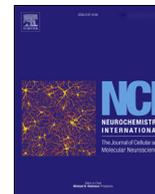




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Mammalian CSAD and GADL1 have distinct biochemical properties and patterns of brain expression

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ABSTRACT

Variants in the gene encoding the enzyme glutamic acid decarboxylase like 1 (GADL1) have been associated with response to lithium therapy. Both GADL1 and the related enzyme cysteine sulfinic acid decarboxylase (CSAD) have been proposed to be involved in the pyridoxal-5'-phosphate (PLP)-dependent biosynthesis of taurine. In the present study, we compared the catalytic properties, inhibitor sensitivity and expression profiles of GADL1 and CSAD in brain tissue. In mouse and human brain we observed distinct patterns of expression of the PLP-dependent decarboxylases CSAD, GADL1 and glutamic acid decarboxylase 67 (GAD67). CSAD levels were highest during prenatal and early postnatal development; GADL1 peaked early in prenatal development, while GAD67 increased rapidly after birth. Both CSAD and GADL1 are being expressed in neurons, whereas only CSAD mRNA was detected in astrocytes. Cysteine sulfinic acid was the preferred substrate for both mouse CSAD and GADL1, although both enzymes also decarboxylated cysteic acid and aspartate. *In silico* screening and molecular docking using the crystal structure of CSAD and *in vitro* assays led to the discovery of eight new enzyme inhibitors with partial selectivity for either CSAD or GADL1. Lithium had minimal effect on their enzyme activities. In conclusion, taurine biosynthesis in vertebrates involves two structurally related PLP-dependent decarboxylases (CSAD and GADL1) that have partially overlapping catalytic properties but different tissue distribution, indicating divergent physiological roles. Development of selective enzyme inhibitors targeting these enzymes is important to further dissect their (patho)physiological roles.

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1. Introduction

Lithium salts are among the most effective pharmacological agents used in psychiatry. Although several molecular targets have been identified, including protein kinases and enzymes involved in phosphoinositide metabolism, lithium's mode of action at the

cellular and molecular level is still being debated (Malhi et al., 2013). A strong genetic association between variants in the human glutamic acid decarboxylase like 1 (GADL1) gene and the response to lithium therapy in bipolar patients was recently reported (Chen et al., 2014). Although these findings were not replicated in other clinical samples (Cristiana et al., 2015), these results are intriguing and warrants a biochemical investigation on GADL1 and the effects of lithium on this enzyme.

Human GADL1 was recently found to function as a cysteine sulfinic acid decarboxylase and postulated to be involved in taurine and possibly also β -alanine and carnosine production *in vivo* (Liu et al., 2012). The sulfur amino acid taurine (2-amino-ethanesulfonic acid) is abundant in mammalian tissues and has been implicated in many physiological functions. Taurine has a regulatory role in maintenance of osmotic pressure and preservation of structural integrity of biological membranes (Hoffmann and

Abbreviations: AADC, Aromatic amino acid decarboxylase; ADC, Aspartate decarboxylase; APS-1, Autoimmune polyendocrine syndrome type 1; BSA, Bovine serum albumin; CA, Cysteic acid; CDO, Cysteine oxidase; CSA, Cysteine sulfinic acid; CSAD, CSA decarboxylase; GABA, Gamma-amino-butanoic acid; GAD, Glutamate decarboxylase; GADL1, Glutamate decarboxylase like 1; HDC, Histidine decarboxylase; ITT, *in vitro* transcription/translation system; OPA, o-phthalaldehyde; MBP, Maltose-binding protein; PCW, Post conception week; PLP, Pyridoxal-5'-phosphate; TEV, Tobacco Etch Virus protease.

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Pedersen, 2006; Schaffer et al., 2010). In the nervous system, taurine may modulate protein phosphorylation (Lombardini, 1994), serve as a trophic factor (Hernandez-Benitez et al., 2010; Pasantes-Morales and Hernandez-Benitez, 2010), or act as a neurotransmitter/neuromodulator (Jia et al., 2008). In several species, taurine deficiency has been linked to specific disease states (Schaffer et al., 2010) and also in humans dietary intake of taurine in the form of energy drinks or vitamin supplements is widespread, although with unclear health implications (Bigard, 2010).

In mammalian tissues taurine is mainly synthesized from cysteine in a three step sequential pathway, involving oxidation by cysteine dioxidase (CDO, E.C. 1.13.11.20), decarboxylation by cysteine sulfenic acid decarboxylase (CSAD, E.C. 4.1.1.29) and finally oxidation of hypotaurine to taurine. Alternatively, taurine may be formed from cysteamine by cysteamine dioxygenase (E.C. 1.13.11.19). The tissue distribution of the various enzymes involved in cysteine metabolism seems to reflect different metabolic demands of these tissues (Stipanuk et al., 2006). Thus, the protein levels of liver CDO, which is rate limiting in the degradation of the potentially toxic amino acid cysteine are tightly regulated in response to cysteine load (Stipanuk et al., 2009).

In comparison, the physiological role and regulation of CSAD and GADL1 are less understood. Mammalian CSAD has been isolated from liver, kidney and brain where it exists as a dimer with a reported subunit molecular mass of approx. 43–55 kDa (Heinamaki et al., 1982; Tang et al., 1996; Tappaz et al., 1998). GADL1 is expressed in muscle and kidney tissue (Liu et al., 2012). However, its pattern of expression in other tissues, including brain is not known. CSAD and GADL1 belong to a large family of pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyze a range of different reactions, such as decarboxylation, transamination, racemization or eliminations using amino acids or related substrates (Toney, 2011). Crystal structures of many PLP-dependent enzymes, including CSAD, have been published (<http://www.rcsb.org/pdb/explore/explore.do?structureId=2JIS>).

In the brain, CSAD has mainly been detected in astrocytes in cerebellum and hippocampus (Reymond et al., 1996a), although there are also reports of CSAD being found in neurons (Chan-Palay et al., 1982). Based on the different tissue distribution of CDO and CSAD, it was proposed that the taurine synthesis pathway is initiated in neurons and completed in astrocytes (Dominy et al., 2004). More recently, taurine biosynthesis from cysteine in murine neurons and astrocytes was reported, indicating that the complete enzymatic machinery for taurine synthesis is present in both cell types (Vitvitsky et al., 2011). However, the identity of the enzymes involved in the synthesis in the two cells types is not known.

A CSAD knockout mouse was recently described (Park et al., 2014). The plasma levels of taurine were reduced by 83% in CSAD^{-/-} mice and most offspring from 2nd generation CSAD^{-/-} mice died shortly after birth, indicating an important physiological role of CSAD.

The aims of our study were (i) to determine the effects of lithium on GADL1 and CSAD, (ii) to compare the substrate specificities of these enzymes, to use this knowledge to find inhibitors of the enzymes and (iii) to study their cellular, regional and temporal patterns of expression in the mammalian brain.

2. Experimental procedure

2.1. Source of materials

Chromatography materials for enzyme purification were purchased from GE Healthcare Life Sciences (Uppsala, Sweden), unless otherwise indicated, and all other reagents were from Sigma (St Louis, MO, USA).

2.2. Molecular modeling/docking of substrates in GADL1 and CSAD

To determine the structural relationships of GADL1, CSAD, and other decarboxylases, we created a homology model of GADL1. The sequence of GADL1 was aligned with that of CSAD in DeepView (Guex and Peitsch, 1997) and submitted to the Swiss-Model server (Schwede et al., 2003) to prepare a homology model of GADL1 (see Fig. 2). A virtual library of 8 million commercially available compounds was obtained from the ZINC database (Irwin et al., 2012) and docked into the active site of CSAD with the Glide software (Friesner et al., 2004) from Schrodinger[®]. A grid centered on the PLP cofactor in the CSAD binding site was defined with dimensions 17 Å in all three dimensions. The compounds were initially docked following the high throughput virtual screening protocol. The top 100,000 compounds were redocked following the standard precision protocol. Finally, the 10,000 top scoring compounds from this procedure were docked into the active site with the extra precision (Friesner et al., 2006) protocol.

