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Original article

Synthesis and biological evaluation of aminomethylphenol derivatives as inhibitors of the murine GABA transporters mGAT1–mGAT4

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Dedicated with best wishes to Prof. Herbert Mayr on the occasion of his 60th birthday.

Abstract

A series of *N*-substituted aminomethylphenol derivatives was synthesized by reductive amination. To study the inhibitory potency of the target compounds at the murine GABA transporters (mGAT1–mGAT4), a [^{3}H]GABA uptake test system in a 96-well format based on HEK cells stably expressing mGAT1–mGAT4 was established and validated. Inhibitory potencies at mGAT1–mGAT4 in the micromolar range and a slight subtype selectivity for mGAT3 were observed for the synthesized aminomethylphenol derivatives. Among the compounds investigated 5-*n*-dodecylaminomethyl-2-methoxyphenol (**21**) was found to be most potent with an IC_{50} value at mGAT3 of about 3 μM.

Keywords: GABA-uptake inhibitors; Antiepileptic; Murine GABA transporters; Aminomethylphenols

1. Introduction

γ-Aminobutyric acid (GABA, **1**, Fig. 1) is the major inhibitory neurotransmitter in the central nervous system. The activity of GABA in the synaptic cleft is thought to be terminated by reuptake in GABA-ergic neurons and astroglial cells. Four distinct subtypes of sodium-dependent transporters mediating the transport of GABA are known [1]. When cloned from murine brain these GABA transporters are generally termed mGAT1, mGAT2, mGAT3, and mGAT4 [2] (for the different nomenclature for various species see Ref. [3]). In the 1970s (*RS*)-nipecotic acid (**2**) and guvacine (**3**, Fig. 1) were found to be inhibitors of GABA uptake in neuronal and astroglial cell cultures [4]. This first generation of GABA uptake inhibitors, however, lacked the capability to cross the blood–brain barrier in sufficient quantities, limiting its pharmacological usefulness. About a decade later the first systemically active GABA uptake inhibitors, such as SK&F-8997-A (**4**), tiagabine (**5**) or NO 711 (**6**) (Fig. 1) were described possessing characteristic lipophilic side chains attached to the ring nitrogen of nipecotic acid and guvacine [1,5–7]. These compounds have been shown to be potent and subtype selective inhibitors of GAT1 [8], possessing anticonvulsant activity in animal models [9]. In the meantime tiagabine has been introduced successfully in the therapy of epilepsy classifying GAT1 as an approved target [10]. The therapeutic potential mediated by mGAT2, mGAT3 and mGAT4 is less clear so far, but an anticonvulsant activity has also been demonstrated for the moderately mGAT4 selective nipecotic acid derivative (*S*)-SNAP-5114 (**7**, Fig. 1), the poorly mGAT2 selective *N*-substituted 4-hydroxy-4-arylpiperidine derivative NNC 05-2045 (**8**, Fig. 1), and the mGAT1/mGAT2 selective compound EF1502 (*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) [11,12].

Interestingly, none of the latter GAT inhibitors, neither NNC 05-2045 nor EF1502, are deduced from classical heterocyclic amino acids as parent structures. Aminomethylphenol

Abbreviations: GABA, γ-aminobutyric acid; mGAT, murine GABA transport protein; DPB, diphenylbutenyl; DPP, Diphenylpropyl; r.t., room temperature; TOE, 2-[tris-(4-methoxyphenyl)methoxy]ethyl.

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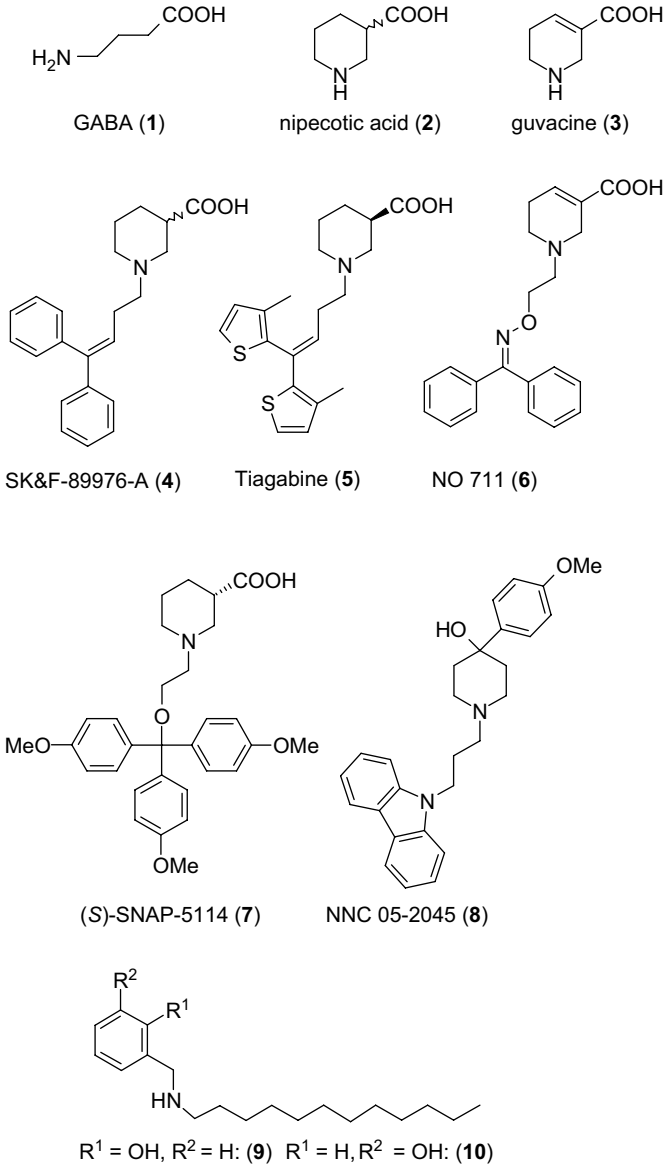

Fig. 1. Structure of GABA as well as of various known GABA uptake inhibitors.

derivatives such as compounds **9** and **10** (Fig. 1) represent another class of non-amino acid derived GABA uptake inhibitors. First reported in the early 1980s, they inhibit neuronal [^{3}H]GABA and glial [^{3}H]β-alanine uptake [13]. As these uptake assays were performed with rat cortex − heterologous expression of GABA transporters was not possible at that time − the full subtype selectivities of these compounds are yet to be determined. To examine these questions further we decided to synthesize a series of *N*-substituted aminomethylphenol derivatives and to determine their inhibitory potencies at mGAT1−mGAT4. In addition, our study was designed to reveal the impact of the position and the acidity of the OH-function as well as the influence of the lipophilic residues attached to the nitrogen on the inhibitory potency of their aminomethylphenyl derivatives at mGAT1−mGAT4. To this end a synthetic strategy based on reductive amination (as outlined in Fig. 2) seemed to be the best option as this method should be highly flexible by allowing a wide variety of phenol derived aldehydes and amines to be combined to yield the corresponding aminomethylphenol derivatives.

