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Guanidino Compounds with native GABA(A) δ Receptor selectivity: A Tale of Homeostatic Compensation in δ-KO mice

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Key Words

$GABA_A$ receptor, GABA-mimetics, extrasynaptic $GABA_A$ Receptors, Guanidinoacetate, Guanidinopropionate

Abbreviations

AGAT: arginine:glycine amidinotransferase (EC 2.1.4.1)

GAMT: guanidinoacetate N-methyltransferase (EC 2.1.1.2.)

CGC: cerebellar granule cell

aCSF: artificial cerebrospinal fluid

GABA: □-aminobutyrate

GABAR: □-aminobutyrate type A receptor

□-GABAR: extrasynaptic □ subunit-containing GABARs

□2-GABAR: synaptic □2 subunit-containing GABARs

□-KO mice: □-GABAR subunit deficient mice

GAA: guanidinoacetate

GES: guanidinoethanesulfonate

□-GBA: □-guanidinobutyrate

β-GPA: β-guanidinopropionate

□-K-□-GVA: □-Keto-□-guanidinovalerate

EC_{50}: half maximal effective concentration

IC_{50}: half maximal inhibitory concentration

I_{max}: maximal current at saturating agonist concentration

GCs: guanidino compounds

Abstract

Altered GABAergic transmission has been implicated in the neurological symptoms of metabolic disorders associated with guanidino compound (GC) accumulation. Building on previous findings that selected GCs act as direct orthosteric $GABA_A$ receptor (GABAR) agonists, we now asked whether these GCs act preferentially on high-affinity extrasynaptic δ subunit-containing receptors (δ-GABAs). Using whole-cell patch clamp recordings from mouse cerebellar granule cells (CGCs) in brain slices of wild-type and δ-subunit knockout (δ-KO) mice, together with 5 nM [^{3}H]muscimol displacement assays on WT and δ-KO forebrains, we compared the actions of four structurally GABA-like GCs — guanidinoacetate (GAA), β-guanidinopropionate (β-GPA), guanidinoethanesulfonate (GES), and δ-guanidinobutyrate (δ-GBA). These compounds activated CGC GABARs in cumulative concentration-response curves and displaced the highly δ-GABAR-selective ligand [^{3}H]muscimol suggesting δ-GABAR-selective orthosteric agonist actions. In δ-KO forebrains, total [^{3}H]muscimol binding was reduced by ~50%, confirming the loss high-agonist-affinity but low-abundance (~5% of total forebrain GABARs) δ-GABARs. δ-KO CGCs showed markedly reduced agonist sensitivity, with EC_{50} values (μM, WT/δ-KO): GABA (2/6) < β-GPA (3/8) < GAA (4/14) < GES (32/72) < δ-GBA (44/219). The modest loss of agonist sensitivity for GABA and the four GABA-mimetic GCs in δ-KO CGCs is consistent with compensatory upregulation of non-δ extrasynaptic GABARs containing only α and β subunits, as previously described (Tretter et al., JBC 2001), explaining the preserved tonic

inhibition in □-KO neurons. Our findings demonstrate that GABA-mimetic GCs preferentially target □-GABARs and suggest that homeostatic compensation by □β-type GABARs is a key adaptive mechanism maintaining inhibitory tone in □-KO CGC neurons.

Introduction

Altered GABAergic transmission has long been implicated in the neurological symptoms associated with metabolic disorders involving the urea and creatine metabolic pathways [1, 2]. Such disorders, including acute and chronic liver and kidney failure [3-6] and congenital enzyme deficiency disorders are often accompanied by neurotoxicity despite the protective function of the blood-brain barrier. The accumulation of uremic and hepatic toxins primarily affects the central nervous system (CNS), causing cognitive impairment, seizures, and other neurological manifestations. Yet, despite decades of research, the identity of these toxins, their molecular targets and mechanisms remain largely elusive. Among the suspected mediators are guanidino compounds, although hyperargininemia and hyperammonemia are also implicated in neurotoxicity [2, 7-10].

Human genetic disorders and their animal models provide specific insight into the molecular mechanism of these metabolic encephalopathies, since congenital enzyme defects generally lead to the accumulation of specific metabolites, with hyperexcitability and epilepsy a frequent phenotype [11]. A notable example is guanidinoacetate (GAA) in N-methyltransferase (GAMT) deficiency, in which the final step in creatine synthesis is blocked (see Fig. 1A, top left). GAMT deficiency leads to a dramatic increase in guanidinoacetate (GAA), in both brain and the periphery while

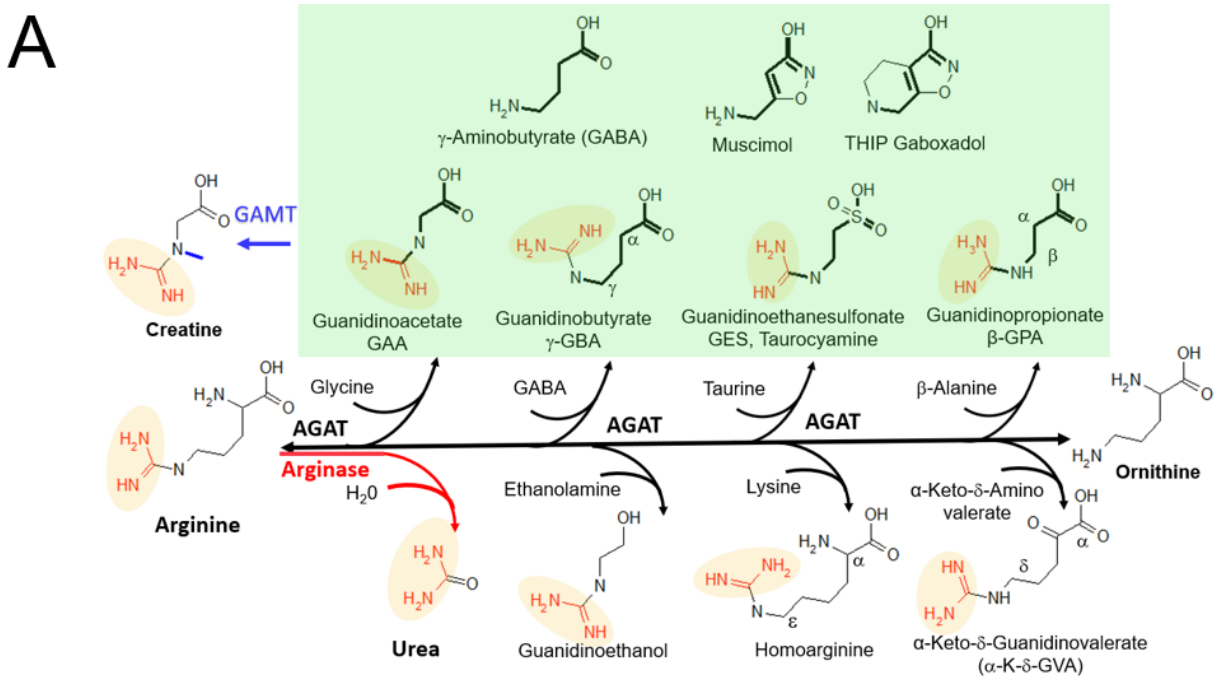
reducing creatine [12]. The neurological symptoms of GAMT deficiency appear to result mainly from GAA accumulation rather than from creatine deficiency, as even prolonged high-dose dietary creatine supplementation restores brain creatine with only limited effect on the neurological phenotype [13].

