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Drosophila Niemann-Pick Type C-2 genes control sterol homeostasis and steroid biosynthesis: a model of human neurodegenerative disease

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Mutations in either of the two human Niemann-Pick type C (NPC) genes, *NPC1* and *NPC2*, cause a fatal neurodegenerative disease associated with abnormal cholesterol accumulation in cells. *npc1a*, the *Drosophila NPC1* ortholog, regulates sterol homeostasis and is essential for molting hormone (20-hydroxyecdysone; 20E) biosynthesis. While only one *npc2* gene is present in yeast, worm, mouse and human genomes, a family of eight *npc2* genes (*npc2a-h*) exists in *Drosophila*. Among the encoded proteins, Npc2a has the broadest expression pattern and is most similar in sequence to vertebrate Npc2. Mutation of *npc2a* results in abnormal sterol distribution in many cells, as in *Drosophila npc1a* or mammalian NPC mutant cells. In contrast to the ecdysteroid-deficient, larval-lethal phenotype of *npc1a* mutants, *npc2a* mutants are viable and fertile with relatively normal ecdysteroid level. Mutants in *npc2b*, another *npc2* gene, are also viable and fertile, with no significant sterol distribution abnormality. However, *npc2a; npc2b* double mutants are not viable but can be rescued by feeding the mutants with 20E or cholesterol, the basic precursor of 20E. We conclude that *npc2a* functions redundantly with *npc2b* in regulating sterol homeostasis and ecdysteroid biosynthesis, probably by controlling the availability of sterol substrate. Moreover, *npc2a; npc2b* double mutants undergo apoptotic neurodegeneration, thus constituting a new fly model of human neurodegenerative disease.

KEY WORDS: Niemann-Pick type C, Sterol, Ecdysteroid, *Drosophila*

INTRODUCTION

Cholesterol, an essential component of eukaryotic cell membranes, also serves as the precursor of many steroid hormones and thus plays vital roles in many developmental processes (Farese and Herz, 1998). Cells in the body maintain proper cholesterol levels through elegant homeostatic regulatory systems. Defects in cholesterol homeostasis and metabolism have been linked directly or indirectly to many disease conditions.

Niemann-Pick type C (NPC) disease is one such cholesterol homeostasis-related disorder characterized by aberrant accumulation of free cholesterol in late endosome and lysosome-like compartments (Patterson, 2003). Normal cells take up exogenous cholesterol through the receptor-mediated low density lipoprotein (LDL) endocytic pathway. LDL-derived free cholesterol must then leave the endosomal compartment, a process that is blocked in NPC disease cells, to move to other membrane compartments, including the endoplasmic reticulum (ER), and to control homeostatic responses (Liscum and Faust, 1987). NPC disease is a progressive neurodegenerative disorder in which the degeneration of cerebellar Purkinje neurons is most prominent (Higashi et al., 1993). Although the link between cholesterol homeostasis defects and neurodegeneration remains enigmatic, the deficiency of oxysterol and/or neurosteroid has recently been implicated as partially responsible for this neurodegeneration (Griffin et al., 2004; Langmade et al., 2006).

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Mutations in either of two different human genes, *NPC1* and *NPC2*, result in Niemann-Pick type C disease, with *NPC1* mutations accounting for about 95% of known cases (Patterson, 2003). The large Npc1 protein has 13 transmembrane domains and a sterol-sensing domain (SSD) (Carstea et al., 1997; Loftus et al., 1997). Npc2, a small, secreted protein that binds cholesterol strongly, was first found as an abundant component of human epididymal fluid and later linked through human genetics to the inherited cause of NPC disease in about 5% of the families studied (Naureckiene et al., 2000). The crystal structure of Npc2 has been determined and found to contain a cavity that genetic analyses show to be the likely binding site for cholesterol (Friedland et al., 2003; Ko et al., 2003). Npc2 may serve as a lysosomal cholesterol transporter, rapidly transporting cholesterol to acceptor membranes (Cheruku et al., 2006). Although Npc1 and Npc2 are different types of cholesterol-binding proteins, they appear to be in a common pathway or process based on the virtually indistinguishable phenotypes of the human patients carrying one or the other homozygous mutation.

To uncover the disease mechanisms as well as the biological function(s) of NPC proteins, useful NPC disease models have been established in yeast, worms, flies and mice (Berger et al., 2005; Higaki et al., 2004; Li et al., 2004; Malathi et al., 2004). We and the L. Pallanck laboratory have previously created *Drosophila* NPC models using *npc1a* (also referred to as *NPC1* – FlyBase) mutations (Fluegel et al., 2006; Huang et al., 2005). *Drosophila* and all other insects are unable to synthesize sterol from simple precursors. In order to synthesize the molting hormone 20-hydroxyecdysone (20E) and to sustain the growth and reproduction of the fly, sterol has to be obtained from food (Clark and Block, 1959). In *Drosophila*, *npc1a* is crucial for sterol homeostasis, as is mammalian *NPC1*. The fly mutants have a molting defect and homozygotes die as first-instar larvae due to a deficiency of the molting hormone 20E, the primary steroid hormone identified in insects to date. 20E plays crucial roles in insect oogenesis, embryogenesis and metamorphosis (Thummel, 1996). *npc1a* mutants can be rescued by feeding them excess 20E or

either of two of its precursors: cholesterol or 7-dehydrocholesterol. Thus, the ecdysteroid deficiency is evidently due to an inability to access sufficient sterol precursor, a somewhat surprising result given the massive accumulations of sterol in punctated structures that are seen in the mutants by filipin staining. The simplest explanation is that the accumulated sterol, stored in multi-lamellar and multivesicular compartments, is not available for 20E synthesis.

Based on the findings in *npc1* mutant worms, flies and mice, we proposed a cholesterol shortage model of NPC disease (Huang et al., 2005). The normal function of Npc1 protein may be to promote delivery of sufficient sterol to the ER and/or mitochondria, organelles in which specific steps of steroidogenesis occur. In the studies reported here, we examined the functions of Npc2 proteins in *Drosophila*. Our results further support the cholesterol shortage model proposed previously.

MATERIALS AND METHODS

Drosophila culture and stocks

Flies were cultured on standard cornmeal medium at 25°C. *npc2a* and *npc2b* excision mutageneses were performed using standard methods, starting with the Bloomington *Drosophila* Stock Center lines KG05307 and KG00996 (Robertson et al., 1988). Three alleles of *npc2a* (*npc2a*^{239}, *npc2a*^{271} and *npc2a*^{376}) and three alleles of *npc2b* (*npc2b*^{18}, *npc2b*^{19} and *npc2b*^{22}) were isolated. All mutants were back-crossed to wild-type (Canton-S) flies three times before further phenotypic characterizations. The molecular lesions were determined using genomic DNA polymerase chain reactions and by sequencing. The coding regions of *npc2a* and *npc2b* are entirely deleted by the mutations in their corresponding sets of alleles.

