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Distinct contributions of β4GalNAcTA and β4GalNAcTB to *Drosophila* glycosphingolipid biosynthesis

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Abstract *Drosophila melanogaster* has two β4-*N*-acetylgalactosaminyltransferases, β4GalNAcTA and β4GalNAcTB, that are able to catalyse the formation of lacdiNAc (GalNAcβ,4GlcNAc). LacdiNAc is found as a structural element of *Drosophila* glycosphingolipids (GSLs) suggesting that β4GalNAcTs contribute to the generation of GSL structures *in vivo*. Mutations in Egghead and Brainaic, enzymes that generate the β4GalNAcT trisaccharide acceptor structure GlcNAcβ,3Manβ,4GlcβCer, are lethal. In contrast, flies doubly mutant for the β4GalNAcTs are viable and fertile. Here, we describe the structural analysis of the GSLs in *β4GalNAcT* mutants and find that in double mutant flies no lacdiNAc structure is generated and the trisaccharide GlcNAcβ,3Manβ,4GlcβCer accumulates. We also find that phosphoethanolamine transfer to GlcNAc in the trisaccharide does not occur, demonstrating that this step is dependent on prior or simultaneous transfer of GalNAc. By comparing GSL structures generated in the *β4GalNAcT* single mutants we show that β4GalNAcTB is the major enzyme for the overall GSL biosynthesis in adult flies. In *β4GalNAcTA* mutants, composition of GSL structures is indistinguishable from wild-type animals. However, in *β4GalNAcTB* mutants precursor structures are accumulating in different steps of GSL biosynthesis, without the complete loss of lacdiNAc, indicating that β4GalNAcTA plays a minor role in generating GSL structures. Together our results demonstrate that both β4GalNAcTs are able to generate lacdiNAc structures in *Drosophila* GSL, although with different contributions *in vivo*, and that the trisaccharide GlcNAcβ,3Manβ,4GlcβCer is sufficient to avoid the major phenotypic consequences associated with the GSL biosynthetic defects in *Brainiac* or *Egghead*.

Keywords *Drosophila* glycosphingolipid · lacdiNAc · Brainiac · Egghead · Glycosyltransferase

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Introduction

The *Drosophila melanogaster* genome encodes three members of the β1,4galactosyltransferase (β4GalT) family [1]. In mammals seven β4GalT homologs exist; six of these catalyse the formation of lacNAc (Galβ,4GlcNAc) on various glycolipid and glycoprotein acceptor structures [2, 3]. The remaining galactosyltransferase acts on xylose residues and is involved in glycosaminoglycan linker

region biosynthesis [4]. An ortholog of this enzyme catalyses the same reaction in *Drosophila* [5, 6]. The two other *Drosophila* β4GalT family members have no correlation to a specific mammalian enzyme and have been shown to encode *N*-acetylgalactosaminyltransferases (GalNAcTs) that synthesise the lacdiNAc (GalNAcβ,4GlcNAc) structural element [1, 7, 8]. Both enzymes are typical type II transmembrane proteins, but only β4GalNAcTA has been found to have clear activity *in vitro* [1, 7]. β4GalNAcTB, on the other hand, requires a cofactor for optimal activity [8] (manuscript in preparation).

The lacdiNAc structural element is found in mammalian glycans, but restricted to a very limited number of proteins and synthesised by protein specific GalNAc transferases [9] only distantly related to the β4GalT family [10, 11]. In invertebrates, lacdiNAc is more abundant and found on both glycoproteins and glycolipids [12]. In *Caenorhabditis elegans*, both glycoproteins and glycosphingolipids (GSLs) carry the disaccharide structure [13, 14], which is synthesised by Ceβ4GalNAcT, able to catalyse the transfer of GalNAc on both types of glycoconjugates [15]. Several insect species have lacdiNAc containing N-glycans [16, 17] and enzymes able to act on glycoprotein acceptors have been identified [18, 19]. In contrast, in *Drosophila*, lacdiNAc has, despite intensive analyses of glycoproteins [20], only been found on GSLs of the arthro-series [21], which represent the common glycolipid series of arthropods and nematodes. These GSLs are characterised by a core structure with mannose linked to glucosylceramide [22, 23]. LacdiNAc occurs in the initial GSL structure, GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer and can also be found in more elongated structures [21].