2.3. Expression vectors

Multiple mRNA transcripts of CSAD and GADL1 have been described, probably due to alternative initiation codons and splicing events (Tappaz et al., 1999). The UniProt database (<http://www.uniprot.org/uniprot/>) lists three human CSAD sequences with 346–520 amino acids, two human GADL1 isoforms with 418–521 amino acids and two mouse GADL1 isoforms with 526–550 amino acids. We obtained cDNA clones corresponding to the 550 amino acids (62 kDa; Q80WP8-2) isoform of mouse GADL1

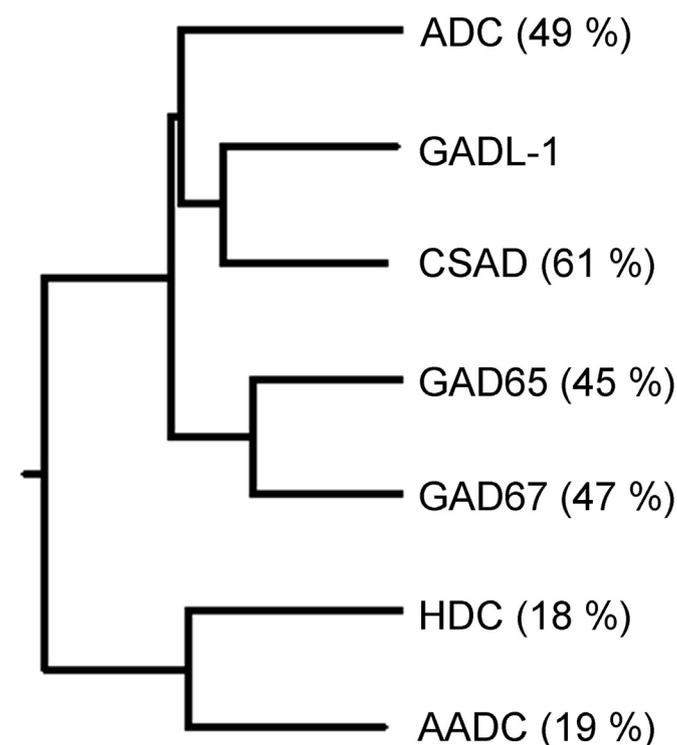


Fig. 1. Phylogenetic tree of PLP-dependent decarboxylases. Amino acid sequences of PLP-dependent decarboxylases were aligned using ClustalW version 2 software (2007) (Larkin et al., 2007). The phylogenetic tree was constructed by the neighbor-joining method based on alignment using the data base accession numbers: Q80WP8 (GADL1), Q9DBE0 (CSAD), P48318 (GAD1), P48320 (GAD2), O88533 (AADC), P23738 (HDC), A7U8C7 (ADC). The percentages of the amino acid sequence similarity to GADL1 are given in parenthesis. All the sequences are from *Mus musculus*, except for ADC, which is from *Tribolium castaneum*.

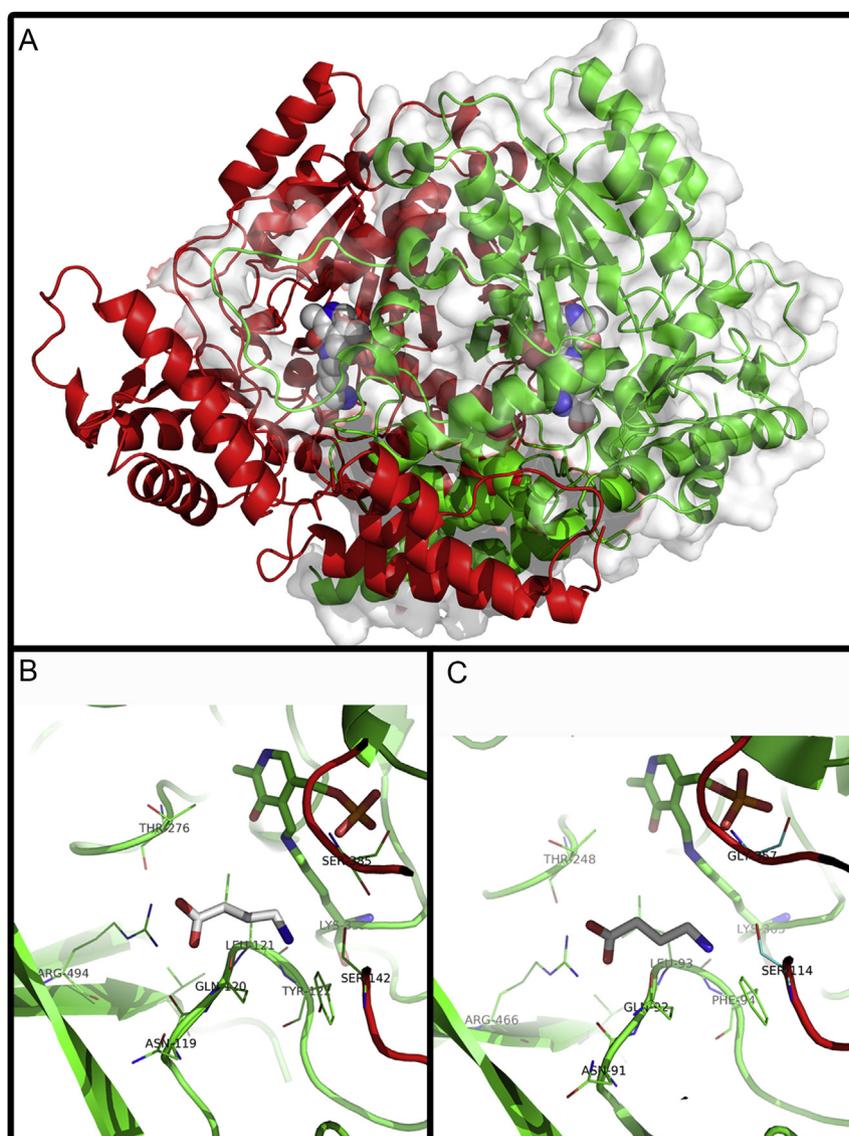


Fig. 2. **A** Homology model of dimeric GADL1 based on CSAD (pdb code 2JIS). One of the subunits (colored green) is represented with transparent surface to get an impression of the overall structure. The PLP cofactor is represented by spheres with size corresponding to the Van der Waal radius, colored by atom type and covalently linked to Lys333 (not shown) in the active site. **B** Representation of the putative substrate binding site in GADL1 with highlighted amino acids in close proximity to the substrate. PLP is shown with a covalent bond to Lys333. The model of GADL1 with GABA in the putative substrate binding site was prepared by aligning the GADL1 homology model with the coordinates of GAD67 in complex with GABA (pdb code 2OKJ). **C** Representation of the substrate binding site in CSAD with highlighted amino acids in close proximity to the substrate; Arg466, Gln92 and Leu93. PLP is shown with a covalent bond to Lys305. The model of CSAD with GABA in the putative substrate binding site was prepared by aligning the CSAD coordinate file (2JIS) with the coordinates of GAD67 in complex with GABA (pdb code 2OKJ).

and the 493 amino acids (55 kDa; Q9DBE0) isoform of mouse CSAD. Three different expression vectors were used using maltose-binding protein (MBP) as a fusion partner; pETM41: for 6×His-MBP-CSAD and 6×His-MBP-GADL1 expression in *Escherichia coli* and pCMV-SPORT 6: for expression of native CSAD and pCRII-Topo for native GADL1 in an *in vitro* transcription/translation system (ITT), essentially as described previously (McKinney et al., 2005). The sequences of all CSAD and GADL1 expression clones were verified by DNA sequencing.

2.4. Cell free transcription and translation of CSAD and GADL1

A rabbit reticulocyte lysate (Promega, Madison, WI) was used to express CSAD and GADL1 where 1 µg of plasmid DNA per reaction (50 µL) and 1 h incubation (30 °C) was used. Enzyme activity was

assayed immediately following removal of unincorporated amino acids by gel filtration using 0.5 mL pre-packed Zeba™ spin columns (Pierce, Rockford, IL, USA), which were preincubated with buffer: 20 mM NaHepes 200 mM NaCl and 10% glycerol. Specific expression levels were determined after gel filtration and separation on 12% SDS-PAGE by integration of [³⁵S] methionine labeled CSAD and GADL1 band intensities using the Personal Molecular Imager™ System and Quantity One Software v. 4.5.2 (Bio-Rad, Hercules, CA, USA).

2.5. Analysis of CSAD and GADL1 expressed in *E. coli*

Heterologous expression of CSAD and GADL1 fusion proteins in *E. coli* was essentially performed as described (Winge et al., 2007) except for the following changes: BL21-CodonPlus (DE3)-RIPL

(Stratagene, La Jolla, CA) was used as expression host and the induction temperature was reduced to 20 °C. Cell pellets were lysed by sonication in 20 mM Hepes pH 7.4, 200 mM NaCl, 10% glycerol, 10 mM benzimidazole, 1 mg/mL lysozyme, 1 µg/mL pepstatin A, 4.6 µg/mL leupeptin. PMSF was added immediately following sonication to a concentration of 1 mM. The cell lysate was fractionated into soluble and insoluble parts by centrifugation at 10,000 × g for 30 min. An equal volume of lysis buffer was added to the pelleted fraction and homogenized. Specific expression levels of fusion proteins were determined by analyzing equal amounts (30 µg) of the soluble fractions by separation on 12% SDS-PAGE. Coomassie stained 6 × HisMBP-CSAD and GADL1 fusion protein band intensities were integrated using the VersaDoc MP 4000 imaging system and Quantity One Software (Bio-Rad, Hercules, CA, USA).

