas the oligomerization domain contains three-stranded antiparallel β-sheets (97).

The crystal structures of human SSADH, both in oxidized and reduced conditions, reveal that the protein is regulated *via* redox-switch modulation, so that the protein is active in the reduced condition while inactive in the oxidized condition. Moreover, when the environment is switched from oxidized to reduced conditions, the activity of the protein is recovered. Under oxidized conditions, a disulfide bond is observed between a catalytic Cys residue (Cys340) and an adjacent Cys residue (Cys342) (Fig. 15). In the oxidized form of human SSADH, a catalytic loop (RNTGQTCVCSN, residues 334 ~ 344) connecting α8 and β13 develops a conformation that completely blocks both the substrate and the cofactor binding sites (Fig. 16). The disulfide-bonded and

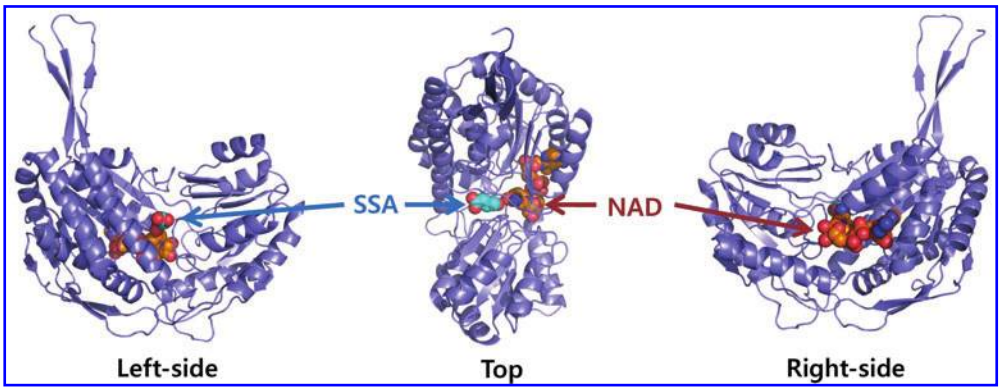

FIG. 14. Monomeric structure of human SSADH. A monomeric protein is presented in the asymmetric unit, and depicted as ribbon representation in which the substrate and NAD bound in the protein are represented with sphere model in cyan and orange colors, respectively. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

closed conformation of the catalytic loop results in an enzyme with an inactive state, both by oxidation of the catalytic residue Cys340, as well as through disabled entries of the substrate and cofactor to the active site (97).

C. Crystal structure of the reduced form of human SSADH

The structure of an active form of human SSADH was determined by supplementation with the strong reducing agent tris(2-carboxyethyl)phosphine during the crystallization procedure (97). In the reduced form, the disulfide bond is broken, and the thiol group of Cys340 moves toward the general base Glu306 constituting an active site environment as observed in other ALDHs (Fig. 17). The reduction of the enzyme also induces large structural changes in the catalytic loop, switching the loop to the open conformation, in which the substrate and the cofactor can obtain access. The crystal structure of C340A SSADH, a mutant that cannot form a disulfide bond even under strong reducing conditions, reveals that the dynamic catalytic loop of the mutant conforms to a structure that is identical to that of the reduced form of human SSADH. This indicates that disruption of the disulfide bond induces the structural alterations of the dynamic catalytic loop, and the open conformation is bioenergetically favored over the closed conformation when the disulfide bond is broken.

