Involvement of sialic acid in the regulation of γ-aminobutyric acid uptake activity of γ-aminobutyric acid acid transporter 1

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The γ-aminobutyric acid (GABA) transporters (GATs) have long been recognized for their key role in the uptake of neurotransmitters. The GAT1 belongs to the family of Na⁺- and Cl⁻-coupled transport proteins, which possess 12 putative transmembrane (TM) domains and three N-glycosylation sites on the extracellular loop between TM domains 3 and 4. Previously, we demonstrated that terminal trimming of N-glycans is important for the GABA uptake activity of GAT1. In this work, we examined the effect of deficiency, removal or oxidation of surface sialic acid residues on GABA uptake activity to investigate their role in the GABA uptake of GAT1. We found that the reduced concentration of sialic acid on N-glycans was paralleled by a decreased GABA uptake activity of GAT1 in Chinese hamster ovary (CHO) Lec3 cells (mutant defective in sialic acid biosynthesis) in comparison to CHO cells. Likewise, either enzymatic removal or chemical oxidation of terminal sialic acids using sialidase or sodium periodate, respectively, resulted in a strong reduction in GAT1 activity. Kinetic analysis revealed that deficiency, removal or oxidation of terminal sialic acids did not affect the K_m GABA values. However, deficiency and removal of terminal sialic acids of GAT1 reduced the V_max GABA values with a reduced apparent affinity for extracellular Na⁺. Oxidation of cell surface sialic acids also strongly reduced V_max without affecting both affinities of GAT1 for GABA and Na⁺, respectively. These results demonstrated for the first time that the terminal sialic acid of N-linked oligosaccharides of GAT1 plays a crucial role in the GABA uptake process.

Keywords: γ-aminobutyric acid / GABA transporter 1 / GABA uptake / sialic acid / neurotransmitter

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA is released from the presynapse into the synaptic cleft, and then binds to post-synaptic receptors leading to the inhibitory potentials (Schuske et al. 2004). The GABA activity is efficiently terminated by GABA transporters (GATs) that quickly remove GABA from the synaptic cleft. Since the excitability of most neurons in the CNS is ruled by the dynamic balance of excitation and inhibition at any time, synaptic inhibition must be tightly regulated. Activation of GABA receptor gates Cl⁻ selective channels leading to a lowering of membrane potential and neuron excitability, whereas the activities of GAT are important for the control of the concentration and dwell time of GABA in the synaptic cleft (Kanner 2006). Since the dysfunction of GABAergic system has been implicated in many diseases of the nervous system, such as epilepsy (Mohler 2006; Cope et al. 2009), depression (Krystal et al. 2002), pain (Otsuka and Yanagisawa 1990), as well as Alzheimer’s disease (Garcia-Alloza et al. 2006), the regulation of GABA activity is of considerable medical interest (Broer 2006; Madsen et al. 2009).

GAT belongs to a family of secondary active systems that are driven by electrochemical gradients of Na⁺ and Cl⁻ ions (Radian and Kanner 1983). They are located in the plasma membranes of neurons and glia cells. Four subtypes of GATs (GAT1–4) have been found so far (Liu et al. 1993). The predominant GAT in GABAergic nerve endings is GAT1. GAT1 is a single polypeptide of 67 kDa with 12 putative transmembrane (TM) domains connected by hydrophilic loops with the amino- and carboxy-termini residing in the cytoplasm. The large extracellular loop between TM domains 3 and 4 contains three conserved N-glycosylation sites (Asn176, Asn181 and Asn184; Guastella et al. 1990). It has been demonstrated that all three N-glycosylation sites are used in vivo and that no additional sites are present (Bennett and Kanner 1997).

N-Glycosylation is a major post-translational modification in eukaryotic cells. Recent results suggest that N-glycosylation may influence many of the physicochemical and the biological properties of glycoproteins, such as folding, stability, targeting, dynamics and ligand binding, as well as cell–matrix and cell–cell interactions (Varki 1993; Schauer 2000; Hedlund et al. 2008). It has also been
suggest that N-glycosylation is involved in the surface expression of neurotransmitter transporters and the regulation of the transport activity. Functional expression of GAT in HeLa cells is abolished by tunicamycin, a potent inhibitor of N-glycosylation (Keynan et al. 1992). In Xenopus oocytes, it was demonstrated that mutations of two of the three N-glycosylation sites led to a reduction in turnover rates and complex changes in the interaction of external Na+ with the transport protein as measured by voltage clamping (Liu et al. 1998). Our previous studies further showed that N-glycans, in particular terminal structures of N-glycans, are involved in the GABA uptake process of GAT1. Deficient N-glycosylation reduced the transport activity of GAT1, which can be partially attributed to a reduced apparent affinity to extracellular Na+ and slowed kinetics of the transport cycle (Cai et al. 2005).