Molecular biology

Eight *npc2*-like genes (*npc2a-h*) were found by BLAST searches of the *Drosophila melanogaster* genome with the sequence of the human NPC2 protein. The cDNAs corresponding to the *npc2* gene family were amplified by RT-PCR and sequenced. The protein sequences were then deduced from the cDNA sequences. The gene structures of all but one (*npc2e*) of the *npc2* genes were predicted correctly in FlyBase. *npc2e* (CG31410) has an extra intron compared to the FlyBase prediction and the correct coding sequence aligns well with other Npc2 proteins. The UAST-*npc2a* and UAST-*npc2b* constructs were made by inserting full-length cDNAs into the *Eco*RI site of the pUAST vector.

Ecdysteroid titer measurement

First-instar wild-type and mutant larvae were collected into 1.5 ml tubes (100 larvae/tube) and kept at –80°C until assayed for whole body ecdysteroid content. Larvae were homogenized by sonication (Sonic Dismembrator, Fisher) and extracted exhaustively with both methanol and ethanol. Pooled solvents for each replicate of 100 larvae were evaporated under low pressure into 2 ml plastic tubes and the dried residue subjected to RIA employing the H22 antiserum (Warren et al., 1988). For each genotype, 600-800 larvae were used.

Sterol and 20E feeding

For *npc2a; npc2b* double mutants, each group of 200 first-instar larvae was placed on apple juice plates with baker's yeast paste containing supplementary sterols, as described previously (Huang et al., 2005), and the lethal phases were determined by larval spiracle and mouth hook development. The final concentrations for the supplements used were: cholesterol, 0.14 mg/g; 7-dehydrocholesterol, 0.14 mg/g; and 20E, 8 μg/g.

Sterol quantitation

The sterol content in larvae was quantified by following a published protocol (Fluegel et al., 2006). The Amplex Red cholesterol assay kit (Molecular Probes) was used to assess sterol content in wandering third-instar animals. Larvae were collected and washed before being weighed and homogenized in 150 mM NaCl, 2 mM EGTA, 50 mM Tris pH 7.5, to make a 100 mg/ml larval homogenate. The homogenate was spun at 5000 rpm for 5 minutes to pellet cuticle debris, and the supernatant was used for sterol content assays. Fluorescence was measured with a fluorescence spectrophotometer with a 560/585 nm filter set.

Filipin staining and immunohistology

For filipin staining of free sterols, tissues were fixed in 4% paraformaldehyde for 30 minutes, washed twice in PBS and stained with 50 μg/ml filipin (Sigma) PBS solution for 30 minutes. Samples were then washed twice with PBS before mounting them in Vectashield mounting medium. For TUNEL analysis, aged brains were dissected and fixed (PBS, 4% paraformaldehyde) for 20 minutes at room temperature. Tissues were washed twice in PBS, once in H_2O plus 0.1% Triton X-100 and 0.1% sodium citrate for 10 minutes, and then twice in PBS. TUNEL analysis was performed by following the manufacturer's instructions (Boehringer Mannheim). TUNEL and neuron double labeling was performed using an antibody against the pan-neuronal marker Elav (Iowa Hybridoma Bank). Synaptotagmin staining (anti-Synaptotagmin, from Dr Hugo Bellen) was performed using standard techniques (Littleton et al., 1993). All images were collected using a compound microscope and a cold CCD camera.

Life span analysis

For each genotype, 10 vials containing a total of 200 flies were passed into fresh vials every 4 days, at which time the number of dead flies was recorded.

Methods for in situ hybridization to detect mRNA in overnight embryo collections and for electron microscopy were described previously (Huang et al., 2005). Malpighian tubules from third-instar larvae were dissected first, then fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, followed by further processing for electron microscopy.

RESULTS

npc2-like genes in *Drosophila melanogaster*

The Npc2 protein has been conserved throughout much of eukaryotic evolution. Only one *npc2* gene is present in yeast, worm, mouse and human genomes. *Drosophila melanogaster*, clearly a highly advanced organism, has a family of eight *npc2*-like genes, which we have named *npc2a-h*. We identified the gene family by BLAST searching with the sequence of human *NPC2*. We further confirmed and corrected the *npc2*-like gene structures by RT-PCR (Fig. 1A; also see Materials and methods). Protein sequences of Npc2a-h (CG7291, CG3153, CG3934, CG12813, CG31410, CG6164, CG11314 and CG11315; Fig. 1B) range from 22 to 36% identical to human NPC2.

Of the eight Npc2-like proteins, Npc2a (also referred to as NPC2 – FlyBase) has the highest sequence identity (36%) and similarity (53%) to human NPC2 (Fig. 1B). Further protein sequence analysis within this protein family reveals that CG3934 (Npc2c), CG12813 (Npc2d) and CG31410 (Npc2e) form a subgroup clustered at cytogenetic locus 85F8 on chromosome III, while CG11314 (Npc2g) and CG11315 (Npc2h) form another subgroup clustered at locus 100A3 on chromosome III (Fig. 1A). Both groups of genes presumably arose from gene duplication events.

Crystal structure determination and mutational analyses have shown that Npc2 has three disulfide bonds and forms a hydrophobic core implicated in cholesterol binding (Friedland et al., 2003; Ko et al., 2003). All six disulfide bond-forming cysteine residues are absolutely conserved in *Drosophila* Npc2a-h proteins. At other positions shown to be functional in mouse Npc2, Npc2a-h proteins have some variation. For example, F66, V96 and Y100 (amino acid numbers correspond to positions in the mature Npc2 protein without the signaling peptide) of mouse Npc2 are located near the hydrophobic core and are involved in cholesterol binding (Ko et al., 2003). V96 is the same or highly similar in seven *Drosophila* Npc2 proteins except Npc2h, F66 is conserved or replaced by the similar amino acid Tyr (Y) in five Npc2 proteins (not in Npc2f, g and h), and Y100 is conserved or replaced by the similar Phe (F) in six Npc2 proteins (not in Npc2c and f) (Fig. 1B). D72 and K75 of mouse Npc2

