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Discovery of Non-Zwitterionic $GABA_A$ Receptor Full Agonists and a Superagonist

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Abstract—Numerous previous studies of $GABA_A$ receptor ligands have suggested that $GABA_A$ receptor agonists must be zwitterionic and feature an intercharge separation similar to that of GABA (approx. 4.7–6 Å). In this communication we demonstrate that appropriately functionalized GABA amides are partial, full, or superagonists, despite their non-zwitterionic structure.

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS) and is an agonist at three receptor subtypes.[1] The $GABA_A$ receptor ($GABA_AR$), a ligand-gated chloride ion channel, is the most abundant of these subtypes in mammalian brain.[2] Since blood–brain barrier permeant $GABA_AR$ agonists could be useful as anti-nociceptive, anxiolytic, anti-epileptic, and hypnotic agents,[3,4] a large number of GABA analogues have been prepared and assessed in binding and qualitative functional assays (microelectrophoresis onto cat spinal interneurons).[5–10] Some of these compounds have been assessed in quantitative functional assays, such as chloride flux and patch-clamp.[11–16]

Although the structural determinants of $GABA_AR$ agonism have eluded precise definition, all $GABA_AR$ agonists (as opposed to agonistic modulators) disclosed to date possess a zwitterionic structure, with a GABA-like distance between charges (4.7–6.0 Å)[15,17] (Scheme 1). In this communication we show for the first time that appropriately functionalized GABA amides can be effective GABA agonists, despite their non-zwitterionic structure.

GABA amides were studied relatively early in the development of $GABA_AR$ pharmacology, and received little attention thereafter. Suckling reported that **1a–d** were 5000- to 11,000-fold less potent than GABA to displace [^{3}H]GABA from human cerebellum membranes; however, no functional assay data on these compounds were reported (Scheme 2).[18]

The most well known GABA amide is progabide, which functions as a GABA prodrug. Unlike GABA and gabamide (**1a**), it has good blood–brain barrier penetrability.[19,20] In brain, rapid enzymatic transformation to SL 75102 occurs, followed by hydrolysis to GABA; the alternative metabolic pathway involving gabamide **1a** also occurs.[20] Progabide and **1a** were shown to be 600- to 300-fold less potent than GABA for displacement of [^{3}H]GABA from rat membranes, but no functional data were reported for these compounds.[21,22] More recently, GABA dipeptides such as GABA-His and GABA-Gly were studied.[23] Chloride flux assays at 0.1 and 1 mM showed that these compounds are not agonists.[24] Taken together, these published studies provide little motivation to further explore GABA amides. However, because of our interest in developing tethered $GABA_AR$ agonists,[25,26] and because functionalization of the GABA carboxyl is one of the few conceivable ligation strategies, we were motivated to re-examine a small collection of GABA amides in a functional assay.

GABA amides **1a–b**, **3a–b**, **4a–b**, and **5a–g** were prepared from *N*-Boc-2-pyrrolidone **2**,[27] as described in Scheme 3.

Ring-opening with amines affords the corresponding *N*-Boc GABA amide derivative, and acidic deprotection

