Abstract

To date, sterylglucosides have been reported to be present in various fungi, plants, and animals. In bacteria, such as Helicobacter pylori, proton NMR spectral analysis of isolated 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) demonstrated the presence of an α-glucosidic linkage. By contrast, in animals, no detailed structural analysis of GlcChol has been reported, in part because animal-derived samples contain a high abundance of glucosylceramides (GlcCers)/galactosylceramides, which exhibit highly similar chromatographic behavior to GlcChol. A key step in vertebrate GlcChol biosynthesis is the transglycosylation reaction catalyzed by glucocerebrosidase (GBA1) or GBA2, utilizing GlcCer as a glucose donor. These steps are expected to produce a β-glucosidic linkage. Impaired GBA1 and GBA2 function is associated with neurological disorders, such as cerebellar ataxia, spastic paraplegia, and Parkinson’s disease. Utilizing a novel three-step chromatographic procedure, we prepared highly enriched GlcChol from embryonic chicken brain, allowing complete structural confirmation of the β-glucosidic linkage by 1H-NMR analysis. Unexpectedly, during purification, two additional sterylglucoside fractions were isolated. NMR and GC/MS analyses confirmed that the plant-type sitosterylglucoside in vertebrate brain is present throughout embryonic development. The aglycon structure of the remaining glucosylceramide (Glx-2) remains elusive due to its low abundance. Together, our results uncovered unexpected aglycon heterogeneity of sterylglucosides in vertebrate brain.

Agycon diversity of brain sterylglucosides: structure determination of cholesteryl- and sitosterylglucoside.

Lipid glycosylation is a common feature in all three domains of life: bacteria, archaea, and eukaryotes. To date, sterol glycosylation has been encountered in bacteria, fungi, plants, and animals [see review (1)], indicating its important role during the evolution of life. While bacteria, such as Helicobacter pylori, produce large amounts of 1-O-cholesteryl-α-D-glucopyranoside (2), animals such as chickens and snakes (3, 4) produce 1-O-cholesteryl-β-D-glucopyranoside (GlcCho), also known as glucosyl-β-D-cholesterol.

Recently, Marques et al. (5) measured elevated levels of GlcCho not only in somal tissues derived from mouse models of Gaucher disease (GD) and Niemann-Pick disease type C, but also in the plasma of GD and Niemann-Pick disease type C patients. It is well-known that in GD, glucosylceramide (GlcCer) accumulates in the lysosomal compartment of macrophages, and that GD is also associated with a homozgyous mutation in glucocerebrosidase (GBA1) (6–9). Heterozygous mutations in GBA1, on the other hand, have been recognized to be a high-risk factor for Parkinson’s disease (10).

The ability of GBA1 and its homolog, GBA2, to hydrolyze GlcCer is well-established. Interestingly, both enzymes also possess transferase activity, catalyzing the transfer of the glucose (Glc) residue from GlcCer to cholesterol (Chol) to yield GlcChol in mammalian cells (5, 11, 12). As a consequence, GlcCer, a key intermediate in sphingolipid metabolism, is the precursor of GlcChol. Similar to GBA1, loss-of-function mutations in GBA2 are associated with

Supplementary key words • brain lipids • cholesterol • glycolipids • mass spectrometry • sterols, glucocerebrosides; • matrix-assisted laser desorption/ionization-tandem mass spectrometry

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Abbreviations: ALS-PDC, amyotrophic lateral sclerosis-parkinsonism dementia complex; CDCl3, deuterated chloroform; CerS, ceramide synthase; Chol, cholesterol; CID, collision-induced dissociation; C,M, chloroform/methanol; GalCer, galactosylceramide; GaCer (d18:1-C12:0), β-D-galactopyranosyl-(1→4)-N-acetyl-D-muramyl-L-alanylamide; GlcCer, glucocerebrosidase; GD, Gaucher disease; Glc, glucose; GlcCer, glucosylceramide; GlcCer (d18:1-C18:0), β-D-glucopyranosyl-(1→1)-N-sulfo-D-glucosyl-erythrosphingosine; GBA, glucocerebrosidase; GD, Gaucher disease; Glc, glucose; GlcCer, glucosylceramide; GlcCer (d18:1-C18:0), β-D-glucopyranosyl-(1→1)-N-sulfo-D-glucosyl-erythrosphingosine; GlcChol, 1-O-cholesteryl-β-D-glucopyranoside (glucosyl-β-D-cholesterol); GlcCho, 1-O-cholesteryl-β-D-glucopyranoside (glucosyl-β-D-cholesterol); GlcSto, 1-O-sitosteryl-β-D-glucopyranoside; (glucosyl-β-D-sitosterol); HILIC, hydrophilic interaction chromatography; HOHAA, homonuclear Hartmann-Hahn; HSQC, heteronuclear single quantum coherence; i.d., inner diameter; LRMS, low-resolution mass spectra; MRM, multiple reaction monitoring; M.W, methanol/water; RP, reversed-phase.

This work was supported by the Special Postdoctoral Researcher Program, RIKEN (to H.A.); the ONO Medical Research Foundation (to H.A.); and a Grant-in-Aid for B, 16H0199 (to H.A.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors declare that they have no conflicts of interest.

Manuscript received 11 August 2016 and in revised form 26 September 2016.

Published, JLR Papers in Press, October 3, 2016
DOI 10.1194/jlr.M071480

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This article is available online at http://www.jlr.org
neurological disorders, such as cerebellar ataxia and spastic paraplegia (13–15). However, the exact molecular mechanism and specific contributions of GBA1 and GBA2 dysfunction to neuronal malfunction and degeneration are not fully elucidated. Nevertheless, the transferase activity of GBA1 and GBA2 represents an intriguing intersection between two major lipid metabolic pathways, namely sphingolipids and sterols. Consequently, sphingolipid-sterol cross-talk may be important in maintaining neuronal homeostasis, all in the context that its deregulation plays a crucial role in the pathogenesis of neurodegenerative disorders, such as GD and Parkinson’s disease.