2. Chemistry

The aminomethylphenol derivatives **21**−**33** depicted in Fig. 2 and Table 2 were selected as target compounds. They are derived from the hydroxybenzaldehydes **11**−**13** and, in order to study the effect of the acidity of the phenol function, also from the fluorinated hydroxybenzaldehydes **14**−**16**. As primary amines for the reductive amination compounds **17**−**20** were selected covering prototypical substituents in addition to aliphatic residues at known GAT inhibitors. For the reductive amination, a mixture of the respective aldehydes (**11**−**16**) and the respective primary amines (**17**−**20**) was first allowed to react for 20 h in the presence of a 3 Å molecular sieve at room

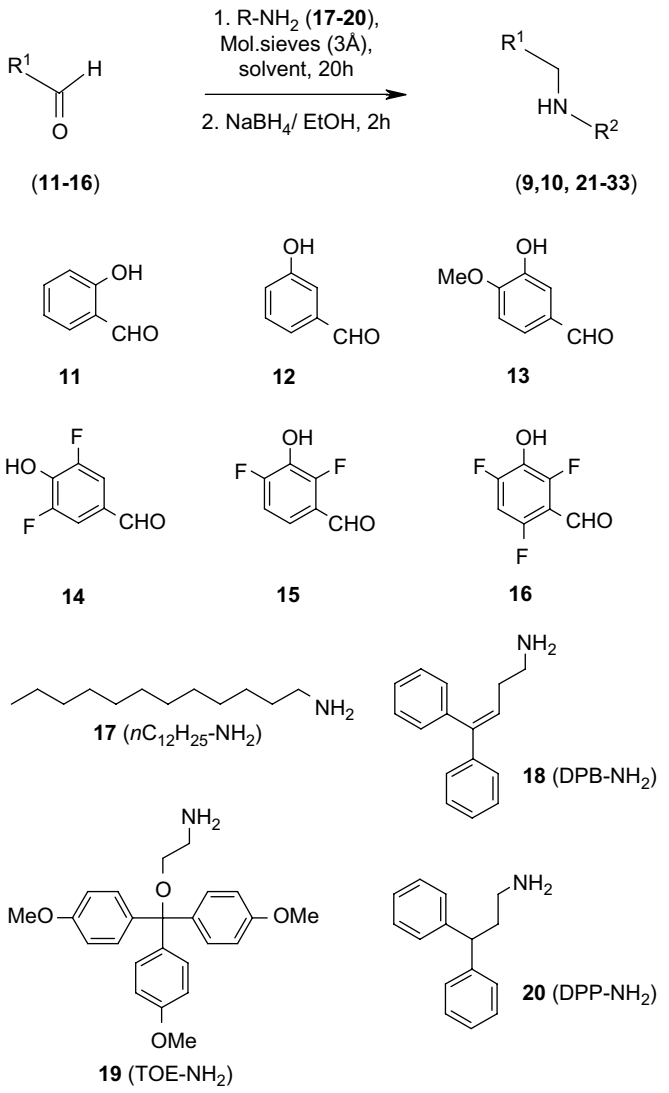

Fig. 2. Structure and synthesis of aminomethylphenol derivatives investigated as GAT inhibitors.

temperature. Following filtration over silica gel and evaporation, the reaction product was then dissolved in ethanol and treated with sodium borohydride at room temperature for 2 h. After purification by column chromatography or recrystallization the desired secondary amines **21**–**33** were obtained in moderate to good yields (Table 2).

3. Biological evaluation

The inhibitory potencies of all the compounds prepared in this way, the known aminomethylphenols **9** and **10**, that had been resynthesized for this study, as well as the new aminomethylphenols **21**–**33**, were determined at the four murine GABA transporter subtypes mGAT1–mGAT4. The study was performed as [^{3}H]GABA uptake assays in a 96-well plate format, based on stably transfected HEK cells. With this new test system we significantly improved a former assay based on a smaller 24-sample format and employing only transiently mGAT1–mGAT4 expressing COS and HEK cells suspended in single test tubes [14]. In order to assess the reliability of the new test system it was validated by a characterisation of the well-known GAT inhibitors **2**–**7** (Fig. 1 and Table 1). The results obtained in our new [^{3}H]GABA uptake assay were in excellent agreement with those obtained in the earlier system [14] with regard to both the rank order of potency and the subtype selectivity of the GAT inhibitors confirming the validity of the new test system.

4. Results and discussion

The *N*-dodecylsubstituted aminomethylphenols **9** and **10**, which have been already described as inhibitors of neuronal [^{3}H]GABA and glial [^{3}H]β-alanine uptake in rat cortex [13], were found to inhibit GABA uptake at mGAT1–mGAT4 with a reasonable potency at all subtypes and to display a slight subtype selectivity for mGAT3. 5-*n*-Dodecylaminomethyl-2-methoxyphenol (**21**) was slightly more potent at each of the four murine GABA transporters than the aforementioned *N*-dodecylsubstituted aminomethylphenols **9** and **10**. Although the potencies of inhibitors obtained from different test systems may be compared with restrictions, the pIC_{50} value of 5.57 (i.e. 2.7 μM) obtained for compound **21** at mGAT3 (Fig. 3) suggests that this new GABA uptake inhibitor is (to the best of our knowledge) one of the most potent for this transporter reported to date.

The other aminomethylphenol derivatives derived from 2-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, and 3-hydroxy-4-methoxybenzaldehyde possessing DPB (**22**–**24**), DPP (**26** and **27**), or TOE moiety (**28**–**30**), respectively, were also found to display a reasonable potency at mGAT1–mGAT4 (pIC_{50} values between 4.1 and 5.1, i.e. IC_{50} values between ~80 and 8 μM). In most cases a slight subtype selectivity for mGAT3 was detected as well. Surprisingly, the fluorinated aminomethylphenol derivatives (**25**, **31** and **33**) were rather poor inhibitors at mGAT1–mGAT4. The fluorine substituted compound **32**, however, appeared to be an exception, being moderately potent at mGAT3 (pIC_{50} value 4.99; i.e. IC_{50} value 10.2 μM; see Table 2).

