Guanidinoacetate (GAA) Is a Potent $GABA_A$ Receptor GABA Mimetic: Implications for Neurological Disease Pathology

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Author Contributions

P.M.: developed concepts, designed and performed electrophysiology experiments, analyzed data and edited. M.W.: developed concepts, designed experiments, analyzed data and wrote original draft. M.U-O.: designed and performed ligand binding experiments, analyzed data and edited. G.S.L.: acquired funding, developed concepts, designed experiments and wrote the manuscript.

Competing Interest Statement

G.S.L. has served as a consultant to Audentes Therapeutics and serves on the Scientific Advisory Board of Taysha Therapeutics in areas unrelated to this work.

Key Words

Guanidinoacetate, guanidino compounds, $GABA_A$ receptor, GABA-mimetic, guanidinoacetate methyltransferase deficiency, arginase deficiency,

Abbreviations

AGAT: arginine:glycine amindinotransferase (EC 2.1.4.1)

ARG1: arginase 1 (EC 3.5.3.1)

BBB: blood-brain barrier

CGC: cerebellar granule cell

CSF: cerebrospinal fluid

GAA: guanidinoacetic acid

γ-GBA: γ-guanidinobutyric acid

GABA: γ-aminobutyric acid

$GABA_AR$: γ-aminobutyric acid type A receptor

GC: guanidino compound

GAMT: guanidinoacetate methyltransferase (EC 2.1.1.2)

GES: guanidinoethanesulfonic acid

EC_{50}: half maximal effective concentration

IC_{50}: half maximal inhibitory concentration

UCD: urea cycle disorder

HE: hepatic encephalopathy

UE: uremic encephalopathy

Abstract (287 words out of 300 max).

Impairment of excretion and enzymatic processing of nitrogen, e.g. due to liver or kidney failure, or with urea cycle and creatine synthesis enzyme defects, surprisingly leads to primarily neurologic symptoms, yet the exact mechanisms remain largely mysterious. In guanidinoacetate N-methyltransferase (GAMT) deficiency, the guanidino compound guanidinoacetate (GAA) increases dramatically, including in the cerebrospinal fluid (CSF), and has been implicated in mediating the neurological symptoms in GAMT-deficient patients. GAA is synthesized by arginine-glycine amidinotransferase (AGAT), a promiscuous enzyme that not only transfers the amidino group from arginine to glycine, but also to primary amines in e.g., GABA and taurine to generate γ-guanidinobutyric acid (γ-GBA) and guanidinoethanesulfonic acid (GES), respectively. We show that GAA, γ-GBA and GES share structural similarities with GABA, evoke $GABA_A$ receptor ($GABA_AR$) mediated currents (whereas creatine [methylated GAA] and arginine failed to evoke discernible currents) in cerebellar granule cells in mouse brain slices and displace the high-affinity GABA-site radioligand [^{3}H]muscimol in total brain homogenate $GABA_ARs$. While γ-GBA and GES are GABA agonists and displace [^{3}H]muscimol (EC_{50}/IC_{50} between 10 and 40 μM), GAA stands out as particularly potent in both activating $GABA_ARs$ (EC_{50} ~6 μM) and also displacing the $GABA_AR$ ligand [^{3}H]muscimol (IC_{50} ~3 μM) at pathophysiologically relevant concentrations. These findings stress the role of substantially elevated GAA as a primary neurotoxic agent in GAMT deficiency and we discuss the potential role of GAA in arginase (and creatine transporter) deficiency which show a much more modest increase in GAA concentrations yet share the unique hyperexcitability neuropathology with GAMT deficiency. We conclude that orthosteric activation of $GABA_ARs$ by GAA, and potentially other $GABA_AR$ mimetic guanidino compounds (GCs) like γ-GBA and GES, interferes with normal inhibitory GABAergic neurotransmission which could mediate, and contribute to, neurotoxicity.

Introduction

Neurological impairment is the primary manifestation of enzyme defects in urea cycle and creatine synthesis pathways, as well as hepatic and uremic encephalopathy (HE and UE). In HE and UE, generally due to liver and kidney failure respectively, toxins like ammonium and glutamine along with guanidino compounds (GCs), accumulate and lead to a poorly understood neuropathology (Hamed, 2019; Häussinger et al., 2022). $GABA_A$ receptors ($GABA_ARs$) are the main inhibitory neurotransmitter receptors in the brain and have long been