While the presence of GlcChol in human and mouse tissue, such as brain, has been inferred by LC-ESI-MS/MS analyses (5), its purification and complete structural analysis based on GlcChol-containing fractions are yet to be reported. Here, we describe the purification of the ster glycoside fraction from embryonic chicken brain. Embryonic chicken brains were selected as starting material due to the well-investigated and comparatively discernable composition of their glycolipid fraction (16, 17). During development of the purification procedure, we placed special emphasis on removing the large excess of chromatographically similar galactosylceramide (GalCer) known to be present in the CNS of vertebrates. Structural analysis of the isolated sterylglucoside fraction revealed the presence of a variety of aglycons. In addition to the major component featuring the expected cholesteryl aglycon, at least two more sterol aglycons were encountered, including the plant-type sitosterol. Our work described here is the first report to describe the complete structure of GlcChol and 1-O-sitosteryl-β-D-glucopyranosid [(GlcSito), also known as glucosylβ-sitosterol] derived from vertebrate brain and to demonstrate that sterylglucosides have a heterogeneous aglycon composition.

MATERIALS AND METHODS

Materials

The GlcChol, GlcSito, and GalCer from bovine brain, deuterated chloroform (CDCl3; 99.96% D), and 2,5-dihydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol-d7, β-D-galactopyranosyl-(1→1)-N-lauroyl-α-erythrosphingosine [GalCer (d18:1-C12:0)], and β-D-glucopyranosyl-(1→1)-N-steroyl-α-erythrosphingosine [GlcCer (d18:1-C18:0)] were purchased from Avanti Polar Lipids (Alabaster, AL). The 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl 2,2,2-trichloroacetimidate was purchased from Tokyo Chemical Industry (Tokyo, Japan) and TMS was from Acros Organics (Geel, Belgium). Cerezyme®, a recombinant human GBA1 used in enzyme replacement therapy for GD (18), was purchased from Genzyme Japan (Tokyo, Japan). For LC-ESI-MS/MS, MS, HPLC-grade acetonitrile and methanol were purchased from Thermo Fisher Scientific (Waltham, MA), chloroform and distilled water were from Kanto Chemical Co., Inc. (Tokyo, Japan), ammonium formate was from Sigma-Aldrich Japan (Tokyo, Japan), and ammonium acetate was from Wako (Osaka, Japan).

Animals

Fertilized Boris Brown chicken eggs were purchased from Inoue Poultry Farm (Sagamihara, Japan) and maintained in a rocking egg incubator at 38°C. Upon reaching the appropriate stage, the embryos were sacrificed, either heads without eyes (6 days old) or brains (8–18 days old) were quickly harvested and immediately frozen in liquid nitrogen (N2). After lyophilization, samples were stored at −80°C until further use.

Enrichment of sterylglucoside

Total lipids were extracted from lyophilized brains (290 brains, ~7.0 g dry weight) of 10- to 12-day-old embryonic chickens using 200 ml of chloroform:methanol (C:M) (C:M at 2:1, v/v; C:M at 1:1, v/v; and C:M at 1:2, v/v). After evaporation, the combined extracts were hydrolyzed for 1 h at 37°C in C:M (2:1, v/v, 150 ml) containing 0.1 M KOH. The reaction mixture was subjected to Folch’s partition, and the lower phase was evaporated to dryness. The resulting lipid film was resuspended in chloroform (50 ml) and applied to a column of silica gel 60 (Kanto Chemical Co.) equilibrated with chloroform. Glycolipids, including GlcChol, were eluted using a stepwise gradient, starting with pure chloroform (250 ml), and then with 250 ml each of C:M (98:2, v/v); C:M (95:5, v/v); C:M (91:9, v/v); and C:M (82:18, v/v). The fractions eluted with C:M (91, v/v) containing sterylglucoside alongside hexosylceramide were pooled and evaporated to dryness. The lipid film was resuspended in 6 ml of methanol:water (M:W) mixture (M:W at 9:1, v/v) and subjected to reversed-phase (RP) column chromatography over silica gel 120 (RP-18; Kanto Chemical Co.), equilibrated with M:W (91, v/v). Elution was facilitated by using a stepwise gradient of M:W (95:5, v/v, 4 ml); M:W (98:2, v/v, 10 ml); and methanol (10 ml). The presence of sterylglucosides was evaluated using TLC, and positive fractions were pooled and dried.

Isolation of sterylglucosides

A portion of the enriched sterylglucoside fraction was further purified by RP-HPLC, as described previously (19) with minor modifications. Briefly, the lipid film was resuspended in a small volume of mobile phase B (M:W at 85:15, v/v), applied to an RP-HPLC Luna C18(2) column [4.6 mm inner diameter (i.d.) × 250 mm, particle size, 3 µm; Phenomenex, Torrance, CA], and eluted with the following gradient of mobile phase A (pure methanol): 2 min, 0%; 13 min, 0–100% linear gradient; 40 min, 100% (washing step); and 15 min, 0% (equilibration). The flow rate was kept constant at 0.6 ml/min, and the column was maintained at room temperature. Lipid detection with short wave UV light at 205 nm was disabled during elution to prevent potential damage of the UV-absorbing double bond at the C5-C6 position of the expected aglycon (see Fig. 2B). The eluent was manually collected into 14 fractions in the following volumes: fraction 1, 15 ml; fractions 2–13, 0.3–0.6 ml, and fraction 14, 22 ml. Subsequently, all fractions were subjected to LC-ESI-MS/ MS analysis, utilizing multiple reaction monitoring (MRM).

