Egghead and Brainiac Are Essential for Glycosphingolipid Biosynthesis in Vivo*

Received for publication, December 9, 2004, and in revised form, December 13, 2004
Published, JBC Papers in Press, December 15, 2004

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The Drosophila genes, brainiac and egghead, encode glycosyltransferases predicted to act sequentially in early steps of glycosphingolipid biosynthesis, and both genes are required for development in Drosophila. egghead encodes a β4-mannosyltransferase, and brainiac encodes a β3-N-acetylglucosaminyltransferase predicted by in vitro analysis to control synthesis of the glycosphingolipid core structure, GlcNAcβ1–3Manβ1–4Glcβ1-Cer, found widely in invertebrates but not vertebrates. In this report we present direct in vivo evidence for this hypothesis. egghead and brainiac mutants lack elongated glycosphingolipids and exhibit accumulation of the truncated precursor glycosphingolipids. Furthermore, we demonstrate that despite fundamental differences in the core structure of mammalian and Drosophila glycosphingolipids, the Drosophila egghead mutant can be rescued by introduction of the mammalian lactosylceramide glycosphingolipid biosynthetic pathway (Galβ1–4Glcβ1-Cer) using a human β4-galactosyltransferase (β4Gal-T6) transgene. Conversely, introduction of egghead in vertebrate cells (Chinese hamster ovary) resulted in near complete blockage of biosynthesis of glycosphingolipids and accumulation of Manβ1–4Glcβ1-Cer. The study demonstrates that glycosphingolipids are essential for development of complex organisms and suggests that the function of the Drosophila glycosphingolipids in development does not depend on the core structure.

Invertebrates, Caenorhabditis elegans and Drosophila melanogaster, have recently attracted considerable attention as model organisms for deciphering specific biological roles of complex carbohydrates. One elegant example of this was a number of studies leading to the identification of a series of glycosylation genes critical for vulval invagination in C. elegans, which were all shown to affect a common biosynthetic pathway for the assembly of the O-linked oligosaccharide linker region common for all proteoglycans (1). Another example was the role of the O-linked fucose glycosylation pathway on the Notch receptor function (2). The Drosophila neurogenic genes brainiac and egghead encode glycosyltransferases essential for epithelial development during oogenesis and in the embryo (3, 4). egghead and brainiac mutants display similar, non-additive defects, which has led to the proposal that they act in the same pathway (3). In previous reports we demonstrated that brainiac encodes a UDP-N-acetylglucosamine:βManβ1,3-N-acetylglucosaminyltransferase (β3GlcNAc-trans), and egghead encodes a GDP-mannose:βGlc β1,4-mannosyltransferase, with putative functions in sequential steps in the biosynthesis of the core structure of arthro-series glycosphingolipids (GlcNAcβ1–3Manβ1–4Glcβ1-Cer) as predicted by in vitro analysis (Fig. 1) (5–7). Loss of either gene is predicted to abrogate glycosphingolipid biosynthesis at the di- or monosaccharide-ceramide step.

Insect, nematode, and vertebrate glycosphingolipids share a common element consisting of Glcβ1-ceramide, after which they differ markedly in structure and complexity (Fig. 1A). Insect and nematode glycosphingolipids are built on Manβ1–4Glcβ1-ceramide (MacCer1) predicted to be catalyzed by Egghead, while vertebrate glycosphingolipids are built on Galβ1–4Glcβ1-ceramide (LacCer) catalyzed by the β4-galactosyltransferases, β4Gal-T5 and -T6 (8, 9). Despite considerable differences in overall structures of glycosphingolipids among insects and vertebrates, it is clear that homologous glycosyltransferase genes conserved throughout evolution catalyze most biosynthetic steps. Egghead is perhaps the only exception suggesting that MacCer-based glycosphingolipids represent a specific functional basis for the diversification of the underlying biosynthetic pathways. Importantly, vertebrate glycosphingolipids based on the LacCer core diverge at the third biosynthetic step to form different classes of structures (Fig. 1A), which are differentially expressed in cells and are differentially expressed during development and differentiation (10). The vertebrate glycosphingolipid lacto-series is initiated by addition of β1,3GlcNAc to LacCer by brainiac orthologs designated β3GnTs (11–15). Interestingly, Drosophila brainiac functions both on the invertebrate and vertebrate precursor substrate MacCer and LacCer, while the vertebrate orthologs appear to only act on LacCer (5).

