A *Drosophila* model of the Niemann-Pick type C lysosome storage disease: *dnpc1a* is required for molting and sterol homeostasis

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Summary

Niemann-Pick type C (NPC) disease is a fatal autosomal-recessive neurodegenerative disorder characterized by the inappropriate accumulation of unesterified cholesterol in aberrant organelles. The disease is due to mutations in either of two genes, *NPC1*, which encodes a transmembrane protein related to the Hedgehog receptor Patched, and *NPC2*, which encodes a secreted cholesterol-binding protein. *Npc1* mutant mice can be partially rescued by treatment with specific steroids. We have created a *Drosophila* NPC model by mutating *dnpc1a*, one of two *Drosophila* genes related to mammalian *NPC1*. Cells throughout the bodies of *dnpc1a* mutants accumulated sterol in a punctate pattern, as in individuals with *NPC1* mutations. The mutants developed only to the first larval stage and were unable to molt. Molting after the normal first instar period was restored to various degrees by feeding the mutants the steroid molting hormone 20-hydroxyecdysone, or the precursors of ecdysone biosynthesis, cholesterol and 7-dehydrocholesterol. *dnpc1a* is normally highly expressed in the ecdysone-producing ring gland. Ring gland-specific expression of *dnpc1a* in otherwise mutant flies allowed development to adulthood, suggesting that the lack of ecdysone in the mutants is the cause of death. We propose that *dnpc1a* mutants have sterols trapped in aberrant organelles, leading to a shortage of sterol in the endoplasmic reticulum and/or mitochondria of ring gland cells, and, consequently, inadequate ecdysone synthesis.

Key words: Niemann-Pick Type C, Sterol, Steroid, Ecdysone, *Drosophila*, Lysosome storage

Introduction

Niemann-Pick Type C disease (NPC) is both a lysosomal storage disorder and a neurodegenerative disease. At the cellular level, the most notable aspect of the disease is a massive accumulation of cholesterol, glycosphingolipids and other lipids in aberrant organelles. The underlying defect appears to be a failure of normal organelle trafficking and a consequent failure of lipid homeostasis (Liscum and Sturley, 2004; Mukherjee and Maxfield, 2004; Sturley et al., 2004). The disease is inherited as an autosomal recessive condition. The disease is caused by mutations in either of two genes, *NPC1* (Carstea et al., 1997) and *NPC2* (Naureckiene et al., 2000).

Neurons in individuals with NPC gene mutations and in a cat model of the disease grow extra dendritic processes and form neurofibrillary tangles (NFTs) (Walkley and Suzuki, 2004), and progressive neurodegeneration is a prominent symptom, particularly in the cerebellum (Higashi et al., 1993). Options for therapy are highly limited at this time (Patterson and Platt, 2004). The outcome for individuals with NPC is usually death in the teenage years. Understanding the origins of NPC is important to find ways to save lives and because it provides an entry point for studying still-mysterious aspects of lipid and transport cell biology.

The two NPC genes encode entirely different types of proteins that probably participate in the same pathway, though the molecular mechanisms that link the two proteins are unknown (Vanier and Millat, 2003). NPC1, a cholesterol binding (Ohgami et al., 2004) 13 transmembrane region protein (Davies and Ioannou, 2000) is required for normal movements of populations of late endosomes and for proper homeostatic regulation of sterol and other lipid levels. Further interest in the NPC1 protein derives from its striking sequence similarity to the Patched protein (Carstea et al., 1997), the receptor for Hedgehog signaling proteins that regulate many aspects of growth and cell fate determination during development (Hooper and Scott, 2005). Another closely related protein, NPC1L1, is crucial for intestinal uptake of cholesterol (Altmann et al., 2004; Davies et al., 2005). NPC1 works in a mysterious partnership with NPC2, a lysosomal protein that can be secreted and that binds strongly to cholesterol (Naureckiene et al., 2000; Friedland et al., 2003; Ko et al., 2003). The central mysteries still remain: what are the molecular functions of NPC1 and 2, how does either one regulate organelle movements and molecular trafficking, and why does loss of either protein lead to neurodegeneration and other symptoms?

The *npc1* gene is well conserved through about a billion years of evolution, allowing studies with a variety of powerful

experimental organisms (Higaki et al., 2004). Useful *NPC1* models have been generated using yeast (Malathi et al., 2004) and worm (Sym et al., 2000), although no sterol trafficking defect has been reported in either model. By mutating one of the two *NPC1*-like fly genes, *dnpc1a* (*NPC1* – FlyBase) we have generated a *Drosophila* model of NPC1 that has a cholesterol accumulation defect similar to that of mammalian NPC mutants. *dnpc1a* mutants accumulate sterol in a punctate pattern in many tissues, implying a conserved role of NPC1 in cholesterol trafficking from fly to mammals.

We find that *dnpc1a* function is crucial for normal steroid hormone metabolism. Neurosteroid treatment has been shown to suppress neurodegeneration in *Npc1* mutant mice (Griffin et al., 2004), implying a neurosteroid hormone deficiency in *Npc1* mice that may parallel the defect in *Drosophila*. Our studies with *dnpc1a* mutants suggest a model for NPC1 function: the protein may allow delivery of sufficient sterol to mitochondria in order for steroid hormones to be made there. More complete understanding of the molecular and cell biology of these pathways may increase the options for NPC therapy.

Materials and methods

Drosophila stocks

Flies were cultured on standard cornmeal medium at 25°C unless otherwise specified. UAS-*dnpc1a-yfp* transgenic stocks were generated by standard methods. The GAL4 driver lines: *tub*-GAL4, 69B-Gal4, 48Y-Gal4, MHC-Gal4, *elav*-Gal4, *Feb36-GAL4*, Aug 21-Gal4 and 2-*286-GAL4* were used in our studies. All other stocks are described in FlyBase and are available from the Bloomington Stock Center. *dnpc1a* excision mutagenesis was performed using the *Δ2-3* chromosome, which carries a stable source of P-transposase (Robertson et al., 1988). Two alleles of *dnpc1a* (*dnpc1a*^{1} and *dnpc1a*^{2}) were isolated. Genomic DNA from *dnpc1a* heterozygotes was amplified with primers flanking the deletion region, and PCR products were sequenced to determine the molecular lesions.

Molecular biology

Full-length *dnpc1a* cDNA was amplified by RT-PCR and subcloned into pBluescript SK. The stop codon was replaced with a *Kpn*I site using PCR and the new full-length cDNA was inserted in frame into the *Kpn*I site of pEYFP-N3 to create a dNPC1a-YFP fusion construct (pEYFP-*dnpc1a*). From this clone, a 4.3 kb *R*I-*Not*I fragment containing full-length *dnpc1a* cDNA and EYFP-coding region was inserted into the pUAST vector to create the pUAST-*dnpc1a-yfp* construct. All constructs requiring PCR amplification were confirmed by sequencing.