2.6. Activity assays

Activities of CSAD, GADL1 and GAD65 were measured using both ITT-expressed protein and *E. coli* expressed protein. A reaction mixture of 100 µl containing varying amounts of purified recombinant proteins and amino-acids was prepared in 60 mM potassium phosphate (pH 7.4) containing 5 mM DTT, 50 mM sucrose and 0.5 or 100 µM PLP. Adding 1 mM of the amino acid substrate started the reaction. Samples of 30 µl were taken at 15–60 min and the reaction stopped by addition of an equal volume of ice-cold ethanol with 5% acetic acid. The samples were then centrifuged at 15,700 × g for 10 min before the supernatant was being transferred to a microtiter plate and analyzed by HPLC. Samples were diluted to 50% in solvent (24% ethanol in 50 mM Na-phosphate pH 6.0), and 4.2% *o*-phthalaldehyde (OPA)-reagent was added before injection into a Zorbax Eclipse XDB-C18 column. Determination of product in the reaction-mixture was based on fluorescence detection of OPA-bound amino acid at 366/455 nm.

In order to perform inhibition assays on the two enzymes, different concentrations of the inhibitors were tested (0.001–50 mM). The assay was performed as described above at 37 °C for 60 min and stopped by addition of equal volume of ice-cold ethanol with 5% acetic acid.

2.7. Gene expression assays

Total RNA was obtained from Clontech laboratories, Mountain View, California, USA (brain, liver, muscle and kidney) and Sciencell, Carlsbad, USA (neurons and astrocytes). Total cDNA was obtained using High Capacity RNA-to-cDNA kit from Applied Biosystems (Foster city, CA). Gene-specific primers (Suppl. Table 1), TaqMan[®] Minor Groove Binder (MGB) probes and Assay-on-Demand[™] PCR reagents were from Applied Biosystems and were designed to be gene-specific and target all reported splice variants of GADL1 and CSAD (Suppl. Table 2). Each real-time PCR reaction was run in triplicate and contained 1 µl TaqMan[®] primer/probe. Cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial diluted standards were used to prepare a standard curve, which was run on the same plate and used to calculate relative gene expression abundance. The results were quantified by the $\Delta\Delta C_t$ method using the housekeeping genes GAPDH and β -actin as endogenous controls to adjust for unequal amounts of RNA and efficiency of cDNA synthesis. None of the samples showed signs of DNA contamination when reverse transcriptase was omitted from the cDNA reaction. The relative gene expression levels are presented as $2^{\Delta\Delta C_t}$ using CSAD as the control.

2.8. Western blot analysis

Frozen mouse tissues (200 µg) were lysed in ice-cold lysis buffer containing protease inhibitors. The lysates were centrifuged at 16,000 × g for 10 min at 4 °C. The supernatants were collected and stored at –80 °C. Protein concentrations were determined and 20 µg of total protein or 10 ng of purified CSAD or GADL1 protein were analyzed on 10% TGX Stain-Free gels (Bio-Rad) for 45 min at 20 mA/gel. The gel was activated and imaged (see “Imaging and data analysis” section below). The activated gel was transferred to an Immun-Blot nitrocellulose membrane (Bio-Rad) in 7 min using the Trans-Blot Turbo Transfer System (Bio-Rad) with Trans-Blot Turbo Midi Transfer Packs. Pre-made western blots of protein samples from mouse brain sampled at 11 different developmental stages (MW-201-D) and adult brain tissues from 14 different species (AW-201) were also obtained from Zymogen Laboratories, San Diego, CA. The membranes were blocked with 5% bovine serum albumin (BSA), 1% glycine and 0.1% Tween-20 in Tris-buffered saline and incubated with custom made affinity purified sheep antibodies generated against purified human CSAD or human GADL1 MBP fusion proteins (James Hastie, University of Dundee). To avoid cross reactivity against these related proteins, the GADL1 antibodies were filtered against purified CSAD and the CSAD antibodies were filtered against purified GADL1. Antigens were detected using horseradish peroxidase-conjugated goat anti-sheep antibody (1:10,000) (Bio-Rad Laboratories, Hercules, CA) as a secondary antibody and enhanced chemiluminescence detection.

We also tested commercial rabbit antibodies against either human CSAD (Sigma–Aldrich HPA039487; 1:2,000, immunogen peptide corresponding to amino acids 52–110 in the CSAD protein sequence), anti-GADL1 (Sigma–Aldrich SAB2103888; 1:1000, immunogen 159–208) or anti-GADL1 (Thermo Scientific, Rockford, IL, PA5-13434; 1:100, immunogen 224–254). The Sigma anti-Glutamic Acid Decarboxylase 65/67 (Anti-GAD65/67) antibody from rabbit (G5163, Immunogen human GAD67 amino acids 579–594) was also used (1:10,000). The commercial CSAD antibodies all reacted against the respective MBP fusion proteins, as well as with the cleaved purified enzymes. Although the antibodies were raised against three different immunogens and marketed to be specific against either of these enzymes, they all showed some cross reactivity with the other enzyme (Suppl. Fig. 1). This lack of specificity is also reflected in the recently published Human protein atlas (<http://www.proteinatlas.org/>), where two commercially available antibodies against each of GADL1 and CSAD show multiple protein bands and little internal consistency.

This is in contrast to our custom made sheep CSAD and GADL1 antibodies that were specific for their respective proteins and were judged to be more reliable in studies of tissues with low levels of expression of these proteins. None of the antibodies showed cross reactivity against hGAD1, whereas the commercial antibody raised against GAD67 showed some cross reactivity against both CSAD and GADL1 (Suppl. Fig. 1).

2.9. Imaging and data analysis

Housekeeping genes as GAPDH are being expressed at different levels in the different tissues (Eaton et al., 2013). It is therefore difficult to find an antibody to use as a loading control when comparing different tissues. Using TGX Stain-Free gels (Bio-Rad) we were able to normalize the western blots with total protein as described in Gurtler et al. (Gurtler et al., 2013). CSAD and GADL1 signals were automatically normalized using ImageLab software with either the intensity values of GAPDH or Stain-Free total lane volumes. The normalized datasets were used for the calculation of CSAD/GADL1 levels. In addition, t-tests (double-sided and paired)

were performed with Microsoft Office Excel for Mac 2011 to evaluate the statistical significance of the calculations.

3. Results

Genome databases contain many sequences within the family of the PLP-dependent enzymes, including GAD65 and GAD67, aromatic amino acid decarboxylase (AADC), histidine decarboxylase (HDC), aspartate decarboxylase (ADC), CSAD and GADL1. Although the terminology “GADL1” probably is based upon its sequence similarity to GAD (45–47% amino acid identity), a sequence comparison shows that it is more similar to ADC (49%) and to CSAD (61%) (Fig. 1). The three-dimensional structure of GADL1 is not known. However, the structures of human CSAD (pdb accession code 2JIS), GAD67 and GAD65 (pdb codes 2OKJ and 2OKK, respectively) are deposited in the protein data bank. This information can be used to model the structure of GADL1.

3.1. Homology modeling

A homology model of GADL1 was prepared with CSAD as template in SwissModel <http://www.rcsb.org/pdb/explore/explore.do?structureid=2JIS> (Fig. 2A). The structure of CSAD was solved in complex with the PLP, without substrate present. However, the structures of GAD67 and GAD65 are of the ternary complexes with both cofactor and product (gamma-amino-butanoic acid, GABA) present in the proposed substrate-binding site. Superimposition of our GADL1 homology model and CSAD with GAD65 and GAD67 thus provided a structural model of GADL1 and CSAD in ternary complex with cofactor and GABA in the postulated substrate-binding site (Fig. 2B and C).

Inspection of this model revealed some amino acid residues that may be critical for enzyme function. A Schiff base linkage (internal aldimine) between PLP and Lys405 of the active site in GAD65 has been described (Fenalti et al., 2007). In our homology model of GADL1 PLP is covalently attached to Lys333. Furthermore, His219 of GADL1 is involved in base stacking with PLP. This residue corresponds to the conserved base-stacking residue His191 in CSAD and His 291 in GAD67 (Qu et al., 1998). This residue is found in two possible conformations in CSAD and the equivalent His in GAD65/67 is postulated to be involved in regulation of enzymatic activity through interactions with the activation loop.