Enzymatic deglucosylation of sterylglucosides

Aglycon release was effected by suspending a portion of the enriched sterylglucoside fraction in a total volume of 20 or 40 µl reaction buffer [50 mM citrate-phosphate buffer (pH 5.3), 0.25% Triton X-100, 0.6% sodium taurocholate, 1–2 µl of 100 µg/µl Cerezyme® in PBS] and incubation at 37°C for 16–20 h. The reaction was terminated by addition of 2 ml of C:M (2:1, v/v) and 460 or 480 µl of water [adjusted to a total volume of 500 µl (including the reaction mixture)] to facilitate lipid extraction after Folch’s partition. The organic layer was separated, dried, and the sterol fraction was purified by TLC on silica gel 60 using hexane:diethyl ether:acetic acid (80:20:1, v/v/v) as an eluent. Lipids were stained by primuline reagent (0.01% primuline, 80% acetone) and visualized by long wave UV detection. The band comigrating with standard Chol (Wako, Osaka, Japan) was collected and extracted by Folch’s partition. The organic layer containing the released aglycons was separated and dried under a stream of N2 gas.
GC/MS analysis
The lipid containing released aglycons was suspended in 25 μl of TMS at room temperature for 30 min. The resulting trimethylsilylated material was subjected to GC/MS analysis on a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with an Ultra capillary column (25 m × 0.2 mm, film thickness of 0.33 μm; Agilent Technologies Inc., Santa Clara, CA). We employed the following temperature gradient: from 180 to 250°C at a heating rate of 20°C/min and from 250 to 300°C at a heating rate of 5°C/min.

MALDI-Spiral TOF/TOF analysis
The highly purified sterylglucoside fraction and authentic GlcChol standard were each dissolved in C:M (1:1, v/v) at a concentration of 1 μg/μl, mixed with MALDI matrix A [10 μg/μl of 2,5-dihydroxybenzoic acid in C:M (1:1, v/v)] and matrix B (1 μg/μl LiCl in water) at a ratio of 1:1 (v/v). From the resulting mixture, 0.6–1.5 μl were spotted onto a MTP 384-hole mirror finish stainless steel plate (JEOL Ltd., Tokyo, Japan) and dried. The samples were analyzed with a JMS-S3000 Spiral TOF (JEOL Ltd., Akishima, Japan) equipped with the TOF/TOF option (20). A Nd:YLF laser pulse of 349 nm was operated at 250 Hz. For product ion mass spectrum acquisition, helium collision gas was introduced. The collision energy was 20 keV to induce high-energy collision-induced dissociation (CID).

LC-ESI-MS/MS analysis
LC-ESI-MS/MS analysis was performed on a semi-micro LC system 100XL (Eksigent, Dublin, CA) fused to a triple quadrupole linear ion trap mass spectrometer, QTRAP4500 (SCIEX, Toronto, Canada). The datasets were analyzed with MultiQuant and Analyst software (SCIEX). Target lipids were monitored in the MRM mode using specific precursor-product ion pairs, as detailed in Table 1. Ionization efficiency of GlcCer (d18:1-C12:0) and GalCer (d18:1-C12:0) were similar under the employed conditions.

Sterylglucosides were analyzed as described previously (19), with minor modifications. Briefly, samples (~200 μg dry weight) dissolved in 10 μl of C:M (2:1, v/v) were diluted 10-fold with mobile phase B [M:W at 85:15 (v/v), 5 mM ammonium acetate] and applied onto a RP column [Luna C18(2) column; 2 mm i.d. × 250 mm, particle size, 3 μm; Phenomenex] maintained at 36°C and at a flow rate of 0.15 ml/min. The samples were then eluted with the following gradients of mobile phase A (methanol:water:formic acid, 89:9:1, v/v/v, with 20 mM ammonium formate): 3.3 min, 0%; 13.4 min, 0–35% linear gradient; 1.3 min, 35–70% linear gradient; 3 min, 70% (washing step); 30 min, 0%, flow rate increased to 0.2 ml/min (equilibration). The mass spectrometer was set to positive ion mode (ion spray voltage, 5,500 V; curtain gas pressure, 30 psi; nebulizer gas pressure, 90 psi; heating gas pressure, 30 psi; temperature, 100°C) utilizing MRM detection for targeted analysis.

NMR spectroscopy
Highly purified sterylglucoside fractions and authentic standards were dissolved in CDCl3 containing tetramethylsilane as an internal chemical shift reference. One-dimensional 1H-NMR and two-dimensional double quantum filtered correlation spectroscopy (DQF-COSY) and homonuclear Hartmann-Hahn (HOHAHA) spectra, as well as 1H-13C multiplicity-edited heteronuclear single quantum coherence (HSQC) spectra were recorded on a DRX-500 spectrometer (Bruker BioSpin, Yokohama, Japan) equipped with a TXI cryogenic probe. Probe temperature was set at 25°C. The NMR data were processed with XWIN-NMR (version 3.5) and the spectra were displayed using XWIN-PLoT (version 3.5).

Synthesis of deuterium-labeled cholesteryl-β-D-glucoside
The synthetic route to deuterium-labeled cholesteryl-β-D-glucoside (GlcChol-d7) is shown in supplemental Fig. S2. Unless stated otherwise, reactions were performed under argon. All solvents and chemicals were purchased as reagent grade from commercial suppliers and used without further purification, unless stated otherwise. Dry solvents were purchased from Kanto Chemical Co. and used as supplied. Analytical TLC and flash column chromatography were performed using the indicated solvent systems on Merck silica gel 60 F256 plates and on Kanto Chemical Co. silica gel 60 N (40–100 mesh), respectively. Low-resolution mass spectra (LRMS) were recorded on an SCIEX 4000 QTRAP mass spectrometer. NMR spectra were obtained on a JEOL ECA-500 spectrometer (1H at 500, 13C at 125 MHz) in the indicated solvents, with chemical shift referenced to residual nondeuterated solvent.