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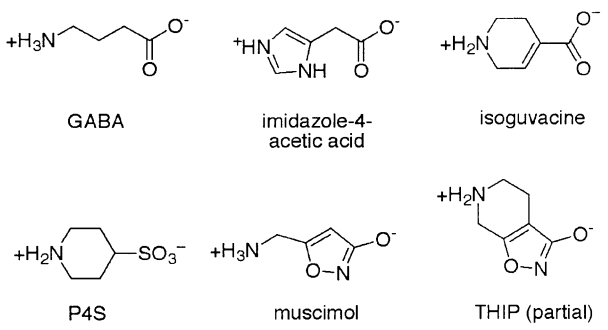
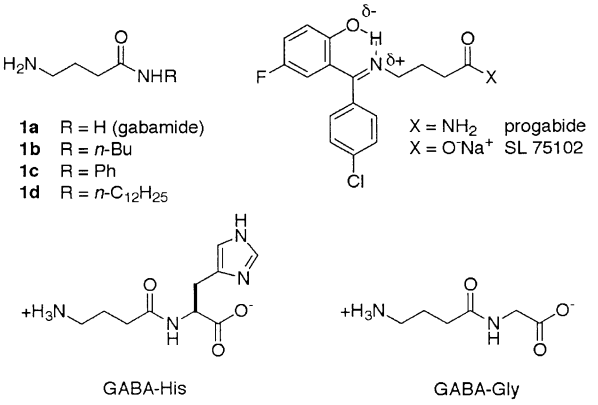
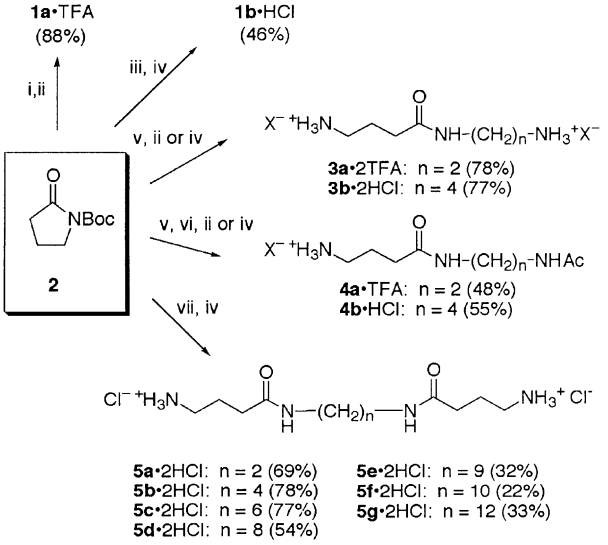
provided **1a**–**b**, **3a**–**b**, and **5a**–**c**. In general, HCl deprotection provided crystalline products; in cases where the HCl salts were not crystalline, the trifluoroacetic acid salts were prepared. To prepare **4a**–**b**, acetylation of the terminal amino group was carried out before acidic deprotection. All compounds gave satisfactory NMR



Scheme 1. Representative $GABA_AR$ full and partial agonists.



Scheme 2. GABA amides (and metabolite SL 75102) examined previously by other workers.



Scheme 3. Synthesis of GABA amides examined in this study: (i) NH_4OH (concd), reflux; (ii) TFA, neat, 10 min; (iii) n-$BuNH_2$ (3 equiv), THF, reflux, 12 h; (iv) concd HCl (aq); (v) $H_2N(CH_2)_nNH_2$ (3 equiv), THF, reflux, 12 h; (vi) Ac_2O, Et_3N, CH_2Cl_2; (vii) $H_2N(CH_2)_nNH_2$ (0.5 equiv), THF, reflux, 12 h.

(^1H, ^{13}C) and mass spectral data. Prior to assay, all drugs were analyzed by HPLC using a modification of Saller's procedure (assay of the dansyl sulfonamide derivatives),[28] to confirm that they were free from trace GABA impurities (<0.1 wt%).[29] These drugs were then analyzed using a standard $^{36}Cl^-$ flux assay in mouse brain synaptoneurosomes (Table 1).[30,31] Simple GABA amides **1a**–**b** proved to be partial agonists; in this regard, they behave similarly to THIP.[32] GABA amides with pendant amines or acetamides were full agonists if the tether consisted of 4 methylenes (**3b** and **4b**; Table 1, Fig. 1).

Interestingly, the shorter homologues (**3a** and **4a**) did not elicit any net chloride uptake at the highest concentration tested (4 mM).[33] Dose–response curves were also obtained for GABA amide dimers **5a**–**c**: **5b** proved to be the most potent (Table 1, Fig. 2). Hill slopes for all the GABA amides were in the range of 1.3–2.1, consistent with the idea that two agonists must bind in order to gate the $GABA_AR$.[2]

The role of tether length on agonism by the GABA amide dimers was further assessed by examining the uptake elicited by 1 mM **5a**–**g** (Fig. 3). As can be seen, chloride uptake above backround is statistically significant only for tether lengths of 2–6 methylenes (**5a**–**c**); at longer tether lengths (8, 9, 10, 12 methylenes) uptake is indistiguishable from backround.