Sialic acids are negatively charged and usually the terminal sugar residues on the oligosaccharide chains of cell surface or serum glycoconjugates. Sialylation is involved in a broad range of biological and pathological processes (Crocker et al. 2007; Hedlund et al. 2008; Schauer 2009), including intercellular adhesion, signaling (Razi and Varki 1998), apoptosis (Zhuo et al. 2008; Earl et al. 2010), cancer cell differentiation (Hedlund et al. 2008), immune (Crocker et al. 2007) and nervous system functioning and development (Potschka et al. 2008; Rutishauser 2008). Structural studies indicate that the presence or the absence of specific terminal sugars may affect hydrophilic or hydrophobic interactions between sugar residues and amino acid residues in the Fc fragment, which in turn may impact antibody effector functions (Raju 2008). The structure and the biological functions of some glycoproteins are determined by sialic acids. One example is that sialic acids influence the conformation of gangliosides and contribute to the supramolecular structures in cell membranes, thus influencing their functions (Siebert et al. 1996).

In this work, the role of sialic acids in the GABA uptake of GAT1 was investigated. For this purpose, GAT1/green fluorescent protein (GFP) fusion protein was expressed in Chinese hamster ovary (CHO), CHO Lec3 and human embryonic kidney 293 (Hek293) cells, separately. The key enzyme of sialic acid biosynthesis is uridine diphosphate (UDP)-N-acetylgalcosamine (GlcNAc) 2-epimerase/N-acetylmannosamine kinase (GNE; Spivak and Roseman 1966; Hinderlich et al. 1997; Stasche et al. 1997). It regulates the cell surface sialylation for the biosynthesis of glycoconjugates (Kepper et al. 1999). CHO Lec3 is a CHO mutant without UDP-GlcNAc 2-epimerase activity resulting in a deficiency in sialic acid biosynthesis (Hinderlich et al. 2001; Hong and Stanley 2003). The GABA uptake activities of GAT1 in both CHO and CHO Lec3 cells were quantitatively compared. Furthermore, the sialidase or sodium periodate (NaIO4) was used to remove or oxidize the sialic acids of GAT1 and followed by studying the effects on GABA uptake. Kinetic analysis was performed with either different GABA concentrations or different Na+ concentrations. We demonstrate in this work that the reduced surface bound sialic acids decreases the GABA transport activity by GAT1, indicating the involvement of sialic acid in the regulation of the GABA uptake activity of GAT1.

Results

Expression of GAT1/GFP fusion proteins in CHO, CHO Lec3 and Hek293 cells

Complementary DNAs encoding GFP-tagged GAT1 were functionally transfected into CHO, CHO Lec3 and Hek293 cells, which do not express endogenous GAT1 and GFP. Stable transfectants were selected by fluorescence-activated cell sorting (FACS). Flow cytometry analysis and fluorescence microscopy (Figure 1A and B) showed the expression of GAT1/GFP on the surface of transfected CHO, CHO Lec3 and Hek293 cells. The expression of GAT1/GFP was determined by western blotting with either anti-GAT1 pAb or anti-GFP pAb following immunoprecipitation with anti-GFP mAb. In transfected CHO cells, the GAT1/GFP fusion protein showed several bands in sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE; Figure 1C and D) as described previously (Cai et al. 2005), two monomeric forms running as a main band of ~110 kDa and a small band of ~90 kDa. The 110 kDa polypeptide contains mature N-glycans of the complex type, whereas the 90 kDa peptide contains only N-glycans of the oligomannosidic type. In transfected CHO Lec3 and Hek293 cells, the GAT1/GFP fusion protein shows the same bands and molecular weights (Figure 1C) as those in CHO cells.

Deficient sialic acid biosynthesis in CHO Lec3 cells results in the decrease in the GABA uptake activity of GAT1/GFP

CHO Lec3 cells are defective in sialic acid biosynthesis. They disclose less sialic acid on their cell surface glycoproteins and glycolipids. To study the role of terminal sialic acid of GAT1 in GABA uptake activity, aliquots of stable CHO and CHO Lec3 transfectants were used for determination of both GAT1 protein and sialic acid amounts, and the other aliquots were used for the GABA uptake assay simultaneously. Figure 2A and B show the total and plasma membrane GAT1/GFP protein expressed in CHO Lec3 cells as well as in CHO cells. The expression of the terminal sialic acids on GAT1/GFP was detected by lectin Maackia amurensis agglutinin (MAA) and Sambucus nigra agglutinin (SNA). In these cell lines, only α2,3-linked sialic acid could be detected by MAA, but not α2,6-linked sialic acid by the use of SNA (data not shown). Normalized to the amount of plasma membrane expressed GAT1 protein, the concentration of the terminal sialic acid of GAT1 in CHO Lec3 cells was only 35.2±13% of that in CHO cells (Figure 2C and D). Remarkably, the GABA uptake activity of CHO Lec3 cells was also reduced to 33.8±5.7% (Figure 2E), in comparison to those in CHO cells. These results suggest that a deficiency in the terminal sialic acid composition of GAT1/GFP protein results in a significant reduction in the GABA uptake activity.

