Supplementary Information

Discovery of a new class of orthosteric antagonists with nanomolar potency at extrasynaptic $GABA_A$ receptors

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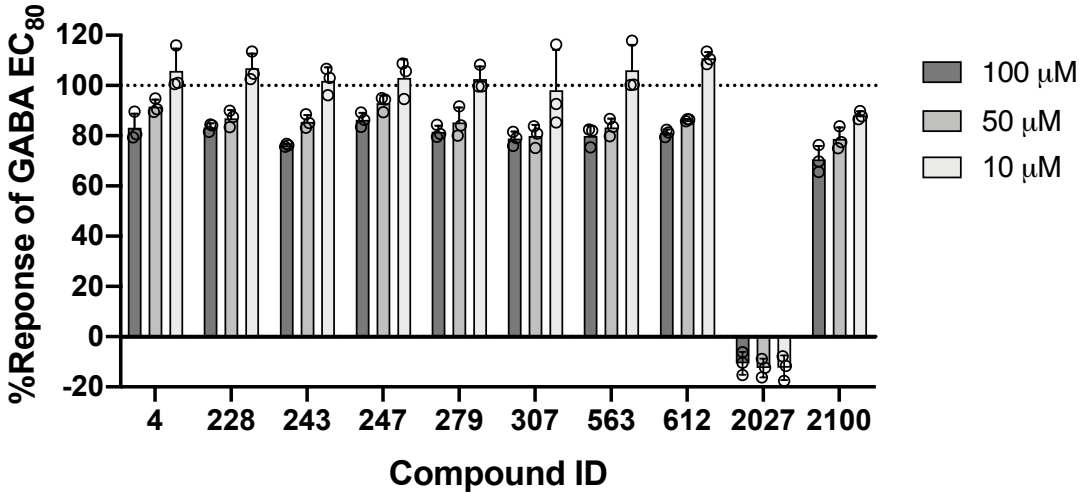
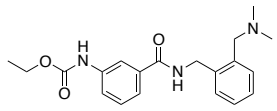
Supplementary Fig. S1

Validation of hits from the primary screening on $\alpha_4\beta_3\delta$ receptors in the FMP assay. Only **2027** was able to inhibit the GABA EC_{80} induced response in a concentration of 10 μM. The shown data is from a single experiment performed in triplicate, shown as means ± SD.



Supplementary Fig. S2

Structures and IDs of the 52 tested analogues of **2027**. Compounds were purchased from Enamine, except for compounds marked with * were purchased from Vitas-M and ** from ChemBridge.



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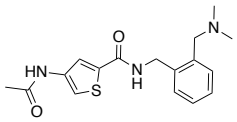

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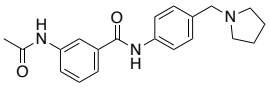

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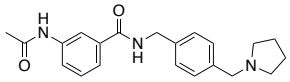

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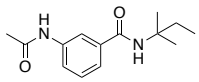

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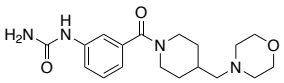

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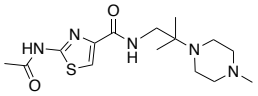

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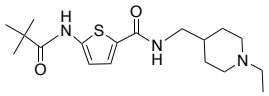

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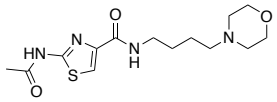

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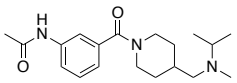

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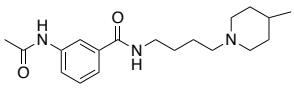

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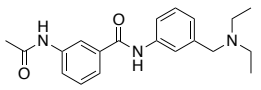

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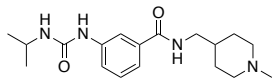

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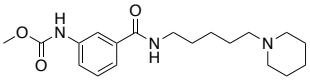