Although **5b** is 33-fold less potent than GABA, it is a 'superagonist' because its maximal uptake (E_{max}) is 49% higher than that achieved for GABA (Fig. 2, Table 1). To establish that the superagonism of **5b** was not an artifact, we took steps to determine whether GABA active transport during the assay caused a sub-optimal GABA E_{max}. The GABA transport inhibitor nipecotic

Table 1. $GABA_AR$ agonism by GABA amides and controls

Drugs	Chloride uptake EC_{50}, μM[a]	Agonism (E_{max})	Hill slope
1a•TFA	317±42	Partial (307±15)	1.4
1b•HCl	340±219	Partial (362±79)	1.5
3a•TFA	>4000[b]	—	
3b•HCl	1140±380	Full (497±76)[c]	1.5
4a•TFA	>4000[b]	—	
4b•HCl	414±134	Full (549±75)[c]	1.7
5a•2HCl	733±208	Partial (264±36)	1.3
5b•2HCl	475±68	Super (578±40)[d]	1.6
5c•2HCl	643±213	Full (433±94)[c]	2.1
GABA	14.3±1.5	Full (389±12)	1.3
THIP	335±111	Partial (252±51)	2.1

[a]Activation of ^{36}Cl uptake into mouse brain synaptoneurosomes. Triplicate experiments were performed, run at 4–9 concentrations per drug. Uptake is expressed in terms of nmol total chloride per min per mg of protein. Protein concentrations were determined according to the method of Bradford.[30] Dose–response curves were constructed using non-linear regression. EC_{50} and E_{max} (maximal uptake) data are expressed as mean±standard error.

[b]No net chloride uptake above background at the highest concentration tested (4 mM).

[c]The difference in E_{max} for **4b** and GABA does not meet the criterion for statistical significance ($p=0.067$, t-test).

[d]E_{max} 49% greater than that of GABA; the results are statistically significant ($p=0.0095$, t-test).

acid (100 μM)[11] was added to chloride flux experiments containing 13 μM and 1 mM GABA. In neither case did addition of nipecotic acid give additional ion flux, indicating that GABA transport does not limit GABA E_{max}, and confirming the 'superagonist' status of **5b**.

To confirm that the effects of **5b** on chloride uptake are indeed mediated by the $GABA_AR$, control experiments were performed (Fig. 4). Picrotoxinin (a $GABA_AR$ non-competitive antagonist) blocks the stimulating effects of **5b** on $^{36}Cl^-$ uptake, confirming that the observed uptake is mediated by the $GABA_AR$ and not another chloride channel. Additional controls with bicuculline (a $GABA_AR$ competitive antagonist) also showed antagonism of chloride uptake stimulated by **5b**, confirming that observed **5b** binds to the $GABA_AR$ agonist site.

Because of the widely-held view that GABA agonists must be zwitterionic and feature a GABA-like intercharge separation of 4.7–6.0 Å, our observation of $GABA_AR$ agonism by non-zwitterionic compounds requires some explanation. First of all, not all the GABA amides we examined show agonist activity. Drugs **3a**, **4a**, and **5d–g** gave no chloride uptake over backround at the highest concentrations tested. Secondly, two of the compounds we examined are partial agonists, including gabamide **1a**, the known metabolite of the GABA prodrug progabide. However, when the appropriate functional group is situated 4–6 methylenes distant from the GABA amide nitrogen, the resulting drugs are full agonists (**3b**, **4b**, and **5c**), or in one case, a superagonist (**5b**). At this point the optimum tether length appears to be four methylenes, and the amide group (**4b** and **5b**) appears to be superior to the ammonium group (**3b**); further optimization of the tether length and pendant group is in progress. Based on the agonism of **4b** and **5a–c**, we propose that proximal to the agonist binding site there exists a site that can weakly bind the pendant amide group. This proposal is further supported by the tether length dependence of agonism in the **5a–g** series (Fig. 3).

Why have GABA amides been left largely unexplored? It appears that a traditional dependence on agonist binding studies for initial screening of GABA analogues, and the demonstrated low potencies of GABA amides to displace [^{3}H]GABA,[18,21] created the impression that GABA amides were not a fertile field in which to find new $GABA_AR$ agonists. However, we believe that the use of binding assays to screen $GABA_AR$ agonists may produce a large number of false negatives, since such studies address binding to high-affinity (nM) sites, which Dunn has shown are *not* involved in channel gating.[34] $GABA_AR$ channel gating is mediated by binding to independently localized low-affinity (μM) agonist sites.[2,34]

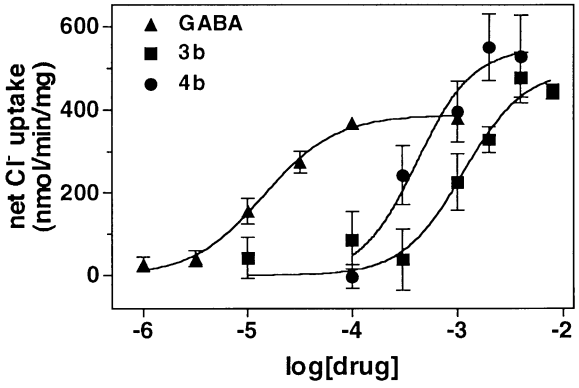


Figure 1. Dose–response curves of GABA, **3b**, and **4b**.