Unambiguous structure–activity relationships could neither be deduced for the position of the hydroxy group in the phenol moiety nor for the substituent attached to the nitrogen. The latter observation may cause some surprise as previous studies have shown that the potency and subtype selectivity at GAT1 of heterocyclic amino acids such as nipecotic acid (**2**) and basic structures not representing heterocyclic amino acids such as 4-*N*-methylamino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol have increased significantly on addition of the DPB-residue [8,15]. The same is true for nipecotic acid (**2**) and the TOE-residue with regard to their activity at mGAT4 [16].

5. Conclusion

Several of the newly synthesized *N*-substituted aminomethylphenol derivatives turned out to be moderately potent inhibitors of the murine GABA transporters mGAT1–mGAT4 displaying a slight preference for mGAT3. The aminomethylphenol derivatives with a fluorinated phenol moiety that were designed for their enhanced acidity showed comparatively weak inhibitory potencies. The position of the hydroxy function, the introduction of an additional methoxy group in the

Table 1
GABA uptake inhibition for known GABA uptake inhibitors at mGAT1–mGAT4 stably expressed in HEK

Compound	mGAT1	mGAT2	mGAT3	mGAT4
(*RS*)-Nip.a.[a] ((*RS*)-**2**)	4.88 ± 0.07	3.10 ± 0.09	4.64 ± 0.07	4.70 ± 0.07
(*R*)-Nip.a.[a] ((*R*)-**2**)	5.07 ± 0.02	3.28 ± 0.05	4.71 ± 0.04	4.79 ± 0.05
(*S*)-Nip.a.[a] ((*S*)-**2**)	4.13 ± 0.05	3.12 ± 0.12	3.71 ± 0.04	3.51 ± 0.06
Guvacine (**3**)	4.87 ± 0.07	3.31 ± 0.03	4.59 ± 0.05	4.59 ± 0.05
SK&F-89976-A (**4**)	6.16 ± 0.05	3.43 ± 0.07	3.71 ± 0.04	3.56 ± 0.06
Tiagabine (**5**)	6.88 ± 0.12	100 μM/52%[b]	100 μM/64%[b]	100 μM/73%[b]
NO 711 (**6**)	6.83 ± 0.06	3.20 ± 0.09	3.62 ± 0.04	3.07 ± 0.05
(*S*)-SNAP-5114 (**7**)	4.07 ± 0.09	100 μM/56%[b]	5.29 ± 0.04	5.81 ± 0.10

The compounds' inhibitory potencies are given as pIC_{50} values (mean ± S.E.M., $N = 3$)[c]

[a] Nipecotic acid.

[b] Remaining [^{3}H]GABA uptake in the presence of 100 μM test compound as compared to a control without inhibitor.

[c] Data show the pIC_{50} ($-\log IC_{50}$) for inhibition of [^{3}H]GABA in uptake experiments performed as described in Section 6. Each value represents a mean ± S.E.M. of three independent experiments (with triplicate samples for each data point).

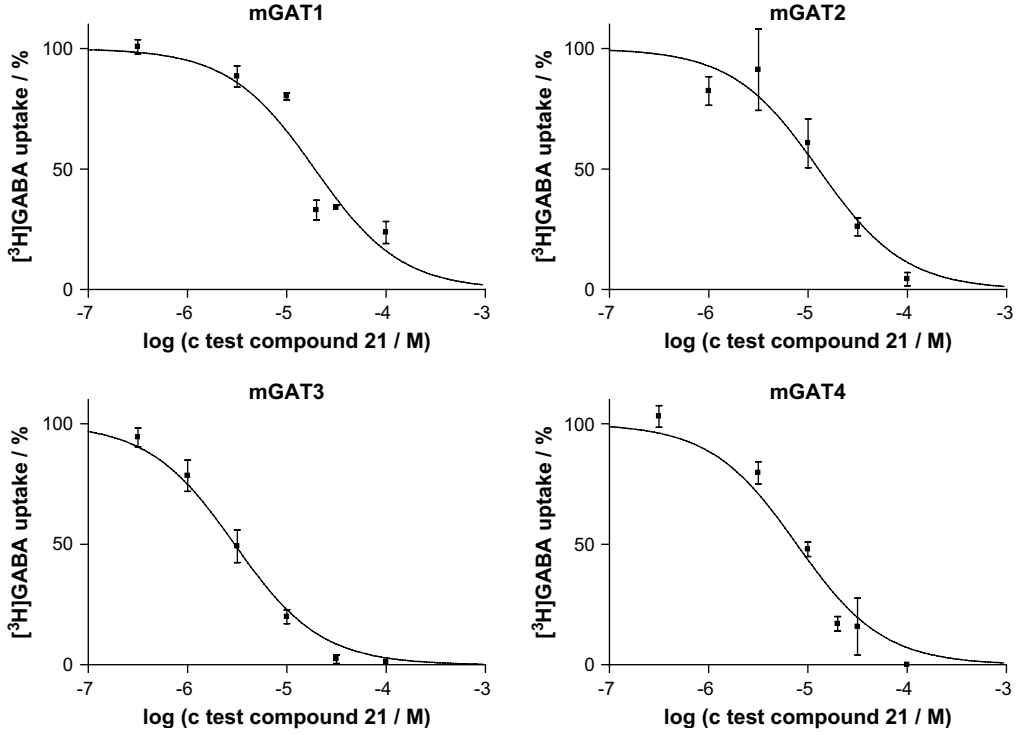

Fig. 3. Inhibition of [^{3}H]GABA uptake by compound **21** at mGAT1–mGAT4 stably expressed in HEK cells. Each inhibition curve with its data points (mean ± S.E.M. from triplicate samples) shows one representative [^{3}H]GABA uptake experiment (out of three independent experiments) performed for mGAT1, mGAT2, mGAT3 and mGAT4 as described in Section 6.2.2.

phenol moiety or variations of the lipophilic residues attached to the amino group in all aminomethylphenol derivatives studied produced only marginal variations of the inhibitory potencies at all four murine GABA transporters. Though potency and subtype selectivity at the individual GABA transporters has still to be improved, this class of GABA uptake inhibitors could nevertheless represent an interesting starting point, especially for the development of new mGAT3 inhibitors.