Reduction in the GABA transport activity of GAT1 by sialidase treatment

In order to verify that the reduced GABA uptake activity of GAT1 in CHO Lec3 cells is not resulted from other mutational effects of the CHO Lec3 cells, sialidase was used to remove the cell surface sialic acids from the GAT1/GFP protein and then the GAT1 activity was determined. Both
GA T1/GFP-transfected CHO and Hek293 cells were treated with sialidase at different doses. Sialidase works efficiently under weak acidic conditions; however, the cell growth was affected when the pH value is <5.0. At pH 5.5, sialidase maintained the enzymatic activity and the growth of the cells was hardly affected (data not shown). Further experiments were performed in a serum-free medium with pH 5.5.

Figure 3A shows that in transfected CHO cells, after the treatment of sialidase with 0.05, 0.2, 0.4, 0.6 and 0.8 U/mL in pH 5.5 and the serum-free medium for 4 h, the GABA uptake activities of GA T1 reduced to 72, 55, 28, 21 and 17%, respectively. Also, Figure 3B shows the same tendency in transfected Hek293 cells. After the treatment of sialidase with 0.05, 0.2, 0.4, 0.6 and 0.8 U/mL under the same conditions, the GABA uptake activities in Hek293 cells reduced to 89, 70, 61, 48 and 34%, respectively. These results indicate that sialidase reduces the GABA uptake activity of GA T1 in a dose-dependent manner.

Removal of terminal sialic acid by sialidase results in strong decrease in the GABA transport activity of GA T1/GFP

Since our results show that sialidase has a direct influence on GA T1 activity, quantitative measurements were further performed with GA T1/GFP stably transfected Hek293 cells, in which the GA T1/GFP protein expressed significantly higher than in CHO cells. After incubation with 1 U/mL of sialidase in the serum-free medium (pH 5.5) for 4 h, the aliquots of cells were used to determine the amount of protein expression...
and sialic acids, whereas the other aliquots were used to measure the GABA uptake activity by GA T1. Figure 4A and B shows that after the treatment of sialidase, the amounts of the total and plasma membrane GA T1/GFP protein of Hek293 cells were in the same range as those of cells without the treatment of sialidase; and the amount of α2,3-linked sialic acid of GA T1 reduced significantly to 13.8 ± 10.5% (Figure 4C and D). The GABA uptake activity was reduced to 22.2 ± 11.5% (Figure 4E), as well, compared with that of cells without sialidase treatment. The terminal sialic acids were removed without a significant influence on the protein expression and stability of GA T1. However, the reduction in sialic acid concentration leads to the marked decrease in the GABA uptake of GA T1. These results indicate that terminal sialic acids on N-glycans are crucial for the GABA uptake activity of GA T1.

Oxidation of terminal sialic acid by sodium periodate also results in strong decrease in the GABA transport activity of GA T1/GFP

In order to clarify the GABA uptake activity is dependent only on the acidic property of sialic acid of the GA T1 protein, chemical oxidation of surface membrane-associated sialic acid residues was performed by using NaIO₄ (Perez et al. 1985).

In order to prevent the formation of the Schiff base from the formed aldehyde with available amino groups, aliquots of cells were further treated with sodium borohydride (NaBH₄). After NaIO₄ treatment, the amounts of the total and plasma membrane bound GA T1 protein of Hek293 cells were not affected compared with those of cells without treatment of NaIO₄ (Figure 5A and B), however, the amount of α2,3-linked sialic acid of GA T1 is reduced to 30.5 ± 10.5% (Figure 5C and D). Concomitantly, the GABA uptake activity was reduced to 25.2 ± 11.5% (Figure 5E), as well, compared with that of cells without NaIO₄ treatment. Meanwhile, after further mild NaBH₄ reduction which converted aldehyde to alcohol, the reduced GABA uptake activity was not changed apparently (Figure 5E). These results give further evidence that terminal sialic acids are necessary for the regulation of the GABA uptake activity of GA T1.
Fig. 5. Quantitative determination of the GABA uptake activity of GAT1/GFP in Hek293 cells after NaIO4 treatment. The GAT1/GFP stably transfected Hek293 cells suspended in PBS (pH 7.4) were incubated with 1 mM NaIO4 in on ice for 15 min. Then aliquots of cells were separated for immunoprecipitation with anti-GFP mAb IgG, cell surface biotinylation, GABA uptake assay and mild NaBH4 reduction followed the GABA uptake assay. Aliquots of each immunoprecipitate and biotinylation probes were analyzed by SDS/PAGE (7.5%) and western blotting. (A) Total expressed GAT1/GFP protein. The aliquots of immunoprecipitate probes were immunoblotted with anti-GFP pAb. (B) Plasma membrane expressed GAT1/GFP protein. Biotinylation probes are immunoblotted with anti-GFP pAb. (C) Sialic acid levels. The other aliquots of immunoprecipitate probes were stained with MAA, GAT1/GFP in untreated Hek293 cells (lane 1); GAT1/GFP in NaIO4-treated Hek293 cells (lane 2). (D) Quantification of the levels of sialic acids of GAT1/GFP in Hek293 transfectants with or without NaIO4 treatment. (E) Quantification of GABA uptake activity in Hek293 transfectants with or without NaIO4 treatment. The total GAT1/GFP (A), plasma membrane expressed GAT1/GFP (B) protein expression and sialic acid levels (C) were quantified with Quantity One software (Bio-Rad). Both sialic acid levels and GABA uptake activity values were normalized to the amount of the plasma membrane GAT1 protein. And both the values of untreated Hek293/GAT1 cells were set at 100%. All other values were expressed relative to these values. The values represent the mean ± SD of three separate experiments.