*This work was supported by the Human Science Frontier Program (RGP0663/2002-C), the Velux Foundation, the Danish Medical Research Council, National Institutes of Health Grants P41 RR05351 and P20 RR16459, and European Community Marie Curie Fellowship IHP HPMF-CT-2000–01083. The costs of publication of this article were defrayed in part by the payment of page charges. This article must advertise HPMP-CT-2000–01083. The costs of publication of this article were defrayed in part by the payment of page charges. This article must be printed in U.S.A.

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1 The abbreviations used are: MacCer, Manβ1–4Glcβ1-ceramide; LacCer, Galβ1–4Glcβ1-ceramide; CHO, Chinese hamster ovary; CHO, Chinese hamster ovary; HPTLC, high performance thin layer chromatography; SPE, solid-phase extraction; MBL, mannann binding lec- tion; DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein; UAS, upstream activating sequence; CDH, ceramide dihexoside; GM2, GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1-Cer; GM3, N-acetyl-neuraminylgalaCeramide.
In this report we present direct evidence that Egghead and Brainiac do function in vivo in the glycosphingolipid pathway and are essential for glycosphingolipid biosynthesis in vivo. Furthermore, we demonstrate that despite the fundamental difference in the structure of core glycosphingolipid, the *Drosophila egghead* mutant can be rescued by introduction of the corresponding enzyme from the human glycosphingolipid bio- synthetic pathway. In contrast the fly glycosphingolipid biosynthetic pathway is not elongated in vertebrate cells. The results show that glycosphingolipids are essential for development of complex organisms and suggest that the function of *Drosophila* glycosphingolipids in development does not depend on the core structure.

**EXPERIMENTAL PROCEDURES**

**Sequencing of egghead Mutants—**Genomic DNA was purified from *egh*¹, *egh*²³¹⁶, and *egh*¹²¹² homologous mutant larvae. A PCR product was generated by standard polymerase chain reaction using primers (5′-AAGCTTCGAGGACCAAAAGC-3′) and (5′-TCTCCCTCTACCATGTTCAAGG-3′) (25 cycles of 95 °C for 45 s, 55 °C for 30 s, 72 °C for 2 min). The generated PCR product was purified and sequenced with primers (5′-CAATCATAATACCGCC-3′), (5′-CACTCAATTGCACT-3′), (5′-GAGAACATCTTTGCGG-3′) using ABI sequencing. Mutations/deletions were verified by generation of a second independent PCR product.

**Enzymatic Activity in Extracts from Mutant Larvae—**Mutant larvae were homogenized in extraction buffer: 100 mM Hepes, 1% Triton X-100, 25 mM CaCl₂, 10 mM NaCl, EDTA-free protease inhibitor mixture (Roche Applied Science), spun 1,000 × g for 10 min, and supernatant used for enzymatic assay.

**Extraction of Glycosphingolipids from Mature Flies, Larvae, and CHO Cells—**Glycosphingolipids were extracted and fractionated by methods similar to those described previously (16). Freeze-dried flies (5–10 g) were homogenized in 50 mM bicine, 1% n-octyl-β-D-glucoside, 10 mM NaCl, EDTA-free protease inhibitor mixture (Roche Applied Science), spun 1,000 × g for 10 min, and supernatant used for enzymatic assay.