Compound 3 (2,3,4,β-tetra-O-acetyl-1-O-cholesteryl-β-D-glucopyranoside; see supplemental Fig. S2) was synthesized as

### Table 1. Analytical conditions used for the analysis by MRM methods

| Glucosylated sterols | Precursor Ion (Q1) | Product Ion (Q3) | Collision Energy (eV) |
|----------------------|------------------|-----------------|----------------------|
| GSX1 (GlcChol)       | 566.4<sup>1</sup> | 369.4<sup>1</sup> | 15                   |
| GSX-2                | 580.3<sup>1</sup> | 383.3<sup>1</sup> | 15                   |
| GSX-3 (GlcSito)      | 594.3<sup>1</sup> | 397.3<sup>1</sup> | 13                   |
| GlcCers              |                  |                 |                      |
| GlcCer (d18:1-C12:0) | 644.4<sup>1</sup> | 264.2 or 464.4<sup>1</sup> | 43 or 21             |
| GlcCer (d18:1-C16:0) | 700.7<sup>1</sup> | 264.2<sup>1</sup> | 45.5                 |
| GlcCer (d18:1-C18:0) | 728.7<sup>1</sup> | 264.2<sup>1</sup> | 48                   |
| GlcCer (d18:1-C24:1) | 810.7<sup>1</sup> | 264.2<sup>1</sup> | 55.5                 |

<sup>1</sup>Precursor ion (Q1) [M + NH₄]<sup>+</sup>.
<sup>2</sup>Precursor ion (Q1) [M + H]<sup>+</sup>.
<sup>3</sup>Product ion (Q3) aglycon-related ion.
<sup>4</sup>Product ion (Q3) long-chain base-related ions.
follows. Cholesterol-d7 (100 mg, 0.25 mmol) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl 2,2,2-trichloroacetimidate (150 mg, 0.3 mmol) were dissolved in dry dichloromethane (5 ml) at −40°C and stirred for 10 min. The reaction was initiated by addition of trimethylsilyl trifluoromethanesulfonate (5 μl) and stirred for 2 h at −40°C. Subsequently, the reaction was quenched with triethylamine (1 ml), and the volume was increased with dichloromethane (5 ml) prior to extraction against water and brine. The organic layer was dried over Na2SO4 and concentrated in vacuo. The residue was subjected to flash chromatography on silica gel (gradient of hexane:ethyl acetate at 10:1 to 1:1, v/v); product elution was monitored by TLC (hexane:ethyl acetate at 3:1, v/v, to give compound 3 as a white amorphous solid (112.5 mg, 0.16 mmol, 63% yield).1H NMR (500 MHz, CDCl3, 25°C): δ = 5.35 (d, 1H, J = 5.2, H-6), 5.25 (dd, 1H, J = 9.2, J = 9.7, Glc H-3), 5.11 (dd, 1H, J = 9.7, J = 9.7, Glc H-4), 4.99 (dd, 1H, J = 9.7, J = 8, Glc H-2), 4.58 (d, 1H, J = 7.7, Glc H-1), 4.25 (dd, 1H, J = 12.3, J = 4.9, Glc H-A), 4.10 (dd, 1H, J = 12, J = 2.3, Glc H-B), 3.68 (n.r., 1H, Glc H-5), 3.48 (m, 1H, H-3), 2.25 (dd, 1H, J = 9.2, J = 9.7, Glc H-3), 5.11 (dd, 1H, J = 9.7, J = 9.7, Glc H-4), 4.99 (dd, 1H, J = 9.2, J = 8.8, Glc H-5), 4.77 (d, 1H, J = 8.8, Glc H-6A), 4.72 (d, 1H, J = 8.8, Glc H-6B), 4.07 (n.r., 1H, Glc H-5), 3.82 (dd, 1H, J = 12.2, J = 1.3, Glc H-6A), 3.66 (dd, 1H, J = 12, J = 12, Glc H-6B), 2.76 (s, 3H, Ac), 2.41 (s, 3H, Ac), 2.29 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.99 (n.r., 1H, Chol H-21), 1.90 (n.r., 1H, Chol H-22A), 1.84 (n.r., 1H, Chol H-12A), 1.82 (n.r., 1H, Chol H-16A), 1.58 (n.r., 1H, Chol H-2B), 1.55 (n.r., 2H, Chol H-15), 1.48 (n.r., 1H, Chol H-11A), 1.47 (n.r., 1H, Chol H-7B), 1.42 (n.r., 1H, Chol H-11B), 1.41 (n.r., 1H, Chol H-8), 1.38 (n.r., 1H, Chol H-20), 1.31 (n.r., 2H, Chol H-23), 1.30 (n.r., 1H, Chol H-22A), 1.25 (n.r., 1H, Chol H-16B), 1.14 (n.r., 1H, Chol H-12B), 1.08 (n.r., 2H, Chol H-24), 1.07 (n.r., 1H, Chol H-17), 1.06 (n.r., 1H, Chol H-1B), 0.99 (n.r., 3H, Chol H-19), 0.97 (n.r., 1H, Chol H-22B), 0.90 (n.r., 1H, Chol H-9), 0.89 (n.r., 3H, Chol H-21), 0.66 (n.r., 3H, Chol H-18); 13C NMR (125 MHz, CDCl3, 25°C): δ = 140.5 (1C, Chol C-5), 121.7 (1C, Chol C-6), 101.2 (1C, Glc C-1), 78.8 (1C, Chol C-3), 76.7 (1C, Glc C-3), 76.3 (1C, Glc C-5), 73.7 (1C, Glc C-2), 70.3 (1C, Glc C-4), 61.6 (1C, Glc C-6), 56.8 (1C, Chol C-14), 56.2 (1C, Chol C-17), 50.3 (1C, Chol C-9), 42.2 (1C, Chol C-13), 39.8 (1C, Chol C-12), 39.2 (1C, Chol C-24), 38.5 (1C, Chol C-4), 37.3 (1C, Chol C-3), 36.6 (1C, Chol C-10), 36.1 (1C, Chol C-22), 35.8 (1C, Chol C-20), 31.9 (1C, Chol C-7), 31.8 (1C, Chol C-8), 29.5 (1C, Chol C-2), 28.1 (1C, Chol C-16), 24.1 (1C, Chol C-15), 23.6 (1C, Chol C-23), 21 (1C, Chol C-11), 18.9 (1C, Chol C-19), 18.3 (1C, Chol C-21), 11.4 (1C, Chol C-18); LRMS (ESI, pos) calcd. for C33H49D7O6 [M + Na]+: 758.44, found: 758.41.