The activation loop of human GAD65 comprises a stretch of 11 residues from Ala432 to Tyr442 (Ala-Gly-Tyr-Leu-Phe-Gln-Pro-Asp-Lys-Gln-Tyr) with Tyr434 being involved in catalysis. This stretch of amino acids corresponds to 361–371 in GADL1 (Ala-Ser-Tyr-Leu-Phe-Gln-Gln-Asp-Lys-Phe-Tyr) including the conserved catalytic tyrosine residue, Tyr363 in GADL1 (corresponding to Tyr335 in human CSAD and Tyr322 in human AADC).

The carboxyl group of the product (GABA) in GAD67 is found to form a salt bridge with Arg567 and a hydrogen bond with Gln190 (Fenalti et al., 2007). These two residues correspond to Arg494 and Gln120 in our homology model and are thus likely to be involved in interactions with the GADL1 substrate. Furthermore, the structure of GAD from *E. coli* in complex with the substrate analogue glutamate points to Thr62 (corresponding to Gln120 in GADL1) as important for substrate binding.

3.2. GADL1 and CSAD form dimers in solution

Using cDNA of the coding reading frame from mouse, the GADL1 and CSAD sequences were cloned into vectors for both mammalian and bacterial expression. The proteins expressed in bacteria were fused to MBP to increase their solubility and facilitate purification. They were expressed and purified as described in Experimental

Procedures. When purifying the proteins on a gel-filtration column after cleavage with TEV, CSAD and GADL1 had apparent molecular masses of 115 kDa and 137 kDa, respectively, suggesting that they are both present as dimers. Although both proteins have a tendency to aggregate in solution, it appears that GADL1 has the highest solubility (results not shown). When analyzed on SDS-PAGE, CSAD and GADL1 appear at almost the same molecular weight, i.e. as 99 kDa as fusion proteins (MBP-CSAD or MBP-GADL1) or as cleaved proteins at 56 kDa (CSAD) and 59 kDa (GADL1), respectively (MBP has a molecular weight of 45 kDa) (Suppl. Fig. 1).

3.3. GADL1 and CSAD have overlapping substrate specificities

When we performed activity assays with the standard protei-nogenic amino acids and some abundant occurring dicarboxylic acids as substrates for CSAD, GAD and GADL1, few compounds displayed significant activity with either enzyme (Suppl. Table 3). However, cysteic acid (CA), homocysteic acid, CSA and Asp were decarboxylated by both GADL1 and CSAD at different rates (Fig. 3, Table 1 and data not shown). Notably, no production of GABA from glutamate was observed for either CSAD or GADL1, whereas, as expected, GAD catalyzed this reaction (data not shown). These data suggest that mouse CSAD and GADL1 are more similar to each other than they are to GAD, as the sequence comparison also suggests (Fig. 1).

3.4. Kinetic properties of CSAD and GADL1

Using different concentrations of CSA and Asp, we determined the kinetic properties of CSAD and GADL1 (Table 1). Whether analyzed as fusion proteins with MBP or as pure, cleaved enzymes, the activity towards CSA was higher for CSAD than for GADL1 ($p = 0.0001$), whereas GADL1 had much higher V_{max} values than CSAD for decarboxylation of Asp ($p = 0.003$). For both substrates, the fusion proteins and the purified cleaved enzymes had similar K_m values, but the V_{max} values were highest for the pure decarboxylases. Using CSA as substrate, the specificity constants (K_{cat}/K_m) were much higher for CSAD than for GADL1, whereas the opposite was true using Asp as substrate. The K_m value for decarboxylation of

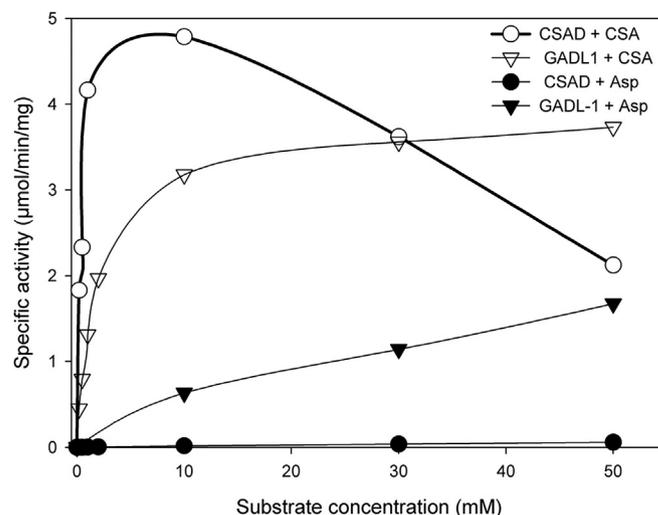


Fig. 3. Substrate dependence and inhibition of CSAD and GADL1. Substrate dependence of purified GADL1 (triangles) and CSAD (circles) using CSA (open symbols) or Asp (filled symbols) as substrates ($n = 10$). The assay was performed as described above at 37 °C for 60 min and stopped by addition of equal volume of ice-cold ethanol with 5% acetic acid.

Table 1
Kinetic parameters of recombinant CSAD and GADL1 using CSA and aspartate as substrates. Activity assays were performed as described in the method-section. The results are presented as means \pm S.E.M. of three separate measurements using different enzyme preparations. The kinetic values were calculated based on predicted subunit masses of 55 kDa (493 aa) for mouse CSAD, 57 kDa (502 aa) for mouse GADL1, 100 kDa for mouse MBP CSAD and 102 kDa for MBP GADL1. Human CSAD has a predicted mass of 55,023 kDa (493 aa) and human GADL1 is predicted to be 59 246 kDa (521 aa). *Substrate inhibition, K_{Si} CSA: 26 \pm 10 mM for cleaved CSAD.

Enzyme form		CSA (0–50 mM)				Aspartate (0–50 mM)			
		V _{max} (μmol/min/mg)	K _m (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (mM ⁻¹ s ⁻¹)	V _{max} (μmol/min/mg)	K _m (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (mM ⁻¹ s ⁻¹)
Fusion proteins	CSAD	0.33 \pm 0.046*	0.14 \pm 0.11	0.55	3.9	0.025 \pm 0.003	23.0 \pm 6.7	0.04	0.0017
	GADL1	0.74 \pm 0.055	1.74 \pm 0.56	1.3	0.75	0.66 \pm 0.02	21.3 \pm 1.5	1.2	0.056
Cleaved proteins	CSAD	7.22 \pm 1.26*	0.20 \pm 0.06	6.6	33	0.16 \pm 0.027	93 \pm 32	0.14	0.0015
	GADL1	3.84 \pm 0.034	1.12 \pm 0.06	3.6	0.94	1.41 \pm 0.10	31.7 \pm 4.7	1.3	0.041

CSA by recombinant mouse CSAD (0.14–0.20 mM) was similar to that reported for purified rat liver CSAD (0.17 mM) (Guion-Rain and Chatagner, 1972). Although the K_m value for decarboxylation of CSA by mouse GADL1 (1.12–1.74 mM) was similar to recently reported values for human GADL1 (1.14 mM) (Liu et al., 2012), the mouse enzymes had 2–3 fold higher specific activities (Table 1). Interestingly, the K_m values were much higher and the specificity constants for decarboxylation of Asp were much lower for mouse GADL1 and CSAD than reported for the recombinant human GADL1 (Liu et al., 2012). A comparison of these constants revealed that compared to GADL1, CSAD had a 1000-fold higher selectivity towards CSA. A pronounced substrate inhibition by CSA was apparent for CSAD (K_{Si} = 26 \pm 10 mM), but this was not observed for GADL1 in this substrate concentration range (Fig. 3). Both GADL1 and CSAD catalyzed decarboxylation of CA to taurine, at rates of 12–13% of that observed for the conversion of CSA to hypotaurine (data not shown). Using equimolar concentrations of CA and CSA (1 mM), CA decarboxylation by the competing substrate was inhibited by 49–56% for both enzymes, while the CSA decarboxylation was inhibited by only 5–15% for GADL1 and CSAD, respectively; illustrating that CSA is the preferred substrate for both enzymes.