Deficiency, removal or oxidation of terminal sialic acid did not change the $K_m$ GABA values of GAT1.

To verify whether the terminal sialic acids of GAT1 influence the affinity of GAT1 for GABA, kinetic analysis was performed with different GABA concentrations. Kinetic parameters of GABA uptake were determined for GAT1/GFP-transfected CHO and CHO Lec3 cells and GAT1/GFP-transfected Hek293 cells with and without treatment with sialidase or NaIO4. The kinetic constants were calculated on the basis of the Michaelis–Menten equation by the double-reciprocal plot analysis. As shown in Figure 6A, the $V_{max}$ GABA values of CHO cells is 1.5 pmol μg protein$^{-1}$ min$^{-1}$, whereas the value of CHO Lec3 cells is reduced to 0.31 pmol μg protein$^{-1}$ min$^{-1}$. However, the $K_m$ GABA values of CHO Lec3 cells (4.4 μM) showed in the same range as that of CHO cells (4.2 μM). And Figure 6B shows that the $V_{max}$ GABA values of untreated Hek293 cells is 3.63 pmol μg protein$^{-1}$ min$^{-1}$, whereas the values of the cells with sialidase and NaIO4 treatment are strongly reduced to 1.22 and 1.02 pmol μg protein$^{-1}$ min$^{-1}$, respectively. However, either the $K_m$ GABA values of Hek293 cells treated with sialidase or NaIO4 (7.5 μM) did not increase comparing to that of untreated Hek293 cells (7.6 μM). These results suggest that a deficiency, removal and oxidation of terminal sialic acids decreased the $V_{max}$ GABA values without affecting the binding affinity of GAT1 to GABA.

Deficiency and removal of terminal sialic acid increased $K_m$ Na$^+$ values of GAT1.

Kinetic analysis was also performed by lowering sodium ion concentrations to further determine the influence of the terminal sialic acids of GAT1 on the affinity of GAT1 for Na$^+$. For solutions with reduced Na$^+$, the NaCl was replaced by KCl to create equal osmotic pressure. Sodium-dependent GABA uptake was thus measured with the lower concentrations of extracellular Na$^+$. Since the GAT1-dependent Na$^+$ uptake is proportional to GABA uptake at the ratio 2:1, the Na$^+$ uptake rate can be determined depending on the value of the GABA...
uptake rate. Figure 7 shows the GABA uptake rate of GAT1 is dependent on the extracellular Na⁺ concentration. The kinetic constants were calculated on the basis of the Michaelis–Menten equation by the double-reciprocal plot analysis. The $K_m$ Na⁺ values are 125 and 1000 mM for CHO and CHO Lec3 cells, respectively. The deficiency of terminal sialic acids on N-glycans in CHO Lec3 cells reduced the apparent affinity significantly (Figure 7A). In Hek293 cells, the $K_m$ Na⁺ value after the removal of cell surface sialic acids by sialidase treatment increased from 205 to 1180 mM, suggesting a reduction in the apparent affinity of GAT1 for Na⁺ (Figure 7B). This result suggests that the reduced GABA uptake activity can at least partially be attributed to a reduced apparent affinity of GAT1 for extracellular Na⁺ and slowed kinetics of the transport cycle.

Oxidation of terminal sialic acid did not change the $K_m$ Na⁺ value of GAT1
Above experiments show that oxidation of the terminal sialic acid of GAT1 reduced also significantly the GABA uptake activity of GAT1. However, kinetic analysis show that NaIO₄ treatment did not change the $K_m$ Na⁺ value indicating that the affinity of GAT1 to sodium ion is not affected by oxidation of sialic acids (Figure 7C). The modification with NaIO₄ remained the negative charge, but removed two terminal exocyclic carbon atoms from membrane sialic acid. This suggests that not only the negative charge, but the structure of sialic acid itself is directly involved in the regulation of the GABA turnover rate of GAT1.