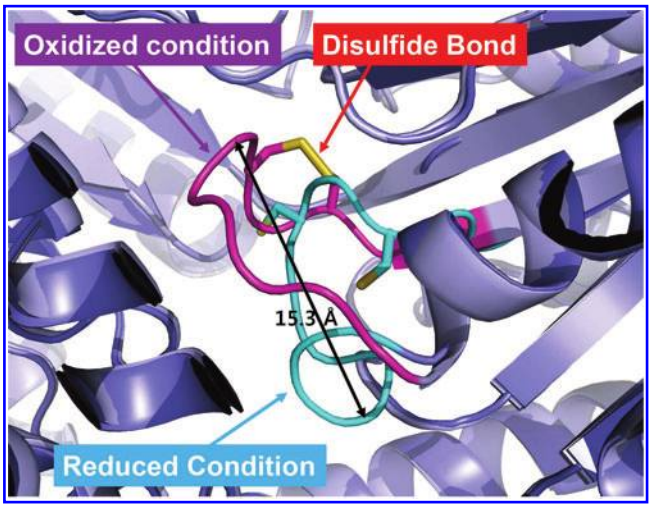

FIG. 15. Structural changes of the dynamic catalytic loop upon redox changes. Structural changes of the dynamic catalytic loop are shown in the figure. The catalytic loops of the oxidized and the reduced forms of the wild-type SSADH (colored magenta and cyan, respectively) are superimposed. The disulfide bond in the oxidized form, and the two cysteine residues in the reduced form of SSADH, are shown in a stick model and labeled accordingly. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Recently, Langendorf and colleagues (106) reported the structural characterization of SSADH encoded by the *Escherichia coli gabD* gene using X-ray crystallographic analysis. In *E. coli*, two genetically distinct forms of SSADH have been described that prevent any accumulation of intracellular SSA. Langendorf and colleagues (106) demonstrated convincing evidence for an electron density cluster in the active site of the enzyme for the cofactor $NADP^+$. Additional structural information revealed that deletion of three specific amino acids in *E. coli* SSADH (as compared to the human protein, which utilizes NAD^+) enables the enzyme to utilize $NADP^+$ and accommodate it within the active site. Both Kim *et al.* (97) and Langendorf and coworkers (106) have mapped a number of human missense mutations onto the human and *E. coli* SSADH proteins, with the specific amino acid residues involved in these mutations superimposing well between species.

D. Redox-switch modulation of human SSADH

Redox-mediated modification of cellular proteins confers a responsiveness to reactive oxygen species and changes in redox status that regulate the initiation of signal transduction pathways and the induction of gene expression across species (8, 34, 86). The redox-switch modulation of human SSADH observed in the crystal structure of the protein was comprehensively examined both *in vivo* and *in vitro*. For these studies, the properties of wild-type SSADH were compared with those of two Cys substituted mutants (C340A and C342A). The C340A is a mutant that is neither active nor able to form a disulfide bond, and the C342A is unable to form a disulfide bond but is still catalytically functional. No demonstrable SSADH activity was detected from all three forms without the presence of a strong reducing agent, indicating that SSADH is inactive under oxidative conditions, and returns to active status when the catalytic residue Cys340 is reduced in the presence of thiol (97).