RNA hybridization

dnpc1a and *dnpc1b* transcripts were detected by in situ hybridization of full-length antisense and sense (control) probes to 0- to 16-hour-old embryos and wandering third instar larvae; detection was with anti-DIG alkaline phosphatase and the CBIP/NBT substrate (Roche).

RNAi

dsRNA was prepared from PCR-generated template that corresponds to nucleotides 3086-3603 of *dnpc1a* cDNA. dsRNA was synthesized with the Ambion Megascript kit. Zero to 1 hour dechorionated embryos were injected under oil with dsRNA at 2 μM.

Lethal phase determination

Each *dnpc1a* allele was balanced with *CyO, P[w+, act-GFP]*. The flies were allowed to lay eggs on apple juice plates, supplemented with baker's yeast paste, at 25°C. Eggs were collected in 2-hour periods, and embryos or larvae were identified as *dnpc1a* homozygotes by the absence of the GFP-marked balancer chromosome. Larval stages were determined by the appearance of the mouth hooks.

Sterol and ecdysone feeding

For *dnpc1a* mutants, each group of 200 first instar larvae was placed on apple juice plates with baker's yeast paste containing supplement sterols, and the lethal phases were determined. The final concentrations for the sterols used were: cholesterol, 0.14 mg/g and 1.4 mg/g; 7-dehydrocholesterol, 0.014 mg/g, 0.14 mg/g and 1.4 mg/g; 20-hydroxyecdysone (20E), 8 μg/g; ergosterol, 0.14 mg/g and 1.4 mg/g; progesterone, 1.4 mg/g; and desmosterol, 1.4 mg/g.

Filipin staining and brain histology

For filipin staining, tissues were fixed in 4% paraformaldehyde for 30 minutes, washed twice in PBS, and stained with 50 μg/ml filipin (Sigma) solution for 30 minutes. Samples were washed twice with PBS before mounted in Vectashield mounting medium. All pictures were taken with a Zeiss compound microscope with a DAPI filter.

The heads of mutant and wild-type flies, obtained 16-24 hours after eclosion, were removed from their bodies. The proboscis and ventral air sac were removed to aid fixation. The heads were placed in Carnoys fixative for 6 hours at room temperature. They were washed in 100% ethanol and embedded in HistoGel (Richard-Allan Scientific). The pellets containing the heads were processed through alcohol, xylene and molten Paraplast. They were embedded in Paraplast and 6 μm sections were cut. The tissue was stained with Haematoxylin and Eosin, and the slides mounted in Permount. Similar methods were used in making brains sections from first-instar larvae.

Electron microscopy

Dissected tissues from wild type and mutants were prepared for TEM using a microwave protocol. We used a Pelco Laboratory microwave (model #3451) equipped with a Cold Spot, Steadytemp chiller/recirculator run at 15°C and vacuum chamber (Ted Pella, Mountain Lakes, CA). Malpighian tubules were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 in the microwave. Following two brief rinses in 0.1 M cacodylate buffer containing 0.3 M sucrose on the bench, the tissue was post-fixed in the microwave using 2% osmium tetroxide in 0.1 M cacodylate buffer containing 0.8% potassium ferricyanide. After rinsing with distilled water, the tissue was dehydrated in an ascending alcohol series in the microwave. The tissue was infiltrated and embedded in Embed 812 resin (Electron Microscopy Sciences, Fort Washington, PA). The tubule samples were oriented in a flat embedding mold and hardened overnight in a 65°C oven. Semi-thin sections (1 to 2 μm) were cut using a Diatome HistoKnife (Diatome, USA) and stained with Toludine Blue. Thin sections (50-70 nm) were cut with a Diatome diamond knife and mounted on Formvar coated grids. The samples were examined on a JEOL 1230 electron microscope at 80 kV and photographed using a Gatan 967 slow-scan cooled CCD camera.

Results

Drosophila NPC1-like genes

A BLAST search of the *Drosophila melanogaster* genome sequence identified two *NPC1*-like genes that we have named *dnpc1a* and *dnpc1b*. *dnpc1a* is predicted to encode a 1287 amino acid protein with 44% similarity and 63% identity to human NPC1, and 38% identity and 56% similarity to human NPC1L1 (Fig. 1A). dNPC1b is a 1254 amino acid protein that shares 38% sequence identity and 55% similarity to human NPC1, and 33% identity and 51% similarity to human NPC1L1 (Fig. 1A). The dNPC1a protein sequence is 39% identical and 58% similar to the dNPC1b sequence.