The biological function of glycolipids in *Drosophila* has been demonstrated by mutants lacking the mannosyltransferase (*egghead*, *egh*) or the GlcNAc transferase (*brainiac*, *brn*) [24–27]. These mutants have very similar lethal developmental phenotypes and show defects in epithelial morphogenesis during oogenesis and embryogenesis [28, 29]. In contrast to this, mutants in the β4GalNAc transferases, show non-lethal, rather mild behavioural and morphological phenotypes, differing for the two transferases [1, 30, 31]. *Drosophila* mutants for *β4GalNAcTA* display an abnormal locomotion phenotype, indicating a role for this enzyme in the neuromuscular system [1, 31], whereas a small proportion of homozygous *β4GalNAcTB* mutant flies exhibit abnormal oogenesis due to defective epidermal growth factor receptor signalling between the oocyte and follicle cells [30]. Flies doubly mutant for the *β4GalNAcTs* are viable [1] and these flies can be maintained as a homozygous stock over many generations indicating fertility is not significantly compromised (N. Haines and K.D. Irvine, unpublished). The lethality of *egh* and *brn* mutants compared to the viability of *β4GalNAcT* double mutants suggests two alternative possibilities: that the essential functions of GSLs can be fulfilled by the trisaccharide GSL structure, or that the β4GalNAcTs do not function in GSL synthesis and that this role is carried out by additional uncharacterized enzymes.

To resolve this issue and to determine the contribution of the two different β4GalNAcTs for extending the GSL trisaccharide in *Drosophila* we have carried out an analysis of GSL structures generated in the single and double *β4GalNAcT* mutants. We find that in double mutants no GSLs larger than the trisaccharide product of Brainiac are synthesised, demonstrating that the β4GalNAcTs are indeed required for GSL synthesis *in vivo*. We find that β4GalNAcTB is the prominent enzyme for lacdiNAc formation on glycolipids: the *β4GalNAcTB* mutant shows a reduction in lacdiNAc containing structures with the accumulation of GalNAc transferase acceptor structures. In contrast, the GSL profile of the *β4GalNAcTA* mutant is essentially identical to that of the wild-type flies.

Material and methods

Extraction, purification and preparation of *Drosophila* GSLs

Drosophila melanogaster flies of wild-type strain (Oregon R) and knock out strains *β4GalNAcTA*$^{4.1}$, *β4GalNAcTB*^{GT} and the double mutant *β4GalNAcTA*$^{4.1}$; *β4GalNAcTB*^{GT} [1] were collected and frozen. 1,5 g of frozen material were extracted by the method of Folch [32]. Therefore, the flies were disrupted in a Dounce homogenizer in 3 vol (4 ml per g wet weight) of ice-cold, deionized water. After sonification of the suspension 4 vol of methanol were added and again homogenized, followed by the addition of 8 vol of chloroform, homogenization and sonification. The 8:4:3 (chloroform/ methanol/ water) extract was vigorously shaken and then centrifuged to remove insoluble material. After centrifugation the upper phase was collected, dried under a nitrogen-stream and re-dissolved in 3:47:48 (chloroform/methanol/water). Salt and hydrophilic contaminants were removed from extractions by reverse-phase chromatography (Sep-Pak® Plus C_{18} columns, Waters Corporation, Milford, MA, USA) [33]. The column was equilibrated with 5 column volume of 3:47:48 and the sample was applied. Subsequently, the column was washed twice with 10 column volumes of water. The glycolipids were eluted with 10 column volumes of 10:10:1 and dried under a nitrogen-stream. For further analysis the samples were dissolved in chloroform/methanol/water (30:60:8).

High-performance thin-layer chromatography

Glycolipid preparations corresponding to 50 mg of flies were spotted onto nanosilica-gel 60 plates (Nano-Durasil-20, Macherey-Nagel, Düren, Germany) and developed in running solvent composed of chloroform/methanol/0.25% aqueous KCl (5:4:1). GSLs were visualised chemically by 0.5% orcinol(w/w)/62.5% methanol/10% H_2SO_4-staining.

Mass spectrometry

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of extracted GSLs was performed on an Ultraflex II TOF/TOF MS (Bruker Daltonics, Bremen, Germany) in the reflector negative-ion mode using 2,5-dihydroxybenzoic acid (20 mg/ml in 30% acetonitrile; Bruker) as matrix and in the reflector positive-ion mode using 6-aza-2-thiothymine (5 mg/ml in water; Sigma) as matrix for sample preparation. Fragment-ion spectra were acquired by laser-induced decay in the LIFT mode as described previously [34].

