The Physiological Function and Pathogenic Role of the *Drosophila* Protein Swiss Cheese

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

AChE	acetylcholine esterase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DIV	days *in vitro*
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EMS	ethyl-methane sulfonate
EOPF	ethyl octylphosphonofluoridate
ER	endoplasmic reticulum
HIV	hours in Vitro
IBMX	3-isobutyl-1-methylxanthine
LCAT	lecithin acyltransferase
MOCDPP	mono-ortho-cresyldiphenylphosphate
NTE	Neuropathy Target Esterase
OP	Organophosphate
OPIDN	Organophosphate Induced Delayed Neuropathy
PCR	polymerase chain reaction
PKA	cAMP dependent protein kinase
Pkare	PKA-related protein kinase
PKI	Protein Kinase Inhibitor
PrKX	Protein Kinase X
PtdCho	phosphatidylcholine
SEM	Standard Error of the Mean
SWS	Swiss Cheese
TOCP	Tri-Ortho-Cresyl Phosphate
WT	wild-type

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ABSTRACT

This dissertation looks at the physiological role of the *Drosophila* protein Swiss Cheese (SWS) and the involvement of Swiss Cheese in the development of a neuropathy associated with pesticide exposure. A brief introductory chapter presents background on the discovery of the SWS protein and the Pesticide induced neuropathy. Chapter 2 describes a new physiological role for SWS as a regulator of a highly conserved kinase, PKA-C3. Evidence is presented that SWS binds this kinase, keeping it membrane bound and inactive. Overactive PKA-C3 in sws^{1} flies is thought to play a role in the degenerative phenotype associated with these mutants, suggesting that activity of PKA-C3 is tightly regulated *in vivo*. Chapter 3 presents evidence that exposure to Organophosphates (OPs) decreases kinase activity and we propose this is due to action of OPs on SWS, which then prevents SWS from releasing and activating PKA-C3. We show that increased levels of SWS protein increase the toxicity of OPs, which argues in favor of a controversial hypothesis that suggests SWS adopts a novel toxic function after being bound by OPs. Chapter 4 presents preliminary data showing that constitutively active PKA-C3 leads to vacuole formation in the brain as does mutation of proposed cyclic nucleotide binding sites on SWS. We interpret this to mean that overactive and constitutively bound PKA-C3 can cause degeneration. This work suggests that PKA-C3 is a protein whose activity is tightly regulated by interactions with SWS and both proteins are important for the maintenance of the adult nervous system in *Drosophila*. Overall this work adds to the understanding of what is required for neuronal integrity over the life of an organism and how disruption of these requirements can lead to neuronal disease.

CHAPTER 1

INTRODUCTION

Overview

Age-related neurodegenerative diseases represent an increasing burden on a societal and personal level, especially as medical technology improves and life expectancy increases. Treatment and diagnosis for neurodegenerative diseases lags behind other disease processes because the basic biological causes of many of these diseases, including similarities and differences between them, are poorly understood. One obstacle to understanding neurodegenerative disease is the complexity of the nervous system; as such, it is essential to employ a simple model organism, such as *Drosophila melanogaster*, to investigate the processes that are important for maintaining healthy and functional neurons over the life of an organism.

Drosophila is an excellent choice for the study of neurodegenerative disease for a variety of reasons. First, the majority of genes known to harbor disease-related mutations in humans have an ortholog in *Drosophila* (Reiter et al., 2001). Further, other important genes associated with disease that have remained elusive can be identified in a non-biased fashion using forward genetic screens. Although the comparative simplicity of fruit flies makes them genetically tractable, their brains are complex enough to allow for the study of learning, cross talk between neurons and glia, identifying neural circuits, and processes associated with aging (Kretzschmar, 2005; Skoulakis and Grammenoudi, 2006; Partridge, 2009). Finally, their relatively short life-span makes fruit flies a practical

genetic system for modeling disease, and for understanding the physiological functions of proteins *in vivo*.

In addition to genetic mutations that cause neurodegeneration, many disorders are sporadic in nature and no gene mutations have been identified. Sporadic disease is often attributed to environmental factors and thought to target the same pathways as specific genetic perturbations; therefore, understanding the effects of environmental toxins can reveal underlying processes that are important for nervous system function and maintenance. For example, exposure to the neurotoxin rotenone (used as a pesticide) leads to Parkinson's-like symptoms because it causes a specific mitochondrial dysfunction that has also been associated with Parkinson's disease (Di Monte et al., 2002; Greenamyre et al., 2003).

This dissertation examines the physiological function of the *Drosophila* protein Swiss Cheese (SWS), as well as the role of Swiss Cheese in the development of a delayed neuropathy induced by organophosphate pesticides. Broadly, this work examines the effect of genetic and environmental perturbations on a single protein required for neuronal health.

Outline

I will begin by giving a general overview of the *sws*^{1} mutant fly phenotype, and what was learned about the function of the SWS protein from this phenotype. Next I will describe what is known about the murine and human homologs of SWS, called Neuropathy Target Esterase (NTE). I will then briefly describe one known function of SWS/NTE in membrane lipid homeostasis, which, while important to the physiological role of SWS in some contexts, is not the main focus of this dissertation. Rather, this dissertation suggests a novel role for SWS as a regulatory subunit for a recently described kinase, PKA-C3. I will, therefore, give a general overview of cAMP-dependent kinase signaling. Finally, a disease thought to result from the action of organophosphate pesticides on the human homolog of SWS will be described.

Experiments described in this introduction involve a variety of different model systems. Accordingly, I will use the species-appropriate name of the protein in which experiments were done: SWS for *Drosophila* experiments and NTE for mouse. In cases where numerous experiments have been done in multiple systems (such as fly and mouse), the term SWS/NTE will be used.

In the appendix section, two review articles will be presented that were written during this dissertation work but do not directly relate to the body of my research.

The *swiss cheese (sws)* fly

The *swiss cheese* mutant was identified in a large histological screen for structural brain defects in Berlin wild-type flies exposed to the chemical mutagen ethyl-methane sulfonate (EMS) (Heisenberg, 1979). EMS predominantly causes G/C-to-A/T DNA transitions (Keightley, 1996). There are 5 extant *sws* mutant alleles that show a decreased life-span when compared to wild-type flies (Kretzschmar et al., 1997). Two splice variants have been described for the X-linked *sws* gene, one is 5.4 kb and the other is 1.7 kb. Both transcripts are expressed in heads, but only the 5.4 kb transcript is seen in bodies (Kretzschmar et al., 1997). All experiments in this dissertation focus on the sws^{1} mutant, which has a point mutation at nucleotide 1616, resulting in an early stop codon. SWS protein is not detected in sws^{1} flies when using polyclonal antibodies raised against a bacterially expressed fragment of SWS that spans the region between the first and second predicted transmembrane domains (Figure 1-1). Consequently, any truncated protein that might be produced in *sws* mutant flies is predicted to undergo rapid degradation. While extremely unlikely, the 170 amino acids present in the fusion protein, but not in the truncated protein produced in sws^{1} *(Muhlig-Versen et al., 2005),* might be the most immunogenic region and would therefore be recognized by the antibody produced. If this scenario occurred, a truncated protein produced in sws^{1} would not be detectable. However, additional evidence supporting that sws^{1} is a functional null (i.e. no functional protein) comes from experiments showing that flies heterozygous for

the mutation over a deficiency[1] show the same decrease in life expectancy seen in homozygous mutants (Kretzschmar et al., 1997). This suggests that there is no contribution of functional SWS protein when the gene mutation in *sws*^{1} is present.

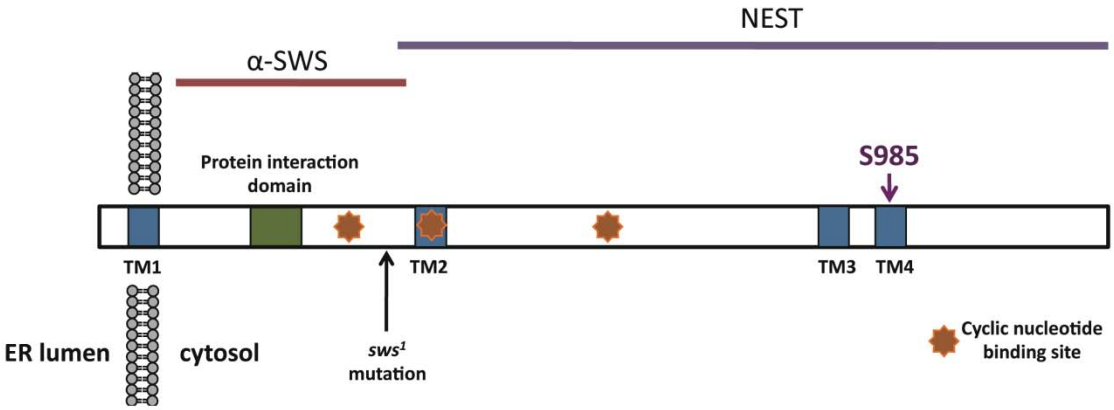

Figure 1-1. Cartoon of the SWS protein. The four predicted transmembrane domains are shown as blue boxes. Proposed cyclic nucleotide binding sites are shown as orange stars. The protein interaction domain is the site with homology to the region of PKA regulatory subunits known to bind catalytic subunits, called the pseudosubstrate region. The location of the point mutation in *sws*^{1} is indicated, as is the active site serine required for esterase activity (S985). The maroon bar labeled α-SWS indicates the fragment against which SWS antibodies were made. The purple bar labeled NEST indicates the minimum protein fragment required for esterase activity (Atkins and Glynn, 2000).

[1] Deficiency lines each have a specific deletion of chromosomal material. In this case, the region surrounding and including the *sws* gene is deleted.

Homozygous *sws*^{1} mutants show progressive vacuole formation in all regions of the brain and a reduced brain-cortex volume as flies age (Figure 1-2). Glial cells show an abnormal phenotype – they hyperwrap neurons and form membranous whirls. These abnormal glial structures are first visible in late pupa and increase with age. Apoptotic cell death of both neurons and glia is also evident in aged *sws*^{1} flies (Kretzschmar et al., 1997).

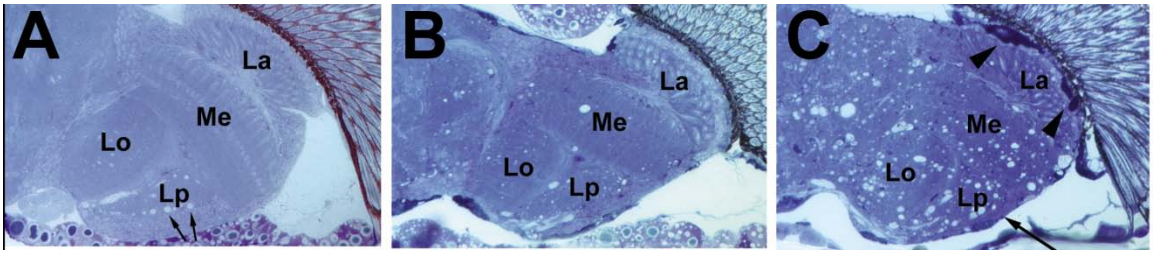

Figure 1-2. Progressive degeneration in *sws*^{1} flies. A-C, Horizontal plastic head sections (1μm) stained with toludine blue. **A.** Wild-type, age 20 d (arrows indicate white areas in the lobula plate that are not vacuoles but cross-sections of giant fibers). **B.** *sws*^{1} 5 d old. **C.** *sws*^{1} 20 d old. Vacuolization and darkly stained structures that result from hyperwrapping of glial cells (arrowheads in C) increase with age. These are accompanied by a thinning of the cortex (long arrow in C).

Because SWS is X-linked, gynandromorph studies with flies mosaic for sex allowed for the generation of flies heterozygous for the *sws*^{1} mutation in some brain areas, but hemizygous mutant in other regions. Hemizygous tissue, but not heterozygous tissue, showed vacuolization, indicating SWS is needed in a cell-autonomous fashion and does not have long-range effects (Kretzschmar et

al., 1997). This observation suggests the glial and neuronal *sws*^{1} phenotypes are independent. This assertion was further confirmed with UAS-SWS rescue constructs driven in each cell type: neuronally driven SWS rescued only neuronal phenotypes, while glial drivers rescued only the glial phenotype (Muhlig-Versen et al., 2005). Interestingly, transient expression of SWS in adulthood, using whole animal heat-shock promoters, did not rescue the phenotype (Muhlig-Versen et al., 2005). This result may indicate that SWS is needed during development and that the adult phenotype is already initiated during development (although *sws* mutants lack a developmental phenotype). Alternatively, the heat-shock promoters used may not have induced SWS expression for long enough or at high enough concentrations to rescue the phenotype. In summary, SWS is a protein with cell-autonomous effects that is needed in both neurons and glia to maintain an intact nervous system with age.

SWS has amino acid homology to the regulatory subunit (RIα) of cAMP-dependent protein kinase (PKA) in both the region that interacts with catalytic subunits and the cAMP-binding sites (Kretzschmar et al., 1997). PKA signaling will be discussed in more detail later in this introduction. SWS is also homologous to the mammalian protein neuropathy target esterase (NTE), described in detail in the next section.

The mammalian homolog of *sws*

The mouse homolog of SWS, called Neuropathy Target Esterase (mNTE), was cloned in 2000 and has 39% amino acid identity with *Drosophila* SWS

(Moser et al., 2000). There is a stretch of 235 amino acids in the C-terminii of both proteins that is 61% conserved. This region includes the highly conserved serine 985 that is known to be important for the initiation of organophosphate-related neuropathy, discussed later. Young mice express mNTE almost ubiquitously in the brain, but its expression becomes more restricted with age, similar to the expression patterns of SWS in *Drosophila* (Moser et al., 2000; Muhlig-Versen et al., 2005). Adult mice express mNTE in groups of large neurons in the cortex, midbrain, thalamus, hippocampus, and cerebellum (Moser et al., 2000). Expression of murine NTE in *Drosophila* rescues the *sws* phenotype to the same extent as UAS-SWS, demonstrating that mammalian NTE is functionally homologous to *Drosophila* SWS (Muhlig-Versen et al., 2005).

A brain-specific deletion of mNTE showed degeneration in the mouse brain similar to that seen in *sws* mutant flies. These mice had progressive vacuole formation in the fibrous regions, but not cell body regions, of the hippocampus and thalamus (Akassoglou et al., 2004).

In mouse, mNTE is important for non-neural tissue development, as evidenced in homozygous mutant mice that die from defects in embryonic vasculogenesis and placental formation (Moser et al., 2004). Surprisingly, NTE is not detected in mouse or chicken glia (Glynn et al., 1998; Winrow et al., 2003). In contrast, the *Drosophila* SWS protein is detected in glia, and a strong glial phenotype is seen in the mutant, as noted above (Kretzschmar et al., 1997; Muhlig-Versen et al., 2005).

Human NTE was cloned from a fetal brain cDNA library, using affinity purification of biotinylated compounds known to bind the conserved serine (Lush et al., 1998). Lush and colleagues found human NTE to be approximately 150-kDa. Like *Drosophila* SWS, it has four predicted transmembrane domains. The most N-terminal transmembrane domain of NTE is the most likely to function as a membrane-spanning region, according to hydrophobicity plots. Recently, mutations in human NTE have been associated with an inherited neuropathy (Rainier et al., 2008), reinforcing the importance of understanding the physiological function of SWS/NTE. Patients with this motor neuron disease have mutations in the esterase domain of NTE (Rainier et al., 2008), that reduce the esterase activity of the protein. This was determined via *in vitro* assays using SWS protein fragments containing mutations that mimic those found in patients (Hein et al., 2010b). It is interesting that reduction of esterase activity by only 30-47% (depending on the point mutation) in humans leads to a neurodegenerative disease (Hein et al., 2010b). By comparison, similar reductions in esterase activity do not lead to neurodegeneration in flies or mice (Kretzschmar et al., 1997; Winrow et al., 2003). SWS activity must be reduced by more than 50% for detection of a phenotype, as heterozygous sws^{1} mutants do not show a phenotype (Kretzschmar et al., 1997). This suggests a disease mechanism that is independent of esterase activity. Further support for an esterase-independent disease mechanism are the recent identification of non-disease-causing mutations in NTE that reduce esterase activity to similar levels as disease-causing mutations (Hein et al., 2010a). These data stongly suggests that disease

progression is not directly related to esterase function of NTE and encourages investigation of alternative physiological functions of NTE/SWS that, when disrupted, lead to neurodegeneration. This thesis presents evidence that SWS functions as a regulatory subunit for the kinase PKA-C3. Furthermore, my experiments show that modification of this function results in neurodegeneration and provides strong evidence that similar dysfunction of NTE may be the mechanism of disease in the inherited neuropathy linked to NTE mutations.

Enzymatic activity and subcellular localization and of SWS/NTE

NTE is an esterase with a serine and two aspartate residues required for activity (Atkins and Glynn, 2000). Mutation of the active site serine in *Drosophila* (S985) abolishes esterase activity (Muhlig-Versen et al., 2005). Prior to the identification of a physiological substrate for NTE, its ability to hydrolyze a synthetic substrate (phenyl valerate) (Johnson, 1977) was used as a surrogate for testing its biological activity. In this way it was shown that membrane insertion was essential for NTE's catalytic activity. Furthermore it was shown that the minimum protein fragment required for esterase activity (called NEST) begins just before the second predicted transmembrane segment and extends to the C-terminus of the protein (Figure 1-1).

Murine NTE has been shown to be membrane-associated and enriched in homogenate fractions containing endoplasmic reticulum (ER), golgi, and plasma

membrane (Richardson et al., 1979). Antibody staining of *Drosophila* SWS shows a pattern that resembles endoplasmic reticulum, colocalizing with an ER marker, though some SWS is also detectable in non-ER vesicles (Muhlig-Versen et al., 2005). In chicken spinal cord and sensory neurons, antibodies directed against chicken NTE show intracellular staining in the cell body region and proximal axon segments of neurons, but never in dendrites or the nucleus (Glynn et al., 1998).

It is thought that NTE undergoes both anterograde and retrograde fast axonal transport, based on the finding that NTE accumulates around both sides of a chicken sciatic nerve ligation (Carrington and Abou-Donia, 1985; Glynn et al., 1998). However, while NTE is most likely transported in a membrane-bound state via vesicles, it should be noted that a soluble form of NTE has also been identified in chicken (Escudero and Vilanova, 1997).

Increased levels of neuronal SWS protein (associated with a two-fold increase in esterase activity) cause its localization to vesicular puncta within neuronal cell bodies, but do not induce noticeable histological degeneration (Muhlig-Versen et al., 2005). In contrast, over-expression of SWS in photoreceptors does cause degeneration in the eye that is not seen with expression of catalytically dead SWS. This suggests that much higher activity of UAS-SWS (as achieved by expression via the strong photoreceptor promoter construct GMR-GAL4) can lead to degeneration. This is also supported by the results obtained after over-expression of SWS in glia where higher levels of

expression (resulting in a 12-fold increase in esterase activity) caused abnormal membranous structures (Muhlig-Versen et al., 2005).

Controversy remains in the field with regard to the exact nature of NTE/SWS's transmembrane segment architecture. Interestingly, *in vitro* studies indicate that NEST remains membrane-associated, despite lacking the first transmembrane segment (Atkins and Glynn, 2000). Importantly, any results using the NEST fragment should be interpreted with caution, since proteolysis studies indicate that full-length NTE remains anchored to the ER membrane via the most N-terminal transmembrane segment, and that none of the other three predicted segment actually span the membrane (Li et al., 2003). Thus, while each of the predicted transmembrane segments in NTE may have the ability to function as such *in vitro*, it remains unclear which of these segments actually span the membrane when the full-length protein is expressed *in vivo*. Contributing to this debate, our laboratory has shown that deletion of the first 80 amino acids of SWS (including the first transmembrane segment) causes a more diffuse localization of the protein when expressed neuronally, as compared to neuronal expression of full-length SWS (Bettencourt Da Cruz, 2006). This observation suggests that membrane localization has been lost with the deletion of the first transmembrane domain.

In summary, SWS/NTE is a membrane-associated protein with esterase activity that is mediated by a highly conserved serine residue.

SWS as a phospholipase

The first physiological function described for SWS/NTE was as an enzyme that catalyzes the hydrolysis of membrane lipids (van Tienhoven et al., 2002). Membrane lipids are known to play important roles in cell structure and signaling (Fernandez-Murray and McMaster, 2007). Specifically, mNTE has been shown to act as a phospholipase resembling calcium-independent phospholipase A2, with which NTE shares sequence similarity in the esterase domain (van Tienhoven et al., 2002; Quistad et al., 2003). Both the yeast homolog of SWS and *Drosophila* SWS catalyze phosphatidylcholine (PtdCho) hydrolysis (Zaccheo et al., 2004; Muhlig-Versen et al., 2005), suggesting SWS's role in lipid regulation is highly conserved. *sws*^{1} flies have increased levels of PtdCho and decreased levels of triacylglycerol, a precursor to PtdCho (Muhlig-Versen et al., 2005). Of note, despite the high levels of PtdCho in *sws*^{1} flies, these animals have reduced levels of cholesterol ester. Because cholesterol ester can be synthesized from PtdCho by the enzyme lecithin acyltransferase (LCAT), SWS has been proposed to function in a similar way as LCAT or perhaps it can modify the actions of the *Drosophila* LCAT homolog (Muhlig-Versen et al., 2005).

The interaction between SWS and PKA

Overview of PKA signaling

Protein kinases are important molecules for mediating the downstream effects of cyclic nucleotides and for maintaining proper cellular communication (Tasken and Aandahl, 2004). A canonical PKA complex consists of a dimer of regulatory subunits, which binds and inhibits two catalytic subunits. In general, PKA signaling is thought to occur after cyclic AMP binds to the regulatory subunits, which then results in a dissociation of the complex and activation of catalytic subunits (Skalhegg and Tasken, 1997) (Figure 1-3).

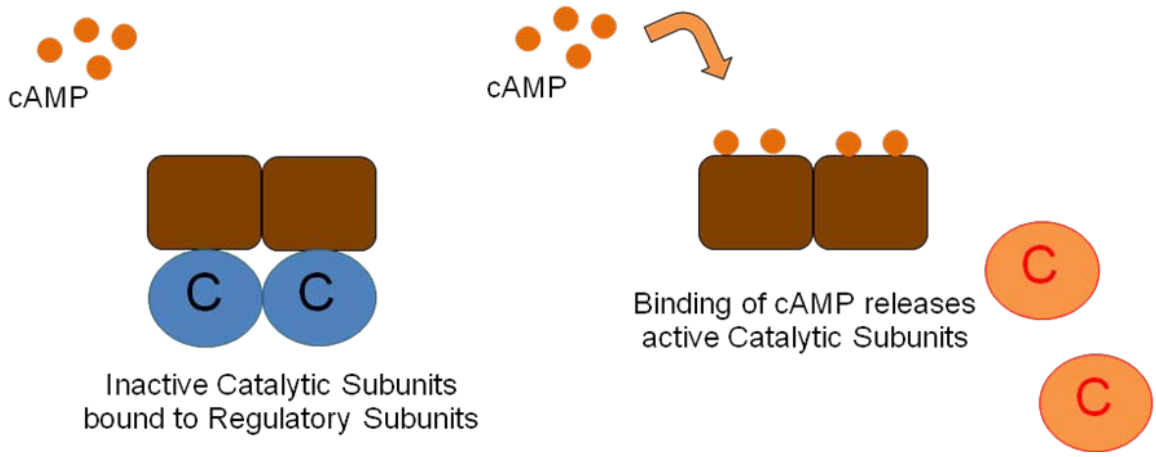

Figure 1-3. Canonical PKA signaling. In the absence of bound cyclic nucleotide (cAMP) the regulatory subunits (brown squares) maintain catalytic subunits in a bound and inactive (blue circles) state. Upon binding of cAMP to the regulatory subunit, the complex dissociates and the catalytic subunits are activated (orange circles).

Adapted from (Skalhegg and Tasken, 1997).

Mammals have three well-known isoforms of PKA catalytic subunits (α, β, and γ). Mammals have four regulatory isoforms: two are mostly cytoplasmic (RIα and RIβ), and two (RIIα and RIIβ) are often membrane-bound via kinase-anchoring proteins (Faux and Scott, 1996). Type-one regulatory subunits (RI) and type-two regulatory subunits (RII) differ also in that RII subunits are true substrates of PKA that can be phosphorylated, while RI is a pseudosubstrate[2] (Faux and Scott, 1996).

There are three known PKA catalytic subunits in *Drosophila*, DC0/PKA-C1, DC1/PKA-C2, DC2/PKA-C3. DC0 is the most similar to mouse Cα subunit. *Drosophila* has two known regulatory subunits RI and RII (Kalderon and Rubin, 1988; Park et al., 2000). *Drosophila* mutants with altered levels of cAMP show learning deficits, suggesting cyclic nucleotide-dependent signaling via PKA is important in complex neural processing (Byers et al., 1981; Livingstone et al., 1984). These cAMP-dependent defects likely relate to abnormal activity of the most highly expressed PKA in flies, PKA-C1. In contrast, the work in this dissertation will focus on the role that PKA-C3 plays in neuronal physiology and pathology.

[2] A pseudosubstrate mimics the substrate of an enzyme and thus inhibits its activity.

Regulation of PKA-C3, a novel conserved catalytic subunit

PKA-C3 is the *Drosophila* homolog of human Protein Kinase X (PrKX) and mouse Pka-related gene (Pkare). Together, these proteins represent a distinct group of recently identified kinases that, interestingly, have more similarity with their homologs across species (i.e., PKA-C3: PrKX: PKare) than with other cAMP-dependent kinases from the same species (Zimmermann et al., 1999; Diskar et al., 2010).

PKA-C3 and PrKX can be bound and inhibited by RI subunits from their respective species (Melendez et al., 1995). The preference of PKA-C3/PrKX for binding RI but not RII regulatory subunits (i.e. only those with pseudosubstrate autoinhibitory domains) is unique among cAMP-dependent kinases (Diskar et al., 2010). Work described in this dissertation shows that *in vivo,* SWS also functions to bind and inhibit PKA-C3. However, it should be noted that this discovery does not completely rule out the possibility that PKA-C3 may bind with both SWS and other canonical regulatory subunits in *Drosophila.*

PrKX and RIα can be dissociated with about half the concentration of cAMP that is required for dissociation of PKA-Cα/RIα (Zimmermann et al., 1999). Interesting, PrKX also seems to phosphorylate the RIIα subunit in a cAMP-independent manner. Phosphorylation of RII by PrKX has been shown to lower

the re-association rate of the RII holoenzyme[3]. In mouse fibroblasts, PrKX localizes to the cytoplasm but translocates to the nucleus in response to cAMP (Zimmermann et al., 1999). However, it is not known yet whether Pkare and PrKX are membrane-tethered when bound to NTE (as I have shown for *Drosophila* PKA-C3 when associated with SWS), and I have not yet addressed whether *Drosophila* PKA-C3 translocates to the nucleus when activated.

Expression and Physiological function of PKA-C3 and homologs

In mouse, the PKA-C3 ortholog (Pkare) is expressed ubiquitously in the adult; in the developing embryo, expression begins around the time of neuronal differentiation (11.5 pc). At this stage, Pkare expression is observed in regions of neuronal differentiation but is distinctly absent from zones of proliferation, including the ventricular zone (Blaschke et al., 2000). This type of expression pattern might indicate that Pkare has a specific role in neural development, but that after the completion of embryogenesis Pkare is more broadly expressed and assumes alternative post-developmental roles.

Potential non-neuronal roles of PKA-C3/Pkare/PrKX are suggested by work in varied systems. For instance, a homolog of PKA-C3 in an aquatic fungus (*Blastocladiella emersonii*) has been shown to regulate cell shape and migration (de Oliveira et al., 1994). In cell lines derived from the collecting tubule of the

[3] A holoenzyme includes all subunits, in the case of PKA, the holoenzyme refers to two regulatory and two catalytic subunits.

human fetal kidney, PrKX has been shown to be important for epithelial cell migration, ureteric bud branching, and cell adhesion (Li et al., 2009). In addition to kidney development, PrKX has been shown to play a role in differentiation of the hematopoietic lineage (Semizarov et al., 1998). Other work in human epithelial cell lines indicates PrKX can phosphorylate Polycystin-1 (Li et al., 2008) and interacts with other proteins that mediate actin dynamics, apoptosis, and protein assembly and translocation (Li et al., 2009).

Taken together, these results suggest that the proteins in the PKA-C3 family have important roles in many tissues and organs, including the developing and mature nervous system. The exact role of PKA-C3 in *Drosophila* is not yet understood, but its regulation and the phenotypes associated with down-regulation of active PKA-C3 will be described in this dissertation.

SWS and Organophosphate Induced Delayed Neuropathy

The mammalian homolog of SWS (NTE) has long been associated with the development of Organophosphate-Induced Delayed Neuropathy (OPIDN). NTE was identified as the target protein of a certain class of organophosphates (OPs) in 1970 (Johnson, 1970), although, as discussed above, its physiological functions are still being elucidated. Controversy surrounds the mechanism by which OPs act on NTE to induce disease. One hypothesis is that OPs inhibit the function of NTE, resulting in OPIDN. Others argue that NTE bound by OPs must undergo a toxic gain-of-function conversion.

Because protein levels can be easily manipulated in flies, they provide an excellent system for investigating these alternative models of OPIDN. Increased levels of SWS can be used to test whether SWS/NTE is protective, which would support the loss-of-function hypothesis; conversely, if extra protein is found to increase toxicity, this outcome would support the gain-of-function hypothesis. The following section will describe the development and symptoms of OPIDN, accompanied by a summary of previous evidence for both the loss-of- and gain-of-function hypotheses.

Organophosphate-Induced Delayed Neuropathy

Organophosphate-Induced Delayed Neuropathy (OPIDN) is a disease that results from exposure to certain organophosphorus (OPs) chemicals that act on SWS/NTE. OPIDN symptoms include degeneration of long axons, paralysis, and reduced nerve conduction velocity (Lotti, 2000; Wijeyesakere S. J., 2010). During prohibition, a large number of American's were afflicted with OPIDN when the organophosphorus compound Tri-Ortho-Cresyl Phosphate (TOCP) was added to a beverage called "Jamaica Ginger" (Smith et al., 1930).

As noted above, not all organophosphates cause OPIDN. While many OPs act on the enzyme acetylcholinesterase (AChE), only those that can bind NTE and act as pseudosubstrates lead to OPIDN (Johnson, 1974; Glynn, 2003). In addition, while OP binding can inhibit the esterase function of NTE, only OPs that undergo a further reaction termed "aging" are able to induce OPIDN and are thus called neuropathic OPs (Johnson, 1990). The aging modification leads to

the permanent inhibition of NTE. In addition, the aging modification includes the loss of a side group (“R”) from the OP (Figure 1-4) and a resulting negative charge on the covalently bound OP-NTE complex (Johnson, 1974; Wijeyesakere S. J., 2010). Additionally, it has been shown that intramolecular transfer of the “R” group to an unknown site on NTE (called the “Z” site) can occur (Atkins and Glynn, 2000), although transfer to the “Z” site is dependent on the specific OP that binds NTE (Atkins and Glynn, 2000). The fact that not all neuropathic OPs induce this intramolecular transfer strongly argues that transfer of the “R” group to the “Z” site is not a requirement for the development of OPIDN.

Figure 1-4. The aging reaction. Neuropathic and non-neuropathic OPs are able to bind NTE. The organophosphorylated enzyme does not have esterase activity. Neuropathic OPs undergo a further “aging” reaction, in which an R group is lost and the active site is left with a negative charge. Because only aging OPs lead to OPIDN, aging is thought to be a critical step in the development of neuropathy.

Adapted from (Wijeyesakere S. J., 2010).

The requirement of OP-induced aging for the development of OPIDN has led to the hypothesis that NTE might acquire a novel toxic-function after OP exposure, since simple inhibition (which is accomplished by non-aging OPs) is

insufficient for the development of this disease (Johnson, 1974; Lotti and Johnson, 1980). Other evidence in support of a gain-of-function mechanism is that pre-dosing of animals with non-aging OPs is protective against later insults with neuropathic OPs (Johnson, 1970). This latter observation implies that occupation of the NTE binding site by OPs that do not cause the aging reaction prevents disease, even though NTE activity would be inhibited in this context.

However, in addition to evidence supporting the gain-of-function model, there is also compelling evidence that loss of SWS/NTE activity can lead to neurodegeneration. As highlighted by the *sws* mutant fly phenotypes described in this dissertation, the loss of SWS function is detrimental to neuronal integrity. Indeed, OPs that bind SWS/NTE do cause reduced esterase activity: neurotoxic OPs inhibit NTE activity by 70-90% (Johnson, 1974) by binding to the active site serine of NTE (S966). Further support for the loss-of-function model comes from Winrow and colleagues (2003), who showed that mice heterozygous for loss-of-function mutations in NTE were more sensitive to injection of the organophosphate ethyl octyphosponofluoridate (EOPF), exhibiting increased production of tears, decreased movement, seizures, and higher mortality rates (Winrow et al., 2003). Curiously, these symptoms are remarkably similar to what would be expected from inhibition of AChE and are not symptoms classically associated with OPIDN. However, EOPF is thought to have a high binding affinity for NTE and a low binding affinity for AChE (Li and Casida, 1997), but the binding affinity of EOPF for AChE has not been reported in the absence of NTE, which is needed to interpret the experiment as described. It is possible that in the

absence (or reduction) of the primary target (such as NTE), binding to a secondary target (such as AChE) could increase. There are also some non-aging OPs that can lead to mild OPIDN-like phenotypes, but only in very high doses that result in a nearly complete inhibition of enzyme activity (Lotti, 2000). These observations support a scenario whereby non-neuropathic OPs that completely inhibit NTE (unsurprisingly) result in a phenotype similar to *sws*/NTE mutants.