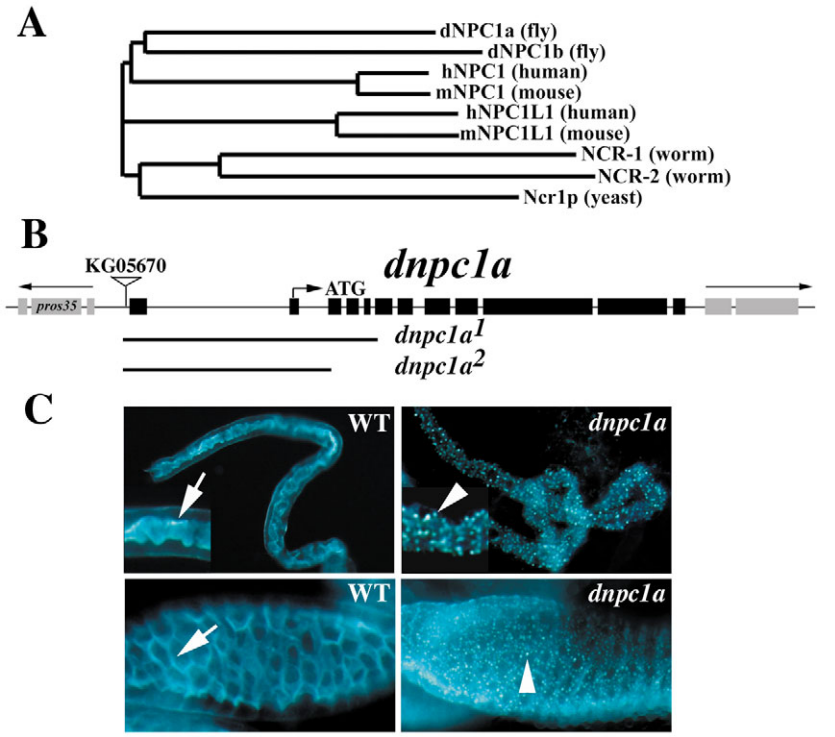

Fig. 1. *dnpc1a* gene and mutant phenotypes. (A) Phylogenetic tree of yeast, worm, fly, mouse and human NPC1 proteins determined using the ClustalW analysis. According to the BDGP prediction (www.flybase.org), *dnpc1b* encodes a 1223 amino acid protein that lacks any signal peptide. However, another putative start codon 93 nucleotides before the BDGP-predicted ATG adds a 31 amino acid peptide that contains a predicted signal peptide and aligns well with the N termini of NPC1 and NPC1L1. (B) The gene structure of *dnpc1a* and deletion regions of two *dnpc1a* alleles. (C) Aberrant sterol accumulation in *dnpc1a* mutants observed using filipin staining. Left column, wild type; right column, *dnpc1a* mutants. Top panels show the Malpighian tubules (inset, magnified view); bottom panels show midguts. (Left) Filipin staining highlights the lumen of the Malpighian tubules (top, arrow) and the cell-cell boundaries of midgut cells (bottom, arrow) in wild-type first-instar larvae. In *dnpc1a* mutants (right), in addition to the normal sites of sterol, punctate accumulations of filipin staining are visible (arrowheads) inside Malpighian tubule and midgut cells.

Expression of *dnpc1a*

As a first step toward unveiling the function of *dnpc1a*, we determined the temporal and spatial expression of *dnpc1a* throughout development by in situ hybridization. During embryonic stages *dnpc1a* is ubiquitously expressed, with higher levels in several tissues. *dnpc1a* RNA is first found in preblastoderm and blastoderm embryos, reflecting its presence in maternally provided mRNA (Fig. 2A). After the blastoderm embryo changes from a syncytium to individual cells, the level of *dnpc1a* RNA increases and becomes further concentrated in the extending germband (Fig. 2B). The highest expression is seen in the hindgut of fully extended germband embryos (Fig. 2C). During germband retraction and before dorsal closure, expression in the aminoserosa is detected (Fig. 2D). Starting at stage 16, strong staining of the putative prothoracic gland cells in the embryo was observed (Fig. 2E). The prothoracic gland cells of the ring gland are the predominant source of ecdysteroids, the molting hormones, in *Drosophila* post-embryonic development.

In wandering third-instar larvae the *dnpc1a* RNA level is highest in the prothoracic gland component of the ring gland (Fig. 2F), and is accompanied by ubiquitous expression in other tissues including brain, garland cells, midgut and imaginal discs (Fig. 2F,G). The observed spatial and temporal pattern of expression of *dnpc1a* suggests that, like *NPC1* in mammals, *dnpc1a* probably functions in all cells. The higher *dnpc1a* RNA in embryonic and larval ring glands implies that *dnpc1a* may be involved in the regulation of ecdysteroids that are produced there.

Compared to *dnpc1a*, *dnpc1b* has a much more restricted expression pattern. *dnpc1b* mRNA can be detected in midgut and hindgut during late embryonic stages; any other signal is weak to undetectable (data not shown).

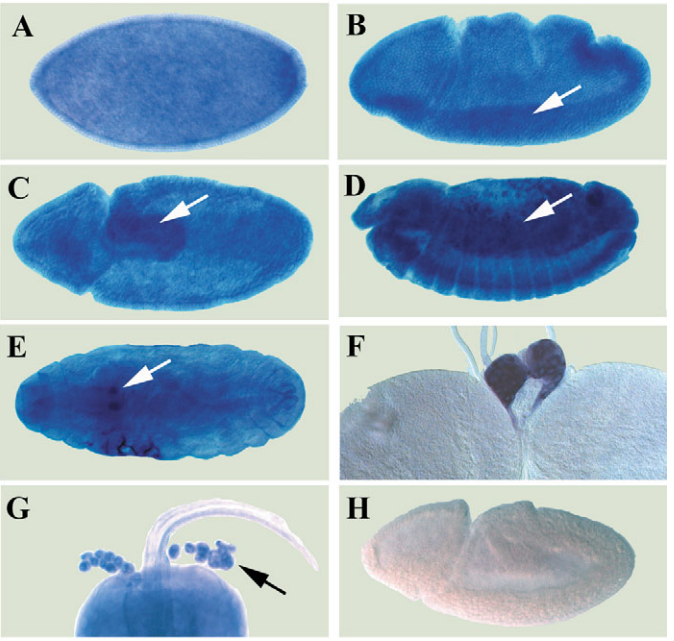

Fig. 2. Localization of *dnpc1a* RNA in embryos and larvae. (A-E) Anterior towards the left. (A) Ubiquitous expression of *dnpc1a* in a cellular blastoderm-stage embryo. (B) Higher level of expression can be seen in gastrulation furrow (arrow) of stage 7 embryo. (C) After germband extension, higher level expression is in the hindgut (arrow). (D) Aminoserosa (arrow) staining can be seen in a stage 14 embryo. (E) Putative prothoratic gland cells (arrow) in stage 16 embryos. (F) High level expression of *dnpc1a* mRNA in the ring gland of wandering third-instar larvae. (G) *dnpc1a* mRNA is also present in the garland cells (arrow). (H) In situ hybridization with *dnpc1a* probe detects no signals in *dnpc1a*^{1} mutant embryo.

dnpc1a mutants die during an extended first larval instar

To explore the role of *dnpc1a* in development further, we used double-stranded RNA (dsRNA) interference and generated loss-of-function mutants. *dnpc1a* dsRNA-injected embryos developed normally during embryogenesis, but most of them (65%, *n*=87) arrested during the first larval stage.

Next, we produced loss-of-function *dnpc1a* mutations. The *dnpc1a* gene is located at cytological band 31B1. A mutation caused by a transposon insertion, KG05670, had been assigned to the *pros35* gene, a gene adjacent to *dnpc1a* and transcribed in the opposite direction (Fig. 1B). The KG05670 transposon is closer to the 5′ UTR of *dnpc1a* than to *pros35* (Fig. 1B). Using KG05670 as a starting strain for imprecise excision, we generated two deletion alleles of *dnpc1a* that cause N-terminal 182 and 45 amino acid deletions. The alleles will be referred to as $dnpc1a^{1}$ and $dnpc1a^{2}$ (Fig. 1B). Based on the nature of the deletions, these two mutations are likely to be null alleles of *dnpc1a*. In situ hybridization with a probe encompassing the coding region detected no signals in those mutant embryos, providing further evidence that they are null alleles (Fig. 2H).

Consistent with the dsRNAi result, flies homozygous for either of the *dnpc1a* alleles, or trans heterozygous for the combination, died as first-instar larvae. The larval lethality can be fully rescued by ubiquitous expression of a *dnpc1a* cDNA-*yfp* fusion construct, confirming that the lethal phenotype is indeed caused by loss of *dnpc1a* function and not loss of *pros35* function. Embryos produced by mothers homozygous for a *dnpc1a* mutation in their germline cells, and fertilized by *dnpc1a* mutant sperm, also died during the first-instar larval stage, showing that *dnpc1a* is essential for larval development but not for embryogenesis (data not shown).