Results

Small glycospingolipids accumulate in the *β4GalNAcTB* and *β4GalNAcT* double mutant

GSLs (Folch upper phases) from wild-type, *β4GalNAcTA* and *β4GalNAcTB* mutants, as well as the double mutant (*β4GalNAcTA*; *β4GalNAcTB*) were analyzed by high-performance thin-layer chromatography (HPTLC) using orcinol/sulfuric acid staining (Fig. 1). The extracts of wild-type flies and *β4GalNAcTA* mutants show similar staining patterns (Fig. 1, lanes 2 and 3), indicating that loss of this gene does not result in a significant change in overall GSL composition. The mutation of *β4GalNAcTB*, on the other hand, resulted in a change of the GSL pool with decreased staining intensity of the high molecular weight GSL species as compared to wild-type and the *β4GalNAcTA* mutant. Additionally, bands with higher mobility, probably representing smaller glycolipid structures, became visible (marked with arrows; Fig. 1 lane 4). The double mutant showed a similar profile to the *β4GalNAcTB* single mutant. The larger GSLs were no longer visible and the smaller structures appeared to be the most prominent GSL species (marked with arrows; Fig. 1 lane 5). Based on the mobility of G_{M1}, the upper most band in the *β4GalNAcTB* and double mutants could represent the trisaccharide precursor of GalNAc transferases. No conclusions on the exact structures accumulating in the different mutants can be made from the TLC staining pattern, but mutation of *β4GalNAcTA* seems to have no significant effect on the glycolipid composition, whereas in the *β4GalNAcTB* and double mutant detectable changes occur, indicating accumulation of smaller glycolipid structures.

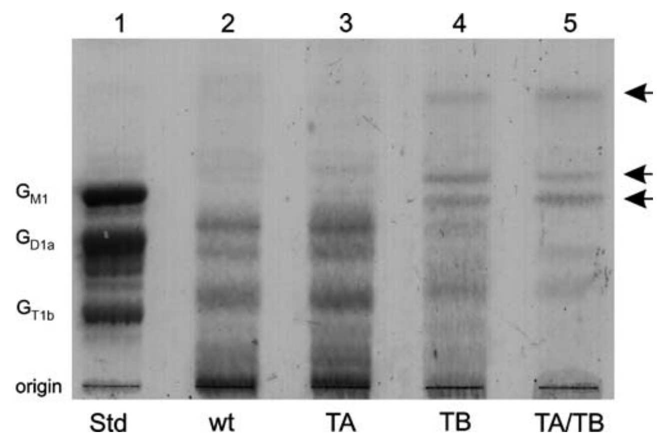

Fig. 1 HPTLC analysis of GSLs from *Drosophila*. GSLs of adult flies were extracted using the method of Folch, and upper phases were resolved by HPTLC. *Lane 1*: type III gangliosides from bovine brain as references, *lane 2*: wild-type, *lane 3*: *β4GalNAcTA* mutant, *lane 4*: *β4GalNAcTB* mutant and *lane 5*: *β4GalNAcTA$^{4.1}$*/*β4GalNAcTB^{GT}* double mutant. The *arrows* point at structures appearing in the *β4GalNAcTB* and double mutant. Based on the relative position to G_{M1} the upper band runs at a position matching the trisaccharide precursor structure expected to accumulate in *β4GalNAcT* mutants

GSL structures from wild-type and *β4GalNAcTA* mutant are identical

To resolve the structures of the different glycolipids synthesised by the different mutants and wild-type flies, GSLs were analysed by MALDI-TOF-MS in negative-ion mode (Fig. 2) and positive-ion mode (Fig. 3). By negative-mode MALDI-TOF-MS, only the PE-modified and glucuronic acid-modified GSLs were registered. In positive-mode, neutral GSLs as well as zwitterionic and acidic GSLs were detected as sodium and/or potassium adducts. Together with the ceramide heterogeneity, this resulted in multiplets of signals for GSLs sharing the same oligosaccharide moiety.

As with HPTLC, the comparison of wild-type and *β4GalNAcTA* mutant GSL patterns did not indicate any significant changes. The major GSL signal was detected at *m/z* 1592.9 (Fig. 2) and corresponded to the $[M-H]^-$ ion of the ceramide pentahexoside modified with phosphoethanolamine (PE), which was corroborated by MALDI-TOF/TOF-MS (Fig. 4a). The fragment ions at *m/z* 731.0 (B_3-ion, according to the nomenclature of Domon and Costello [35]) and *m/z* 1186.5 (Y_3-ion) indicate the attachment of the PE to the innermost *N*-acetylhexosamine. The GSL mass is consistent with a ceramide composition of C14:1 tetradecasphingenine and C20:0 arachidic acid, which is the major ceramide found in insect GSLs [21, 22]. Next to this major ceramide species, the complex GSLs exhibited ceramides with a 28 Da lower mass, which presumably corresponds to ceramides with two methylene groups (C_2H_4) less (*m/z*