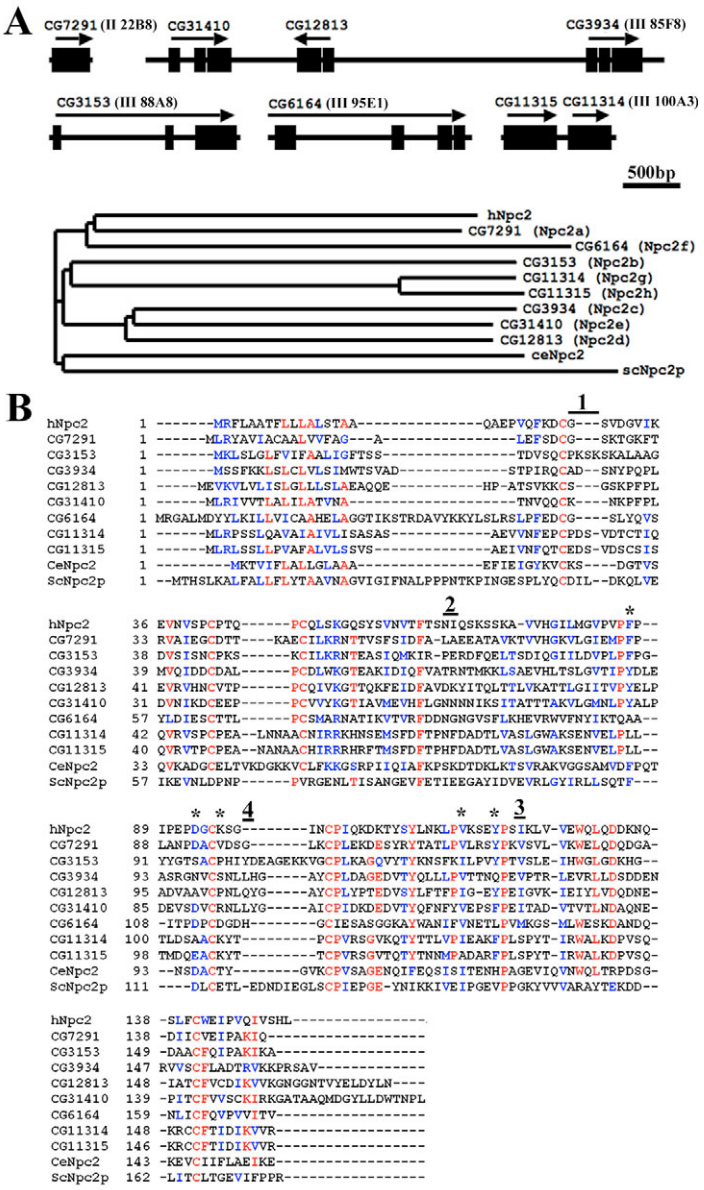

Fig. 1. The *npc2* gene family in *Drosophila melanogaster*. (**A**) The gene structure and the phylogenetic tree of eight *npc2*-like genes. Two gene clusters contain five *npc2*-like genes (CG31410, CG12813 and CG3934; CG11314 and CG11315), which can be identified based upon protein sequence similarities and gene location. (**B**) Protein sequence alignment of Npc2 proteins. hNpc2, *homo sapiens* NPC2; CeNpc2, *Caenorhabdtis elegans* Npc2 (NCR-2); ScNpc2p, *Saccharomyces cerevisiae* Npc2. 1, 2, 3 indicate the positions of three intron positions described in the text. 4 marks the intron position of *C. elegans npc2* (*ncr-2*). The asterisks denote the five key residues previously found to be important for Npc2 function.

are not required for cholesterol binding but are necessary for normal Npc2 function. D72 is conserved or replaced by the related amino acid Glu (E) in four of the *Drosophila* Npc2 proteins (Npc2a, e, f and h), while K75 is conserved in only three Npc2 proteins (Npc2e, g and h) (Fig. 1B). The variations of these key residues in Npc2 proteins may allow retention of cholesterol-binding ability while adding some capability to bind to sterols other than cholesterol. Evidence for functional conservation despite the changes in key residues is provided by the rescue of mammalian *Npc2*-mutant cells with an introduced yeast *NPC2* gene, which also has changes in encoded key residues such as K75 and Y100 (Fig. 1B) (Berger et al., 2005).

Gene duplication and gene structure evolution of *npc2*-like genes

The *npc2*-like gene family is present in other sequenced *Drosophila* genomes, such as *D. yakuba*, *D. pseudoobscura* and *D. virilis*, as well as genomes from other insect species, including *Anopheles gambiae* (at least eight *npc2*-like genes), *Bombyx mori* (at least three *npc2*-like genes) and *Tribolium casteneum* (at least three *npc2*-like genes). Together these data suggest possible multiple rounds of gene duplication events within Class Insecta.

The gene structures of *Drosophila melanogaster npc2a-h* reveal a pattern of evolution in the generation of introns within the coding region. Three genes (*npc2a*, *g* and *h*) have no intron. Two genes, *npc2b* and *d*, each have one intron in the same position (position 1 in Fig. 1B). Two others, *npc2c* and *e*, have two introns in the same positions (positions 1 and 2 in Fig. 1B). The eighth gene, *npc2f*, has three introns (positions 1, 2 and 3 in Fig. 1B). Interestingly, the intron positions (positions 1, 2 and 3 in Fig. 1B) in the *D. melanogaster npc2*-like gene family are almost identical to the intron positions of the vertebrate *npc2* genes, including those from human, mouse, rat, chimpanzee, cow and zebrafish. By contrast, the intron position in *ncr-2*, the *Caenorhabditis elegans* homolog of *npc2*, is different (position 4 in Fig. 1B). Together, the chromosomal clustering of *npc2* genes and the similarity of intron positions support the concept that the generation of the *npc2* gene family was a result of multiround gene duplication events.

Pattern of *npc2a-h* transcription in time and space

To address the potential roles of different NPC2-like proteins, the temporal and spatial expression patterns of the *npc2a-h* genes during embryonic stages was determined using whole-mount in situ hybridization. The data revealed that *npc2a* has the broadest expression pattern, whereas other *npc2* genes are either not detectably expressed or expressed in restricted locations (Fig. 2). The *npc2a* gene provides a substantial maternal contribution of RNA, and is also ubiquitously expressed at all stages examined. High levels of *npc2a* expression were found in midgut, salivary gland and ventral nerve cord (Fig. 2A-D). *npc2b* is expressed at the highest levels in the trachea and hypopharynx (Fig. 2I). *npc2g* is specifically expressed in head mesoderm and fat body (Fig. 2G,H). *npc2d* and *npc2h* transcripts could be detected only in salivary gland (Fig. 2F), while *npc2e* is expressed in hindgut (not shown). The expression of *npc2c* and *npc2f* was not detected by in situ hybridization at any time during embryogenesis.

As *npc1a* is highly expressed in the ring gland, and ring gland expression of *npc1a* is important for ecdysteroid biosynthesis, the expression of *npc2a-h* in ring glands was examined. Brains and imaginal discs from wandering third-instar larvae were also examined. In contrast to *npc1a*, none of the *npc2a-h* genes was highly expressed in ring glands. We could detect moderate levels of gene expression in larval ring glands, brains and imaginal discs for several *npc2* genes, including *npc2a* and *npc2b* (Fig. 2K,L and data not shown).

Npc2a is required for sterol homeostasis

Because *npc2a* has the broadest expression pattern among the eight genes studied, and the highest protein sequence similarity to vertebrate Npc2, we focused initially on characterizing *npc2a*