3.5. Effects of metal ions

As reported for pig brain CSAD (Tang et al., 1996), mouse CSAD was activated by addition of manganese ions (52% stimulation in the presence of 0.4 mM MnCl₂, p = 0.003), but this effect was not present for GADL1 using similar assay conditions. The enzyme activities of CSAD and GADL1 were essentially unaffected by Li⁺ using final concentrations of LiCl from 0.05 to 40 mM, however, for GADL1 a weak stimulation was observed in the presence of 0.2–0.4 mM Li⁺ (p = 0.03) (Fig. 4A).

3.6. Inhibitors of CSAD and GADL1

To further characterize the substrate and inhibitor specificities of GADL1 and CSAD, we performed *in silico* docking into the active site of CSAD of a subset of the ZINC database of different compounds [28] and tested the top 20 compounds *in vitro*. As shown in Table 2 and Fig. 4B, bis-carboxymethyl-trithiocarbonate (Compound A) was the compound with highest affinity for both enzymes (K_i \approx 70 and 60 μM for CSAD and GADL1, respectively). Removing the two terminal carboxyl oxygens and substituting the adjacent thioether into an ether increased the selectivity for GADL1 over CSAD (compound B). Removing the central thioether of Compound A gave rise to Compound C with selectivity for CSAD over GADL1, although at a significantly lower affinity for both. Compound E was the most selective CSAD inhibitor of the compounds tested. Although none of the compounds were potent inhibitors of either enzyme, their relative selectivity might be exploited to produce even more specific compounds.

Glutamate was completely inactive as inhibitor of either CSAD

or GADL1, indicating that none of these enzymes are involved in glutamate metabolism. In contrast to the recently reported insensitivity of CSAD towards inhibition by L-cysteine [40], the latter compound completely inhibited the CSAD activity (>95%) of both

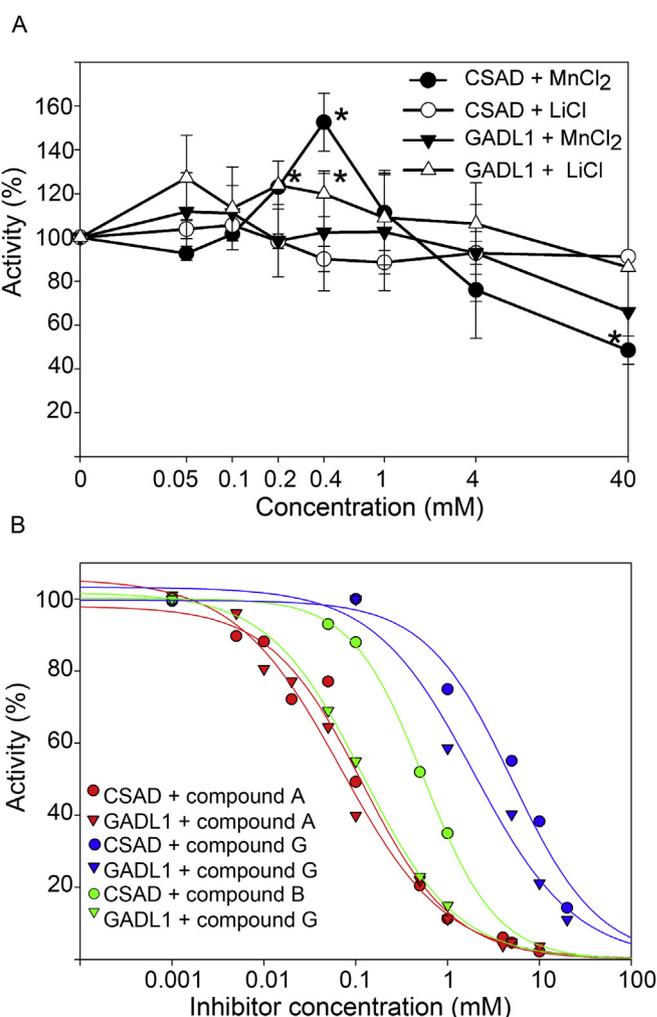
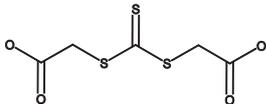
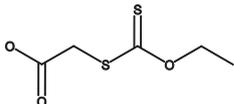
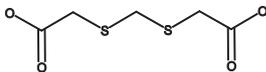
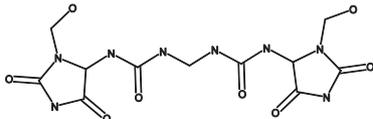
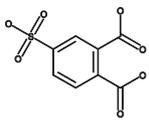
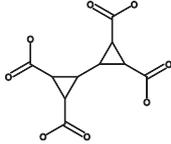
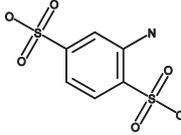
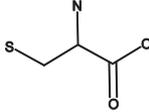


Fig. 4. A Effect on CSAD (circles) and GADL1 (triangles) by MnCl₂ (closed symbols) and LiCl (open symbols). * Significantly different from control (t-test, p < 0.05). Data are expressed as average \pm STDEV (n = 3) B Inhibition of CSAD (circles) and GADL1 (triangles). The different compounds used are Bis-(carboxymethyl)-trithiocarbonate (Red, compound A), Ethylxanthogenacetic acid (Green, compound B) and 2,5-disulfoaniline (Blue, compound G). Different concentrations of the compounds were tested (0.05–40 mM for the salts and 0.001–50 mM for the inhibitors) using a standard concentration of CSA (100 μM). The assay was performed as described above at 37 °C for 60 min and stopped by addition of equal volume of ice-cold ethanol with 5% acetic acid. Data are expressed as average \pm STDEV (n = 3).

Table 2

Specificity of CSAD and GADL1 inhibitors. Different concentrations of the inhibitors were tested (0.1–50 mM). The assay was performed as described above at 37 °C for 60 min and stopped by addition of equal volume of ice-cold ethanol with 5% acetic acid.

Compound	Name	Zinc ^a	CSAD IC ₅₀ ± S.E. (mM)	GADL1 IC ₅₀ ± S.E. (mM)	CSAD K _i ± S.E. (mM)	GADL1 K _i ± S.E. (mM)	Selectivity	2D structure
A	Bis-(carboxymethyl)-trithiocarbonate	01661333	0.12 ± 0.029	0.068 ± 0.013	0.07 ± 0.04	0.06 ± 0.03	1.09	
B	Ethylxanthogenacetic acid	01760604	0.43 ± 0.23	0.098 ± 0.012	0.25 ± 0.28	0.09 ± 0.02	2.71	
C	MethyleneBis thioglycolic acid	01635711	1.6 ± 0.53	2.7 ± 0.21	0.93 ± 0.66	2.55 ± 0.44	0.36	
D	Imidazolidinyl urea	04245708	2.72 ± 0.92	1.30 ± 0.52	1.59 ± 1.14	1.23 ± 1.00	1.29	
E	4 sulfophtalic acid	01693949	0.97 ± 0.3	7.4 ± 0.94	0.57 ± 0.37	7 ± 1.89	0.08	
F	Bi(cyclopropane)-2,2,3,3 tetracarboxylic acid	25722391	7.3 ± 1.3	10.0 ± 0.35	4.26 ± 1.68	9.46 ± 0.81	0.45	
G	2,5-disulfoaniline	01687023	5.3 ± 1.0	2.4 ± 0.6	3.09 ± 1.29	2.27 ± 1.17	1.36	
H	L-cysteine	00895042	1.44 ± 0.45	0.92 ± 0.10	0.84 ± 0.56	0.87 ± 0.20	0.97	

^a Accession number in Zinc database (Irwin et al. 2012)

GADL1 and CSAD at concentrations above 10 mM. Kinetic studies showed that the inhibitors were competitive against CSA (data not shown). Furthermore, as the most potent inhibitors were substrate analogs and the inhibition constants were unaffected by 10-fold dilution of target enzyme or the addition of 0.1 mg BSA in the assay, they probably inhibit GADL1 and CSAD by binding to the enzymes' active sites. It is unlikely that the enzyme inhibition is due to the formation of colloidal aggregates by the inhibitors (Feng et al., 2007), or other non-specific effects.