Quantification of sterylglucosides in animal tissue

The hophilized embryonic chicken tissue (~110 mg) at the desired developmental stage was homogenized and total lipids were extracted with a C:M (2:1, v/v, 5–10 ml) mixture spiked with 5 pmol/ml hophilized isotope of GalCer (d18:1-C12:0) and GlcChol-d7, each as internal standards. Extracts were dried under a flow of N2 gas and hydrolyzed for 2 h at room temperature in C:M (2:1, v/v, 2 ml) containing 0.1 M KOH. After neutralization with 1 M acetic acid (~100 μl), the reaction mixture was subjected to Folch’s partition and the lower phase was dried under a flow of N2 gas. The resulting lipid film was suspended in C:M (2:1, v/v) at a concentration of ~200 μg hophilized tissue/μl, diluted 10-fold with mobile phase B or A, and aliquots (10 μl) were subjected to RPLC-ESI-MS/MS or HILIC-ESI-MS/MS analysis, respectively. Peak areas were integrated and quantified relative to the associated internal standard.

High-performance TLC

The extracted lipids were dissolved in C:M (2:1, v/v), applied to a high-performance TLC plate (silica gel 60; Merck Millipore, Darmstadt, Germany) impregnated with boric acid, and developed with C:M:2.5 M NH4OH (65:35:8, v/v/v). The carbohydrate portion of the lipids was visualized with orcinol reagent (120°C, 10 min). GlcChol, GlcCer, and GalCer were identified by comigration with their respective commercial standards, such as GlcChol, GlcCer (d18:1-C18:9), and GalCer from bovine brain.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA) to calculate mean ± SEM values.

RESULTS

Purification of sterylglucosides

A multi-step chromatographic purification protocol was developed to isolate the sterylglucoside fraction from CNS tissue in order to facilitate a complete structure analysis. After hydrolysis of the glycophospholipid fraction, sterylglucosides were enriched by normal phase chromatography, utilizing a C:M gradient during elution, together with the large excess of GalCer known to be present in vertebbrate brain. Removal of the large excess of GalCer was achieved by RP chromatography utilizing a M-W gradient (Fig. 1A).
Fig. 1. Purification of GlcChol from embryonic chicken brain. A: High-performance TLC analysis of the sterylglucoside elution profile from a C18 silica gel column during enrichment, visualized by orcinol staining. GSX, GlcChol-positive fractions, were combined for further purification. B: RPLC-ESI-MS/MS spectra of GSX fraction during neutral-loss scan of \([M+NH_4]^+\). Total ion count (TIC) chromatogram, individual MS/MS spectra of indicated peaks. C: Elution profile of purified sterylglucosides from RP-HPLC, as monitored by RPLC-ESI-MS/MS. Monitored precursor-product ion pairs are 566/369, 580/383, and 594/397, depicted with open circles (solid line), filled circles (solid line), and open triangles (dotted line), respectively.
Monitoring by boronated TLC revealed the successful separation of the chromatographically similar GalCer fraction. Fractions (13–17) corresponding to the standard GlcChol in the Rf value were pooled for further treatment and were designated as GSX.

To confirm the presence of glycosylated sterols, GSX was subjected to RPLC-ESI-MS/MS analysis by neutral-loss scan. Fragmentation under CID conditions favored elimination of the carbohydrate moiety, together with the cationic site. This is elucidated through formation of a resonance-stabilized homo-allylic carbocation of the aglycon (supplemental Fig. S1). Surprisingly, three distinct peaks were detected, indicating the presence of multiple compounds (Fig. 1B). Each major peak corresponded to the individual molecular-related ion [M + NH₄]⁺ at m/z 566, 580, and 594, respectively. Utilizing a large-scale column with similar chromatographic conditions, GSX was further separated into three major fractions, termed GSX-1, GSX-2, and GSX-3. Fractions were monitored by RPLC-ESI-MS/MS (Fig. 1C) and corresponding fractions were pooled as indicated.

In order to gain initial structural insight into GSX-1, GSX-1 was subjected to MALDI-Spiral TOF/TOF (Fig. 2). The prominent ion at m/z 242.3 (Fig. 2A) was identified with tetrabutylammonium cation and its intrinsic positive charge facilitated excellent ion yield in positive mode. The presence of the prominent ion in all tested samples at similar ion yield suggests that this contaminant was instrumentally entered into the final purification step. The predicted lithium adduct of GlcChol ([C₃₃H₅₆O₆ Li]⁻, 548.41) at [M + Li]⁺ m/z 555.5 was subjected to high-energy CID. The product ion spectrum (Fig. 2C) revealed a dominant ion at m/z 187.1, corresponding to [hexose + Li]⁺. The charge remote fragmentation pattern matched the fingerprint of the Chol standard (23), with the expected constant shift of 162 Da (Fig. 2D) supporting the presence of a sterylhexoside.