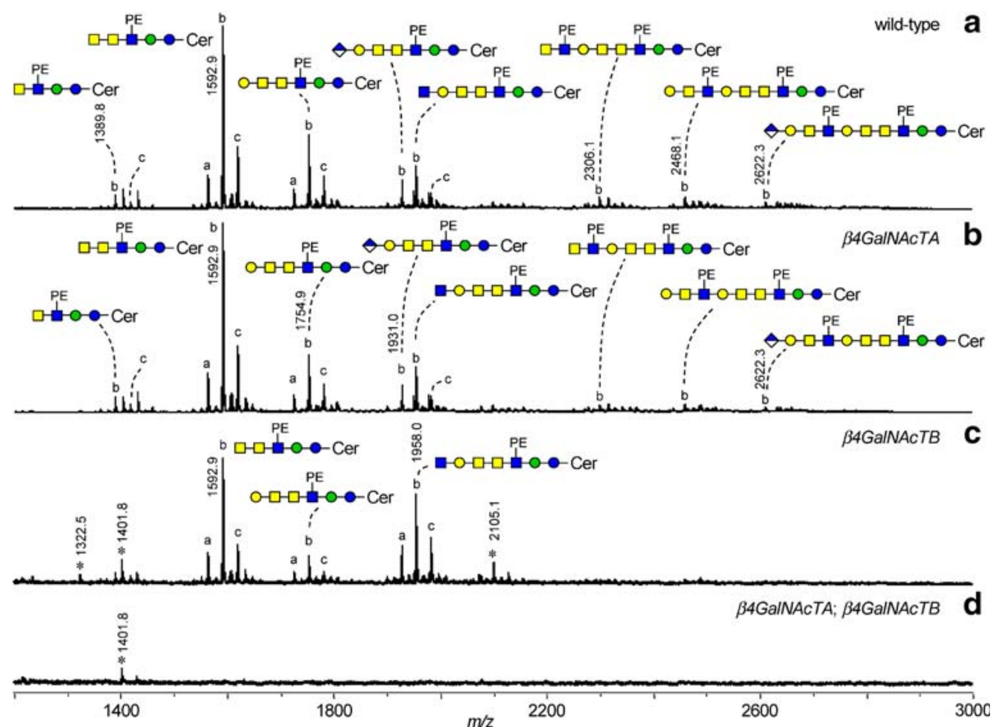

Fig. 2 Negative-mode MALDI-TOF-MS of glycolipids from *Drosophila* wild-type and mutants. Folch upper phases were analyzed by negative-mode MALDI-TOF-MS in the reflectron mode using 2,5-dihydroxybenzoic acid matrix for sample preparation. A more complete list of registered masses and assigned glycolipid structures is given in Table 1. **b** GSL with ceramide composition of C14:1 tetradecasphingenine and C20:0 arachidic acid; **a**, **c** GSL with ceramide mass which is 28 Da (2 methylene groups, C_2H_4) lower (**a**) or higher (**c**) than for **b**; *blue circle*, glucose; *green circle*, mannose; *yellow circle*, galactose; *yellow square*, *N*-acetylgalactosamine; *blue square*, *N*-acetylglucosamine; *white/blue diamond*, glucuronic acid; *Cer*, ceramide; *asterisk*, no GSL

1564.8 for PE-containing ceramide pentahexoside; Fig. 2; Table 1). A further ceramide species showed a 28 Da higher mass, which is expected to reflect two additional methylene groups (*m/z* 1620.9; Table 1).

The pattern of zwitterionic, PE-containing GSLs detected was largely in accordance with the results of Seppo *et al.* for GSL species from *Drosophila* embryos [21], as summarized in Table 1. In addition to the described