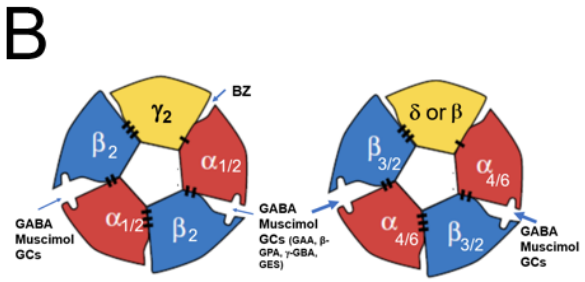
The creatine precursor GAA is formed by arginine:glycine amidinotransferase (AGAT) which transfers an amidino group from arginine (its rate-limiting substrate) to glycine. Due to its broad substrate specificity, AGAT can also generate other guanidino compounds, including □-guanidinopropionic acid (□-GPA), □-guanidinobutyric acid (□-GBA) and guanidinoethanesulfonic acid (GES) - from their precursor amino acids □-alanine, GABA and taurine, respectively (see Fig. 1A) [14, 15]. Under conditions such as arginase deficiency, where arginine accumulates, mass-action effects might drive overproduction of GAA and related GCs see Fig. 1 [16]. Conversely, □-GBA, β-GPA and GES (but not GAA) can be hydrolyzed by a guanidino acid hydrolase, which might have evolved to detoxify these potentially harmful GCs [15]. Because GABA and GABA mimetic guanidino compounds are highly polar, they do not cross the blood-brain barrier in the absence of active transport mechanisms. Therefore, endogenous brain synthesis of GCs by the enzyme AGAT (Fig. 1) may be critical for their brain accumulation. Indeed, GAA activates GABARs at concentrations matching those found in the CSF in GAMT deficiency, implicating GAA agonist action in the associated neuropathology [1, 17].

β-GPA is a potent creatine transporter blocker [18, 19], competing with creatine for transport with an EC_{50} of ~ 13 □M in recombinant (HEK) cell expression [20]. This inhibition reduces intracellular ATP, which might underlie β-GPA's anti-hyperglycemic and exercise mimetic actions [21, 22]. Given the high ATP demand of cancer cells, β-GPA is currently under investigation as a potential cancer therapeutic

[23]. GES has been shown to be a taurine transporter substrate with a K_m of ~150 □M. Oral GES administration has been used to study the effects of (peripheral) taurine depletion [24].

Mammalian GABARs are heteropentameric complexes, with classical synaptic GABARs comprising the majority of all brain GABARs [25]. Synaptic GABARs most frequently contain two □1 subunits, two β2 subunits and one □2 subunit arranged in a pseudo-symmetric □1β2□1β2□2 configuration [26] (see Fig. 1B). Incorporation of a □2 subunit is necessary for (post)synaptic □2-GABARs localization and confers relatively low GABA sensitivity, such that □2-GABAs are primarily activated by brief bursts of high (mM) GABA concentrations of GABA released into the synaptic cleft [27, 28] (Fig. 1C). In contrast, extrasynaptic GABARs lack the □2 subunit, which is replaced by the □ subunit – or by a third β subunit [29]. Additionally, □-GABARs contain either □4 (forebrain) or □6 in cerebellar granule cells [30, 31] (Fig. 1C). The absence of □2 results in GABARs with markedly higher GABA/agonist sensitivity [32], which is further increased by □ subunit incorporation [33, 34].





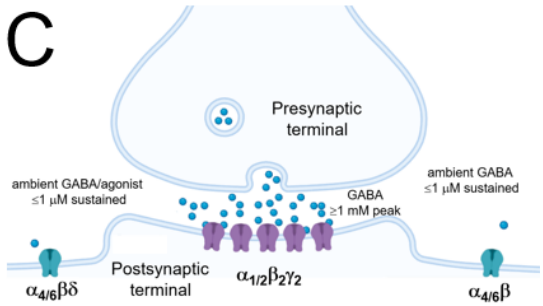


Figure 1. *Structures and biosynthesis of GABA-like guanidino compounds (***A***)* Biosynthetic pathways involving the enzymes arginase, AGAT and GAMT. The GABA backbone structure is in bold, and the amidino group is in red and shaded in yellow. GAA, □-GBA, GES and β-GPA are grouped with GABA (and the conformationally restricted GABA analogs THIP and muscimol) and shaded in green, all of which activate native GABARs and displace [^{3}H]muscimol from mouse brain native (WT and □-KO) GABARs. Based on structural requirements, urea, guanidinoethanol, homoarginine and □-K-□-GVA are unlikely to bind at reasonable concentrations to the GABA/muscimol site; we confirmed absence of detectable CGC GABAR activity at concentrations up to 1 mM for arginine, creatine and □-K-□-GVA as well as absence of [^{3}H]muscimol (5 nM) displacement at concentrations up to 0.3 mM in mouse forebrain. Muscimol and THIP are conformationally restricted high affinity orthosteric GABA ligands, with particularly high affinity for extrasynaptic □ subunit-containing GABARs. (**B**) Subunit arrangement of $GABA_AR$ showing two orthosteric agonist at β/□ subunit interfaces, with the classical benzodiazepine (BZ) binding site at the □/□2 subunit interface. In □-containing GABARs, the □ subunit replaces the □2 subunit. In GABARs composed of only □ and □ subunits, there is a third β subunit at the same position (marked in yellow). Agonist/GABA binding triggers conformational changes that open the central Cl^- permeable pore. (**C**) Dichotomy between synaptic (low affinity) and high affinity extrasynaptic $GABA_A$ receptors. Most classic synaptic GABA receptors are composed of □1, β2 and □2 subunits and mediate inhibitory synaptic transmission triggered by brief (milliseconds) bursts of high (□1 mM) GABA concentrations in the synaptic cleft. In marked contrast,