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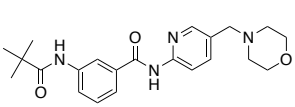

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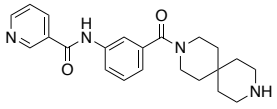

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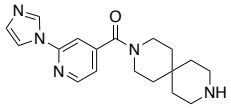

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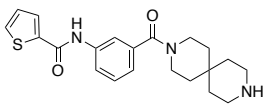

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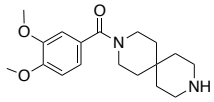

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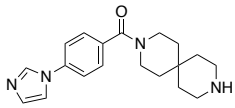

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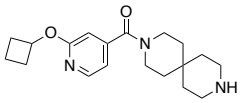

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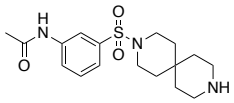

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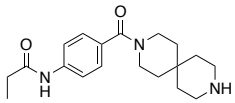

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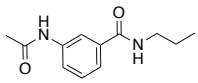

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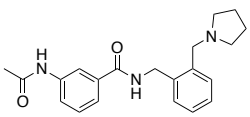

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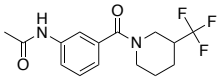

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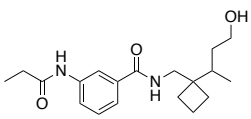

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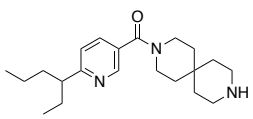