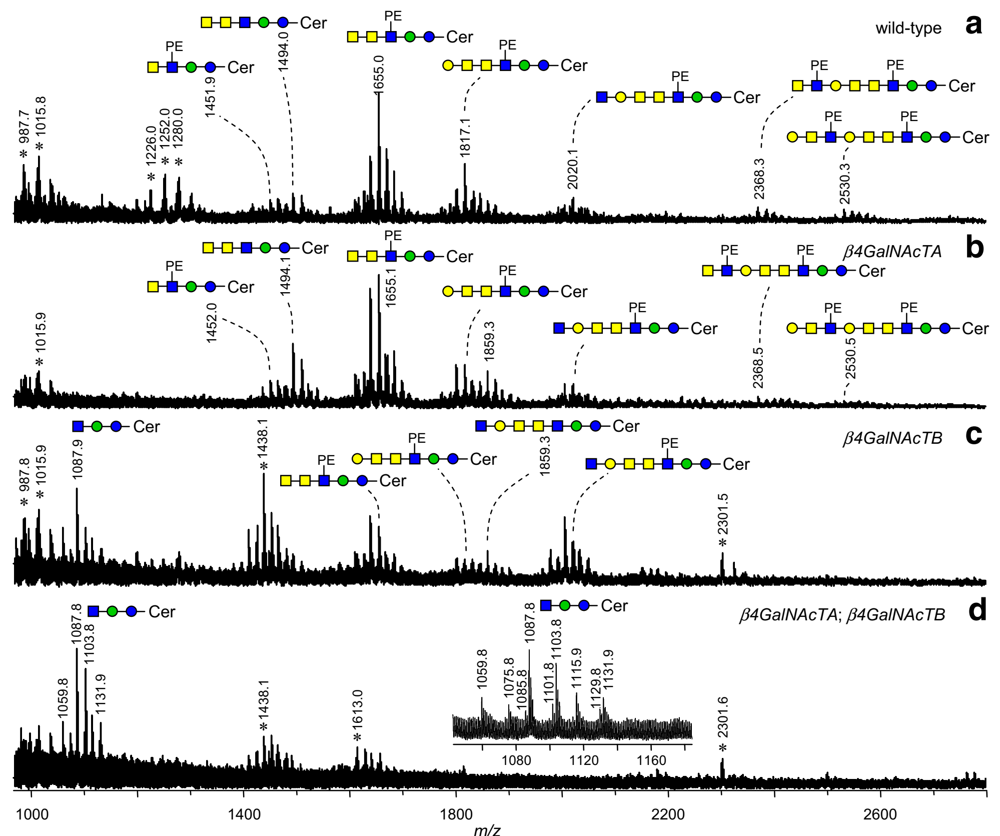

Fig. 3 Positive-mode MALDI-TOF-MS of glycolipids from *Drosophila* wild-type and mutants. Folch upper phases were analyzed by positive-mode MALDI-TOF-MS in the reflectron mode using 6-aza-2-thiothymine matrix for sample preparation. A list of registered masses for assigned neutral glycolipid structures is given in Table 1. For key see legend Fig. 2. **a** wild type, **b** *β4GalNAcTA*, **c** *β4GalNAcTB*, **d** *β4GalNAcTA*; *β4GalNAcTB*

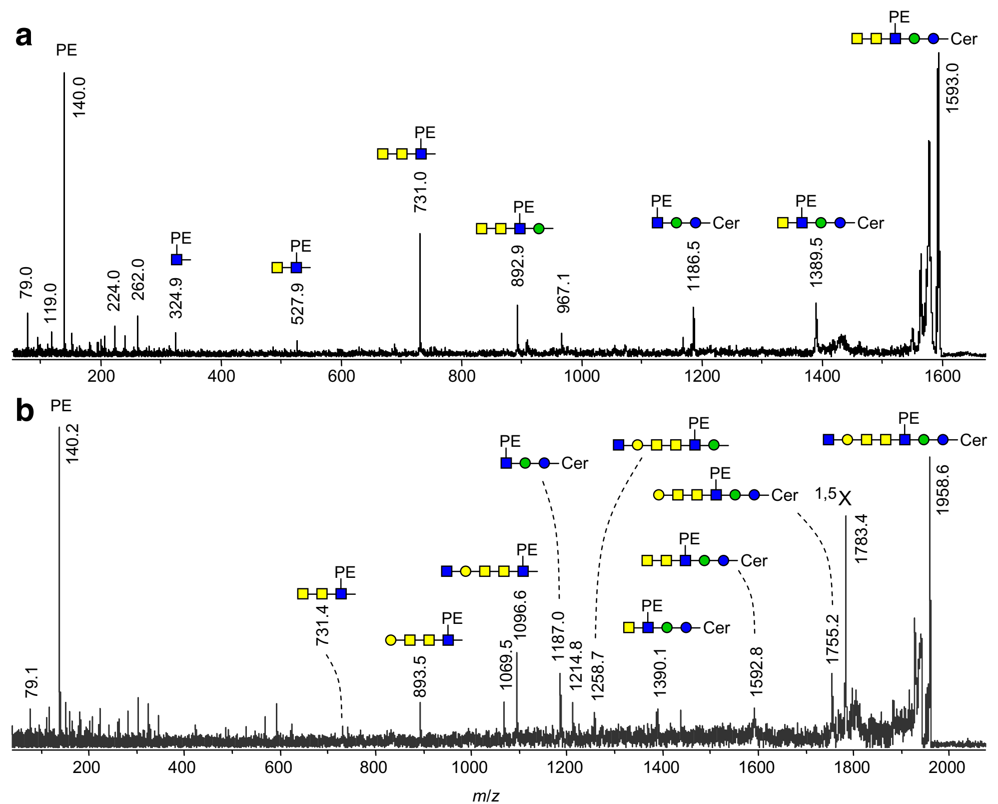

Fig. 4 MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species. Zwitterionic glycolipid species **a** with a pentasaccharide glycan moiety (*m/z* 1592; from wild-type), and **b** with a heptasaccharide glycan moiety (*m/z* 1958; from the *β4GalNAcTA* mutant) were analyzed by MALDI-TOF/TOF-MS in deprotonated form using 2,5-dihydroxybenzoic acid as a matrix. For key see legend Fig. 2. $^{1,5}X$, cross-ring cleavage of the terminal *N*-acetylglucosamine