**In Vitro Glycosylation Assays—**CHO cells—

**Stable Expression of Egghead in Chinese Hamster Ovary Cells—**The *egghead* cDNA, isolated from High Five™ cells, was stably expressed in Sf9 cells as described (19). Egghead enzyme assays were performed as described previously (6) in reaction mixtures containing 25 mM Hepes-KOH (pH 7.4), 10 mM MgCl₂, 1% n-octyl-β-D-glucoside (Sigma), and 100 μM GDP-[¹⁴C]Man (2,000–4,000 cpm/nmol) (Amersham Biosciences). Assays with *egghead* were performed in the same reaction mixture except for addition of UDP-[¹⁴C]GlcNAc (3,000 cpm/nmol)/UDP-GlcNAc (Amersham Biosciences) and *MnCl₂*. Enzyme sources were microsomal fractions of baculovirus-infected High Five cells prepared essentially as described (6). Reaction products were separated on octadecyl-silica (Supelco) and analyzed either by scintillation counting and/or by high performance thin layer chromatography followed by detection with orcinol.

**Generation of Monoclonal Antibody Recognizing Manβ1-4Glcβ1-Cer—**For production of the anti-MacCer monoclonal antibody BALB/c mice were immunized three times with 10 μg of purified MacCer isolated from High Five™ cells as described (5). Hybridomas were selected by immunocytoLOGY on air-dried, acetone-fixed CHO cells stably transduced with full-length egghead cDNA and screened using Northern blot analyses of RNA from Sf9 cells as described (19). 1.37-kb *egghead* full, pVL-brainiac full, pVL-MacCer T2-full, and pVL-MAC T5-full were co-transfected with Baculo-Gold™ DNA (Pharmingen) in Sf9 cells as described (19). Egghead enzyme assays were performed as described previously (6) in reaction mixtures containing 25 mM Hepes-KOH (pH 7.4), 10 mM MgCl₂, 1% n-octyl-β-D-glucoside (Sigma), and 100 μM GDP-[¹⁴C]Man (2,000–4,000 cpm/nmol) (Amersham Biosciences). Assays with *egghead* were performed in the same reaction mixture except for addition of UDP-[¹⁴C]GlcNAc (3,000 cpm/nmol)/UDP-GlcNAc (Amersham Biosciences) and *MnCl₂*. Assays with *egghead* were performed with UDP-[¹⁴C]Gal/UDP-Gal. Enzyme sources were microsomal fractions of baculovirus-infected High Five cells prepared essentially as described (6). Reaction products were separated on octadecyl-silica (Supelco) and analyzed either by scintillation counting and/or by high performance thin layer chromatography followed by detection with orcinol.

**Stable Expression of Egghead in Chinese Hamster Ovary Cells—**The 1.37-kb *egghead*-Myf-fragment was used for baculo constructs cloned into the BamHI/XbaI sites of pcDNA3+ (18). Chinese hamster ovary (CHO-K1) cells were stably transfected with the pcDNA3-egghead-Myf as described previously and clones selected with anti-Myc antibodies (Invitrogen) (6). Two rounds of screening and cloning were performed by limiting dilution cloning using immunoreactivity with anti-Myc monoclonal antibody.

**Immunolabeling—**CHO cells were grown to subconfluence in the appropriate media as recommended by American Type Culture Collection. Cells were incubated with 10% newborn calf serum (Invitrogen) before fixation in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with undiluted anti-MacCer hybridoma supernatants for 18 h at 4 °C and detected with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (F261, Dako). Immunostaining with soluble mannan binding lectin (MBL) was performed on non-fixed cells using purified MBL from human serum detected with an anti-MBL monoclonal antibody (generous gift from P. Garred, Copenhagen University Hospital, Copenhagen, Denmark) and fluores-
cein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin. 

*Drosophila* ovaries were dissected, fixed in 4% formaldehyde in phosphate-buffered saline, blocked in 0.1% bovine serum albumin, 0.05% Tween 20 phosphate-buffered saline and incubated with undiluted anti-MacCer antibody and detected with Cy5 anti mouse antibodies from Jackson ImmunoResearch Laboratories. Ovaries were mounted in 80% glycerol. DAPI was included in the washes to reveal nuclei.