Table 2
Physical data and inhibitory potency of aminomethylphenol derivatives (given as pIC_{50} values; mean ± S.E.M., $N = 3$) at mGAT1–mGAT4[c]

Product[x]	Starting material		Physical data				GABA uptake inhibition (pIC_{50} values)			
	Aldehyde[y]	Amine[z]	Mp (°C)	Yield (%)	Empirical formula	Analysis	mGAT1	mGAT2	mGAT3	mGAT4
9	**11**	n-$C_{12}H_{25}$–NH_2	–	–	$C_{19}H_{33}NO$	CHN	4.23 ± 0.16	4.54 ± 0.03	4.73 ± 0.09	4.20 ± 0.07[b]
10	**12**	n-$C_{12}H_{25}$–NH_2	–	–	$C_{19}H_{33}NO$	CHN	4.68 ± 0.11	4.82 ± 0.08	5.25 ± 0.15	4.60 ± 0.16
21	**13**	n-$C_{12}H_{25}$–NH_2	76–77	22	$C_{20}H_{35}NO_2$	CHN	4.91 ± 0.11	4.90 ± 0.07	5.57 ± 0.04	5.06 ± 0.04
22	**11**	DPB–NH_2	Oil	66	$C_{23}H_{23}NO$	CHN	4.48 ± 0.03	4.84 ± 0.04	5.11 ± 0.07	4.83 ± 0.03
23	**12**	DPB–NH_2	129–130	57	$C_{23}H_{23}NO$	CHN	4.54 ± 0.06	4.75 ± 0.05	5.10 ± 0.07	4.68 ± 0.02
24	**13**	DPB–NH_2	81–82	70	$C_{24}H_{25}NO_2$	CHN	4.73 ± 0.02	4.71 ± 0.05	4.92 ± 0.02	4.64 ± 0.09
25	**14**	DPB–NH_2	160–161	31	$C_{23}H_{21}F_2NO$	CHN	100 μM/70%[a]	100 μM/82%[a]	100 μM/66%[a]	100 μM/76%[a]
26	**11**	DPP–NH_2	Oil	69	$C_{22}H_{23}NO$	CHN	4.28 ± 0.07[b]	4.74 ± 0.05	4.61 ± 0.05	4.60 ± 0.03
27	**12**	DPP–NH_2	144–145	69	$C_{22}H_{23}NO$	CHN	4.06 ± 0.11[b]	4.32 ± 0.03	4.42 ± 0.07	4.30 ± 0.10[b]
28	**11**	TOE–NH_2	Oil	86	$C_{31}H_{33}NO_5$	CHN	4.57 ± 0.09	4.93 ± 0.04	5.08 ± 0.02	4.79 ± 0.04
29	**12**	TOE–NH_2	62–63	80	$C_{31}H_{33}NO_5$	CHN	4.36 ± 0.10	4.89 ± 0.02	5.02 ± 0.09	5.11 ± 0.10
30	**13**	TOE–NH_2	122–123	75	$C_{32}H_{35}NO_6$	CHN	4.59 ± 0.06	4.68 ± 0.08	5.05 ± 0.09	4.84 ± 0.08
31	**14**	TOE–NH_2	199–200	54	$C_{31}H_{31}F_2NO_5$	CHN	100 μM/59%[a]	100 μM/64%[a]	100 μM/51%[a]	100 μM/67%[a]
32	**15**	TOE–NH_2	151–152	56	$C_{31}H_{31}F_2NO_5$	CHN	4.07 ± 0.10[b]	100 μM/54%[a]	4.99 ± 0.04	100 μM/82%[a]
33	**16**	TOE–NH_2	128–129	62	$C_{31}H_{31}F_3NO_5$	CHN	100 μM/87%[a]	100 μM/89%[a]	100 μM/56%[a]	100 μM/64%[a]

x,y,z: Compound numbers of products and starting material according to Fig. 2.

[a] Remaining [^{3}H]GABA uptake in the presence of 100 μM test compound as compared to a control without inhibitor.

[b] R^2 obtained for curve fitting <0.8 (mean of three inhibition curves).

[c] Data show the pIC_{50} ($-\log IC_{50}$) for inhibition of [^{3}H]GABA in uptake experiments performed as described in Section 6.2.2. Each value represents a mean ± S.E.M. of three independent experiments (with triplicate samples for each data point); examples for representative inhibition curves obtained for test compound **21** are shown in Fig. 3.

6. Experimental section

6.1. Chemistry

6.1.1. General

Tetrahydrofuran was distilled from sodium under nitrogen. All other solvents for column chromatography and recrystallization were distilled prior to use. Purchased chemicals were used without further purification. TLC plates were made from silica gel 60 F_{254} on aluminium sheets (Merck). Flash column chromatography (CC) was carried out with Merck silica gel 60 (mesh 0.040–0.063 mm) as stationary phase. Melting points: m.p. (uncorrected) was determined with a Büchi 510 Melting Point apparatus. NMR spectroscopy: ^{1}H NMR spectra were recorded at room temperature with a JNMR-GX (400 MHz, Jeol) using TMS as internal standard and integrated with the NMR software Nuts (2D Version 5.097, Acorn NMR, 1995). IR spectroscopy: FT-IR Spectrometer 1600 and Paragon 1000 (Perkin Elmer); oils were measured as film, solid samples as KBr pellets. Mass spectrometry: Mass Spectrometer 5989 A with 59,980 B particle beam LC/MS interface (Hewlett Packard); analysis was carried out using chemical ionization (CH_5^+) unless stated otherwise. HRMS was obtained on JMS GCMate II (Jeol). Elementary analysis: Elementaranalysator Rapid (Heraeus).

6.1.2. General procedures

6.1.2.1. General procedure 1 (GP1). To a solution of the hydroxybenzaldehyde (1.0 equiv.) and the primary amine (1.0 equiv.) in EtOH (10 ml mmol^{-1}) molecular sieve (3 Å) was added and the mixture was stirred at r.t. for 20 h. After filtration over silica gel the solvent was evaporated. The resulting residue was dissolved in EtOH (10 ml mmol^{-1}), $NaBH_4$ (1.2 equiv.) was added and the solution was stirred at r.t. for 2 h. Then EtOH was evaporated and the obtained residue was dissolved in EtOAc (10 ml mmol^{-1}) and washed three times with brine. The organic layer was separated, dried ($MgSO_4$), and concentrated in vacuo. Products were purified by CC.