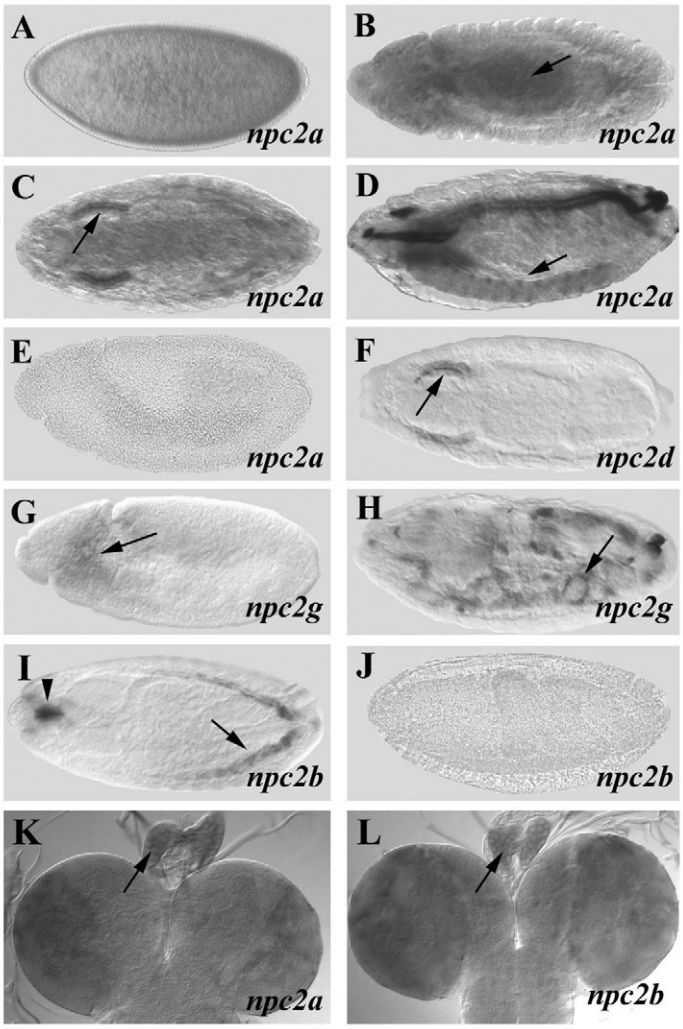

Fig. 2. Transcription patterns of *npc2*-like genes ascertained with in situ hybridization in *Drosophila melanogaster*. *npc2a* is deposited maternally (**A**) and is broadly expressed with a higher level of expression in many tissues, including the midgut (arrow in **B**), salivary gland (arrow in **C**) and ventral nerve cord (arrow in **D**). (**E**) *npc2a* in situ staining signal was not detected in the homozygous *npc2a* mutant embryos. (**F**) The salivary gland expression of *npc2d*. (**G**,**H**) *npc2g* was specifically expressed in head mesoderm (arrow in G) and fat body (arrow in H). (**I**) *npc2b* was specifically expressed in trachea (arrow) and hypopharynx (arrowhead). (**J**) *npc2b* in situ staining signal was not detected in the homozygous *npc2*b mutant. (**K**,**L**) *npc2a* and *npc2b* were expressed in larval brain hemispheres and ring gland (arrows), respectively.

function using mutant phenotypic analysis. Through P element imprecise excision we generated three deletion alleles ($npc2a^{239}$, $npc2a^{271}$ and $npc2a^{376}$; Fig. 3A). The whole coding region of *npc2a* was completely deleted in each of the three alleles, yet homozygous mutant animals were viable and adults were fertile. Each allele was tested in trans to several different genetic deficiencies that remove the gene, and these genetic combinations were also viable and fertile. Whole-mount in situ hybridization with an *npc2a* antisense probe did not detect any RNA signal in homozygous *npc2a* mutant embryos, indicating that they are bona fide *npc2a* mutants (Fig. 2E).

We next examined the sterol distribution in *npc2a* mutants using filipin staining. Filipin, which stains non-esterified sterols, is often used to study sterol accumulation in *NPC1* and *NPC2* mutant mammalian cells. We previously used filipin successfully to determine the sterol distribution in *Drosophila npc1a* mutants and found that homozygous mutants have an abnormal sterol distribution similar to that found in mammalian NPC mutants. This is most easily seen by light microscopy as a punctate pattern of filipin-stained particles, and with electron microscopy as multi-lamellar structures (Huang et al., 2005).

In *npc2a/npc2a* mutant tissues, including salivary gland, midgut, Malpighian tubules, imaginal discs, brains, trachea and ovaries, a punctate pattern of filipin fluorescence was found (Fig. 3B-G). Most tissues had many such spots of accumulated sterol, except trachea, where we found fewer puncta. The filipin staining phenotype was similar to that of *Drosophila npc1a* mutant tissues and mammalian NPC mutant cells, indicating a conserved role for *Drosophila npc2a* in regulating efficient intracellular sterol trafficking. The sterol distribution abnormality in *npc2a/npc2a* mutants could be fully rescued by ubiquitous expression of a *UAST-npc2a* transgene (see below), indicating that this phenotype is indeed due to *npc2a* mutation.

We further examined the structure of mutant *npc2a/npc2a* cells using electron microscopy. Large multi-lamellar body and multi-vesicular body structures were found in *npc2a* mutant Malpighian tubules (Fig. 4), just as in homozygous *npc1a* mutants. The multi-lamellar structures were often clustered together to form large inclusions with or without electron-dense materials within (Fig. 4B and C, respectively). The similarities in cellular phenotypes and ultrastructural defects of *npc1a* and *npc2a* mutants further suggest the conserved roles of NPC genes in regulating intracellular sterol trafficking from *Drosophila* to mammals. As the homozygous mutants survive to adulthood, while *npc1a/npc1a* flies do not, there must be important differences between *npc1* and *npc2a* phenotypes, and accumulation of sterol is not, by itself, adequate to cause death.

Ecdysteroid deficiency in *npc1a* but not *npc2a* mutants

The apparently similar defects in sterol distribution in *Drosophila npc1a* and *npc2a* mutants raise the question: why do *npc1a* mutants die as first-instar larvae, while *npc2a* mutants are viable and ultimately fertile? We have suggested previously that the first-instar larval lethality of *npc1a* is due to ecdysteroid deficiency, although this was inferred rather than measured directly (Huang et al., 2005). The difference in phenotypes between *npc1a* and *npc2a* homozygotes could reflect different ecdysteroid levels.

We have now directly measured ecdysteroid levels during the first-instar stages (38 hours after egg laying) of wild-type, *npc1a/npc1a* and *npc2a/npc2a* larvae. Compared to wild type, the *npc1a* mutant had low ecdysteroid titers (16.7±0.9 pg/100 mutant larvae versus 87.7±4.4 pg/100 wild-type larvae). The *npc2a/npc2a* mutant larvae had somewhat lower than normal ecdysteroid levels (53.3±3.6 pg/100 mutant larvae versus 73.8±4.1 pg/100 wild-type larvae) (Garen et al., 1977; Kraminsky et al., 1980; Neubueser et al., 2005). These results could explain why *npc1a* mutants die as first-instar larvae, i.e. cannot molt, while *npc2a* mutants are viable and are fertile as adults. Furthermore, the data support our previous hypothesis that the first-instar lethality of *npc1a/npc1a* mutants is due to ecdysteroid deficiency.