It was recently shown that mice dosed with the neuropathic OP mono-ortho-cresyldiphenylphosphate (MOCDPP) exhibited histological abnormalities resembling the pathology seen in NTE conditional knock-out mice (i.e., lacking NTE specifically in the nervous system) (Read et al., 2009). This group also showed that wild-type mice exposed to OPs (MODCPP) for one week had increased levels of PtdCho. They suggested that altered phosphatidyl choline metabolism underlies the putative loss-of-function effects of OPs on NTE (Read et al., 2009), although PtdCho levels return to normal by two weeks. In contrast, Hou and colleagues (2009) showed that treating wild-type mice with either an aging or non-aging OP resulted in nearly identical changes in membrane lipid composition (Hou et al., 2009), which argues against changes in phospholipid metabolism as a specific mechanism for induction of OPIDN.

As stated earlier, Rainier and colleagues recently found that mutations in the human NTE gene can cause spastic weakness in the lower extremities that resembles OPIDN. The NTE mutations found in these patients resulted in altered esterase function (esterase activity of approximately 37-40%), as shown by testing their activity with recombinant versions of the human NTE catalytic

domain (Hein et al., 2010b). While this observation initially appears to support a loss-of-function mechanism for disease, other asymptomatic subjects with mutations in NTE also showed reductions in esterase activity of approximately 40% (Hein et al., 2010a), indicating that disease is not well correlated with levels of esterase activity.

Although reports supporting a loss-of-function mechanism out-number those supporting a gain-of-function effect, many of the loss-of-function reports are based on correlations between the phenotypes caused by non-functional SWS protein and by OP treatment. However, these studies did not specifically address how OP exposure alters protein function *in vivo*. In this dissertation, I have examined the effects of SWS protein levels on development of OPIDN-like phenotypes. Based on my results, I propose a novel mechanism that explains the toxic gain-of-function effects that result after OP exposure.

Concluding Remarks

The results presented in Chapter 2 of this dissertation suggest a new physiological function for the *Drosophila* protein SWS as a novel regulatory subunit for the kinase PKA-C3. They also suggest that disruption of SWS leads to neuronal degeneration by abnormally increasing the activity of PKA-C3. I present evidence in Chapter 3 that a toxic gain-of-function mechanism underlies the induction of OPIDN, and that the interaction between SWS and PKA-C3 is involved in this gain-of-function effect. Chapter 4 describes preliminary studies that test the requirement for proper levels of PKA-C3 activity to prevent neuronal

degeneration, and that support cyclic nucleotide binding as a possible mechanism for the release and activation of PKA-C3 from SWS. Overall, this dissertation adds to our knowledge of the role of SWS in the development of the neurodegenerative disease organophosphate-induced delayed neuropathy, and how the physiological function of SWS contributes to the health of neurons over time. In addition, my results are also important to better understand the mechanisms that may lead to the inherited human disease (from mutation of NTE) and consequently to develop possible treatment strategies for this devastating condition.

CHAPTER 2

Swiss Cheese, a protein involved in progressive neurodegeneration acts as a non-canonical regulatory subunit for PKA-C3

Abbreviated title: SWS inhibits the PKA-C3 catalytic subunit

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JSW performed experiments for Figures 1A-D, 2C-D, 4C-D, 6A-E, 7A-D, 8A-B, 13, and 12. JSW and ABC both contributed to experiments in Figure 5. ABC created constructs for the yeast two-hybrid experiments and performed the original yeast two-hybrid screen. ABC also created the UAS-SWS^{R133A} construct. DK performed experiments for Figures 3A-F, 4A-B and alignments in Figures 9, 10, 11, 14, and 15.

Chapter 3 is a manuscript as it appears in the original paper published in the Journal of Neuroscience, October 22, 2008.

Summary

The *Drosophila* Swiss Cheese (SWS) protein and its vertebrate orthologue Neuropathy Target Esterase (NTE) are required for neuronal survival and glial integrity. In humans, NTE is the target of Organophosphorus compounds, which cause a paralyzing axonal degeneration and recently mutations in NTE have been shown to cause a Hereditary Spastic Paraplegia called NTE-related Motor-Neuron Disorder. SWS and NTE are concentrated in the endoplasmic reticulum and both have been shown to have an esterase function against an artificial substrate. However, the functional mechanisms and the pathways in which SWS/NTE are involved in are still widely unknown. Here we show that SWS interacts specifically with the C3 catalytic subunit of cAMP activated protein kinase (PKA-C3) which, together with orthologues in mouse (Pkare) and human (PrKX), forms a novel class of catalytic subunits of unknown function. This interaction requires a domain of SWS which shows homology to regulatory subunits of PKA and, like conventional regulatory subunits, the binding of SWS to the PKA-C3 inhibits is function. Consistent with this result, expression of additional PKA-C3 induces degeneration and enhances the neurodegenerative phenotype in *sws* mutants. We also show that the complex formation with the membrane-bound SWS tethers PKA-C3 to membranes. We therefore propose a model in which SWS acts as a non-canonical subunit for PKA-C3, whereby the complex formation regulates the localization and kinase activity of PKA-C3, and that disruption of this regulation can induce neurodegeneration.

Introduction

swiss-cheese (sws) mutant flies show age-dependent neurodegeneration, glial hyperwrapping, and neuronal apoptosis . SWS is the orthologue of vertebrate Neuropathy Target Esterase (NTE; see also supplementary figure 1) (Kretzschmar et al., 1997; Lush et al., 1998; Moser et al., 2000) which plays an important role in organophosphate-induced delayed neuropathy (OPIDN), occurring after intoxication with Organophosphorus compounds (Glynn, 2000; Moretto, 2000) found in pesticides and nerve agents (Lotti and Moretto, 2005). OPIDN, which has first been described after a poisoning epidemic in the southern United States (Smith et al, 1930), is characterized by degeneration of long axons in the central and peripheral nervous systems (Ehrich and Jortner, 2001). Recently, mutations in human NTE have also been shown to cause a Hereditary Spastic Paraplegia called NTE-related Motor-Neuron Disorder (Rainier et al., 2008). Mice lacking NTE show severe growth retardation and die around day 9 of embryonic development (Moser et al., 2004), while neuronal specific NTE knock-out mice show a strikingly similar phenotype to *sws* mutants, including vacuolization, abnormal myelin figures, and neuronal death (Akassoglou et al., 2004). We have also shown that these proteins are functionally conserved because mouse NTE can completely replace SWS in *Drosophila* (Muhlig-Versen et al., 2005).

Both, SWS and mouse NTE are widely expressed in the nervous system with a more restricted pattern to large neurons in older animals (Moser et al., 2000; Muhlig-Versen et al., 2005). Both have been localized to the endoplasmic reticulum (ER) (Akassoglou et al.,

2004; Muhlig-Versen et al., 2005) and Li et al. (2003) described that NTE transfected into COS cells is inserted into ER membranes with most of it exposed on the cytoplasmic face.

NTE and SWS exhibit esterase activity against the artificial substrate phenyl-valerate (Johnson, 1977; Muhlig-Versen et al., 2005), which requires a serine residue within a highly conserved domain. A point mutation in this serine in SWS abolishes esterase activity and interferes with the function of SWS *in vivo* (Muhlig-Versen et al., 2005). In addition, SWS contains several regions that show homology to the regulatory subunit of cAMP-dependent protein kinase (PKA). One of these regions contains a tandem cyclic nucleotide binding site found in canonical regulatory subunits, while a third consists of a single cyclic nucleotide binding site (see supplementary figure 2 and 3). The fourth region shows homology to the motif required for the interaction of the regulatory subunit with the catalytic subunit of PKA, including the pseudo-substrate site. PKA holoenzymes are tetramers consisting of two catalytic and two regulatory subunits, which are activated by dissociation of the regulatory subunits allowing the catalytic subunits to unfold their kinase activity (Francis et al., 2002; Taylor et al., 2005). The binding between the subunits is mediated by the pseudo-substrate site which resembles the R-R-X-S-X consensus site found in PKA substrates (Poteet-Smith et al., 1997). In this study, we show that SWS acts similar to regulatory subunits specifically binding PKA-C3, and we show that this interaction plays a role in the neurodegenerative phenotype of *sws*.

Results

Mutations in the interaction domain of SWS interfere with its biological function

SWS is a 1425aa long transmembrane protein with a highly conserved C-terminal domain that is involved in its function as serine esterase, and a N-terminal region that shows homology to the regulatory subunit of PKA (Kretzschmar et al., 1997), including cyclic nucleotide binding sites and a domain which in PKA is involved in binding the catalytic subunit (see Fig. 2-2*A, B*). This region, which from now on will be referred to as interaction domain, contains a site resembling the pseudo-substrate site of the regulatory subunit of PKA (PKA-R). As described above, this site is similar to the R-R-X-S-X site found in substrates of PKA and has been shown to mediate binding between the catalytic and regulatory subunits of the PKA complex. Whereas the serine residue in this motif, which is the target for phosphorylation, is maintained in the type RII subunits, it is replaced by alanine in vertebrate RI subunits and by glycine in the fly R1 subunit. The SWS pseudo-substrate site contains an asparagine at this position (see Fig. 2-2*B*). The two arginines, which are shared by all types of regulatory subunits (RIa, RIb, RIIa, RIIb) and by the PKA inhibitor PKI, are also conserved in SWS. Mutations in these arginines have been shown to decrease the interaction and inhibitory potency of R1a (Buechler et al., 1993; Poteet-Smith et al., 1997). To investigate the functional importance of the interaction domain, and specifically the pseudo-substrate site in SWS, we mutated the second arginine residue (R^{133}) to an alanine. To determine whether this mutant construct (SWS^{R133A}) is functional, we expressed SWS^{R133A} pan-neuronally using the *Appl*-GAL4 driver (Torroja et al., 1999) in *sws* mutant flies. Determining the degree of

neurodegeneration by measuring the area of vacuoles in brain sections from 14d old flies, we observed a reduced rescue capability of SWS^{R133A} compared to the wild type SWS construct expressed under the same conditions (equal expression levels of the SWS constructs were confirmed by Western Blot, supplementary figure 4). Control sws^1 flies, carrying the *Appl*-GAL4 driver but no SWS construct, showed a mean vacuole area of $52^{\pm}6.8$ mm^2 (Fig 2-1*A, D*). Compared to these flies, sws^1 mutant flies expressing SWS^{R133A} pan-neuronally showed significantly less vacuolization (Fig. 2-1*B*; $30^{\pm}2.4$ mm^2; $p<0.01$). However, the rescue ability of the SWS^{R133A} construct was significantly less than seen after expression of the wild type construct. Due to the cell-autonomous requirement of SWS in neurons and glia, neuronal expression even of the wild type SWS in sws^1 mutants can only prevent neuronal degeneration while the glial phenotype still persists (Muhlig-Versen et al., 2005). Therefore a few vacuoles are still detectable after expression of the wild type construct, but these are much less than with SWS^{R133A} (Fig. 2-1*C* and *D*; $16^{\pm}3.7$ mm^2; $p<0.05$ to SWS^{R133A}). These results show that the R to A mutation in the pseudo-substrate site does interfere with the function of the SWS protein *in vivo*.

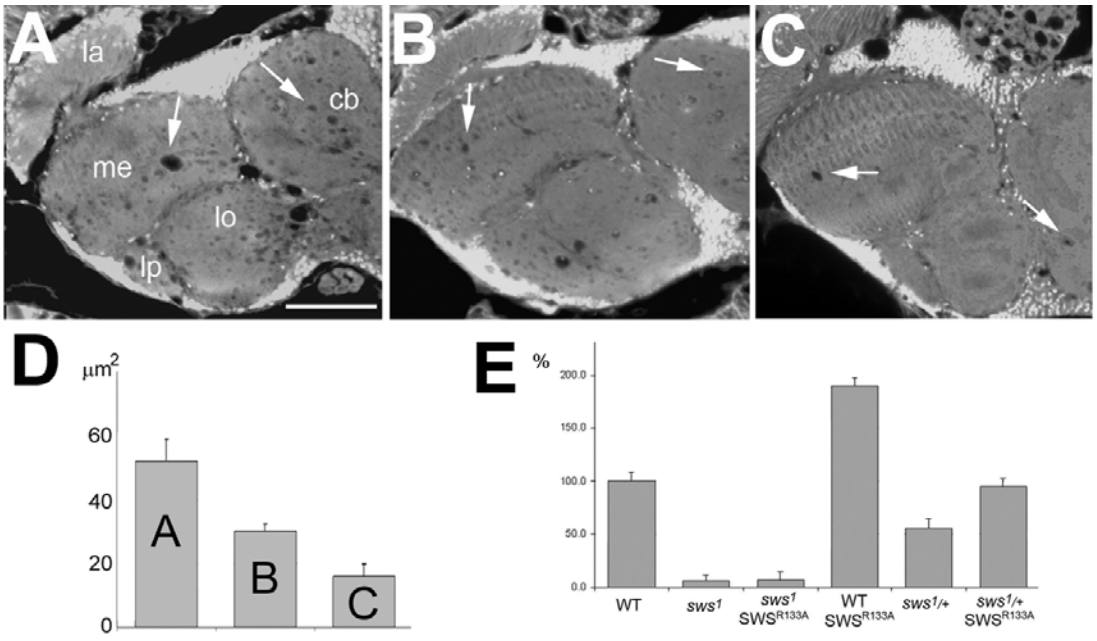

Figure 2-1. Expression of SWS^{R133A} in the nervous system of *sws*^{1} mutant flies does only partially rescue the degenerative phenotype and affects esterase activity. *A*, A *sws*^{1} fly shows the characteristic vacuolization in the neuropil (arrows). *B*, Expressing SWS^{R133A} pan-neuronally in these flies leads to a significant reduction in vacuole formation. *C*, Expressing wild type SWS under the same conditions almost completely reverts the mutant phenotype. *D*, Mean area of vacuoles in mm^2 in the three genotypes shown in *A, B,* and *C*. Number of measured flies was; n=24 for *Appl*-GAL4, *sws*^{1} (A), n=28 for *Appl*-GAL4, *sws*^{1}; UAS-SWS^{R133A} (B), and n=34 for *Appl*-GAL4, *sws*^{1}; UAS- SWS (C). SEMs are indicated. All sections are horizontal paraffin sections through the heads of 14day old flies. *E,* Esterase activity in fly head homogenates revealed that expressing the SWS^{R133A} construct pan-neuronally did not restore the esterase function in *sws*^{1}. In contrast, this construct showed esterase function in a wild type background and heterozygous *sws*^{1}/+ flies, approximately doubling the activity. All values are expressed relative to wild type (100%). SEMs are indicated. Scale bar for *A*-*C*=50µm. la=lamina, me=medulla, lo=lobula, lb=lobula plate, cb=central brain.

In addition to its rescue ability, as measured by vacuolization, we also determined whether the R to A mutation affects the esterase activity of SWS. For these experiments, we used head homogenates from flies expressing SWS^{R133A} in neurons (using *Appl*-GAL4) and measured the hydrolyzing activity against the artificial substrate phenyl valerate, a standard method to detect NTE activity. Expression of SWS^{R133A} in the sws^{1} mutant background appeared not to restore the esterase activity (Fig. 2-1*E*), although it has an intact esterase domain. Interestingly, the mutated construct did show esterase activity in a wild type or heterozygote sws^{1} mutant background, about doubling the endogenous esterase activity. In comparison expression of the wild type construct restored the activity in *sws* mutants (116%) and also doubled the activity (197%) in wild type (Muhlig-Versen et al., 2005). This shows that the mutation in the pseudo-substrate site does not affect the esterase activity per se, however, SWS appears to require an interaction mediated via the pseudo-substrate binding site to exhibit esterase activity.

SWS interacts specifically with the C3 subunit of PKA

The requirement of the pseudo-substrate site for wild type SWS function suggested that this domain is important for an interaction with a partner. We therefore used the Two-Hybrid System to isolate direct binding partners of SWS. In addition to the full-length SWS protein, we also used two smaller fragments (SWS^{60-544} and SWS^{60-241}; Fig. 2-2*A*) that both contained the interaction domain but no transmembrane domain to screen an expression library from adult *Drosophila*. With all three baits we isolated cDNAs for the C3 catalytic subunit of PKA (PKA-C3). The longest isolated PKA-C3 clone encoded aa138-583 of the longer (583aa) isoform (corresponding to aa55-500 of the smaller isoform), containing the

entire protein kinase domain (aa274-528, http://flybase.bio.indiana.edu). The smallest one encoded aa246-482. Interestingly, we isolated only one positive clone with the full-length SWS construct, which also encoded PKA-C3.

To determine whether the binding of SWS to PKA-C3 is indeed mediated by the pseudo-substrate site, we created bait fragments with the R^{133} to A mutation. Co-transfection of $SWS^{60\text{-}241R133A}$ with the PKA-C3 clone resulted in colonies that did not grow as well as colonies formed by PKA-C3 and SWS without the R to A mutation (compare the first and last row in Fig. 2-2*C*). A similar result was obtained with the $SWS^{60\text{-}544R133A}$ construct (first and last row, Fig. 2-2*D*). This shows that as with the vertebrate regulatory PKA subunit (Poteet-Smith et al., 1997), a point mutation in the conserved arginine in SWS significantly reduces the binding of SWS to the C3 catalytic PKA subunit.

Besides C3 two other catalytic PKA subunits have been identified in *Drosophila* (Melendez et al., 1995). Although we only isolated clones for the C3 subunit in our Two Hybrid screens, we verified that SWS does not interact with the other two catalytic subunits. For this purpose, we created pMyr transformation constructs for all three catalytic PKA subunits using full-length cDNAs (kindly provided by D. Kalderon, Columbia University). As shown in figure 2-2*C* and *D,* only SWS and PKA-C3 produced colonies under the restrictive conditions, whereas co-transfection with PKA-C1 or PKA-C2 (second and third row) did not result in colonies under the same conditions. This strongly suggests that SWS specifically interacts with the C3 subunit.

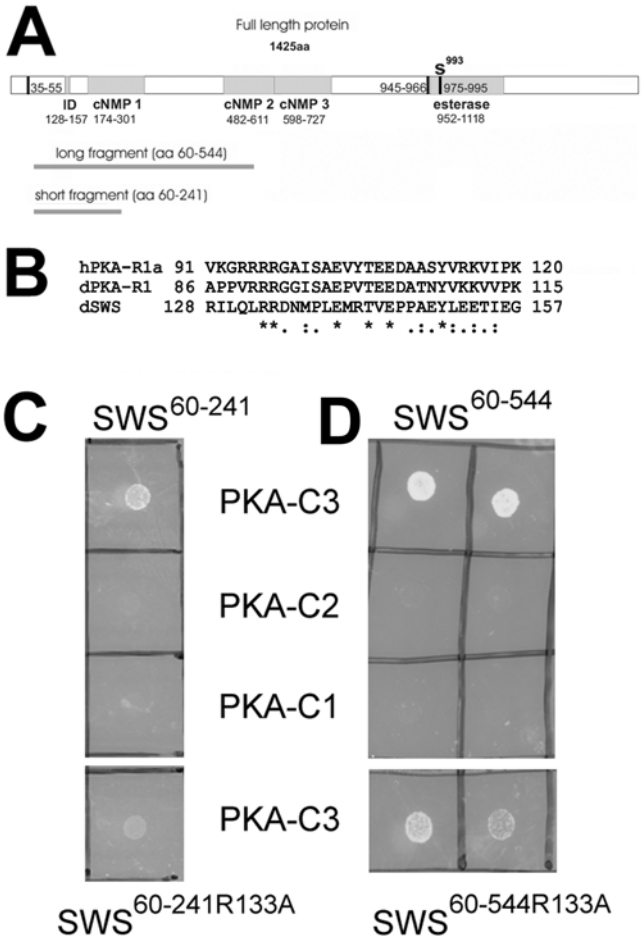

Figure 2-2. SWS binds specifically to PKA-C3. *A,* The different fragments used in the Yeast Two Hybrid experiments are shown as dark grey lines above a schematic of the SWS protein. The predicted transmembrane (TM) domains are shown as vertical black bars and the interaction domain (ID), cyclic nucleotide binding sites (cNMP1-3), and esterase domain are indicated as grey boxes. The active site serine which is localized in the putative third transmembrane domain within the esterase domain is indicated by an S. *B*, Sequence comparison of the interaction domain of SWS with the human and *Drosophila* R1 regulatory subunits. Identical amino acids are indicated by asterisks, highly conserved amino acids by colons, and conserved amino acids by dots. *C*, Using the smaller SWS (SWS^{60-241}) fragment we obtained colonies at the restrictive temperature when co-transfected with PKA-C3 (first row) but not after co-transfection with PKA-C2 or PKA-C1 (second and third row). Using the same fragment with a mutation in the conserved arginine^{133} still produced colonies after co-transfection with PKA-C3 at the restrictive temperature but these colonies grew less well (fourth row). *D*, The same results were obtained in co-transfection studies using the larger SWS^{60-544} fragment.

SWS tethers PKA-C3 to membranes

The interaction of SWS and PKA-C3 in the yeast Two-Hybrid system strongly suggested that SWS and PKA-C3 are also found in a complex *in vivo*. We therefore performed immunohistochemistry on adult brains using our anti-SWS rabbit antiserum and an anti-PKA-C3 rat antiserum for co-localization studies (Melendez et al., 1995). We have recently shown that SWS is expressed in most or all neurons (Muhlig-Versen et al., 2005) and PKA-C3 can also be detected at low levels in most or all neurons (Fig. 2-3*A*). In addition to this weak pan-neuronal expression, we found stronger expression in some neurons, which also appeared to contain higher levels of SWS (arrowheads in Fig. 2-3*A*-C). Interestingly, PKA-C3 was highly expressed in a few very large neurons (asterisks in Fig. 2-3*A*-C). In addition, we detected that PKA-C3 and SWS co-localize on a sub-cellular level because both can be found in the same vesicles (arrows, Fig. 2-*3A*-*C*). As mentioned above, SWS and NTE both have been shown to be enriched in the ER (Akassoglou et al., 2004; Muhlig-Versen et al., 2005), suggesting that the PKA-C3 immunopositive vesicles might be part of the ER. To test this hypothesis, we performed co-immunostainings with anti-PKA-C3 (Fig. 2-3*D*) and GRP78 (Fig. 2-3*E*), a marker for the ER. As shown in Fig. 2-3*F*, some vesicles indeed contained both protein (white arrows) but others were only positive for PKA-C3 (green arrowheads) or GRP78 (red arrowheads). These results show that some PKA-C3 is localized to the ER, but like SWS it is also found in other parts of the cell, and indeed SWS and PKA-C3 seam to co-localize to a much higher degree than PKA-C3 and GRP78.

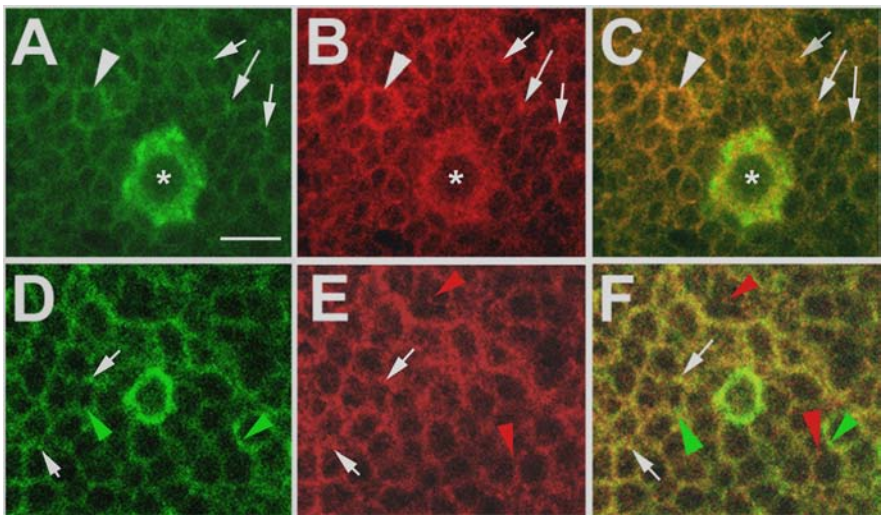

Figure 2-3. SWS and PKA-C3 co-localize in neurons. *A-C*, Brain whole-mounts stained with anti-PKA-C3 (green) and anti-SWS (red) show expression in most or all neurons with stronger expression of both proteins in some neurons (arrowheads), including a few large neurons, which highly express PKA-C3 (asterisks). In addition, both proteins can be co-localized to the same vesicles (arrows). *D-E*, In some vesicles PKA-C3 (green) co-localizes with the ER marker GRP78 (red), although both proteins can also be found separately (green arrowheads and red arrowheads). Thickness of the optical sections was 0.1mm. Scale bar=5μm.

Both, SWS and NTE contain several predicted transmembrane domains and SWS could therefore tether PKA-C3, which by itself is not a transmembrane protein, to membranes. To determine whether SWS does affect the sub-cellular location of PKA-C3, we used the PKA-C3 antiserum on brain whole mounts from *sws*^{1} mutant flies. Whereas a mostly vesicular pattern of PKA-C3 is detected in the wild type background (Fig. 2-4*A*), its sub-cellular localization becomes less concentrated to vesicles in *sws*^{1} mutant brains (Fig. 2-4*B*). To verify that SWS does indeed recruit PKA-C3 to membranes, we performed Western Blots form membrane and cytosolic fractions prepared from *yw* control and *sws*^{1}

mutant heads. First, we confirmed that SWS is confined to membranes and as shown in Fig 2-4*C*, the 160kD SWS protein is exclusively detected in membrane fractions from control flies. We could also detect a putative PKA-C3 band of 66kD (the predicted size for PKA-C3) in control membrane fractions, which was missing in membrane fractions prepared from *sws*^{1} flies (asterisks in Fig. 2-4*D*). To verify that this band does correspond to PKA-C3, we created flies that contained a PKA-C3 construct under the control of the UAS sequence and expressed it in neurons using *Appl*-GAL4. Membrane fractions derived from these flies (PKA m) did indeed show increased levels of this band. Although PKA-C3 was depleted form the membranes of *sws*^{1} flies, we could not detect an accumulation of the 66kD band in the cytosolic fraction of *sws*^{1} (nor did we see it in control flies). However, a smaller band of about 50kD was increased in *sws*^{1} cytosolic fractions compared to controls, which could be a degradation product of PKA-C3. To determine whether SWS affects the levels of PKA-C3, we performed Western Blot with head lysates which showed that the lack of SWS does not affect PKA-C3 levels of PKA-C3 in general (supplementary figure 3, lane 4 and 5). We also induced this construct in the eye (via GMR-GAL4) or pan-neuronally (via *elav*-GAL4), which again resulted in the increase of the 66kD band. These results show that the loss of SWS does not affect PKA-C3 levels but changes its sub-cellular localization, depleting it form membranes.

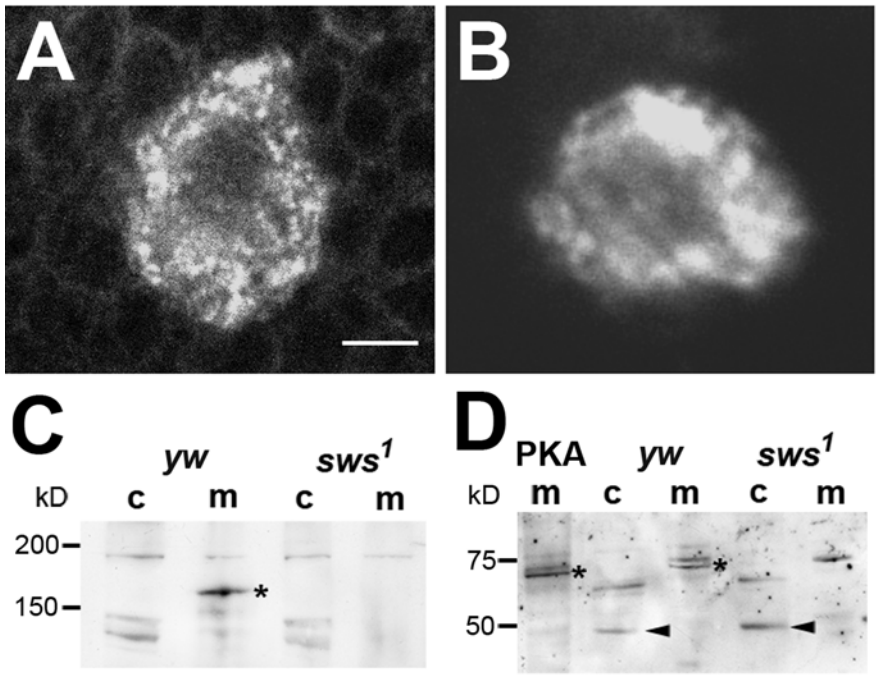

Figure 2-4. PKA-C3 is mislocalized in the absence of SWS. *A*, Whereas PKA-C3 can be found in a mostly punctuate pattern in *yw* control flies, its pattern is less distinct in a *sws*^{1} mutant fly (*B*). *C*, In Western Blots, the SWS protein (asterisks), which contains several transmembrane domains, is exclusively found in membrane fractions (m) from *yw* control flies, while it is missing in *sws*^{1} mutants. *D*, PKA-C3 is detected as a 66kD band in membrane fractions from *yw* control flies and, in increased amount in Appl-GAL4/UAS-PKA-C3 (mPKA) flies, whereas it is not detectable in membrane fractions from *sws*^{1} mutant flies. Cytosolic fractions (c) reveal an increase in a 50kD band in *sws*^{1}, which could be a degradation product of PKA-C3. Scale bar for *A* and *B*=2µm.

SWS inhibits the catalytic activity of PKA

To investigate the functional consequences of the interaction between SWS and PKA-C3, we tested whether SWS regulates the catalytic activity of PKA-C3. For this experiment, we employed a PKA activity assay based on the phosphorylation of the kemptide peptide *in vitro*. Measuring the overall PKA activity in fly head homogenates in

the presence of cAMP, we found a significant increase in the PKA activity of *sws*^{1} mutant flies (1.15$^{\pm}$0.16 p/q) versus controls (0.82$^{\pm}$0.10 p/q; p<0.01, Fig. 2-5). In contrast, expression of additional SWS in neurons via *elav*-GAL4 reduced the activity to 0.55$^{\pm}$0.36 p/q although not quite statistically significant (p=0.054). To determine the activity with the intrinsic cyclic nucleotide concentration present in the lysates, we repeated these measurements without adding cAMP and also found a significant increase in the kinase activity of *sws*^{1} mutant flies compared to control flies (0.83$^{\pm}$0.11 p/q; versus 0.52$^{\pm}$0.04 p/q, p<0.02, *yw*: n=16, *sws*^{1}; n=19). These results strongly suggest that SWS binding to PKA-C3 inhibits the catalytic function of PKA, similar to the inhibitory function described for canonical regulatory subunits of PKA.

Somewhat surprisingly, pan-neuronal expression of PKA-C3 via *Appl*-GAL4 in the presence of cAMP (or *elav*-GAL4, data not shown) did not significantly increase the overall kinase activity (0.9$^{\pm}$0.1 versus 0.83$^{\pm}$0.1 p/q, Fig. 2-5) although the expression of PKA-C3 was confirmed by Western Blots (supplementary figure 5). It is possible that the additional expression of only this isoform is not sufficient to significantly change the overall level of PKA activity. Alternatively, the additional PKA-C3 is inactive because it is bound by SWS.

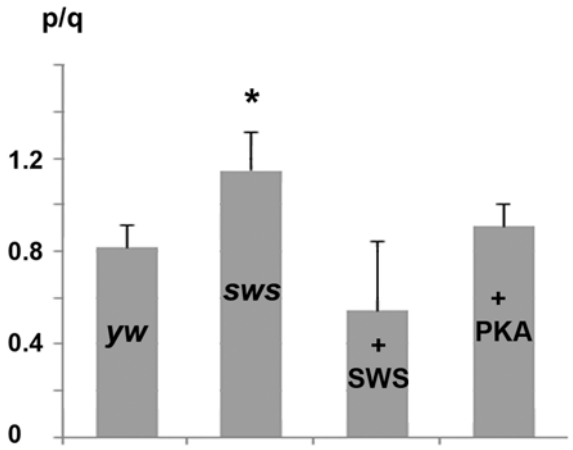

Figure 2-5. PKA activity in fly head homogenates. In the Presence of cAMP (0.4 mM) PKA activity is increased by about 30% in *sws*^{1} extracts compared to *yw* control flies, while expressing additional SWS in wild type reduces PKA activity (although not statistically significant; p=0.054). Expression of additional PKA-C3 via *Appl*-GAL4 did not increase the overall PKA activity compared to *yw* controls. n=16 for *yw*, n=21 for *sws*^{1}, n=13 for *elav*-GAL4/UAS-SWS, and n=15 for *Appl*-GAL4/UAS-PKA/C3. p/q=quotient of the luminosity value of phosphorylated to unphosphorylated kemptide peptide. Values were normalized to ng protein in the lysates. SEMs are indicated.

