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Evolution of Developmental Control Mechanisms

# A neurodegenerative disease affecting synaptic connections in *Drosophila* mutant for the tumor suppressor morphogen Patched

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ABSTRACT

The tumor suppressor morphogen, Patched (Ptc), has an extensive homology to the Niemann–Pick-C 1 (NPC1) protein. The NPC disease is a paediatric, progressive and fatal neurodegenerative disorder thought to be due to an abnormal accumulation of cholesterol in neurons. Here, we report that *patched* mutant adults develop a progressive neurodegenerative disease and their brain contains membranous and lamellar inclusions. There is also a significant reduction in the number of synaptic terminals in the brain of the mutant adults. Interestingly, feeding cholesterol to wild type flies generates inclusions in the brain, but does not cause the disease. However, feeding cholesterol to mutant flies increases synaptic connections and suppresses the disease. Our results suggest that sequestration of cholesterol in the mutant brain in the form of membranous material and inclusions affects available pool of cholesterol for cellular functions. This, in turn, negatively affects the synaptic number and contributes to the disease-state. Consistent with this, in *ptc* mutants there is a reduction in the pool of cholesterol esters, and cholesterol-mediated suppression of the disease accompanies an increase in cholesterol esters. We further show that Ptc does not function directly in this process since gain of function for Hedgehog also induces the same disease with a reduction in the level of cholesterol esters. We believe that loss of function for *ptc* causes neurodegeneration via two distinct ways: de-repression of genes that interfere with lipid trafficking, and de-repression of genes outside of the lipid trafficking; the functions of both classes of genes ultimately converge on synaptic connections.



## Introduction

Several recent studies have shown that *Drosophila* can be used as an effective model system to understand human diseases. For instance, fly models for several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Fragile-X syndrome, and several spinocerebellar ataxias have been reported (reviewed in Bonini and Fortini, 2003). Ongoing genetic studies with *Drosophila* neurodegenerative disease models promise to enhance our understanding of disease pathogenesis and generate target lists for future investigations and drug development. Such diseases can also be very helpful in understanding the brain itself. The work described in this paper stems from our unexpected result that the loss of function for *patched* (*ptc*) causes a progressive neurodegenerative disease in *Drosophila*.

The *ptc* gene encodes a seven-pass transmembrane protein and serves as a receptor for the Hedgehog family of signaling proteins (reviewed in Goodrich and Scott, 1998; Bhat, 1999). In humans, Ptc is a tumor suppressor protein and the loss of function for *ptc* leads to nevoid basal cell carcinoma, medulloblastoma, and several other tumors and developmental defects (Hahn et al., 1996; Johnson et al., 1996). In *Drosophila*, the loss of function for *ptc* results in defects in neuroblast formation and identity specification in the ventral nerve cord, as well as defects in segmentation of the embryonic ectoderm and headcase development (Bhat, 1996; Bhat and Schedl, 1997; Bejsovec and Wieschaus, 1993; Shyamala and Bhat, 2002). Previous results indicate that Ptc represses Smoothened (Smo), a G-protein-coupled transmembrane protein and the effector of Hh-signaling, from activating downstream target genes (Goodrich and Scott, 1998; Bhat, 1999). In cells where such an activation of downstream genes is required, the interaction of Hh with Ptc relieves the repression of Smo by Ptc, thus allowing Smo to activate downstream genes (such as *wingless* and also *ptc* itself).

Ptc has extensive homology to the Niemann Pick C 1 (NPC1) protein. For example, both Ptc and NPC1 proteins are seven-pass membrane proteins of similar sizes, both have sterol-sensing domains located approximately at the middle of the protein, the two proteins also have two of the what are known as permease domains (present in MexD and AcrB bacterial proteins), placed at the C-terminal portion of the protein; the two proteins have significant homology in these domains, including the transmembrane domains (Loftus et al., 1997). However, any functional sharing between these proteins has not been explored and it remains unknown if this structural similarity

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translates into any functional sharing in developmental processes or disease or it is just a coincidence.

The NPC disease is a fatal, inherited, paediatric, and progressive neurodegenerative disorder thought to be due to the abnormal accumulation of cholesterol in neurons (Blanchette-Mackie et al., 1988; Suzuki et al., 1995; Carstea et al., 1997; Loftus et al., 1997). A faulty cholesterol homeostasis is thought to play a role in the development of this disease. The disease is either caused by mutations in the *NPC1* gene, which encodes a transmembrane protein (Carstea et al., 1997; Loftus et al., 1997), or in the *HE1/NPC2* gene, which encodes a cholesterol-binding lysosomal protein (Naureckiene et al., 2000; see also Okamura et al., 1999). The exact mechanism, by which these proteins prevent this neurodegenerative disease, or the role of cholesterol in the manifestation of the disease-state, is not understood. While it is thought that in NPC, formation of inclusions in neurons is causing neuronal death, resulting in a disease-state, no direct evidence exists to link the inclusions to disease-state. Additionally, it is not clear if the inclusions are actually caused/constituted by cholesterol. A recent study suggests that in the case of Huntington's disease, formation of inclusion bodies is a cellular response to decrease the mutant protein and prevent the poisoning of other neurons (Arrasate et al., 2004).