Redundant roles of *npc2a* and *npc2b* in sterol homeostasis and ecdysteroid biosynthesis

The ecdysteroid titer results do not explain why apparently similar defects in sterol distribution are associated with a shortage of sterol substrate for ecdysteroid biosynthesis in *npc1a* but not *npc2a*

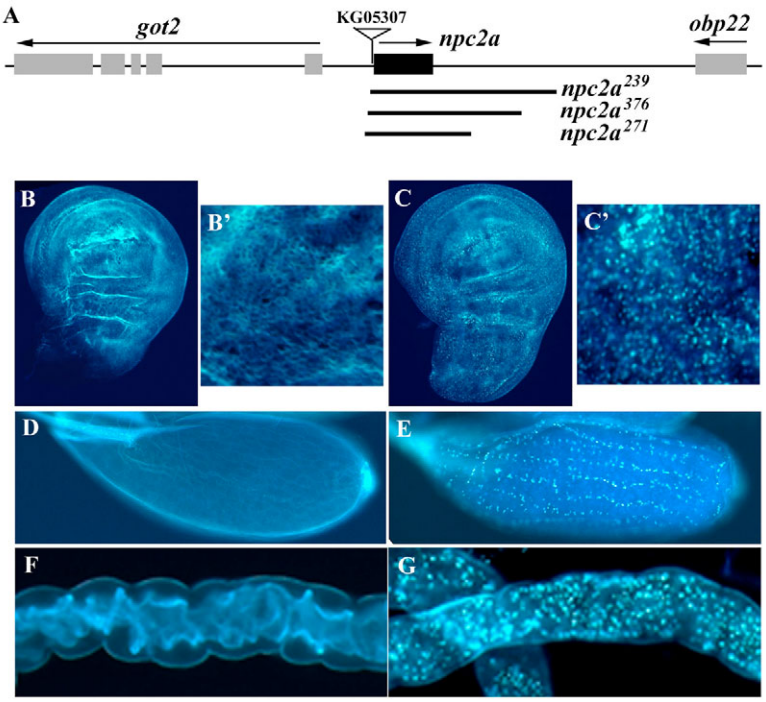

Fig. 3. *npc2a* mutants and their sterol accumulation phenotypes. (**A**) The gene structure of *npc2a* and the chromosome intervals deleted in three *Drosophila npc2a* alleles. (**B-G**) Filipin staining reveals the sterol distribution patterns in wild type (B,D,F) and *npc2a* mutants (C,E,G). B and C are filipin-stained wing imaginal discs from third-instar larvae: wild type (B) and *npc2a* mutant (C). The magnified views (B′,C′) show that in wild type, sterol is located mainly at cell-cell boundaries, whereas in *npc2a* mutants sterol accumulates in a punctate pattern that is not restricted to those boundaries. (D,E) Aberrant sterol accumulation was observed in a striped pattern in *npc2a* (E) but not wild-type eggs (D). (F,G) Filipin staining highlighted the lumen of the Malpighian tubules in wild type. In *npc2a* mutants, massive punctate accumulations of filipin staining were visible inside Malpighian tubules.

mutants. There are at least two possibilities. First, *npc1a* and *npc2a* may function differently in ecdysteroidogenesis, so that only *npc1a* but not *npc2a* is involved in sterol transport to the mitochondria. This could be true despite the apparently similar overall accumulation of sterol in filipin-stained compartments. Alternatively, the difference could be due to redundant functions of the multiple *npc2* genes. Perhaps in the *npc2a/npc2a* mutants a substantial amount of sterol reaches the mitochondria, transported by other Npc2 family protein-mediated processes.

In order to test the gene redundancy hypothesis and to examine possible functions of a second *npc2* gene, the function of *npc2b* was analyzed. *npc2b* is expressed in the tracheal system and hypopharynx (Fig. 2G), so we paid particular attention to the possible redundancy of *npc2a* and *npc2b* in these tissues. We found that *npc2a* and *npc1a* mutants have quite different patterns of sterol accumulation in larval trachea. Punctate filipin staining was readily observed in *npc1a* mutants but few sterol particles accumulated in the trachea of *npc2a* mutants (Fig. 5A,C).

To determine whether sterol accumulation in *npc2a* mutants is prevented by *npc2b*, we generated three *npc2b* deletion alleles ($npc2b^{18}$, $npc2b^{19}$ and $npc2b^{22}$) of *npc2b* by imprecise P element excision. The whole coding region of *npc2b* was completely deleted in these three alleles. In homozygous *npc2b* mutant animals, no in situ hybridization signal could be detected with an *npc2b* antisense probe, so as expected the new alleles of *npc2b* were nulls (Fig. 2). Like *npc2a* homozygotes, *npc2b* homozygotes and flies carrying an *npc2b* allele in trans to a genetic deficiency were viable and fertile.

No sterol accumulation was observed in any *npc2b* mutant tissues, including the trachea, where we know the gene is preferentially transcribed (Fig. 5B). However, *npc2a/npc2a; npc2b/npc2b* doubly homozygous mutants had a large number of filipin-stained puncta in the trachea. The level of sterol particles was similar to sterol accumulation in *npc1a/npc1a* mutants (Fig. 5D). We conclude that *npc2a* and *npc2b* function redundantly in sterol trafficking, at least in trachea.

Although both single mutants were viable, fertile and were not developmentally delayed, *npc2a; npc2b* double mutants died as larvae or pupae and the third-larval instar was prolonged. Aside from a small percentage of animals (about 17%) that died in the first or second larval stage, the majority of *npc2a/npc2a; npc2b/npc2b* double mutants molted to the third instar quite normally. They remained in the third instar for 3-6 days, compared with about 2 days for wild-type animals. Twenty-six percent died while still in the third-instar stage, while the remaining 57% formed pupae (Fig. 6A). About a tenth of the mutant pupae developed to the adult stage, but they were sick and usually died within 2 weeks (Fig. 6A). For this reason we were not able to establish homozygous double mutant stocks.

Most of the *npc2a/npc2a; npc2b/npc2b* double mutants could be rescued by feeding them a diet enriched with cholesterol, 7-dehydrocholesterol or 20E (Fig. 6A). The prolonged third instar of the double mutants, together with the results of the rescue experiment, suggests that the ecdysteroid level is relatively low in the double mutants. In the presence of sufficient substrate, *npc2a/npc2a; npc2b/npc2b* mutants were evidently able to synthesize enough ecdysteroid for fairly normal development.

As with *npc1a* mutants, the insufficiency of sterol substrate appeared to be the main problem for *npc2a/npc2a; npc2b/npc2b* double mutants. The similarity of the double *npc2* homozygotes to *npc1a* homozygotes suggests that *npc1a* has irreplaceable functions, while the two *npc2* genes tested to date have somewhat redundant functions. Both Npc1 and Npc2 are necessary to regulate sterol homeostasis and carry out adequate biosynthesis of 20E.