A dNPC1a function in sterol metabolism

To address whether *Drosophila dnpc1a* plays a role in cholesterol trafficking like its mammalian homolog *NPC1*, the distribution of sterol in wild-type and *dnpc1a* mutant larvae was examined using filipin staining. Filipin stains free 3-β-hydroxysterols (Friend and Bearer, 1981), including ergosterol and cholesterol, so the filipin-staining pattern may reflect the localization of ergosterol, cholesterol and perhaps other sterols. *Drosophila* is unable to synthesize its own sterol, instead obtaining sterol from its food (Clark and Block, 1959). Ergosterol is abundant in fungi, yeast and plants, and can substitute for cholesterol to sustain the growth and reproduction of the fly, so ergosterol is likely to be a major sterol source for laboratory flies that live on a yeast-rich medium.

Owing to the small size of first-instar larvae, the staining pattern was first examined in large tissues from this stage: Malpighian tubules and midgut. In wild-type animals, the fluorescent filipin signal highlights the lumen of Malpighian tubules and the cell-cell boundaries of the midgut (Fig. 1C). In *dnpc1a* mutants, in addition to the normal localization, a punctate pattern of fluorescence was observed inside cells in both tissues, reflecting sterol trapped in aberrant subcellular structures (Fig. 1C). This subcellular sterol accumulation phenotype is similar to that of mammalian *NPC* cells, suggesting a conserved role of *dnpc1a* in intracellular sterol trafficking.

The first instar arrest of *dnpc1a* mutants can be prevented by ecdysone in the food

At 25°C, wild-type first-instar larvae normally molt to second instar ~48 hours after egg laying (AEL), i.e. about 1 day after hatching. Homozygous *dnpc1a* mutants remained as first-instar larvae for a prolonged period before dying 90-192 hours AEL (Fig. 3A,B). One possible cause of the arrested development is a failure to molt. Molting is normally controlled by a pulse of ecdysone, a steroid that serves as the molting hormone. Ecdysone is synthesized from cholesterol that is, in turn, derived from diet sterols (yeast ergosterol/plant sterol) (Gilbert et al., 2002). Flies reared on the ergosterol biosynthesis-defective yeast mutant, *erg-6*, die during a prolonged first instar, similar to that of *dnpc1a* (Parkin and Burnet, 1986).

Thus, the first-instar arrest of *dnpc1a* mutants may well be due to a defect in ecdysone production. The high level of *dnpc1a* transcription normally present in the ecdysone-producing organ, the ring gland, is consistent with this hypothesis. Alternatively, the *dnpc1a* mutant phenotype may be due to a defect in the response to ecdysone. To distinguish these two possibilities, we performed 20-hydroxyecdysone-feeding experiments. 20-Hydroxyecdysone (20E) converted from ecdysone (E) is the active molting hormone in vivo. If the

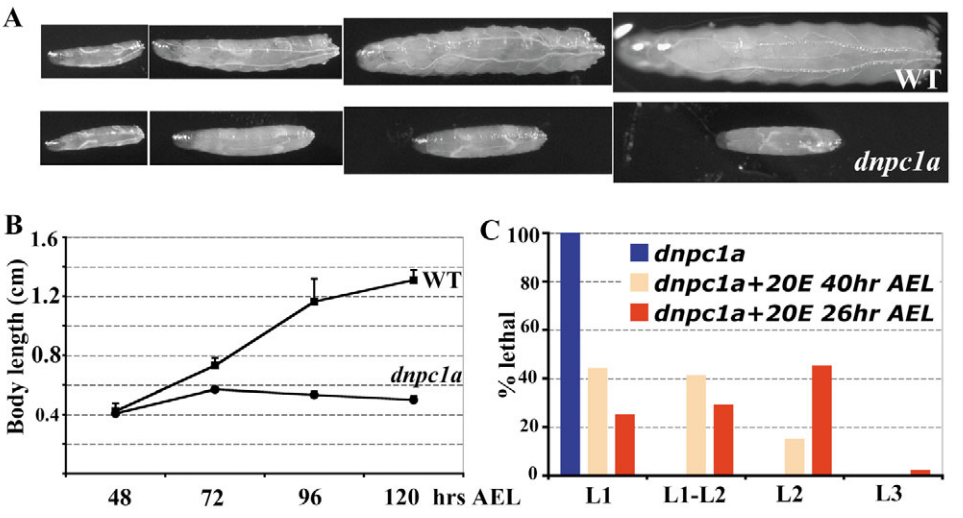

Fig. 3. *dnpc1a* mutants are arrested during the first instar and can be partially rescued by ecdysone feeding. (A) Comparison of different sizes of larvae of wild type (top) versus *dnpc1a* mutants (bottom) at different times points after egg laying (AEL) shown in B. (B) Graphic view of the body length of the wild type (*n*=50) and *dnpc1a* mutant larvae (*n*=50) at different times points after egg laying. (C) 20-Hydroxyecdysone feeding of developing larvae at two different time points can postpone the lethal stages of *dnpc1a* mutants. *x*-axis, stages of development.

defect is in ecdysone production, feeding 20E should rescue *dnpc1a* mutants. By contrast, if the defect is in the response to ecdysone, feeding the larvae 20E would probably not fix the first larvae arrest of *dnpc1a* mutants. In any case, any rescue accomplished by adding 20E to the food would indicate that a cause of death is inability to molt and possibly a hormone deficiency.

Without 20E, 100% of *dnpc1a* homozygotes died during the first-instar stage. Feeding the *dnpc1a* mutants 8 μg 20E per gram of medium starting in the early first instar (26 hours AEL) prevented much of the first-instar arrest: 25% of the animals died at first instar, 29% died during the first to second instar transition (with the double pairs of mouth hooks characteristic of that transition; not shown), 45% died in the second instar and 2% died in the third instar (Fig. 3C). If the feeding with 20E was initiated late in the first instar (40 hours AEL), the rescue was similar but weaker (Fig. 3C). The results indicate that the first instar arrest of *dnpc1a* mutants is likely to be a consequence of insufficient ecdysone.

High cholesterol or 7-dehydrocholesterol in the medium rescues *dnpc1a* mutants

For insect ecdysone biosynthesis, the substrate cholesterol is first converted to 7-dehydrocholesterol, probably by a microsomal/endoplasmic reticulum (ER)-localized P450 enzyme (Gilbert et al., 2002). The 7-dehydrocholesterol must translocate to the ring gland mitochondria, and then move into the internal mitochondrial membrane for further chemical modifications that produce ecdysone (Gilbert et al., 2002).