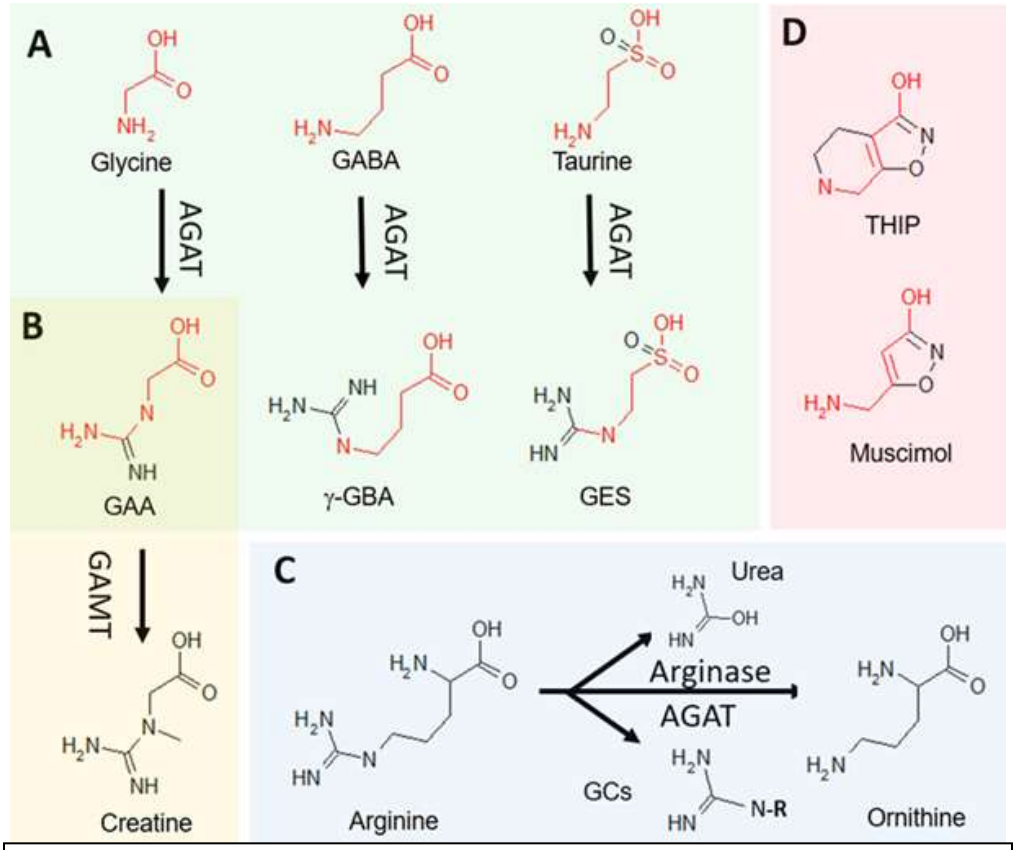

Fig. 1. The creatine synthesis pathway with chemical structures of γ-aminobutyric acid (GABA) & potential guanidino GABA analogs. Shown in red is GABA & the GABA backbone structure in potential GABA analogs. (**A**) AGAT is a nonspecific (promiscuous) enzyme & converts primary amines in glycine (main substrate) but also GABA, taurine, β-alanine & lysine (the latter not shown) into their respective amidino derivatives (GAA, γ-GBA, GES, β-GPA). (**B**) GAMT catalyzes the methylation of GAA using S-adenosyl-L-methionine (SAM) as the methyl donor to generate creatine. (**C**) Arginine is the main physiological AGAT amidino group donor and is converted into ornithine by both AGAT & arginase 1. Arginine is the limiting substrate for GAA synthesis, and therefore dietary arginine restriction can limit AGAT activity & GAA synthesis. (**D**) Muscimol and THIP (Gaboxadol) are conformationally restricted GABA analogs with high affinity (selectivity) for extrasynaptic $GABA_ARs$ and are potent neurotoxins. Unlike GABA, muscimol & THIP are thought to passively cross the BBB due do their ring structures conferring increased hydrophobicity (Benkherouf et al., 2019). In the absence of active transport systems, most small hydrophilic compounds like GABA, GAA, γ-GBA & GES are expected to have limited brain access due to their low BBB permeability.

suggested of being involved in the neuropathology of hepatic/uremic encephalopathy (Cauli et al., 2009; Schafer and Jones, 1982; Sergeeva, 2013), certain urea cycle disorders (UCD) (i.e. arginase deficiency [ARG1]) and creatine deficiency disorders like guanidinoacetate methyltransferase (GAMT) deficiency (Fernandes-Pires and Braissant, 2022; Neu et al., 2002; Schulze et al., 2016). Elevated ammonium, with free NH_3 likely permeating through the blood-brain barrier (BBB), at least in part causes the poorly understood toxicity of hepatic encephalopathy (Cooper and Jeitner, 2016; Goldbecker et al., 2010). However, it appears likely that ammonium is only one component in a multifactorial disease process, and in creatine pathway enzymopathies (i.e. arginine:glycine amidino transferase [AGAT] and GAMT enzyme deficiencies) and

creatine transporter SLC6A8 defects, hyperammonemia is generally absent (Sharer et al., 2017). In arginase (ARG1) deficiency, elevated arginine rather than hyperammonemia is thought to be the major treatment challenge (Burrage et al., 2015). Thus, mechanisms other than sequela from hyperammonemia must account for neuropathology in GAMT, AGAT, SLC6A8 and ARG1 deficiencies. Furthermore, there are unique hyperexcitability/epilepsy phenotypes present in GAMT (Khaikin et al., 2018; Stöckler-Ipsiroglu et al., 2014) and ARG1 deficiencies (Amayreh et al., 2014; Deignan et al., 2010; Huemer et al., 2016; Jichlinski et al., 2018), as well as in some patients with functional mutations of the creatine transporter SLC6A8 (Fernandes-Pires and Braissant, 2022) that to date have eluded explanation. The creatine biosynthesis pathway consists of AGAT to synthesize the intermediate GAA by transferring the amidino group from arginine (the limiting factor) to glycine. GAA is subsequently methylated by GAMT to generate creatine (Fig. 1A, B, C). Both AGAT and GAMT are expressed in the brain (AGAT mainly in neurons, GAMT mainly in oligodendrocytes) and therefore GAA and other small hydrophilic GCs synthesized by AGAT can be produced locally from precursors within the brain (see Fig. 1A) without the necessity of these GCs crossing the BBB (Braissant et al., 2010; Fernandes-Pires and Braissant, 2022; Hanna-El-Daher and Braissant, 2016). AGAT has been shown to be a fairly nonspecific or promiscuous enzyme, converting not only glycine but other primary amines like GABA, taurine, β-alanine, and lysine into their amidino (guanidino) derivatives, i.e. γ-guanidinobutyric acid (γ-GBA), guanidinoethanesulfonic acid (GES), β-guanidinopropionic acid (β-GPA) and homoarginine, respectively (Watanabe et al., 1994) with γ-GBA and GES being potential structural GABA analogs (Fig. 1A). GCs, increased in the brain in e.g., ARG1 and GAMT deficiencies, have long been suspected of being neurotoxic (Deignan et al., 2010; Khoja et al., 2022; Torremans et al., 2005). The significant guanidinoacetate (GAA) accumulation in GAMT deficiency is considered neuropathogenic (Hanna-El-Daher et al., 2015; Hanna-El-Daher and Braissant, 2016; Stöckler-Ipsiroglu et al., 2014). In GAMT deficient patients and mouse models, GAA accumulates in bodily fluids including the CSF where it is elevated up to 1000-fold, from about 10-100 nM in controls to up to ~30 μM in those afflicted with GAMT deficiency (Hanna-El-Daher et al., 2015; Schütz and Stöckler, 2007; Stöckler-Ipsiroglu et al., 2014). While treatment of GAMT patients with sustained high dose oral creatine with ornithine and protein restriction does lower GAA levels, it remains elevated at > 1 μM in the CSF; while this reduction is associated with improved symptoms, it apparently fails to produce complete remission of symptoms (Stöckler-Ipsiroglu et al., 2014). Therefore, it is possible that even minor GAA increases in the brain could induce neurotoxicity.