NMR analysis of GSX-1 revealed a low-field pattern in the range of 4.5–3.3 ppm (Fig. 3A), which is typically associated with glucopyranosides. The ¹H chemical shifts of the hexoside portion match well with those of the GlcChol reference (Table 2). The large splitting (7.8 Hz) of the anomeric proton (Glc H-1) at 4.44 ppm is archetypical for an axial-axial J coupling consistent with β-glycosidic linkage. In the low-field region, the aglycon exhibits a deshielded vinyl proton at 5.38 ppm (Chol H-6) consistent with the presence of a Chol C5-C6 double bond and the bridging proton at 3.58 ppm (Chol H-3). Complete superposition (Fig. 3D) of the

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**Fig. 2.** Analysis of GSX-1 by MALDI-Spiral TOF/TOF in positive-ion mode. A: MS1 spectrum. B: Proposed structure of GSX-1. C: High-energy collision-induced MS/MS spectra of GSX-1, selected precursor ion [M + Li]⁺ m/z 555.5. D: High-energy collision-induced MS/MS spectra of Chol, selected precursor ion [M + Li]⁺ m/z 393.4.
high-field portion of the $^1$H-$^{13}$C HSQC correlation spectra of GSX-1 (Fig. 3B) with an authentic GlcChol sample (Fig. 3C) confirms the presence of the expected $\beta$-cholesteryl aglycon. This concludes the complete structure determination of natural GlcChol from embryonic chicken brain.

**Identification of minor sterylglucosides**

The low content of the highly purified fractions GSX-2 and GSX-3 precluded their NMR spectroscopic analysis. However, to gain initial structural insight, we subjected the partially purified fraction GSX to NMR analysis (Fig. 4). In the low-field region, the HOHAHA spectrum (Fig. 4A) confirmed the presence of glucopyranoside as the sole carbohydrate. The most notable HSQC cross-peak (Fig. 4B), not corresponding to the Chol aglycon of GSX-1, is located at $^{13}$C: $\delta = 45.9$ ppm/$^1$H: $\delta = 0.93$ ppm. The signal phase and chemical shift are characteristic of an additional methine branching point in the side chain of sterols. The methylene and methyl cross-peaks at $^1$C: $\delta = 22.9$ ppm/$^1$H: $\delta = 1.28$ ppm and $^{13}$C: $\delta = 12.2$ ppm/$^1$H: $\delta = 0.85$ ppm, respectively, support the presence of an ethyl side chain, as in sitosterol. Comparison of the orphan HSQC cross-peaks
As expected, Chol eluted at 19.3 min (Fig. 5), followed by GC/MS analysis of the liberated aglycons. Fragment ions of the main component corresponded well to the expected GlcChol, while the two minor components exhibited an increased mass, greater by 14 and 28 Da. Charge-mediated as well as charge-remote fragmentation (24) of the further-purified main component and Chol standard by MALDI-Spiral TOF/TOF and Fourier transform ion cyclotron resonance mass spectrometry (23, 25), together with GC/MS analysis of the liberated aglycon, confirmed the presence of a β-cholesterol residue. Complete superposition of the 2D NMR spectral data from natural and authentic GlcChol proves that a glucopyranoside residue is present and is linked via a β-glycosidic linkage to a β-cholesterol aglycon.

The minor component, with a mass difference of +28 Da compared with GlcChol, is consistent with the presence of two additional methylene groups. Indeed, 2D NMR and GC/MS spectral data corresponded well to authentic stiteryl-β-glucopyranoside. The minor component (GSX-2), with a mass difference of +14 Da compared with GlcChol, suggests either the presence of an additional methylene or keto group. The GC elution profile of GSX-2 is consistent with sterols featuring an additional methylene group compared with Chol like campesterol, but its EI MS fingerprint did not match well with authentic campesterol (Fig. 5B, supplementary Fig. S3A). Specifically, the intensity ratio between m/z 129.1 and its complementary fragment [M-129] at m/z 343.2 generated during EI-induced retro-Diels-Alder reaction has been suggested to reflect the relative stability of the formed oxonium and C-4 carbonium ion, respectively (26). Elevated intensity of [M-129] is thus associated with resonance stabilization of the C-4 carbonium ion via the adjacent double bond or by electron-donating C-4 methylation. The prominent intensity of m/z 129 in GSX-2 thus suggests reduced C-4 carbonium ion stability compared with campesterol, possibly due to an altered position of the double bond. Nevertheless, fragments m/z 254 and 213 indicate the presence of a double bond in the B or C ring, while suggesting that the putative additional methylene or keto group is associated with the sterol side chain.

### DISCUSSION

To date, the evidence that GlcChol is present in vertebrate brain was based on LC-ESI-MS/MS data (5). Isolation of sufficient GlcChol from brain for mandatory NMR structural analysis has been hampered by the abundant copresence of GalCer, which exhibits highly similar chromatographic behavior to GlcChol. In the present study, we achieved separation of GlcChol from GalCer using a newly established two-step chromatographic setup. Untargeted LC-ESI-MS/MS analysis of the putative GalCer-free GlcChol-rich fraction (GSX) isolated from embryonic chicken brain revealed the presence of at least three distinct sterylglucosides. Fragment ions of the main component corresponded well to the expected GlcChol, the two minor components exhibited an increased mass, greater by 14 and 28 Da. Charge-mediated as well as charge-remote fragmentation (24) of the further-purified main component and Chol standard by MALDI-Spiral TOF/TOF and Fourier transform ion cyclotron resonance mass spectrometry (23, 25), together with GC/MS analysis of the liberated aglycon, confirmed the presence of a β-cholesterol residue. Complete superposition of the 2D NMR spectral data from natural and authentic GlcChol proves that a glucopyranoside residue is present and is linked via a β-glycosidic linkage to a β-cholesterol aglycon.