The aberrant sterol accumulation and the apparent shortage of cholesterol-derived ecdysone in *dnpc1a* mutants seem to create a paradox. The cells have abundant, in fact excessive, sterol that should be sufficient for ecdysone biosynthesis. Perhaps the abnormal sterol accumulation leads to a local shortage of sterol precursor available for ecdysone biosynthesis. Alternatively, it could be that sterol accumulation is somehow toxic and inhibits the ecdysone biosynthesis machinery. To distinguish these possibilities, we increased the sterol concentration in the yeast paste. Although the main sterol in yeast paste is ergosterol (~0.3 mg/g), yeast paste medium also contains a trace of cholesterol (~0.6 μg/g) (Xu and Nes, 1988). The first-instar arrest of the *dnpc1a* mutant was significantly suppressed by increasing cholesterol in the food from a trace amount to 0.14 mg/g or 1.4 mg/g (Fig. 4A).

Sterol availability is evidently limiting in *dnpc1a* mutants, suggesting that the accumulated mass of sterol in the mutant cells is not available for steroid synthesis. A high level of cholesterol added to the media bypasses the sterol defect, perhaps by allowing sterol to reach the endoplasmic reticulum (ER) or mitochondria directly to nourish ecdysone biosynthesis.

Ergosterol is able to support the growth and reproduction of *Drosophila* (Clark and Block, 1959). Curiously, adding the level of ergosterol to the medium that allowed rescue by cholesterol (1.4 mg/g or 0.14 mg/g) did not have any rescuing activity (Fig. 4A). This may indicate that cholesterol and ergosterol are moved into or within cells along at least partly different paths, or that the ergosterol is more susceptible to the diversion into aberrant organelles in the mutant cells.

7-dehydrocholesterol is the first metabolic product on the path from cholesterol to ecdysone. Feeding *dnpc1a* mutants

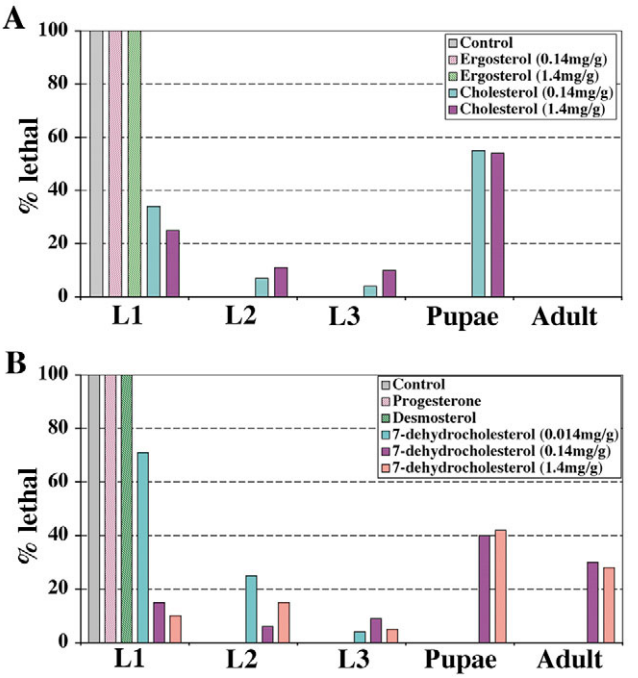

Fig. 4. High media level of cholesterol or 7-dehydrocholesterol can rescue *dnpc1a*. (A) Cholesterol, but not ergosterol, can partially rescue *dnpc1a* mutants. (B) 7-Dehydrocholesterol can rescue *dnpc1a* mutants to adulthood. Desmosterol and progesterone do not rescue. *x*-axis, stages of development.

with a high level of 7-dehydrocholesterol was even more effective in suppressing the first-instar lethal phenotype of the *dnpc1a* mutant than cholesterol feeding (Fig. 4B). A significant percentage of rescued flies even reach adulthood, although they usually died within a day or two after eclosion (Fig. 4B). By contrast, feeding the *dnpc1a* mutants with desmosterol, a sterol that can be used to make ecdysone by some insects but not by *Drosophila melanogaster* (Gilbert et al., 2002), or with progesterone, a human steroid hormone derived from cholesterol, did not rescue at all (Fig. 4B).

Cholesterol and 7-dehydrocholesterol are much more potent rescuing agents than 20-hydroxyecdysone, suggesting that in the presence of enough proper substrate, *dnpc1a* mutant larvae were able to synthesis their own ecdysone and quite possibly control the timing and amount of hormone production. 20-Hydroxyecdysone may rescue more poorly because the mutant larvae cannot control the time of exposure, location or amount of ecdysone. The results also indicate that the enzymatic machinery for ecdysone biosynthesis is probably fine in *dnpc1a* mutants.

No obvious neurodegeneration in *dnpc1a* mutants

As the *Drosophila dnpc1a* model recapitulates the sterol accumulation phenotypes of NPC disease, we investigated whether the flies also have a neurodegeneration problem, another characteristic of mammalian NPC disease. *Drosophila* neurodegeneration mutants often have a short life span and numerous large vacuoles in brain (Min and Benzer, 1999; Palladino et al., 2002; Tschape et al., 2003). Although *dnpc1a* mutants die during the first instar, 7-dehydrocholesterol treatment can extend the mutant lifespan to adulthood. This

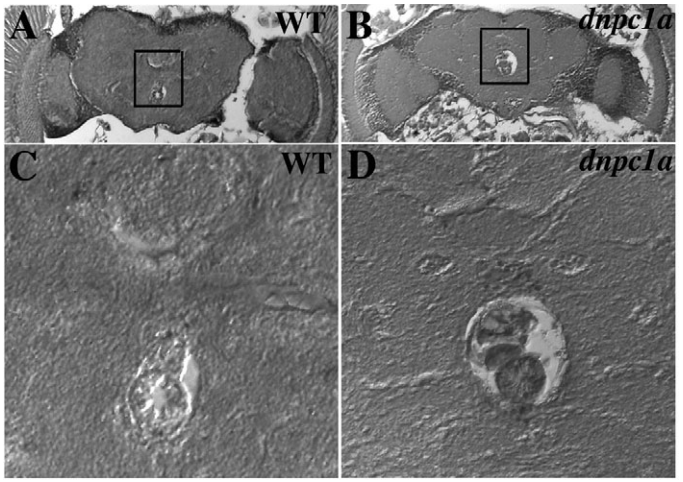

Fig. 5. No significant difference in the brain sections of wild type and *dnpc1a* mutants. The comparison of the brain histology between a wild-type adult animal (A) and a 7-dehydrocholesterol feeding-treated *dnpc1a* mutant adult (B) reveals no significant difference. (C,D) Enlarged views of the central brain regions boxed in A,B, respectively. No obvious characteristic neurodegenerative vacuoles are observed in *dnpc1a* mutants.