Whether the much smaller increase in GAA observed in ARG1 deficiency (Amayreh et al., 2014; Huemer et al., 2016; Ingoglia et al., 2021), perhaps along with other GCs (Deignan et al., 2010), could contribute to hyperexcitability symptoms is currently debated (Huemer et al., 2016; Ingoglia et al., 2021). Arginine is a direct precursor of GAA, whose synthesis is highly dependent on arginine levels and therefore high levels of plasma arginine may cause increased brain GAA levels (Ingoglia et al., 2021). As brain cells lack enzymes of the urea cycle, elevated peripheral arginine could drive increased brain synthesis of GAA and other GCs (Fig. 4), likely by arginine crossing the BBB through monoamine transporters (Verrey et al., 2004). Hyperargininemia itself has also served as a focus as a potentially causative agent: arginine is significantly elevated not only in plasma but also in the CSF with reference levels of 18±5 μM compared to 75±33 μM in those afflicted with ARG1 deficiency (Deignan et al., 2010). Arginase- as well as GAMT- deficient patients and mouse models also show elevated GC levels (e.g. GAA, γ-GBA, homoarginine and others) in serum (Ingoglia et al., 2021) and CSF despite treatment (i.e. arginine/protein restriction along with the addition of sodium benzoate in some) (Deignan et al., 2010; Deignan et al., 2008; Marescau et al., 1990; Torremans et al., 2005).

Herein we show that GAA concentrations as low as 300 nM can activate native GABA receptors (GAA EC_{50} of ~ 6 μM) in mouse cerebellar granule cells in brain slices and leads to displacement of 5nM [^{3}H] muscimol (GAA IC_{50} ~ 3 μM) in brain homogenates. We also demonstrate that besides GAA the structurally similar, closely related GCs GES and γ-GBA activate GABA currents in cerebellar granule cells in brain slices and displace the orthosteric GABA-site ligand [^{3}H]muscimol from $GABA_A$Rs in mouse brain homogenates. Neither arginine (up to 1 mM, hyperargininemia being pathognomonic in ARG1 deficiency) nor creatine (up to 10 mM) evoked any discernible GABA currents in cerebellar granule cells (Fig. 2D, E). These findings implicate GAA and other GCs in the neuropathophysiology that occurs in GAMT deficiency (Torremans et al., 2005), and also ARG1 deficiency where hyperargininemia could drive increased brain synthesis of GAA and other GCs through AGAT activity. Our findings suggest that orthosteric activation of $GABA_A$R by GAA and the structurally related GCs GES and γ-GBA are a potential mechanism mediating (and contributing to) neurotoxicity.

Materials and Methods

Animal Procedures

All procedures were in accordance with protocols approved by the University of California at Los Angeles (UCLA) Chancellor's Animal Research Committee (Animal Welfare Assurance number: ARC-2019-032). Wild-type (C57BL/6, WT) mice (age 2-8 months of age, both sexes, total ~35 mice) were used for the studies. The mice were housed in 12:12 h light:dark cycle in static plastic cages in groups of 4-5 mice per cage having *ad libitum* access to chow (PicoLab 20 Rodent Chow #5053, PMI Nutrition International, Arden Hills, Minnesota, USA) and water. Mice, after brief isoflurane anesthesia, were killed by decapitation and brains were removed. The cerebellum was separated with a scalpel from the rest of the brain, frozen on dry ice and stored at -80 ºC. All transported samples were shipped on dry ice.

Brain Slice Preparation and Electrophysiology

Parasagittal cerebellar slices were prepared using standard techniques (Hanchar et al., 2005; Santhakumar et al., 2006). The cerebellum was removed from the cranium of 6-10 weeks old mice (both sexes), submerged in cold (<4ºC) artificial cerebrospinal fluid (aCSF), and 285-300 µm thick slices were prepared using a vibratome (Leica VT-1000s, Buffalo Grove, IL, USA). The slicing solution consisted of the following (in mM): 85 NaCl, 75 sucrose, 24 $NaHCO_3$, 25 glucose, 4 $MgCl_2$, 2.5 KCl, 1.25 NaH_2PO_4, and 0.5 $CaCl_2$ (all purchased from Sigma-Aldrich, St Louis, MO, USA). Cerebellar slices were stored in 35ºC aCSF for 30 minutes and then brought to room temperature for subsequent electrophysiological experiments. The aCSF for storage and electrophysiological recordings was saturated with 95% O_2 and 5% CO_2 and consisted of the following (in mM): 119 NaCl, 26 $NaHCO_3$, 11 glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, and 1 NaH_2PO_4 (pH 7.3-7.4) (all chemicals purchased from Sigma-Aldrich, St Louis, MO, USA). Whole cell pipette solution consisted of (in mM): 100 KCl, 5 NaCl, 40 HEPES, 4 $MgCl_2$, 4 ATP and 0.4 GTP, titrated to pH 7.4 with KOH. Cerebellar granule cells (CGCs) were visualized using an upright microscope (Zeiss, White Plains, NY, USA) with a 60X water immersion lens equipped with an infrared-DIC enhancement. Pipette resistances were 10-12 MΩ. Recordings from CGCs were performed using a Multiclamp 700B amplifier (Axon Instruments, Inc., Foster City, CA, USA) and were filtered at 4 kHz and digitized at 10 kHz. Neurons were voltage-clamped at -70 mV and recordings were performed at 22-24ºC with glutamate receptor-mediated transmission blocked by 10 µM DNQX and action potentials blocked by 0.3 µM TTX. Electrophysiologic studies included 5 to 6 mice per group.

Reagents

[Methylene-^{3}H]muscimol (22 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). GABA, γ-guandinobutyric acid (γ-GBA), arginine and creatine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Guanidinoacetic acid (GAA) was purchased from Pfaltz & Bauer (Waterbury, CT, USA), while guanidinoethanesulfonic acid (GES, taurocyamine) was purchased from Cayman Chemical (Ann Arbor, MI, USA). GAA, GES, γ-GBA, and THIP were prepared as 10 mM stock solutions either in aCSF for electrophysiology, or in assay buffer for [^{3}H]muscimol displacement studies.