Unlike fast-desensitizing synaptic □2-GABARs, extrasynaptic GABARs exhibit sustained activation in the continuous presence of low (□1 μM) ambient GABA. Their high GABA/agonist sensitivity and lack - or very slow and incomplete - desensitization enable them to mediate a constant (tonic) form of inhibition. This sustained activity of extrasynaptic GABARs more than compensates for their low abundance, with □4β□-GABARs constituting ~5% of total GABAR in the mouse forebrain [25] and ~20% □6□□ GABARs in the cerebellum [31]. Thus despite their low abundance, the combination of high GABA/agonist sensitivity combined with persistent activity renders extrasynaptic GABARs critical regulators of neuronal excitability [35] - notwithstanding the absence of obvious hyperexcitability in □-KO mice, presumable due to homeostatic compensatory plasticity.

Here we demonstrate and confirm [17] that the GABA structural analogs GAA, β-GPA, □-GBA, and GES are agonists on native GABARs in WT and less potently also in □-KO CGCs. (Fig. 2A).

Agonist actions were confirmed by displacement of the orthosteric radioligand [^{3}H]muscimol at 5 nM, a highly □-GABAR–selective concentration [36]. In WT membranes GAA, β-GPA, □-GBA, and GES displaced around 50% □-GABAR specific [^{3}H]muscimol binding that is lacking in the □-KO forebrain, with potencies comparable to those observed with electrophysiology, consistent with the notion that this reflects □-GABARs selectivity. Interestingly, [^{3}H]muscimol displacement by GAA, β-GPA, GES and GABA of the remaining 50% (likely artefactual) high-affinity [^{3}H]muscimol binding in □-KO brains suggests comparable potency to this poorly characterized fraction of high affinity [^{3}H]muscimol binding [36]. A notable exception is □-GBA, with loss of displacement potency and reduced maximal currents in □-KO CGCs. While □ subunit deficiency significantly reduces agonist potency, the effect is unexpectedly modest and surprisingly no reduction (with the exception of □-GBA) in maximal current amplitude, for not only GAA, β-GPA, GES, but also GABA itself. Given that synaptic receptors are expected to be largely desensitized, this likely reflects an under-appreciated compensatory expression of fairly agonist sensitive “binary” α6β extrasynaptic receptors [31], to replace extrasynaptic □6β□ GABARs in □-KO cerebellar granule cells (CGCs).

Materials and Methods

Animal (mouse) Methods

Animal care and experimental procedures were as described [17, 37] and conducted according to the guidelines of the UCLA Chancellor’s Animal Care and Use

Committee (ARC) (Animal Welfare Assurance number A3196-01 and animal protocol ARC-2019-032) and kept according to the National Institutes of Health guidelines with temperature and humidity control. We used C57BL/6 wildtype mice and □-KO mice (a gift from Istvan Mody at UCLA) on the C57BL/6 background (both sexes, 6-16 weeks of age, total about 115 mice). The mice were housed in conventional cages in 12:12 h light:dark cycle in groups of 4-5 mice per cage having *ad libitum* access to rodent lab chow (PicoLab Rodent Diet 20, #5053) and water. Brains were removed after brief isoflurane anesthesia, the cerebellum was used for preparing cerebellar brain slices, and forebrains were stored at -80ºC and shipped on dry ice for [^{3}H]muscimol displacement experiments. Wild type C57Bl/6 mice were bred within our mouse colony as were □-KO mice.

Brain Slice Preparation and Electrophysiology

Slices were prepared using standard techniques: Cerebella were removed from the cranium, submerged in cold (<4ºC) artificial cerebrospinal fluid (aCSF), and ~290 □M thick slices were cut with a (Leica VT-1000s, Deerfield, IL, USA) vibratome. The slicing solution contained (in mM): 85 NaCl, 0.5 $CaCl_2$, 4 $MgCl_2$, 2.5 KCl, 1.25 NaH_2PO_4, 75 sucrose, 24 $NaHCO_3$, 25 glucose. Slices were oxygenated with 95% O_2 and 5% CO_2 in artificial cerebrospinal fluid (aCSF) for storage and electrophysiological recordings containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$ and 11 glucose. Whole cell pipette solution consisted of (in mM): 100 KCl, 5 NaCl, 4 $MgCl_2$, 4 ATP and 0.4 GTP, 40 HEPES adjusted to pH 7.4 with KOH. All salts/chemicals were obtained from Millipore-Sigma (Saint Louis, MO, USA). Cerebellar granule cells (CGCs) were visualized using an infrared-DIC enhancement equipped upright microscope (Zeiss, White Plains, NY) and whole cell CGC recordings performed with a

Multiclamp 700B amplifier (Axon Instruments, Inc., Foster City, CA, USA) at -70 mV at room temperature (22-24ºC). Action potentials and glutamate receptor-mediated transmission were blocked by 0.3 µM TTX and 10 µM DNQX respectively.

Reagents

GABA, □-GBA and β-GPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Guanidinoacetic acid (GAA) was from Pfaltz & Bauer (Waterbury, CT, USA) and GES (taurocyamine) was purchased from Cayman Chemical (Ann Arbor, MI, USA). [Methylene-^{3}H]muscimol (22 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). GABA, GAA, β-GPA, GES, □-GBA were prepared as 100 mM or 10 mM stock solutions either in aCSF for electrophysiology, or in assay buffer for [^{3}H]muscimol displacement studies. Gabazine (SR95531), TTX, and DNQX were obtained from Tocris Bioscience, Inc. (Minneapolis, MN, USA) or from Hello Bio (Princeton, NJ, USA).