*Fly Strains—Armadillo-Gal4 (II), actin-Gal4, and tubulin-Gal4 are described in flybase (fly.bio.Indiana.edu/flybase.htm). Brnt<sup>+/−</sup> was described in Goode et al. (21) and egh mutations in Goode et al. (3). We further characterized three egh mutant alleles at the molecular level. For isolation of genomic DNA and characterization of the enzymatic activity of egh and brn mutants, animals of the correct genotype were identified as follows: egh and brn alleles were balanced over a GFP-expressing FM7 balancer chromosome. Larvae were sexed, and mutant males were picked on the basis of their lack of GFP expression. In the case of the egh<sup>9PP4</sup> and brn<sup>69G6</sup> alleles, the cuticular marker yellow (y) present on these chromosomes was also used to identify mutants by the color of the head skeleton.

*Genetic Mosaic Analysis—brn and egh mutant alleles were recombined onto FRT18 and mitotic recombination clones were induced in adult females by heat shock for 60 min at 38 °C. The genotypes used are as follows: y w brnt<sup>+/−</sup> / FRT18y UbiGFP FRT18; hs-FLP<sup>+/−</sup> (II), y egh<sup>9PP4</sup> / FRT18y UbiGFP FRT18; hs-FLP<sup>+/−</sup> (II), egh<sup>69G6</sup> / FRT18y UbiGFP FRT18; hs-FLP<sup>+/−</sup> (II). egh<sup>9PP4</sup> / FRT18y UbiGFP FRT18; hs-FLP<sup>+/−</sup> (II). Clones were marked by the loss of GFP expression in follicular epithelial cells of the Drosophila ovaries.

*Rescue of the eggh Head Mutant Flies—pUAS-b4GalT6 was constructed by cloning full-length cDNA into pUAST. The same construct was used for the in vitro glycosylation assay described above verifying the presence of truncated 1-Cer (data not shown). The anti-MacCer antibody produced only background labeling in the wild-type cells adjacent to the clones presumably reflecting low level of expression of the immediate precursor substrate for Brainiac (and subsequent enzymes), as co-expression of multiple intermediate species is a common feature found for glycosphingolipids. Taken together, these observations confirm the predicted functions of Egghead and Brainiac as enzymes required for sequential elongation steps of glycosphingolipid biosynthesis in vivo.

*egghead Mutants Are Rescued by Vertebrate βGal-GalT6—The finding that egh mutants lack MacCer synthase activity provided an opportunity to assess the significance of the core structure in glycosphingolipid function. We therefore asked whether expression of the human glycosyltransferase, βGal-T6, which functions as a LacCer synthase (Fig. 1A), could rescue glycosphingolipid biosynthesis in egghead mutant animals. That this might be possible was plausible because Brainiac can elongate both Manβ1–4Glcβ1 and a Galβ1–4Glcβ1 substrate in vitro (Fig. 2A, lanes 1 and 4; see also Ref. 5). We first verified that the lethality of egh<sup>69PP4</sup> mutant could be rescued by ubiquitous expression of an eggh cDNA in transgenic flies. Egh<sup>69PP4</sup> actin-Gal4 UAS-egh flies were recovered at 66% of the frequency of F7 actin-Gal4 UAS-egh control flies. We next tested whether expression of the human β4GalT6 cDNA (full coding Golgi-retained form) also rescued egh<sup>69PP4</sup> mutant flies to viability. For four different UAS-β4GalT6 transgenes, egh<sup>69PP4</sup> armadillo-Gal4;UAS-β4GalT6 flies were recovered at frequencies ranging from 76% to >100% of control males (note that PM7 males are weak and are recovered at less than the expected mendelian ratio, so it is possible for a healthy egh genotype to be recovered at over 100% of the level of the FM7 controls in this experiment). For UAS line III-1, which gave 80% recovery with armadillo-Gal4, recovery of mutant animals increased to >100% with the stronger tubulin-Gal4 driver, indicating that the level of transgene expression can affect the degree of rescue obtained.