DNQX, TTX, THIP and Gabazine (SR95531) were purchased from Tocris Cookson, Inc. (Part of Bio-Techne, Minneapolis, MN, USA).

Preparation of Brain Membranes

Mouse brain (*sans* cerebellum) membranes were prepared using a modification of the method of Squires and Saederup (Squires and Saederup, 2000) essentially as described by Uusi-Oukari et al. (Uusi-Oukari et al., 2014). The resulting membrane pellets were suspended in 50 mM Tris-HCl, pH 7.4 (assay buffer) and frozen at -70ºC. Before a binding experiment, the suspension was thawed and washed once by centrifugation/resuspension in assay buffer. Binding studies included 4 (γ-GBA, GES) or 5 (GAA) mice per group.

The binding of 5 nM [^{3}H]muscimol was measured in assay buffer at room temperature (RT, 22ºC) in a total volume of 300 µl using triplicates with each displacement curve from an individual mouse brain. Non-specific binding was determined in the presence of 100 µM GABA. The incubation (40 min) was terminated by filtration of the samples with a Brandel Cell Harvester (Model M-24, Gaithersburg, MD, USA) onto Whatman GF/B filters (Whatman International Ltd., Maidstone, UK). The samples were rinsed twice with 4-5 ml of ice-cold assay buffer. Filtration and rinsing steps took a total time of ~15 s. Air-dried filters were immersed in 3 ml of Optiphase HiSafe 3 scintillation fluid (Wallac, Turku, Finland) and radioactivity determined in a Hidex 600 SL liquid scintillation counter (Hidex, Turku, Finland).

Data analysis

[^{3}H]muscimol displacement and electrophysiology data were analyzed using curve fits to individual activation and displacement curves using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Individual EC_{50} and IC_{50} were used to generate averages and standard error of mean (SEM). To determine GAA, γ-GBA and GES statistical differences p-values were calculated using a one-way ANOVA test with Tukey post-hoc

comparison (both with standard function in Phyton 3.9 and Graph-Pad Prism 8). The absence of previous studies of similar nature did not allow us to perform sample size estimation and power analysis. No outlier tests were performed, no outliers were excluded, no exclusion criteria were pre-determined and there was no blinding. Only high quality stable electrophysiological recordings without apparent changes of baseline currents were included for data analysis.