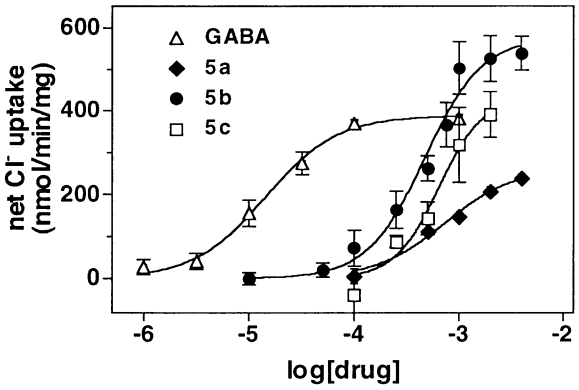


Figure 2. Dose–response curves of GABA and **5a–c**.

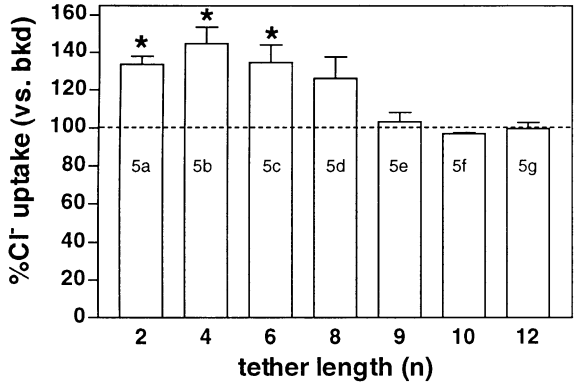


Figure 3. Effect of tether length of GABA amide dimers (n = number of methylenes) on chloride flux (at 1 mM drug), expressed as % uptake versus backround (bkd, 100%, dotted line). An asterisk signifies that uptake is significantly different than backround ($p < 0.05$, t-test).

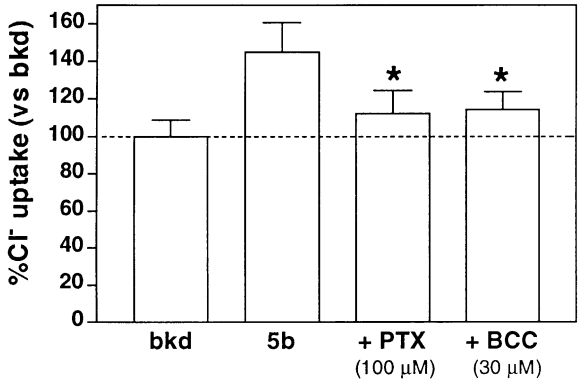


Figure 4. Effects of non-competitive (picrotoxinin, PTX) and competitive (bicuculline, BCC) inhibitors on ion flux elicited by **5b** (1 mM); bkd represents background uptake in the absence of agonist. Asterisks indicate that the agonist-induced uptake in the presence of blocker is significantly less than that observed with 1 mM **5b** alone ($p < 0.05$, t-test).

Finally, to the best of our knowledge, **5b** is the only known superagonist for the $GABA_AR$; its E_{max} is 49% greater than that of GABA. Although **5b** is 33-fold less potent than GABA, its EC_{50} value is comparable to that of THIP, a drug which has undergone extensive evaluation as an anti-nociceptive, anti-epileptic, and hypnotic agent.[3,4] Because **5b** is a superagonist, it will elicit more chloride uptake than THIP at any given concentration (data not shown). Will GABA amides like **4b** and **5b** cross the blood–brain barrier? Will they, like progabide and **1a**, be subject to rapid enzymatic hydrolysis in brain? By what mechanism does **5b** exert its superagonism? Experiments to address these questions are underway.

Acknowledgements

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