Discussion
In this study, we demonstrated for the first time that the terminal sialic acids of GAT1 are essential for GABA uptake. Deficiency, removal or oxidation of surface sialic acid residues on N-glycans of GAT1/GFP, respectively, resulted in a strong reduction in GAT1 activity. Kinetic analysis revealed that the negative charge of the terminal sialic acids of GAT1 is responsible for the GABA uptake activity by the apparent affinity for extracellular Na⁺. Meanwhile, the disruption of the structure of sialic acids also strongly reduced $V_{\text{max}}$ without affecting both affinities of GAT1 to GABA and Na⁺.

Previously, we have shown the need of N-glycans for the biological significance of the membrane glycoproteins, e.g. dipeptidyl peptidase IV (Fan et al. 1997), as well as GAT1 (Cai et al. 2005), and demonstrated a deficiency of N-glycosylation brought about by site-directed mutagenesis results in a reduction in the GABA uptake activity of GAT1. The GAT1 mutants containing mutations in any two of the three N-glycosylation positions showed a significantly reduced GABA uptake activity, whereas GABA uptake activity could be hardly detected after all three N-glycosylation sites of GAT1 were mutated. These data suggest that the N-glycans of GAT1 play a crucial role in the GABA transport function of GAT1 (Cai et al. 2005). It has been demonstrated that purified GAT proteins can be functionally reconstructed into liposomes, which indicates that no other protein is needed for GABA uptake activity (Radian and Kanner 1985). Taken together, we suggest that the reduced GAT1 activity resulted from deficiency of its own N-glycosylation, but not due to other cell surface glycoconjugates. Further experiments demonstrated that the GABA uptake
uptake could also be reduced by treatment with 1-deoxynojirimycin (dMM), which inhibits N-glycosylation processing resulting in a mannose rich type of N-glycans. It suggests an involvement of the terminal structures, including sialic acid, of the N-glycans in the regulation of the GABA transport activity of GA T1. Kinetic analysis demonstrated that deficient N-glycan trimming decreased the $V_{\text{max}}$ values of GABA uptake by GA T1, whereas the $K_m$ GABA values were not affected (Cai et al. 2005). It is indicated that the turnover rate of the transporter is affected, but not the substrate-binding process. Voltage-clamp experiments revealed that the deficiency of N-glycans leads to the reduction in the affinity of GA T1 to Na$^+$ (Liu et al. 1998; Cai et al. 2005). In this event, the oligosaccharides of GA T1 play a pivotal role in the regulation of GABA uptake activity by affecting the affinity with sodium ions.

It is widely known that the oligosaccharides turn into hybrid or complex forms after N-glycan trimming. Sialylation, which occurs on glycolipids and N- and O-glycans, stands out among the terminal positions of the glycan chains. Sialic acids are negatively charged and are usually the terminal sugar residues on the oligosaccharide chains of cell surface or serum glycoconjugates, where they play an important role in key cellular and molecular interactions (Schauer 2009). It was reported that sialic acid is directly involved in the symport transport of amino acid transmitters (Zaleska and Erecinska 1987). In this work, the role of sialic acids terminated N-glycans in the GABA transport by GA T1 was further revealed. In our studies, GFP-tagged GA T1 protein and anti-GFP antibody were used for quantitative determination of the expression of GA T1/GFP fusion protein, since both our own and commercially available anti-GA T1 antibodies were not suitable due to their weak and unstable binding activity to the protein. Meanwhile, GFP tag has been reported not to influence the intracellular distribution and characteristic function of GA T1 (Chiu et al. 2002). For the quantitative analysis of terminal sialic acid concentration and GABA transport activity, the cell surface expression of GA T1 in different cell lines was determined by cell surface biotinylation and the resulting values were used for normalization. This is a well-established method for the quantitative analysis of cell surface proteins since the biotinylation reagent does not penetrate the cell membrane (Law et al. 2000).

First, quantitative analysis was performed in stably transfected CHO and CHO Lec3 cells with GA T1/GFP fusion protein. CHO Lec3 cells are deficient in sialic acid residues on cell surface glycoproteins due to mutations in the GNE gene, which abolished UDP-GlcNAc 2-epimerase activity (Hinderlich et al. 2001; Hong and Stanley 2003). These cells were cultured in a serum-free medium to prevent sialic acid incorporation on glycoproteins during experiments. The expression of GA T1 protein and the sialic acid, as well as the GABA uptake activity of GA T1 were quantified in both CHO and CHO Lec3 transfectants. We found that GA T1/GFP expressed on the cell surface in CHO Lec3 was in the same range as in CHO cells, however, after normalization the GABA uptake activity of GA T1 in CHO Lec3 cells decreased strongly with a reduced amount of cell surface sialic acid (Figure 2). These results suggest that the reduced sialic acid concentrations on GA T1 resulted in the decrease in GABA uptake activity by GA T1 in CHO Lec3 cells.