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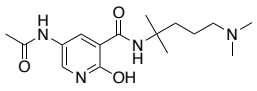	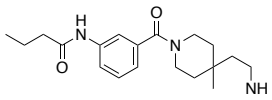	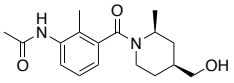	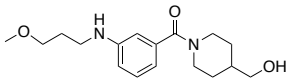
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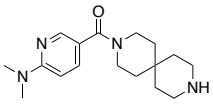	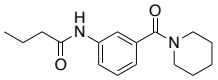	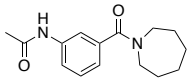	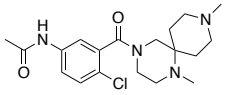
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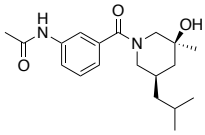	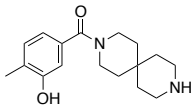	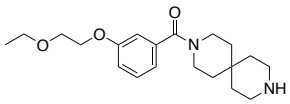	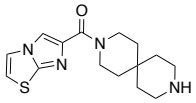
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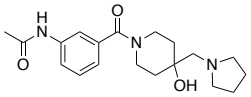	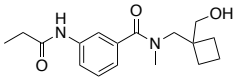	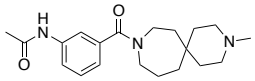	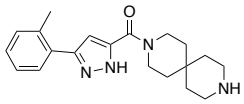
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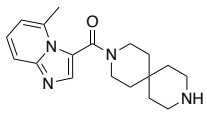	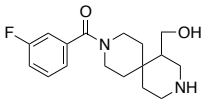	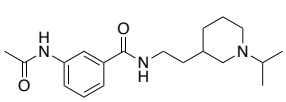	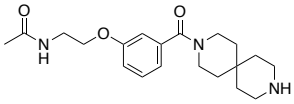
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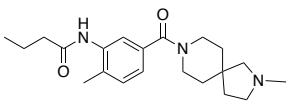	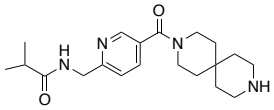	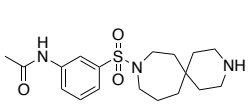	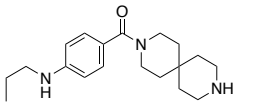
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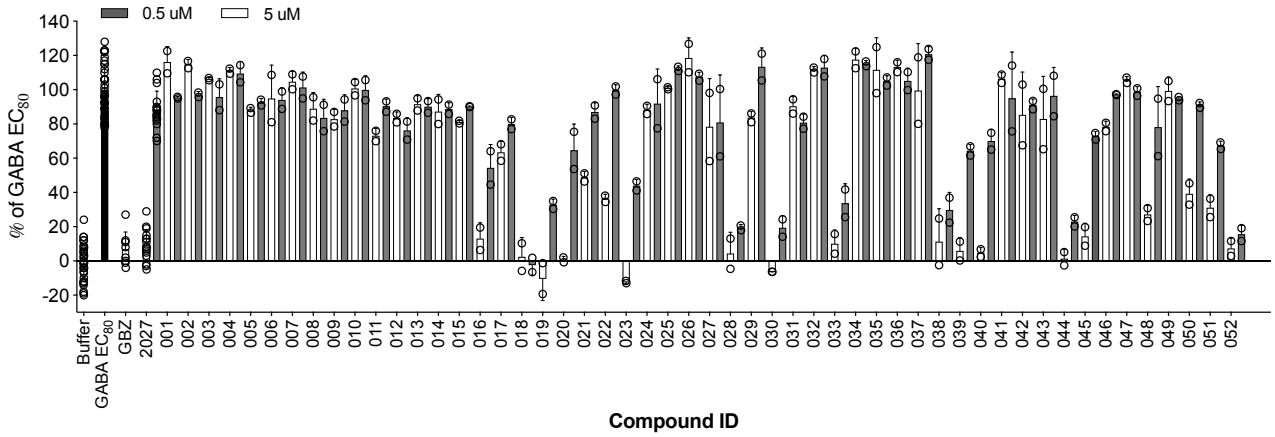
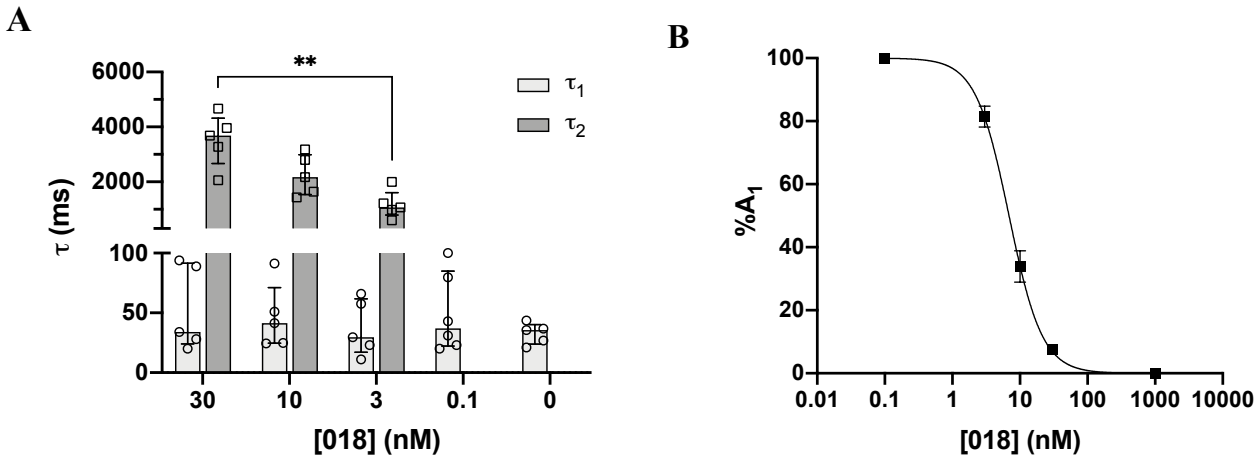
Supplementary Fig. S3

Results of the testing of the 52 analogues of **2027** at $\alpha_4\beta_1\delta$ receptors in the FMP assay. Compounds were tested in concentrations of 0.5 μM and 5 μM in duplicates in a single experiment. Gabazine (GBZ) in a concentration of 5 μM was included as a positive control. Compound ID refer to supplementary Fig. S2. The shown data is given as means ± SD from a single experiment.