[^{3}H]Muscimol Displacement Experiments

Brain (*sans* cerebellum) membranes from WT and □-KO mice were prepared as described [38] and stored frozen at -70ºC. After thawing, membranes were washed once in assay buffer (50 mM Tris-HCl, pH 7.4) and incubated for 40 min with 5 nM [^{3}H]muscimol in assay buffer in a total volume of 300 µl at room temperature. Non-specific binding was determined by adding 100 µM GABA. After incubation, membranes were collected by filtration through Whatman GF/B filters (Whatman International Ltd., Maidstone, UK) with a Model M-24 Brandel Cell Harvester (Gaithersburg, MD, USA) followed by two quick washes with 4-5 ml of ice-cold assay buffer. Three milliliters of Optiphase HiSafe 3 scintillation fluid (Wallac, Turku, Finland)

were added to air-dried filters and GABAR bound [^{3}H]muscimol radioactivity determined in a Hidex 600 SL liquid scintillation counter (Hidex, Turku, Finland).

Data Analysis

GraphPad Prism (versions 7-10) software (GraphPad, San Diego, CA, USA), Microsoft Excel and Igor Pro were used for [^{3}H]muscimol displacement and electrophysiology data analysis. Data are presented as mean ± standard error of mean (SEM) with statistical comparisons performed by Student's t-test (unpaired, two-tailed) in Microsoft Excel and one-way ANOVA with Tukey's multiple-comparison test in GraphPad Prism.

Results

Because the guanidino compound GAA likely exerts its neurotoxicity through actions at GABARs [1, 37], we studied four structural GABA analogous GCs - GAA, □-GBA, □-GPA, GES - for their ability to evoke GABAR-mediated currents in CGCs from both WT and □-GABAR-deficient mice, as we suspected that GABA-mimetic actions are mediated primarily by high-agonist-affinity □ subunit-containing GABARs.

GABARs are frequently studied using recombinantly expressed receptors with defined subunit combinations. However recombinant systems have notable limitations. In particular, □-GABARs are difficult to express functionally, often resulting in GABARs composed of only □ and β subunits which do not fully reflect the high GABA, THIP and muscimol sensitivity observed in native □-GABARs. [34, 36, 39]. Furthermore, key modulatory proteins such as Neuroligin-2 [28], Shisa7 [40, 41] and TMEM132B, which are known to change pharmacological and functional properties

[40, 42, 43] as well as endogenous bound neurosteroids [26] are likely missing in recombinant expression systems.

To circumvent these limitations, we examined native GABARs in CGCs from acutely prepared brain slices. CGCs, which constitute roughly 50% of all neurons in the mammalian brain, express well-characterized synaptic GABARs (mainly □1□2□2 and □6□□2 receptors) as well as extrasynaptic □6β□ GABARs. Notably, cerebellar extrasynaptic □-GABARs comprise ~20% of total cerebellar GABARs [28, 31] – significantly higher than the ~5% of extrasynaptic □4□□ GABARs found in the forebrain [30]. This high abundance should make CGCs uniquely suited for studying the relative contributions of synaptic and extrasynaptic GABARs by comparing agonist-evoked responses to GABA and guanidino GABA mimetics in WT and □-KO CGCs.

To measure the activation of native GABAR-mediated currents, we performed whole cell patch-clamp recordings from CGC using cumulative concentration-response experiments in which progressively higher agonist concentrations were applied. Under continuous agonist exposure, synaptic GABARs are expected to desensitize; thus evoked currents are primarily mediated by non-desensitizing extrasynaptic GABARs. As illustrated in Figure 2, such slow perfusion conditions result in steady-state GABA currents that are arguably most relevant for GABA-mimetic compounds, which - like ambient GABA - are likely found in the extracellular space

and detected by the high-affinity extracellular binding sites of extrasynaptic GABARs.