Rescued egh<sup>69PP4</sup> armadillo-Gal4;UAS-β4GalT6 animals were normal in appearance, and both males and females were fertile, indicating that the transgene also rescued the female sterility caused by egh mutants (3, 4). Therefore we were able to establish a homozygous viable strain of rescued animals. Glycosphingolipids were isolated from the rescued flies and their composition was analyzed by high performance thin layer chromatography and 1H NMR. Analysis of the glycosphingolipids from the rescued animals by thin layer chromatography showed accumulation of a disaccharide glycosphingolipid, as the intensity of the ceramide dihexoside (CDH) band was elevated compared with wild-type animals, and some of the trisaccharide (CTH) form was also seen (Fig. 2B). We next asked whether brainiac (brn) and eggh mutant animals were blocked in glycosphingolipid biosynthesis in vivo, as would be predicted on the basis of their in vitro enzymatic functions, if no redundancy in these enzyme functions or alternate biosynthetic pathways exist. Characterization of glycosphingolipids from mutant larvae by thin layer chromatography showed accumulation of the truncated product Glcβ1-Cer in all four egh mutants, whereas MacCer accumulated in the brn mutant (Fig. 1C). We produced a monoclonal antibody that specifically recognizes MacCer but not further elongated glycosphingolipids, to provide a tool to visualize this biosynthetic intermediate in vivo. The specificity of the antibody was tested by immunostaining of glycosphingolipids separated by thin layer chromatography. The antibody detected MacCer but not LacCer or GlcNAcβ1–3Manβ1–4Glcβ1-Cer (data not shown). The antibody was then used to test for the presence of MacCer in clones of cells lacking Egghead or Brainiac activity in the *Drosophila* ovary. Clones of cells lacking Brainiac activity, which accumulate MacCer, showed strong labeling (Fig. 1D). In contrast, cells lacking Egghead activity, which we expect to be blocked at the Glcβ1-Cer step, showed no labeling above background with this antibody (Fig. 1D). This indicates that Egghead and Brainiac are present and active in the follicular epithelial cells of egg chambers. The anti-MacCer antibody produced only background levels of labeling in the wild-type cells adjacent to the clones presumably reflecting low level of expression of the immediate precursor substrate for Brainiac (and subsequent enzymes), as co-expression of multiple intermediate species is a common feature found for glycosphingolipids. Taken together, these observations confirm the predicted functions of Egghead and Brainiac as enzymes required for sequential elongation steps of glycosphingolipid biosynthesis in vivo.