Results

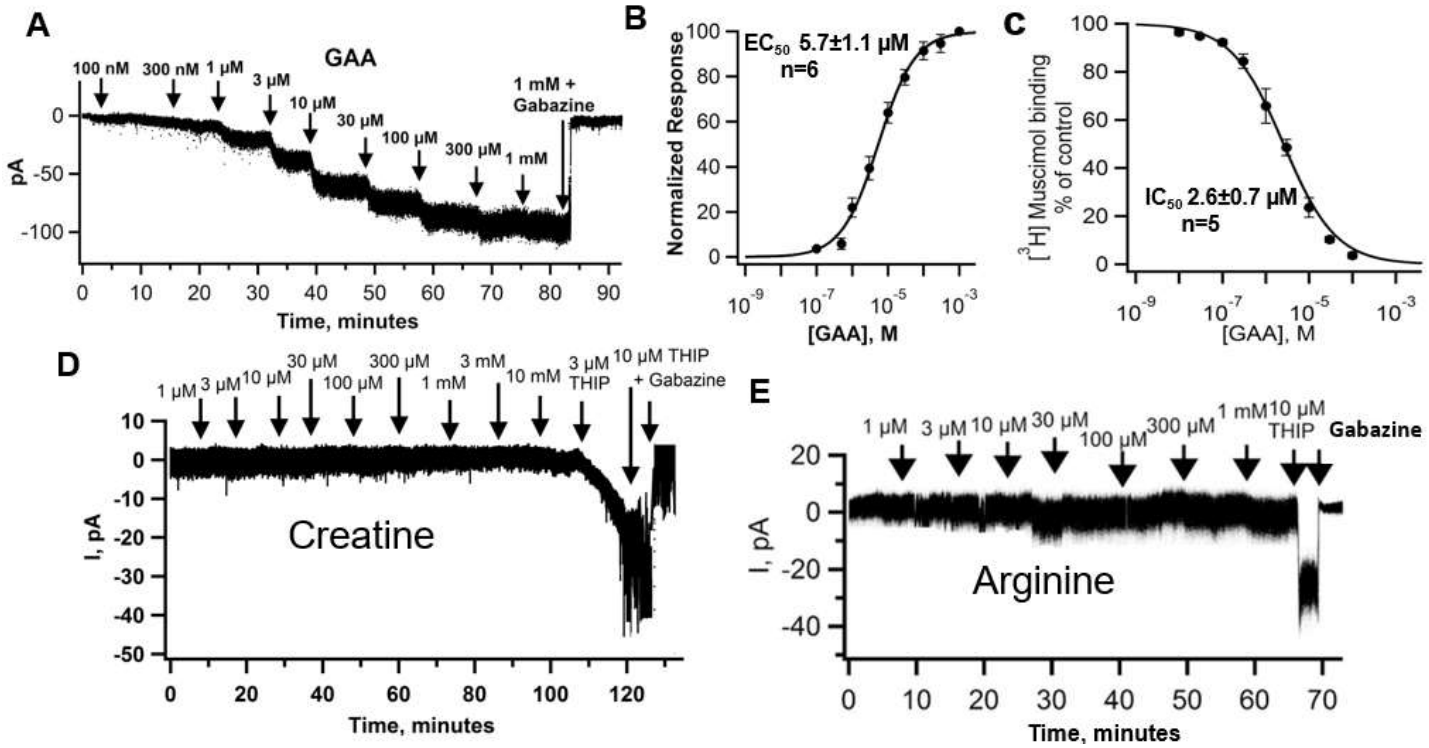

Fig. 2 Guanidinoacetic acid (GAA) at low, pathologically relevant, concentrations acts as a GABA mimetic, and displaces [^{3}H]muscimol in mouse brains. (**A**) Cumulative GAA concentration-response curve using whole-cell patch clamp recording in a mouse cerebellar granule cell (CGCs) held at -70 mV in the presence of 10 µM of the glutamate receptor blocker DNQX (6,7-Dinitroquinoxaline-2,3-dione) with action potentials blocked by 0.3 µM TTX (Tetrodotoxin). GAA at concentrations as low as 300 nM increases the $GABA_AR$ current. After application of 1 mM GAA, the current was blocked by the $GABA_A$ specific blocker Gabazine (SR95531). (**B**) GAA concentration-response curve with an average EC_{50} value of 5.7±1.1 µM, Hill Slope (h) 0.89±0.1 (n=6) in WT CGCs (**C**) Displacement of 5 nM [^{3}H]muscimol by guanidinoacetic acid (GAA) in mouse brain (*sans* cerebellum) GAA IC_{50}=2.6±0.7 µM, (n=5 brains). Creatine (up to 1 mM) (**D**) and arginine (up to 10 mM) (**E**) fail to evoke discernible currents in mouse cerebellar granule cells (representative recordings out of 5 similar recordings). As a positive control that $GABA_AR$ are present and accessible, we used 3 or 10 µM of the GABA analog and mimetic THIP (a.k.a. gaboxadol) at the end of each recording followed by gabazine (SR95531) block. Data reported are mean ± SEM, error bars are SEM.

Guanidino compounds (GCs) have long been suspected as neurotoxins and $GABA_AR$ as potential targets for hepatic and uremic toxins in urea cycle and creatine synthesis enzymatic disorders, as well as in hepatic and uremic encephalopathy. Guanidinoacetate, which structurally resembles GABA (Fig. 1B), accumulates in GAMT deficiency and has been suggested to be a GABA agonist and mimetic (Neu et al., 2002). Based on these findings we decided to investigate the actions of GAA on the ability to induce $GABA_AR$-Cl^- currents on native $GABA_AR$ in brain slices. To test this we used patch-clamp electrophysiology on mouse cerebellar granule cells and recorded cumulative GAA concentration-response curves. GAA perfused onto the brain slices at concentrations between 300 nM and 1 mM induced currents that were blocked by 1 mM gabazine (a.k.a. SR95531), a competitive and selective $GABA_AR$ antagonist (see Fig. 2A). Summary data show that under our recording conditions the half maximal concentration for GAA current activation (EC_{50}) is 5.7±1.1 µM [n=6] (Fig.

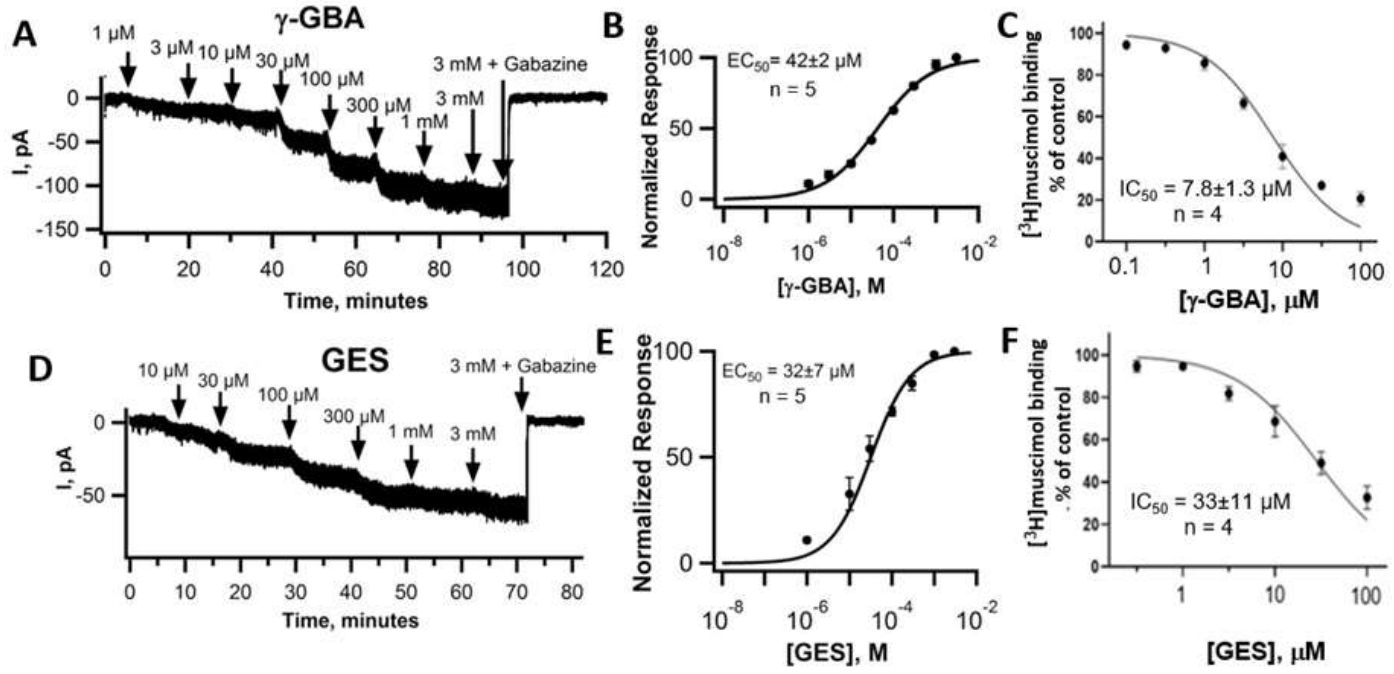

Fig. 3. γ-guanidinobutyric acid (γ-GBA) and guanidinoethanesulfonic acid (GES) both evoke $GABA_A$ currents and displace [^{3}H]muscimol from $GABA_A$Rs. (**A, D**) Cumulative γ-GBA and GES concentration-response curves using whole-cell patch clamp recording in mouse cerebellar granule cells (CGC) held at -70 mV in the presence of 10 µM of the glutamate receptor blocker DNQX and 0.1 µM TTX (Tetrodotoxin). After application of 3 mM GES or γ-GBA the current was blocked by a $GABA_AR$ specific blocker gabazine added to the bath. (**B, E**) Summary data with EC_{50} values of 42±2 µM, Hill slope 0.74±0.05 [n=5] for γ-GBA and 32±7 µM, Hill Slope 1.0±0.1 [n=5] for GES. (**C, F**) 5 nM [^{3}H]Muscimol binding is displaced by γ-GBA (IC_{50}=7.8±1.3 µM, n=4) and GES (IC_{50}=33±11 µM, n=4). Data reported are mean ± SEM and error bars are SEM.