#### Developmentally dependent presence of sterylglucosides

To allow accurate quantification of sterylglucosides, a deuterated GlcChol (GlcChol-d7) derivative was prepared, as detailed in the Materials and Methods. Lipid extracts of embryonic chicken brains aged from E6 (heads without eyes) to E18, spiked with the internal standards, GlcChol-d7 and GalCer (d18:1-C12:0), were subjected to LC-ESI-MS/MS analysis. MRMR monitoring of sterylglucosides, specifically GlcChol, GlcSito, and GSX-2 (Fig. 6), revealed the continued presence of all three sterylglucosides during embryonic development. GlcCer was monitored alongside the sterylglucosides. The abundance of GlcChol detected in stage E6 embryonic chicken brain was comparable to GlcCer (d18:1-C16:0). In contrast to the continued decrease of GlcCer (d18:1-C16:0) during development, GlcChol content decreased only until stage E10 and subsequently steadily increased until E18, resulting in about a three times excess of GlcChol compared with GlcCer (d18:1-C16:0). Similarly, GSX-2 and GlcSito continuously decreased until stage E10 and subsequently stabilized to a low amount. At all time points analyzed, GSX-2 and GlcSito fractions were digested completely by Cerezyme®. By contrast, at stages E16 and E18, about 10% of the GlcChol fraction was Cerezyme® resistant, consistent with previous reports (5).

### TABLE 2.

| GlcChol 1H (ppm) | GlcChol 1H (ppm) |
|-----------------|-----------------|
|                  |                  |
|                  |                  |

(Table 3) with authentic GlcSito revealed good agreement, further supporting the presence of a sitosteryl aglycon.

Next, the GSX fraction was subjected to enzymatic deglycosylation, followed by GC/MS analysis of the liberated aglycons (Fig. 5). As expected, Chol eluted at 19.3 min (peak 1) and represented the major sterol. Additionally, two more sterols eluted at 21.4 and 23.5 min, termed peak 2 and peak 3, respectively. The elution time and fragmentation pattern of Fig. 5A peak 3 (Fig. 5C) coincided well with authentic sitosterol (supplemental Fig. S3B), strongly supporting the presence of GlcSito. The elution time and molecular ion [M] of Fig. 5A peak 2 (Fig. 5B) corresponded to authentic campesterol (supplemental Fig. S3A), although their fragmentation pattern matched poorly. For example, the intensity ratio between m/z 129 and its complementary fragment [M-129] at m/z 343 differed greatly between authentic campesterol and peak 2 (supplemental Fig. S3A, Fig. 5B). Moreover, fragment m/z 192.3 was only present in peak 2, while m/z 457, which is typically associated with loss of a methyl radical, was absent. However, the lack of any diagnostic NMR signal associated with campesterol or its C-24 epimer, dihydrobrassicasterol, might be attributed to its low abundance in the GSX fraction.

#### 1H chemical shifts of hexose residue in GSX-1 and of Glc residue in the reference compound GlcChol

| GlcChol 1H (ppm) | GlcChol 1H (ppm) |
|-----------------|-----------------|
|                  |                  |
|                  |                  |

NMR peaks are overlapping.

#### Structural analysis of GlcChol

To date, the evidence that GlcChol is present in vertebrate brain was based on LC-ESI-MS/MS data (5). Isolation of sufficient GlcChol from brain for mandatory NMR structural analysis has been hampered by the abundant copresence of GalCer, which exhibits highly similar chromatographic behavior to GlcChol. In the present study, we achieved separation of GlcChol from GalCer using a newly established two-step chromatographic setup. Untargeted LC-ESI-MS/MS analysis of the putative GalCer-free GlcChol-rich fraction (GSX) isolated from embryonic chicken brain revealed the presence of at least three distinct sterylglucosides. Fragment ions of the main component corresponded well to the expected GlcChol, the two minor components exhibited an increased mass, greater by 14 and 28 Da. Charge-mediated as well as charge-remote fragmentation (24) of the further-purified main component and Chol standard by MALDI-Spiral TOF/TOF and Fourier transform ion cyclotron resonance mass spectrometry (23, 25), together with GC/MS analysis of the liberated aglycon, confirmed the presence of a β-cholesterol residue. Complete superposition of the 2D NMR spectral data from natural and authentic GlcChol proves that a glucopyranoside residue is present and is linked via a β-glycosidic linkage to a β-cholesterol aglycon.

The minor component, with a mass difference of +28 Da compared with GlcChol, is consistent with the presence of two additional methylene groups. Indeed, 2D NMR and GC/MS spectral data corresponded well to authentic sitosteryl-β-glucopyranoside. The minor component (GSX-2), with a mass difference of +14 Da compared with GlcChol, suggests either the presence of an additional methylene or keto group. The GC elution profile of GSX-2 is consistent with sterols featuring an additional methylene group compared with Chol like campesterol, but its EI MS fingerprint did not match well with authentic campesterol (Fig. 5B, supplementary Fig. S3A). Specifically, the intensity ratio between m/z 129.1 and its complementary fragment [M-129] at m/z 343.2 generated during EI-induced retro-Diels-Alder reaction has been suggested to reflect the relative stability of the formed oxonium and C-4 carbonium ion, respectively (26). Elevated intensity of [M-129] is thus associated with resonance stabilization of the C-4 carbonium ion via the adjacent double bond or by electron-donating C-4 methylation. The prominent intensity of m/z 129 in GSX-2 thus suggests reduced C-4 carbonium ion stability compared with campesterol, possibly due to an altered position of the double bond. Nevertheless, fragments m/z 254 and 213 indicate the presence of a double bond in the B or C ring, while suggesting that the putative additional methylene or keto group is associated with the sterol side chain.
The developmental profile of each GlcCer species in embryonic chicken brain differed markedly. While the metabolic importance of this fatty acid-specific profile of GlcCers is currently unclear, its origin might be related to the strong correlation observed between ceramide species and the expression levels of their respective ceramide synthases, as reported during postnatal rat brain development (27). The steady decline of GlcCer (d18:1-C16:0) might indicate a steady reduction of ceramide synthase (CerS)6 expression levels, while the increase in GlcCer (d18:1-C18:0) could correspond to increased expression of CerS1. The same trends of CerS6 and CerS1 expression levels have been reported during postnatal rat brain development (P1 to P21). The late stage increase of GlcCer (d18:1-C24:1) could be associated with CerS2 [a key enzyme in myelin biosynthesis (28)] expression levels and coincides well with the known onset of myelination in late stage chicken embryos. In contrast, the blood-brain barrier development occurs between P1 and P30 in chicken and is likely not associated with the observed GlcCer and sterylglucoside embryonic development profile.