provided an opportunity to examine the adult brain and search for possible neurodegeneration. We sectioned brains from 7-dehydrocholesterol-treated *dnpc1a* sick adult escapers before they died. The gross brain morphology is fine in the mutants and there were no evidence of neurodegenerative vacuoles in the brain sections (Fig. 5). To further investigate possible neurodegeneration that might be missed in animals partially rescued with sterol, brains from 96 hour AEL first-instar *dnpc1a* mutants were examined directly. Again the gross brain morphology was fine in the mutants, with no evidence of typical neurodegenerative vacuoles (data not shown). We cannot exclude the possibility that neurodegeneration may happen in small subset(s) of neurons.

dnpc1a is required in ring gland for larval development

The rescue by ecdysone suggests a hormone deficiency, and the rescue by specific sterols suggests a sterol deficiency. The apparent sterol deficiency could be due to either less sterol uptake from the food, causing a global shortage, or less sterol available for ecdysone production in ring gland cells, causing a local shortage in that tissue. The former possibility seems less likely as abundant sterol accumulates in aberrant organelles in most or all tissues. The 'shortage' of sterol in any tissue, it appears, is mainly a problem of accessing the sterol.

We investigated which tissue requires *npc1a* function in order for normal molting to occur. If *dnpc1a* is required in tissues where sterol is absorbed from food, such as midgut, the primary defect is probably a global shortage of sterol. If *dnpc1a* is required within cells that make hormone, then the defect is very likely to be due to a local shortage and the failure to make enough hormone. If *dnpc1a* is required in tissues that undergo metamorphosis, the primary defect is probably in the response to hormone or in a sterol-related function other than hormone synthesis, or both.

We used the UAS-Gal4 system to drive tissue-specific expression of a functional *dNPC1a-YFP* fusion gene in otherwise *dnpc1a* mutant flies. Tub-Gal4, a Gal4 driver that activates target genes in all tissues, was combined with *UAS-dnpc1a-yfp*. This pair of transgenes fully rescued *dnpc1a* mutants so that they developed into fertile adults, and also prevented abnormal sterol accumulation in all tissues (Table 1). 69B-Gal4, which drives *dNPC1a-YFP* expression in ring gland, brain, embryonic epidermis, imaginal discs and testis, also fully restores development of *dnpc1a* mutants into fertile adults (Table 1).

The most informative experiment came from using Gal4 drivers that produce ring gland-specific expression of *dNPC1a-YFP* (Timmons et al., 1997). Either the 2-286 or Feb36 Gal4 driver allowed otherwise *dnpc1a* mutants to enjoy robust adult viability. The subcellular accumulation of sterol in many tissues other than the ring gland was not reduced under these conditions (Table 1 and Fig. 6B,D,H), providing confirmation that the main ectopic expression of the rescuing gene is in the ring gland. The ring gland is composed of the prothoracic gland (producing ecdysone hormone), the corpora allata (producing juvenile hormone) and the corpora cardiaca. The Feb36 Gal4 driver drives expression in the prothoracic gland and corpora allata. Moreover, a corpora allata-specific Gal4 driver (Aug21) did not provide any rescuing activity, so *dnpc1a* is required in

Table 1. *dnpc1a* is required in ring gland for larval survival

Gal4 driver	Tissue specificity	*dnpc1a* animals rescue to (% *n*)*	Number of larvae (*n*)
Tubulin	Ubiquitous	Adult (90)	120
69B	Ring gland, brain, embryonic epiderm, imaginal discs, testis	Adult (ND)	>40
2-286	Ring gland, salivary glands	Adult (87)†	100
Feb36	Ring gland (prothoracic gland and corpora allanta), salivary glands, trachea, midgut, malpighian tubules	3rd instar and adult (75)†	60
Aug21	Corpora allanta, few neurons	None	>50
Elav	Pan neuronal, salivary glands	None	>50
48Y	Embryonic endoderm	None	>50
MHC	All muscles	None	>50

*The survival stage of *dnpc1a/dnpc1a; Gal4 driver/UAS-dnpc1a-yfp* animals were determined.
†Male semi-sterile.
Gal4 drivers were used to target expression of *UAS-dnpc1a-yfp* to specific tissues/cells in *dnpc1a* mutants.
The percentage of rescued (% *n*) is calculated by dividing the number of rescued animals with total number (*n*) of the same genotype animals examined.
ND, not determined.

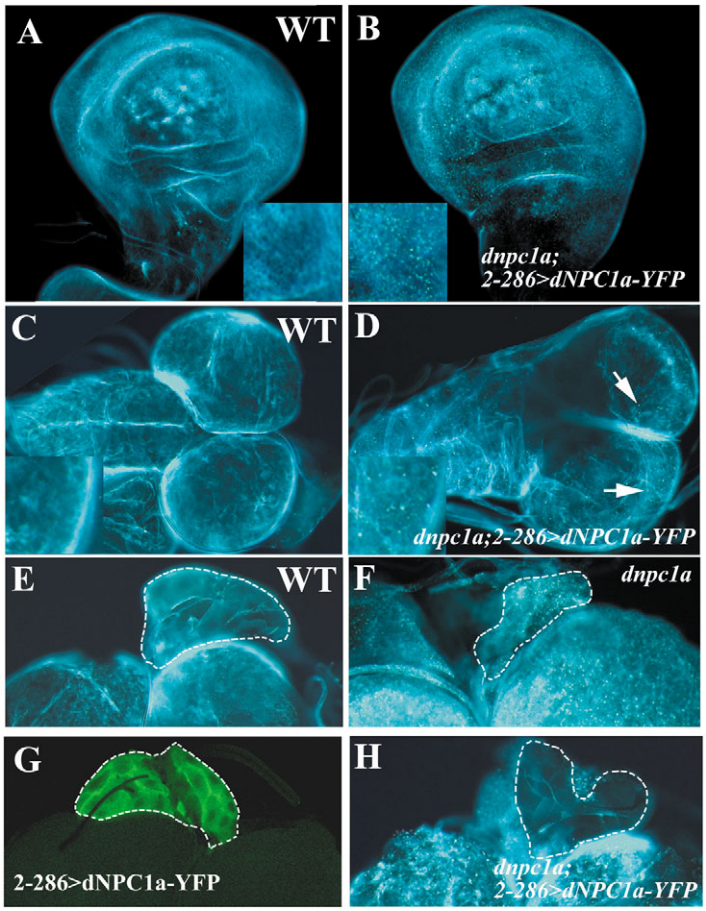

Fig. 6. Sterol accumulation in *dnpc1a* mutants. (A,B) Filipin-stained wing imaginal discs from wild type (A) and *dnpc1a* mutant (B) third-instar larvae. Insets show magnified views; sterol is mainly located at cell-cell boundaries in wild type, while it accumulates in a punctate pattern in *dnpc1a* mutants. (C,D) Filipin-stained brains from wild-type (C) or *dnpc1a* mutant (D) third-instar larvae. There is no punctate accumulation present in wild type (C), but some accumulation can be seen in *dnpc1a* mutants (arrows in D). Insets show magnified views. (E-H) Third-instar ring glands (outlined) in different backgrounds. (E) Wild-type ring gland filipin staining. (F) Filipin staining of ring gland in ecdysone-rescued *dnpc1a* mutants. Punctate sterol accumulation can be seen in both ring gland and brain lobes. (G) The 2-286 driver specifically induces the expression of *dnpc1a*-YFP in ring gland but not brain lobes. In order to see the brain lobes, the signal was boosted. (H) *dnpc1a* mutant rescued by 2-286 driving *dnpc1a*-YFP. The ring gland shows a wild-type filipin staining pattern, while the brain lobes still have punctate sterol accumulation.