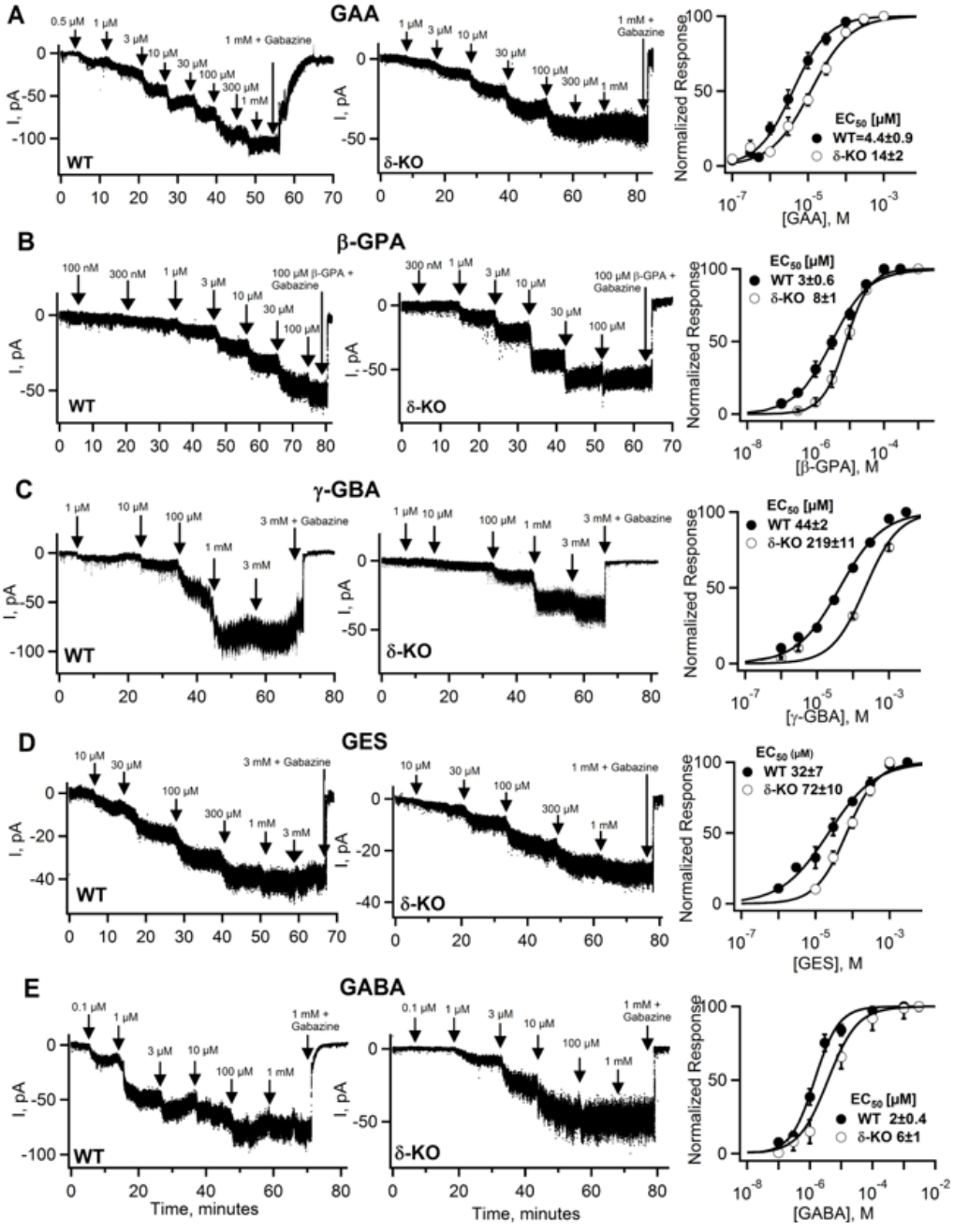


Figure 2. *The structural GABA analogs GAA, □-GBA, β-GPA, GES, like GABA, evoke GABA receptor currents in mouse cerebellar granule cells (CGC) from WT and □-KO mice.* Whole-cell patch clamp recordings of CGCs at a holding potential of -70 mV in the presence of the glutamate receptor blocker DNQX (10 μM) with action potentials blocked by 0.3 μM TTX. Currents in WT and □-KO CGCs were evoked by perfusion of increasing concentrations (indicated by arrows) of (**A**) GAA, (**B**) β-GPA, (**C**) □-GBA, (**D**) GES and (**E**) GABA for 5-10 min, until the currents reach a new steady state. After application of a saturating agonist concentration, responses were blocked by the GABAR-specific antagonist gabazine (SR95531) to confirm mediation by CGC GABARs. Concentration-response curves (right panels) were generated by fitting current amplitudes with the Hill equation to determine EC_{50} values and Hill slope coefficients. Statistical comparisons between WT and □-KOs currents were performed using Student's t-test with EC_{50} values determined by fitting each individual experiment and showed a moderate yet significant loss of agonist potency in □-KO CGCs. For β-GPA the curve was significantly shallower in WT when compared to □-KO CGCs (p=0.003) and only □-GBA showed significantly reduced current amplitude in the □-KO (p=0.017) (see Table 1 for data and

276

277 Perfusion of increasing concentrations of GAA, β-GPA, □-GBA and GES evoked
278 currents

	WT EC_{50} in μM (n)	□-KO EC_{50} in μM (n)	pEC_{50} p value	WT slope	□-KO slope	slope p-value	WT I_{max} pA	□-KO I_{max} pA	I_{max} p-value
GAA	4.4±0.9 (7)	14±2 (7)	0.002 **	1.1±0.16	0.94±0.1	0.299	-60±18	-50±10	0.67
β-GPA	3.2±0.6 (5)	8.2±1.4 (6)	0.008 **	0.85±0.1	1.3±0.2	0.003**	-45±9	-74±19	0.18

□-GBA	44±2.3 (6)	219±11 (5)	$3x10^{-9}$ **	0.81±0.1	0.95±0.1	0.17	-75±12	-32±7	0.017*
GES	32±7 (5)	72±10 (5)	0.017 *	0.89±0.3	0.83±0.2	0.8	-35±7	-29±6	0.48
GABA	1.8±0.1(11)	6.1±1 (8)	0.0002 **	1.5±0.3	1.1±0.1	0.3	-66±5	-65±18	0.92

in both WT and in □-KO CGCs, similar to those observed with GABA, and these currents were completely blocked by the GABAR specific antagonist gabazine. Representative current traces for GAA, □-GBA, GES, β-GPA and GABA in both WT and □-KO CGCs are shown in Fig. 2A-E, and potency comparisons of electrophysiological recordings from WT and □-KO CGCs are summarized in Figs. 3A,B. The EC_{50} values for half maximal activation, Hill slope, as well as the maximal currents evoked (with number of experiments in parentheses and variability expressed as ± SEM [standard error of mean]) are presented in Table 1.

Table 1. *Analysis of EC_{50} and I_{max} values of GABA and the GABA-structural analogs GAA, β-GPA, δ-GBA, and GES of wild type and δ-KO CGCs GABA currents.* EC_{50} values, Hill slopes and maximal currents (I_{max}) were determined from curve fits of individual experiments, with representative traces shown in Fig. 2. Also shown are maximal evoked currents (I_{max}) at a saturating agonist concentration. Mean EC_{50}, Hill slope and I_{max} values ± standard error of mean (SEM) are tabulated. Statistical comparisons between WT and δ-KOs currents (EC_{50} converted to pEC_{50}s for t-test) were performed using Student's t-test (unpaired, two-tailed) in Microsoft Excel with

288 Among the tested compounds, GABA shows the highest potency (WT EC_{50} ~ 2
289 µM), followed closely by β-GPA and GAA (WT EC_{50} ~3 and 4 µM respectively) with GES
290 and δ-GBA being less potent (WT EC_{50} ~32 and 44 µM respectively) (Fig. 3). As
291 expected from the loss of high-affinity δ-GABARs, all compounds showed significantly
292 decreased potency in δ-KO CGCs, with δ-GBA exhibiting the most pronounced loss of