PKA-C3 modulates the neurodegenerative phenotype of *sws*

To investigate whether PKA-C3 plays a role in the neurodegenerative phenotype observed in *sws*^{1} mutant flies, we genetically altered the levels of PKA-C3 in *sws*^{1}. Comparing *sws*^{1} flies with or without additional expression of PKA-C3 (via *Appl*-GAL4), we found a significant increase of the degenerative phenotype in the presence of additional PKA-C3 (Fig. 2-6*B,* Fig. 2-6*A sws*^{1} alone). As shown in figure 6*C*, *sws*^{1} flies over-expressing PKA-C3 showed a mean vacuole area of $79^{\pm}8.2$ mm^{2} compared to $52^{\pm}6.8$ mm^{2} in *sws*^{1} ($p<0.05$). Next we asked if lower levels of PKA-C3 can suppress the *sws*

phenotype. Whereas combining *sws*^{1} with the Df(3L)brm^{11} deficiency which removed one copy of the PKA-C3 gene did not reduced vacuole formation in the central brain (54$^{\pm}$9.2 mm^{2}), measurements in another part of the brain, the lamina cortex, revealed a significant suppression. Vacuole formation in this area was reduced from 39$^{\pm}$4.1 mm^{2} in *sws*^{1} (Fig. 6*E*) to 22$^{\pm}$3.7 mm^{2} ($p<0.05$) in *sws*^{1} flies heterozygous for the deficiency (Fig. 2-6*D*). These measurements were performed in 7d old flies because the severe phenotype in this area often results in a loss of contact between the eye and the lamina cortex in 14d old *sws*^{1} flies, preventing the measurement of vacuoles. These genetic interactions show that the interaction of PKA-C3 with SWS plays a role in the progressive neurodegeneration observed in *sws*^{1}. To determine whether this effect is specific for PKA-C3, we also removed one copy of PKA-C1 using flies heterozygous for the PKA-C1^{C2} allele (homozygous PKA-C1^{C2} is lethal), which did not affect the vacuole formation in *sws*^{1} (data not shown), neither in the central brain nor in the lamina cortex.

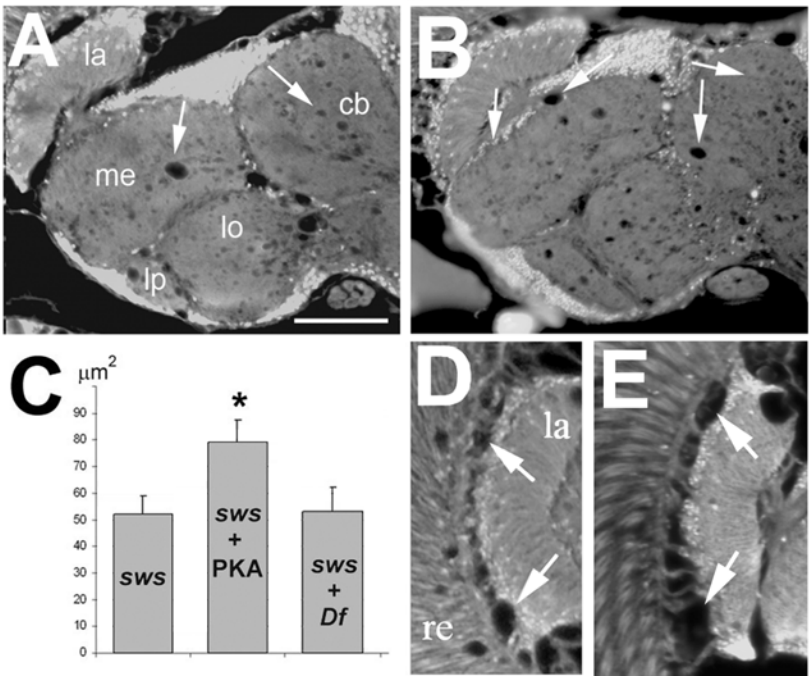

Figure 2-6. Over-expression of PKA-C3 enhances the neurodegenerative phenotype of *sws*. Compared to the *sws*^{1} mutant fly (*A*), flies expressing PKA-C3 via the *APPL*-GAL4 driver (*B*) showed a significant increase in vacuolization. *C,* This is confirmed by measuring the mean area of vacuoles in mm^{2} in *sws*^{1} (n=24) and *sws*^{1}; UAS-PKA-C3 flies (n=33). *sws*^{1} flies carrying one copy of the deficiency Df(2)brm^{11} showed no significant difference in the mean area of vacuoles in the central brain (n=22). Sections in *A* and *B* are horizontal paraffin sections through the heads of 14day old flies. *D*, Vacuoles (arrows) in the lamina cortex of a 7d old *sws*^{1} fly heterozygous for Df(2)brm^{11}. In this region Df(2)brm^{11} significantly reduces the area of vacuoles compared to *sws*^{1} without the deficiency (*E*) (n=17 for *sws*^{1}, n=13 for *sws*^{1}; Df(2)brm^{11}). Scale bar for *A*-*C*=50µm. la=lamina, me=medulla, lo=lobula, lb=lobula plate, cb=central brain, re=retina.

Additional expression of PKA-C3 does not affect the esterase activity of SWS

As described above, we found that the SWS construct with the mutation in the interaction site (SWS^{R133A}) showed no esterase activity when expressed in *sws*^{1} flies,

suggesting that the interaction of SWS with PKA-C3 might regulate its esterase activity. We therefore tested whether increased amounts of PKA-C3 affect the esterase activity of SWS. Comparing the esterase activity levels in homogenates prepared from control flies versus flies expressing additional PKA-C3 pan-neuronally revealed that elevated levels of PKA-C3 did not change the esterase activity of SWS significantly ($108^{\pm}10$ versus $100^{\pm}10$). This suggests that binding to PKA-C3, although required to activate the esterase activity of SWS, complex formation per se is not sufficient to activate the esterase function.

Hyperactive PKA-C3 in the *sws* mutant contributes to the degenerative phenotype

The hyperactive PKA in *sws* flies and the enhanced degenerative phenotype in the presence of additional PKA-C3, suggested that increased PKA activity could play a role in the progressive neurodegeneration observed in *sws*. To test this hypothesis, we investigated whether additional expression of PKA-C3 in the nervous system can by itself induce a neurodegenerative phenotype. Whereas we could not detect any signs of degeneration in 1d old male *Appl*-GAL4/Y; UAS-PKA-C3/+ flies (Fig. 2-7*A*), these flies showed a few, mostly large vacuoles after 30d of aging (arrows, Fig. 2-7*B*). In approximately 10% of these flies, we also found severe vacuolization in the lamina (arrowhead, Fig. 2-7*C*). Aged females, carrying one copy of each *Appl*-GAL4 and UAS-PKA-C3 did not show a phenotype probably due to effects of dosage compensation (Lucchesi, 1996) on the X-chromosomal *Appl*-GAL4. Nevertheless, this shows that expression of PKA-C3 in neurons can induce a degenerative phenotype, although it is quite weak. However, as we present in figure 2-5*A* these flies only exhibited a slight increase in their kinase activity (which was not significant) and therefore a weak phenotype may be expected. As noted above, the lack of a significant increase in kinase activity

could be due to the fact that additionally expressed PKA-C3 is catalytically inactive because it is inhibited by binding to SWS. Therefore, we repeated these experiments with PKA-C3 over-expressing flies that lack on copy of the *sws* gene, thereby reducing the amount of the proposed PKA-C3 inhibitor. Although heterozygous sws^{1} flies do not show a degenerative phenotype due to the recessive nature of the mutation, they did enhance the degeneration caused by PKA-C3 over-expression, now resulting in vacuolization in aged females (Fig. 2-7*D*; males could not be tested because *sws* is localized on the X).

To get additional support that the neurodegenerative phenotype in *sws* is partially due to the missing inhibition of PKA-C3, and not to the possible effects of PKA-C3 on the esterase function of SWS, we performed rescue experiments with an SWS construct that has no esterase activity (Muhlig-Versen et al., 2005). As shown in figure 2-8, *Appl*-GAL4 induced expression of this construct (SWS^{S985D}) resulted in a partial rescue with a vacuole area of $29.4^{\pm}4.4$ mm^2 (Fig. 2-8*B*) in contrast to control sws^{1} flies with $52^{\pm}6.8$ mm^2 (Fig. 2-8*A*) and sws^{1} flies expressing wild type SWS with $16^{\pm}3.7$ mm^2 (see Fig. 2-1*D*; both $p<0.05$). This shows that a catalytically inactive SWS construct, which however has an intact PKA-C3 interaction domain, can partially rescue the neurodegenerative phenotype. Together with the results, that over-expression of PKA-C3 induces an age-dependent degenerative phenotype, which is enhanced by reducing the level of its inhibitor SWS, this strongly suggests that the missing inhibition of PKA-C3 is partially responsible for the progressive neurodegeneration observed in *sws* mutant flies.

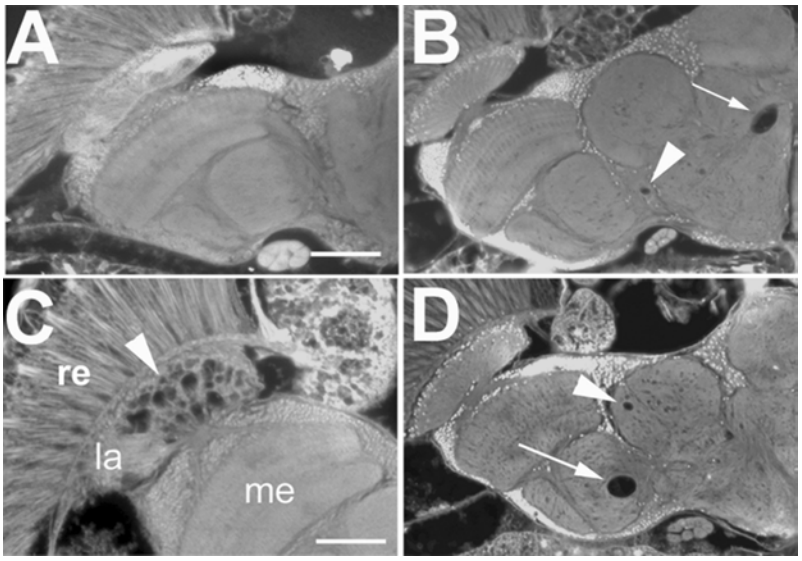

Figure 2-7. Neuronal expression of PKA-C3 induces vacuolization. *A,* A 1d old male fly hemizygous for *Appl*-GAL4 and heterozygous for UAS-PKA-C3 shows no degeneration while a 30d old one (*B*) shows some, sometimes quite large vacuoles (arrowhead and arrow). *C,* In about 10% of these aged flies the lamina shows severe vacuolization (arrowhead). *D*, Removing one copy of *sws* in 30d old females heterozygous for *Appl*-GAL4 and UAS-PKA-C3 also results in the formation of vacuoles similar to the ones seen in males, while these heterozygous females do not show vacuoles without removing one copy of *sws* (data not shown). All sections are horizontal paraffin head sections. Scale bar for *A, B, D*=50µm, *C*=25µm. re=retina, la=lamina.

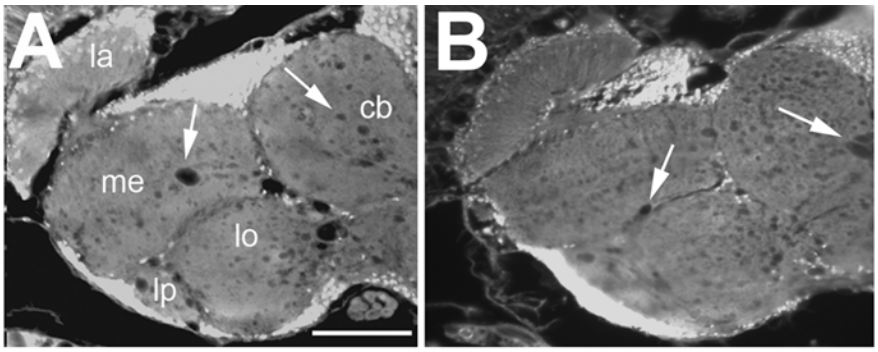

Figure 2-8. Expression of a catalytically inactive SWS construct results in a partial rescue. *A*, *sws*^{1}. *B,* Pan-neuronal expression of SWS^{S985D}, which has no esterase activity (Muhlig-Versen et al., 2005) but an intact PKA-C3 interaction domain in the nervous system of *sws*^{1} mutant flies can partially rescue the degenerative phenotype. Mean area of vacuoles in mm^{2} in *Appl*-GAL4, *sws*^{1} flies (n=24) and *Appl*-GAL4, *sws*^{1}; UAS-SWS^{S985D} (n=38). Sections are horizontal paraffin sections through the heads of 14day old flies. SEMs are indicated. Scale bar=50µm. la=lamina, me=medulla, lo=lobula, lb=lobula plate, cb=central brain.

Discussion

Our results show that SWS binds specifically to PKA-C3, inhibiting its catalytic function, similar to the inhibitory function of canonical regulatory subunits. The binding is mediated by the N-terminally localized interaction domain of SWS which contains a site, the pseudo-substrate site, that is shared by all types of canonical regulatory subunits (Poteet-Smith et al., 1997). As we have shown, a mutation in one of the conserved arginines in the pseudo-substrate site reduces the binding capacity of SWS for PKA-C3, similar to the decreased binding observed with comparable mutations in vertebrate canonical regulatory subunits (Buechler et al., 1993; Poteet-Smith et al., 1997). In

addition, we verified that the interaction between SWS and PKA-C3 is of biological relevance because expression of the construct with the mutated pseudo-substrate site (SWS^{R133A}) only partially restores the function of SWS in *sws*^{1} mutant flies. Surprisingly, we found that SWS^{R133A} does not exhibit significant esterase activity in the *sws*^{1} background, although it has an intact esterase domain, suggesting that the interaction between SWS and PKA-C3 has a dual function: regulating the kinase activity of PKA-C3 and the esterase activity of SWS.

In addition, the binding of PKA-C3 to the transmembrane protein SWS also regulates the sub-cellular localization of PKA-C3; while PKA-C3 is found in membrane fractions in wild type flies it is missing in membranes from *sw*s mutants. For canonical PKA complexes it has been shown for that the spatial regulation plays an important role for the function because it ensures that PKA is exposed to localized cAMP gradients within a cell, which then allows accurate substrate selection (Michel and Scott, 2002). Although the targeting of canonical PKA complexes to different cellular compartments is also regulated by the regulatory subunits, in canonical complexes it is controlled by binding of the regulatory subunit to various A kinase-anchoring proteins (Feliciello et al., 2001; Michel and Scott, 2002) and not a direct consequence of the localization of the regulatory subunit itself.

The regulatory subunits of PKA are not only controlling the localization of the complex but also the activation of its kinase function by binding cAMP, which results in the release of the catalytic subunit (Taylor et al., 2005). As shown in supplementary figure 2 and 3, SWS as well as its human and mouse orthologues, contain three cyclic nucleotide binding sites; a tandem binding site similar to the one found in canonical regulatory subunits and

an additional single site. Interestingly, this single site resembles more a cGMP binding site because, both SWS and NTE contain a threonine instead of an alanine within this motif (highlighted in supplementary figure 3*A*), which is typical for cAMP binding sites (Shabb et al., 1990). In addition, the third binding site in SWS also has a threonine (whereas NTE has an alanine, highlighted in supplementary figure 3*B*), suggesting that the complex formation of SWS and PKA-C3 may be regulated by cGMP. However, it has been shown that cyclic nucleotide binding sites can also bind to other ligands, including heme in the bacterial transcription factor cooA subfamily (Lanzilotta et al., 2000) and ortho-chlorophenolic compounds in the bacterial transcriptional regulators CprK (Kannan et al., 2007). Future experiments will determine whether and which compound the three cyclic nucleotide binding sites of SWS can bind.

In addition to identifying this novel PKA complex our results also describe for the first time a function for the unique C3 subunit. PKA-C3 has been identified as a catalytic subunit by its homology to the well-characterized PKA-C1 subunit (Kalderon and Rubin, 1988). Comparing PKA-C3 with the other two catalytic subunits found in *Drosophila* showed that it is closer related to the C1 subunit ($7e^{-103}$) than to C2 ($3e^{-70}$; see supplementary figure 6) but studies expressing PKA-C3 under the control of the PKA-C1 promoter have indicated that PKA-C3 is not functionally redundant with PKA-C1 (Melendez et al., 1995).

PKA-C3 shares 62% identity in the catalytic core region with the recently isolated X-linked protein kinases in mouse (Pkare) and human (PrKX) (Blaschke et al., 2000; Diskar et al., 2007). Notably, the homology between the human and fly proteins is actually higher than between the different catalytic subunits from any one species (Zimmermann et al.,

1999). Comparing PKA-C3 with human PrKX provided an E-value of $7e^{-125}$ and PKA-C1 has an even higher homology to human PKA-Ca ($7e^{-174}$; see supplementary figure 7). It was therefore suggested that these unique catalytic subunits represent a novel class of evolutionary conserved PKAs.

Here we show, that PKA-C3 is expressed in the nervous system, with very high levels in distinct large neurons, suggesting a function in the adult nervous system. This was verified by our results that additional PKA-C3 enhances the neuronal degeneration observed in *sws*^{1}, while removing on copy of the PKA-C3 gene suppresses degeneration, confirming a neuronal function. This neuroprotective function could be solely mediated by its effects on SWS, alternatively PKA-C3 can directly induce neurodegeneration. The latter is supported by our findings that over-expression of PKA-C3 in neurons resulted in a weak but readily detectable degenerative phenotype, while it did not affect the esterase function of SWS. In addition, an SWS construct without esterase activity but an intact pseudo-substrate site, allowing binding and inhibition of PKA-C3, can still partially rescue the degenerative phenotype of *sws*^{1}. Finally, removing one copy of the proposed inhibitor SWS in PKA-C3 over-expressing flies, enhanced the PKA-C3 induced neurodegeneration. Therefore, we propose that SWS acts as a non-canonical regulatory subunit that inhibits the catalytic activity of PKA-C3, and that the disruption of this inhibitory mechanism results in the uncontrolled release of active PKA-C3 which damages the nervous system.

Because mouse Pkare mRNA is also expressed in many regions of the adult nervous system, including hypothalamus, hippocampus, cerebellum, and olfactory bulb (Blaschke et al., 2000) while the human Pkare protein was shown to be highly expressed in brain (Li et al., 2005). Together with our data, that PKA-C3 plays a role in progressive

neurodegeneration, this suggests that this novel class of catalytic subunits may have a specific function in the maintenance of the nervous system conserved between flies and mammals.

Materials and Methods

Drosophila stocks and UAS-lines. The sws^1 allele has been described in (Kretzschmar et al., 1997). If not stated differently, yw, the genetic background of all the transgenic lines and sws^1 was used as control. Actin-GAL4, GMR-GAL4, elav-GAL4, and $Df(3L)brm^{11}$ were provided by the Bloomington Stock Center and Appl-GAL4 by L. Torroja (Universidad Autonoma de Madrid, Spain). Stocks were maintained and raised under standard conditions. To create the UAS-SWS^{R133A} construct, we used the pUAST-sws construct described in (Muhlig-Versen et al., 2005) and replaced the $arginine^{133}$ in the PKA pseudo-substrate site by alanine, using primers that substituted the CGG triplet by GAA. The SWS^{S985D} construct has been described in (Muhlig-Versen et al., 2005). The UAS-PKA-C3 line was created by inserting the PKA-C3 coding region (the PKA-C3 cDNA was kindly provided by D. Kalderon, Columbia University, New York) into the pUAST vector (Brand and Perrimon, 1993).

Tissue sections and whole mount preparations for immunohistochemistry

Paraffin sections were performed as described in (Bettencourt da Cruz et al., 2005). Adult brains were dissected on ice and fixed in 4% parformaldehyde over night. Immunohistochemistry was performed following the protocol of Buchner et al. (in Ashburner, 1989). The anti-SWS rabbit antisera was used 1:100, anti-PKA-C3 rat anitsera was used 1: 1000, and mouse-anti GRP78 (Stressgen) 1:1000 and applied over night at 4^oC. Cy2 and Cy3 secondary antibodies were obtained from Jackson ImmunoResearch. Preparations were observed with an Olympus Fluoview confocal 300 microscope and optical sections taken with a thickness of 0.1mm.

Determination of vacuole size. To analyze the neurodegenerative phenotype of different genotypes, we photographed sections at the level of the great commissure. For a double blind analysis, pictures were taken and numbered and the area of the vacuoles in the central brain was then calculated in Photoshop as total pixel number, which was subsequently converted into mm^2 (Bettencourt da Cruz et al., 2005). As controls, we used flies from the same cross which did not carry the UAS construct and flies which had a balancer chromosome instead of the deletion chromosome. Both controls showed similar values and were therefore pooled. Statistics were done using the SPSS program and independent samples t-tests. Levene's test was used to determine variance and p-values.

Yeast Two Hybrid screens. The Two-Hybrid-Screens were performed using the CytoTrap Vector kit and the CytoTrap® XR Adult *Drosophila* cDNA Library from Stratagene following the instruction manual. As bait, we used the full-length SWS protein and two fragments that did not contain any of the predicted transmembrane domains. Both of these shortened fragments contained the pseudo-substrate site as predicted from sequence similarity with the regulatory subunit of PKA. The longer fragment consisted of amino acids 60-544, the smaller one of amino acids 60-241. cDNAs for the PKA-C1 and PKA-C2 catalytic subunit were kindly provided by D. Kalderon and inserted into the pMyr vector (Columbia University, New York).

Western Blots

Membrane and cytosolic fractions were prepared from the different genotypes following the protocol of Orgad et al. (Orgad et al., 1987). Approximately 300 heads were used for

each preparation, protein amounts determined by Bradford assays (Bradford, 1976) and 12mg loaded per lane. Gels and blots were performed as described in (Tschape et al., 2002). Anti-SWS was used at 1:500 and anti-PKA 1:4000 (generated by D. Kalderon and kindly provided by B. Biteau, University of Rochester, New York). When head lysates were used directly in Western Blots, five heads were loaded per lane and a loading control performed using anti-actin 1:500 (Developmental Studies Hybridoma Bank, University of Iowa).

PKA activity assays. The PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase kit from Promega was used for PKA activity measurement. Five fly heads were homogenized in 50µl extraction buffer and centrifuged at 14,000xg for five minutes. 2 µl of the supernatant was immediately used for activity measurement assays for each sample, while 20ml were used to perform Bradford assays (Bradford, 1976). Activity measurements were performed using 0.04mg/ml PepTag A1 peptide, and 250mM isobutylmethylxanthine (IBMX) either with or without cAMP (0.4 mM) added to the reaction. The reaction was performed according to the kit protocol and evaluated by luminosity measurement of the gel picture by determining the p/q quotient of the luminosity value of phosphorylated to unphosphorylated kemptide peptide. Values were normalized per ng amount of protein in the lysate, as determined by the Bradford assays. As positive control, we used 2mg/ml PKA provided by the kit, negative controls contained water instead of fly head lysate. Incubation time was 30min.

Esterase assays. Frozen flies were homogenized (5% w/w) in 50 mM Tris-HCl, 1mM EDTA. After brief centrifugation (500xg; 5min) the homogenates were assayed as

described by Johnson (1977). NTE-like activity is operationally defined as that portion of phenyl valerate hydrolyzing activity which is resistant to paraoxon (40 mM; a non-neuropathic OP) but sensitive to mipafox (50 mM; a neuropathic OP). The homogenates were pre-incubated (37^{o}C; 20 min) with paraoxon in the presence or absence of mipafox before substrate, phenyl valerate, was added and reaction allowed to proceed for another 20 min. Activity was determined as the amount of phenol liberated and expressed as the NTE-like activity (i.e., the difference in the activity measured in the presence and absence of mipafox) per mg homogenate protein. Homogenates were prepared from approximately 200 flies (2-5 days old) and measurements done in duplicates.

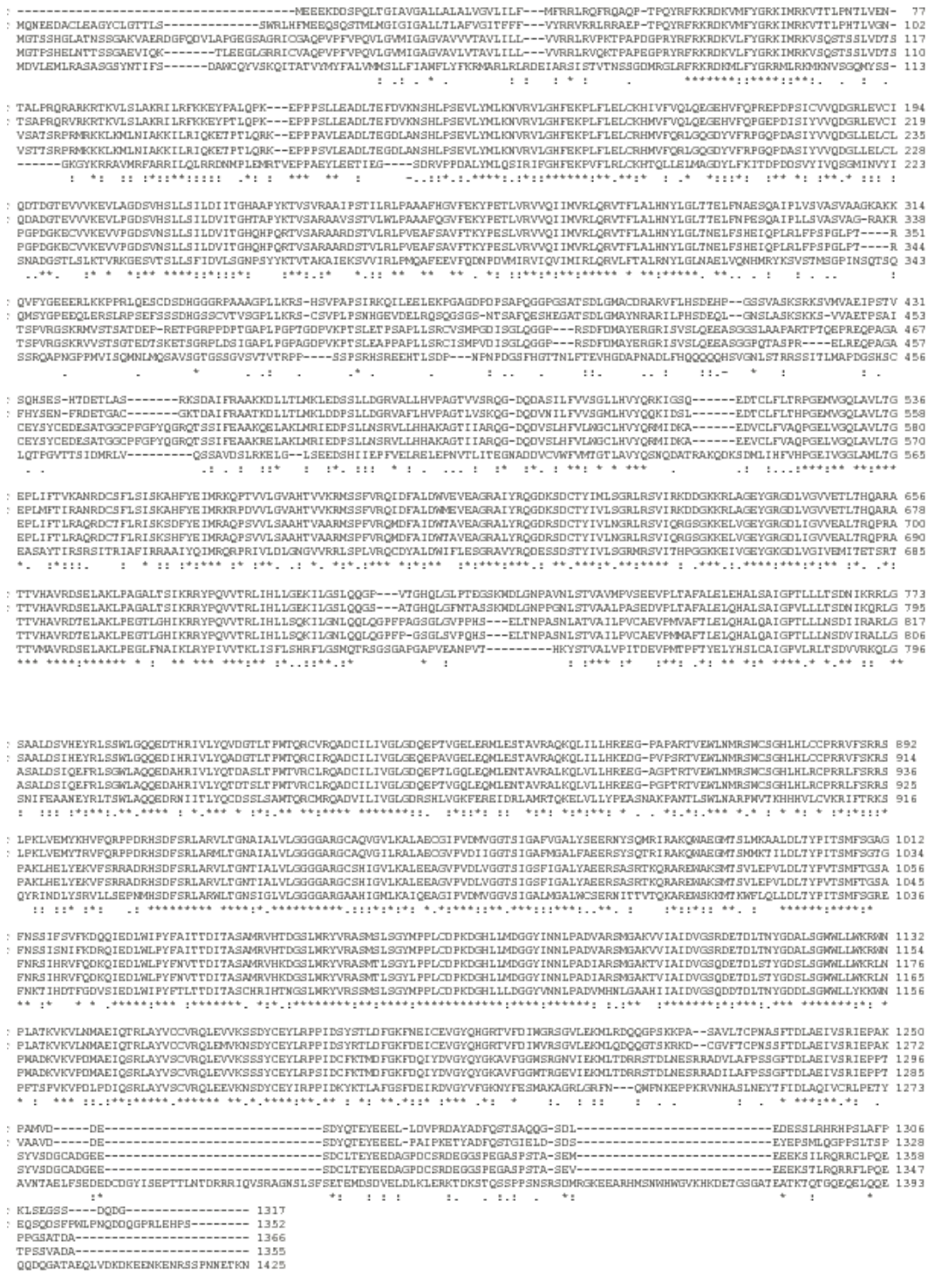

Figure 2-9. Supplementary figure 1. Sequence alignment of SWS, mouse and human NTE, and the recently isolated mouse and human NTE-related (NTE-r). NTE and NTE-r are also referred to as Patatin-like phospholipase domain containing protein 6 and 7, respectively. All NTE proteins show a homology to SWS with an e-value of 0.0. Identical amino acids are indicated by asterisks, highly conserved amino acids by colons, and conserved amino acids by dots.

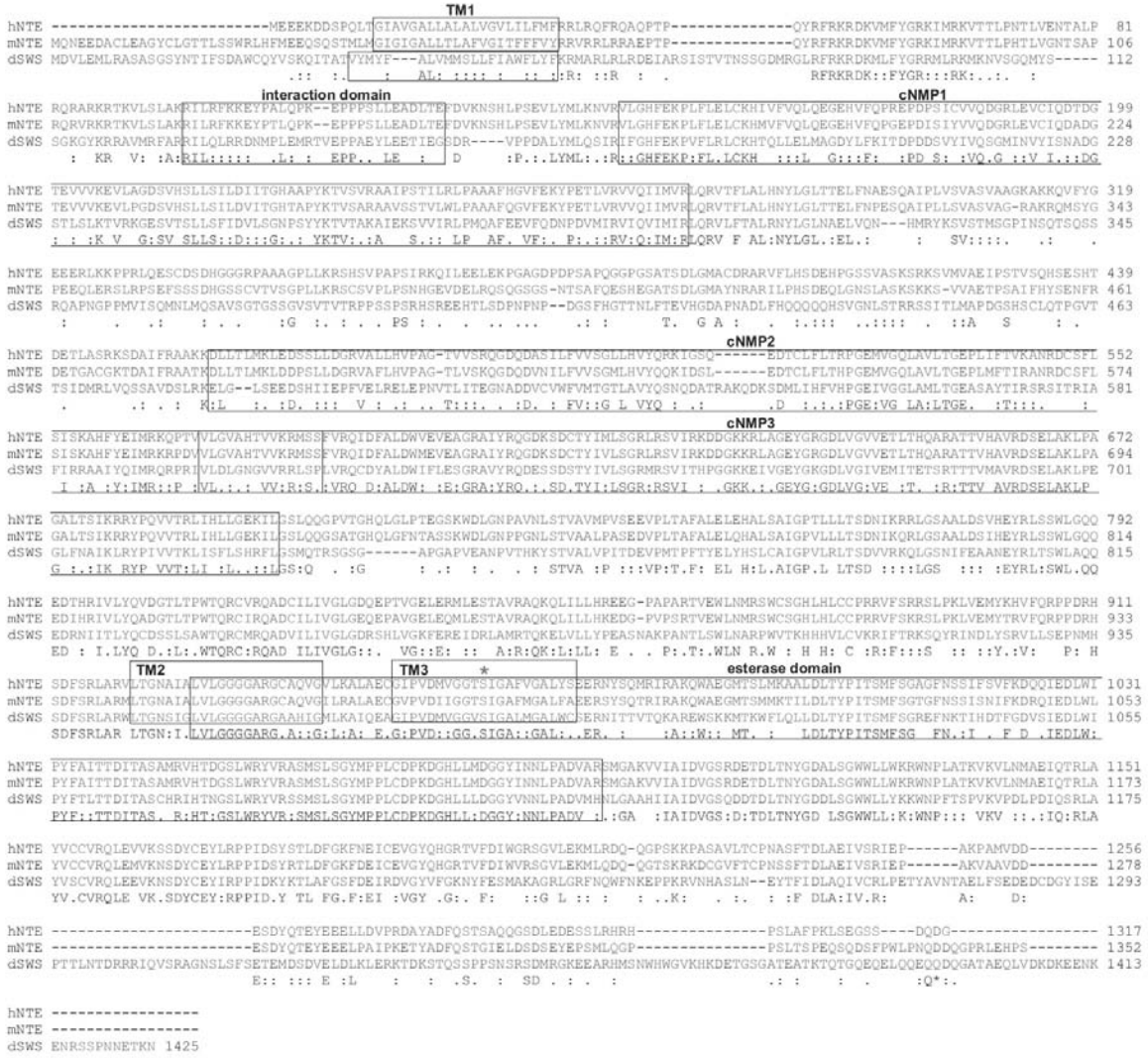

Figure 2-10. Supplementary figure 2. Alignment of SWS, mouse and human NTE-r showing the localization of the transmembrane domains (TM), the interaction domain (ID), the three cyclic nucleotide binding sites (cNMP1-3), and the esterase domain (also called patatin domain). The active sites serine localized in the putative third transmembrane domain is indicated by an asterisk. Identical amino acids are indicated by letters, highly conserved amino acids by colons, and conserved amino acids by dots.