Nz_28 which exhibits two PE moieties [GalNAcβ,4(PE-6) GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6) GlcNAcβ,3Manβ,4GlcβCer], a possible variant of this structure exhibiting an additional hexose was detected. This latter GSL species (Nz_29) has not been described before in *Drosophila*. Nz_28 and Nz_29 were analyzed by MALDI-TOF/TOF-MS and fragmentation spectra were interpreted in a comparative manner in order to obtain

Table 1 Registered GSLs species

GSL	Structure	Registered mass (*m/z*)
Zwitterionic		
Nz4	GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1389.8
Nz5	GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1592.9
Nz6	Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1754.9
Nz7	GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1958.0
Nz_27	(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2081.0
Nz8	GalNAcβ,4GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2161.1
Nz_28	GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2284.1
Nz_29	GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4(Hex1-)GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2446.2
Acidic		
Az6	GlcAβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1931.0
Az9	GlcAβ,3Galβ,3GalNAcβ,4GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ, 4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2499.2
Az_29	GlcAβ,3Galβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ, 4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2622.3
Neutral		
N3	GlcNAcβ,3Manβ,4GlcβCer	1087.9
N5	GalNAcα,4GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer	1494.1
N7	GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer	1859.3

Zwitterionic and acidic GSLs are listed as $[M-H]^-$ species and neutral GSLs as $[M+Na]^+$. Various other adducts were registered, which are not listed in this table

information on the position of the additional hexose (Fig. 5). The non-reducing end fragments observed in the region below *m/z* 1000 were largely the same for the two GSLs, indicating that the hexose modification was not attached to the terminal GalNAcβ,4(PE-6)GlcNAcβ, 3Galβ,3GalNAc-moiety. Two ions at *m/z* 1258.4 and *m/z* 1461.3 were observed for the hexose-modified GSL (Nz_29; Fig. 5a), but not for Nz_28 (Fig. 5b), which exhibited corresponding ions of a 162 Da lower mass at *m/z* 1096.9 and *m/z* 1299.9. This difference indicated the attachment of the hexose residue to the internal β-linked GalNAc residue, resulting in the Nz_29 structure as listed in Table 1.

Besides the zwitterionic GSLs with PE-moieties, wild-type and *β4GalNAcTA* mutant exhibited acidic and neutral GSLs, which were likewise analyzed by MALDI-TOF/TOF-MS (data not shown). A group of glucuronic acid-containing GSLs were detected (Table 1), which is in accordance with previous results for *Drosophila* and other dipterans [21, 22]. Furthermore, the neutral GSLs GalNAcα,4GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer (N5) and GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4GlcNAcβ, 3Manβ,4GlcβCer (N7) were registered (Table 1).

Precursor structures accumulate in β4GalNAcTB and β4GalNAcTA/TB double mutants

The negative-mode MALDI-TOF mass spectrum of the GSLs of the *β4GalNAcTB* mutant showed an enrichment of the GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6) GlcNAcβ,3Manβ,4GlcβCer species (Nz7 at *m/z* 1958.0) when compared to wild-type and *β4GalNAcTA* mutant profiles (Fig. 2c, Table 1). The structure of this GSL was corroborated by MALDI-TOF/TOF-MS (Fig. 4b). Positive ion-mode MALDI-TOF-MS of *β4GalNAcTB* mutant GSLs moreover revealed the accumulation of a N3 species GlcNAcβ,3Manβ,4GlcβCer (at *m/z* 1087.9; Fig. 3c, Table 1). This GSL was characterized by MALDI-TOF/TOF-MS of the sodium adduct with laser-induced decay (Fig. 6). The oligosaccharide sequence was indicated by a series of Y-ions at *m/z* 884.7 ($[Hex_2Cer+Na]^+$), at *m/z* 722.6 ($[Hex_1Cer+Na]^+$), and at *m/z* 560.2 ($[Cer+Na]^+$). The HexNAc–Hex–Hex–Cer sequence was additionally indicated by the B-ion series with signals at *m/z* 226.1 ($HexNAc+Na]^+$), *m/z* 388.1 ($HexNAc_1Hex_1+Na]^+$), and *m/z* 550.2 ($HexNAc_1Hex_2+Na]^+$). The accumulation of the N3 and N7 GSL species terminating with an *N*-acetylglucosamine in the *β4GalNAcTB* mutant indicated the major role of β4GalNAcTB in these two elongation steps. The GSL profiles of wild-type and *β4GalNAcTA* mutant were largely the same and gave, therefore, no evidence for a role of β4GalNAcTA in GSL biosynthesis. Such a role was inferred from the absence of lacdiNAc in the double mutant; whereas the *β4GalNAcTB* mutant still had some elongated arthro-series GSLs (Fig. 2c), these structures were completely absent in the double mutant (Fig. 2d). The only GSL detected was N3 (GlcNAcβ,3Manβ,4GlcβCer at *m/z* 1087.8; Fig. 3d). From these data it can be concluded that on the background of a *β4GalNAcTB* mutant, β4GalNAcTA