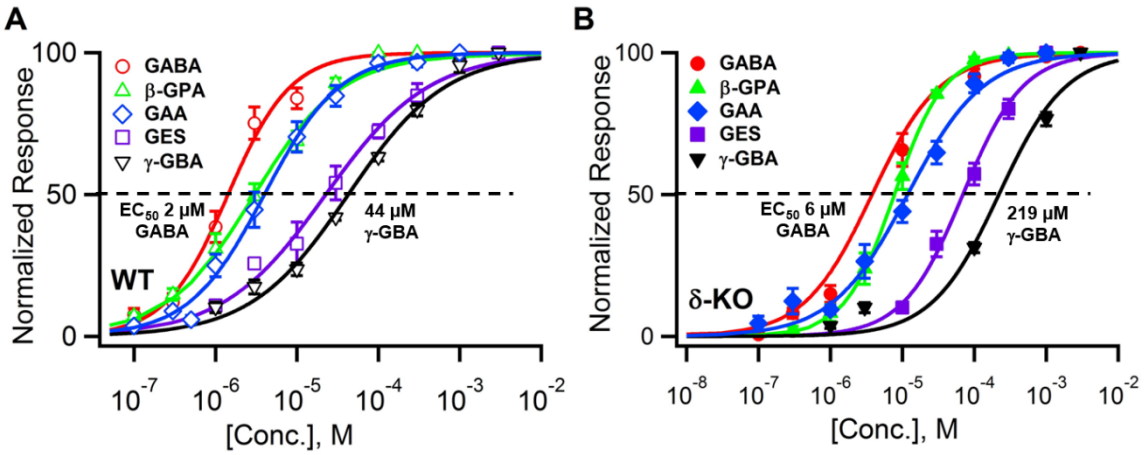


Figure 3. *Comparison of GAA, β-GPA, GES δ-GBA and GABA potency in WT (**A**) and δ-KO cerebellar granule cells (CGC) (**B**).* The EC_{50} values in WT CGCs range from 2 µM for GABA to 44 µM for δ-GBA, and from 6 µM for GABA to 219 µM for δ-GBA in δ-KO CGCs. See Table 1 for tabulation of number of experiments (n) and EC_{50} values. Error bars are standard error of mean (SEM). One-way ANOVA with Tukey's multiple-comparison test indicated that most pairwise comparisons of EC_{50} values were highly significant ($p<0.001$), except for GABA vs GAA in WT ($p=0.03$), and not significant ($p>0.05$) for GABA vs. β-GPA, GAA vs. β-GPA in both

293 agonist-sensitivity in δ-KO CGCs (Fig. 2).

294 Maximal current amplitudes varied substantially across CGCs and, except for
295 δ-GBA, did not differ significantly between WT and δ-KO CGCs. δ-GBA, was the only

compound to evoke significantly reduced maximal currents (p=0.017) in □-KO CGCs (see Table 1).

By physically separating the cerebellum from the rest of the brain allowed us to investigate the two major □-GABAR isoforms: cerebellar □6β□ receptors, examined by whole-cell recordings from cerebellar granule cells, and forebrain □4β□ receptors, analyzed in [^{3}H]muscimol displacement assays. This approach allowed us to confirm that the tested guanidino compounds are GABA analogs and mimetics at the orthosteric GABA site in both major □-GABAR subtypes [17, 37], and that effects are not unique to □6β□-GABARs expressed in CGCs.

Despite the low abundance of □-receptors in the forebrain (~5% of total GABARs), we observed a highly significant ~50% reduction in high-affinity [^{3}H]muscimol binding sites in □-KO brains (Fig. 4B). The origin of the residual 50% of high-affinity [^{3}H]muscimol binding in the □-KO forebrain homogenates is unclear, since it is absent in □-KO forebrain autoradiographic sections [44] and lacks functional equivalents (see Discussion).

Interestingly, the residual high-affinity binding in □-KO mouse brains did not differ significantly from WT for GAA, □-GPA, GES and GABA displacement (with IC_{50} values given in Fig. 4A). Thus, WT and □-KO IC_{50}s closely reflect the displacement sensitivity for □-GABARs. In contrast, significantly higher concentrations of □-GBA were required to displace (5 nM) [^{3}H]muscimol from □-KO forebrain membranes (WT IC_{50}=8.5±0.9 μM, □-KO IC_{50}=26±5 μM, p=0.0035**). This shows that □-GBA displays increased □-selectivity, with a □-specific (WT minus □KO) □-GBA displacement IC_{50} of 3.5±1.2 μM (Fig. 4A, upper right panel).