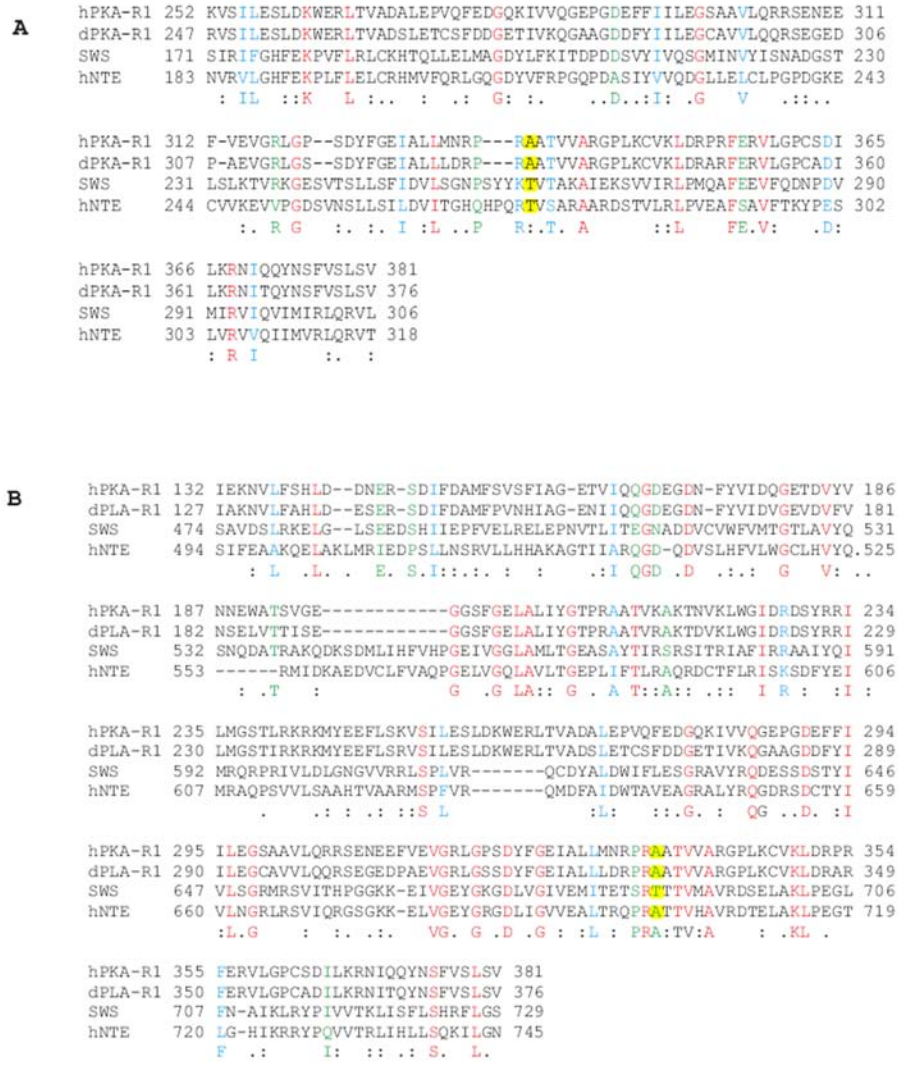

Figure 2-11. Supplementary figure 3. Sequence alignment of the cyclic nucleotide binding regions in human and fly regulatory subunits as well as SWS and NTE. *A*, SWS and NTE contain a single, N-terminally localized cyclic nucleotide binding region that shows homology to the second cAMP binding domain in canonical regulatory subunits. *B*, Comparison of the tandem cyclic nucleotide binding region. Residues conserved in all four sequences are shown in red, residues conserved between the canonical regulatory subunits and either SWS or NTE are shown in blue. The alanines/threonines found in consensus sites for cAMP or cGMP respectively are highlighted.

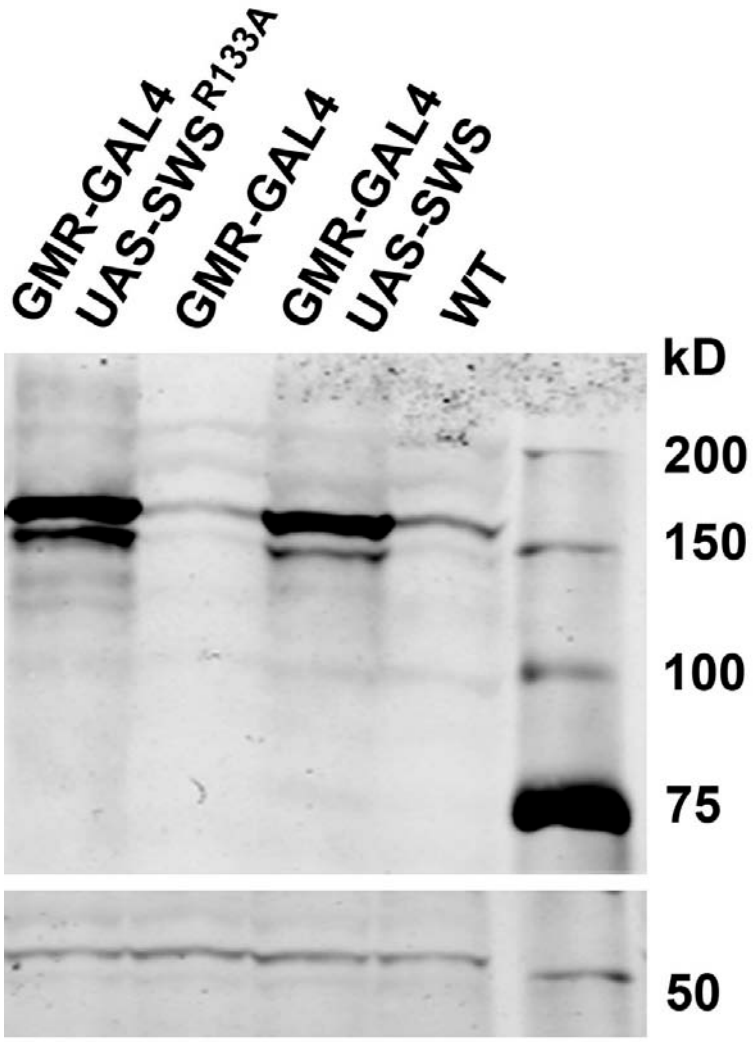

Figure 2-12. Supplementary figure 4. Western Blots reveal a similar expression of SWS^{R133A} and wild type SWS. Expression of both constructs via GMR-GAL4 resulted in an equally increased level of the mutated (SWS^{R133A}, lane 1) and wild type protein (Lane 3) compared to control flies containing only the GMR promoter construct (lane 2) and wild type flies (lane 4). A loading control using anti-actin is shown below.

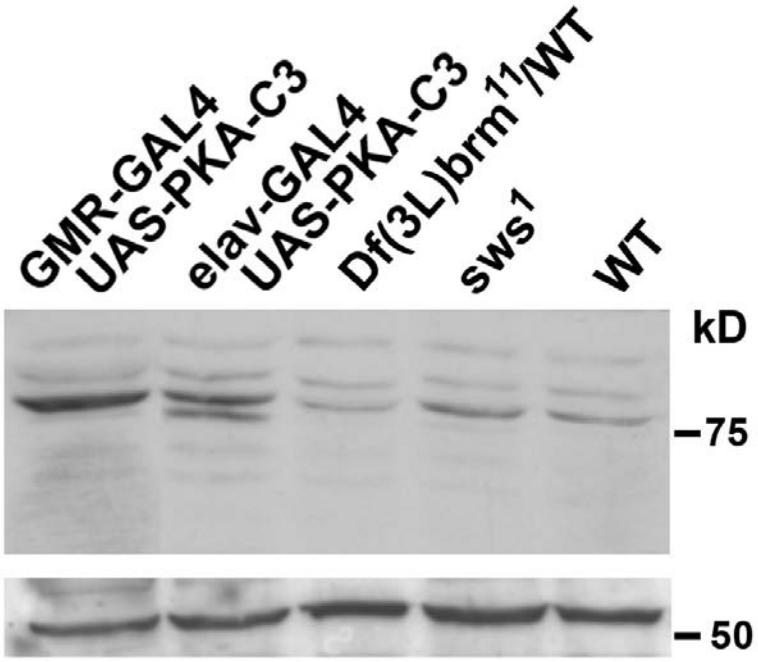

Figure 2-13. Supplementary figure 5. Western blot showing expression levels of PKA-C3. Induction of PKA-C3 with GMR-GAL4 (lane 1) and *elav*-GAL4 (lane 2) results in increased expression levels of PKA-C3, whereas flies heterozygous for the deficiency DF(3L)brm^{11} (lane 3) show decreased levels compared to wild type (lane 5). *sws*^{1} mutant flies (lane 4) show expression levels similar to wild type.

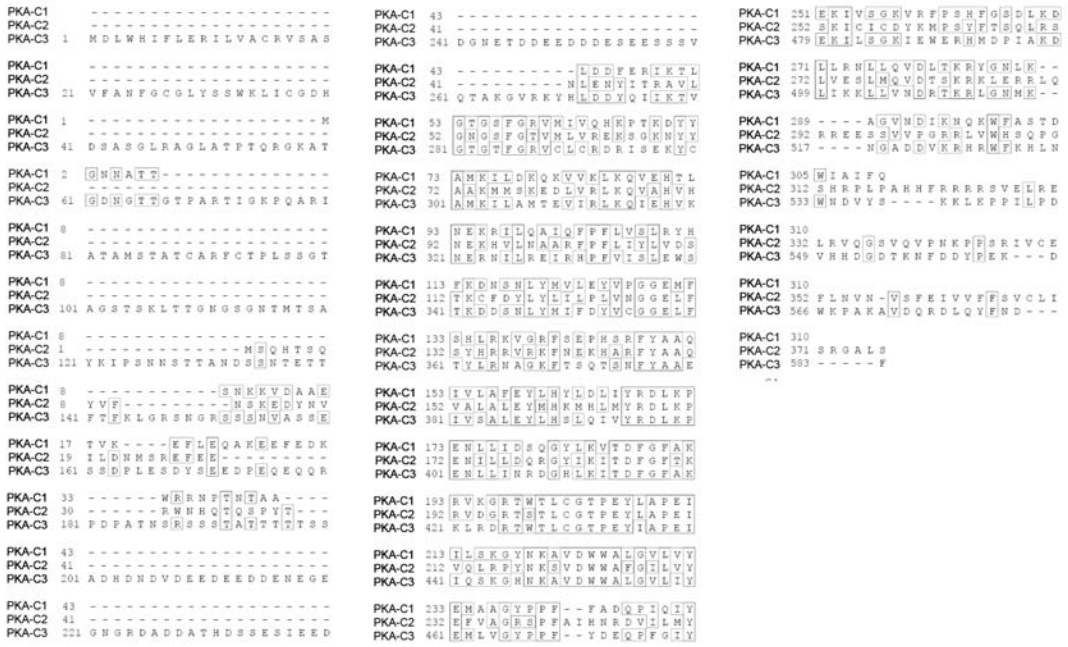

Figure 2-14. Supplementary figure 6. Alignment of the three *Drosophila* catalytic subunits of PKA. Conserved residues are boxed. PKA-C3 is more closely related to PKA-C1 (E-value $7e^{-103}$) than to PKA-C2 (E-value $3e^{-70}$).

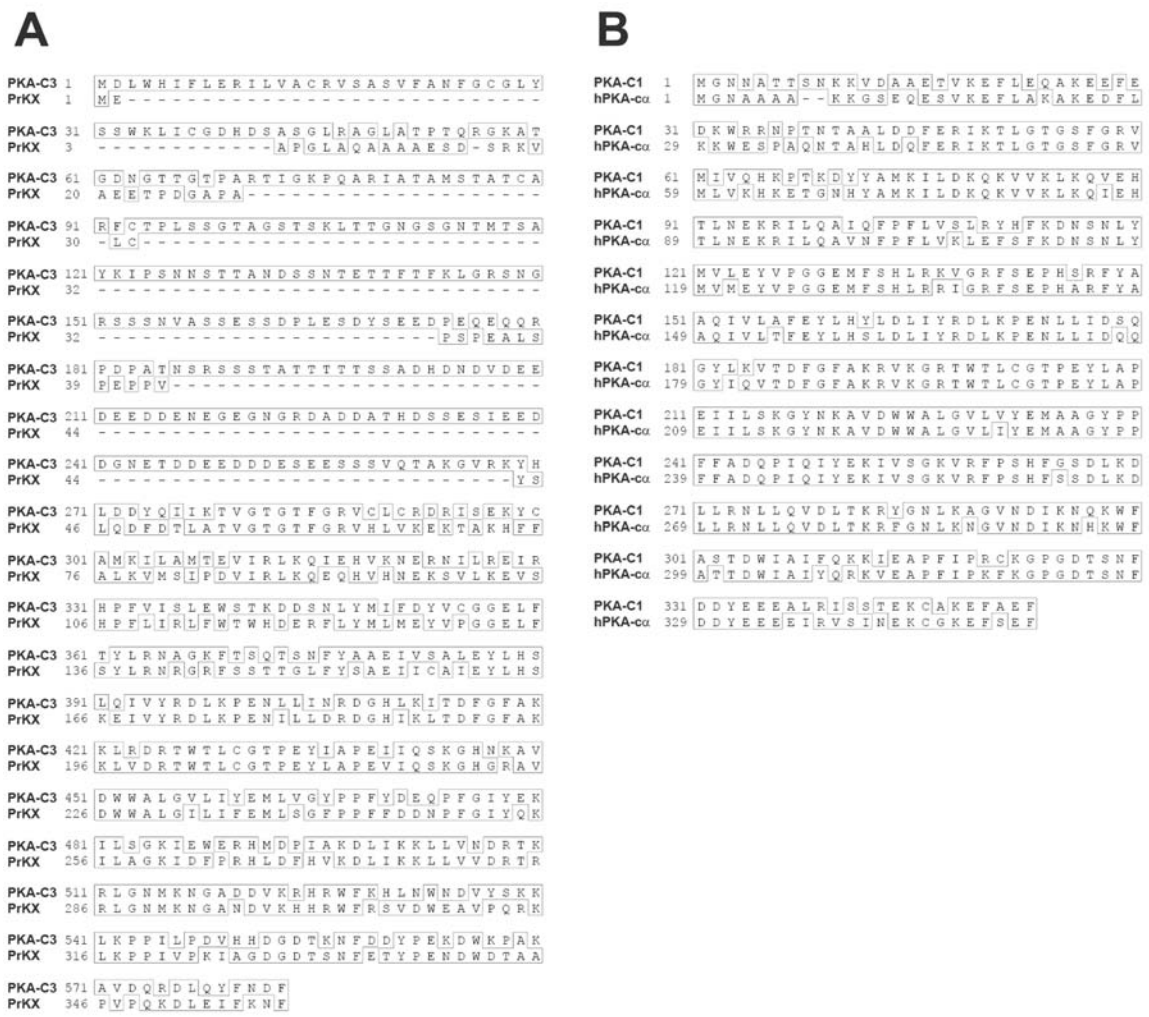

Figure 2-15. Supplementary figure 7. Alignment of *Drosophila* catalytic subunits with their most related human counterpart. *A*, Sequence comparison of PKA-C3 and human PrKX. Conserved amino acids are boxed (E-value $2e^{-125}$). *B*, Sequence comparison of PKA-C1 with human PKA-Ca (E-value $3e^{-174}$). PKA-C2 is equally related to human PKA-Ca (E-value $2e^{-74}$) and human PKA-Cb (E-value $4e^{-75}$).

Genotype	Vacuolation	Esterase activity	Kinase activity
wild type	none	normal	normal
***sws*^{1}**	set as standard	none	increased
wild type + SWS	none	increased	slightly decreased
***sws*^{1} + SWS**	severely decreased	normal	ND
wild type + SWS^{R133A}	ND	increased	ND
***sws*^{1} + SWS^{R133A}**	decreased	severely decreased	ND
wild type + PKA-C3	(weak degeneration)	normal	normal
hetero. *sws*^{1} + PKA-C3	(increased to above)	ND	ND
***sws*^{1} + PKA-C3**	increased	ND	ND
***sws*^{1} - one copy PKA-C3**	decreased	ND	ND
wild type + SWS^{S985D}	none	normal	ND
***sws*^{1} + SWS^{S985D}**	decreased	none	ND

Table 2-1. Supplementary figure 6. Table summarizing the effects of different genotypes on vacuole formation, SWS esterase activity, and kinase activity.

Acknowledgements

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Chapter 3

sws as a model for OPIDN

Unpublished data

Introduction

Organophosphate-induced delayed neuropathy (OPIDN) is a degenerative disease that results from exposure to organophosphate pesticides (OPs). Symptoms include neuronal degeneration and cell death; leading to limb weakness, abnormal reflexes and paralysis (Smith et al., 1930; Abou-Donia, 2003; Lori D. White, 2004) with a characteristic 6 -14 day delay from exposure to onset. Outbreaks of OPIDN have occurred in humans after accidental ingestion of the organophosphorus compound Tri-Ortho-Cresyl Phosphate (TOCP). The most well known epidemic occurred during prohibition in the United States, when a beverage called Jamaica Ginger was contaminated with TOCP resulting in over 50,000 cases of OPIDN (Smith et al., 1930).

Many OPs bind and inhibit acetylcholineesterase (AChE), but some also target a different enzyme called neuropathy target esterase (NTE) in mammals and Swiss Cheese (SWS) in *Drosophila* (Johnson, 1970; Lotti, 2000). The enzymatic activity of NTE/SWS is important for maintaining homeostasis of membrane lipids, particularly by degrading phosphatidylcholine (Zaccheo et al., 2004; Muhlig-Versen et al., 2005). Inhibition of this phospholipase activity has been proposed to play a role in the development of OPIDN (Read et al., 2009).

Because the effects of OPs on AChE are acutely toxic, only those patients who survive initial toxicity (often via antidote treatment) develop delayed neuropathy. Alternatively, those who are exposed to an OP like TOCP, whose preferred target is NTE, will not have acute symptoms, but only exhibit delayed

neuropathy. However, not all OPs that bind NTE/SWS lead to OPIDN. Only those OPs that undergo the so-called “aging reaction” lead to OPIDN, which has led to the theory that “aged” NTE/SWS adopts a new toxic function, distinct from the physiological function of the protein (Glynn, 2003). Evidence for this theory include studies showing that SWS/NTE can be inhibited by non-aging OPs without causing neuropathy (Lotti and Johnson, 1980; Johnson, 1990). Perhaps even more compelling evidence comes from studies in which exposure to non-aging OPs was found to be protective against the neuropathy causing aging OPs (Johnson, 1970).

An alternative argument is that a simple loss of physiological function may account for the development of OPIDN. SWS and NTE are essential nervous system proteins; flies lacking the SWS protein show a dramatic progressive degeneration (Kretzschmar et al., 1997), and similar phenotypes result from brain-specific knock-outs of NTE in mice (Akassoglou et al., 2004). It has been noted that these symptoms are quite similar to what is seen histologically after OP exposure, which (like mutations in SWS/NTE) leads to reduced phospholipase processing (Read et al., 2009). However, other groups have shown that both neuropathic and non-neuropathic OPs lead to similar changes in membrane lipids (Hou et al., 2009), which would argue against this effect being the mechanism for OPIDN. Humans with mutations in NTE have a distinct motor neuron disease resembling spastic paraplegia (Rainier et al., 2008). Nevertheless, the requirement for functional SWS/NTE to maintain healthy neurons and glia does not rule out the possibility that the aged,

organophosphorylated protein might have a novel toxic function, leading to OPIDN neuropathy.

In addition to phospholipase activity, SWS has recently been shown to have a second function as a regulatory subunit for the third catalytic subunit of Protein Kinase A (PKA-C3). In *sws*^{1} mutant flies, a point mutation creates an early stop codon and thus a non-functional protein. These flies show an increase in kinase activity, suggesting that SWS negatively regulates kinase activity (Bettencourt da Cruz et al., 2008). Overactive PKA-C3 seems to be partially responsible for the degeneration seen in *sws*^{1} mutants, as elevated levels of PKA-C3 increases vacuole formation in *sws*^{1} mutants and can also cause degeneration, albeit subtle, in wild type flies.

Based on the involvement of PKA-C3 in the degenerative phenotype in the *sws*^{1} mutant, the aim of this chapter was to see if PKA-C3 activity could also be affected in OPIDN.

Data in this chapter support a gain-of-function scenario of OP-modified SWS/NTE. In particular I have shown that increased levels of SWS protein increase the toxic effects of TOCP on cells in culture and in adult *Drosophila*. I have also shown that the interaction between SWS and PKA-C3 is affected by TOCP, leading to reduced PKA-C3 activity, while increasing the levels of PKA-C3 in *Drosophila* neurons is somewhat protective against the affects of TOCP. I therefore propose that TOCP renders SWS unable to release PKA-C3, thus keeping PKA-C3 away from its normal downstream targets. The resulting

misregulation of PKA-C3 targets could then account for the gain-of-function effect seen after OP exposure. I also hypothesize that only those OPs that age SWS/NTE can result in changes that lead to constitutive binding of PKA-C3 to SWS.

Results

The organophosphorus compound tri-ortho-cresyl phosphate inhibits SWS esterase activity in flies.

To establish *Drosophila* as a model for OP-induced neuronal degeneration, flies were treated with the organophosphorus compound tri-ortho cresyl phosphate (TOCP). TOCP was chosen because it induces OPIDN in humans (Smith et al., 1930), and causes OPIDN-like phenotypes in chickens (Carrington and Abou-Donia, 1983). In contrast to most other organophosphates, which primarily target AChE, TOCP appears to be specific for NTE, with little or no effect on AChE (Lotti, 2000). We first determined an appropriate dose by treating flies with 0.08 mg/ml, 0.8 mg/ml, 8 mg/ml, and 25 mg/ml of TOCP, which is approximately 0.5 to 150 times the neuropathic oral dose for humans (with a fly weighing 1mg and drinking about 1μl in 16h, (Ja et al., 2007)). Two weeks after exposure, the two lower concentrations did not result in an increased lethality compared to mock-treated flies (12% and 8% versus 10% in untreated flies), whereas a dose of 8mg/ml resulted in 27% lethality, which was further increased to 60% at 25 mg/ml. We therefore used doses between 8 mg/ml and 25 mg/ml, depending on the specific assay. For assays in which flies were not allowed to

age and thus lethality over time was less of an issue, 32 mg/ml was used to achieve the maximum effects of TOCP.

To induce OPIDN in chickens, NTE esterase activity must be at least 70% inhibited (Johnson, 1982; Johnson, 1990; Lotti, 1992; Quistad et al., 2002). We therefore measured whether TOCP at the selected concentration range can inhibit SWS esterase activity by feeding 1-5d old flies with an intermediate dose of 16mg/ml TOCP, for 24 hours. We then measured the activity of SWS against the established synthetic substrate phenyl valerate (Johnson, 1977). In wild-type flies, exposure to TOCP reduces SWS esterase activity by about 80% (Figure 3-1a), a reduction that, as mentioned above, leads to degenerative phenotypes in other species. To confirm that TOCP indeed acts on SWS, we also studied the effects of TOCP on flies lacking SWS (*sws*^{1}) and flies expressing additional SWS. As previously shown (Muhlig-Versen et al., 2005), *sws*^{1} mutant flies do not exhibit significant esterase activity, and consequently TOCP treatment did not have a detectable effect. When UAS-SWS was over-expressed with the pan-neuronal driver *elav*-Gal4, esterase activity increased by approximately 1.6 fold in untreated flies, and 60% of this activity was inhibited by TOCP. Thus, flies over-expressing SWS have more than twice the amount of esterase activity after TOCP exposure when compared to wild-type flies exposed to TOCP. To verify that TOCP exposure did not result in inhibition of AChE, we also measured AChE activity in each of these fly lines, but even at the highest dose of 36mg/ml we could not detect any effects on AChE activity. Importantly, even in the *sws*^{1} mutant (where the primary target SWS is lacking), AChE was not inhibited by

TOCP (Fig. 3-1B). Therefore, it is highly unlikely that AChE-related effects play a role in the following experiments.

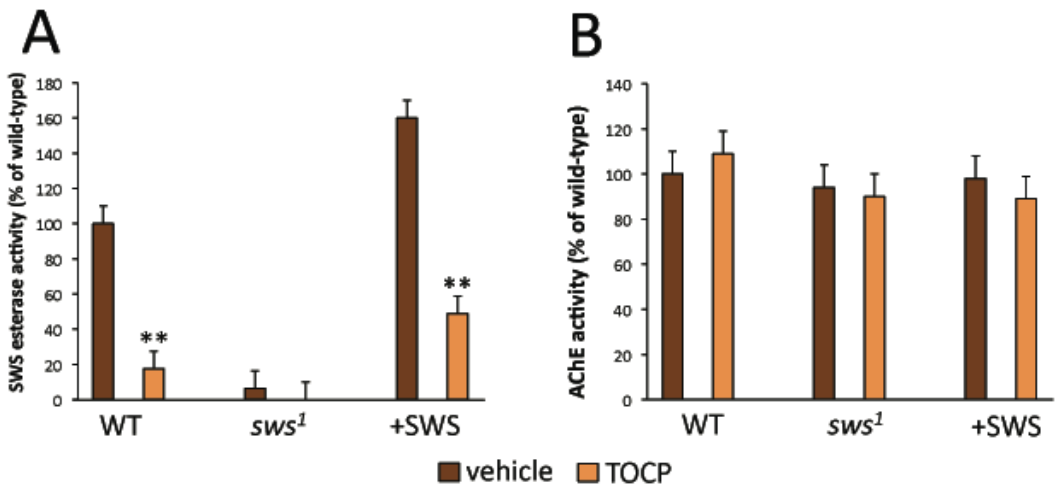

Figure 3-1. Ingestion of the neuropathic organophosphorus ester Tri-Ortho-Cresyl Phosphate inhibits SWS esterase activity but not acetylcholine esterase activity in adult *Drosophila*. **A.** Activity of SWS esterase in fly head homogenates is decreased by TOCP in wild-type, unaltered in *sws*^{1} mutants, and increased with over-expressed SWS protein. **B.** Activity of acetylcholine esterase is not affected by genotype or TOCP exposure. All values are expressed relative to wild-type (100%). Two independent experiments were performed for each assay. **A-B**. SEMs are indicated. Independent t-tests were performed to compare vehicle-treatment to TOCP-treatment for each genotype. ** indicates $p<0.01$.

Drosophila exposed to TOCP develop an OPIDN-like phenotype

To determine whether TOCP can induce symptoms similar to OPIDN in *Drosophila*, we used fast phototaxis behavior to assess ataxia-like symptoms. Fourteen days after a single exposure to 8 mg/ml TOCP, flies showed a reduced performance index compared with vehicle-treated flies (67.3 $\pm$ 2.219 versus 60.8

± 1.647 p=0.069) (Figure 3-2A). While this effect is not statistically significant, additional experiments will hopefully lead to a significant difference, showing that this effect is biologically relevant. To visualize histological signs of degeneration in the brain, 1day old flies were treated with 32 mg/ml TOCP for 48 hours and examined when they were 7 days old. Paraffin sections of brains from TOCP-exposed flies revealed some degeneration in 12 out of 16 brains, although the level of degeneration was quite variable. Additionally, some brains from untreated flies occasionally showed small holes (5 out of 16), possibly due to restricted nutrition during vehicle/toxin administration (i.e. glucose lacks complete nutritional requirements (Tatum, 1939).

Due to the sensitivity of mammalian motor neurons to OP exposure, I also examined the thoracic ganglia, which contains *Drosophila* motor neurons and indeed, I detected vacuoles following TOCP treatment. Many vacuoles were located in the cortex region of the thoracic ganglia (the location of the cell bodies of motor neurons) suggesting that the observed vacuoles are due to neuronal death. Although cell death has been described in OPIDN (Mou et al., 2006), the characteristic feature of this syndrome is axonal degeneration (Lotti, 2000). Accordingly, to examine axonal degeneration in flies, I performed studies in primary neuronal cell cultures in which neurite length can be easily measured.

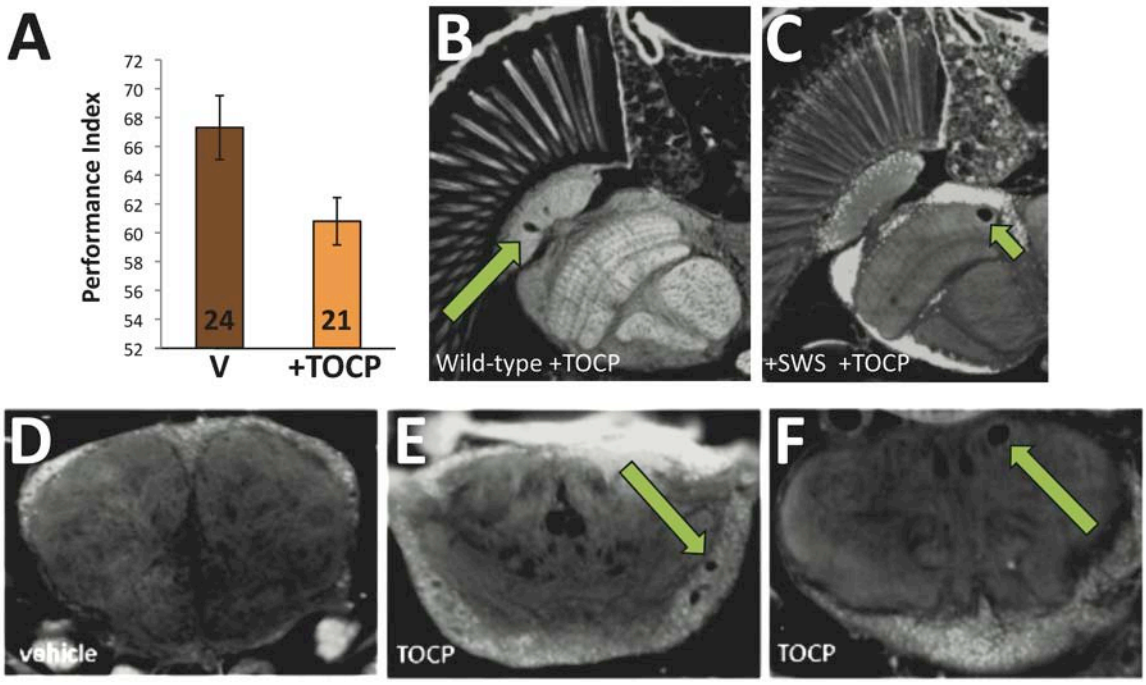

Figure 3-2. TOCP exposure causes histopathology defects in wild-type adult *Drosophila*. **A.** Fast phototaxis assays reveal that fourteen days after TOCP exposure treated flies perform less well than those exposed to vehicle. A t-test was used to compare performance index of vehicle-treated versus TOCP-treated flies (p=0.069). Error bars represent SEMs. N (labeled inside bars) = independent experiments. **B.** Example of wild-type fly that developed vacuoles (arrow) after consuming 32 mg/ml TOCP. **C.** Example of a fly expressing increased levels of SWS pan neuronally that developed vacuoles (arrow) after consuming 32 mg/ml TOCP. **D.** Thoracic ganglia of a wild-type fly that was exposed to vehicle. **E.** TOCP-treated (32 mg/ml) wild-type fly that shows vacuole formation in the cell body region of the thoracic ganglia (arrow), indicating cell death. **F.** TOCP-treated (32 mg/ml) wild-type fly that shows vacuole formation in the neuropil region (arrow) that is consistent with axon degeneration.

TOCP exposure causes degeneration of neuronal processes

To determine an appropriate concentration of TOCP and to investigate the dose-dependency of its effects, I cultured primary neurons for 12 hours and then treated them with TOCP, in concentrations ranging from 1.5 μg/ml to 50 μg/ml. After 6 hours, cells were imaged, and I measured the length of the longest neurite from each cell. As shown in Figure 3-3A, neurite length decreased with increasing doses of TOCP, revealing a dose-dependent response. This decrease in neurite length could either be due to the inhibition of outgrowth by TOCP treatment or to the active retraction of neurites. To distinguish between these possibilities, I imaged cells in real time during exposure to TOCP. Primary neurons were plated on glass coverslips and allowed to adhere to the coverslip for 20 minutes before image collection every 10 minutes. After 100min, TOCP was added at 14 μg/ml, an intermediate concentration to permit detection of both increased and decreased changes in neurite length (as needed in later experiments) compared to wild-type neurons. I then imaged the cells for another 100-200min, depending on how long they remained in focus. Within 50 minutes of TOCP exposure, cell processes began to thin and fragment in 21 out of 23 cells, showing characteristic blebs of degenerating axons (Figure 3-3E). Blebbing and fragmentation of axons is associated with axon degeneration, but not axon retraction (Luo and O'Leary, 2005), therefore for the remainder of this chapter all changes in neurite length caused by TOCP will be referred to as “degeneration”.

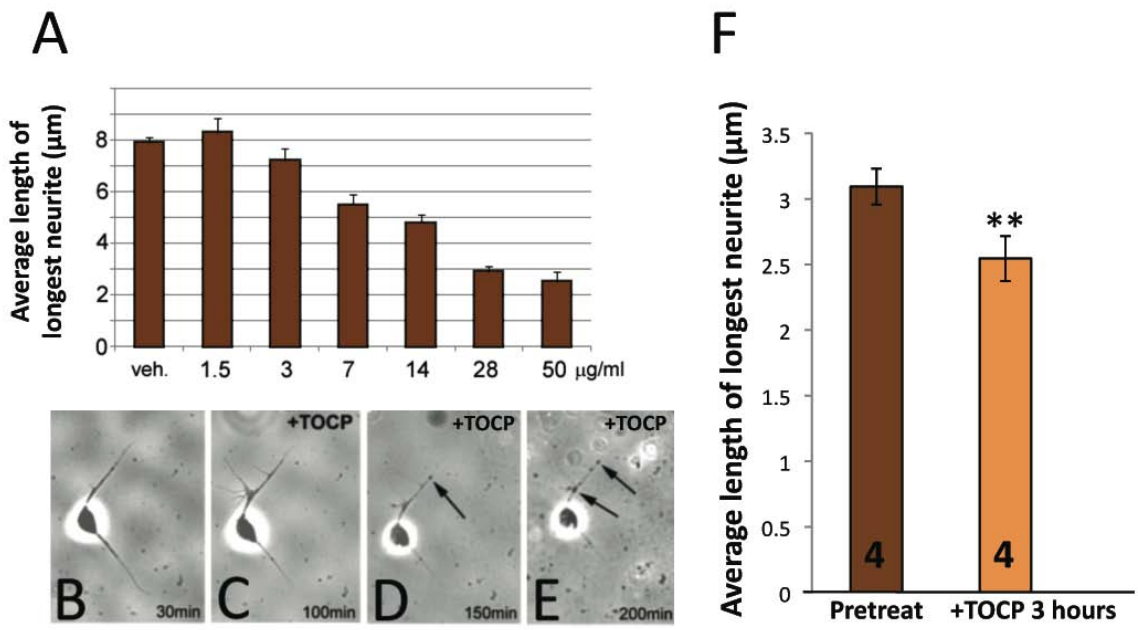

Figure 3-3: TOCP causes *Drosophila* neurons to retract. **A.** Dose-response curve of changes in average length in longest neurite per cell with increasing concentrations of TOCP; neurite lengths are shorter with increased concentrations of TOCP. **(B-E)** Frames from a movie of primary D*rosophila* neurons in culture. **B.** 30 mins after the start of imaging (1 hour *in vitro* total), the cell has put out processes. **C.** After 100 mins of imaging the neuron has elaborate processes and TOCP is added. **D.** Neurites (arrow) begin to retract and **E.** fragmentation of process occurs (arrows). **F.** Comparison of average length of longest neurite prior to treatment and after another 3 hours in TOCP, showed that retraction occurred. A t-test comparing this data indicates $p<0.01$. N = independent experiments, indicated inside each bar of the graph. 200 neurons were measured pretreatment and 172 treated neurons were measured. Error bars indicate SEMs.

