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SPECIAL REPORT

1-(3-(9H-Carbazol-9-yl)-1-propyl)-4-(2-methoxyphenyl)-4-piperidinol, a novel subtype selective inhibitor of the mouse type II GABA-transporter

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The selectivity of new derivatives of the γ-aminobutyric acid (GABA)-uptake inhibitor, tiagabine was characterized at the four cloned mouse GABA transporters (mGAT1 through mGAT4) by measuring [^{3}H]-GABA uptake into stably transfected baby hamster kidney cells. While tiagabine is a highly selective inhibitor of mGAT1 ($K_i = 0.11 \pm 0.02$ μM), these derivatives exhibited low potencies at mGAT1 but differential activities at mGAT2, mGAT3 and mGAT4. In particular, 1-(3-(9H-carbazol-9-yl)-1-propyl)-4-(2-methoxyphenyl)-4-piperidinol (NNC 05-2090) was a potent inhibitor of mGAT2 ($K_i = 1.4 \pm 0.3$ μM) showing at least 10 fold selectivity over mGAT1, mGAT3 and mGAT4. NNC 05-2090 is the first subtype selective inhibitor of mGAT2 and may represent a novel useful tool for investigating the physiological roles of GAT2 in the brain and periphery.

Keywords: γ-Aminobutyric acid (GABA) uptake; lipophilic GABA uptake inhibitor; BGT-1; tiagabine; 1-(3-(9H-carbazol-9-yl)-1-propyl)-4-(2-methoxyphenyl)-4-piperidinol (NNC 05-2090)

Introduction Inactivation of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in synapses of the central nervous system is primarily mediated by re-uptake into the presynaptic terminals via high affinity sodium-dependent GABA uptake carriers (Krogsgaard-Larsen *et al*., 1987). This process can be inhibited by the GABA uptake inhibitor, nipecotic acid and more potently by its lipophilic derivatives such as (**R**)-N-(4,4-bis(3-methyl-2-thienyl)but-3-en-1-yl) nipecotic acid (tiagabine) (Krogsgaard-Larsen *et al*., 1987; Suzdak, 1993). Molecular cloning of the GABA transporters has revealed the existence of a family of four subtypes (Uhl & Hartig, 1992). When cloned from murine brain these subtypes were termed mGAT1 through mGAT4 (Liu *et al*., 1993). An alternative nomenclature has been used for the human homologues (as well as for the rat homologues) where mGAT1 correspond to hGAT-1, mGAT2 to hBGT-1, mGAT3 to hGAT-2 and mGAT4 to hGAT-3 (Uhl & Hartig, 1992). Subtypes mGAT1, mGAT3 and mGAT4 transport GABA with a high affinity whereas mGAT2 display a lower affinity for GABA and also utilize the osmolyte betaine as a substrate (Liu *et al*., 1993). Tiagabine and related analogues are potent and highly selective inhibitors of hGAT-1 (and mGAT1) while (±)-1-(2-[tris(4-methoxyphenyl)methoxy]ethyl-3-piperidinecarboxylic acid (SNAP-5114) has been shown to be a moderately selective inhibitor of hGAT-3 (which are homologous to mGAT4) (Borden *et al*., 1994; Dhar *et al*., 1994). However, no potent inhibitors of mGAT2 or mGAT3 have been found previously. In the present study, a novel subtype selective inhibitor of mGAT2, NNC 05-2090 is described which is 2000 fold more potent than nipecotic acid to mGAT2.