2B). To ensure that GAA effects on GABA receptors were due to direct effects and indeed mimic GABA at the GABA binding site, and are not due to allosteric or indirect effects (e.g. by blocking GABA or taurine transporters and thereby indirectly increasing extracellular GABA or taurine concentrations), we performed displacement studies utilizing the high affinity $GABA_AR$ GABA-site ligand [^{3}H]muscimol (Fig. 1D) in mouse brain homogenates. These data demonstrate that GAA effectively displaces 5 nM [^{3}H]muscimol with an half maximal inhibitory concentration (IC_{50}) of 2.6 ± 0.7 µM [n=5] (Fig. 2C) in whole mouse brain homogenates.

In marked contrast to GAA, creatine at concentrations up to 10 mM lead to no discernible current activation, showing that methylation of GAA (i.e., creatine) by GAMT (Fig. 1B) completely prevented its GABA-mimetic actions (Fig. 2D). Similarly, arginine, a suspected toxin in hyperargininemia and ARG1 deficiency, and potential driver of increased GAA synthesis in the brain (Amayreh et al., 2014; Ingoglia et al., 2021), at concentrations up to 1 mM had no discernible effects on GABA currents (Fig. 2E). As a positive control that the GABA site is accessible we used 3 µM and/or 10 µM THIP, another conformationally restricted GABA analog (Fig. 1D) to evoke GABA-Cl^- currents that are blocked by gabazine (Fig. 2D, E).

Besides GAA we also investigated the GABA analogs γ-GBA and GES, the amidino analogs of GABA and taurine (Fig. 1A) respectively. Just like GAA, both γ-GBA and GES are able to evoke $GABA_AR$ mediated Cl^- currents in CGCs (Fig. 3A, D) although with lower potency (EC_{50} 42±2 µM for γ-GBA [n=5] and 32±7 µM for GES [n=5]) (Fig. 3B, E). Both GES- and γ-GBA-mediated $GABA_AR$-Cl^- current responses are blocked by the specific GABA competitive antagonist gabazine. The direct action of GES and γ-GBA on the $GABA_AR$ was confirmed with 5 nM [^{3}H]muscimol displacement (Fig. 3). The room temperature assays demonstrate that both γ-GBA and GES are able to displace [^{3}H]muscimol from the GABA binding site with an IC_{50} of ~8 µM for γ-GBA and ~33 µM for GES (Fig. 3C, F).

EC_{50} and IC_{50} values from individual concentration-response and [^{3}H]muscimol displacement curves were used to generate averages and standard errors of mean in Figs 2 and 3. ANOVA was used to compare EC_{50} values of GAA, γ-GBA and GES on native $GABA_AR$ activation and IC_{50} of [^{3}H]muscimol displacement. Adjusted p-values derived from one-way ANOVA with Tukey post-hoc comparision are for EC_{50}: GAA vs. GES p=0.0015; GAA vs. γ-GBA p=0.001; γ-GBA vs. GES p=0.27 [df(drugs)=2, F=21.2]. For [^{3}H]muscimol displacement IC_{50} comparisons

adjusted p-values from ANOVA are GAA vs. GES p=0.014 ; GAA vs. γ-GBA p=0.81; and γ-GBA vs. GES p=0.074 [df(drugs)=2, F=6.8].