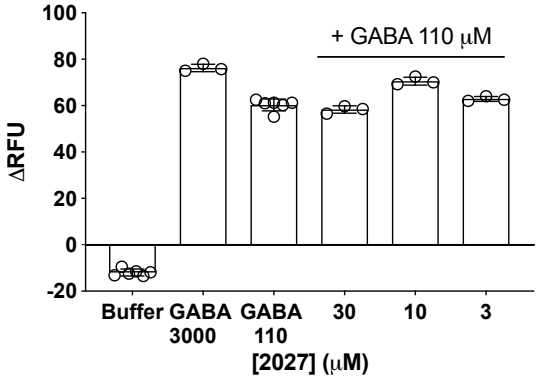
Supplementary Fig. S4

Compound **018** shows slow dissociation kinetics independent of the GABA concentration. (**A**) Time constants, τ, for currents induced by 100 μM GABA with or without preincubation of **018**. The constants were determined by fitting to a monoexponential function for no **018** preapplication and 1 nM **018** preapplication and to a biexponential function for the remaining cells except for 3 cells applied 3 nM **018** and 1 cell applied 30 nM **018** which could be fitted to both a mono- and biexponential function. F-test showed that these cells were best fitted to the biexponential function (F-test, $P<0.05$). Statistical analysis was performed using Kruskal-Wallis ANOVA followed by Dunn's multiple comparison. τ_1 was compared to 10 nM **018** (100 μM GABA alone). Data are shown as medians with interquartile range (25% and 75%). (**B**) Concentration-dependent decrease in $\%A_1$ for 100 μM GABA, giving a functional K_B of 6.9 nM [5.99:7.91], n=5-6. Data are given as means ± SEM. Further details are given in Table S1.



Supplementary Fig. S5

Compound **2027** does not quench the fluorescent signal induced by BGT1-mediated GABA uptake in the FMP assay (performed essentially as previously described in methods section and based on Al-Khawaja et al. [43]). As shown, the fluorescent signal induced by uptake of 110 μM GABA by human BGT1 transiently expressed in δ-HEK cells was not decreased/quenched by **2027** in any of the tested concentrations. Data are shown as means ± SD from a representative experiment performed in triplicate (n=2).



Supplementary Table S1

Summary of time constant for 100 μM GABA with or without preapplication of **018** and the contribution from the fast current amplitude to the total amplitude (%A_1).

018 (nM)	τ_1 (ms)		τ_2 (ms)		**%A_1**	**n**
0	35.0	[24.0;40.0][a]		-	-	5
0.1	37.1	[22.3;84.9][a]		-	-	6
3	29.6	[22.4;61.8]	1070	[793;1607]	81.5	5
10	41.4	[24.6;71.2]	2170	[1535;2990]	33.9	5
30	34.0	[24.0;91.5]	3685	[2670;4315]	7.5	5

If not stated, current traces were fitted to a biexponential function. 3 cells applied with 3 nM **018** and 1 cell applied with 30 nM **018** could be fitted to both a mono- and biexponential function. F-test showed that they were best fitted to the biexponential function (F-test, $P<0.05$). Data are given as medians followed by 25-75% quartiles in squared brackets with *n* denoting the number of tested cells. Comparison of the determined time constant are shown and described in supplementary Fig. S4. [a]Current traces could only be fitted to a mono-exponential function.

Supplementary Table S2

The permeability study of **018** and **2027** shows high degree of recovery both before and after cell lysis indicating that sticking to the cells was no issue.

Compound	Mean recovery %		Mean total recovery %	
	Apical to basal	Basal to apical	Apical to basal	Basal to apical
018	97.93	101.17	99.59	101.17
2027	87.93	104.62	89.44	104.62