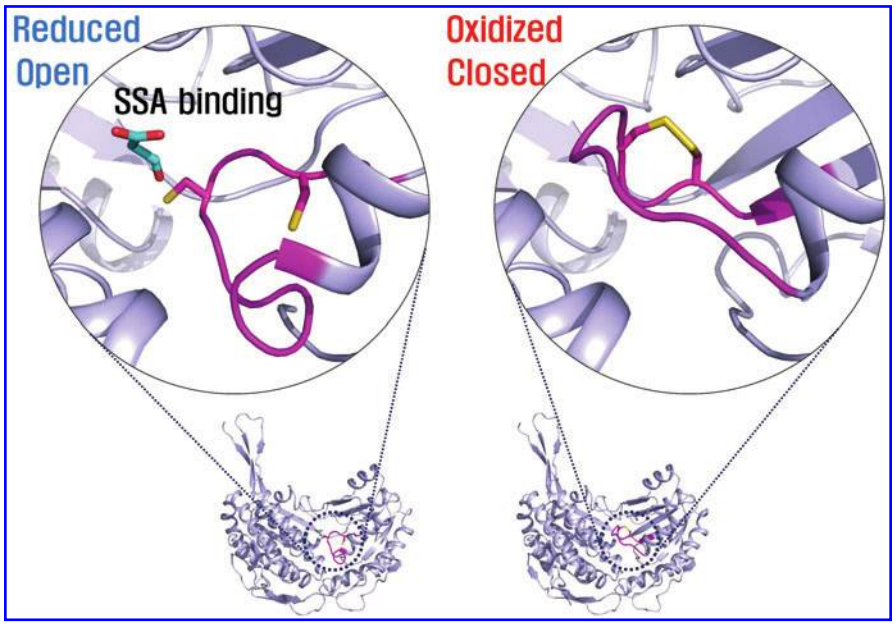

FIG. 16. Blockade of the SSA binding site by the dynamic catalytic loop in the oxidized form. The catalytic loop of the oxidized form of SSADH is presented with magenta color. SSA substrate bound to the reduced form of the protein is shown with stick model in cyan color. The disulfide bond formation in the oxidized form of SSADH blocks the binding of SSA. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

The data of Kim and colleagues (97) further demonstrated that human SSADH senses reactive oxygen species. When reduced wild-type SSADH was treated with hydrogen peroxide, the protein was almost completely inactivated, and recovered its activity when the environment was switched back to a reduced state. The results indicate that wild-type SSADH is inactivated by oxidative stress through a disulfide bond formation between the catalytic residue Cys340 and an adjacent Cys residue Cys342, which enables the enzyme to be reactivated upon the environmental return to reducing conditions. The sensitivity of the protein was further confirmed *in vivo*. These results suggest that the redox-switch modulation could be a physiological control mechanism for human SSADH (and likely other mammalian SSADHs), which regulates its activity in the relatively oxidized environment of the mitochondrial matrix and allows the enzyme to either respond to, or be protected from, oxidative stress. Considering the role of SSADH in the GABA metabolic pathway, the cellular concentration of GABA in human brain might be partially controlled by the redox status of the mitochondrial matrix as well as oxidative stress.

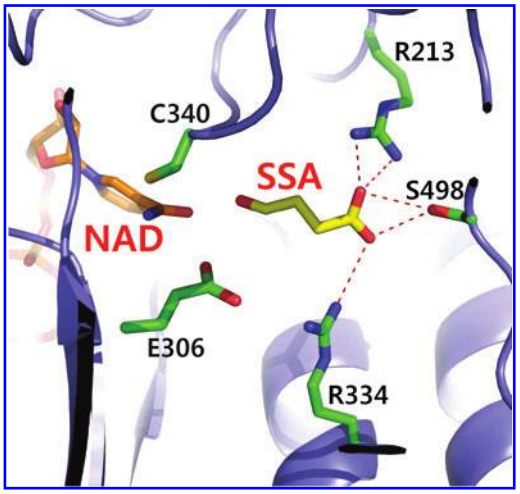

FIG. 17. Substrate binding properties of human SSADH. SSA and NAD are shown as a stick model with yellow and orange colors, respectively. Residues involved in the binding of SSA and catalytic residues are presented as stick model with green color, and labeled accordingly. The hydrogen bonds involved in SSA binding are presented with *red dotted lines*. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

E. SSA and NAD$^+$ binding specificity

Unlike other ALDHs that usually contain a hydrophobic tunnel to accept hydrophobic alkanal and aromatic aldehyde substrates, SSADH (employing a hydrophilic SSA substrate) shows a very high substrate specificity (157). The high fidelity substrate binding mode of the enzyme was demonstrated through evaluation of the crystal structure of the C340A SSADH mutant bound with its biological substrate, SSA. SSADH uses conserved hydrophilic residues such as Arg213, Arg334, and Ser498 to constitute an optimum environment to accept SSA as a substrate that contains the hydrophilic carboxyl end (Fig. 17) (97).