**RESULTS**

**egghead and brainiac Mutants Produce Truncated Glycosphingolipids—In vitro studies predicted that the enzymes encoded by the egghead and brainiac genes would be required for glycosphingolipid biosynthesis in vivo. To confirm this, we tested four different egghead (egh) mutants. As a first step, we sequenced three of the egh alleles to determine the nature of their molecular lesions. egh<sup>69PP4</sup> resulted from an 11 nucleotide deletion which caused a frameshift at amino acid 97 and deletion of most of the coding sequence. This allele is expected to cause a complete loss of enzymatic activity as the active site has been deleted. egh<sup>69PP4</sup> resulted from a 15-base pair deletion that removed amino acids 113–117, of which two are conserved. egh<sup>7</sup> resulted from a single nucleotide change that changes the conserved methionine at position 308 to lysine (M308K). Extracts were prepared from larvae mutant for these alleles as well as egh<sup>64h6</sup> and tested for mannosyltransferase activity with n-octyl glucoside (Fig. 1B). All four mutants were devoid of significant detectable mannosyltransferase activity. We next asked whether brainiac (brn) and egghead mutants were blocked in glycosphingolipid biosynthesis in vivo, as would be predicted on the basis of their in vitro enzymatic functions, if no redundancy in these enzyme functions or alternate biosynthetic pathways exist. Characterization of glycosphingolipids from mutant larvae by thin layer chromatography showed accumulation of the truncated product Glcβ1-Cer in all four egh mutants, whereas MacCer accumulated in the brn mutant (Fig. 1C). We produced a monoclonal antibody that specifically recognizes MacCer but not further elongated glycosphingolipids, to provide a tool to visualize this biosynthetic intermediate in vivo. The specificity of the antibody was tested by immunostaining of glycosphingolipids separated by thin layer chromatography. The antibody detected MacCer but not LacCer or GlcNAcβ1–3Manβ1–4Glcβ1-Cer
required to fully assess the impact the Gal for Man substitution imposes. To further confirm that the in vivo function of the β4Gal-T6 rescue depends on elongation by Brainiac, we tested whether we could rescue egh9PP4 brn1.6P6 double mutants with β4Gal-T6. In the absence of Brainiac no rescue was obtained, indicating that elongation of the LacCer core by Brainiac is...
required for glycosphingolipid function in the rescued 
mutant flies. These experiments indicate that replacing the 
MacCer core with LacCer is compatible with glycosphingolipid 
function in the fly.

Introduction of Egghead into Mammalian Cells—Three 
pathways of vertebrate glycosphingolipid biosynthesis are 
defined by the nature of third residue added to the LacCer core 
(Fig. 1A). Three different enzymes are responsible for the 
defining steps. The neo/lacto-series contains GlcNAc in a β3 
linkage to LacCer. This resembles insect glycosphingolipids, which 
have GlcNAc in a β1,3 linkage to MacCer. However, the two 
mammalian enzymes that add GlcNAc in a β1,3 linkage to 
LacCer cannot elongate a MacCer substrate in vitro (Fig. 2A, 
lanes 2 and 3). CHO-K1 cells mainly express the ganglioside 
glycosphingolipid, GM3. Stable transfection of CHO-K1 cells to 
express egghead resulted in accumulation of MacCer, visual- 
ized by TLC (Fig. 3A). The intensity of the CDH band was 
higher in egghead-expressing cells, and this was shown by 
NMR to reflect accumulation of MacCer without further elon- 
gation (Fig. 3B). Egghead transfected cells, but not control 
CHO cells, bound the MBL (Fig. 3D), consistent with the 
prediction that they produce MacCer. These observations indicate 
that expression of Egghead can to a considerable extent 
override endogenous glycosyltransferases in mammalian cells and 
lead to synthesis of a truncated MacCer product.

Anti-MacCer antibody strongly labeled CHO cells trans- 
fected to express Egghead but not control cells transfected to 
express the vertebrate β3GnT2 enzyme (Fig. 3D). This indi- 
cates that Egghead can redirect mammalian glycosphingolipid 
biosynthesis to produce MacCer, which is not further elong- 
ated. Egghead therefore may serve as a competitive biosyn- 
thetic inhibitor of early glycosphingolipid synthesis in verte- 
bilates. Although the Egghead-expressing CHO cells contain a 
low level of residual GM3, the reduced production of normal 
glycosphingolipids did not appear to cause defects in the 
growth, survival, or morphology of these cells in culture.

DISCUSSION

In this report we have presented direct evidence that Egg- 
head and Brainiac are enzymes essential for glycosphingolipid 
biosynthesis in vivo, and thus demonstrate that glycosphingo- 
lipids are essential for Drosophila development. Furthermore, 
we show that substituting Egghead for the vertebrate LecCer 
synthase can provide the essential glycosphingolipid functions 
required to support development of egghead mutants, despite 
the fact the core structure of vertebrate and insect glycosphingo- 
lipids are different. In contrast introduction of the Drosophila 
glycosphingolipid pathway into mammalian cells can interfere 
with the normal biosynthesis because the vertebrate enzymes 
cannot elongate the MacCer insect glycosphingolipid core.