Degeneration was quantified by comparing primary cultures of *Drosophila* neurons that were grown for 12 hours and then treated with 14 µg/ml TOCP for 3

hours, with neurons that were fixed at the time of treatment. Exposure to TOCP resulted in the average neurite length of treated cells being significantly shorter than the neurite length of cells before treatment (2.54 μm ± 0.17 versus 3.09 μm ± 0.13, respectively, p = 0.01), verifying that these neurons indeed showed neurite degeneration in response to TOCP.

This result demonstrates that TOCP is able to cause degeneration of *Drosophila* neuronal processes *in vitro* and is not simply stopping the outgrowth of processes, which establishes *Drosophila* as a viable model for the study of OPIDN. Because protein levels can be easily altered in *Drosophila* neurons, I used this system to investigate how levels of SWS protein alter the effects of TOCP. As described above, it has been suggested that “aging” OPs engender organophosphorylated SWS/NTE with a new and toxic function (Johnson, 1970). To test this gain-of-function hypothesis, I over-expressed SWS in neurons, which should then be more sensitive to the effects of OPs, if this hypothesis is correct. In contrast, neurons with reduced levels of SWS should be protected from OP exposure. Primary neurons from wild-type flies, flies over expressing SWS, and flies heterozygous for the *sws*^{1} mutation were cultured for 12 hours and then exposed to 14 μg/ml TOCP. Neurite length was determined at two time points: at 3 hours after treatment, and after 96 hours *in vitro* (HIV). Because of genotypic variability in the length of neurites observed in untreated cultures, values were normalized to the effects of TOCP seen in wild-type neurons. Values higher than one indicate a greater difference between vehicle-treated and TOCP-treated cells and thus demonstrate greater sensitivity to treatment. The converse is true for

values less than one. As shown in Figure 3-4A, cells expressing excess SWS have increased sensitivity to TOCP compared with wild-type at 3 hours (relative difference 1.47 $\pm$ 0.16 versus 1 $\pm$ 0.09, p=0.03) and at 4 days (1.15 $\pm$ 0.02 versus 1 $\pm$ 0.09, $p < 0.01$). The decrease in relative difference between wild-type cells at 96 hours could be due to some recovery from TOCP exposure. However, when I examined neurite length over time, in treated and untreated cells (Figure 3-4B), it appeared more likely that this effect reflected a reduction in health of vehicle-treated cells at 96 HIV. Also, the difference between untreated and treated wild-type cells became so large by 96 HIV that if differential effects of TOCP, based on genotype, did not greatly increase over this time frame, then the *relative* difference would decrease. Neurons heterozygous for sws^{1} were protected from TOCP compared to WT at 3 hours (0.53 $\pm$ 0.25, p = 0.1) and 96 hours (0.828 $\pm$ 0.065, p=0.016). The different effects of TOCP on neurons with either excess or reduced levels of SWS indicate that TOCP can lead to a toxic gain-of-function effect, whereby excess SWS bound by OPs exerts a novel toxic effect on cells.

To determine if a toxic gain-of-function effect could be confirmed *in vivo* I used our fast phototaxis assays (Figure 3-4C) to calculate a performance index for how proficiently flies move towards a light source. Although, this assay can detect both muscular and neuronal degeneration (Greene et al., 2003; Carmine-Simmen et al., 2009), I suspect all defects in OP treated flies are neuronal, as muscle degeneration is not associated with OP exposure. Prior to TOCP exposure, flies with increased neuronal SWS already showed reduced performance index in this test at 14 days (17.5% worse than wild-type, $p<0.01$ as

measured by ANOVA), and after TOCP exposure, they were affected to a great extent than wild type flies (26% versus 10%). To determine if the effect of TOCP on flies expressing increased SWS is significantly more than effects of TOCP on WT flies linear regression analysis is needed. This observation (pending statistical confirmation) would support my results from cell culture experiments, in which TOCP induced a toxic gain-of-function that led to shorter neurites. As described above, in control flies expressing SWS with a mutation in the OP binding domain, no effects of TOCP were seen (66.4 ± 4.1 versus 71 ± 2.9, $p = 0.37$) (Figure 3-4C).

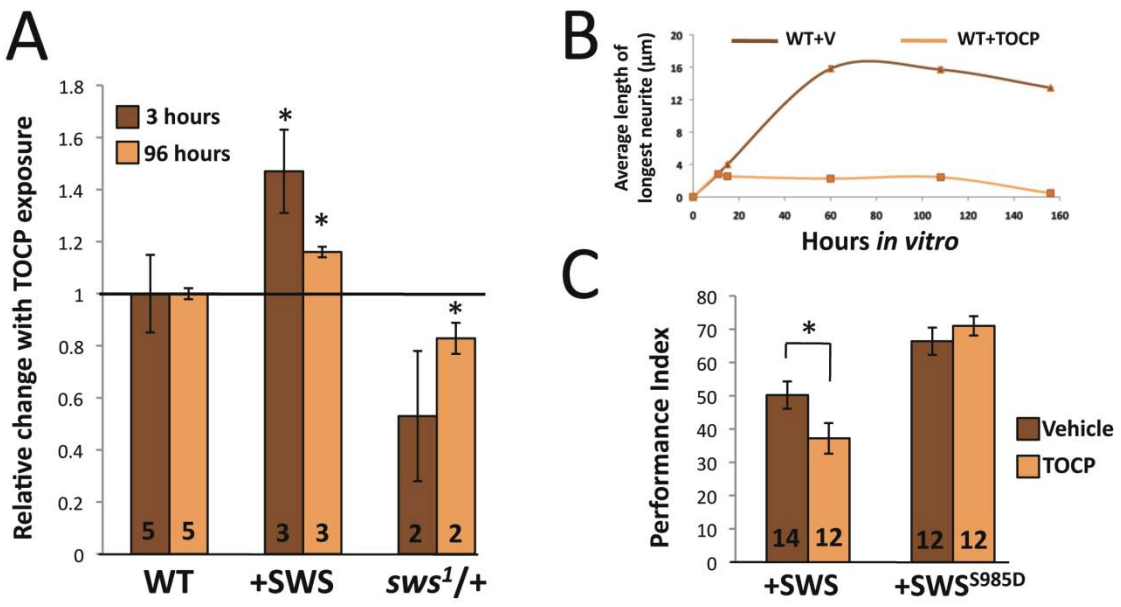

Figure 3-4: Increases in SWS protein levels increase the effects of TOCP exposure. **A.** Relative changes in the average length of the longest neurite (per cell) with increased and decreased levels of SWS protein, as compared to wild-type cells, at 3 hours and 4 days after exposure. At least 30 cells (across experiments – indicated by N) were measured from each genotype and treatment type at each time point. Neurons with additional SWS showed a greater change with TOCP exposure, while those with reduced SWS showed less change. All values are expressed relative to the change in wild-type cells following TOCP exposure, which has been set at 1 (indicated by black line). T-tests compared the relative change for each genotype upon OP exposure to the relative change of wild-type cells after OP exposure. * indicates $p<0.05$. **B.** Average length of longest neurite per cell for wild-type vehicle-treated (brown line) and TOCP-treated (orange line) cells in culture for 160 hours. **C.** Performance index of flies in fast phototaxis assay shows TOCP affects behavior of adult flies. Flies with increased SWS levels had reduced performance even when treated with vehicle, and this effect is further decreased with TOCP. Flies expressing the SWS protein with a mutation in the TOCP-binding domain showed no defect prior to treatment and were unaffected by TOCP. N = independent experiments indicated inside each bar of graph. Between 90-231 cells per genotype was counted for each treatment. SEMs are indicated.

TOCP affects kinase levels in *Drosophila* and mammalian cells

We recently showed that SWS can function as a regulatory subunit for PKA, specifically binding and inhibiting the C3 catalytic subunit (Bettencourt da Cruz et al., 2008). To determine if TOCP can modify the interaction between SWS and PKA-C3, kinase activity assays were performed on adult *Drosophila* that were fed either 16 or 32mg/ml TOCP. Because the data from these treatment groups was not significantly different they were combined. As shown in Figure 3-5A, I detected significantly reduced kinase activity in head lysates from flies that consumed TOCP, compared with control flies fed sucrose solution (0.63 ± 0.04 vs 0.72 ± 0.03, $p=0.03$).

To determine if OPs might similarly affect mammalian cells, I first confirmed that mammalian (mouse) NTE also interacts with PKA-C3. In yeast two hybrid assays, a direct interaction between murine NTE and *Drosophila* PKA-C3 could be detected (Figure 3-5B), while interactions with the other *Drosophila* catalytic subunit, C1 and C2 were not detected (as described for SWS). As *Drosophila* PKA-C3 shares 62.4% sequence identity with its mammalian homolog Pkare (Zimmermann et al., 1999), I suspected that mNTE might regulate Pkare activity, and that this interaction could be affected by TOCP. Therefore, I performed kinase activity measurements using primary neurons from the rat hippocampus after treating them with TOCP or with the non-neuropathic OP paraoxon (PO) (Figure 3-5C). Indeed, these cells also showed a dramatic decrease in PKA activity after treatment with TOCP (exhibiting approximately half the activity of ETOH-treated control cells, $p = 0.01$), but not after exposure to the

non-neuropathic organophosphate paraoxon (p = 0.7). To confirm that the reduced kinase activity was not due to cell death, cells were visualized prior to harvesting and appeared to be alive, though cells had reduced process lengths in both OP-treated groups (about 40% shorter in randomly measured cells). Likewise, alcohol dehydrogenase (ADH) assays showed no significant difference between cells treated with vehicle versus TOCP (p=0.67), suggesting cells were still alive and enzymatically active at the time of harvesting. However, due to my lack of experience with ADH assays, I will need additional evidence that cell death is not contributing to reduced kinase activity levels seen in my mammalian cells cultures. *In vivo* work with paraoxon was not possible, as flies succumb to this compound at doses far below what would be necessary to detect SWS inhibition (presumably due to inhibition of AChE).

Cells with increased levels of PKA-C3 are resistant to TOCP treatment.

To determine if increased PKA-C3 levels could protect cells from TOCP treatment UAS-PKA-C3 was over-expressed in all *Drosophila* neurons using the *elav*-Gal4 driver. Primary neuronal cultures were prepared from these flies and treated with 14 μg/mL of TOCP after 12 HIV. Cells were imaged after 4 DIV (Figure 3-6A). Neurons from flies with increased levels of PKA-C3 had a 20% smaller response to TOCP treatment when compared to wild-type cells (0.84 ± 0.023 versus 1 ± 0.021, $p < 0.01$). This strongly suggests that degeneration is mediated, at least in part, by a loss of PKA-C3, as increasing PKA-C3 levels ameliorates the effects of TOCP. To determine if PKA-C3 could be playing a role in the decreased performance index measured with the fast phototaxis assay

observed after TOCP treatment, flies expressing SWS with a mutation in the PKA-C3 binding site were treated with TOCP and tested with this assay. Interestingly we saw these flies were not affected by TOCP (69.1 $\pm$ 4.8 versus 65.3 $\pm$ 3.9, p = 0.54) (Figure 3-6B).

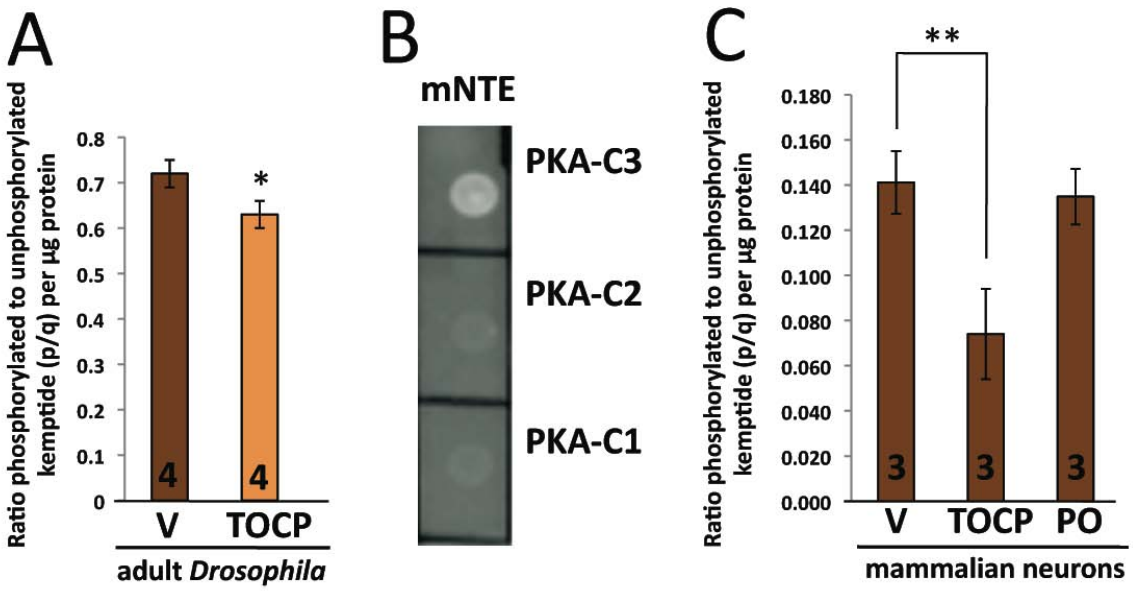

Figure 3-5: TOCP causes reduction in PKA activity in adult flies and cultured mammalian neurons. **A**. Adult *Drosophila* show a reduction in PKA levels after consuming 16 mg/ml or 32 mg/ml TOCP (combined) compared with vehicle. **B.** The murine homolog of SWS (mNTE) interacts with *Drosophila* PKA-C3, but not PKA-C1 or PKA-C2. C. The neuropathic compound TOCP, but not non-aging PO, reduces PKA activity in cultured rat hippocampal neurons. N = independent experiments indicated inside each bar of graph. SEMs are indicated. T-test were used to compare each treatment (TOCP or PO) with vehicle-treated flies (A) and cells (B).

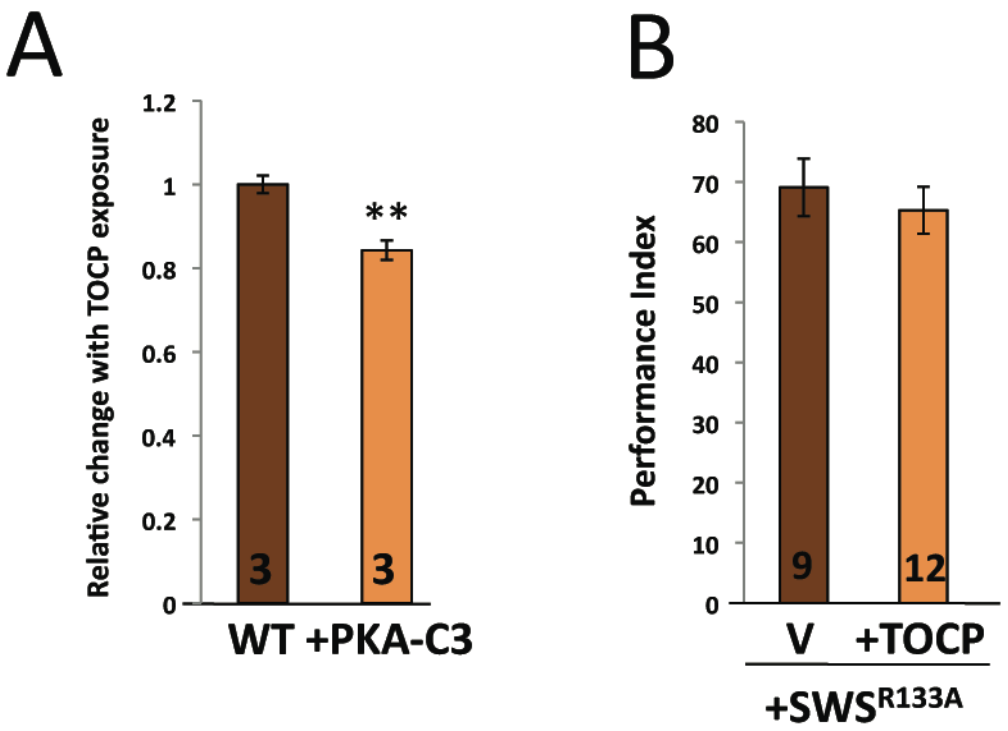

Figure 3-6: Over-expression of PKA-C3 protects against the effects of TOCP, while mutant SWS (that is unable to bind PKA-C3) is resistant to TOCP. **A.** Increased PKA-C3 expression protects *Drosophila* neurons from neurite degeneration caused by TOCP (at least 90 cells for each genotype and treatment type were counted across multiple experiments). T-tests were performed comparing the relative change of neurite length after TOCP exposure from WT neurons versus neurons over-expressing PKA-C3. **B.** Fast phototaxis assays show that neuronal over-expression of SWS^{R133A} (which lacks the ability to bind PKA-C3) rendered the flies resistant to TOCP. T-tests compared the performance index of vehicle-treated flies to those treated with TOCP. **A-B.** N (indicated in bars) = independent experiments. SEMs are indicated. ** indicates $p<0.01$.

Discussion

The work presented here establishes *Drosophila melanogaster* as a useful model for the study of organophosphate-induced delayed neuropathy. Treatment of *Drosophila* with the organophosphate TOCP inhibits SWS esterase activity and causes axon retraction/degeneration, the histological sign classically associated with OPIDN. Additionally, I used a behavioral assay to test the effects of OP exposure *in vivo*. Both my *in vitro* and *in vivo* data show that flies (or neurons) with increased levels of SWS protein show increased sensitivity to the effects of TOCP, which argues that a toxic gain-of-function is induced by TOCP. This result is in contrast to the results of Winrow and colleagues (2003), who reported that mice with heterozygous deletions of NTE were more susceptible to the aging OP ethyl octylphosphonofluoridate (EOPF). Phenotypes observed after treatment with EOPF included increased tear production and seizures, which are known effects of cholinergic inhibition (Zimmer et al., 1998; Lotti, 2000). While EOPF is thought to be a specific inhibitor of NTE, it does have a limited ability to bind AChE (Casida and Quistad, 2004). In addition, it has not been ruled out that EOPF could bind AChE when there are reduced levels of the primary target NTE, as would be the case in mice that are heterozygous knocks-outs and could account for the discrepancy between my results and those described by Winrow et al. (2003).

Inhibition of SWS/NTE can also be accomplished by OPs that do not cause OPIDN, indicating that the loss of esterase activity alone does not lead to

neuropathy. Although, Read et al. showed that OP exposure mimics the phenotype of animals with inactive protein (Read et al., 2009), this observation proved the importance of functional protein, but did not rule out the possibility of a toxic gain-of-function effect caused by OP binding. Conversely, many groups argue that disruption of membrane lipids is responsible for the development of OPIDN. An important argument against this hypothesis comes from data showing that alterations in membrane lipids occur after exposure to both aging and non-aging OPs (Hou et al., 2009), again indicating that this effect is not the sole mechanism for OPIDN.

Although we did find significant effects in the behavioral assays using student's t-tests using ANOVA to compare flies of all genotypes (WT, UAS-SWS, SWS^{S985D}, SWS^{R133A}) revealed no statistically significant difference in treated versus untreated flies. Therefore the data presented in this thesis still require more thorough statistical analysis. However, that TOCP exposure results in a significant change in the performance of only UAS-SWS flies and not wild type (with the student's t-tests), would suggest that these flies are more sensitive to the effects of TOCP. Hopefully, more experiments will confirm the significance of these results. It is possible that I will need to repeat these experiments with higher concentrations of TOCP to get more robust results.

The behavioral experiments also indicated that flies with mutations in the PKA-C3 binding site of SWS may be more resistant to the effects of TOCP, which suggested that OPIDN could be associated with changes in the regulatory function of SWS on PKA-C3. My experiments designed to test the action of OPs

on PKA activity levels revealed, somewhat surprisingly, that the activity of PKA was reduced after TOCP exposure. While the reduction in PKA activity was modest, PKA-C3 makes up only a small portion of the total PKA activity in flies. Activity of all three PKA catalytic subunits should be detected by this assay; however, because PKA-C3 binds to a direct target of TOCP, I suspect that PKA-C3 is the predominant kinase affected by TOCP. The ability of TOCP to reduce kinase activity suggests that the binding of TOCP to SWS acts to lock PKA-C3 in an inactive state, either by some conformational change or by simply blocking the binding of cyclic nucleotide. Still, there are other ways to interpret this data. OPs could be causing a direct reduction in kinase activity independent of SWS. However, I think this is unlikely because preliminary experiments suggest that in *sws* null flies there is no affect of TOCP treatment on kinase activity. TOCP could affect SWS and/or PKA-C3 protein levels, which would also lead to reduced kinase activity. I expect that this is at least not the case for SWS as Glynn and colleagues (1998) showed that OP exposure did not noticeably change the ability of antibodies to detect NTE (qualitatively or quantitatively) suggesting that OP exposure does not change the amount of NTE protein (Glynn et al., 1998). Nevertheless, this could be addressed by western blotting of head lysates from flies that have been exposed to TOCP by probing for both SWS and PKA-C3. An alternative method would be to compare protein levels of SWS and PKA-C3 in mock and TOCP treated COS cells that have been transfected with constructs to express both proteins (it would first have to be confirmed that the treatment affects kinase activity in these assays). A benefit to this method is that the

concentration of TOCP can be more tightly controlled in cell culture media than can be done with feeding of TOCP to adult flies.

Currently, little is known about the function of PKA-C3 in *Drosophila* or its homologs in mammals, but based on the expression patterns in mammals, it has been hypothesized that PKA-C3 plays a role in neuronal differentiation (Blaschke et al., 2000). My work argues that PKA-C3 also has an important role in the adult nervous system. Previous studies have suggested that TOCP might also have direct actions on other kinases, indicated by increased levels of phosphorylated cyclin-dependent kinase 5 (Wang et al., 2006). While we have no evidence suggesting that TOCP directly acts on PKA-C3, this alternative model will need to be addressed. Enhanced phosphorylation of cytoskeletal proteins has also been observed in OPIDN (Abou-Donia, 1993) but the specific kinases involved in this effect are unknown. It is unlikely that this can be connected to PKA activity, given our results. An alternative model is that PKA-C3 can activate a phosphatase, so that when PKA-C3 is inactive, increased phosphorylation of its downstream targets results.

In our behavioral assays, we saw that mock treated flies with increased levels of neuronal SWS already performed poorly when compared to wild-type controls. This result is consistent with our model that inhibition of PKA-C3 activity results in behavioral deficits; in this case the excess SWS would reduce the activity of PKA-C3 by acting as additional regulatory subunits. Assuming this model, it is surprising that untreated flies with neuronal over-expression of SWS^{S985D} (a version of SWS that lacks esterase activity, but should retain the

ability to bind PKA-C3) do not mimic those with over-expression of UAS-SWS. This observation suggests that SWS^{S985D} is unable to bind PKA-C3, possibly due to allosteric change resulting from the mutation of Serine 985. The ability of SWS^{S985D} to bind PKA-C3 should therefore be tested using yeast-two hybrid and co-immunoprecipitation experiments.

Glial phenotypes in response to OP exposure have been reported, but are proposed to be secondary to axonal damage (Uga et al., 1977). In *Drosophila* glia express SWS protein and require it for proper function, but it is unknown if PKA-C3 is also expressed in glia, or if SWS functions solely as a phospholipase in these cells. This scenario would explain the selective effects of OPs on neurons, while glia appear to be only indirectly affected by these compounds.

In summary, I propose that neuropathic OPs do interfere with the phospholipase function of SWS/NTE, (supporting conventional models) but their inhibiting effect on this function are not sufficient to induce OPIDN. My data suggest that a decrease in PKA-C3/PrKX activity (by increased binding to SWS) is required for the development of OPIDN. Although this supports a gain-of-function model, this would not involve a totally new function of SWS/NTE in the presence of a toxin, but SWS would gain toxicity by binding its normal partner (PKA-C3) in an unregulated manner. SWS is a protein with two known physiological functions: it was first discovered to be a phospholipase capable of hydrolyzing lipids, and later found to serve as a regulatory subunit for *Drosophila* PKA-C3. My results indicate that NTE likely plays the same role in mammals. While previous work has proposed that the phospholipase function of SWS is

disrupted in OPIDN, my work adds an additional novel mechanism that would include the PKA regulatory function of SWS/NTE .

Materials and Methods

Drosophila stocks and UAS lines. The *sws*^{1} allele has been described by (Kretzschmar et al., 1997). A recombination of *sws*^{1} with the Appl-Gal4 line was used in all experiments. To obtain heterozygous *sws*^{1} larvae, homozygous *sws*^{1} flies were crossed with Canton-S and females were selected since *sws*^{1} is X-linked. Appl-Gal4 without *sws*^{1} was used as control for kinase assays and the wild-type strain Canton-S was used for all other controls. UAS-PKA-C3 and UAS-SWS^{R133A} have been described by Bettencourt da Cruz et al. (2008). UAS-SWS and UAS-SWS^{S985D} have been described by Muhlig-Versen et al. (2005). Canton S and *elav*-GAL4 were provided by the Bloomington Stock Center (Indiana University, Bloomington, IN) and Appl-GAL4 was provided by L. Torroja (Universidad Autonoma de Madrid, Spain).

Tissue sections. Paraffin sections were performed as described in (Bettencourt da Cruz et al., 2005). For sectioning of thoracic ganglia flies were mounted with heads down in the histology collar and transverse sections were made of the thorax.

Esterase/AChE assays. SWS esterase assays were done as described in Bettencourt da Cruz et al. (2008). AChE assays were done as described in (Schuh et al., 2002). 5 whole flies were homogenized in 250μl of buffer and 20μl of this solution was used in each independent measurement.

Fast phototaxis assay. Flies were starved overnight and then tested in a countercurrent apparatus as described by Seymour Benzer (1967). Flies were allowed six seconds to move in response to light on each trial and 5 consecutive trials were performed. 10-20 flies were used in each test group.

Primary culture of Drosophila neurons. Cultures were prepared as described in (Wu et al., 1983) but were plated onto glass coverslips coated with 2μg/ml Laminin (Sigma #L2020) and 0.2mg/ml Concavalin A (Sigma #C2010) and media for cultures was supplemented with 50μg/ml insulin and 5μg/ml 20 Hydroxy Ecdysone (Sigma H-5142). After culture neurons were allowed to adhere to the coverslips and grow for 12 hours before TOCP treatment. Culture media was removed and fresh media was added every 48 hours.

Analysis of neurite length. Images were taken using transmitted light on an EVOS gen 1 Microscope (AMG). The NeuronJ plugin for ImageJ (Abramoff, 2004) was then used to measure the longest process on each cell in each image. If it was unclear visually which process was longest all were measured and only the largest number was used. Analysis was done blind to genotype and treatment of cells and only cells with all processes in the field of the image were measured.

Time-lapse imaging. 30 mins after plating cells culture dishes were moved to the stage of an inverted Leica DM IRB microscope. Every 10 min for 16 hours, a phase contrast image of each selected cell (maximum of 6 per dish) was acquired using MetaMorph software (Molecular Devices, Inc).

Administration of TOCP to adult flies. Flies were starved of food and water for 4 hours and then transferred onto a 5% sucrose solution with desired concentration of TOCP. All vials were kept in the dark due to the light sensitivity of TOCP. For PKA assays flies remained on TOCP for 24 hours and then were used in the assay. For fast phototaxis assay flies were transferred back to normal food after 16 hours and aged for 14 days. For thoracic ganglia studies flies were put on fresh TOCP daily for 6 days and then immediately put onto histology collars. For brain sections flies were treated with TOCP for 6 days and then aged an additional two weeks.

PKA activity measurements. PKA activity measurements on fly heads were done as described in Bettencourt da Cruz et al. (2008). For measurements on mammalian neurons, primary hippocampal cultures were prepared from E18 embryonic rats, as described previously (Kaech and Banker, 2006) and grown directly on Poly-L-Lysine coated dishes at a density of $2x10^6$ without a glial feeder layer. 3 hours after plating cells were treated with OP for 24 hours. Prior to assays cells were observed under the microscope to ensure they survived the treatment. Then media was removed and cells were scrapped from culture dishes in 200μl PKA extraction buffer (PepTag® Assay, promega), homogenized and centrifuged at 14,000g for 5 min. Supernatant (13μl) was immediately used for activity measurement assays for each sample, whereas (100μl) were used to perform Bradford assays (Bradford, 1976). Measurements were normalized to total protein in the sample using Bradford's method.

Protein Interaction Assays. The yeast two-hybrid system was utilized to directly test protein-protein interactions following the instruction manual for the CytoTrap Vector kit from Stratagene. Briefly, full length cDNA for murine Neuropathy Target Esterase (Moser et al., 2000) was amplified with polymerase chain reaction and cloned into the pSos vector using unique sites generated by primers. This construct was co-transformed independently with each of the three Drosophila PKA catalytic subunits cloned in the pMyr vector (Bettencourt da Cruz et al., 2008). The interaction between SWS and PKA-C3 was used as a positive control, while SWS and PKA-C1 co-transformation was used a negative control, as judged by lack of yeast growth at non-permissive temperature.

Chapter 4

Additional studies on the regulation of PKA-C3

Unpublished results

Development of a constitutively active version of PKA-C3

Experimental Rationale

As described in Chapter 2, I propose that increased PKA-C3 activity contributes to the degenerative phenotype seen in *sws*^{1} mutants. My data shows that *sws*^{1} mutant flies have increased levels of kinase activity compared to wild-type flies. I also showed that neuronal expression of PKA-C3 in *sws*^{1} mutant flies further increased vacuole formation in the brain, compared to the *sws*^{1} phenotype, leading to the hypothesis that increased PKA-C3 activity contributes to neurodegeneration. If this hypothesis is valid, then increased levels of PKA-C3 activity in wild-type flies should lead to vacuole formation, but, as presented in Chapter 2, neuronal expression of UAS-PKA-C3 yielded only low levels of degeneration. Reducing the amount of SWS protein by removing one copy of *sws,* increased the level of degeneration in fly brains, suggesting that SWS levels are related to vacuole formation. One explanation for these results is that SWS levels in wild-type flies are high enough to compensate for the increased amount of UAS-PKA-C3 expressed using the neuronal driver *Appl*-GAL4. This model seems plausible, as flies with increased neuronal expression of PKA-C3 protein did not show increased kinase activity compared with wild-type flies (Figure 2-5), indicating that the UAS-PKA-C3 construct is either inactive or can be bound and inactivated by regulatory subunits (specifically the regulatory subunit SWS). To overcome these problems, I created a constitutively active version of UAS-PKA-C3. I hypothesize that expression of this constitutively active version of PKA-C3 should lead to higher levels of neuronal degeneration, compared with expression

of the wild-type PKA-C3 construct used in Chapter 2, if PKA-C3 activity is indeed responsible for the observed neurodegeneration.

Materials and Methods

Orellana and McKnight identified two point mutations (His^{87}-to-Glu and Trp^{196}-to-Arg) in the murine catalytic subunit Cα that blocked its interaction with regulatory subunits, but not its kinase activity, thus creating a constitutively active condition (Orellana et al., 1993). These sites are well conserved in *Drosophila* PKA-C3, so primers were designed to induce the following amino acid changes: His^{235}-to-Glu and Trp^{427}-to-Arg (Table 4-1). Mutations were made using the QuickChange Site-directed mutagenesis kit from Stratagene and verified by sequencing. Additionally, an HA-tag was added to the 3' end of the DNA sequence using primers (Table 4-1). Purified DNA was sent to Best Gene (Chino Hills, CA) for production of transgenic fly lines. Four individual insertions were identified for UAS-PKA-$C3^{active}$. Two lines (lines 1 and 4) were checked for protein expression by Western blotting, as described in Chapter 2, and tested for kinase activity using Kemptide kinase assays, as described in Chapters 2 and 3.

Results and Discussion

Preliminary kinase activity assays indicated that neuronal expression of UAS-PKA-$C3^{active}$ (line 4) did not increase kinase activity in fly head lysates, above that induced by expression of UAS-PKA-C3. Therefore, all further experiments focused on the UAS-PKA-$C3^{active}$ (line 1), which, when neuronally expressed, did increase levels of kinase activity in head lysates, compared with

the *Appl*-Gal4 driver alone and *Appl*-Gal4 expressing UAS-PKA-C3 (Figure 4-1). The modest increase in kinase activity measured after expression of UAS-PKA-C3 is not surprising, because the bulk of kinase activity measured in this assay is likely due to the activity of PKA-C1 and C2. Because this experiment was only done one time, no statistical analysis could be performed. Expression of UAS-PKA-C3^{active} with the stronger driver GMR-Gal4 yielded higher protein levels in head lysates than expression of UAS-PKA-C3^{active} line 4. This increased protein expression in line 1 compared to line 4 might be responsible for higher kinase activity levels seen in heads expressing UAS-PKA-C3^{active} line 1 (Figure 4-1).