3.7. CSAD and GADL1 expression patterns

The tissue distribution of CSAD has been controversial (Tang et al., 1996; Tappaz et al., 1999; Vitvitsky et al., 2011). Since many studies were done before the discovery of GADL1, it was important to establish whether the reported discrepancies could be due to contamination of CSAD preparations with GADL1. For this purpose, we systematically compared the mRNA and protein levels for CSAD and GADL1 in mouse tissues. The mRNA levels were determined by real-time reverse transcriptase (RT)-PCR quantification using specific DNA probes (Suppl. Table 1). As shown in Fig. 5A, the level of CSAD mRNA was much higher in mouse liver and kidney than in total brain extracts ($p = 1 \times 10^{-6}$) and 7 times ($p = 4 \times 10^{-10}$)

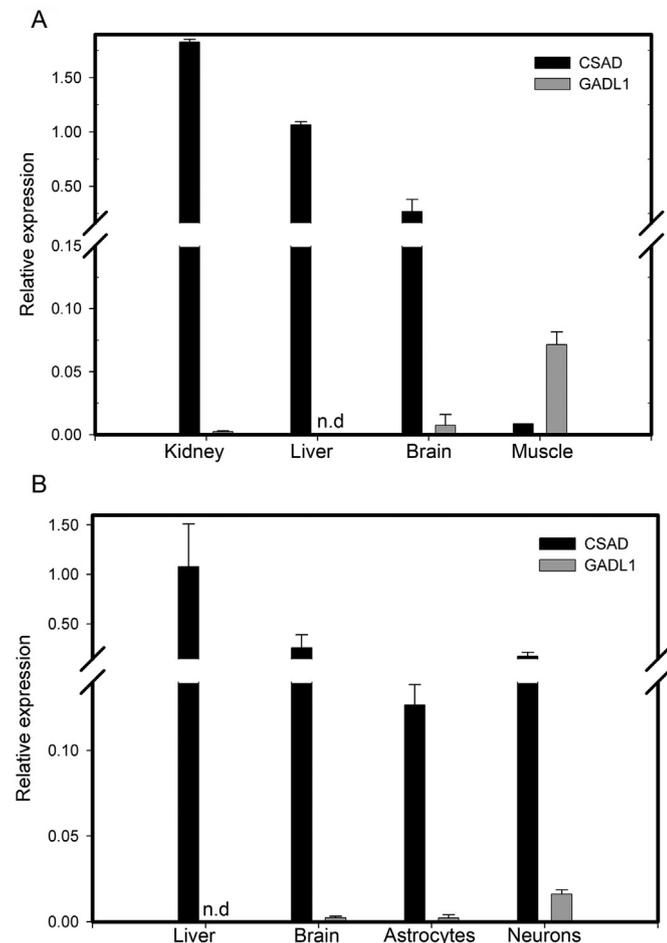


Fig. 5. Gene expression of CSAD and GADL1 in different tissues. **A** Adult mouse tissues. **B** Adult human tissues. Total poly-A + RNA was reverse transcribed and the cDNA amplified by real-time PCR using specific primers for CSAD or GADL1. The PCR products were detected with TaqMan[®] detection assay. PCR values were normalized to those produced with primers for GAPDH and β -actin and presented as average \pm STDEV ($n = 3$). All values were significantly different between tissues and proteins ($p < 0.05$).

higher, respectively), and almost no CSAD mRNA was detected in the muscle, as reported previously (Ide et al., 2002; Park et al., 2002; Reymond et al., 2000). For GADL1 only low levels of mRNA were observed in adult brain compared with CSAD ($p = 0.001$), but was not detected in liver and kidney samples. However, in contrast to CSAD, mRNA of GADL1 was detected in skeletal muscle ($p = 0.005$), as was also reported by Liu et al. (Liu et al., 2012). These results were also confirmed by western blot experiments (Fig. 6), although the amount of CSAD detected in kidney was lower than in brain and liver ($p = 0.01$ and 0.0005).

Although taurine is found in both astrocytes and neurons, CSAD is more abundant in non-neuronal cells (Tappaz et al., 1998). It has been proposed that astrocytes produce taurine via CSAD, while

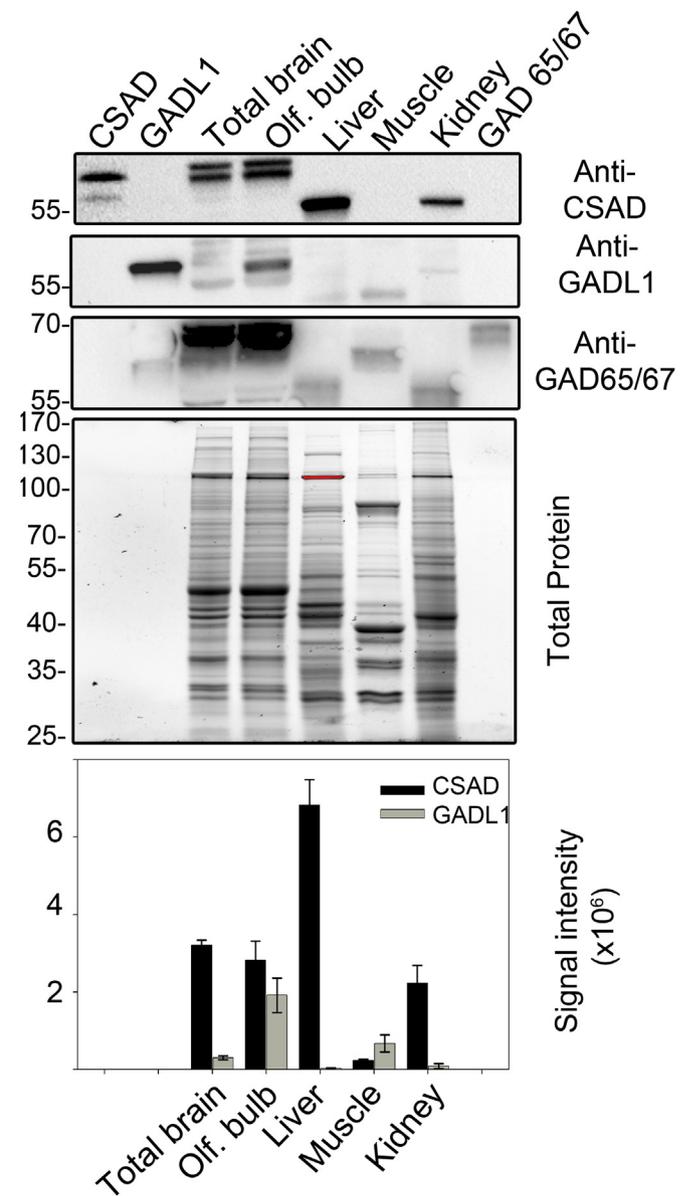


Fig. 6. Protein expression of CSAD and GADL1 in different tissues. Representative western blot image of CSAD, GADL1 and GAPDH in mouse brain, olfactory bulb, liver, muscle and kidney (20 μ g, 3 weeks old, $n = 3$). Recombinant mCSAD, mGADL1 and hGADL1 were used as controls. A representative image of the stain free gel is shown under the immunoblots showing the total protein in the samples. The signals from 3 different samples are expressed as average \pm STDEV in the bar chart. * Significantly different $p < 0.05$. Signals were normalized against both GAPDH and total protein (using stain-free gels from Bio-Rad).

neurons synthesizes taurine via the cysteamine dioxygenase pathway (Vitvitsky et al., 2011). To examine whether CSAD or GADL1 could be involved in taurine biosynthesis in either neurons or astrocytes, CSAD and GADL1 mRNA levels were measured in both cell types. As shown in Fig. 5B, CSAD was expressed at lower levels in human astrocytes than in neuronal cells ($p = 0.0001$), whereas GADL1 was only expressed in neuronal cells. Although the level of GADL1 mRNA was six-fold lower than those of CSAD ($p = 1 \times 10^{-7}$), this enzyme might account for some of the CSAD-activity in neurons (Vitvitsky et al., 2011). The Human Protein Atlas <http://www.proteinatlas.org/> also shows that both proteins are mainly present in neurons. It also indicates the presence of GADL1 in astrocytes in certain tissues such as hippocampus and cerebral cortex. However, these results are uncertain as the antibody used in their study also showed cross reactivity towards CSAD (results not shown).

Publicly available databases, such as the Allen Brain Atlas (<http://www.brain-map.org/>), were also consulted to study the *in situ* RNA hybridization patterns of GAD65/67, CSAD, and GADL1. A comparison of their expression levels showed that although GAD65/67 and CSAD in general are expressed at higher levels than GADL1, certain brain regions, such as the olfactory bulb, have higher levels of GADL1 than CSAD (Suppl. Fig. 2A). At protein levels, GADL1 appeared to be expressed at the same level as CSAD in the olfactory bulb, but at very low levels in total brain lysates ($p = 0.003$) (Fig. 6).