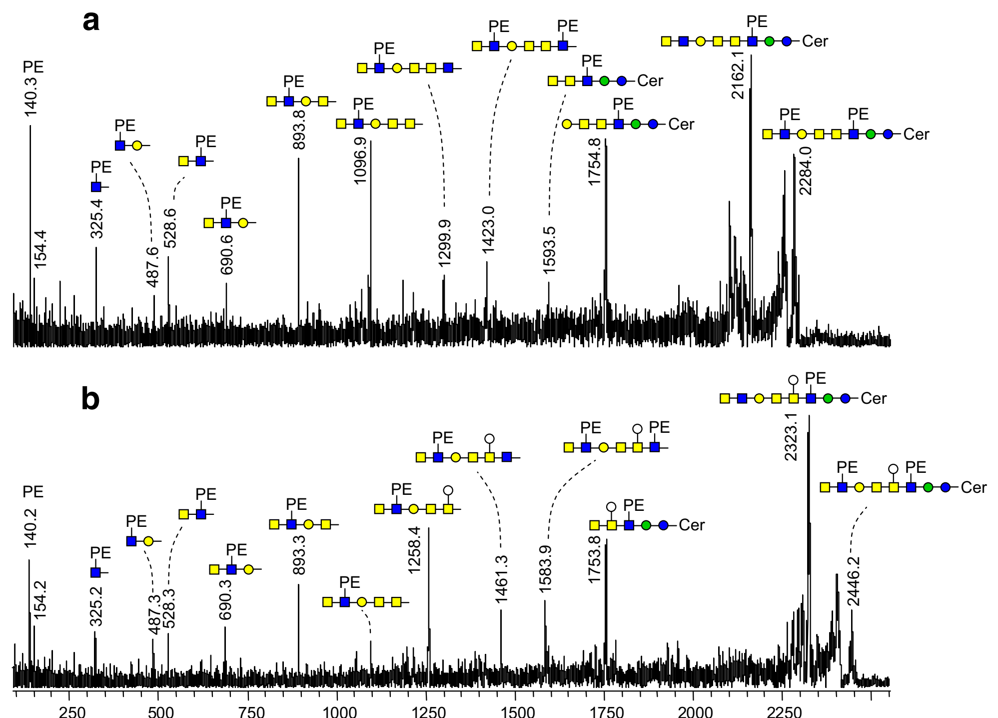

Fig. 5 MALDI-TOF/TOF-MS analyses of two zwitterionic glycolipids containing eight and nine monosaccharide residues. Zwitterionic glycolipid species (**a**) with an octasaccharide glycan moiety (*m/z* 2284; from wild-type), and (**b**) with a nonasaccharide glycan moiety (*m/z* 2446; from wild-type) were analyzed by MALDI-TOF/TOF-MS in deprotonated form using 2,5-dihydroxybenzoic acid as a matrix. For key see legend Fig. 2. *Empty circle*, hexose

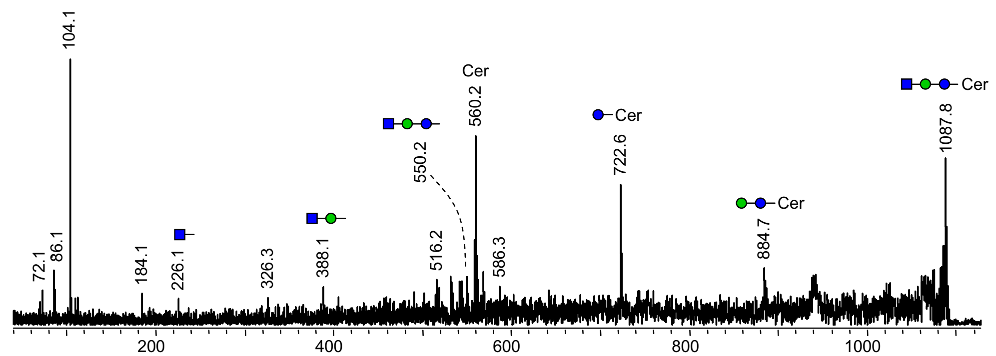

Fig. 6 MALDI-TOF/TOF-MS analysis of the neutral glycolipid enriched in *β4GalNAcTB* knockout mutants. The neutral ceramide trisaccharide (sodium adduct at *m/z* 1087.8; *β4GalNAcTA*/*β4GalNAcTB* double mutant) was analyzed by MALDI-TOF/TOF-MS in the positive-ion mode using 6-aza-2-thiothymine as matrix substance. For key see legend Fig. 2

is responsible for the residual production of complex GSLs longer than the GlcNAcβ,3Manβ,4GlcβCer structure (Fig. 2c). Only the mutation of both *β4GalNAcTA* and *β4GalNAcTB* appears to completely abolish the production of GSLs elongated beyond the ceramide trihexoside structure (Fig. 3d).