Kinase activity assays showed that head lysate from flies over-expressing UAS-PKA-C3^{active} with the GMR-GAL4 driver had 40% more kinase activity, compared to head lysate from flies with the driver alone (p/q = 1.46 $\pm$ 0.13 versus 0.89 $\pm$ 0.06, p>0.01) (Figure 4-2). Values were normalized to protein levels in each sample using Bradford's method (Bradford, 1976). These assays were carried out in the presence of cAMP, which should not affect the activity of PKA-C3^{active}, as it should not require cAMP-mediated release from regulatory subunits for activity. In the absence of cAMP, a slight increase in kinase activity was seen in head lysate from flies over-expressing UAS-PKA-C3^{active} compared with driver alone (p/q = 0.32 $\pm$ 0.08 versus 0.20 $\pm$ 0.04), but with three experiments performed so far, this increase was not statistically significant. Due to the low levels of kinase activity detected in the absence of cAMP, these experiments should be repeated using increased amounts of head lysate in each assay. The rationale behind performing experiments with and without cyclic nucleotide was

to determine if expression of UAS-PKA-C3^{active} did increase constitutively active kinase. Expression of UAS-PKA-C3^{active} should increase kinase activity in both assays (with and without cAMP) because it should not require cAMP for activity (but will remain active in the presence of cAMP). Conversely, UAS-PKA-C3 should only increase kinase activity in the presence of cAMP, because it requires cAMP for release from regulatory subunits and activation. However, in the presence of cAMP, all catalytic subunits of PKA should be activated (not just PKA-C3), and these large effects are likely to overwhelm detection of UAS-PKA-C3^{active} expression. Interestingly, this was not the case; differences between the activity of UAS-PKA-C3^{active} and UAS-PKA-C3 were detected with cAMP, but I was unable to detect differences in the absence of cAMP. This result might suggest that UAS-PKA-C3^{active} is not constitutively active, as increased activity does appear to require cAMP. It is also possible that UAS-PKA-C3^{active} has a lower binding affinity to SWS, and thus is activated at lower cAMP concentrations than un-mutated UAS-PKA-C3. If UAS-PKA-C3^{active} is constitutively active, I would expect that in the absence of cAMP, it would be localized to the cytoplasm, suggesting that UAS-PKA-C3^{active} is not bound to SWS. However, the subcellular localization of UAS-PKA-C3^{active} (with or without cAMP) remains to be examined.

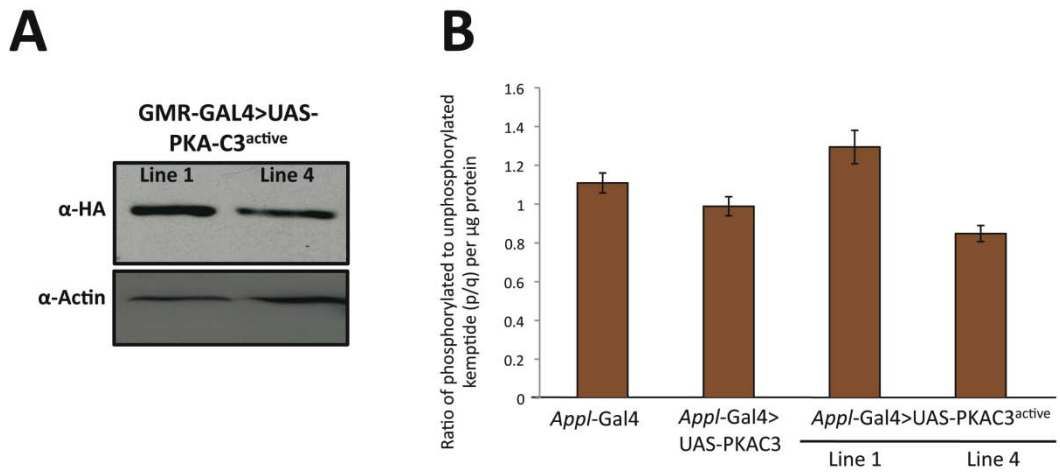

Figure 4-1: Protein levels and changes in kinase activity after expression of UAS-PKA-C3 that has been mutated to yield constitutive activity. **A.** Western blots of lysates from 10 fly heads per lane, comparing the expression level of the two UAS-PKA-C3^{active} insertion lines; line 1 has higher levels of protein than line 4, as detected with anti-HA antibodies. Actin loading control shows total protein levels are similar in each lane (or even higher for line 4). **B.** Expression of UAS-PKA-C3^{active} line 1 shows increased kinase activity in head lysates, compared to driver alone or expression of either an un-mutated version of UAS-PKA-C3 or a different insertion line of UAS-PKA-C3^{active} (line 4). Error bars represent SEM and come from variation within triplicate measurements of a single lysate preparation.

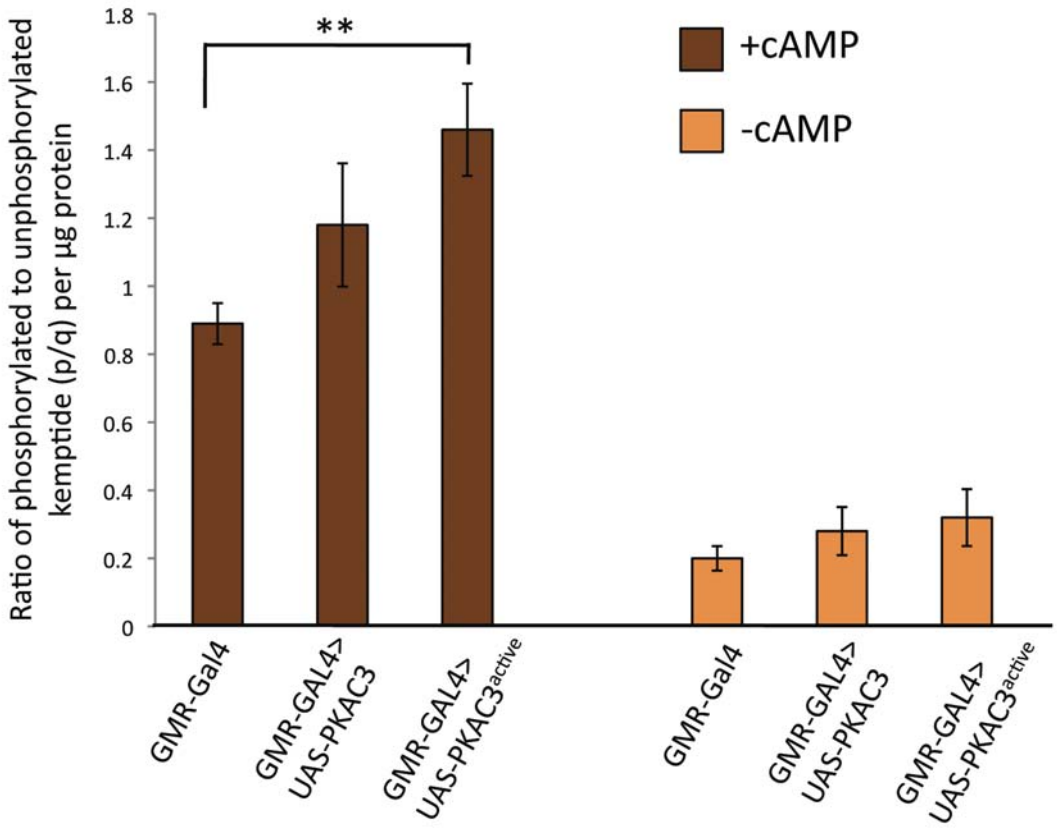

Figure 4-2: Kinase activity assays (with and without cAMP) show increased activity in head lysates with GMR-Gal4>UAS-PKA-C3^{active}, in the presence of cAMP. GMR-Gal4>UAS-PKA-C3 heads did not show increased kinase activity levels compared to heads with just the GMR-Gal4 driver, in the presence or absence of cAMP. Three independent experiments were performed with and without cAMP, for each of the three genotypes. Separate Student's t-tests were performed comparing values from GMR-Gal4 flies to flies expressing UAS-PKA-C3 or UAS-PKA-C3^{active}. T-test's were performed comparing values from flies expressing UAS-PKA-C3 versus UAS-PKA-C3^{active}. Only the difference between GMR-Gal4 and GMR-Gal4>UAS-PKA-C3^{active} was statistically significant. Error bars represent SEMs.

As presented in Chapter 2, I propose that increased PKA-C3 activity contributes to the neurodegenerative phenotype seen in *sws*^{1} mutants, and thus should cause degeneration in wild-type flies as well. If degeneration is related to kinase activity, then PKA-C3^{active} should cause more degeneration in wild-type flies than over-expression of un-mutated PKA-C3. To test if PKA-C3^{active} can cause vacuole formation in the brain, 14 day-old flies expressing neuronal UAS-PKA-C3^{active} were examined histologically for signs of degeneration. Preliminary results indicate that neuronal expression of UAS-PKA-C3^{active} does lead to vacuole formation. 100% of flies expressing the UAS-PKA-C3^{active} (line 1) construct driven by *Appl*-GAL4 on the X-chromosome, had vacuole formation at 14 days (N=4) (Figure 4-3), and one fly had very large holes in the optic lobe (Figure 4-3C). 100% of flies expressing the line 4 insertion also contained holes (N=8), but they were noticeably smaller than those seen when expressing insertion line 1 (compare Figure 4A with 4B and C). As noted above, neuronal expression of the line 4 construct did not significantly increase kinase activity in heads, compared with flies expressing wild-type UAS-PKA-C3, perhaps due to a lower protein expression level (Figure 4-1A). While preliminary, these results suggest that increased levels of PKA-C3 increase neuronal degeneration, presumably through the hyper-phosphorylation of downstream targets of PKA-C3. Identification of these downstream targets would be a very interesting next step that remains to be completed.

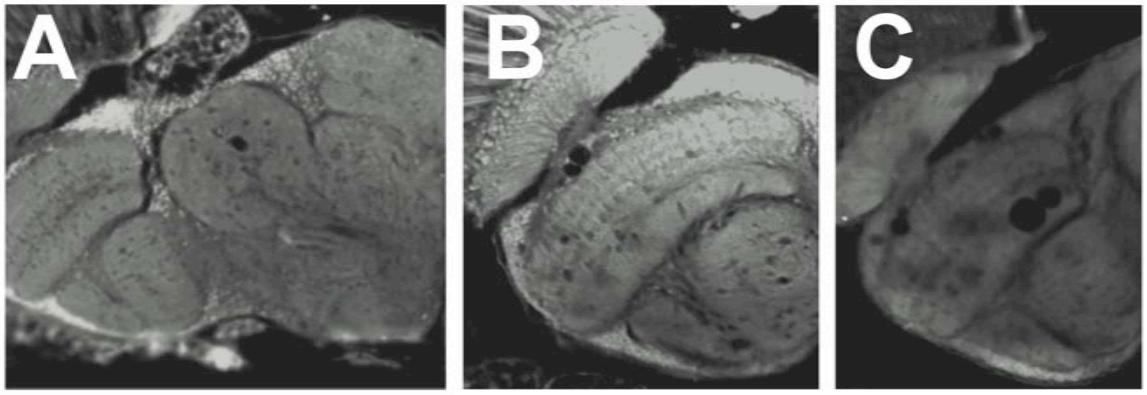

Figure 4-3: Neuronal expression of UAS-PKA-C3^{active} leads to vacuole formation. **A.** Paraffin sections of *Appl*-GAL4>UAS-PKA-C3^{active} (line 4) at 14 days shows low levels of vacuolization. **B-C.** Paraffin sectioning of *Appl*-GAL4>UAS-PKA-C3 (line 1) shows moderate vacuole formation at 14 days.

Cyclic nucleotide regulation of PKA-C3 release from SWS

Experimental rational

SWS has homology to the regulatory subunit of PKA, in both the domain responsible for binding catalytic subunits and the cyclic nucleotide-binding domains. Studies in Chapter 2 showed that SWS and PKA-C3 interact, as determined by yeast two-hybrid assays, and that the proteins co-localize, shown via immunohistochemistry. In addition, they both partition to the same membrane fractions, when SWS is present. Chapter 2 also showed that in the absence of SWS, PKA-C3 is no longer found in membrane fractions while kinase activity levels are increased. Taken together, these results suggest that SWS acts as a regulatory subunit for PKA-C3. In order for SWS to function as a true regulatory subunit (as opposed to an inhibitor), some mechanism to release

PKA-C3 is required. In canonical PKA signaling, catalytic subunit release occurs when cyclic nucleotides bind the regulatory subunit. Based on the presence of cyclic nucleotide-binding sites in SWS, I hypothesized that cyclic nucleotide-binding could also be the mechanism for release of PKA-C3 from SWS. To test this hypothesis, membrane and cytosol fractions were prepared from *Drosophila* head lysate, and were used to determine if cyclic nucleotides could alter subcellular localization of PKA-C3. Head lysates were incubated with cAMP, cGMP or both cyclic nucleotides, to determine if the localization of PKA-C3 changed with cyclic nucleotide exposure. If cyclic nucleotide binding to SWS is the mechanism for PKA-C3 release, then the addition of cyclic nucleotide should cause a change in the localization of PKA-C3, since it should promote a dissociation from membrane-anchored SWS. Both cAMP and cGMP were tested, as SWS has two regions with sequence similarity to cGMP binding sites in mammals and one site more similar to sites that bind cAMP (Shabb et al., 1990).

Materials and Methods

Experiments in Chapter 2 used a PKA-C3 antibody provided by D. Kalderon. Unfortunately, the small quantity of antibody provided was not enough to perform the experiments outlined in this chapter. To circumvent this technical issue, I created constructs with a myc-tagged version of UAS-PKA-C3 with the appropriate primers (Table 4-1), and used this construct to produce transgenic flies. To investigate nucleotide-induced changes in the localization of PKA-C3, I prepared lysates from fly heads expressing UAS-PKA-C3^{myc} with the neuronal driver *elav*-GAL4 and incubated them with 0.9mM cyclic nucleotide for 60 mins at

4 degrees. Lysates were prepared in the presence of protease inhibitors and 5μM Protein Kinase Inhibitor (PKI) to prevent re-association with regulatory subunits. After incubation with cyclic nucleotides, membrane and cytosol fractions were prepared as described in Chapter 2.

Results and Discussion

First, I confirmed that in the presence of SWS protein, PKA-C3^{myc} was localized to the membrane, as seen for untagged PKA-C3. PKA-C3^{myc} was detected in membrane fractions, and a weak band was even detected in the cytosol of flies expressing the myc-tagged construct (Figure 4-4A shows four bands in the *elav*-GAL4>UAS-PKA-C3^{myc} lane versus 3 bands in the *sws*^{1} *Appl*-GAL4>UAS-PKA-C3^{myc} lane, arrow). In contrast, no PKA-C3^{myc} was seen in membrane fractions when expressed in *sws*^{1} flies, though a weak protein band was seen in the cytosol fraction. This result suggests that in the absence of the regulatory subunit SWS, all PKA-C3^{myc} goes to the nucleus, or is rapidly degraded. I suspect the latter possibility is unlikely, because *sws*^{1} flies have increased levels of kinase activity, suggesting that the catalytic subunit is not degraded. Although protein levels appear lower in head lysates of *sws*^{1} *Appl*-GAL4>UAS-PKA-C3^{myc} flies, I should still detect PKA-C3^{myc}, since these flies were males and due to the X-linkage (and dosage compensation) of *Appl*-GAL4, PKA-C3^{myc} protein expression levels should be twice that of the *elav*-GAL4>UAS-PKA-C3^{myc} flies. It will be important to repeat this experiment with identical drivers and with higher protein levels, to ensure that no PKA-C3^{myc} is present in membrane fractions from *sws*^{1} *Appl*-GAL4>UAS-PKA-C3^{myc} heads. These

experiments were not carried out in the presence of PKI and should be repeated. It will also be important to examine nuclear preparations from *sws*^{1} flies, to determine if high levels of PKA-C3 can be detected, as would be expected.

I then examined whether the presence of cyclic nucleotide induces changes in the localization of PKA-C3^{myc}. A robust band around 67kD (arrow), the expected size of PKA-C3, was detected in the cytosol fraction of all cyclic nucleotide-treated head lysates from flies expressing neuronal UAS-PKA-C3^{myc} (Figure 4-4B). It is unclear if the lower band in these blots (approximately 60kD, grey arrowhead) is also an authentic PKA-C3^{myc} band, representing a truncated protein, or if this is a non-specific band. Neither cytosol nor membrane fractions from untreated PKA-C3^{myc} lysates showed any immunoreactive bands of the correct size, possibly due to degradation of the sample; thus, this experiment still lacks an important control. Because we lack a control for the localization of PKA-C3^{myc} in the absence of cyclic nucleotide (but presence of PKI), we must consider the possibility that PKA-C3^{myc} is shifted to the cytosol, not because of cyclic nucleotide activity, but because PKI is competitively binding and shifting the localization of PKA-C3^{myc}. If PKI turns out to be responsible for the localization of PKA-C3^{myc} in this assay, future experiments must be performed without PKI.

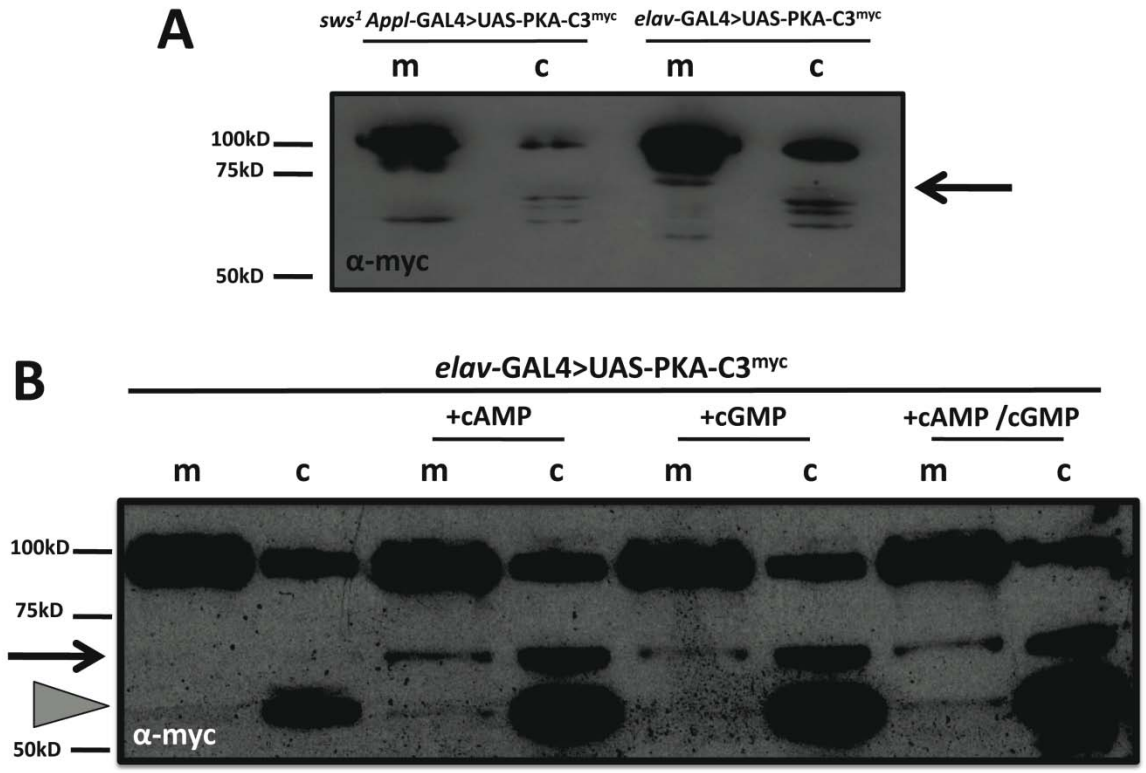

Figure 4-4. Western blots show that PKA-C3^{myc} is localized to the membrane in flies with wild-type background, but not in *sws*^{1} mutants or in the presence of cyclic nucleotide. **A.** Western blot on membrane (m) and cytosol (c) fractions from *sws*^{1} *Appl*-GAL4>UAS-PKA-C3^{myc} and *elav*-GAL4>UAS-PKA-C3^{myc} head lysates. PKA-C3 is approximately 67kD and is seen in the wild-type m and c lanes (arrow), but is absent from both fractions in the *sws*^{1} background. **B.** Western blot on *elav*-GAL4>UAS-PKA-C3^{myc} head lysates exposed to cAMP, cGMP or both show that any cyclic nucleotide leads to accumulation of PKA-C3^{myc} (arrow at 67kD, grey arrowhead indicates possible non-specific band) in the cytoplasm, while in vehicle treated cytosol fractions (lane 2), no accumulation of PKA-C3^{myc} is detected.

Additional controls will also be necessary to confirm that changes in PKA-C3 localization are related to the action of cyclic nucleotide specifically on SWS

proteins, and not on a possible alternative regulatory subunit of PKA-C3. To test if cyclic nucleotides can act specifically on SWS, experiments need to be repeated using a SWS protein in which its cAMP binding-sites have been mutated (described in the next section). Expressing this construct in *sws*^{1} flies should abolish any effects of cyclic nucleotide, if indeed they are mediated by SWS. The SWS construct lacking a functional PKA-C3 binding domain (SWS^{R133A}) could also be used as a control. As shown in Chapter 2, SWS^{R133A} has reduced binding to PKA-C3 in yeast two-hybrid experiments. By this model, PKA-C3^{myc} localization in head lysates from *sws*^{1} flies expressing UAS-SWS^{R133A} should not change with cyclic nucleotide-exposure, as PKA-C3 should not be bound to this mutated version of SWS. In this scenario, all PKA-C3^{myc} should be cytoplasmic (or possibly nuclear), though there is a possibility that catalytic subunits could be degraded or could bind other regulatory subunits in the absence of functional binding sites in SWS. For example, it has been shown that Pkare is capable of binding RI subunits (Diskar et al., 2010) and at this point we cannot rule out that PKA-C3 could also bind RI in flies.

It will also be important to test if the results presented in this section can be confirmed with endogenous protein, to exclude the possibility that tagged and over-expressed PKA-C3 behaves differently from endogenous PKA-C3. For this reason, we have recently created our own PKA-C3 antibodies that are currently being tested.

An alternative method to test the ability of cyclic nucleotide to bind SWS and regulate the release of PKA-C3 would be to perform *in vitro* experiments with

purified protein. First, a co-immunoprecipitation of SWS and PKA-C3 from *Drosophila* head lysates without cyclic nucleotides would be performed to confirm the feasibility of this approach. Next, cyclic nucleotides could be incubated with pulled-down protein complexes prior to elution. If cyclic nucleotide is shown to trigger the release of PKA-C3, I would expect to find PKA-C3 in wash fractions instead of being co-localized with SWS in the final elute. These experiments would determine if cyclic nucleotides directly regulate the release of PKA-C3 from SWS, as opposed to affecting interactions of PKA-C3 with alternative regulatory subunits.

It would also be beneficial to confirm that cyclic nucleotides are binding directly to SWS by testing the ability of immunoprecipitated SWS to bind radiolabeled cAMP and/or cGMP. In addition, it would be interesting to determine the lowest concentration of cyclic nucleotide required to release PKA-C3 from SWS. Ideally, I would like to determine which cyclic nucleotide acts on SWS to trigger the release of PKA-C3 *in vivo*. Cellular concentrations of cAMP and cGMP in invertebrate cells are quite different, with concentrations of cAMP being approximately 10-fold higher than cGMP concentrations (Morton and Truman, 1985). Thus, even if cGMP is able to release PKA-C3 from SWS at half the concentration of cAMP, this would not confirm that cGMP indeed acts on SWS to release PKA-C3 *in vivo,* since cGMP is presumably present at a much lower concentration than cAMP in cells. Work in *Manduca sexta* has also shown that the relative efficacy of cAMP and cGMP in activating particular kinases can be altered, when comparing results from cell homogenates with those from intact

tissue (Morton and Truman, 1988). Furthermore, my preliminary studies do not address the possibility that SWS could be anchored in a microdomain with drastically different concentrations of cyclic nucleotide (Zaccolo and Pozzan, 2002), which would further complicate the question of which cyclic nucleotide acts on SWS *in vivo*.

Mutation of cyclic nucleotide binding sites in SWS

Experimental Rational

As previously described, SWS has similarity to the regulatory subunits of PKA in the cyclic nucleotide-binding sites. To determine if these sites are important for the physiological function of SWS, all three potential cyclic nucleotide-binding sites were mutated. Additionally, if mutation of cyclic nucleotide-binding sites does lead to permanent binding of PKA-C3, and if that is indeed the mechanism for disease in OPIDN, expression of this version of SWS should replicate the OPIDN phenotype. To address this hypothesis, I first created a construct in which all cyclic nucleotide-binding sites were mutated, called UAS-SWS$^{\Delta cAMP}$. I created constructs containing each single mutation and all combinations of the three sites possible as well, but transgenic flies have not been created with these constructs. These constructs should allow elucidation of the requirement of each site, for the function of SWS, as a regulatory subunit. Experiments using these constructs in cell culture would be prudent prior to creation of fly lines.

Materials and Methods

Mutations were made using the QuickChange Multi Site-directed mutagenesis kit from Stratagene and verified by sequencing. Primers used for each mutation are listed in Table 4-1. To abolish cNMP binding at site three, two amino acids that are only nine amino acids apart must be mutated, therefore these primers have overlapping sequence, and thus these mutations had to be made using sequential PCR reactions. A 3' HA-tag was added to this construct by subcloning in the 3' end of a previously made HA-tagged SWS construct (made by Alexandre da Cruz). Transgenic flies were created as described above.

Results and Discussion

Western blots were performed to determine protein expression levels from various transgenic lines obtained via P-element transformation. As shown in Figure 4-5D, line Z had protein expression levels equivalent to the other two lines (A and K) when expressed via *Appl*-Gal4, although lysate for the Z line was obtained from fewer flies, indicating a higher expression level per fly, compared to other insertion lines. Therefore, line Z was chosen to cross with driver lines for histological examination. Using either the neuronal driver *elav*-Gal4 (Figure 4-5B) or *Appl*-GAL4 (Figure 4-5C) to drive the UAS-SWS$^{\Delta cAMP}$ Z insertion line, vacuoles could be detected in *Drosophila* brains at 14 days. No such vacuoles can be detected in brains from flies expressing neuronal UAS-SWS at 14 days (Figure 4-5A). Male flies hemizygous for the *Appl*-GAL4 driver, were used for histological analysis. I would expect these flies to have twice the expression levels of UAS constructs, and indeed I did see degeneration in more of these flies, compared to those heterozygous for the *elav*-GAL4 driver (83% versus 71%, N=18 and 32

respectively). Even in hemizygous *Appl*-GAL4 males expressing UAS-SWS$^{\Delta cAMP}$, there was variability in the phenotype. I will now age flies longer to determine if the severity of the phenotype increases, and also if the phenotype becomes more consistent across flies examined. The ability of SWS$^{\Delta cAMP}$ to act in a dominant way is interesting, as we propose that permanent binding of the SWS and PKA-C3 complex might lead to dominant effects after OP exposure. Since OP exposure is transient, but has long lasting effects it would be interesting to determine if SWS$^{\Delta cAMP}$ could function similarly. To address this, heat shock driver-lines could be used to induce transient expression and determine whether this leads to histological signs of degeneration and for how long these effects last. Additionally, heat shock driver-lines could be used to induce expression of UAS-SWS$^{\Delta cAMP}$ in primary cultures of *Drosophila* neurons to determine if the neurite retraction described in Chapter 3 could be replicated.

Importantly, I have not yet shown that expression of SWS$^{\Delta cAMP}$ reduces PKA activity levels in flies. PKA activity assays must be done on flies over-expressing the UAS-SWS$^{\Delta cAMP}$ construct. Additionally, the phenotypes described here may be the result of a toxic effect of SWS$^{\Delta cAMP}$ that does not involve PKA-C3. If combined expression of UAS-SWS$^{\Delta cAMP}$ and UAS-PKA-C3 ameliorates the vacuolization shown after expression of UAS-SWS$^{\Delta cAMP}$ alone, this would argue that the lack of PKA-C3 is indeed responsible for the degeneration observed. These experiments might require expression of the constitutively active version of PKA-C3, to avoid binding of all excess PKA-C3 by UAS-SWS$^{\Delta cAMP}$. Additionally, if RNAi mediated knock-down of PKA-C3 shows a

similar phenotype as UAS-SWS$^{\Delta cAMP}$ expression, this would further support our hypothesis that the degeneration is due to the loss of PKA-C3.

Different protein expression levels of UAS-SWS and UAS-SWS$^{\Delta cAMP}$ could explain the data presented here, and direct comparison of UAS-SWS and UAS-SWS$^{\Delta cAMP}$ expression levels has not been done. While this has not been directly addressed, data in chapter 2 showed that UAS-SWS, under the control of GMR-Gal4, expresses protein at expected amounts (Figure 2-12). Figure 4-5D shows expression of UAS-SWS$^{\Delta cAMP}$, under the control of *App*l-Gal4, which if anything, appear lower than expected for the number of heads per lane. Until direct comparisons with the same drivers are made this possibility cannot be conclusively addressed, but I expect that the expression levels of UAS-SWS$^{\Delta cAMP}$ are not responsible for the neurodegenerative phenotype observed.

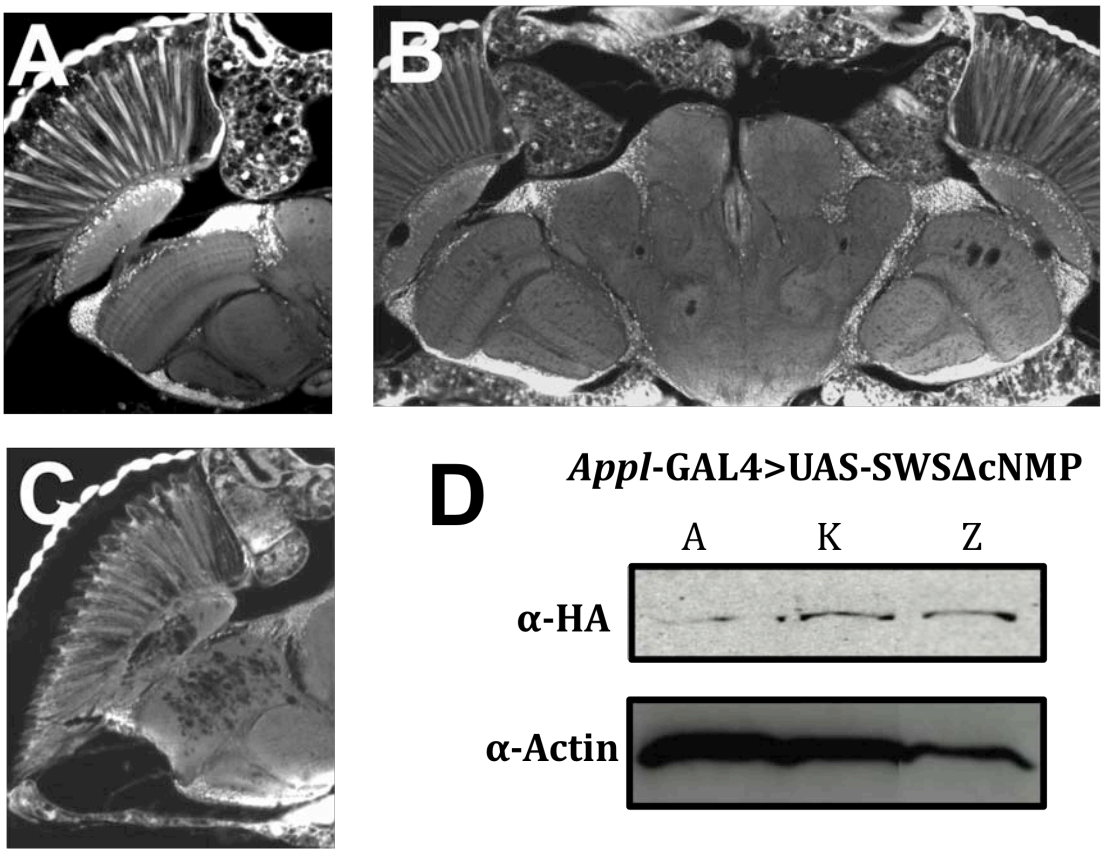

Figure 4-5. Expression of SWS$^{\Delta cAMP}$ results in neuronal degeneration. A-C Paraffin sections of heads from 14 day old flies. A. Neuronal expression of un-mutated SWS does not cause neuronal degeneration. B. An *elav*-GAL4>UAS-SWS$^{\Delta cAMP}$ expressing fly showed vacuole formation, mostly in the optic lobes. C. *Appl*-GAL4>UAS-SWS$^{\Delta cAMP}$ expressing males showed vacuoles in the optic lobes, which can be quite severe in some flies. D. Western blots showed varying expression levels in different insertion lines (induced by *Appl*-GAL4). For A and K lines 25 males fly heads were loaded onto the western. For line Z 8 female and 10 male heads were used. A loading control using anti-actin is shown below.