3.8. Developmental pattern of CSAD and GADL1 in mouse brain

In some tissues, CSAD expression has been reported to increase with age (Tappaz et al., 1998), while in zebrafish an important role of CSAD in early embryogenesis was recently reported (Chang et al., 2013). To investigate the pattern of GADL1 and CSAD expression during development and compare it with GAD expression, we immunoblotted total brain extracts from mice at 11 different ages, from embryonic day 17 (E17) to 12 months. As shown in Fig. 7A, striking differences were observed between the staining patterns of the three antibodies. CSAD expression decreased with age, whereas the expression of GAD increased and was strongest in adult brain, consistent with its reported developmental pattern (Kim et al., 2006; Ohkuma et al., 1986). However, when using specific antibodies for GADL1, three weak bands were visible; a high molecular weight species (65 kDa), that had similar developmental pattern as CSAD, and two bands at 58–60 kDa that gradually disappeared after E17. This protein intensity pattern corresponds well with mRNA levels from the Human Brain transcriptome database (<http://hbatlas.org/>), as shown in Suppl. Fig. 2B (Kang et al., 2011). Although the detection of GADL1 in the total brain lysate was weak, we cannot rule out the presence of the protein in specific parts of the brain, such as was seen in the olfactory bulb.

3.9. Cross-species characterization of brain CSAD and GADL1

CSAD purified from many sources and species has been reported with subunit molecular masses of 43, 51, 53, or 55 kDa (Pasantes-Morales et al., 1976; Skoldberg et al., 2004; Tang et al., 1996; Weinstein and Griffith, 1987; Wu, 1982). To examine cross-species reactivity of the CSAD antibodies, and compare their protein levels, total brain homogenates from 14 different species of adult age were analyzed by western blotting (Fig. 7B). Consistent with the strong sequence similarities between mammalian CSAD enzymes, comparable labeling intensity was obtained for most of these species. Only one major CSAD band appeared at around 58 kDa (Fig. 7B). In accordance with the low abundance and mainly embryonic expression pattern of GADL1 expression, much weaker bands appeared when the same samples were analyzed with anti-GADL1 antibodies, making the comparison of this enzyme across

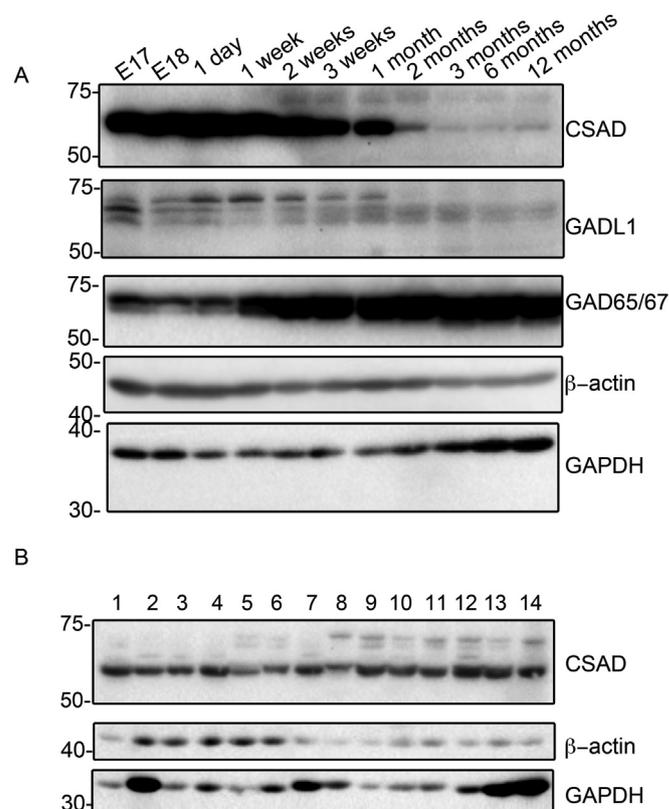


Fig. 7. Protein expression of CSAD and GADL1 in brain. **A** Representative western blot image of CSAD, GADL1, GAD65/67 and Beta-actin/GAPDH in total brain lysates (70 μ g) from mice at different developmental stages and different ages (embryonic day 17 to 12 months old, Zyagen Laboratories). **B** Representative western blot image of CSAD and Beta-actin/GAPDH in total brain lysates (70 μ g) from 14 different species (Zyagen Laboratories) using custom-made CSAD antibody 1. Human (55 years), 2. Mouse (10 weeks), 3. Rat (10 weeks), 4. Dog (2 years), 5. Rabbit (15 weeks), 6. Guinea Pig (15 weeks), 7. Hamster (10 weeks), 8. Chicken (1 year), 9. Bovine (3 years), 10. Sheep (3.5 years), 11. Porcine (3 years), 12. Equine (5 years), 13. Monkey Cynomolgus (4 years), 14. Mini Pig (2 years).

species more subject to ambiguity (results not shown).

4. Discussion

Here we report a systematic comparison of murine CSAD and GADL1 structures, biochemical properties and tissue expression patterns.

4.1. Biochemical properties of CSAD and GADL1

In order to characterize these two proteins we first developed a new expression and purification protocol. Although both enzymes appeared to have a preference for decarboxylation of CSA, they were also active against other substrates, with different substrate specificities and sensitivity towards inhibitors. Glutamate was completely inactive as substrate or inhibitor of either CSAD or GADL1, indicating that none of these enzymes are involved in glutamate metabolism. Thus, it is also unlikely that the observed strong association between genetic variants GADL1 and response to lithium treatment ($p = 2.52 \times 10^{-37}$) is related to altered production of the inhibitory transmitter γ -aminobutyric acid, as recently suggested (Chen et al., 2014). The effects of metal ions on the enzyme activities were tested using a wide range of concentrations including the therapeutic range of Li^+ used in human pharmacotherapy. The minimal effect of lithium on either CSAD or GADL1

activity further argues against a direct relationship between GADL1 genetic variants and the response to lithium therapy. As lithium is eliminated via kidneys, it has been speculated that the association between GADL1 variants and response to lithium therapy could be mediated by renal GADL1 (Birnbaum et al., 2014). However, as the response to lithium therapy was independent on renal clearance of lithium (Chen et al., 2014) and the kidney levels of GADL1 are very low, we consider this to be unlikely.

Alternative substrates and physiological functions of GADL1 should also be considered. The decarboxylation product of Asp (β -alanine) produced by GADL1 or CSAD (Fig. 3) is a constituent of the dipeptide carnosine. As for taurine, carnosine and its metabolic derivatives may have antioxidant properties and are found in many tissues, including muscle and brain (Baldyrev and Abe, 1999). A role of GADL1 in carnosine production was supported by recent genome wide association studies showing a strong association between human blood levels of N-acetyl carnosine and variants in the GADL1 gene ($p = 3.4 \times 10^{-18}$ for rs7643891) (Shin et al., 2014). Indeed, the tissue distribution of GADL1 seems to be compatible with a physiological role of carnosine in muscle and possibly also brain function. The role of β -alanine in brain function is less clear. However, it is interesting that the plasma levels of this amino acid is altered by selective serotonin reuptake inhibitors, indirectly linking it to brain (dys)function (Woo et al., 2015).

4.2. Expression of CSAD and GADL1

The most striking difference between the enzymes was found in their patterns of tissue expression of both mRNA and protein, where GADL1 was mainly found in muscle and brain, in particularly olfactory bulb, and CSAD was found in liver, kidney and brain tissues, confirming previous reports (Guion-Rain and Chatagner, 1972; Liu et al., 2012; Park et al., 2014, 2002; Reymond et al., 1996a; Reymond et al., 2000; Tang et al., 1996; Tappaz et al., 1994, 1998; Weinstein and Griffith, 1987).

The recent observations that human (Liu et al., 2012) and mouse GADL1 decarboxylate CSA at comparable rates to CSAD (Table 1), raised the possibility that the enzyme “CSADII” described by Tang and coworkers (Tang et al., 1996) is identical to GADL1. This would explain the reported discrepancies between CSAD enzyme activity and mRNA levels in different tissues (Tappaz et al., 1999). However, the low expression levels of GADL1 in adult neurons (Table 3, Fig. 5) indicate that this enzyme probably cannot account for much of the CSAD activity previously reported (Tang et al., 1996; Tappaz et al., 1999). The relative expression levels and catalytic activities of CSAD and GADL1 are in accordance with the recent study of Park et al., who found that CSAD knockout mice had a 83% reduction of plasma taurine levels and a similar reduction in liver and brain taurine levels in offspring of CSAD $^{-/-}$ mice (Park et al., 2014). Based on our estimates of catalytic activity and expression levels, GADL1 probably accounts for a small fraction of the remaining

CSAD activity in CSAD knockout mice, while cysteamine dioxygenase (EC 1.133.11.19) also may contribute to taurine synthesis.