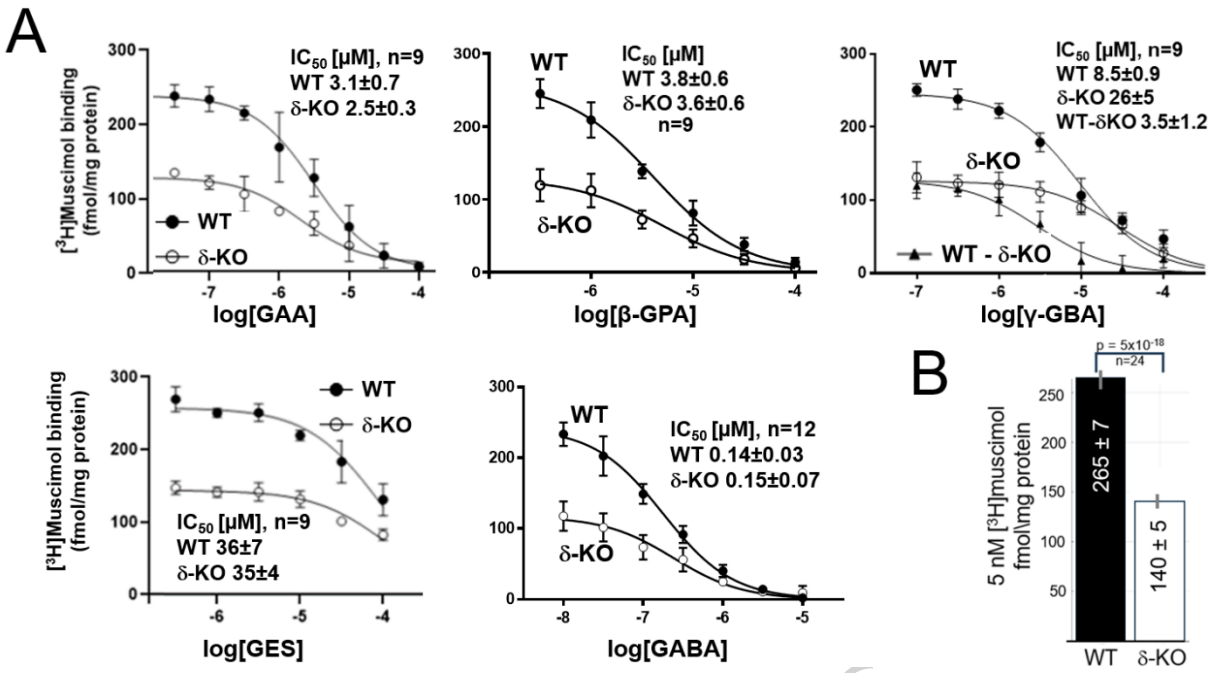


Figure 4. *[^{3}H]muscimol displacement from WT and □-KO mouse forebrain membranes by GABA, GAA, □-GBA, β-GPA, GES.* (**A**) Displacement curves, with specific binding normalized to protein concentrations show a consistent ~50% reduction of 5 nM [^{3}H]muscimol binding in □-KO forebrains compared with WT. Half-maximal concentrations for displacement (IC_{50}) were calculated and are shown in the figures with standard deviations (SD). Since the IC_{50} for □-GBA is significantly higher in □-KO forebrain membranes when compared to WT, we calculated the □-specific □-GBA IC_{50} component by subtracting □-KO from WT values (WT-□KO). [^{3}H]muscimol displacement requires significantly higher concentrations of □-GBA in □-KO brains (p=0.0035) when compared to WT, without significant differences between WT and □-KO brains in displacement potency for GAA (p=0.38) and GABA (p=0.41), β-GPA (p=0.48) and GES (p=0.91) (WT, wild type; KO, knockout; data are expressed as mean ± standard deviation). (**B**) Bar graph illustrating the highly significant (p=5x10^{-18}) reduction of total [^{3}H]muscimol

Discussion

The goal of this study was to identify the molecular targets of GABA structural analog guanidino compounds and test the hypothesis that high-affinity extrasynaptic □-GBARs are their primary sites of actions (see Fig. 1A). To this end, we compared the effects of four GABA-mimetic compound on native receptors in wild-type (WT) and □-subunit knockout (□-KO) mouse cerebellar granule cells (CGCs) using whole-cell recordings and cumulative concentration–response curves (Fig. 2 and 3), and also

performed [^{3}H]muscimol radioligand displacement assays in the mouse forebrain (Fig. 4). We have previously shown that GAA, δ-GBA and GES are orthosteric GABA agonists [17] and in this study also included, β-GPA, best known for its ability to block creatine transporters (see below).

Because extrasynaptic δ-GABARs are highly sensitive to GABA and activated by low ambient extracellular (typically ≤1 μM), we anticipated that, in the absence of highly agonist-sensitive δ-GABARs, structural GABA mimetics, would activate GABAR in δ-KO CGCs only at much higher concentrations. Indeed, for all compounds tested (including GABA itself), δ-GABARs deficiency resulted in significantly higher EC_{50} values (see Fig. 2 and Table 1). The potency loss is also evident in the original traces: currents evoked at threshold concentrations for β-GPA, δ-GBA and GES are absent or smaller in δ-KO CGCs, consistent with the loss of high-affinity δ-GABAR-mediated responses. The absence of δ-GABARs is further reflected in steeper Hill slopes for β-GPA, δ-GBA and GES – indicative of the loss of a high-affinity current component - although this reached statistical significance only for β-GPA (see Fig. 2B, C, D and Table 1). Nevertheless, given the ~1000-fold difference in sensitivity to extracellular (≪1 μM) versus synaptic (mM range) GABA, the magnitude of the potency shifts for all compounds were surprisingly small.

A plausible explanation for the surprisingly modest losses of agonist sensitivity and similar maximal evoked steady-state currents (Table 1) is that the absence of δ-GABARs triggers a homeostatic upregulation of fairly agonist-sensitive α6β-containing GABAR composed of only α6 and β subunits. As previously reported, 24% of all GABARs were estimated to be composed of only α6 and β subunits in the δ-KO cerebellum, which is close to the estimated total number of δ-GABARs in WT cerebellar granule cells [31] - suggesting that α6β receptors may essentially

substitute for the loss of extrasynaptic □6β□ in □-KO CGCs. Under our conditions, with continuous agonist exposure, abundant synaptic receptors are expected to be fully desensitized and thus unlikely to contribute meaningfully to GABA-evoked currents. Our findings therefore support the interpretation that potency differences between WT and □-KO CGCs reflect the smaller sensitivity gap between extrasynaptic □6β□ and □6β GABARs (with □6β GABARs replacing high-affinity □6β□ GABARs in □-KO CGCs), rather than the expected ~1000-fold difference between □6β□ and synaptic □β□ GABARs. This interpretation is consistent with reports that recombinantly expressed GABARs composed solely of □ and β subunits generally show much higher GABA/agonist sensitivity when compared to synaptic □2-GABARs [32], although still significantly lower than □-GABARs [33, 34].

To complement our electrophysiological data findings in CGCs, we performed [^{3}H]muscimol displacement assays. Muscimol is a high-affinity GABA analog and mimetic that has been used for decades as a specific general GABAR ligand [45]. Both muscimol and THIP are reported to be highly selective for □-GABARs [34, 36] and their high potency for □-GABARs provides the molecular basis for the pronounced behavioral insensitivity of □-KO and □4-KO mice to THIP [46, 47] and muscimol [47]. Notably, autoradiography studies showed an essentially complete loss of high-affinity (6 nM) [^{3}H]muscimol binding in the □-KO forebrain [44], in contrast to our findings (Fig. 4), which show that ~50% of 5 nM [^{3}H]muscimol binding remains in the □-KO in forebrain homogenates. The nature of this residual high-affinity binding in in □-KO forebrain homogenates is uncertain, but has also been observed in homogenates of recombinantly expressed □2-GABARs, whose [^{3}H]muscimol binding affinities are between 1-10 nM, whereas functional (EC_{50}) muscimol responses that require muscimol concentrations ~1000-fold higher (i.e. 1-10 μM) [48]. This contrasts sharply

with functional muscimol δ-GABA responses with an EC_{50} of ~1 nM for δ4β3δ receptors [36]. Such high-affinity [^{3}H]muscimol binding to non-δ GABARs in homogenates has therefore been proposed to represent non-functional desensitized synaptic GABAR [49] or artefacts arising from homogenization or freezing [36, 50].