Drosophila lacking zygotic activity of the 
egg and 
mutants die as pupae. Drosophila lacking maternal and zygotic activity 
of these enzymes are devoid of elongated glycosphingolipids 
and have a more severe defect, dying as embryos with a defect 
in correct specification of neural and epidermal cell types. 
Elongated glycosphingolipids also appear to be required for 
normal development of the mouse embryo. Mice mutant for the 
glucosylceramide synthase enzyme controlling the ultimate 
glycosphingolipid precursor die during gastrulation due to apo- 
ptosis in all germ layers but particularly in ectoderm (22). The 
mouse embryos lacking glucosylceramide synthase die at an 
earlier stage of embryogenesis than Drosophila egg and 
mutations. This may reflect a difference in the position at which 
truncation occurs. For example, elevated ceramide levels are 
known to be pro-apoptotic (23). Accordingly, knockdown of 
the Glcβ1-Cer synthase in Drosophila by RNAi also leads to 
increased apoptosis, thought to be due to elevated ceramide lev- 
els (24). Whether ceramide levels are responsible for apoptosis 
in the mouse mutants in vivo remains to be determined.

Egghead and Brainiac are expressed and required during 
oogenesis (3, 4, 21, 25). In the absence of their function, devel- 
opment of the ovarian follicles is defective. We note that earlier 
reports suggested that the activity of these genes was limited to 
the germ line, because phenotypes were not observed in so- 
matic mutant clones in the follicular epithelia. Using the Mac- 
Cer antibody on genetic mosaics we show that Egghead and 
Brainiac are both present and active in the follicular epithelia.

Interestingly, the orthologs of brainiac and egg do not appear to 
be essential for development of the nematode C. elegans (26). 
Instead, both genes are required for susceptibility to the crystal 
(5B) toxin from the bacterium Bacillus thuringiensis. It there- 
fore appears that Drosophila has acquired functions for glyco- 
sphingolipids that are not shared among all invertebrates and 
that Drosophila presents an excellent model for studies of such 
functions in vivo.
The phenotypes associated with brn and egh mutants initially suggested a role of these in Notch receptor modulation similar to but distinct from fringe. Given the demonstrated function of Brainiac and Egghead in glycosphingolipid biosynthesis it is tempting to suggest that extended glycosphingolipids in Drosophila might play a direct role in modulation of receptor functions in a manner similar to the effects of GM3 on the epidermal growth factor receptor (27–29). Alternatively, extended glycosphingolipids might play an indirect role on signaling by virtue of their contribution to the formation of lipid rafts and the recruitment of receptors to rafts. Another appealing possibility is that glycosphingolipids influence the cleavage of membrane-bound ligands, such as the activation of the epidermal growth factor receptor ligands Spitz, Gurken, and Keren by the Rhomboid family of secretases (30). Of special interest in this context is the possibility that glycosphingolipids could influence Rhomboid-2 cleavage of Gurken in oogenesis. Likewise in the case of Notch signaling, glycosphingolipid could affect the γ-secretase, which is organized in lipid rafts and cleaves the intracellular tail of Notch (31). The availability of Drosophila lacking elongated glycosphingolipids will provide an opportunity to investigate the functions of glycosphingolipids in cell signaling in vivo. In considering possible modes by which glycosphingolipids may act, it is intriguing that they can do so apparently normally when their core structure has been altered by replacing MacCer with LacCer. This observation provides a starting point for further humanization of the biosynthetic pathway by further replacement of Brainiac with enzymes responsible for the next steps in the mammalian lacto-, ganglio-, or globo-series biosynthetic pathways. Conversely, vertebrate cells with MacCer-based glycosphingolipids provide a unique genetic tool to address structure-function relationships for glycosphingolipids.

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J. Biol. Chem. 2005, 280:4858-4863. doi: 10.1074/jbc.C400571200 originally published online December 15, 2004

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