The binding mode of NAD$^+$ to the enzyme was elucidated by examining the crystal structure of the C340A SSADH mutant bound with NAD$^+$. While the ADP moiety of the NAD$^+$ molecule was added to the structure using the well-defined electron density, the nicotinamide ring and ribose portions of the cofactor could not be modeled as there was no electron density for them. This phenomenon is also observed in other ALDHs, in which the nicotinamide ring and ribose portions of the cofactor are mobile producing a poor electron density (40). In human SSADH, the adenine base is positioned in a hydrophobic pocket that is constituted by several conserved hydrophobic residues such as Ile201, Ala264, Gly268, and Leu292. For stabilization of the ribose ring, the hydroxyl groups of the ring are hydrogen bonded to the side chains of Lys228 and Glu231, and the backbone carbonyl group of

Thr202 (97). Diphosphate oxygen atom AO1 accepts hydrogen bonds from the side chains of Ser285 and Thr288. The NAD^+ molecule is unable to access its binding site in the closed form of the enzyme in which the Val341 residue located in the midst of the disulfide bond-forming two Cys residues moves 5.03 Å toward the NAD^+ binding site, resulting in a complete blockage of the tunnel in which the nicotinamide ring binds.

F. Nonredox regulation of E. coli *SSADH*

As is the case for human SSADH, an SSADH coding gene, *gabD*, is located within the *gab* operon in *E. coli*, which comprises *gabT* (γ-aminobutyrate transferase), *gabD* (SSADH), *gabP* (GABA permease), and *gabC* (a regulatory gene), and the products of the *gab* operon are involved in GABA degradation (9, 125, 132). *E. coli* SSADH (EcSSADH) shares 54% amino acid identity with human SSADH, and lacks an N-terminal signal sequence. EcSSADH is located in the cytosol and the GABA shunt functions to utilize GABA as the sole nitrogen source in *E. coli*. Because the location of the protein (cytosol *vs*. mitochondria) and function of the GABA shunt are different between human and *E. coli*, it was of interest to investigate whether the EcSSADH is redox-switch regulated as well, which was elucidated through analysis of the EcSSADH crystal structure.

The catalytic loop of EcSSADH, unlike that of human SSADH, does not undergo disulfide bond-mediated structural changes upon alterations in the environmental redox status (Fig. 18). Although EcSSADH contains two Cys residues (Cys289 and Cys291) at the corresponding positions of Cys340 and Cys342 of human SSADH, the two Cys residues do not form a disulfide bond under oxidized conditions, indicating that, unlike human SSADH, EcSSADH may not be redox regulated (1). Activity recovery assays employing recombinant proteins further confirmed the nonredox regulation of EcSSADH. Detailed structural analysis demonstrated that the catalytic loop of EcSSADH (RNAGQTCVCAN, residues 283 ~ 293) is more tightly stabilized than that of human SSADH (RNTGQTCVCSN, residues 334 ~ 344) with the surrounding environment, indicating that the redox-switch modulation is strongly related to structural stability.