Tissue-specific requirement of *npc2*

Our results agree well with the hypothesis that Npc1 and Npc2 promote efficient intracellular sterol trafficking for ecdysone biosynthesis. To further pinpoint the roles of Npc2, we examined the sterol level in *npc2* mutants and possible tissue-specific requirements for *npc2*. Despite the altered filipin staining patterns

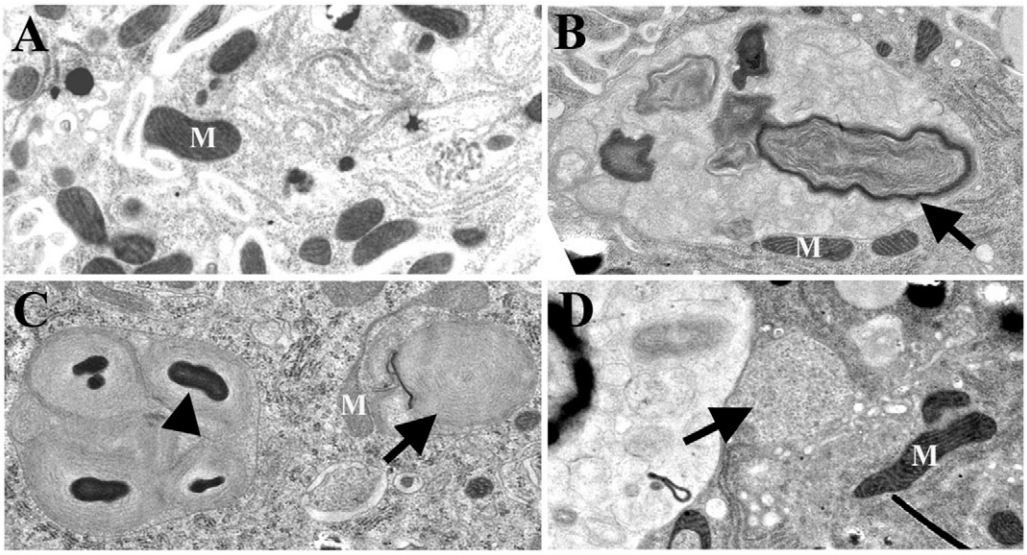

Fig. 4. Ultrastructural defects in third-instar larval Malpighian tubules of *npc2a* mutants. (**A**) Wild-type *Drosophila melanogaster*; (**B-D**) *npc2a* mutants. Large multi-lamellar structures (arrows in B and C) and multivesicular bodies (arrow in D) are present in *npc2a* mutants but not wild-type tubules. The multi-lamellar structures were often clustered together to form large inclusions with or without electron-dense whorls within (arrowhead in C and arrow in B, respectively). M, mitochondria.

in many tissues, the overall level of sterol was not much different in *Drosophila npc1a* mutants compared to controls (Fluegel et al., 2006). We measured sterol levels in *npc2* mutants and found a similar result: the overall level of sterol was not significantly changed in *npc2a/npc2a* or *npc2b/npc2b* single mutants or in *npc2a/npc2a;npc2b/npc2b* double mutants (Fig. 6B).

As *npc1a* is required in the ring gland for ecdysone biosynthesis, we analyzed whether *npc2* genes are also important in this same crucial tissue or in others. We focused our analyses on the ring gland, nervous system and trachea. We used the Gal4 system to address tissue-specific requirements for *npc2a* and *npc2b*. Ubiquitous expression of *UAST-npc2a* or *UAST-npc2b* could rescue the lethality: 83% of the double mutants survived to adulthood in the presence of *tub-Gal4>UAST-npc2a* and 80% survived to adulthood in the presence of *tub-Gal4>UAST-npc2b*. Only 5% survived in double mutants lacking any transgene. The pattern of punctate filipin-stained sterol accumulation in trachea of double mutants was similarly rescued by the transgenes (data not shown).

Expression of UAST-*npc2a* or UAST-*npc2b* only in the ring gland, using the *2-286-Gal4* driver, rescued the lethality of the double mutant: 78% of the double mutants survived to adulthood in the presence of *2-286-Gal4>UAST-npc2a* and 86% survived to adulthood in the presence of *2-286-Gal4>UAST-npc2b*. These findings are consistent with the conclusion that a defect in ecdysone biosynthesis is the main cause of the larval lethal phenotype. By contrast, pan-neuronal expression of UAST-*npc2a* or UAST-*npc2b* did not show any rescuing activity.

Neuronal phenotypes of *npc2* mutants

In addition to cellular defects in cholesterol homeostasis, mammalian NPC mutants have neuronal and behavioral defects, including region-specific neurodegeneration, ataxia, dementia and early death. We examined *Drosophila npc2* mutants in detail to search for potential neuronal phenotypes.

Drosophila neurodegenerative mutants are often associated with a short life span and numerous large vacuoles in the brain (Min and Benzer, 1999; Palladino et al., 2002). We assessed the adult life span of *npc2a* mutants. *npc2a* mutants displayed a slightly shorter life span compared with wild type (Fig. 7A). For example, by day 52 more than 60% of the wild-type flies were still alive compared with fewer than 10% of the *npc2a/npc2a* mutants. Fifty percent of the *npc2a* mutants died by day 36, a time when more than 90% of the wild-type flies remained alive.

We sectioned adult brains from 30-day-old *npc2a/npc2a* and wild-type animals to look for the presence of large vacuoles indicative of neurodegeneration. We found no evidence of any neurodegenerative vacuoles (data not shown). Reasoning that subtle neurodegeneration may not cause the formation of large vacuoles, we next used TUNEL staining to look for apoptotic cells in adult brains. Compared with wild type, we found few TUNEL-positive cells in 30-day-old *npc2a/npc2a* mutant brains (Fig. 7B). By contrast, many TUNEL-positive cells were present in 7-day-old *npc2a/npc2a; npc2b/npc2b* double homozygous brains and in tracheal cells that extended along the top of the brains (Fig. 7B). We double-stained mutant flies with antibodies against the pan-neuronal marker Elav and for TUNEL-positive cells. Most of the TUNEL-positive cells were neurons (Fig. 7C). Similar TUNEL-positive cells, indicative of neurodegeneration, were found in *Drosophila npc1a* mutants (data not shown). Thus *Drosophila npc1/2* mutants faithfully display cholesterol accumulation and neurodegenerative phenotypes analogous to those of mammalian NPC mutants.

Mammalian Npc1 has been found in axons as well as presynaptic nerve terminals, and *Npc1/Npc1* mutant mice have mild morphological changes in presynaptic nerve terminal (Karten et al., 2006). For this reason, Synaptotagmin staining of third-instar larvae was performed to examine neuromuscular junction (NMJ) structure and axon morphology. We found no difference in NMJ morphology in *npc2a/npc2a* mutants, but axonal transport defects were detected at a low frequency (two to three sites per animal). These defects took the form of accumulated Synaptotagmin within axon tracts (Fig. 7C). The significance of this phenotype for neural function remains to be learned.

DISCUSSION

NPC disease is characterized by aberrant lysosomal storage of cholesterol and other lipids and by massive degeneration of Purkinje neurons in the cerebellum and, to a lesser degree, other neurons. Major intracellular trafficking defects involving at least the late endosomes and lysosomes that contain Npc1 protein are also observed. The link between the trafficking defects, sterol