Weinstein and Griffith reported that there was almost no detectable CSAD mRNA in the brain, whereas they could still measure enzyme activity (Weinstein and Griffith, 1987). It was explained by fast turnover of the mRNA, but it could also be due to the existence of several CSAD enzymes with different mRNA sequences (Suppl. Table 2). Western blotting revealed multiple molecular species. This could be due to alternatively spliced variants, as there is evidence for alternative splicing for CSAD and GADL1. The identity and roles of such putative splice variants should be further investigated.

Taurine has mainly been detected in neurons in the cerebellum and hippocampus (Ottersen et al., 1988; Zhang and Ottersen, 1992), and more in fetal tissues than in adults (Terauchi et al., 1998). However, it is unclear whether biosynthesis of taurine occurs exclusively in neurons or glial cells, or both cell types. The use of different antisera may have contributed to the diverging results reported from different research groups. Chan-Palay and coworkers found an exclusive localization of CSAD in rat neurons (Chan-Palay et al., 1982), results that were later confirmed by others (Kuriyama et al., 1985; Magnusson et al., 1988; Ohkuma et al., 1986; Taber et al., 1986; Wu et al., 1987). However, other research groups were not able to detect CSAD in neurons, only in astrocytes (Reymond et al., 1996b; Tappaz et al., 1992). There are several theories for these results. It was recently proposed that taurine is independently produced from cysteine by either astrocytes or neurons (Vitvitsky et al., 2011), while previous reports indicated that taurine is produced by a cooperative exchange of taurine precursors between astrocytes and neurons as each cell type contains incomplete but complementary components of this pathway (Dominy et al., 2004).

As commercially available antibodies against CSAD do not fully discriminate between CSAD and GADL1, the finding of CSAD in both neurons and astrocytes could potentially be due to cross-reactivity. However, the RT-PCR data reported here using gene specific probes establish that CSAD is present in both neurons and astrocytes, consistent with the view that taurine biosynthesis takes place independently in both neurons and astrocytes, and that this activity cannot be attributed to GADL1, as very low levels of GADL1 were detected in neurons, and not in astrocytes (Table 3, Fig. 5B). However, although our data suggest an expression of GADL1 only in the neurons, The Human Protein Atlas suggest protein expression in both neurons and glia cells. Non-specific antibodies, as previously discussed, could explain these discrepancies.

4.3. Developmental patterns

Our mRNA and protein measurements were compared with data deposited in the Allen Brain Atlas (<http://www.brain-map.org/>) and The Human Protein Atlas (<http://www.proteinatlas.org/>). They confirm that the PLP-dependent decarboxylases GADL1, CSAD, GAD65 and GAD67 all have different expression patterns in humans, as well as in mice, which is consistent with distinct physiological roles of these proteins. In particular, CSAD and GADL1 have strikingly different patterns of expression in the human brain, where mRNA levels of GADL1 are lower than CSAD in most brain regions, except olfactory cortex and ventral striatum (Fig. 7A). In the latter region, GADL1 mRNA levels appear to be almost 10 fold higher than for CSAD, indicating a specific physiological role of GADL1 in these structures. Our data also suggest that levels of RNA and protein are lower for GADL1 than for CSAD in most human and mouse tissues (Figs. 5 and 6), except for muscle and olfactory bulb. This is consistent with data in the databases Braineac (www.braineac.org/), Allen Brain Atlas and The Human Protein Atlas.

Table 3

Gene Expression assay with different mouse and human tissues. Total RNA from different tissues was analyzed using RT-PCR and normalized against GAPDH. Results are average \pm stdev for three preparations.

Species	Tissue	CSAD (Δ CtGAPDH)	GADL1 (Δ CtGAPDH)
Human	Brain	-8.07 ± 0.78	-14.73 ± 0.47
	Liver	-5.96 ± 0.61	n.d.
	Astrocytes	-9.02 ± 0.08	-15.56 ± 0.36
	Neurons	-8.33 ± 0.44	-11.9 ± 0.54
Mouse	Brain	-8.06 ± 0.62	-13.83 ± 1.45
	Liver	-6.04 ± 0.55	n.d.
	Kidney	-5.17 ± 0.02	-14.86 ± 0.42
	Muscle	-12.93 ± 0.24	-9.85 ± 0.15

The presence of GADL1 in olfactory bulb is particularly interesting as taurine is known to be abundant in this brain structure, exceeding glutamate and GABA in concentration, and has been shown to be important in early stages of olfactory maturation (Steullet et al., 2000).

The highest level of human CSAD mRNA is found during prenatal development, with a maximum around 17 weeks post conception (pcw), although it is also detected in many brain regions in adults (Ohkuma et al., 1986). In contrast, GADL1 appears to be exclusively expressed in brain cortical areas during embryonic development, with highest levels during 12–14 pcw. Except for these weeks, CSAD had a much higher level of expression than GADL1 (Brainspan and Human Brain Atlas). CSAD appears to have the highest level in the intermediate zone, important for neural migration, whereas GADL1 has the highest level in the subplate zone, important in establishing correct wiring and functional maturation of the cerebral cortex. The subplate neurons disappear during postnatal development, and this could explain the low levels of GADL1 in adult brain. When analyzing lysates from total mouse brain, we obtained similar results (Fig. 7A), although three lanes of protein bands were observed. These protein bands could indicate different splice variants. This has not previously been investigated for GADL1, although it has been reported that alternative splicing is frequent during early embryonic development in mice (Revil et al., 2010). Distinct developmental patterns of expression have also been described for other PLP-dependent decarboxylases, such as GAD67 (as shown in Fig. 7A) and AADC (Blechingberg et al., 2010; Liu et al., 2010). Temporal and organ specific splicing events, resulting in many different mRNA and protein species from these genes, indicates a fine tuned physiological regulation of expression, but has also complicated previous studies of these enzymes (Blechingberg et al., 2010; Liu et al., 2010).

4.4. Animal models

The physiological roles of CSAD and particularly GADL1 are not well established. It was recently reported that CSAD is expressed during early embryonic development of the Zebrafish and that knockdown of *Csad* significantly reduced embryonic taurine levels, leading to increased early mortality and cardiac abnormalities (Chang et al., 2013). This is in line with previous studies on taurine transporter (TauT) knockout mice, which showed abnormalities of kidney, liver, brain, retina and olfactory function (Warskulat et al., 2007). In contrast, the CSAD knockout mice had a less severe phenotype (Park et al., 2014).

Unlike CSAD, GADL1 is absent from the Zebrafish genome. Considering the predominantly embryonic pattern of GADL1 expression observed in mice and humans, it is tempting to speculate that GADL1 may have a specific role during embryonic development and that this evolutionary more recent enzyme has replaced some CSAD function in early brain development.

Selective knockdown and knockout studies of GADL1 in combination with CSAD may be needed to resolve the metabolic role of these enzymes. However, since both *CSAD* and *GADL1* appear to be transcribed to multiple tissue-specific transcripts with different in-frame start codons, comprehensive knockdown experiments of these enzymes may be technically challenging (Chang et al., 2013).

4.5. PLP-dependent decarboxylases in human disease

Several PLP-dependent decarboxylases act as autoantigens in different autoimmune disorders, e.g. GAD65 in type 1 diabetes (Baekkeskov et al., 1990), GAD65 and GAD67 in the neurological condition Stiff Person Syndrome (Bjork et al., 1994), and AADC (Rorsman et al., 1995), histidine decarboxylase and CSAD

(Skoldberg et al., 2004) in APS-1. Interestingly, autoantibodies typically recognize evolutionary conserved epitopes on these enzymes and act as strong inhibitors of the enzymatic activity, presumably by binding to epitopes close to the substrate binding or active sites of the enzymes targeted. We have previously observed that serum from a subgroup of APS-1 patients (3 out of 83 individuals) contain antibodies reacting with both CSAD (Skoldberg et al., 2004) and GADL1 protein produced from a mouse cDNA library (data not shown). Although APS-1 patients with these autoantibodies have multiple autoimmune manifestations, it is unclear whether they have any pathogenic significance.

5. Conclusion

Purified murine CSAD and GADL1 both decarboxylate CSA, CA, homocysteic acid and Asp, although with very different affinities. However, we cannot rule out that these enzymes may have still other physiological substrates. The novel enzyme inhibitors identified by *in silico* and *in vitro* screening had relatively low affinities, but could be used as templates to obtain more selective compounds. The two proteins are located in different tissues. The possible role GADL1 in olfactory bulb and embryonic brain development should be investigated further.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuint.2015.08.013>.

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