It is not clear whether the reduction in GA T1 activity in CHO Lec3 cells is resulted from the change in the biochemical properties or other interference of the mutation. In order to exclude this possibility, further experiments were performed with sialidase to simply remove the sialic acids from cell surface sialoglycoconjugates, including GA T1 protein. Our results showed that GA T1 expressed both in CHO and Hek293 cells displayed a significant reduction in GABA uptake activity after sialidase treatment in a dose-dependent manner (Figure 3). In order to quantitatively analyze the sialic acids of GA T1 and total and plasma membrane GA T1 protein, a certain amount of GA T1 is necessary for immunoprecipitation, immunoblot and lectin staining. The higher expression of GA T1 on the cell surface requires fewer cells to be used and thus, less sialidase for the analysis. Therefore, Hek293 transfectants were used for further quantification analysis due to their high expression levels of GA T1/GFP protein on the surface. Figure 4 showed that after treatment of the Hek293/GA T1 cells with 1 U/mL of sialidase for 4 h, almost 80% of sialic acid was removed, and the GABA uptake activity was correlative reduced to ~22% (Figure 4). This result indicates directly that the reduced GABA uptake activity is directly attributed to the reduction in sialic acid on the N-glycans.

It has been demonstrated that the GABA transport process is driven by the gradient of Na$^+$ and Cl$^-$ with a stoichiometry that results in an electrogenic substrate transport. The affinity of GA T1 to Na$^+$ determines the turnover rate of GABA transport (Nelson 1998). Furthermore, voltage-clamp experiments revealed that a deficiency in the terminal part of the N-glycans of GA T1 by mutations did not affect the affinity of GA T1 to GABA, whereas reduced significantly the affinity of GA T1 to extracellular sodium ions (Cai et al. 2005). Our kinetic analysis with different GABA concentrations shows that deficiency or oxidation of sialic acids on N-glycans decreased the $V_{\text{max}}$ values of GABA uptake by GA T1, whereas the $K_m$ GABA values were not affected. This result is consistent with our former findings with deficient N-glycosylation and dMM treatment. It is clear that the deficient cell surface sialic acid reduces the GABA uptake turnover rate, however, has no influence on the GABA binding.

It is well known that sialic acids are bulky hydrophilic and electronegatively charged monosaccharides on animal cells, which favor the affinity to inorganic cations. In addition to their negative charge, sialic acids also make a contribution to recognition of the variety of ligands, such as hormones, lectins, antibodies and microorganisms (Schauer 2009). In order to clarify whether sialic acids are involved in the GA T1 activity based on only their negative charge, we determined the influence of oxidation of surface sialic acids on the GABA uptake activity by NaIO$_4$. NaIO$_4$ treatment of GA T1/GFP stably transfected Hek293 cells led to the oxidation of the cell surface sialic acid residues with formation of 7- or 8-aldehydic derivative that remains acidic groups (Perez et al. 1985). Thus, the electronegative charge of GA T1 was not impaired. However, the GABA uptake activity of GA T1 was inhibited as a consequence of a direct effect of the removal of
the two terminal exocyclic carbon atoms from membrane sialic acid with NaIO₄. After oxidation, it is possible that the free aldehyde form Schiff base with available amino groups which thus cause inactivation of GA T₁. In order to exclude this possibility, NaBH₄ treatment was further performed to convert aldehyde to alcohol so that the Schiff base structure could not be formed. Furthermore, the GABA uptake activity of GA T₁ remained the low level as that of cells treated only with NaIO₄. It suggests that the unique structure of sialic acid may be the essential component of GA T₁ in GABA uptake process.

To further clarify the molecular mechanism of the sialic acid in the GABA uptake by GA T₁, the affinity of GA T₁ for sodium ion was measured. The CHO Lec3 cells and sialidase-or NaIO₄-treated Hek293 cells are too fragile for the patch-clamp methods. Since the GA T₁-dependent Na⁺ transport is proportional to GABA transport in the ratio 2:1, sodium-dependent GABA uptake was kinetic analyzed with different sodium ions concentrations. Sodium ions were replaced with potassium ions in the buffer to compensate the loss of osmotic pressure, and the GABA uptake activities were normalized to the plasma membrane expressed GA T₁ amounts.

We found that both deficiency (CHO Lec3) and removal of sialic acids (by sialidase) reduced the affinity of GA T₁ for sodium ions significantly, which is in agreement with our former results of patch-clamp experiments with deficient N-glycosylation of GA T₁. It is reported that the removal of the surface sialic acids affected only the Na⁺-dependent transport of amino acids but not the uptake of leucine, which is Na⁺-independent (Zaleska and Erecinska 1987). Our kinetic data provide strong evidence that the absence of sialic acids reduces the affinity of GA T₁ for Na⁺; consequently, the GABA transport activity of GA T₁ is reduced. However, the oxidation of sialic acid by NaIO₄ does not affect the affinity of sodium ion for GA T₁, whereas the oxidized residues still carry negative charge after structural disruption of sialic acids. It suggests that the negative charge of sialic acids is not the only factor involved in the GABA transport by GA T₁. The structure of sialic acids on N-glycans of GA T₁ protein itself is required for the GABA uptake activity of GA T₁.