Since the residual high-affinity [^{3}H]muscimol binding in the δ-KO forebrain lacks any evidence for functional significance, it seems prudent to focus on the ~50% [^{3}H]muscimol displacement component in the WT forebrain, that is lacking in the δ-KO forebrain. Interestingly, we obtained very similar IC_{50} values for [^{3}H]muscimol displacement in both WT and δ-KO for GABA, GAA, GES, and β-GPA (except for δ-GBA).This indicates that both the δ-dependent and the residual non-δ-dependent components show very similar half maximal IC_{50} [^{3}H]muscimol displacement (see Fig. 4A). Notably, these IC_{50} values align closely with our functional EC_{50} values supporting the interpretation that both the cumulated concentration-response electrophysiology and high-affinity (5 nM) [^{3}H]muscimol binding assays primarily reflect high-affinity extrasynaptic GABARs. Binding assays often yield higher apparent potencies than functional measurements, but in our case IC_{50} values for [^{3}H]muscimol displacement were only modestly lower—largest for GABA at just over 10-fold—than EC_{50} values from CGC recordings. This small difference may reflect that both assays were performed at room temperature rather than on ice, the high agonist sensitivity of cerebellar δ6βδ-GABARs compared with α4-containing receptors, and the fact that we did not calculate K_i values (expected to be lower) using the Cheng–Prusoff equation, as the K_D for [^{3}H]muscimol at α4βδ receptors is unknown.

The combined evidence that the four guanidino compounds studied here and elsewhere ([17, 37] are (1) close structural GABA analogs (Fig. 1A), (2) activate functional GABA-like currents in CGCs, (3) which are completely blocked by the

specific blocker gabazine (SR55731) (Fig. 2) and (4) completely displace [^{3}H]muscimol at relevant and comparable concentrations (Fig. 4), strongly suggests that GAA, β-GPA, GES and □-GBA are GABA mimetics acting on orthosteric GABA site in GABARs (Fig. 1C). From a simple structure–activity perspective, the four guanidino compounds tested here preserve the core electrostatic and spatial features of GABA: a terminal positively charged group (either a primary amine, as in GABA, or a protonated amidino/guanidino group) paired with a terminal negatively charged group (a carboxylate in GABA, or, in the case of GES, a sulfonate), separated by an unbranched, flexible 2–4 atom aliphatic linker (Fig. 1A). This supports two key inferences. First, a negatively charged sulfonyl group can serve as an effective bioisostere for the GABA carboxylate at the orthosteric site, as also seen with homotaurine, which is structurally identical to GABA except for containing a sulfonate instead of a carboxylate group, a potent orthosteric GABA mimetic [37]. Second, replacement of the protonated terminal $-NH_3^+$ group in GABA with a protonated guanidino/amidino moiety (GAA, β-GPA, □-GBA) maintains high apparent affinity and efficacy. Together, these observations argue that the orthosteric GABA pocket primarily recognizes the correct charge separation and linker geometry, rather than strictly requiring a primary amine on one end and a carboxylate on the other.

β-GPA is a high-affinity substrate for the creatine transporter, with an EC_{50} of ~13 μM.- considerably lower than that of creatine itself (EC_{50} of ~ 35 □M) [20] - and under investigation as competitive creatine transport blocker cancer drug [23]. In this study, β-GPA displayed even higher potency on GABARs, activating tonic CGC currents (EC_{50} ~3 μM, Fig.2 & 3) and displacing [^{3}H]muscimol in the forebrain (IC_{50} ~4 μM. Fig. 4). As a highly effective transporter substrate, β-GPA may gain access to the brain through the creatine transporters expressed at the blood-brain barrier, similar

to the mechanism by which creatine enters the CNS in deficiency syndromes. Such transporter-mediated uptake could allow β-GPA to interfere with GABAergic neurotransmission centrally. Even if brain penetration were limited, β-GPA and other GCs could act on peripheral GABARs, including those on immune cells, potentially mediating anti-inflammatory effects akin to effects proposed for taurine and homotaurine [37].

□-Selectivity of guanidino compounds may help explain the paradoxical hyperexcitability phenotype seen in GAMT deficiency, where the resulting massive increase in GAA (including in the brain), might activate GABARs at disease relevant concentrations [17]. Activation of □-GABARs on inhibitory interneurons can reduce their firing, leading to disinhibition of downstream excitatory neurons **-** a key mechanism by which activation of tonic inhibition by GABA agonists, or □-subunit gain of function mutations, can produce network-level excitatory effects, despite acting through inhibitory receptors [51, 52].

Overall, our data support the notion that structural GABA analogs and mimetics exhibit much higher affinity for extrasynaptic □4/6β□ GABARs – and, to a somewhat lesser extent, also for extrasynaptic □β receptors - compared to synaptic □2-GABAR. In □-KO GCGs, the modest reduction in agonist sensitivity and preservation of tonic currents are consistent with homeostatic upregulation of high-affinity □6β receptors. These findings reinforce the view that higher GABA agonist affinity for extrasynaptic receptors is a common feature of orthosteric GABAR ligands, including guanidino compound GABA structural analogs like GAA, β-GPA, GES and □-GBA.

Declarations

Ethics approval and consent to participate: Animal care and experimental procedures were conducted according to the guidelines of the UCLA Chancellor's Animal Care and Use Committee (ARC) (Animal Welfare Assurance number A3196-01 and animal protocol ARC-2019-032). Consent to participate: not applicable.

Consent for publication: All authors have read and approved the final manuscript.

Availability of data and materials: The data that support the findings of this study are available from authors upon reasonable request**.**

Competing interests: G.S.L. has served as a consultant to Astellas Gene Therapies and serves on the Scientific Advisory Board of Taysha Therapeutics in areas unrelated to this work.

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