Methods The cDNAs encoding the murine GABA transporters (kindly provided by Dr N. Nelson) were subcloned into the mammalian expression vector pZEM (Foster *et al*., 1991; Liu *et al*., 1993). Baby hamster kidney (BHK) cells stably expressing mGAT1 through mGAT4, respectively, were cultured in Dulbecco's modified Eagles media supplemented with 5% foetal bovine serum, 2 mM glutamine, 0.05 mg ml^{-1} gentamycin and 2 μM methotrexate in a humidified atmosphere (95% air, 5% CO_2) at 37°C. The cells were seeded in 24-well plates and assayed when they were approximately 2/3 confluent. Cells were washed with 2 × 1 ml assay buffer (composition in mM: NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, D-glucose 11 and HEPES 10, pH 7.4) and subsequently incubated in buffer for 20 min at 37°C. Incubation with [^{3}H]-GABA (1 nM of [^{3}H]-GABA (Amersham, Arlington Heights, U.S.A.) with a specific activity of 89 Ci mmol^{-1} was diluted with unlabelled GABA to a final concentration of 0.5 μM) lasted for a subsequent period of 3 min or 10 min in the case of mGAT2. The cells were washed quickly with 2 × 2 ml ice-cold assay buffer, solubilized with 1 ml of NaOH (1 M) and transferred to scintillation vials. The total amount of [^{3}H]-GABA taken up accounted for less than 5% of the total amount of substrate available. Tiagabine, 9-(3-(4-(4-chlorophenyl-4-hydroxy-1-piperidinyl)propyl)-9H-pyrido[3,4-b]-indole-3-carboxylic acid ethyl ester (NNC 05-0341), 9-(3-(4-phenyl-4-hydroxy-1-piperidinyl)propyl)-9H-pyrido[3,4-b]-indole-3-carboxylic acid ethyl ester (NNC 05-1965), 1-(3-(9H-carbazol-9-yl)-1-propyl)-4-(4-chlorophenyl)-4-piperidinol (NNC 05-1973), 1-(3-(9H-carbazol-9-yl)-1-propyl)-4-(4-methoxyphenyl)-4-piperidinol (NNC 05-2045), NNC 05-2090 and SNAP-5114 were synthesized at Novo Nordisk A/S, Denmark. All other reagents or chemicals were from Sigma (St. Louis, U.S.A.).

Results and discussion Mouse GABA transporters were expressed in BHK cell lines and used for characterizing the subtype selectivity of new lipophilic inhibitors of GABA uptake. Eadie-Hofstee analysis revealed a saturable single affinity site for [^{3}H]-GABA uptake into BHK cells expressing mGAT1, mGAT2, mGAT3 and mGAT4 (Hill coefficients were 0.91 ± 0.05, 0.99 ± 0.01, 0.95 ± 0.03 and 0.94 ± 0.03, respectively) with respective apparent affinities (K_m) of 12 ± 3, 95 ± 8, 8 ± 1 and 13 ± 3 μM and a maximum velocity for transport (V_{max}) of 0.66 ± 0.06, 0.70 ± 0.08, 0.48 ± 0.03 and 0.90 ± 0.26 nmol mg^{-1} protein min^{-1}, respectively (data not shown). In Table 1 the apparent K_i values of GABA uptake inhibitors as well as the apparent affinities of novel lipophilic inhibitors are shown. Tiagabine and SNAP 5114 are selective inhibitors of mGAT1 and mGAT4, respectively which is in

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Table 1 Apparent affinities of GABA uptake inhibitors at mGAT1 through to mGAT4

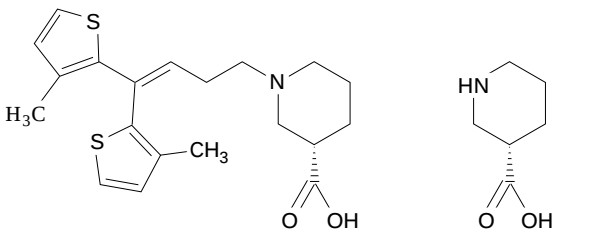
	K_i (μM)			
	mGAT1 (hGAT-1)	*mGAT2 (hBGT-1)*	*mGAT3 (hGAT-2)*	*mGAT4 (hGAT-3)*
GABA	13±3	97±12	9.2±1.8	12±2
Nipecotic acid	70±9	2785±507	124±21	201±45
Tiagabine	0.11±0.02	>100	>100	>100
SNAP-5114	>30	22±2	20±3	6.6±0.2
NNC 05-0341	20±7	4.1±1.4	7.4±2.6	2.8±0.3
NNC 05-1965	23±2	2.6±0.3	10±3	2.8±0.7
NNC 05-1973	43±9	2.4±0.5	34±2	5.2±0.1
NNC 05-2045	27±2	1.6±0.4	14±4	6.1±1.3
NNC 05-2090	19±2	1.4±0.3	41±11	15±4