The involvement of sialic acid can be further explained with a widely accepted theory of GA T₁-mediated GABA transport, an alternating access mechanism, in which conformational changes alternate to expose a central binding site for one or more substrates to either side of the membrane (Jardetzky 1966). An alternating access transport model was developed in Xenopus oocyte membranes. The model assumes two predominantly states of GA T₁, Eᵢᵣ and Eᵢᵤ (Hilgemann and Lu 1999), and the complete transport cycle is proposed in four steps (Kanner and Zomot 2008). Our results can be well-interpreted within this model. We assumed that the negative charge of sialic acid influences the binding of GABA and Na⁺ with GA T₁, whereas the structure of sialic acid is involved in the conformational changes of GA T₁ during the translocation cycle. The impaired structure of sialic acids could disturb the correct conformational changes of GA T₁, which results in a blocked GABA translocation.

In conclusion, deficiency in sialic acid biosynthesis leading to a decreased sialic acid concentration, enzymatic removal or chemical oxidation of sialic acids results in reduction in the GABA uptake activity of GA T₁. In consideration of the previous results of GA T₁ N-glycosylation mutants (Cai et al. 2005) and functional reconstruction of purified GA T₁ protein (Radian and Kanner 1985), we conclude that the reduction in GA T₁ activity specifically due to the removal or oxidation of terminal sialic acids in the N-glycans of GA T₁, but not the lack/oxidation of sialic acid of other surface glycoconjugates. Reduced GABA transport activity caused by defective sialic acids can be partially attributed to a reduced affinity of GA T₁ to Na⁺ and slowed kinetics of the transport cycle. Furthermore, not only is the negative charge involved, but also the unique structure of sialic acid itself is crucial for the GABA uptake process. It also brings out the possibility that terminal sialic acid of GA T₁ is involved in two main steps of the GABA transport mechanism: substrate and Na⁺ binding and translocation.

Materials and methods

DNA construct

The GA T₁/GFP fusion protein was constructed as described previously (Cai et al. 2005).

Cell culture and transfection

CHO and Hek293 cell lines were obtained from ATCC (USA) and CHO Lec3 cell line was kindly provided by Dr. Pamela Stanley. CHO and CHO Lec3 cells were maintained in alpha-modified Eagle’s medium (MEM alpha) with 10% fetal bovine serum and 100,000 U L⁻¹ of penicillin/streptomycin. Hek293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 100,000 U L⁻¹ of penicillin/streptomycin. The stable transfections of GA T₁/GFP fusion protein were performed using SuperFect (Qiagen, Hilden, Germany) as reagents according to the protocols of Qiagen. After 48 h transfection, the transfectants were selected with 600 mg L⁻¹ of geneticin (G418; Biochrom) for 1–3 weeks. The stable transfectants expressing GA T₁/GFP fusion proteins were selected by flow cytometry with the FACS Vantage cell-sorter (Becton Dickinson, Erembodegem, Belgium) and then cultured with 400 mg L⁻¹ of G418. For CHO Lec3 cells, serum-free growth was accomplished by gradually reducing the fetal calf serum concentration to zero.

Flow cytometry and fluorescence microscopy

CHO, CHO Lec3 and Hek293 transfectants were monitored by flow cytometry and fluorescence microscopy for cell surface expression of GA T₁/GFP fusion proteins.

Immunoprecipitation and western blotting

Solubilization of GA T₁/GFP protein was performed at 4°C or on ice. About 10⁷ cells were collected, washed once with phosphate-buffered saline (PBS), and resuspended in tris-buffered saline (TBS) buffer (50 mM Tris, pH 7.3, 150 mM NaCl). Suspected cells were sonicated at 4°C for 15 min. Cell debris was removed by centrifugation at 6000 × g for 10 min at 4°C, and a turbid supernatant solution containing cell membrane was obtained. After centrifugation at 100,000 × g
at 4°C for 30 min, the crude membrane fractions were solubilized in TBS containing 1% n-dodecyl-β-d-maltoside and stirred for at least 4 h at 4°C. The lysate was centrifuged at 18,000 × g at 4°C for 1 h. Total protein concentrations of the supernatant were measured with BCA™ Protein Assay Kit (Thermo). Quantified aliquots of the supernatants were incubated with protein-G-Sepharose-bound anti-GFP IgG for 12 h at 4°C. After intensive wash, immunoprecipitates were eluted by boiling for 3 min in SDS sample buffer. The aliquots of supernatant were divided into half and then subjected to SDS/PAGE according to Laemmli (1970), and the separated proteins were transferred to a nitrocellulose membrane (Millipore) by western blotting. One blot membrane was used for immunostaining of GA T1 protein with the anti-GA T1 or anti-GFP polyclonal antiserum. Subsequently, blots were incubated with horseradish peroxidase–conjugated anti-rabbit (IgG) (DakoCytomation) and then visualized using AEC and substrate buffer (Calbiochem).