6.1.2.2. General procedure 2 (GP2). To a solution of the hydroxybenzaldehyde (1.0 equiv.) and the primary amine (1.0 equiv.) in EtOH (10 ml mmol^{-1}) molecular sieve (3 Å) was added and the mixture stirred at r.t. for 20 h. After filtration over silica gel the solvent was evaporated. The resulting residue was dissolved in EtOH (10 ml mmol^{-1}), $NaBH_4$ (1.2 equiv.) was added and the solution was stirred at r.t. for 2 h. Then EtOH was evaporated almost completely and H_2O (10 ml) was added, and the precipitate was recrystallized from a suitable solvent.

6.1.3. Individual procedures

*6.1.3.1. 2,4,6-Trifluoro-3-hydroxybenzaldehyde (**16**).* In analogy to literature [16] from 1.33 g (9.0 mmol) 2,4,6-trifluorophenol, 1.086 g (10.0 mmol) trimethylsilyl chloride, and 0.715 g (10.5 mmol) imidazole. CC (petroleum ether/EtOAc = 6/4). Yield: 230 mg (22%); orange crystals, m.p. 158–159 °C; TLC: $R_f = 0.27$ (petroleum ether/EtOAc = 6/4); ^{1}H NMR ($CDCl_3$): $\delta = 5.19$ (s, 1H, OH), 6.83 (m, 1H, H_{aromat}), 10.25 (s, 1H, CHO); IR: $\tilde{\nu} = 3078, 1672, 1504, 1249, 1121, 1048$ cm^{-1}; MS: *m/z* (%): 177 (100) $[M+1]^+$, 145 (5), 127 (5); HRMS (70 eV) calcd for $C_7H_3F_3O_2$: 176.0085, found: 176.0129.

*6.1.3.2. 2-[Tris-(4-methoxyphenyl)methoxy]ethylamine (**19**).* Tris-(4-methoxyphenyl)methanol [18] (2.80 g, 8.0 mmol, 1 equiv.) and 0.15 ml conc. H_2SO_4 in dry toluene (15 ml) were heated for 5 min at 65 °C. After addition of *N*-hydroxyethylphthalimide (2.29 g, 12.0 mmol, 1.5 equiv.) the mixture was stirred at r.t. for 3 h. CH_2Cl_2 (10 ml) and H_2O (10 ml) were added. The solid material was filtered off. The filtrate was extracted three times with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by CC (petroleum ether/EtOAc = 6/4) to give 2-{2-[Tris-(4-methoxyphenyl)methoxy]ethyl}isoindol-1,3-dione. Yield: 4.19 g (76%); yellow powder, m.p. 105–106 °C; TLC: $R_f = 0.27$ (petroleum ether/EtOAc = 6/4); ^{1}H NMR ($CDCl_3$): $\delta = 3.35$ (t, $J = 5.6$ Hz, 2H, CH_2CH_2), 3.74 (s, 9H, OCH_3), 3.90 (t, $J = 5.6$ Hz, 2H, CH_2CH_2), 6.71–6.84 (m, 6H, H_{aromat}), 7.22–7.26 (m, 6H, H_{aromat}), 7.71–7.85 (m, 4H, H_{aromat}); IR: $\tilde{\nu} = 2953, 2834, 1712, 1508, 1390, 1248, 1176, 1032$ cm^{-1}; MS (EI, 70 eV) *m/z* (%): 523 (4) $[M^+]$, 333 (100), 174 (12), 135 (8); $C_{32}H_{29}NO_6$ (523.59).

To 2-{2-[Tris-(4-methoxyphenyl)methoxy]ethyl}isoindol-1,3-dione (2.1 g, 4.0 mmol, 1 equiv.) hydrazine-hydrate (2.0 g in 40 ml MeOH, 40.0 mmol, 10 equiv.) was added. The solution was stirred at r.t. for 7 h. The solid material was filtered off, and the filtrate was concentrated in vacuo. H_2O (40 ml) was added, and the mixture was extracted four times with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, filtered and concentrated in vacuo to give **19**. Yield: 1.10 g (70%); yellow oil; TLC: $R_f = 0.24$ (CH_2Cl_2/MeOH/NH_3 (25%) = 100/2/1); ^{1}H NMR ($CDCl_3$): $\delta = 2.86$ (t, $J = 5.4$ Hz, 2H, CH_2CH_2), 3.13 (t, $J = 5.5$ Hz, 2H, CH_2CH_2), 3.79 (s, 9H, OCH_3), 6.83 (m, 6H, H_{aromat}), 7.32 (m, 6H, H_{aromat}); IR: $\tilde{\nu} = 3380, 2932, 2835, 1607, 1504, 1302, 1249, 1175, 1035$ cm^{-1}; MS (EI, 70 eV) *m/z* (%): 393 (2) $[M^+]$, 333 (100), 243 (5), 135 (11); $C_{19}H_{33}NO$ (291.48).

*6.1.3.3. 5-n-Dodecylaminomethyl-2-methoxyphenol (**21**).* According to GP1 from 3-hydroxy-4-methoxybenzaldehyde (**13**) (1.52 g, 10.0 mmol) and *n*-dodecylamine (**17**) (4.63 g, 25.0 mmol). CC: (CH_2Cl_2/MeOH/NH_3 (25%) = 90/9/1). Yield: 697 mg (22%); colourless crystals, m.p. 76–77 °C; TLC: $R_f = 0.35$ (CH_2Cl_2/MeOH/NH_3 (25%) = 90/9/1); ^{1}H NMR ($CDCl_3$): $\delta = 0.88$ (t, $J = 7$ Hz, 3H, CH_3), 1.25–1.31 (m, 18H, 9×CH_2), 1.54 (t, $J = 7$ Hz, 2H, $NHCH_2CH_2$), 2.62 (t, $J = 7$ Hz, 2H, $ArCH_2NHCH_2$), 3.72 (s, 2H, $ArCH_2NH$), 3.88 (s, 3H, OCH_3), 6.69–7.15 (m, 3H, H_{aromat}); IR: $\tilde{\nu} = 3318, 2916, 2852, 2353, 1504, 1284, 1223, 1131, 1034$ cm^{-1}; MS *m/z* (%): 322 (100) $[M+1]^+$, 166 (7), 137 (27); $C_{20}H_{35}NO_2$ (321.50).