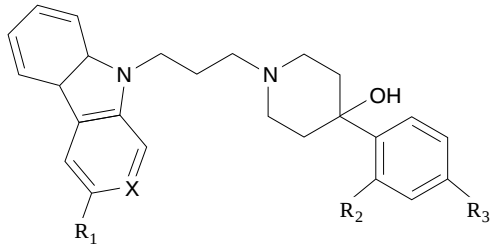
The values (mean±s.e.mean) are the apparent potencies (K_i) of the various GABA uptake inhibitors for preventing [^{3}H]-GABA uptake into BHK cells expressing mGAT1, mGAT2, mGAT3 and mGAT4, respectively. In parentheses, the nomenclature of the human homologues is indicated. The experiments ($n=3-6$) were performed in duplicate and the results were calculated by non-linear regression analysis of dose-response curves with at least 4 data points. The dose-response curves were fitted to a sigmoidal curve by use of the GraphPad Prism programme (GraphPad Software, San Diego, U.S.A.) and converted to K_i values with the Cheng-Prusoff equation. Non-specific uptake of [^{3}H]-GABA was defined as the residual uptake observed in the presence of a large excess of GABA (1 mM, except for mGAT2 where the concentration was 3 mM) and accounted for 4–8% of total uptake. No specific [^{3}H]-GABA-uptake was observed with non-transfected BHK cells (data not shown).

accordance with data obtained using the human homologues, hGAT-1 and hGAT3 (Borden *et al*., 1994; Dhar *et al*., 1994). The five new compounds were all potent inhibitors of mGAT2 (Table 1). While NNC 05-0341 showed no selectivity between mGAT2, mGAT3 and mGAT4, NNC 05-1965, NNC 05-1973 and NNC 05-2045 displayed some selectivity for mGAT2 and mGAT4 (Table 1). Interestingly, NNC 05-2090 (Figure 1) was a potent inhibitor of mGAT2 ($K_i=1.4$ μM) showing at least 10 fold selectivity over other GABA transporters (Table 1).

Since the maximal rates of GABA uptake in the individual cell lines were in the same order of magnitude it seemed unlikely that distortions of the transmembrane gradients for GABA and/or ions due to the activity of transporters could influence the selectivity of NNC 05-2090 for mGAT2. Furthermore, the potencies of GABA, nipecotic acid and SNAP-5114 at mGATs (Table 1) were quite similar to the values obtained with the human and rat homologues (Borden *et al*., 1994; Dhar *et al*., 1994). Structurally, NNC 05-2090 differs from most of the currently available lipophilic GABA uptake inhibitors by virtue of the modified nipecotic acid moiety of NNC-05-2090 (Figure 1). This modification appears to eliminate the high affinity of tiagabine and related structures for GAT-1 (Borden *et al*., 1994) and to alter the GABA transport selectivity (Table 1). Very few inhibitors of GABA transport have been shown to have activity at mGAT2 (or at the human or rat homologues of mGAT2) and none of these are selective for any particular subtype (Borden *et al*., 1994; 1995; Dhar *et al*., 1994). Accordingly, the most potent inhibitor of hBGT-1 (i.e., mGAT2) obtained to date is (−)-2-phenyl-2-[(dimethylamino)ethoxy]-(1R)-1,7,7-trimethylbicycloheptane (EGYT 3886) which has been described as a moderately potent but non-selective inhibitor of the cloned GABA transporters (the IC_{50} ranged from 26 to 46 μM and was 39±6 μM for the human analogue of mGAT2) (Dhar *et al*., 1994). Thus, NNC 05-2090 is a significantly more potent subtype selective inhibitor of mGAT2 as compared to the most potent compounds available.

By use of GAT-1 selective inhibitors of GABA transport it has been shown that this subtype plays a major role in maintaining the resting levels of GABA low in the brain (Borden *et al*., 1995) and blockade of GAT-1 exhibits substantial anticonvulsant effects in rodents and man (Suzdak, 1993). The differential regional distribution of subtypes of GABA transporters in the central nervous system (Liu *et al*., 1993) also suggests that additional GABA transporters are involved in the fine tuning of GABAergic neurotransmission. However, the exact physiological roles of the individual subtypes remain to be determined. In this regard, NNC 05-2090 may prove useful for delineating the roles of mGAT2 in GABAergic transmission and osmoregulation.





	R_1	R_2	R_3	X
NNC 05-0341	$CO_2C_2H_5$	H	Cl	N
NNC 05-1965	$CO_2C_2H_5$	H	H	N
NNC 05-1973	H	H	Cl	CH
NNC 05-2045	H	H	OCH_3	CH
NNC 05-2090	H	OCH_3	H	CH

Figure 1 The chemical structures of tiagabine, nipecotic acid and novel lipohilic inhibitors of GABA uptake.

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