Discussion

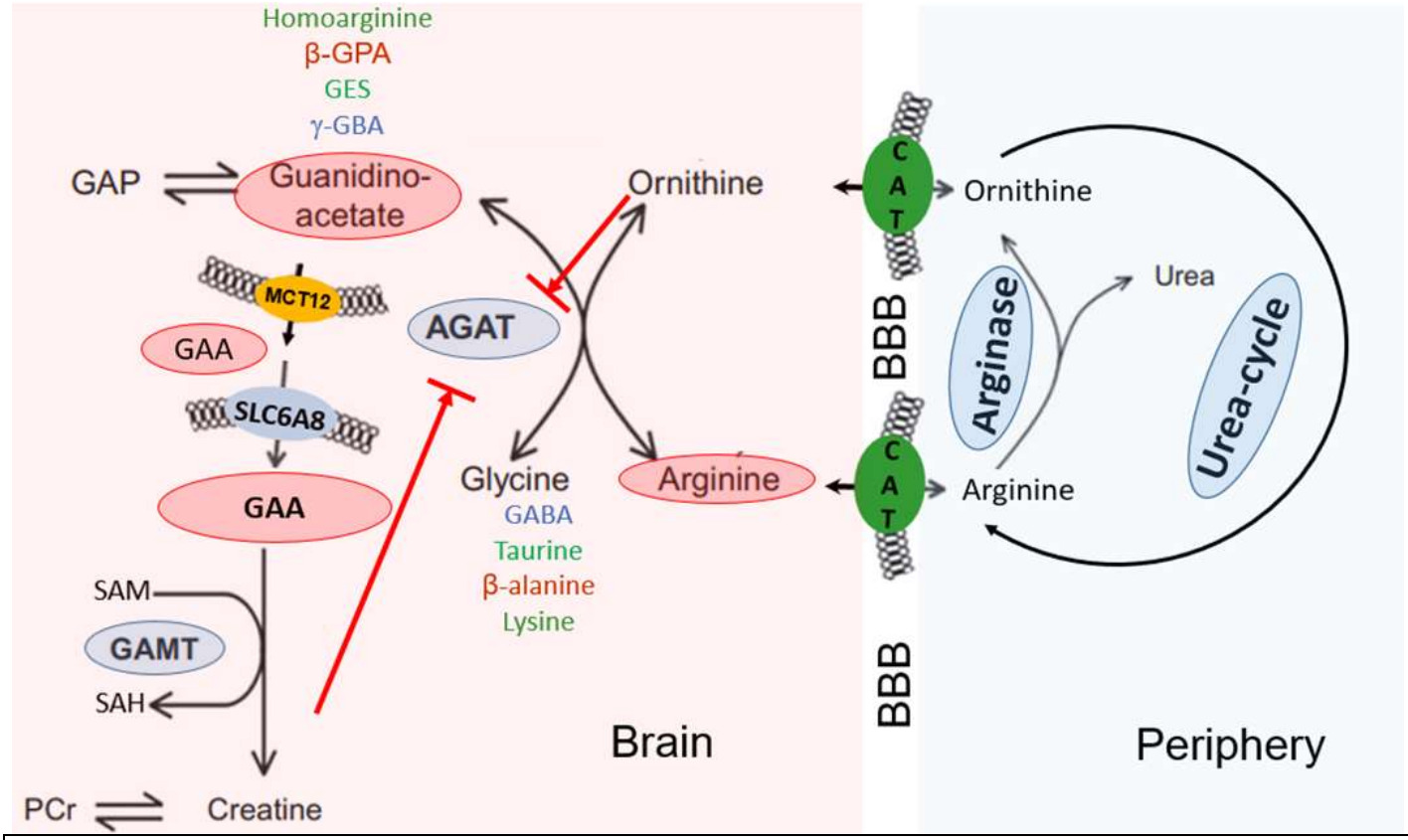

Fig. 4. Creatine metabolic pathway. Both AGAT & GAMT are required for creatine synthesis while the creatine transporter (SLC6A8) is required for uptake of creatine & GAA into cells. In the brain, AGAT is expressed mainly in neurons while GAMT expression is highest in oligodendrocytes. This suggests that GAA synthesized in AGAT positive neurons is taken up into GAMT expressing cells by the creatine transporter (SLC6A8), and thus extracellular GAA may also accumulate in SLC6A8 deficiency. Monocarboxylate transporter 12 (MCT12) has been recently identified as a GAA efflux transporter (Jomura et al., 2020). AGAT is a promiscuous enzyme generating GAA from glycine but also converts other compounds with primary amino groups (e.g. GABA, taurine, β-alanine, lysine) into guanidino compounds (Watanabe et al., 1994) (see Fig. 1). While the brain under normal conditions relies primarily on endogenous creatine synthesis, massive daily creatine supplementation (up to 400 mg/kg/d) for extended periods (months) leads to a normalization of brain creatine levels in GAMT and AGAT deficiency; this resolves AGAT deficiency manifestations if treated early in life, however it is only partially effective in GAMT deficiency. It has been shown in GAMT-deficient mice that phosphorylated guanidinoacetate (GAP) can substitute for the lack of phosphocreatine (PCr) (Kan et al., 2004). The unique clinical manifestations of AGAT deficiency are therefore likely due to lack of both creatine and GAA. This highlights increased GAA (rather than the lack of creatine) in mediating neurotoxicity in GAMT deficiency. Active transport mechanisms, in particular the cationic amino acid transporters (CAT), allow arginine and ornithine to cross the BBB.

Urea cycle (UCD) and creatine synthesis disorders (CSDs) are genetic conditions in which enzymes (and transporters) involved in either nitrogen metabolism or creatine biosynthesis are malfunctioning due to mutations, and like in HE and UE, manifest primarily in neurological pathology. Depending on which of the individual UCD and CSD enzymes or transporters is dysfunctional, the clinical presentation, as well as the biochemical fingerprint, vary, and this might provide an opportunity in deducing general mechanisms of neurological impairment and help with better future treatment options.

The two enzymes required for creatine synthesis (AGAT, GAMT) and the creatine transporter (SLC6A8) are expressed in the brain, and therefore the mammalian brain is largely independent of dietary or peripherally synthesized creatine (Fernandes-Pires and Braissant, 2022; Hanna-El-Daher and Braissant, 2016). While BBB permeability for creatine via SLC6A8 is low, it can be overcome by massive and prolonged creatine supplementation; the BBB permeability for GAA is likely even lower, consistent with the finding that GAA is about 10 times less efficiently transported than creatine by the creatine transporter (SLC6A8) (Jomura et al., 2022) and the observation that dietary GAA does not accumulate in the brain (Ostojic, 2021; Ostojic and Ostojic, 2018). This implies that GAA neurotoxicity likely arises through GAA produced within the brain itself through GAMT enzyme block (i.e., GAMT deficiency) or through increased AGAT activity driven by increased brain arginine concentrations (i.e., ARG1 deficiency) (Fig. 4). Brain arginine is not directed to urea cycle intermediates as these enzymes are not present in the brain; along with brain AGAT expression this might explain why in GAMT-deficient mice GAA accumulation is more pronounced in the brain when compared to plasma and to other GCs (including γ-GBA) which are increased in the brain, but not in the plasma (Torremans et al., 2005). It is therefore possible that arginine-driven GAA/GC synthesis through AGAT is more pronounced in the brain than in the periphery (see Fig. 4).

We show in figure 2 that GAA concentrations at exactly the pathological concentrations found in the CSF in GAMT deficiency (1-30 µM) activate GABA currents (GAA EC_{50} ~6 µM) in cerebellar granule cell neurons while creatine (up to 10 mM) and arginine (up to 1 mM) do not evoke any currents (Fig. 3 D, E); these findings make $GABA_A$ receptors plausible targets for GAA neurotoxicity. GAA has previously been shown to be a GABA analog and while it was concluded for this to happen at pathophysiologically relevant concentrations, most assays

required around >100 μM GAA (e.g., on recombinantly expressed α1β2γ2 $GABA_AR$ or in cultured cortical (globus pallidus) neurons for half maximal activation (Neu et al., 2002). Even higher GAA concentrations (i.e. mM range) were required to evoke currents in cultured cerebellar granule cell neurons, and it was concluded the very high extracellular GAA concentrations required to activate $GABA_ARs$ are not likely to be reached in GAMT deficiency (Cupello et al., 2008). One possible explanation for these discrepancies between the high potency of GAA we show here and the lower potency in the two cited studies is that GAA binds to and activates highly GABA- and GABA analog- (i.e. THIP, muscimol) sensitive extrasynaptic δ-subunit-containing $GABA_ARs$ (Benkherouf et al., 2019; Meera et al., 2011). Consistent with the finding that $GABA_AR$ δ subunits are only expressed in mature animals, it has been shown that cultured CGCs (usually derived from neonatal mice) do not express δ subunits, which are needed for highly GABA-sensitive δ-$GABA_ARs$ (Gatta et al., 2009). Consistent with the notion that $GABA_ARs$ are critical GAA targets is that in GAMT-deficient mice higher doses of the $GABA_AR$ pore blocker picrotoxin are required to induce seizures when compared to WT mice (Schulze et al., 2016). In contrast to GAA, creatine up to 10 mM, has no discernable GABA agonist activity (Fig. 2D) demonstrating that GAA methylation by GAMT (Fig. 1) completely abolishes $GABA_AR$ agonist activity.