the ecdysone-producing prothoracic gland cells of the ring gland. By contrast, many non-ring gland GAL4 drivers we tested, such as the pan-neural driver, *elav*-Gal4, the endoderm (midgut)-specific driver 48Y-Gal4 and the muscle-specific driver MHC-Gal4, were not able to rescue the development of *dnpc1a* mutants beyond first larval instar (Table 1).

These results indicate that *dnpc1a* is required in the prothoracic gland component of ring gland for ecdysone biosynthesis, supporting the hypothesis that *dnpc1a* mutants cannot avail themselves of the sterol in the aberrant organelles and therefore cannot make adequate molting hormone. The mutant flies rescued with ring gland expression of *dnpc1a* provided a good opportunity to examine *dnpc1a* functions in other tissues at later stages.

dnpc1a larval and adult phenotypes in flies with ring gland-specific expression of *dNPC1a-YFP*

dnpc1a mutants with ring gland-specific expression of *dNPC1a-YFP* driven by the 2-286 driver were used to examine phenotypes in third-instar larvae and adults. The tissues examined, brain, imaginal discs, trachea, ovaries, testis and Malpighian tubules, are in some cases too small to dissect and study in detail in first-instar larvae. In all mutant tissues examined, normal filipin staining was seen at cell-cell boundaries and surfaces, plus abnormal sterols accumulated as in mutant first-instar larvae (Fig. 6). We directly compared sterol accumulation in the third instar ring glands and brains from wild type, *dnpc1a* mutants rescued by ecdysone feeding and *dnpc1a* mutants rescued by ring gland-specific expression of *dnpc1a-YFP*. As expected, no sterol accumulated in wild-type ring glands and brains (Fig. 6C,E). *dnpc1a* mutants rescued by ecdysone feeding have sterol accumulation in both ring glands and brains (Fig. 6D,F), while *dnpc1a* mutants rescued by ring gland-specific expression of dNPC1a-YFP have no sterol accumulation in the ring glands but have sterol accumulation in the brains (Fig. 6G,H).

Among all the mutant tissues examined, Malpighian tubules, which serve a function similar to that of mammalian kidneys, had the most robust sterol accumulation phenotype (Fig. 7A,B). To examine the structures of the punctate sterol accumulations at higher resolution, adult Malpighian tubules from wild-type and *dnpc1a* mutants were analyzed by electron microscopy. Large multi-lamellar structures (0.5-2 μm) were present in mutant Malpighian tubule cells but never in wild-type cells (Fig. 7C,D). More than 80% of the multi-lamellar structures were clustered together to form aggregates 1-4 μm across. The multi-lamellar structures are likely to correspond to the sterol accumulation observed in the light microscope after filipin staining. Multi-lamellar structures have been observed in samples from individuals with *NPC1* mutations (Pellissier et al., 1976). Excess sterol that accumulates because of lipid trafficking defects may be stored in similar aberrant organelles in *Drosophila* and mammals. Our sterol supplementation data suggest that the sterol in those multi-lamellar organelles is not available for synthesizing steroid hormones.

Discussion

Mutations in *NPC1* cause large amounts of cholesterol and other lipids to accumulate in aberrant organelles in most, if not all, cells. The consequence for humans is typically, and tragically, neurodegeneration and other pathology leading to death in the teenage years. However, it is not clear to what extent sterol accumulation is the cause of pathology as opposed to an indicator of an intracellular trafficking process gone awry. The *Drosophila* model of NPC disease makes a strong case for the connection between NPC1 function and steroid production, reinforcing earlier studies with mice that had suggested a

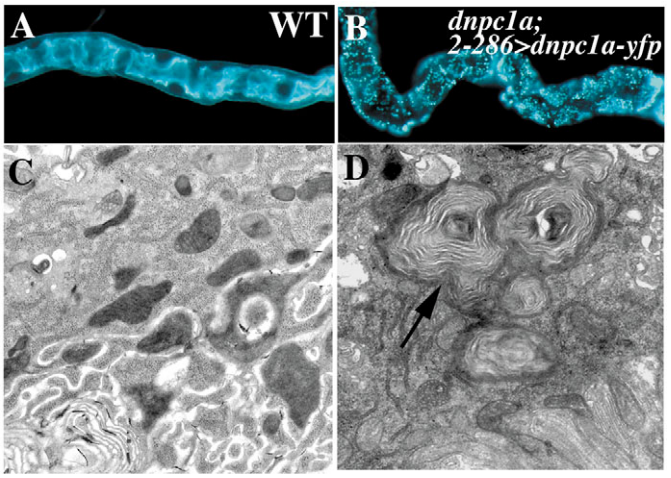

Fig. 7. Ultrastructural defects in adult Malpighian tubules of *dnpc1a* mutants. (A,C) Wild type; (B,D), *dnpc1a* mutants. (A,B) Filipin staining to show the sterol distribution in wild type and *dnpc1a* mutants. (C,D) Electron microscopy pictures to show large multi-lamellar structures (arrow in D) present in *dnpc1a* mutants but not wild-type samples.

connection between NPC disease and neurosteroid production. The fly model offers the opportunity to study tissue-specific functions of Npc genes, and holds the promise of identifying interacting genes and proteins.

Steroid hormone production as a key outcome of NPC1 function

Although NPC1 is conserved through evolution, NPC1 mutants display specific and distinct phenotypes in different model organisms. From studies of worm, fly, and mouse NPC1 mutants, a possible evolutionarily conserved NPC1 function emerges: the protein is needed for cholesterol-derived hormone production. Owing to the diverse roles of steroid hormones in different organisms, NPC1 mutants have quite different phenotypes. The nematode *C. elegans* has two *npc1*-like genes: *ncr-1* and *ncr-2*. Double *ncr-1;ncr-2* mutants have a dauer constitutive phenotype. The dauer state is probably due to the shortage of a sterol-derived dauer formation-inhibiting hormone(s), named Gamravali, although its molecular identity is not known (Li et al., 2004; Matyash et al., 2004). In *Drosophila*, ecdysone is for molting and *dnpc1a* mutants are defective in larval molting owing to ecdysone deficiency. In mammals, neurosteroid is required for proper CNS function and *Npc1* mutant mice have a low level of neurosteroid accompanied with neurodegeneration and neurological dysfunctions, which can be partially suppressed by neurosteroid treatment (Griffin et al., 2004).