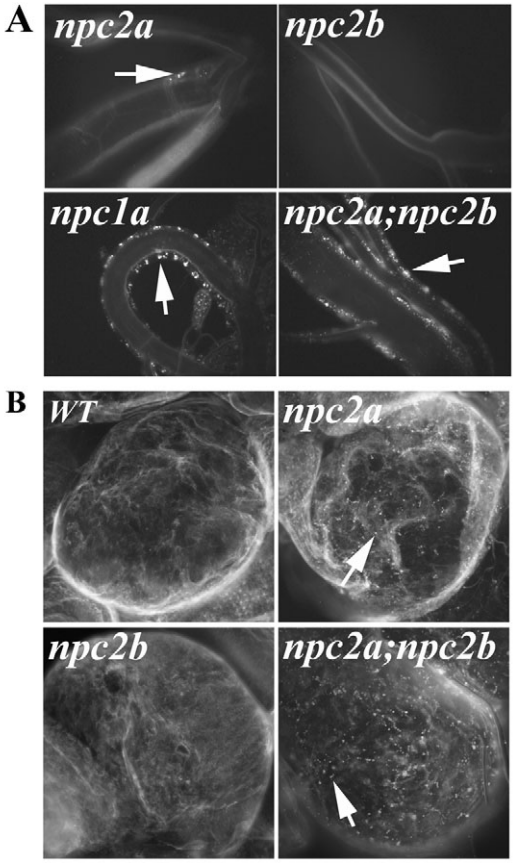

Fig. 5. *npc2a* and *npc2b* act redundantly in regulating sterol homeostasis in *Drosophila*. Filipin staining patterns of third-instar larval tracheas (**A**) and brains (**B**) in different genetic backgrounds. (A) *npc2a* and *npc2b* act redundantly in regulating sterol homeostasis in trachea. A small number of filipin-stained particles of sterol accumulation (arrow) was found in *npc2a* animals. By contrast, there was no sterol accumulation phenotype in *npc2b* mutants or in wild-type animals (not shown). However, massive sterol accumulation (arrow) was found in *npc1a* animals as well as *npc2a; npc2b* double mutants. (B) In brains, the punctated filipin-stained pattern (arrows) was found in both *npc2a* single and *npc2a; npc2b* double mutants but not wild type or *npc2b* single mutants.

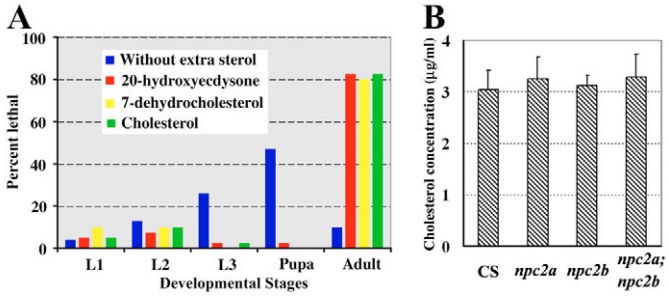

Fig. 6. Sterol requirement and sterol content of *npc2* mutants. (**A**) Rescue of mutant *Drosophila melanogaster* by food supplementation with 20E or other sterols. The particular developmental stage in which the mutants died is shown as a percentage of the total. The *x*-axis indicates the developmental stage and the *y*-axis is the percentage of lethal. Without dietary supplements, nearly all mutants died by the pupal stage. Supplementation with 20E caused substantial rescue, allowing survival of more than 80% of the mutant animals until adulthood. Similarly, cholesterol and its immediate precursor 7-dehydrocholesterol allowed about 80% of the mutant animals to survive to adulthood. (**B**) The total sterol content of third-instar larvae was not changed in *npc2* mutants. Three samples were measured for each genotype and error bars represent standard deviation.

homeostasis defects, and neurodegenerative pathology is still a mystery, and there is considerable debate about which defect is primary, i.e. initiating.

In cells treated with the drug U18666A, which causes a phenotype much like NPC disease, the trafficking defects are readily visible before sterol accumulation (Ko et al., 2001). The trafficking defect may occur earlier than sterol accumulation and compartmentalization in the diseased state as well. The neurodegeneration could then be a consequence of either sterol accumulation or of other outcomes of defective trafficking. Evidence in favor of the latter idea comes from monitoring the degeneration of cerebellar Purkinje neurons in *Npc1/Npc1* mutant mice (Ko et al., 2005). The Purkinje cells that die are not those that have the highest cholesterol accumulation. Other outcomes of defective trafficking may therefore kill neurons, such as a failure to transport sterol substrate to ER/mitochondria for steroid hormone synthesis, as we have suggested in a model of proposed cholesterol shortage (Huang et al., 2005).

Here we show that *Drosophila npc2a* and *npc2b* play redundant roles in regulating sterol homeostasis and 20E biosynthesis. The mutant phenotypes of *npc2a; npc2b* double-homozygous mutants support the proposed cholesterol-shortage model. Moreover, the apoptotic neurodegeneration observed in the fly mutants suggests a further similarity to mammalian NPC disease, and opens up the possibility of applying model organism genetics to understanding the disease process more completely and perhaps devising treatments.

Redundancy of Npc2 proteins in *Drosophila*

A single gene encoding the cholesterol-binding protein Npc2 is present in many eukaryotic species, with the notable exception that a family of Npc2-like proteins arose within insects or their ancestors. The gene structure analysis of the *Drosophila npc2*-like gene family clearly indicates that the *npc2*-like genes were formed by multiple rounds of gene duplication. Why do insects have so many Npc2-like proteins and what are their roles?

In general, gene duplication allows the evolution of new gene functions. In that case, one copy can retain the original function of its ancestor and the other can gain new biological functions through further mutation. The prominent sterol accumulation phenotype in many tissues of the *npc2a* mutant, the broad expression of *npc2a*, and the high degree of sequence identity between Npc2a and human NPC2 compared with the other seven Npc2-like proteins, all suggest that *npc2a* functions similarly to vertebrate *npc2*. From that perspective, the mystery is about the roles of Npc2b-h. Our study of *npc2b* demonstrates that *npc2b* is especially highly transcribed in trachea, and in that tissue it is partially redundant to *npc2a* with respect to sterol homeostasis. This is an incomplete answer to the origin of the gene duplications, because it is not clear why two genes are required. Other *npc2* genes (*npc2c-h*) may also function partially redundantly with *npc2a* because *npc2a; npc2b* double mutants have a weaker phenotype than *npc1a* mutants (larval/pupal lethal versus first-instar lethal). As insects are cholesterol auxotrophs and need external sterol sources for growth (Clark and Block, 1959), it is possible that some of the Npc2-like proteins may be involved in sterol uptake.