Final Conclusions

All together, these experiments suggest that PKA-C3 activity is tightly regulated. Overactive PKA-C3 leads to degeneration, as does mutations in SWS that should yield constitutive binding and therefore inactivation of PKA-C3. Much work remains to verify this data. Perhaps most importantly, downstream targets of PKA-C3 remain unknown. This is an important avenue, as clearly mis-regulation of these targets has important implications for neuronal health. I have not ruled out that PKA-C3 can bind to regulatory subunits others than SWS *in vivo*. This is important, because binding of PKA-C3 with regulatory subunits other than SWS would confound the results of experiments examining cyclic nucleotide regulation of PKA-C3. Until it is confirmed that PKA-C3 is only bound by SWS, I cannot assume that all effects of cyclic nucleotide on PKA-C3 are regulated by SWS.

Table 4-1. Primers used for Mutagenesis		
Mutation	**Primer Sequence (5' to 3')**	**tag**
SWS MUTATIONS		
cNMP site 1 F	TTGAAGACGGTCAGGAA**GAG**CGAGTCGGTGACAT	N/A
cNMP site 1 R	ATGTCACCGACTCG**CTC**TTCCTGACCGTCTTCAA	N/A
cNMP site 2 F	GCATCCCGGCGAAATCGTT**GAA**GGACTGGCCATGC	N/A
cNMP site 2 R	GCATGGCCAGTCC**TTC**AACGATTTCGCCGGGATGC	N/A
cNMP site 3A F	GCAAGGGGGATCTTGTG**GAG**ATCGTTGAAATGATCACG	N/A
cNMP site 3A R	CGTGATCATTTCAACGAT**CTC**CACAAGATCCCCCTTGC	N/A
cNMP site 3B F	ACGGAAACGTCG**TGG**ACCACGACGGTGATGG	N/A
cNMP site 3B R	CCATCACCGTCGTGGT**CCA**CGACGTTTCCGT	N/A
PKA-C3 MUTATIONS		
PKA-C3 3'myc	CGCGCGGCCGCCTACAGATCCTCTTCAGAGATGAGTTTC TGCTCGAAATCATTGAAGTACTGCAAATCTCTTTGG	myc
PKA-C3 3'HA	CGCGCGGCCTAGGCGTAATCGGGCACATCGTAGGGGTA GAAATCATTGAAGTACTGCAAATCTCTTTGG	HA
PKA-C3 H235R F	CGTCTCAAACAGATTGAG**CAG**GTCAAGAACGAGCGG	N/A
PKA-C3 H235R R	CCGCTCGTTCTTGAC**CTG**CTCAATCTGTTTGAGACG	N/A
PKA-C3 W427R F	GCTGAGGGATCGCACT**AGG**ACATTGTGTGGAACG	N/A
PKA-C3 W427R R	CGTTCCACACAATGT**CCT**AGTGCGATCCCTCAGC	N/A

Table 4-1. Primers used for mutagenesis experiments.

Chapter 5

Summary and Future Directions

Overview

The work presented in this dissertation describes evidence for a new physiological role of the *Drosophila* protein SWS: namely, that it acts as a regulatory subunit for a recently described kinase, PKA-C3. I showed that flies have increased kinase activity levels in the absence of SWS protein, suggesting that this increased activity is partially responsible for the degenerative phenotype seen in *sws*^{1} mutant flies. Evidence supporting the hypothesis that organophosphate exposure modifies the interaction between SWS and PKA-C3 is presented in chapter 3. Finally, preliminary data in chapter 4 describes the effects of hyper- and hypoactive PKA-C3. In this section, I will summarize each of the three data chapters presented in this dissertation, and discuss remaining questions and possible experimental approaches to answer these questions.

Summary of Chapter 2: SWS inhibits the PKA-C3 catalytic subunit

Experiments presented in chapter 2 show that the region of SWS with homology to the regulatory subunit of PKA, called the protein interaction domain or pseudosubstrate domain, has physiological importance. Neuronally expressed SWS with a mutated pseudosubstrate domain is not able to fully rescue the *sws*^{1} phenotype, indicating that this domain is important for the physiological function of SWS. Yeast two-hybrid studies reveal that the pseudosubstrate domain is required for SWS to bind the kinase PKA-C3. Both SWS and PKA-C3 can be found in membrane fractions of head lysates from wild-type flies. However, in the

absence of SWS, PKA-C3 loses this membrane localization, suggesting that SWS is responsible for tethering PKA-C3 to the membrane. Flies lacking functional SWS have increased kinase activity levels, which I suggest is due to the loss of SWS-dependent regulation of PKA-C3, supporting the model that SWS acts to inhibit the catalytic activity of PKA-C3. Increasing the level of PKA-C3 in *sws*^{1} mutants (by neuronal expression of UAS-PKA-C3) further increases brain vacuolization, suggesting that increased PKA-C3 activity leads to neurodegeneration. I also show that neuronal expression of UAS-PKA-C3 in wild-type flies leads to moderate degeneration. I propose that this degeneration is modest due to the presence of high levels of SWS protein that inhibit additional PKA-C3. This topic is revisited in chapter 4.

An interesting finding in this chapter is that expression of SWS constructs with mutations in the pseudosubstrate region in flies lacking endogenous SWS resulted in reduced esterase activity, despite the intact esterase domain in this construct. In contrast, expression of the same construct in wild-type flies doubled esterase activity, indicating that this construct has esterase activity, but that perhaps this activity is regulated in some way by its interaction with PKA-C3. To test this hypothesis, PKA-C3 was over-expressed in the wild-type background, but this manipulation did not increase esterase activity. One explanation for this lack of effect is that this over-expressed PKA-C3 was immediately bound by excess SWS and thus was not catalytically active. This experiment could be repeated with the constitutively active version of PKA-C3 described in Chapter 4 to address this issue. If PKA-C3 is required for the regulation of SWS esterase

activity, it would explain why SWS with mutations in the pseudosubstrate region lacks esterase activity.

To determine if PKA-C3 has some role in the regulation of SWS esterase activity, the phosphorylation of SWS could be investigated. This approach assumes that PKA-C3 would act on SWS by phosphorylation, as would be expected from its homology with other catalytic subunits. SWS could be immunoprecipitated and stained with antibodies against phosphoserine. However, since it is likely that other kinases can also act on SWS, this experiment would not conclusively demonstrate the phosphorylation of SWS by PKA-C3. Another way to demonstrate a direct action of PKA-C3 on SWS would be to test whether phosphorylation of SWS was reduced after RNAi-mediated knock down of PKA-C3. However, this experiment would still not reveal a direct action of PKA-C3 on SWS, as PKA-C3 could be responsible for the repression of a phosphatase or activation of another kinase that in turn regulates SWS phosphorylation. Phosphorylation levels of SWS in flies that express SWS with mutations in cyclic nucleotide binding sites (which are presumed to constitutively bind PKA-C3) should also be examined. If PKA-C3 is responsible for the phosphorylation of SWS, then I predict that flies expressing this construct would have reduced levels of phosphorylation, due to reduced activity of PKA-C3.

If phosphorylation of SWS could be correlated with the levels of active PKA-C3, it would be interesting to determine how phosphorylation of SWS is related to SWS esterase activity. To determine what serine residues in SWS are phosphorylated, potential sites could be sequentially mutated, and the altered

forms of SWS co-expressed in COS cells with PKA-C3. Immunoprecipitated SWS protein could then be probed in western blots with phosphoserine antibodies to identify whether specific mutations in SWS reduce its serine phosphorylation, indicating these residues are normally phosphorylated. After identifying proposed sites of phosphorylation, the key residues within these sites could be mutated to mimic phosphorylation (Chang et al., In Press) followed by a determination of how these mutations modify the esterase activity of SWS protein.

Based on previously described results that mutations in the pseudosubstrate region may alter esterase activity, another interesting possibility is that SWS may function as a dimer or in a complex. In the course of my dissertation work, I performed several experiments designed to address this issue, but they were unsuccessful. Lysates from *Drosophila* heads were separated on polyacrylamide gels under non-reducing conditions (i.e. without boiling or addition of reducing agents) and probed with anti-SWS antibodies in western blots, but large protein complexes could not be resolved. Under reducing conditions, SWS monomers normally migrate at 165kD, so a dimer would be predicted to be ~330kD. If other proteins associate with SWS in a complex, their apparent size in western blots could be even larger. To further address this issue, true native gels, in which native membrane protein complexes can be detected (Schagger et al., 1994), may be required.

An alterative method to test if SWS functions as a dimer would be to express tagged and truncated versions of UAS-SWS in *Drosophila* and

determine if these can be co-immunoprecipitated with full-length SWS protein. This approach assumes that truncated SWS would be as capable of forming dimers as full-length SWS, which is unknown. A second approach to consider would be the co-expression of two different versions of tagged UAS-SWS proteins: for example, a myc-tagged and an HA-tagged version. Antibodies against one tag could be used to co-immunoprecipitate the complexes, which could then be detected in western blots by probing with antibodies against the other tag.

Summary of Chapter 3: *sws* as a model for OPIDN

Data presented in Chapter 3 provide evidence that *Drosophila* is a viable model for the neurodegenerative syndrome that can develop after exposure to organophosphates. First, I showed that exposure to the organophosphorus-compound TOCP inhibits SWS esterase activity in flies, as has been shown in mammals, indicating that TOCP is an appropriate OP to use in these studies. Flies show reduced performance on behavioral tests and developed vacuoles in the brain and thoracic ganglia after exposure TOCP. TOCP treatment also reduced neurite length in primary neuronal cultures from *Drosophila*. Collectively, these phenotypes show that TOCP exposure produces measurable deleterious effects on the *Drosophila* nervous system. Next, this chapter addressed how levels of SWS protein affected behavior and neurite degeneration upon OP exposure. High levels of SWS protein were detrimental when combined with TOCP exposure, as measured by both *in vitro* and *in vivo* assays. Conversely, reduced levels of SWS protein protected flies against the effects of TOCP, as

measured by neurite degeneration. Together, these data argue in favor of the controversial hypothesis that when SWS is bound by neuropathic OPs, it undergoes a toxic gain-of-function transformation, resulting in the progressive disease OPIDN. I provide evidence that the interaction between SWS and PKA-C3 is modified after TOCP exposure and suggest this could be a possible mechanism for the gain-of-function effects. Using both adult *Drosophila* and primary hippocampal neuron cultures from rats, I also present evidence that kinase activity levels are reduced after TOCP exposure. I suggest that TOCP acts by blocking the ability of SWS to release PKA-C3, and I present evidence that over-expression of UAS-PKA-C3 is protective against the effects of TOCP on neurite length.

I also showed (using yeast two-hybrid studies) that murine NTE interacts with *Drosophila* PKA-C3. Due to the high sequence similarity between PKA-C3 and mouse Pkare, I expect that NTE and Pkare also interact, which would suggest that the function of SWS as a kinase regulatory subunit is conserved in mammals. More biochemical work using the murine proteins will be required to confirm this proposed interaction between Pkare and mNTE. Co-immunoprecipitation of mNTE and Pkare from mammalian cells would strongly support this hypothesis.

Summary Chapter 4: Additional studies on the regulation of PKA-C3

In Chapter 4, I present preliminary data suggesting that expression of a constitutively active version of PKA-C3 leads to vacuole formation in the *Drosophila* brain. Mutating the proposed cyclic nucleotide binding sites in SWS, which I suggest would reduce activity of PKA-C3, also leads to histological signs of degeneration. These data indicate that both hyperactive and inactive PKA-C3 lead to neuronal damage, suggesting that levels of PKA-C3 must be tightly regulated. Perhaps tight regulation could already be inferred by the fact that PKA-C3, unlike other kinases, binds a unique regulatory subunit: SWS. While this model does not rule out the possibility that PKA-C3 can also bind other regulatory subunits, it suggests that PKA-C3 might have an additional layer of regulation, compared to other kinases. It should also be noted that the appearance of vacuoles in the brain (detected histologically) does not confirm that the mechanism for degeneration is the same for hyper- and hypoactive PKA-C3. It is entirely possible that this an example of a “gain-of-toxic function versus loss-of-function” scenario, whereby constitutively bound PKA-C3 is unable to perform its normal physiological functions, while hyperactive PKA-C3 can promote the promiscuous phosphorylation of proteins that have nothing to do with physiological targets. A well known example of aberrant phosphorylation that leads to neuronal dysfunction is the hyperphosphorylation of the protein tau in Alzheimer’s disease (Chung, 2009). This hyperphosphorylation is thought to result from the combined effects of increased protein kinase levels (Swatton et al., 2004) and decreased protein phosphatase levels (Gong et al., 1993),

generating hyperphosphorylated tau that forms neurofibrillary tangles. Although I have no evidence that mutations in sws result in the hyperphosphorylation of tau or other cytoskeletal proteins, I cannot at present rule out this possibility. It is known that axonal transport is highly dependent on functional cytoskeletal proteins (Cowan et al., 2010), while disruptions in axonal transport are seen in mouse models of hereditary spastic paraplegia (Kasher et al., 2009). Since mutations in human NTE also lead to hereditary spastic paraplegia, it is possible that disruptions in axonal transport might also contribute to the degeneration associated with *sws* mutations.

Outlook

As described in the introductory chapter, *Drosophila* is a simple model system that is well suited to study neurodegeneration. Fly models already exist for many neurodegenerative diseases that recapitulate aspects of the human disease. Here I propose that sws^{1} flies can be used as a model for the disease OPIDN. Additionally, because of recent evidence showing that the human homolog of SWS (NTE) is involved in a progressive motor neuron disease, I propose that the work presented in this dissertation may provide the basis for identifying novel factors leading to motor neuron diseases, such as ALS. The involvement of SWS in both a genetic disease and a disease caused by environmental toxins argues that understanding the physiological function of this protein will be critical to the development of new therapies to treat these conditions.

Future Directions

Based on the experiments presented in this dissertation, I believe there are three important avenues of research that can now be pursued. Each of these future directions will be discussed in the following section.

Downstream Targets of PKA-C3

As noted above, I presented evidence that both hyper- and hypoactive PKA-C3 lead to neurodegeneration. Determining the downstream targets of PKA-C3 will be essential to define its normal functions under physiological conditions, and to determine the mechanism responsible for the development of OPIDN. Finding the authentic downstream targets of PKA-C3 presents a considerable challenge, since so little is known about the function of this kinase. One approach would be to adapt existing programs for predicting the types of proteins that are likely to be phosphorylated by particular kinases (e.g. the Kinexus Bioinformatics program). Alternatively, a modifier screen for other mutations that enhance the phenotypes seen with either constitutively active, constitutively bound PKA-C3, or PKA-C3 RNAi could also elucidate interesting interaction candidates.

The function of SWS and PKA-C3 in glial cells

Flies null for SWS protein have a distinct glial phenotype, whereby glia wrap neurons many more times than in wild-type flies (Kretzschmar et al., 1997). It is unclear if mammalian glia require SWS, because a glial specific knock-out for NTE has not been created, and NTE-null mice are embryonic lethal (Moser et al., 2004). Because the wrapping of glia in invertebrates is distinctly different from

that in vertebrates (invertebrate glia do not form myelin), I do not assume that the same glial defects would be seen in mammals lacking NTE; however, it would be quite interesting to determine if NTE is expressed by specific subsets of mammalian glia, and whether selectively knocking out this expression induces phenotypes analogous to those seen in *sws*^{1} flies.

Another interesting question is whether or not PKA-C3 is found in glial cells in either flies or mammals. In mice, Pkare is strongly expressed in the brain and in specific neurons (Blaschke et al., 2000), but it is unclear if it is present in glial cells. The absence of Pkare staining in the ventricular zone indicates that there are some glia (including ependymal cells) that do not express Pkare, but its expression might be restricted to other subsets of glia. We know that SWS is required for proper glial function in flies (Kretzschmar et al., 1997), but it is unclear if SWS simply functions as a phospholipase in glia or whether its regulation of PKA-C3 plays an additional role. It would be interesting to see if SWS containing mutations in the PKA binding domain are able to rescue the glial phenotype in *Drosophila*. Conversely, the lack of primary glial phenotypes after OP exposure suggests that PKA-C3 may not be important for glial function (a topic for future investigations).

The role of SWS and PKA-C3 in motor neuron disease and ALS

Mutations in NTE have been identified in humans with NTE-related motor neuron disease (NTE-MND) (Rainier et al., 2008) and more recently in patients with Amyotrophic Lateral Sclerosis (ALS) (Rainier et al., 2009), further

highlighting the importance of NTE in the nervous system. As described previously, mutations associated with NTE-MND suggest that disruption of the esterase function of NTE is insufficient to account for this disease (Hein et al., 2010a). I propose that the regulation of PKA-C3 by SWS/NTE could be related to development of NTE-MND, and that the results of my studies in *Drosophila* provide the basis for testing this hypothesis. Similarly, a subset of ALS patients were recently found to be heterozygous for mutations in NTE (Rainier et al., 2009), though it is currently unknown how these mutations affect esterase activity. Because ALS develops in heterozygous patients, I suspect that the underlying defect causing the disease is not related to the esterase activity of NTE, as heterozygous deletion of SWS/NTE does not cause overt degeneration in flies or mice (Kretzschmar et al., 1997; Winrow et al., 2003). Thus, the involvement of NTE in the development of multiple neurodegenerative conditions argues that the work presented here could have broad implications for understanding human disease; specifically degeneration of motor neurons. However, we must determine the physiological functions of this protein thoroughly before we can understand how mutations affecting these functions lead to disease.

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Appendix 1

Alzheimer's Disease and Tauopathy studies in flies and worms

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The writing of this review was a collaborative effort between JSW and DK.

Appendix 1 is a manuscript as it appears in the original paper published in Neurobiology of Disease, 2010.

Abstract

Progressive dementias like Alzheimer's Disease (AD) and other tauopathies are an increasing threat to human health worldwide. Although significant progress has been made in understanding the pathogenesis of these diseases using cell culture and mouse models, the complexity of these diseases has still prevented a comprehensive understanding of their underlying causes. As with other neurological diseases, invertebrate models have provided novel genetic approaches for investigating the molecular pathways that are affected in tauopathies, including AD. This review focuses on transgenic models that have been established in *Drosophila melanogaster* and *Caenorhabtidis elegans* to investigate these diseases, and the insights that have been gained from these studies. Also included is a brief description of the endogenous versions of human "disease genes" (like tau and the Amyloid Precursor Protein) that are expressed in invertebrates, and an overview of results that have been obtained from animals lacking or overexpressing these genes. These diverse models can be used to advance our knowledge about how these proteins acquire a pathogenic function and how disrupting their normal functions may contribute to neurological pathologies. They also provide powerful assays for identifying molecular and genetic interactions that are important in developing or preventing the deleterious effects.

Introduction

Alzheimer's Disease (AD) is the most common form of dementia, accounting for 60-80% of all cases and it is currently the 6th leading cause of death (Alzheimer's Association; www.alz.org). Worldwide, approximately 26 million people suffer from this devastating disease, and with the increase in life span this number is predicted to quadruple over the next four decades (American Health Assistance Foundation; www.ahaf.org). Starting with slight memory loss and confusion, AD eventually leads to severe mental impairment, often accompanied by changes in personality. Although a number of drugs have been developed for treatment to slow the progression of cognitive decline, there is currently no cure for AD.

Histologically, AD is characterized by the formation of amyloid plaques and neurofibrillary tangles (NFTs). The latter primarily consist of tau, a microtubule-binding protein that, when hyperphosphorylated aggregates into insoluble fibrillar deposits in the form of NFTs (Mandelkow and Mandelkow, 1998). These pathological tau inclusions are also found in several other neurodegenerative diseases, including frontal temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration, progressive supra nuclear palsy, and Pick's disease, accordingly this group of diseases is referred to as tauopathies (Gendron and Petrucelli, 2009). In contrast, the main component of amyloid plaques is, as the name implies, Aβ amyloid, which are small peptides consisting of 40 or 42 amino acids (Selkoe,

2000). These peptides are cleaved from the larger Amyloid Precursor Protein (APP) by proteases called b-secretase and γ-secretase (De Strooper and Annaert, 2000, Turner et al., 2003). Whereas β-secretase activity is encoded by a single protein, called the b-site APP-cleaving enzyme or BACE, γ-secretase is a protein complex consisting of presenilin (Psn), nicastrin, aph.1, and pen2 (De Strooper, 2003). That the cleavage of APP and the production of Aβ is indeed a crucial factor in AD was shown by the identification of mutations in Psn-1, Psn-2, and APP that promote the production of Aβ and lead to early-onset familial AD (FAD) (Haass and De Strooper, 1999). Alternatively, APP can be cleaved by α- and γ-secretase, which does not result in the production of toxic peptides (De Strooper and Annaert, 2000).

Although studies on patients afflicted with FAD have contributed significantly to our understanding of the pathogenesis of AD, it has also become apparent that other genes contribute to the more prevalent sporadic forms of the disease. However, studies to identify such factors in humans have obvious limitations, due to methodological difficulties and ethical concerns. Therefore, animal models are an essential experimental system to identify and understand the function of candidate genes and delineate the genetic pathways in which they play a role. In addition, animal models can be used to systematically investigate the disease mechanisms from the earliest stages, whereas human studies must rely on postmortem tissue that typically represents late stages of the disease.

Invertebrates, and especially the well-established model organisms *Drosophila* and *Caenorhabditis elegans*, provide many experimental advantages for this type of analysis. Their anatomy, development, and behavior have been thoroughly studied and they are amenable to a variety of genetic and molecular methods, including the relatively easy generation of transgenic animals and the possibility to perform large-scale mutagenesis screens. Therefore, it is not surprising they have also successfully been used in recent years to investigate the mechanisms of neurodegenerative diseases, including AD and other tauopathies (Bilen and Bonini, 2005, Wu and Luo, 2005, Lu and Vogel, 2009, Teschendorf and Link, 2009).

Modeling Amyloid Toxicity

As noted above, accumulation of Aβ into senile plaques is one of the hallmarks of AD (Glenner and Wong, 1984; Wong et al., 1985) and together with findings that mutations leading to increased Aβ production have been described in familial AD (Chartier-Harlin et al., 1991, Goate et al., 1991, Levy-Lahad et al., 1995, Sherrington et al., 1996) this provided the basis for the amyloid hypothesis, which proposed that Aβ accumulation is the driving factor for disease progression and pathology. For the vast majority of AD cases, however, the cause of amyloid plaque formation is still undefined, and the intrinsic pathogenicity of Aβ peptides remains controversial.

To investigate the toxic function of Aβ peptides in *Drosophila*, several transgenic fly models have been created that specifically express either Aβ40 or Aβ42 (Finelli et al., 2004, Iijima et al., 2004, Crowther et al., 2005). Both of these peptides are found in human plaques, but the Aβ42 peptide has been found to be the more toxic species (Findeis, 2007). Confirming the increased toxicity of this peptide, only expression of Aβ42 resulted in amyloid deposits and degeneration in the fly eye (Finelli et al., 2004) and brain (Iijima et al., 2004; Crowther et al., 2005). Surprisingly however, both peptides induced defects in an associative olfactory learning assay when expressed pan-neuronally (Iijima et al., 2004). Whereas the performance of young flies expressing either protein was not different from controls, their performance became increasingly worse when aged for 6-7 days, although there was no significant difference between flies expressing Aβ42 versus Aβ40. These experiments clearly showed that Aβ-peptides alone are sufficient to induce AD-like phenotypes. In addition, they suggested that learning defects do not require visible plaque formation, which is similar to results obtained by Crowther et al. in a climbing assay (Crowther et al., 2005). In this study the deficits were also observed before the occurrence of large extracellular deposits and instead correlated with the intracellular accumulation of Aβ (in this case Aβ42 and the arctic mutation which is found in patients with early onset familial AD). More recently, Iijima et al. used the fly model to investigate the effects of different aggregation rates by expressing Aβ42 with the arctic mutation, which increases aggregation, or an artificial mutation shown to decrease aggregation (Iijima et al., 2008). Expressing the arctic

mutation resulted in higher levels of Aβ42 oligomers compared to the normal Aβ42, while the artificial mutation reduced the formation of oligomers. Notably, these differences in aggregation tendency correlated with the deleterious effects on life span and locomotion. However, both mutations increased short-term memory deficits compared to flies expressing normal Aβ42, with the artificial mutation causing an even earlier onset than the arctic mutation. This further supported the earlier results by this group, which suggested that the aggregation propensity does not determine the severity of memory deficits. Interestingly, each form of Aβ42 had distinct defects on neuronal degeneration, with the arctic mutation causing mostly vacuoles in the cortex (where all neuronal cell bodies are located, similar to the gray matter in vertebrates). In contrast, the artificial mutation induced vacuolization in the neuropil, which is comprised of neuritis (equivalent to white matter). The specificity of these pathologies correlated with the localization of aggregates, because Aβ42arctic showed large deposits in the cell bodies, whereas the artificial mutation primarily resulted in deposits in neurites. This finding suggested that although aggregation levels can affect some phenotypes, differences in aggregation rates alone do not determine pathogenicity.

Similar results were obtained in *C. elegans,* where expression of Aβ42 in muscle cells induced the formation of amyloid containing inclusions (Link, 1995). As in flies, however expressing variants of Aβ42 with mutations that prevented deposit formation did not reduce toxicity (Fay et al., 1998), providing additional support for a model in which their pathogenicity is not solely due to the levels of

aggregation. Although transgenic worm models expressing Aβ42 in neurons also exhibit amyloid aggregates, the resulting phenotypes are very subtle (Link, 2006, Wu et al., 2006). Although expression of Aβ peptides in these models does certainly not completely recapitulate the disease process, these studies have provided important insights about the toxicity of specific peptide species. However, a caveat of inducing only the expression of Aβ peptides is that features requiring the expression of the entire APP protein cannot be recapitulated nor can these models be used to investigate genetic factors or therapeutic drugs that affect processing.

For these purposes the fly lines that express full-length human APP_{695} (Fossgreen et al., 1998), the predominant form in the nervous system, might prove more useful. To ensure b-cleavage of APP in this model, a human BACE construct was co-expressed with APP_{695}, which together with endogenous fly γ-secretase produced toxic Aβ fragments (Greeve et al., 2004). Histological analysis revealed the formation of amyloid deposits and age-dependent degeneration in these flies, in addition to a decreased life span. Surprisingly, the same phenotypes were induced after expression of APP_{695} alone, suggesting that flies posses an endogenous BACE-like enzyme. Indeed Western Blots confirmed the production of an Aβ fragment in these flies, though it was slightly larger than the Aβ produced by co-expression of human BACE. Flies expressing full-length APP were also used to investigate the effects of altering the processing pattern of APP by either genetic or pharmacological means (Greeve et al., 2004). Increasing the levels of endogenous presenilin (dPsn) or a variant

of dPsnthat contained mutations linked to familial AD, enhanced the phenotypes in this model, whereas removing one copy of the presenilin gene had the opposite effect. Similarly, treating these flies with BACE or γ-secretase inhibitors ameliorated the phenotypes, consistent with the model that the toxic effects are due to the production of Aβ peptides from the full-length protein.

Drosophila models have also addressed aspects of late onset AD, especially the role of ubiquilin. Genetic variants of human Ubiquilin 1 have been associated with a higher risk for late onset AD and it has been shown that Ubiquilin 1 can bind to Presenilin in cell culture (Bertram et al., 2005, Kamboh et al., 2006). Li et al showed that an RNAi knock-down of *Drosophila* Ubiquilin (dUbqln) in the brain resulted in neuronal degeneration and shortened life span. When dUbqln was reduced in the eye it enhanced the eye degeneration caused by expression of dPsn while overexpression suppressed the small eye phenotype (Li et al., 2007). In addition, this group reported that co-expression of dUbqln reduced the amount of full-length APP and the AICD. In contrast, Ganguly et al. showed that loss of dUbqln suppressed dPsn induced eye degeneration and that overexpression of dUbqln caused degeneration (Ganguly et al., 2008). They also showed that expression of human Ubiquilin variants associated with increased risk for late onset AD induced a more severe degeneration compared to wild type human Ubiquilin. While the opposing effects on degeneration do not allow mechanistic insights at this point, the results from both groups suggest that Ubiquilin is involved in the regulation of Psn. And

indeed the binding of dUbqln to dPsn was verified in this model *in vivo* (Ganguly et al., 2008).

To identify novel modifiers of AD phenotypes, genetic interaction screens have been performed using both APP/presenilin (van de Hoef et al., 2009) and Aβ42 expressing flies (Cao et al., 2008). Approximately 200 candidates genes were identified from these screens, including genes involved in vesicle transport, protein degradation, stress response, and chromatin structure. The interacting candidates also included the g-subunit of AMP-activated protein kinase (AMPK) (Cao et al., 2008), a protein complex involved in energy metabolism and cholesterol homeostasis. Interestingly, a mutant in AMPKγ called *loechrig (loe)* genetically interacts with APPL (Amyloid Precursor Protein-like), the sole APP protein in flies (Luo et al., 1990). Loe mutant flies showed an age-dependent degeneration of the CNS which was significantly increased when combined with a knock-out in APPL (Tschape et al., 2002).

Screens with Aβ42 transgenic worms, have also identified genes associated with stress responses and lifespan as candidate interacting partners (Cohen et al., 2006, Fonte et al., 2008), including orthologues for insulin growth factor 1 and the FOXO transcription factor. Future studies of these candidates identified in flies and worms could yield valuable insights into the pathways affected by Aβ. In addition, they could provide new targets for therapeutic intervention.

Physiological functions of APP proteins

Although the importance of APP in the pathogenesis of AD has been acknowledged for a long time, the normal physiological function of this protein and the potential role that disrupting these functions may play in AD are still not well understood (Anliker and Muller, 2006, Senechal et al., 2006, Jacobsen and Iverfeldt, 2009).

One of the first phenotypes observed in APP transgenic fly models was the occurrence of a blistered wing (Fossgreen et al., 1998). It was therefore proposed that the ectopic expression of APP in the wing disc might interfere with interactions between the cell layers of the wing, supporting studies in cell culture that have suggested that APP proteins are involved in cell adhesion (Jacobsen and Iverfeldt, 2009). Their effects on cell-cell interactions could also be the underlying cause of defects in neuronal outgrowth and post-developmental arborization induced by overexpression of APP and APPL in *Drosophila* (Li et al., 2004, Leyssen et al., 2005). Likewise, deletion of APPL resulted in decreased neurite outgrowth (Li et al., 2004), further supporting a role for APP proteins in neurite outgrowth. APPL has also been shown to affect synaptogenesis because APPL deficient flies reveal a reduced number of synaptic boutons at the neuromuscular junction, while an increase in APPL levels results in additional synaptic boutons (Torroja et al., 1999b). These structural changes appear to have corresponding functional consequences as well, because flies lacking APPL exhibited smaller excitatory junction potentials (EJPs) compared to wild type (Ashley et al., 2005). Moreover, patch clamp experiments using cultured

neurons derived from these flies showed an increase in neuronal excitability, presumably due to the activation of K^+ channels (Li et al., 2004). Whether these synaptic defects are a direct cause of the behavioral defects observed in APPL lacking flies (Luo et al., 1992) still needs to be determined. However, these behavioral abnormalities were rescued by expression of human APP_{695}, confirming a functional conservation of human and fly APP/APPL (Luo et al., 1992).

Intriguingly, there is now increasing evidence that synaptic dysfunctions caused by the intracellular accumulation of Aβ is a major aspect of AD (Selkoe, 2008, Nimmrich and Ebert, 2009) and it has also been shown that human APP can affect neuronal excitability (Turner et al., 2003). Therefore, further studies into the functions of invertebrate APP proteins like APPL and APL-1, the sole *C. elegans* APP protein (Daigle and Li, 1993), might help to elucidate the normal physiological roles of these proteins in addition to their toxic effects when mutated or otherwise affected. Intriguingly, even some of the pathogenic features of human APP appear to be conserved in fly APPL. Despite significant sequence differences in the region corresponding to Aβ, amyloid fragments derived from APPL can accumulate into deposits and induce behavioral deficits and neurodegeneration (Carmine-Simmen et al., 2009). This study also showed that APPL can be cleaved by an endogenous β-secretase-like enzyme as well as by human BACE1. Therefore, *Drosophila* can now be used for the initial identification of factors that influence secretase activities without the need and possible complications of ectopically expressed proteins.

Modeling Tauopathies

The second hallmark of AD is the accumulation of hyperphosphorylated forms of tau into Neurofibrillary tangles (NFTs). In fact NFTs correlate more with the progression and severity of AD than amyloid plaques (Arriagada et al., 1992). NFTs are also found in several other neurodegenerative disorders, and although the tau gene appears unaffected in AD, mutations in tau have been shown to cause frontotemporal dementia FTDP-17 (Hutton et al., 1998, Spillantini et al., 1998). Like other microtubule-associated proteins (MAPs), tau binds and regulates the stability and organization of microtubules, a process that is controlled by its phosphorylation level (Lee et al., 2001). Hyperphosphorylation of tau not only interferes with its interaction with tubulin but also increases its ability to form NFTs and to sequester normal tau into these aggregates (Alonso et al., 2008). Moreover, tau mutations in FTDP-17 increase its phosphorylation and impair its ability to bind microtubules (Gendron and Petrucelli, 2009, Iqbal et al., 2009), underscoring the importance of hyperphosphorylation in the toxicity of tau.