Fig. 4. NMR spectroscopic analysis of partially purified GSX fraction from embryonic chicken brain. A: Low-field region $^1$H NMR and 2D HOHAHA correlation spectra. B: High-field region $^1$H NMR and 2D multiplicity-edited $^1$H-$^{13}$C HSQC correlation spectra (CH$_3$, CH, red; CH$_2$, black; arrows indicate unique signals of GlcSito). C: The 2D multiplicity-edited $^1$H-$^{13}$C HSQC correlation spectra of authentic GlcSito standard. D: Structure and aglycon numbering of GlcSito.
Throughout embryonic development, GlcChol levels in the chicken brain exceeded GlcChol levels in egg yolk (data not shown). This is in line with previous reports showing that GlcChol can be synthesized via transglucosylation from GlcCer by GBA1 or GBA2 in the lysosomal compartments or at the cytosolic surface of the endoplasmic reticulum and/or Golgi, respectively (5, 12). By contrast, the content of GlcSito and GSX-2 in egg yolk exceeded their abundance in embryonic chicken brain at all stages analyzed. The presence of diet-derived plant sterols, such as campesterol and sitosterol, in murine brain has been reported previously (29, 30). Consistent with these reports, the presence of Chol, campesterol, and sitosterol was detected in both embryonic chicken brain and egg yolk (data not shown), while the presence or absence of the free unknown GSX-2 aglycon in egg yolk and embryonic chicken brain could not be confirmed.

These findings have functional implications. The consumption of cycad (the seed of *Cycas circinalis*), a traditional indigenous food source in Guam, is one of the strongest epidemiological links to the Guamanian neurological disorder, amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) (31). Sterylglucosides in cycad seeds and flour, such as GlcSito (main sterylglucoside component),

| Carbon Number | Multiplicity | $^{13}$C $\delta$ (ppm) | $^{1}$H $\delta$ (ppm) | $^{13}$C $\delta$ (ppm) | $^{1}$H $\delta$ (ppm) |
|---------------|--------------|-------------------------|------------------------|-------------------------|------------------------|
| 29            | CH$_{3}$     | 12.2                    | 0.85                   | 12.1                    | 0.85                   |
| 26            | CH$_{3}$     | 19.1                    | 0.81                   | 19.1                    | 0.81                   |
| 27            | CH$_{3}$     | 20.0                    | 0.84                   | 19.9                    | 0.84                   |
| 28            | CH$_{2}$     | 22.9                    | 1.28                   | 23.1                    | 1.26                   |
| 25            | CH$_{2}$     | 26.1                    | 1.17                   | 26.1                    | 1.16                   |
| 22            | CH$_{2}$     | 29.1                    | 1.66                   | 29.1                    | 1.66                   |
| 21            | CH$_{2}$     | 33.7                    | 1.32                   | 33.9                    | 1.33                   |
| 24            | CH$_{1}$     | 45.9                    | 0.93                   | 45.9                    | 0.93                   |

Fig. 5. GC/MS analysis of aglycons released by GBA1 treatment from partially purified GSX fraction. A: Total ion chromatogram (asterisks indicate silica impurity). B: Mass spectrum of peak 2. C: Mass spectrum of peak 3, which is similar to TMS-sitosterol.
Fig. 6. Concentrations of sterylglucosides and selected GlcCer species in embryonic chicken brain at indicated developmental stage. Numerical values were averaged over three to four experiments. Data (mean ± SEM) were analyzed using an unpaired t test. **P < 0.01, and ***P < 0.001 compared with the concentration of each lipid in E6 heads without eyes.

campesterol-/dihydrobrassicasterolglucoside, and stigmas-teryglucoside, have been demonstrated to be neurotoxins in vitro (31, 32) and in vivo (32–34). Rodents fed with washed cycad flour or GlcSito recapitulated multiple key features of ALS-PDC, suggesting that plant-type sterylglucosides are a potential factor involved in the development of neurodegenerative disorders in vivo. Taking into consideration that Marques et al. (5) demonstrated the presence of GlcChol in human plasma, it would be of great interest to quantify sterylglucoside levels in the plasma of ALS-PDC patients in the future, based on the above detailed LC-MS/MS method. Nevertheless, direct uptake and incorporation into brain of plant-type sterylglucosides have not been demonstrated. Similarly, acceptor preference of GBA1 and GBA2 toward other sterols, including plant sterols or even polyphenols, have not been reported to date. It remains to be determined whether GlcSito and GSX-2 are derived from egg yolk or are de novo synthesized in embryonic chicken brain.

The authors thank the Support Unit for Bio-Material Analysis; RIKEN BSI Research Resources Center; and especially Ms. Abe, Ms. Otsuki, and Mr. Usui for technical help with MALDI/MS (autoflex speed TOF/TOF, Bruker Daltonics).

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