*6.1.3.4. 2-[(4,4-Diphenylbut-3-enylamino)methyl]phenol (**22**).* According to GP1 from 2-hydroxybenzaldehyde (**11**)

(122 mg, 1.0 mmol) and 4,4-diphenylbut-3-enylamine (**18**) (260 mg, 1.0 mmol) [5,18,19]. CC: (petroleum ether/EtOAc/$NEtMe_2$ = 50/50/1). Yield: 216 mg (66%); yellow oil; TLC: R_f = 0.42 (petroleum ether/EtOAc/$NEtMe_2$ = 50/50/1); ^{1}H NMR ($CDCl_3$): δ = 2.38 (q, J = 6.9 Hz, 2H, $NHCH_2CH_2$), 2.78 (t, J = 6.9 Hz, 2H, $NHCH_2CH_2$), 3.92 (s, 2H, $ArCH_2NH$), 6.05 (t, J = 6.9 Hz, 1H, CH=), 6.76–7.38 (m, 14H, H_{aromat}); IR: $\tilde{\nu}$ = 3321, 3053, 2843, 1589, 1493, 1258, 1104 cm^{-1}; MS *m/z* (%): 330 (100) $[M+1]^+$, 236 (3), 224 (5), 136 (15), 107 (5); $C_{23}H_{23}NO$ (329.44).

*6.1.3.5. 3-[(4,4-Diphenylbut-3-enylamino)methyl]phenol (**23**).* According to GP1 from 3-hydroxybenzaldehyde (**12**) (122 mg, 1.0 mmol) and 4,4-diphenylbut-3-enylamine (**18**) (260 mg, 1.0 mmol) [5,18,19]. CC: (CH_2Cl_2/MeOH/NH_3 (25%) = 100/5/1). Yield: 186 mg (57%); colourless crystals, m.p. 129–130 °C; TLC: R_f = 0.29 (CH_2Cl_2/MeOH/NH_3 (25%) = 100/5/1); ^{1}H NMR ($CDCl_3$): δ = 2.38 (q, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 2.78 (t, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 3.92 (s, 2H, $ArCH_2NH$), 6.06 (t, J = 7.5 Hz, 1H, CH=), 6.75–7.38 (m, 14H, H_{aromat}); IR: $\tilde{\nu}$ = 3282, 2856, 2573, 1580, 1481, 1343, 1278, 1102 cm^{-1}; MS *m/z* (%): 330 (100), 136 (25), 107 (4); $C_{23}H_{23}NO$ (329.44).

*6.1.3.6. 5-[(4,4-Diphenylbut-3-enylamino)methyl]-2-methoxyphenol (**24**).* According to GP1 from 3-hydroxy-4-methoxybenzaldehyde (**13**) (152 mg, 1.0 mmol) and 4,4-diphenylbut-3-enylamine (**18**) (260 mg, 1.0 mmol) [5,18,19]. CC: (CH_2Cl_2/MeOH/NH_3 (25%) = 100/3/1) Yield: 251 mg (70%); colourless crystals, m.p. 81–82 °C; TLC: R_f = 0.16 (CH_2Cl_2/MeOH/NH_3 (25%) = 100/3/1); ^{1}H NMR ($CDCl_3$): δ = 2.35 (q, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 2.73 (t, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 3.65 (s, 2H, $ArCH_2NH$), 3.87 (s, 3H, OCH_3), 6.07 (t, J = 7.5 Hz, 1H, CH=), 6.77–7.36 (m, 13H, H_{aromat}); IR: $\tilde{\nu}$ = 3287, 2928, 2857, 1503, 1437, 1276, 1224, 1131, 1028 cm^{-1}; MS *m/z* (%): 360 (100) $[M+1]^+$, 166 (69), 137 (55); $C_{24}H_{25}NO$ (359.47).

*6.1.3.7. 4-[(4,4-Diphenylbut-3-enylamino)methyl]-2,6-difluorophenol (**25**).* According to GP2 from 3,5-difluoro-4-hydroxybenzaldehyde (**14**) (158 mg, 1.0 mmol) [17] and 4,4-diphenylbut-3-enylamine (**18**) (260 mg, 1.0 mmol) [5,18,19]. Recrystallization from EtOH. Yield: 112 mg (31%); colourless crystals, m.p. 160–161 °C; TLC: R_f = 0.28 (CH_2Cl_2/MeOH/NH_3 (25%) = 90/10/1); ^{1}H NMR ($CDCl_3$): δ = 2.41 (q, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 2.80 (t, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 3.69 (s, 2H, $ArCH_2$), 6.07 (t, J = 7.2 Hz, 1H, CH=), 6.77 (d, J = 7.8 Hz, 2H, H_{aromat}), 7.13–7.39 (m, 10H, H_{aromat}); IR: $\tilde{\nu}$ = 3442, 3024, 2362, 1612, 1505, 1445, 1334, 1013 cm^{-1}; $C_{23}H_{21}F_2NO$ (346.42).

*6.1.3.8. 2-[(3,3-Diphenylpropylamino)methyl]phenol (**26**).* According to GP1 from 2-hydroxybenzaldehyde (**11**) (122 mg, 1.0 mmol) and 3,3-diphenylpropylamine (**20**) (230 mg, 1.0 mmol). CC: (petroleum ether/EtOAc/$NEtMe_2$ = 70/30/3). Yield: 220 mg (69%); yellow oil; TLC: R_f = 0.33 (petroleum ether/EtOAc/$NEtMe_2$ = 70/30/3); ^{1}H NMR ($CDCl_3$): δ = 2.30 (q, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 2.66 (t, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 3.91 (s, 2H, $ArCH_2$), 3.99 (t, J = 7.5 Hz, 1H, $NHCH_2CH_2CH$), 6.75–7.28 (m, 14H, H_{aromat}); IR: $\tilde{\nu}$ = 3334, 3024, 2913, 2850, 1590, 1450, 1260, 1104 cm^{-1}; MS *m/z* (%): 318 (100) $[M+1]^+$, 212 (13), 107 (17); $C_{22}H_{23}NO$ (317.43).

*6.1.3.9. 3-[(3,3-Diphenylpropylamino)methyl]phenol (**27**).* According to GP1 from 3-hydroxybenzaldehyde (**12**) (122 mg, 1.0 mmol) and 3,3-diphenylpropylamine (**20**) (230 mg, 1.0 mmol). CC: (CH_2Cl_2/MeOH/$NEtMe_2$ = 100/3/3). Yield: 219 mg (69%); colourless crystals, m.p. 144–145 °C; TLC: R_f = 0.33 (CH_2Cl_2/MeOH/$NEtMe_2$ = 100/3/3); ^{1}H NMR ($CDCl_3$): δ = 2.29 (q, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 2.62 (t, J = 7.5 Hz, 2H, $NHCH_2CH_2$), 3.62 (s, 2H, $ArCH_2$), 3.98 (t, J = 7.5 Hz, 1H, $NHCH_2CH_2CH$), 6.61–7.26 (m, 14H, H_{aromat}); IR: $\tilde{\nu}$ = 3446, 3278, 3023, 2905, 2848, 1598, 1450, 1275 cm^{-1}; MS (EI, 70 eV) *m/z* (%): 317 (32) $[M^+]$, 136 (57), 107 (100); $C_{22}H_{23}NO$ (317.43).