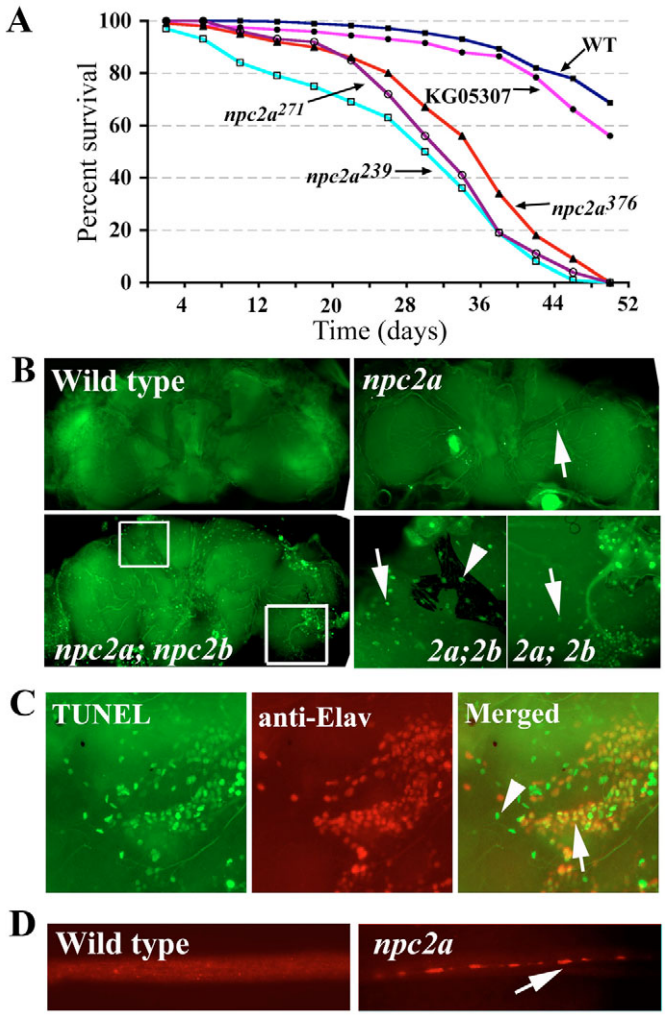

Fig. 7. Neurodegeneration in *npc2a; npc2b* double mutant *Drosophila*. (**A**) Survival data for flies of different genotypes. All mutant designations refer to homozygous animals. KG05307 indicates the starting strain for generating *npc2a* mutants. The *x*-axis indicates the time in days and the *y*-axis shows the percentage of flies surviving. (**B**) Evidence for apoptotic cell death in the nervous system of mutant flies. Wild-type brains (upper left) had little or no TUNEL staining, so there was little normal cell death. A small number of cells were stained by TUNEL in *npc2a* mutants (upper right, arrow). Lower left and, magnified, lower right: *npc2a; npc2b* double mutants had far more frequent death of neurons (arrow) and tracheal cells (arrowhead). (**C**) The apoptotic cells (labeled by TUNEL) in *npc2a; npc2b* double mutants included neurons (labeled by anti-Elav, arrow in the merged panel) and non-neurons (arrowhead in the merged panel). (**D**) Synaptotagmin staining of wild-type and *npc2a* axon bundles. The accumulation of Synaptotagmin (arrow) within axon tracts was observed in a small number of axons in *npc2a* mutants but not wild type.

The pattern of introns in the *Drosophila npc2* gene family provides additional insight into their evolution by suggesting a possible sequence of gene duplication events. The intron-less *npc2* genes (*npc2a*, *g*, *h*) may have come first, as the vertebrate genes also lack introns. Next to arise would be *npc2* genes like *npc2b* and *d* that have a single intron in position 1. An additional intron appears at position 2 in *npc2c* and *e*, and the most elaborate gene, *npc2f*, has a third intron in position 3. Alternatively, the ancient gene may have had three introns, and the other genes have been generated by successive loss of introns. As the intron positions in vertebrate *NPC2* genes are almost identical to those in *Drosophila npc2* genes, one can speculate that they were generated in the same order through evolution.

The cholesterol-shortage model of NPC disease

As a classical lysosomal storage disease, NPC disease is characterized by the accumulation of large amounts of free cholesterol and other lipids in lysosome-like compartments. The search for the causes of this pathology focused mainly on potential cytotoxic effects caused by the accumulation of cholesterol and other lipids (Patterson and Platt, 2004). However, cholesterol-lowering drug treatments did not alleviate NPC disease progression and sometimes made it worse, arguing strongly against the original sterol-excess theory of the disease (Akaboshi and Ohno, 1995; Somers et al., 2001). To elucidate the molecular and cell biology of NPC protein functions, and shed light on the causes of NPC pathology, NPC models have been established in yeast, worms, flies and mice.

Our studies of *Drosophila npc2* genes are consistent with the sterol-shortage model proposed previously (Huang et al., 2005). In this model, sterols are trapped in aberrant organelles in NPC mutant cells, and therefore insufficient amounts of sterol reach the ER or mitochondria. In mammals, the lack of sufficient sterol in the ER triggers a homeostatic activation of transcription of genes that encode machinery for the synthesis and import of sterol, thus setting in motion a sustaining cycle of excess sterol, leading to more excess sterol. In flies and mice, the failure to bring sufficient sterol substrate to the ER/mitochondria could deprive cells of the ability to synthesize adequate steroid hormone. The consequences are different between mammals and flies, because the actions of steroids are quite different. In flies the principal steroid hormone is 20E, the molting hormone, so the defect is a failure to molt. In mammals the cerebellar Purkinje neurons are known to produce multiple neurosteroids, although their functions are far from clear (Tsutsui et al., 1999). *Npc1/Npc1* mutant mice are deficient in neurosteroids, and administration of supplementary allopregnanolone reduces the symptoms of NPC disease (Griffin et al., 2004). Thus, both fly and mouse NPC mutants are steroid hormone deficient and both mutants can be rescued by exogenous steroid hormone treatment, suggesting strongly that cholesterol and the consequent steroid shortages play a central role in NPC disease.

npc1a and *npc2* define a new kind of gene involved in 20E biosynthesis

Our studies reveal a new layer of ecdysteroid biosynthesis regulation, i.e. sterol substrate availability. The regulation of ecdysteroid biosynthesis and the downstream events that mediate ecdysteroid hormone action have been studied continuously for several decades using genetic and biochemical approaches (Gilbert et al., 2002). To date, many genes that affect 20E biosynthesis have been identified and characterized, and these can be grouped into four functional classes. The first class of genes includes upstream factors such as prothoracicotropic hormone (PTTH) that control whether the prothoracic gland should synthesize ecdysone or not. A PTTH mutant has not been isolated in *Drosophila*, but studies in other insects have clearly demonstrated the essential function of PTTH in ecdysteroid biosynthesis (Gilbert et al., 2002). The larval arrest phenotypes resulting from ablating *Drosophila* neurons that produce PTTH are consistent with a role in governing ecdysteroid biosynthesis (X.H. and M.P.S., unpublished).

The second class of genes consists of the yet-to-be-identified PTTH receptor and the Ras signaling cascade that transduces the PTTH signal. Ras appears to act through its downstream effector Raf to control ecdysteroid biosynthesis (Caldwell et al., 2005). The third class of genes includes nuclear transcription factors and regulators, such as *ftz-f1*, *ecd*, *woc* and *mld* (Gaziova et al., 2004; Neubueser et al., 2005; Parvy et al., 2005; Wismar et al., 2000). The targets of these proteins are not well defined but may include the fourth class of genes, the Halloween genes (e.g. *dib*, *sad*, *phm*, *shd*, *spo* and *spo2*) that encode p450 enzymes that mediate the conversion of cholesterol to 20E through multi-step reactions in the ER and mitochondria (Chavez et al., 2000; Gilbert and Warren, 2005; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2002).

The present study, together with our previous study on *Drosophila npc1a*, defines a fifth class of genes functioning to ensure a sufficient supply of sterol substrates for 20E biosynthesis. This class of mutants has intact 20E biosynthetic enzymes, as shown indirectly by our feeding and rescue experiments, but has insufficient sterol substrate for 20E production. Therefore, the ecdysteroid-deficient mutant phenotype can be suppressed by excess cholesterol or 7-dehydrocholesterol, as in *npc1a* or *npc2* (*a* and *b*) mutants. Other members of this gene class may include some START domain-containing proteins as well as PBR, which are implicated in transporting sterol into mitochondria for steroid biosynthesis in mammals (Stocco, 2001).

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