A mouse model for the NPC disease has also been developed (Loftus et al., 1997), but the molecular basis for the disease is not clear in this system as well. In *Drosophila*, there are two *NPC1* genes, *dNPC1a* and *dNPC1b*. Mutants for either of the two genes die at the larval stage (Fluegel et al., 2006; Huang et al., 2005). The study from Huang et al. (2005) reported that while the brains in these rescued mutants were normal, the malphigian tubule (the kidney equivalent in flies) had the highest sterol accumulation and contained large multi-lamellar inclusions. However, a recent paper by Phillips et al. (2008) reported that null mutants for *dNPC1a* gene mimic human NPC patients with progressive motor defects and reduced life span; the brains of these mutants were reportedly contain higher levels of cholesterol and multi-lamellar inclusions. The reasons for discrepancy between the earlier study by Huang et al. (2005) and this study is not clear at this moment.

In a modifier screen, we had previously isolated a hypomorphic mutation of *ptc*, $ptc^{headless}$ ($ptc^{hdl}$), which, in combination with several alleles of *ptc*, produced few adult escapers (Bhat, unpublished results, see also Shyamala and Bhat, 2002). We also found that a viable GAL4 insertion line ($ptc^{gal4}$), which in combination with null alleles of *ptc*, produced viable adults (Shyamala and Bhat, 2002). These *ptc* mutant adults developed a progressive NPC-like neurodegenerative disease and their brain contained lamellar inclusions. The levels of cholesterol esters in these individuals were also reduced. We also observed a significant reduction in the number of synaptic terminals in the brain of the mutant adults. Our results show that feeding cholesterol enhances the synaptic connections and suppresses the disease; this also restores the levels of cholesterol esters. The results further show that Ptc does not function directly in this process but via repressing Smoothened (Smo). Thus, the gain of function for Hedgehog also induces the same disease, and the disease and the loss of synaptic connections in *ptc* mutants can be suppressed by simultaneously reducing the dosage of *smo*. These results provide novel insight into the role of synaptic connections and their maintenance in neural diseases.

## Materials and methods

### *Fly strains, genetics*

The $ptc^{gal4}$ line has been previously described. It is an insertion of the *gal4* gene in the regulatory sequence of the *ptc* gene. $ptc^{hdl}$ allele was isolated in an F1 modifier screen (Bhat, unpublished; see Shyamala and Bhat, 2002). Other stocks used: Df (2R) NP3/CyO ($ptc^{df}$), *hs-hh*, $smo^{1}$, $ptc^{df}$, $smo^{1}$ and $ptc^{H84}$, $smo^{1}$. Other *ptc* alleles used: $ptc^{IN108}$, $ptc^{H84}$, $ptc^{S2}$, $ptc^{IIR87}$, $ptc^{IF85}$, $ptc^{IIC84}$, $ptc^{9B28}$ and *UAS-ptc*. For wild type, Oregon R, Canton-S flies and $white^{1118}$ were used. For analysis, unless otherwise stated, we used $ptc^{gal4}/ptc^{df}$. All the experiments were done at 22 °C except for those involving *Hs-hh*, which were done at 18 °C and 29 °C (see text for details).

### *Locomotor activity assay and determining the progression of the disease in ptc mutants*

Four Newly eclosed females and four newly eclosed males of wild type or *ptc* mutants were placed in a cornmeal–agar vial and their locomotor activity and death were recorded every three days for four weeks. Vials were changed once in every three days. The testing of the flies for stage 1 or 2/3 defects was done by transferring flies to new vials to avoid any interference due to stickiness in older vials. One or two trials were conducted double blind in all experiments. For rescue of the phenotype, we used a *UAS-ptc* transgene (Shyamala and Bhat, 2002). This transgene was introduced to $ptc^{gal4}/ptc^{df}$ background and the adults were monitored for the progression of the disease. Following are the details of the staging in our locomotor assay:

Stage 1: sluggishness and difficulty walking, particularly affected is the movement and coordination of the legs.
Stage 2: walking becomes unsteady, and flies are unable to hold on to smooth surfaces against gravity, they frequently fall down and during walking and unable to get up on their legs.
Stage 3: flies become paralyzed and do not move.

Please refer to the details and movie clips shown in Supplementary data.

### *Cholesterol feeding experiment I*

To determine if overloading the cholesterol metabolism in wild type would result in the formation of inclusions in the brain and cause behavioral defects, newly eclosed wild type adults were fed with a small amount of yeast paste containing 5% cholesterol (W/W; Sigma, stored at −70 °C) placed on top of the food medium once a day (the vials were changed once every day) and their locomotor activity was monitored once a day. Cholesterol was manually mixed with yeast paste thoroughly until detectable cholesterol crystals were not observed. It is possible that the cholesterol is not dissolved in the food; our idea is to make it thoroughly dispersed in the food. For EM analysis, flies fed with yeast paste with or without cholesterol for 4–5 days were used.

### *Cholesterol feeding experiment II*

To determine the effect of feeding cholesterol to *ptc* mutant flies, newly eclosed four females and four males of wild type or *ptc* mutants were placed in a vial of 1% Agarose containing 1% sucrose solution. A small amount of yeast paste with or without 5% cholesterol was placed on top of the Agarose medium (this feeding regimen in wild type also causes inclusion formation similar to the feeding regimen in experiment I discussed above). Flies were transferred into new vials once every three days. The number of dead flies was recorded on the first, fifth, ninth, and fourteenth day when the experiment was terminated. The testing of the flies for stage 1 or 2/3 defects was done in new vials.

### *Cholesterol (total, free and esters) measurement experiments in wild type and ptc mutants*

Wild type and *ptc* mutants, aged three weeks, were used in this experiment. Three separate experiments with triplicates for wild type