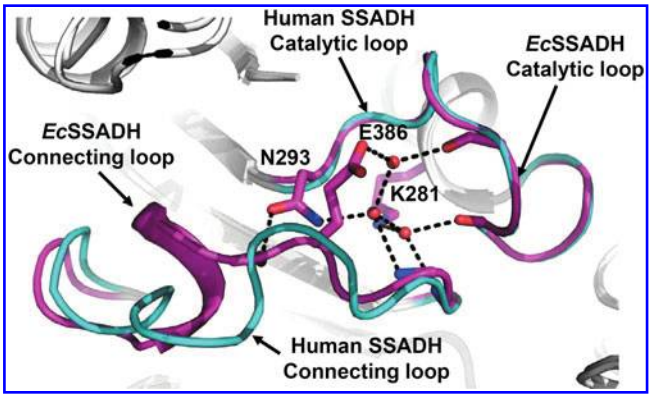

FIG. 18. Comparison of the catalytic loops between human and EcSSADH. Stabilization of the catalytic loops between human and EcSSADH is compared. Unlike human SSADH, the catalytic loop of *E. coli* SSADH is well stabilized by the strong hydrogen bonds with the neighboring loop, resulting in no disulfide bond formation. The catalytic loop and the connecting loop (β15–β16) of EcSSADH are shown in magenta, and those of human SSADH are in cyan. Three water molecules involved in a hydrogen bond network for the stabilization of the EcSSADH catalytic loop are shown in red spheres; hydrogen bonds that form between the catalytic loop and the connecting loop (β15-β16) are depicted with black dotted lines. EcSSADH = *Escherichia coli* SSADH. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Why do highly homologous and identical function proteins have different regulatory mechanisms? EcSSADH is located in the cytosol of *E. coli*, whereas human SSADH is located within the mitochondrial matrix, where the milieu is relatively more oxidizing as compared with the cytosol (85). Moreover, in *E. coli* the GABA shunt is proposed to function to utilize GABA as the sole nitrogen source, whereas in the human brain GABA is used as a main inhibitory neurotransmitter, and its cellular concentration needs to be tightly regulated. Accordingly, it would appear that the presence of a redox or nonredox regulatory scheme for SSADH is organism dependent, and that the choice of regulatory mechanism might be determined by the cellular localization and function of the protein in the organism.

VII. Conclusions and Final Remarks

In the early 1900s, Sir Archibald Garrod presented the now famous Croonian lectures describing inborn errors of metabolism (195). This innovative physician presented the first cohesive overview of disorders such as pentosuria, cystinuria, alkaptonuria, and albinism (among others) as heritable disorders of monogenic nature (172). SSADH deficiency represents yet another of the heritable monogenic disorders, as originally described by Garrod, but it is clear for this disorder (as for many other heritable diseases) that monogenic translates into exceptionally complex metabolic and molecular phenotypes and outcomes. Clinicians, biochemists, molecular biologists, protein chemists, neuroscientists, and crystallographers have now contributed substantively to our understanding of this disorder, revealing a level of complexity, regulation, and functional alterations well beyond the GABA system, to include monoamines (202), intermediary metabolism, bioenergetics, oxidative stress mechanisms, neurosteroid alterations (14, 78, 176), secondary effects on amino acid catabolism, and a host of other processes. This seems to be the norm today for most monogenic disorders (for example, heritable phenylketonuria) (171), and accumulating evidence is revealing the interrelatedness of multiple metabolic pathways [*e.g.*, fat metabolism and oxidative phosphorylation (186)]. Where does this disease complexity leave us with regard to patient treatment and quality of life in SSADH deficiency? Unfortunately, we are still working our way forward in the pathophysiology of this disease, and trying to bring seemingly unrelated data sets together to form a cohesive treatment strategy. This approach may remain, for years to come, our best way of understanding and treating this seemingly straightforward monogenic disorder of GABA metabolism.

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Abbreviations Used

3βHSD = 3-β-hydroxysteroid dehydrogenase
4-HNE = 4-hydroxynonenal
AGAT = arginine:glycine amidinotransferase
ALDH = aldehyde dehydrogenase
Aldh5a1 = aldehyde dehydrogenase 5a1
Ca = calcium
cAMP = cyclic adenosine monophosphate
Cl = chloride
CNS = central nervous system
CSF = cerebrospinal fluid
Cys = cysteine
D-2-HG = D-2-hydroxyglutarate
DA = dopamine
DHA = docosahexaenoic acid
DHHA = 4,5-dihydroxyhexanoic acid
ECoG = electrocorticography
EcSSADH = *Escherichia coli* succinic semialdehyde dehydrogenase
EEG = electroencephalogram
GABA = gamma-aminobutyric acid
GAT = GABA transporter
GHB = gamma-hydroxybutyric acid
GSH = glutathione
HC = homocarnosine
IPSC = inhibitory postsynaptic current
KD = ketogenic diet
LF = left-frontal
LP = left-parietal
MAPK = mitogen-activated protein kinase
MRI = magnetic resonance imaging
MSLT = multiple sleep latency test
REM = rapid eye movement (sleep)
RF = right-frontal
RP = right-parietal
SCoA = coenzyme A
SSA = succinic semialdehyde
SSADH = succinic semialdehyde dehydrogenase
TBPS = tert-butylbicyclophosphorothionate
TCA = tricarboxylic acid
TMS = transcranial magnetic stimulation
UTR = untranslated region

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