Relations of NPC1 function to steroid synthesis

Given the evident steroid hormone synthesis defect in *dnpc1a* flies and the neurosteroid deficiency in *Npc1/Npc1* mice, the role of NPC1 in steroid hormone biosynthesis becomes a central mystery. The steroid biosynthesis pathways are generally very similar in vertebrates and insects. In mammals, the substrate, cholesterol, is delivered to the outer mitochondrial membrane and then to the inner membrane. In the insect steroid synthesis pathway, dietary cholesterol is first converted to 7-dehydrocholesterol in the ER, then translocated to the mitochondria by an unknown mechanism (Gilbert et al., 2002). Once in the inner mitochondrial compartment, the sterol (cholesterol in vertebrates, 7-dehydrocholesterol in insects) is converted into different steroid hormones through a series of enzymatic reactions carried out by P450 enzymes. NPC1 protein has not been observed in mitochondria (Higgins et al., 1999; Neufeld et al., 1999; Ko et al., 2001), so it may function in a delivery process. Together, our studies and others point in one direction: the need for NPC1 to ensure that sufficient intracellular cholesterol substrate is available for steroid hormone biosynthesis. In NPC mutants, cholesterol accumulates in aberrant endosome or lysosome-like compartments. We hypothesize that this trapping process may cause or reflect a deficiency of cholesterol in other compartments, such as ER and/or mitochondria, resulting in deficient steroid hormone synthesis.

A recent study showed that mitochondria from whole brain preparations of *Npc1* mutant mice contain more cholesterol than similar extracts from wild-type animals (Yu et al., 2005). This apparent contradiction to the cholesterol deficiency model could be due to pooling different cell types, such as diverse neurons that do not synthesize cholesterol, with cells that do, thus masking a sterol deficiency in the mitochondria of critical cell types. Alternatively, standard mitochondria preparations usually contain some endosomes and lysosomes. The cholesterol richness of endosomes and lysosomes might have masked a cholesterol deficit in mitochondria. The masking may be particularly severe in *npc1* mutants as there is massive accumulation of cholesterol in mutant endosomes and lysosomes.

Mammals synthesize numerous steroid hormones that have a wide range of important physiological functions. No defect in general steroidogenesis has been observed in NPC1-deficient humans or mice (Soccio and Breslow, 2004); only neurosteroids are lacking from *Npc1* mutant mice (Griffin et al., 2004). Why is neurosteroid biosynthesis particularly sensitive to the loss of NPC1 function? The answer may come from the source of cholesterol for mitochondria and the route for moving cholesterol to mitochondria. In mammals, the source of cholesterol for mitochondrial steroidogenesis varies between cell type or condition: some cells use HDL cholesterol esters in lipid droplets, others use LDL cholesterol arriving via the endosomal pathway, and still others obtain cholesterol by de novo synthesis from acetate (Soccio and Breslow, 2004). The differing sources and pathways may make NPC1 more important for steroid synthesis in some cell types than in others.

Critical requirement for NPC1 in different tissues in different organisms

Purkinje neurons, which undergo prominent neurodegeneration in NPC disease, are the main cells for neurosteroid biosynthesis in the brain (Tsutsui et al., 1999). Using chimeric mice in which some cells have *Npc1* function and others do not, we have shown that *Npc1* is required within Purkinje neurons for their survival (Ko et al., 2005). The apparent cell autonomous role of NPC1 in Purkinje cells raises a question: if NPC1 is required only for neurosteroid biosynthesis, why do wild-type cells fail to help their mutant

neighbors? One explanation could be an unidentified function of NPC1 in Purkinje cells in addition to controlling neurosteroid biosynthesis. Alternatively, there may be a cell-autonomous autocrine neurosteroid signal in Purkinje cells.

Like mammalian *NPC1*, *Drosophila dnpc1a* is widely expressed, but our results show that the crucial function of *dnpc1a* for development into an adult is restricted to a single tissue (the ring gland), and to a specific biological process (ecdysone biosynthesis).

NPC: a sterol 'shortage' disease?

If the steroid synthesis hypothesis about NPC is correct, the disease should be regarded more as a sterol shortage disease than a sterol excess disease, because the accumulated sterol embedded in multi-lamellar membranes is evidently unavailable for further sterol metabolism. The cholesterol shortage model clearly differs from models in which the lipid accumulation itself is the cause of disease pathology. In fact, individuals with NPC live for many years carrying significant accumulations of lipids, including sterols and gangliosides, in many cells. Adult *Drosophila dnpc1a* homozygotes, rescued by ring gland *dnpc1a* expression, seem fully functional except for male sterility, despite the punctate sterol accumulation in many of their tissues. It remains unclear how much damage the accumulated sterol and other lipids impart.

The sterol shortage model is also supported by studies of mammalian NPC1 disease, where the transcriptional program for sterol biosynthesis involving the SREBP transcription factor is triggered in cells that are replete with sterol (Liscum and Faust, 1987). Normally such sterol abundance would leave SREBP tethered in the ER. SREBP is activated by a regulatory system that senses sterol level in the ER and, when sterol seems low, allows SREBP to move into the Golgi and then to the nucleus where it triggers transcription of genes for sterol synthesis, such as HMG CoA reductase, and genes for sterol import, such as LDL receptor (Brown and Goldstein, 1997). The activation of SREBP in *Npc1* mutant cells, despite the abundant sterol, shows that the accumulated sterol is invisible to the cells' regulatory machinery.

Our studies suggest possible new directions for improving NPC disease therapy. Previous therapeutic efforts in lowering cholesterol level and limiting dietary cholesterol supply have been unsuccessful (Akaboshi and Ohno, 1995; Somers et al., 2001), as would be expected if the accumulation of sterol in cells is not the primary cause of pathology. Instead, our results point in the opposite direction: the disease might be usefully treated by increasing the cholesterol level in the mitochondria of critical types of neurons.

The evolutionary conservation of sterol accumulation in *Npc1* mutants of flies and mammals implies a common trafficking function that existed at least half a billion years ago. The further possible similarity in disease mechanisms that is implied by the steroid deficit in *Drosophila* and mice NPC model may permit advances to be made in understanding mammalian disease by employing classical genetic approaches in flies.

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