**Determination of sialic acid of GAT1/GFP**

The other blot membrane was obtained as described in section ‘immunoprecipitation and western blotting’ and then stained with MAA (DIG Glycan Differentiation Kit, Roche) to detect sialic acids α2,3-linked to galactose of GAT1/GFP. The staining procedure was done according to the manufacturer.

**Cell-surface biotinylation**

The biotin-labeling reaction was performed according to manufacturer’s protocol (Pierce). At least 5 × 10⁶ cells were washed three times with cold PBS (pH 8.0) and then suspended in a freshly prepared solution of Sulfo-NHS-LC-Biotin (1.5 mg mL⁻¹ of 10 mM Hepes buffer pH 9.0, 2 mM CaCl₂, 150 mM NaCl) at room temperature for 30 min. The cells were washed three times with 100 mM glucose. Cell lysate was then incubated with 60 µL of streptavidin agarose beads (Pierce) at 4°C overnight. After an extensive wash, the membrane proteins attached to beads were boiled for 4 min in SDS sample buffer, and then subjected to western blotting analysis.

**Sialidase treatment**

Transfected CHO and Hek293 cells were incubated with different concentrations of sialidase in MEM alpha (pH 5.5) and DMEM (pH 5.5) for 4 h at 37°C, respectively. Then aliquots of cells were used for measurement of [³H] GABA uptake and the rest of cells were solubilized for immunoprecipitation, western blotting and glycan differentiation analysis.

**Sodium periodate treatment**

Transfected Hek293 cells (2 × 10⁶ cells mL⁻¹) suspended in PBS were incubated with NaIO₄ (1 mM) on ice for 15 min. Then, aliquots of cells were used for measurement of [³H] GABA uptake, and the rest of cells were solubilized for immunoprecipitation, western blotting and glycan differentiation analysis.

**Mild NaBH₄ reduction**

Following periodate oxidation, aliquots of cells were treated by NaBH₄ to reduce the aldehydes generated by periodate. After two washes in cold PBS, periodate-treated cells were resuspended in PBS with 10 mM freshly prepared NaBH₄ at room temperature for 20 min, washed in cold PBS, and then subjected to the GABA uptake assay.

**Measurement of [³H] GABA uptake**

[³H] GABA uptake assays were performed as described previously (Cai et al. 2005). Cells were washed three times with wash buffer (128 mM NaCl, 5.2 mM KCl, 2.1 mM CaCl₂, 2.9 mM MgSO₄, 5 mM dextrose and 10 mM Hepes, pH 7.4) and then incubated with 200 µL of wash buffer containing 3.7 × 10⁴ Bq [³H] GABA (Perkin Elmer), 10 µM cold GABA, 3.7 × 10⁴ Bq [¹⁴C] sucrose (Perkin Elmer) and 100 µM cold sucrose for 15 min at room temperature. The uptake was stopped by washing cells three times with cold wash buffer, followed by solubilization of the cells with 100 µL of 0.5% (w/v) SDS solution for 1 h at 4°C.

Aliquots were used for measurement of the remaining [³H] GABA and [¹⁴C] sucrose. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay reagent (Pierce). The GABA uptake activity was measured as pM µg protein⁻¹ min⁻¹.

**Quantitative analysis**

Stably transfected cells were separated in aliquots for immunoprecipitation with anti-GFP mAb Igs, cell surface biotinylation and GABA uptake assay. Aliquots of each immunoprecipitate and biotinylation probes were analyzed by SDS/PAGE (7.5%) and then transferred onto the nitrocellulose membrane by western blotting followed with immunostaining and lectin staining as described above. The immunoblots and MAA stained blots were analyzed by imager scanning and both total and plasma membrane GAT1/GFP protein amounts and sialic acid concentrations were further quantified according to the band densities using Quantity One software (Bio-Rad). The relative sialic acid concentration and the GABA uptake activity were normalized to the amount of plasma membrane GAT1. All the values of transfected CHO or untreated Hek293 cells were set at 100%. All other values were expressed relative to these values.

**Statistical analysis**

Data were from three or more separate experiments and were expressed as mean ± SD. P-values were calculated with paired, two-tailed Student’s test.

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Conflict of interest statement
None declared.

Abbreviations
CHO, Chinese hamster ovary; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; dMM, 1-deoxynojirimycin; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GNE, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosaminidase; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GNE, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosaminidase; Hek293 cell, human embryonic kidney 293 cell; MAA, Maackia amurensis agglutinin; MEM, modified Eagle's medium; NaBH₄, sodium borohydride; NaIO₄, sodium periodate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SNA, Sambucus nigra agglutinin; TBS, tris-buffered saline; UDP-GlcNAc, uridine diphosphate-N-acetylglycosamine.

References
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