*6.1.3.10. 2-({2-[Tris-(4-methoxyphenyl)methoxy]ethylamino}-methyl)phenol (**28**).* According to GP1 from 2-hydroxybenzaldehyde (**11**) (92 mg, 0.75 mmol) and 2-[tris-(4-methoxyphenyl)methoxy]ethylamine (**19**) (295 mg, 0.75 mmol). CC: (petroleum ether/EtOAc/$NEtMe_2$ = 65/35/2). Yield: 323 mg (86%); glassy solid; TLC: R_f = 0.18 (petroleum ether/EtOAc/$NEtMe_2$ = 65/35/2); ^{1}H NMR ($CDCl_3$): δ = 2.82 (t, J = 5.1 Hz, 2H, CH_2CH_2), 3.27 (t, J = 5.1 Hz, 2H, CH_2CH_2), 3.78 (s, 9H, OCH_3), 3.94 (s, 2H, $ArCH_2$), 6.76–7.31 (m, 16H, H_{aromat}); IR: $\tilde{\nu}$ = 3430, 2931, 2836, 1608, 1506, 1250, 1175, 1034 cm^{-1}; MS *m/z* (%): 333 (100), 227 (16), 168 (22), 107 (34); $C_{31}H_{33}NO_5$ (499.61).

*6.1.3.11. 3-({2-[Tris-(4-methoxyphenyl)methoxy]ethylamino}-methyl)phenol (**29**).* According to GP1 from 3-hydroxybenzaldehyde (**12**) (92 mg, 0.75 mmol) and 2-[tris-(4-methoxyphenyl)methoxy]ethylamine (**19**) (295 mg, 0.75 mmol). CC: (EtOAc/$NEtMe_2$ = 100/2). Yield: 298 mg (80%); colourless crystals, m.p. 62–63 °C; TLC: R_f = 0.12 (EtOAc/$NEtMe_2$ = 100/2); ^{1}H NMR ($CDCl_3$): δ = 2.83 (t, J = 5.4 Hz, 2H, CH_2CH_2), 3.27 (t, J = 5.4 Hz, 2H, CH_2CH_2), 3.67 (s, 2H, $ArCH_2$), 3.77 (s, 9H, OCH_3), 6.71–7.32 (m, 16H, H_{aromat}); IR: $\tilde{\nu}$ = 3407, 2931, 2834, 1607, 1508, 1249, 1175, 103 cm^{-1}; MS *m/z* (%): 333 (22), 227 (83), 168 (100), 145 (21), 127 (22), 107 (24); $C_{31}H_{33}NO_5 \cdot 0.5H_2O$ (508.62).

*6.1.3.12. 5-({2-[Tris-(4-methoxyphenyl)methoxy]ethylamino}-methyl)-2-methoxyphenol (**30**).* According to GP1 from 3-hydroxy-4-methoxybenzaldehyde (**13**) (114 mg, 0.75 mmol) and 2-[tris-(4-methoxyphenyl)methoxy]ethylamine (**19**) (295 mg, 0.75 mmol). CC: (EtOAc/$NEtMe_2$ = 100/2). Yield: 297 mg (75%); colourless crystals, m.p. 122–123 °C; TLC: R_f = 0.12 (EtOAc/$NEtMe_2$ = 100/2); ^{1}H NMR ($CDCl_3$): δ = 2.80 (t, J = 5.4 Hz, 2H, CH_2CH_2), 3.23 (t, J = 5.4 Hz, 2H, CH_2CH_2), 3.68 (s, 2H, $ArCH_2$), 3.78 (s, 9H, OCH_3), 3.88 (s, 3H, OCH_3), 6.80–7.32 (m, 15H, H_{aromat}); IR: $\tilde{\nu}$ = 3431, 2954, 2834, 1608,

1507, 1247, 1177, 1096, 1036 cm^{-1}; MS *m/z* (%): 333 (40), 257 (40), 225 (45), 198 (36), 137 (92), 105 (100); $C_{32}H_{35}NO_6$ (519.55).

*6.1.3.13. 2,6-Difluoro-4-({2-[tris-(4-methoxyphenyl)methoxy] ethylamino}methyl)phenol (**31**).* According to GP2 from 3,5-difluoro-4-hydroxybenzaldehyde (**14**) (158 mg, 1.0 mmol) and 2-[tris-(4-methoxyphenyl)methoxy]ethylamine (**19**) (394 mg, 1.0 mmol). Reduction with $NaBH_4$ in isopropanol instead of EtOH as solvent, recrystallization from EtOH/Et_2O (3/1). Yield: 144 mg (54%); white powder, m.p. 199–200 °C; TLC: $R_f = 0.30$ (CH_2Cl_2/MeOH/NH_3 (25%) = 90/10/1); ^1H NMR ($CDCl_3$): $\delta = 2.86$ (t, $J = 5.3$ Hz, 2H, CH_2CH_2), 3.33 (t, $J = 5.3$ Hz,2H, CH_2CH_2), 3.58 (s, 2H, $ArCH_2$), 6.67 (d, $J = 8.4$ Hz, 2H, H_{aromat}), 6.84 (d, $J = 6.6$ Hz, 6H, H_{aromat}), 7.29 (d, $J = 6.6$ Hz, 6H, H_{aromat}); IR: $\tilde{\nu}$ = 3446, 2951, 2834, 2360, 1608, 1508, 1463, 1327, 1252, 1176, 1035 cm^{-1}; $C_{31}H_{31}F_2NO_5$ (535.59).