While AGAT transfers the amidino group from arginine to glycine, this is a non-specific enzyme that can use as substrate other amino acids with primary amines like GABA and taurine (Watanabe et al., 1994), both (like glycine) present at mM concentration in the cytoplasm of mammalian cells (Cooper and Jeitner, 2016). It has been shown both in GAMT deficiency as well as in ARG1 deficiency there is an increase in GCs (Deignan et al., 2010; Torremans et al., 2005) which may be driven by mass action through the accumulation of GAA and arginine (see Fig. 4).

Like GAA, taurine and GABA amidino-derivatives (GES and γ-GBA) are also GABA analogs (see Fig. 1 for structures). Both GES and γ-GBA, like GAA, not only evoke tonic GABA currents in cerebellar granule cells with an EC_{50} of ~30-40 μM (Fig. 3), they also displace [^{3}H]muscimol at reasonable concentrations (IC_{50} ~ 8 μM for γ-GBA and ~33 μM for GES), suggesting that they are indeed GABA mimetics on $GABA_ARs$. Taurine itself has been previously shown to be a potent activator of extrasynaptic $GABA_ARs$ in the thalamus (Jia et al., 2008); it has been concluded that taurine can act as an endogenous activator of GABA currents, based on the observation that application of GES, previously suggested to be an inhibitor of taurine uptake (Huxtable, 1992), increased GABA currents (Jia et al., 2008). Our findings that GES not only activates GABA currents but also

displaces [^{3}H]muscimol at similar concentrations (Fig. 3) suggests that GES, just like high concentrations of taurine itself, is an orthosteric $GABA_AR$ agonist.

One of the unique clinical hallmarks in GAMT deficiency (and to a lesser extent in ARG1 deficiency and creatine transporter mutations) is hyper-excitability including seizures, which seems paradoxical if GAA (and potentially other GCs) agonistic actions on $GABA_ARs$ is the critical precipitating factor. GABA is the main CNS inhibitory neurotransmitter and activation of $GABA_ARs$ generally result in a reduction of neuronal excitability. However, sustained activation of $GABA_ARs$ is known to lead to $GABA_AR$ downregulation which may explain the observed hyper-excitability phenotype in GAMT deficiency. This may be analogous to rats that are subjected to repeated high doses of ethanol, which leads to $GABA_AR$ downregulation and a characteristic hyper-excitability phenotype (Olsen and Spigelman, 2012). There are alternative explanations, like that GAA as well as γ-GBA or GES interfere with normal GABAergic transmission in ways that lead to hyperexcitability. Activation of (in particular) classical synaptic $GABA_ARs$ is usually accompanied by desensitization (in which $GABA_ARs$ revert into a closed conformation in the continued presence of agonists). It is notable that we do not observe any discernible desensitization under our electrophysiology recording conditions using cumulative concentration-response curves, with application of increasing GAA, γ-GBA and GES concentrations.

While the lack of creatine synthesis may contribute to the observed phenotypes in creatine deficiency disorders, it has been shown that phospho-GAA [GAP in Fig. 4 which is a phosphagen in some primitive organisms (Ellington, 2001; Huxtable, 1992)] is highly elevated in GAMT-deficient mice (Schmidt et al., 2004) and compensates for lack of phosphocreatine (Kan et al., 2004); this is a likely explanation for the surprising observation that lack of creatine in GAMT-deficient mice has no impact on exercise performance (Lygate et al., 2013). In contrast, AGAT deficiency, where both GAA and creatine are absent, presents as a unique neuropathology manifesting in intellectual disability and autism, along with pronounced myopathy (Stöckler-Ipsiroglu et al., 2015). Also, unlike GAMT deficiency, AGAT deficiency is, with early detection and sustained high dose creatine supplementation, an entirely treatable disease (Stöckler-Ipsiroglu et al., 2015), underscoring the critical role that elevated GAA (rather than the absence of creatine) plays in mediating neurotoxicity in GAMT deficiency (Hanna-El-Daher et al., 2015; Hanna-El-Daher and Braissant, 2016).

In ARG1 deficiency there is also an increase in GAA (Amayreh et al., 2014; Huemer et al., 2016; Ingoglia et al., 2021), although to a much lesser extent compared to untreated GAA deficiency: GAA levels approach

those found in GAMT-deficient patients with optimal treatment [i.e. ~1-2 μM GAA in CSF (Stöckler-Ipsiroglu et al., 2014)] but still clearly within the range that we report here for $GABA_A$Rs activation and [^{3}H]muscimol displacement which occurs at concentrations as low as 300 nM (Fig. 2A-C). In addition, there are multiple other guanidino compounds (including γ-GBA and GES) and elevated taurine found in arginase deficiency (Cantero et al., 2016; Deignan et al., 2010); the combined presence of these could have synergistic effects on $GABA_A$Rs.

The overall concept here is that small molecules which accumulate in creatine and urea cycle disorders, HE, and UE, could interfere with brain neurotransmission. This can occur for example by mimicking neurotransmitters (like GABA or glutamate) or interfering with neurotransmitter transporters. Evolution has protected the brain by evolving the BBB so that small metabolites at physiological concentrations do not usually interfere with information processing in the brain, while active transporters expressed at the BBB ensure that critical metabolites like amino acids and nutrients can enter the brain. Our work presented here suggests that the GCs GAA, γ-GBA and GES, likely synthesized from precursors within the brain, are GABA mimetics and interfere with GABAergic neurotransmission, the latter two to a lesser extent than GAA itself, while arginine does not have an effect. A better understanding of the exact molecular mechanism will hopefully inform better future treatment options, including sophisticated gene therapy approaches to restore enzyme function in the brain (Khoja et al., 2022).

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