Discussion

The analysis of GSLs structures in *Drosophila β4GalNAcTA* and *β4GalNAcTB* mutants show that in the absence of both enzymes GSLs are not further extended after the transfer of GlcNAc by Brainiac. This result demonstrates that the two described β4GalNAc transferases are the only enzymes able to catalyze the synthesis of lacdiNAc on *Drosophila* glycolipids. Furthermore, the accumulated precursor structure carries no PE modification on the terminal GlcNAc residue, suggesting that PE is transferred co-ordinately with the transfer of GalNAc or after the action of the GalNAc transferases. The detection of the neutral GSL species GalNAcα,4GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer (N5) and GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer (N7) in wild-type flies and both single mutants indicates that modification of GlcNAc with PE probably occurs independently, after the transfer of GalNAc.

In this study a new GSL structure, related to the octasaccharide with two PE groups, having an additional branching hexose, was detected. Branched structures have not been described in insects, but based on characterized GSL structures in *C. elegans* [36] the hexose residue could be a β6-linked glucose residue. Alternatively, one of the *Drosophila* β3-galactosyltransferases acting on GalNAc residues [37] could branch the GSL structure in this position.

The loss of complex GSLs in the *β4GalNAcT* double mutant flies suggests that extended glycan structures with and without terminal glucuronic acid residues are of limited importance for *Drosophila* development. The absence (in the double mutant) results in a mild phenotype [1], at least compared to the lethal phenotype of *brainiac* and *egghead* mutants [29]. Considering these differences, the trisaccharide ceramide product of Brainiac by itself is already sufficient to prevent the occurrence of the *brainiac* phenotype.

Nevertheless, *β4GalNAcTA* mutants show defects in behaviour and in the neuromuscular system [1, 31], whilst a small proportion of *β4GalNAcTB* mutants display a defect in epithelial morphogenesis [30]. These different phenotypes indicate that the enzymes have distinct functional roles.

We have found that glycolipid biosynthesis in the *β4GalNAcTA* mutant is not significantly different from wild-type. However, as elongated GSLs are still detectable in the *β4GalNAcTB* single mutant, but not in the double *β4GalNAcTA*; *β4GalNAcTB* mutant, β4GalNAcTA seems able to transfer GalNAc to GSL and may contribute to the generation of these structures in wild-type flies. The phenotypes ascribed to the *β4GalNAcTA* mutants could therefore be due to loss of GSL structures. Perhaps β4GalNAcTA plays a role in generating GSLs in only a limited number of cells rather than significantly contributing to the overall glycolipid profile of the complete fly. Arguing against this is the finding that both *β4GalNAcTA* and *β4GalNAcTB* are widely expressed in *Drosophila* [1] suggesting that *β4GalNAcTA* function is unlikely to be defined by its expression pattern. Another explanation would be that the main function of *β4GalNAcTA* is not in glycolipid biosynthesis but in the generation of a structure thus far not identified.

β4GalNAcTB can correct the behaviour phenotype of the *β4GalNAcTA* mutant when ubiquitously expressed [30]. This suggests that β4GalNAcTB is capable of generating the structures that are normally synthesised by β4GalNAcTA and its failure to do so in the *β4GalNAcTA* mutant is because it is not expressed in the required cells. Although *β4GalNAcTB* clearly plays a major role in glycolipid synthesis it remains uncertain if the ability of this enzyme to rescue the *β4GalNAcTA* phenotype is due to its ability to generate lacdiNAc-containing GSLs. On the other hand, over-expression of *β4GalNAcTA* cannot completely overcome the *β4GalNAcTB* mutant phenotype [30]. This

correlates with our observation that in the *β4GalNAcTB* mutant the overall GSL biosynthesis defect is much more severe than in the *β4GalNAcTA* mutant. This may indicate that β4GalNAcTA is a less efficient in glycolipid biosynthesis than β4GalNAcTB.

Both β4GalNAcTs show genetic interaction with *egghead* and *brainiac* [30], the phenotypes of both mutants become more severe in combination with a double *egghead*/*brainiac* heterozygotes. This suggests that these enzymes all contribute to the generation of the same glycan structure in *Drosophila* and that the mutant phenotypes arise directly from loss of GSL. However, β4GalNAcTA and β4GalNAcTB are not functionally exchangeable and, considering the minor role we have identified for β4GalNAcTA in generating GSL structures, it thus remains possible that these enzymes synthesize lacdiNAc in other glycoconjugates than GSLs; loss of such structures could underlie aspects of the mutant phenotypes.

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