*6.1.3.14. 2,6-Difluoro-3-({2-[tris-(4-methoxyphenyl)methoxy] ethylamino}methyl)phenol (**32**).* According to GP2 from 2,4-difluoro-3-hydroxybenzaldehyde (**15**) (158 mg, 1.0 mmol) [17] and 2-[tris-(4-methoxyphenyl)methoxy]ethylamine (**19**) (394 mg, 1.0 mmol). Recrystallization from EtOH/ether (3/1). Yield: 158 mg (56%); white powder, m.p. 151–152 °C; TLC: $R_f = 0.30$ (CH_2Cl_2/MeOH/NH_3 (25%) = 90/10/1); ^1H NMR ($CDCl_3$): $\delta = 2.83$ (t, $J = 5.2$ Hz, 2H, CH_2CH_2), 3.28 (t, $J = 5.2$ Hz, 2H, CH_2CH_2), 3.72 (s, 2H, $ArCH_2$), 3.78 (s, 9H, OCH_3), 6.71–7.32 (m, 14H, H_{aromat}); IR: $\tilde{\nu}$ = 3440, 2930, 2835, 1608, 1509, 1252, 1175, 1034 cm^{-1}; $C_{31}H_{31}F_2NO_5 \cdot 0.25H_2O$ (540.19).

*6.1.3.15. 2,4,6-Trifluoro-3-({2-[tris-(4-methoxyphenyl)methoxy]ethylamino}methyl)phenol (**33**).* According to GP1 from 2,4,6-trifluoro-3-hydroxybenzaldehyde (**16**) (175 mg, 1.0 mmol) and 2-[tris-(4-methoxyphenyl)methoxy]ethylamine (**19**) (394 mg, 1.0 mmol). CC: (CH_2Cl_2/MeOH = 100/3) Yield: 342 mg (62%); white powder, m.p. 128–129 °C; TLC: $R_f = 0.30$ (CH_2Cl_2/MeOH = 100/3); ^1H NMR ($CDCl_3$): $\delta = 2.88$ (t, $J = 5.1$ Hz, 2H, CH_2CH_2), 3.32 (t, $J = 5.1$ Hz, 2H, CH_2CH_2), 3.70 (s, 2H, $ArCH_2$), 3.77 (s, 9H, OCH_3), 6.46–7.35 (m, 13H, H_{aromat}); IR: $\tilde{\nu}$ = 3436, 2934, 2836, 1608, 1506, 1250 cm^{-1}; $C_{31}H_{31}F_2NO_5 \cdot H_2O$ (553.60).

6.2. Biological evaluation

6.2.1. Cell culture and stable transfection

All the media, sera, additives and antibiotics for cell culture were purchased from PAA (Cölbe, Germany). The following vector constructs were used to express the murine GABA transporters as previously described [14]: pRC-CMV2 containing the cDNA for mGAT1 (obtained from Prof. H. Lüddens) and pcDNA3.1(+) for mGAT2, mGAT3, and mGAT4 (cDNA for these transporters was obtained from Prof. N. Nelson). HEK cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (normal DMEM) in a humidified atmosphere (92% air, 8% CO_2) at 37 °C. Transfections with linearised plasmids (mGAT1, mGAT3 and mGAT4: *Sca*I, mGAT2: *Mfe*I) were performed using FuGENE6 (Roche, Mannheim, Germany) according to manufacturer's protocol: About 3.5×10^6 HEK cells were plated in normal DMEM. On the next day the cells were treated with a mixture of 40 μl FuGENE6 and 13.3 μg cDNA (mGAT1, mGAT3, mGAT4) or 60 μl FuGENE6 and 10.0 μg cDNA (mGAT2). Two days after transfection the cells were split in DMEM supplemented with 10% fetal calf serum and 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 500 μg ml^{-1} geneticin (selection medium). Having reached a confluence of about 30% stably transfected cells were plated 1/10,000 in selection medium. About two weeks later single colonies of stably transfected cells were isolated and further cultivated in selection medium.

6.2.2. [^{3}H]GABA uptake

Cells grown in 145 cm^2 plates to a confluence of 70–90% were treated with 4 ml trypsin/EDTA for about 30 s. Afterwards 8 ml normal medium was added and the resulting cell suspension centrifuged 5 min at 500*g*. The cells were washed for three times in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4) by centrifugation as described above and finally resuspended in Krebs buffer (2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 11 mM glucose, 25 mM Tris, 119 mM NaCl, pH 7.2). The uptake assays were performed with aliquots of the resulting cell suspension (about 100,000 cells per well for mGAT1, mGAT3, mGAT4 and about 200,000 cells per well for mGAT2) in a total volume of 250 μl in 96 well 2.2 ml polyethylene deep well plates (Abgene, Epsom, UK). The cells were equilibrated for 25 min in Krebs buffer in the presence of the test compound at 37 °C in a gently shaking water bath. Due to the poor solubility of the test compounds all samples contained 1% DMSO. (The highest concentration investigated was 100 μM. Since many of the test compounds tended to precipitate at this concentration it cannot be ruled out absolutely that the inhibition assays are affected by precipitation at 100 μM of the test compounds. However, the inhibition curves obtained gave no indication of any distorting effects.) After addition of 25 μl of a solution containing [^{3}H]GABA (3 TBq mmol, Amersham Biosciences, Freiburg, Germany) and unlabeled GABA in Krebs (final concentration 8 nM [^{3}H]GABA and 32 nM unlabeled GABA for mGAT1, mGAT3, mGAT4 and 20 nM [^{3}H]GABA and 20 nM unlabeled GABA for mGAT2) the cells were incubated for further 4 min (mGAT1, mGAT3, mGAT4) or 10 min (mGAT2), respectively. The incubation was stopped by filtration through Whatman GF/C filters pre-soaked for 1 h in 0.9% NaCl by means of a Brandell MWXR-96TI cell harvester (Brandell, Gaithersburg, MD, USA) under reduced pressure (not below 250 mbar). The filters were rinsed four times with cold 0.9% NaCl and subsequently transferred to 96-well sample plates (Perkin Elmer LAS, Boston, MA, USA). After addition of 200 μl Rotiszint Eco Plus (Roth, Karlsruhe, Germany) per well the radioactivity was determined in a microbeta liquid scintillation counter (Perkin Elmer LAS,

Boston, MA, USA). Non-specific uptake was defined in parallel experiments with 10 μM NO 711 (mGAT1) or 1 mM GABA (mGAT2, mGAT3, mGAT4) and was subtracted from total uptake (no inhibitor) to yield specific uptake. IC_{50} values were determined by non-linear regression using Prism 4 (''one site competition''; bottom and top were fixed to 0 and 100%, respectively). The results are expressed as means ± S.E.M. of at least three separate experiments, each carried out in triplicate. Protein was determined in an aliquot of the final cell suspension according to Bradford [20] using BSA as a standard.

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