The first evidence that *Drosophila* could provide a model for tau toxicity actually came from experiments in which bovine tau was used as an axonal marker (Murray et al., 1998). Subsequently, Williams et al. found that expressing this construct in sensory neurons resulted in a significant degeneration of their axonal projections within the thoracic ganglia, although they initially grew out

normally during development (Williams et al., 2000). Together with findings that the pan-neuronal induction of this construct resulted in disrupted axonal transport (Torroja et al., 1999a), these results set the stage for using flies as a model for tauopathies. To establish such a model, Feany and colleagues expressed both wild type human tau and a variant containing a mutation associated with FTDP-17 (R406W) (Wittmann et al., 2001). While expression of either construct in the CNS led to abnormally phosphorylated tau and induced progressive degeneration and early death, the effects were stronger for the mutant form. Surprisingly, however, neither construct induced NFTs, a result that was also confirmed for another tau FTDP-17 mutation (V337M). In contrast, pan-neuronal expression of FTDP-17 tau mutations in the worm did result in the accumulation of insoluble, phosphorylated tau aggregates (Kraemer et al., 2003). Similar to the fly model, the mutant tau form induced more severe phenotypes than wild type tau, including uncoordinated movement and age-dependent neurodegeneration, although the severity of these phenotypes did not correlate with the levels of tau phosphorylation. Together, these results suggested that while NFTs may be themselves toxic, other non-filamentous forms of tau may also be detrimental. That NFTs do not always correlate with neuropathic phenotypes has also been shown in a mouse model expressing mutant human tau. These animals develop NFTs and show memory defects and neuronal loss, but whereas the neurodegenerative and cognitive defects improved after subsequent suppression of tau, the NFTs continued to accumulate (Santacruz et al., 2005).

Tau phosphorylation and neurotoxicity

Though many studies have connected tau hyperphosphorylation with its toxicity, the importance of specific phosphorylation sites for the development of tauopathies is not clear. A variety of kinases and phosphatases, including glycogen synthase-3 (GSK-3b), cdk5, protein kinase A (PKA), and microtubule-affinity regulating kinase (MARK) have been shown to regulate tau phosphorylation in biochemical studies (Lee et al., 2001). However, their function *in vivo* is less clear. In fly models, the pathogenicity of tau also appears to be affected by its phosphorylation state. Expressing human tau with mutations in 14 known phosphorylation sites in the eye abolished its neurodegenerative effect (Steinhilb et al., 2007b), while a construct that was pseudophosphorylated at these sites caused a more severe degeneration than wild type human tau (Khurana et al., 2006). In addition, several groups have shown that tau hyperphosphorylation in flies is mediated at least in part by GSK-3β, which is encoded by the shaggy (sgg) gene in *Drosophila*. As described above, pan-neuronal expression of wild-type human tau in flies induced degeneration but did not cause NFTs (Wittmann et al., 2001). However, when tau was co-expressed with sgg, this not only increased the phorphorylation of tau and exacerbated the degenerative phenotype of the eye, but also resulted in the formation of NFTs (Jackson et al., 2002). A similar result was obtained by Chau et al. who showed that co-expression of either sgg or cdk-5 enhanced both tau phosphorylation and

its degenerative effect (Chau et al., 2006). In addition, the interaction with sgg was confirmed in studies on axonal transport defects caused by human tau expression in motorneurons. In this case, co-expression of constitutively active SGG enhanced the transport defects, while treatment with GSK-3β inhibitors suppressed the phenotype (Mudher et al., 2004).

Other groups have used the fly model to investigate the role of MARK in tau phosphorylation and toxicity. Overexpressing PAR-1, the fly orthologue of MARK, elevated tau phosphorylation and enhanced its toxic effects, while removing the PAR-1 phosphorylation sites in tau abolished its toxicity (Nishimura et al., 2004). This group also showed that phosphorylation of tau by PAR-1 is a prerequisite for downstream phosphorylation events, most likely including tau phosphorylation by SGG and cdk5. In contrast, Chatterjee et al. recently showed that while mutating the PAR-1 sites in tau did indeed decrease its degenerative effect on photoreceptors, it did not prevent phosphorylation of tau by SGG. In addition, a mutant form of tau that was resistant to SGG phosphorylation retained its deleterious effects, although it did not form aggregates (Chatterjee et al., 2009). Interestingly, mutations in the SGG sites of tau increased its microtubule binding affinity of tau. This suggests that either decreased binding to microtubules (as observed in the case of the FTDP-17 mutations) or increased binding (as described above) can have toxic effect, underscoring the importance of a precisely regulated interaction between tau and microtubule.

In summary, these studies indicate that the mechanisms conferring tau toxicity are quite complex. Hyperphosphorylation certainly appears to be an

important factor, whereby tau-associated toxicity may be mediated by the orchestrated phosphorylation of several sites, rather than phosphorylation of a specific single site (Steinhilb et al., 2007a). However, as the results by Chatterjee et al. suggest, hyperphosphorylation seems to be only one mechanism that can confer tau toxicity: This indicates that other modifications that affect the interaction of tau with microtubules can induce neurodegeneration. Furthermore, these effects might not be restricted to interactions between tau and microtubule, as recent studies have shown that tau can also affect the actin cytoskeleton. Phosphorylated tau can induce the accumulation of filamentous F-actin, resembling the Hirano bodies found in patients with AD or Pick's Disease, while co-expression of tau with actin in the eye increases the severity of its degenerative phenotype (Fulga et al., 2007). It is not yet known whether this interaction is due to a direct effect of tau on actin or an indirect one, possibly mediated by an interaction between the microtubule and actin cytoskeleton (Sider et al., 1999). Intriguingly, another study suggested that phosphorylation and other posttranslational modifications of tau may depend on the cell type. Comparing the phosphorylation pattern of tau expressed in photoreceptors versus CNS neurons revealed that little or no phosphorylation was detected at two SGG/GSK-3β sites (T212/S214) in the retinal tau, whereas these sites were clearly phosphorylated in tau expressed in the CNS (Grammenoudi et al., 2006). Cell type also appeared to affect the levels of specific tau species, because two variants were detected in the eye but only one in CNS neurons. Such cell-specific effects on tau could provide an explanation for the varying sensitivity of

different neurons in tauopathy diseases. Using the *Drosophila* model, which allows selective expression in a variety of different CNS neurons, could therefore provide important insights how different neuronal subtypes process and modify tau.

Genetic interaction studies

One of the biggest advantages of fly and worm models is the comparable ease with which they can be used to identify interacting proteins. Using an RNAi based screen in *C. elegans*, Kraemer et al. identified 75 putative modifiers that affected the uncoordinated phenotype induced by the expression of human tau (Kraemer et al., 2006). Besides several kinases and phosphatases, including GSK-3β, they identified proteins involved in protein folding, stress response, and protease activity, but also several proteins of unknown function. Two of these, sut-1 and sut-2, were studied in more detail; sut-1 encodes a nematode specific protein that was shown to interact with UNC-34, an *enabled* protein family member, suggesting that sut-1 may also be involved in the regulation of the cytoskeleton (Kraemer and Schellenberg, 2007). The other candidate, sut-2, encodes a conserved zinc-finger protein that can interact with ZYG-12, a HOOK2 orthologe, which plays a role in the transport of aggregated proteins to the aggresome (Guthrie et al., 2009). Aggresomes have been shown to contain misfolded and aggregated proteins and appear to form when the proteasome pathway is overwhelmed by these proteins. sut-2 might therefore play a role in a

cellular defense mechanism against tau toxcicity by transporting aggregated tau to the aggresome.

Excessive protein accumulation can also be regulated by the proteasome pathway or clearance by autophagy. That these pathways also modulate tau levels was shown in the fly model, because the induction of autophagy by rapamycin reduced the degenerative phenotype of wild type or mutant (R406W) human tau when expressed in the eye (Berger et al., 2006). To investigate the role of the proteasome pathway in tau degradation, Blard et al. expressed a dominant-negative form of the 20S proteasome b6 subunit along with wild type human tau and showed that this resulted in increased tau accumulation, including tau that was hyperphosphorylated by SGG/GSK-3β (Blard et al., 2006). Surprisingly, a hyperphosphorylated variant of tau, that was resistant to proteasome degradation, accumulated when a dominant-negative of SGG was co-expressed, indicating that phosphorylation by another kinase results in a degradation resistant variant of tau.

In addition, genetic interaction test have linked tau-induced neurodegeneration to both cell-cycle regulation and oxidative stress responses. Abnormal activation of the cell-cycle accompanied tau^{R406W} or tau^{V337M}-induced retinal degeneration, and co-expression of genes that promote the cell-cycle (Cyclin A, Cyclin B, or Cyclin D) enhanced this phenotype (Khurana et al., 2006). In contrast, blocking cell-cycle progression by co-expressing the cdk2 inhibitor Dacapo (the fly homolog of p21/p27) or the E2F1 inhibitor Rbf1 (Retinoblastoma factor 1) reduced the neurodegenerative effects of tau. Furthermore, the authors

showed that these effects are mediated through the TOR (Target Of Rapamycin) kinase pathway, which activates cell-cycle progression in both flies and mammalian cells. Enhanced cell-cycle activation also appears to be the underlying mechanism for how oxidative stress increases neuronal degeneration. Interfering with antioxidant defense mechanisms by removing one copy of the superoxide dismutase (SOD) gene or the thioredoxin reductase (Trxr) gene aggravated the degenerative phenotype induced by tau, although it did not affect its pattern of phosphorylation (Dias-Santagata et al., 2007). However, these genetic manipulations did significantly increase the number of foci that are immunopositive for a marker of cell proliferation (proliferating cell nuclear antigen). Likewise, tau-induced toxicity correlated with the activation of the JNK pathway, a well-characterized response to oxidative damage.

Using the rough eye phenotype, Shulman and Feany performed an unbiased genetic modifier screen for genes that altered the neurotoxic effect of tau (Shulman and Feany, 2003). Several phosphatases and kinases were identified, including PAR-1, which surprisingly suppressed the degenerative phenotype when overexpressed. In addition, they found quite a few novel candidates genes encoding transcription factors, cation transporters, and several unknown proteins. Using an innovative cross-species approach, Karsten et al. first used a microarray experiment to identify differentially expressed genes in wild type mice versus mice expressing mutant tau, and then confirmed their interactions with tau in the fly model (Karsten et al., 2006). One of the genes isolated in this assay was puromycin-sensitive aminopeptidase (PSA), whereby a

loss of function mutant form of PSA enhanced tau-induced retinal degeneration. Because co-expression of fly PSA reduced the levels of tau in flies, while human PSA was shown to proteolyze tau *in vitro*, it was suggested that PSA plays a role in tau degradation.

Cellular processes affected by tau

Although hyperphosphorylation and other abnormalities of tau are known to induce toxic gain of function effects, it is also likely that the loss of its normal function has deleterious consequences. *Drosophila* tau (dtau) is highly homologous to human tau, and it is expressed both in the developing and adult nervous system, where it is most prominent in photoreceptors (Heidary and Fortini, 2001). Surprisingly, the loss of dtau does not result in lethality, and so far the only phenotype observed in these flies is a defect in oocyte polarity (Tian and Deng, 2009). The lack of more severe phenotypes is possibly due to redundancy with other microtubule-associated proteins, because three other MAPs have been described in flies that may serve overlapping functions. At least one of them, the MAP1B orthologue *futsch*, is widely expressed in the nervous system and plays a role in dentritic and axonal outgrowth (Hummel et al., 2000). Interestingly, several hypomorphic alleles of *futsch (futsch^{olk})* show a progressive degeneration in the adult, primarily restricted to the olfactory system (Bettencourt da Cruz et al., 2005). In support of the model that these other MAPs serve redundant functions with tau, expressing tau in the nervous system of *futsch^{olk}*

flies suppressed the degenerative phenotype. These studies also indicate that other MAPs, besides tau, could play a role in neurodegenerative diseases.

Overexpressing wild type dtau in the retina resulted in a pattern of degeneration resembling the phenotype caused by expressing wild type human tau (Chen et al., 2007), suggesting that the toxic properties of the fly and human protein are conserved. Likewise, expression of dtau or htau in larval motoneurons resulted in impaired axonal transport and the accumulation of vesicular aggregates accompanied by slower crawling behavior and reduced peristaltic contractions (Ubhi et al., 2007). In addition, both constructs caused a significant reduction in boutons at the larval neuromuscular junction, while htau was also shown to reduce the number of mitochondria in the remaining boutons and perturb synaptic transmission (Chee et al., 2006). In an independent study, Mershin et al. expressed dtau and htau specifically in mushroom bodies, the centers for associative learning and memory in *Drosophila* (Heisenberg, 2003), and demonstrated that both induced a decrease in olfactory learning by 25-30% (Mershin et al., 2004). Because no neurodegeneration could be detected in the mushroom bodies, these learning defects appear to be a consequence of tau accumulation on neuronal function rather than the induction of neuronal death.

Synergistic effects between tau and APP proteins

In the context of AD, it has long been suggested that tau and APP/Aβ might act synergistically in inducing toxic effects. In *Drosophila*, support for this

hypothesis was provided by studies investigating an interaction between the endogenous APPL protein and bovine tau (Torroja et al., 1999a). Pan-neuronal expression of either tau or APPL alone resulted in the accumulation of vesicles in larval motoneurons (an effect also seen after expression of human APP (Gunawardena and Goldstein, 2001)), while this phenotype became significantly more severe when both were co-expressed. Moreover, these flies exhibited defects in cuticle hardening and wing expansion in 99% of the eclosing adult flies, while malformations of this type were only occasionally observed when only one construct was expressed (app. 7% for APPL, and 0.5% for tau). In other studies, co-expression of Aβ42 with tau in the eye enhanced the degenerative phenotype compared to tau alone, which was accompanied by a substantially greater accumulation of filamentous F-actin (Fulga et al., 2007). Studies in flies have also provided further evidence for the hypothesis that this interaction is mediated by the effects of APP on tau phosphorylation (Bloom et al., 2005). Induction of Aβ42 and tau in motoneurons not only enhanced the bouton phenotypes and larval crawling defects caused by tau alone but also increased the phosphorylation level of tau (Folwell et al., 2009). These histological and behavioral defects were reverted when the animals were treated with LiCl, a common inhibitor of GSK-3β, indicating that Aβ may regulate tau phosphorylation via GSK-3b/sgg. However, similar experiments by Wang et al. suggest that the effects of APP on tau phosphorylation can be mediated by PAR-1 (Wang et al., 2007). Specifically, they showed that PAR-1 is phosphorylated by the tumor suppressor protein LKB-1, which in turn promotes PAR-1 dependent

phosphorylation of tau. Expression of full-length APP increased the phosphorylation of both PAR-1 and tau, an effect that was dependent on the presence of LKB-1. Again using eye degeneration as a convenient assay, they also showed that LKB-1 affects the toxic function of tau; knocking-down LKB-1 expression suppressed the degeneration caused by PAR-1 or APP/tau expression, whereas overexpression of LKB-1 enhanced the toxic effects induced by PAR-1 and tau. Together with the observation that expression of APP enhanced the PAR-1 phenotype in an LKB-1 dependent manner, these results suggest a pathway in which APP activates LKB-1, which in turn phosphorylates PAR-1, leading to hyperphosphorylation of tau. As in other models, hyperphosphorylation of tau then results in aggregate formation and degeneration.

Concluding remarks

The successful establishment of fly and worm models that mimic many of the prominent phenotypes described for Alzheimer's Disease and other tauopathies has opened the door to use invertebrate models and the experimental advantages they offer to delineate the molecular and genetic interactions underlying AD and related tauopathies. In particular, the foregoing studies have demonstrated that many of the neurotoxic effects associated with tau and amyloid peptides can be recapitulated in these organisms. This discovery provides a strong rational for using these models for systematic

genetic interaction screens to identify the molecular pathways that are perturbed in these diseases. In turn, promising novel genes and pathways described in invertebrates can then be targeted in mammalian model systems to confirm their relevance in human disease. Combining the advantages of invertebrate and mammalian models can therefore provide a powerful experimental approach for determining the causes of these devastating diseases, and provide new targets for developing novel diagnostic and therapeutic strategies.

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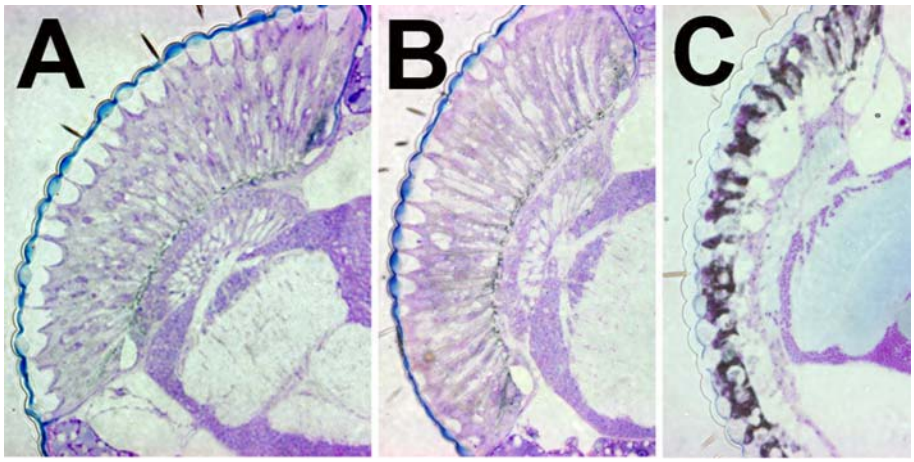

Fig. 1. Retinal degeneration caused by human APP^{695}. A) 25d old control fly. B) At 1d of adult life a transgenic fly expressing APP^{695} in the eye does not show significant degeneration whereas a fly aged for 25d reveals severe retinal degeneration. (C).

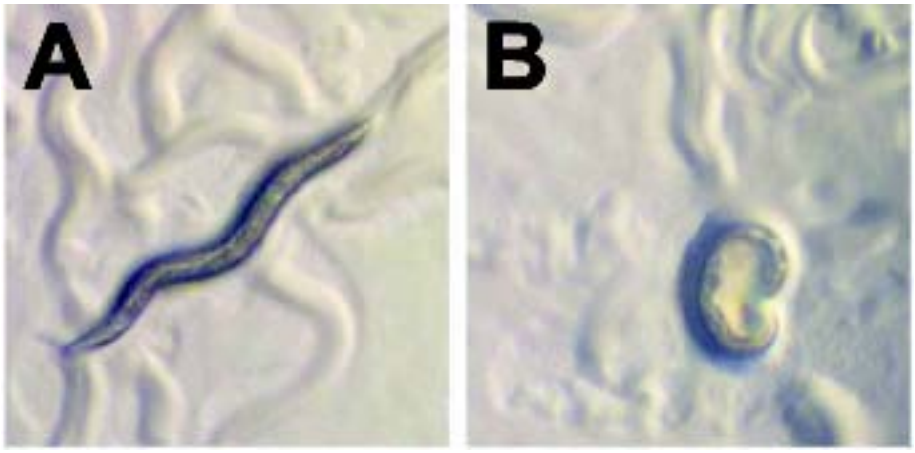

Fig. 2. Transgenic *C. elegans* expressing tau are uncoordinated. A) A wild type *C. elegans* shows the characteristic sinusoidal movement which is disrupted in an animal expressing human tau^{M337V} (B). Pictures kindly provided by B. Kraemer, University of Washington.

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APPENDIX 2

The Cell Giveth and the Cell Taketh Away: An Overview of Notch Pathway Activation by Endocytic Trafficking of Ligands and Receptors

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Abstract

Notch signaling is firmly established as a form of cell-to-cell communication that is critical throughout development. Dysregulation of Notch has been linked to cancer and developmental disorders, making it an important target for therapeutic intervention. One aspect of this pathway that sets it apart from others is its apparent reliance on endocytosis by signal-sending *and* signal-receiving cells. The subtle details of endocytosis-mediated molecular processing within both ligand- and receptor-presenting cells that are required for the Notch signal to maintain fidelity remain unclear. The endosomal system has long been known to play an important role in terminating signal transduction by directing lysosomal trafficking and degradation of cell surface receptors. More recently, endocytic trafficking has also been shown to be critical for activation of signaling. This review highlights four models of endocytic processing in the context of the Notch pathway. In ligand-presenting cells, endocytosis may be required for pre-processing of ligands to make them competent for interaction with Notch receptors and/or for exerting a pulling force on the ligand/Notch complex. In receptor-presenting cells, endocytosis may be a prerequisite for Notch cleavage and thus activation and/or it could be a means of limiting ligand-independent Notch activation. Recent advances in our understanding of how and why endocytosis of Notch receptors and ligands is required for activation and termination of signaling during normal development and in disease states are discussed.

Introduction

In the past decade, endocytic trafficking has been shown to be a critical component of many signaling pathways — including the well-studied Notch signaling pathway, which is essential for a wide range of developmental processes. Many features of the Notch signal transduction cascade have been elucidated, but a key question that remains to be fully answered is why endocytic trafficking is required in both signal sending and receiving cells for the pathway to function. This brief review will cover what is known about the role of endocytosis in Notch signaling and will highlight questions remaining in the field.

Several recent reviews provide an in depth description of the core features of Notch signaling (Kopan and Ilagan, 2009; Tien et al., 2009). Briefly, Notch proteins are single pass transmembrane receptors that transduce signals via a unique mechanism involving receptor proteolysis, resulting in the release of an active intracellular Notch fragment. The extracellular domain of the prototypical Notch receptor contains tandem arrays of Epidermal Growth Factor (EGF[1])-like repeats and a conserved negative regulatory region (NRR) that consists of three Lin12/Notch repeats (LNRs) and a heterodimerization (HD) domain (Fig. 1A). The NRR functions to prevent ligand-

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Abbreviations used:
EGF –Epidermal Growth Factor
NRR – negative regulatory region (structural domain of Notch that shields S2 cleavage site in absence of ligand binding and activation)
LNR – Lin12/Notch repeat
HD – heterodimerization domain
NECD – Notch extracellular domain
NICD – Notch intracellular domain (the portion of Notch that traffics and signals to the nucleus)
CSL – CBF/Su(H)/Lag-1
DSL – Delta/Serrate/Lag-2 (family of ligands that activate Notch)
Lgd - lethal giant disks
Hrs - hepatocyte growth factor-regulated tyrosine kinase substrate
ESCRT – endosomal sorting complexes required for transport
S1 cleavage – (furin-mediated cleavage resulting in heterodimerization of Notch)
S2 cleavage – (ADAM/TACE cleavage resulting in release of NECD fragment)
S3 and S4 cleavage – (γ-secretase cleavages resulting in release of NICD fragment)

independent activation of Notch, as illustrated by the fact that mutations within this domain generate a constitutively active receptor, leading to developmental defects and cancers (Greenwald and Seydoux, 1990; Weng et al., 2004). During intracellular receptor maturation, mammalian Notch is cleaved at the S1 site within the HD domain by furin, or a related member of the proprotein convertase family. This generates extracellular and transmembrane subunits that are held together by the HD domain (Fig. 1A). Furin cleavage is not required for mammalian Notch to reach the cell surface (Bush et al., 2001), but it is required for activation of Notch signaling. Curiously, *Drosophila* Notch lacks a consensus furin cleavage site and only the uncleaved form is detected on the cell surface (Kidd and Lieber, 2002), suggesting that pathway activation differs between vertebrates and invertebrates.

Ligands of the Delta/Serrate/Lag-2 (DSL) family activate Notch. These ligands are membrane-anchored proteins and thus receptor activation requires cell-cell contact in most circumstances. Ligand binding triggers a sequential cascade of cleavages within Notch, named S2, S3 and S4 (Fig. 1B). The S2 site, which resides within the carboxyl (C)-terminal portion of the HD domain, is cleaved by members of the ADAM/TACE metalloprotease family (Mumm et al., 2000). This cleavage releases the Notch extracellular domain (NECD) from the heterodimer (Figure 1B) (Kopan et al., 1996; Struhl and Adachi, 1998). A recent structural analysis showed that the S2 cleavage site is normally masked by extensive interdomain interactions within the NRR (Gordon et al., 2007). Thus, ligand induced conformational changes in the Notch receptor are presumably required to expose the S2 site for ADAM-mediated cleavage. S2 cleavage is a prerequisite for subsequent intramembranous cleavage of Notch at the S3 and S4 sites by the γ-secretase complex. These cleavages release the Notch intracellular domain (NICD) (Struhl and Adachi, 2000; Fiuza and Arias, 2007). The NICD then

translocates to the nucleus, where it interacts with members of the CBF1/Su(H)/Lag-1 (CSL) family of transcription factors, displacing co-repressors and recruiting co-activators to activate transcription of Notch target genes (Fortini and Artavanis-Tsakonas, 1994; Fiuza and Arias, 2007).

Studies in *Drosophila* have shown that dynamin-dependent endocytosis is essential in both the ligand- and receptor-presenting cells for successful transduction of Notch signals (Seugnet et al., 1997), and several models have since been proposed to explain this requirement (illustrated in Figure 2). First, endocytosis has been proposed to direct DSL ligands to an intracellular compartment where they undergo essential post-translational modifications prior to recycling to the cell surface for receptor activation (Fig. 2A) (Wang and Struhl, 2004, 2005). Alternatively, or perhaps in addition, endocytosis of DSL ligand bound to the Notch receptor may be necessary to provide a pulling force that dissociates the Notch heterodimer and/or induces a conformational change, thereby exposing the S2 ADAM cleavage site (Fig. 2B) (Parks et al., 2000; Nichols et al., 2007). In the signal-receiving cell, Notch endocytosis may be required for γ-secretase cleavage of Notch, perhaps because the enzyme complex is primarily active in an intracellular compartment (Fig. 2C) (Gupta-Rossi et al., 2004). Finally, Notch receptor endocytosis and lysosomal targeting may be required to prevent “accidental” ligand-independent activation of Notch (Fig. 2D) (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). In the following sections, we present evidence for and against each of these models, which are not mutually exclusive.

Ligand endocytosis: required for ligand activation?

As summarized above, the requirement for endocytosis in signal sending cells might reflect a need to internalize the ligand prior to receptor interaction in order to generate an

active ligand and/or a need to internalize the ligand-receptor complex in order to activate signaling. There is good evidence supporting both hypotheses and, indeed, endocytosis in the signal-presenting cell may serve multiple functions. The observation that DSL ligand activation requires a specialized endocytic pathway, rather than simple bulk endocytosis, supports the first model. However, the precise effect endocytosis is having on ligand activity remains unresolved. Evidence and proposed mechanisms for this model are discussed in this section.

Endocytosis of DSL ligand is triggered by monoubiquitination of its cytoplasmic tail, by the E3 ubiquitin ligases Neuralized and Mindbomb (Wang and Struhl, 2004). Ubiquitination targets DSL ligands for Epsin-mediated endocytosis, which is essential for signaling. Epsin proteins facilitate membrane curvature in addition to targeting membrane proteins for endocytosis. DSL ligands generated in Epsin mutant cells are efficiently transported to the cell surface, but cannot signal to their neighbors (Wang and Struhl, 2004). Because bulk endocytosis, which does not require Epsin function, is insufficient to facilitate DSL-Notch signaling, these results have been interpreted to suggest that Epsin targets ligands to a special endocytic compartment that they must enter to acquire signaling activity.

Recent studies suggest that ligand recycling may be a prerequisite for receptor binding. An ubiquitination-defective mutant form of the mouse Delta homolog, Dll1, can be internalized, albeit not as efficiently as the wild type species, but is unable to recycle back to the cell surface or to bind and activate Notch in neighboring cells (Heuss et al., 2008). While these studies demonstrate the importance of ligand recycling for receptor binding and activation, the mechanism by which this generates a more potent ligand remains a mystery. One possibility is that DSL ligands undergo post-translational

modification of the extracellular domain within a specialized intracellular compartment, and that this enhances receptor binding (Fig. 2A). Consistent with this possibility, a potential DSL cleavage fragment is detected in wild type cells, but not in Epsin mutant cells, which cannot endocytose ligands (Wang and Struhl, 2004). A second possibility is that DSL ligands are initially diffuse over the entire surface of the cell, but are recycled to the outside of the cell in a clustered state, and that this enhances the strength of ligand-receptor interactions. If endocytosis-mediated clustering is occurring, interactions of monomers within endocytic vesicles may promote multimer formation, or monomers could become concentrated and interact upon recycling to localized plasma membrane domains. This model is supported by the finding that soluble forms of Delta are unable to activate Notch unless they are preclustered (Hicks et al., 2002). Finally, ligands may be recycled to specific lipid microdomains that contain essential cofactors, or are otherwise optimized for signal transmission. Lipid rafts are one example of specialized membrane domains that are believed to function as signaling platforms (Lajoie et al., 2009). Mouse Dll1 is partially localized to lipid raft microdomains, whereas an ubiquitin-defective, recycling incompetent form of Dll1 is not (Heuss et al., 2008). The latter ligand is unable to signal, consistent with the idea that localization to particular microdomains is important for signal transmission and is dependent on endocytosis.

It seems likely from these results that at least some DSL ligands must travel through the endocytic pathway in order to become active Notch ligands. This theory does not rule out the potential requirement for endocytosis during interaction with the Notch receptor. It is possible that DSL ligands undergo two rounds of endocytosis, the first to generate an active ligand and the second to generate an active receptor, as discussed below.

Ligand endocytosis: a means of mechanical strain?

A second hypothesis for why endocytosis in the signal-presenting cell is essential is that ligand induced mechanical uncoupling of the extracellular and transmembrane domains of Notch is critical for efficient DSL-Notch signaling. Such a model features the endocytosis of DSL bound to Notch by the ligand-presenting cell, which, in turn generates a pulling force necessary for mechanical uncoupling of the ligand-bound Notch extracellular domain (NECD) from the Notch transmembrane domain (Fig. 2B). This uncoupling subsequently unmasks the S2 ADAM cleavage site and allows Notch receptor processing and, thus, intracellular signaling.

The initial clues for ligand-endocytosis-mediated Notch activation were provided by the observation that endocytosis-deficient clones of cells in *Drosophila* behave in a manner consistent with an inability to either send or receive Notch signals (Seugnet et al., 1997; Parks et al., 2000). In addition, signaling required the separation of the NECD and NICD into cellular compartments within the signal-sending (called 'trans-endocytosis") and signal-receiving cells, respectively, in such clones (Parks et al., 2000). Trans-endocytosis is also seen during Notch activation in mammals (Parks et al., 2000; Nichols et al., 2007): NECD and ligand colocalize in intracellular vesicles of the ligand-sending cell.

A potential structural basis for the mechanical force uncoupling model was recently discovered in the crystal structure of a modified human Notch protein (Gordon et al., 2007). The crystal structure provided clues to the mechanism of auto-inhibition by the extracellular LNR domains that suppress ligand-independent signaling at the plasma membrane (Weinmaster, 1997). The presence of the globular LNR domains in a solvent-inaccessible pocket surrounding the critical S2 cleavage site supports a model in which

major conformational changes are necessary to expose the S2 site to allow for metalloprotease cleavage, which is a prerequisite for the subsequent γ-secretase mediated cleavage that generates the active NICD signaling molecule. It is unlikely that minor allosteric effects would be sufficient to disrupt the broad and stable interactions of the S2 cleavage site and LNR domains.

If force generated by the signal-sending cell is required to activate Notch on the receiving cell, then one would predict that endocytosis-deficient DSL ligands would fail to activate Notch. Analysis of endocytosis-incompetent *delta* mutants is indeed consistent with this model (Parks et al., 2006). Delta molecules lacking their intracellular domain (*delta*ΔICD) can traffic to the plasma membrane but are unable to activate Notch. Endocytic localization of *delta*ΔICD is not observed in these situations and it is reasonable to infer that trans-endocytosis fails to occur. The finding that *delta*ΔICD expressing cells retain their ability to aggregate with Notch expressing cells suggests that DSL endocytosis is not required for ligand-receptor interactions *in vivo*, and hence, that these interactions are not sufficient to activate signaling (with some caveats, see discussion below). Further evidence that *delta*Δ*ICD* can bind endogenous Notch receptors was provided by the demonstration of its ability to act as a dominant negative in several in vivo contexts, including *Drosophila*, *Xenopus* and chicken (Chitnis et al., 1995; Sun and Artavanis-Tsakonas, 1996; Henrique et al., 1997). In these systems *delta*Δ*ICD* induces an overabundance of neuronal tissue — the classic 'neurogenic' phenotype that is characteristic of impaired Notch signaling.

These observations appear superficially at odds with earlier cell culture experiments. Trans-endocytosis observed in *Drosophila* cell culture was shown to involve translocation of full-length Notch into Delta and Serrate expressing cells in the presence

of canonical Notch signal activation (Klueg et al., 1998). However, saturation of processing and signaling machinery in the context of overexpression experiments is a potentially serious confound to these experiments. There is, in fact, evidence that some limiting components of the *Drosophila* delta-notch signaling pathway exist that can be titrated out in overexpression studies (Selkoe and Kopan, 2003). It is therefore possible that trans-endocytosis of the NECD fragment also occurred in the above experiments, (and triggered the signal activation) but was not observed due to abundance of unprocessed/full-length Notch.

In an attempt to address this issue more rigorously, Nichols et al. performed a quantitative analysis of the NECD-to-NICD ratio in the ligand-presenting cells in a mammalian cell culture system expressing wild type proteins (Nichols et al., 2007). Their careful examination of relative amounts of mammalian NECD and NICD that undergo trans-endocytosis in the presence or absence of pharmacological inhibition of S2 or S3 cleavage showed that the majority of trans-endocytosed Notch consists of NECD independent of NICD. These results are consistent with the finding that separation of the two halves of Notch is sufficient to activate signaling (Rand et al., 2000). Still unresolved, however, is whether trans-endocytosis of S1-cleaved Notch occurs prior to or plays a causal role in S2 cleavage. There is good evidence that S2 cleavage does not occur until after ligand binding (Mumm et al., 2000), but the temporal relationship between dissociation of S1-cleaved heterodimeric forms of Notch and S2 processing has yet to be elucidated. It is possible to have NECD trans-endocytosis in the absence of S2 cleavage, but this may not represent the normal sequence of events. Biochemical experiments that follow the short peptide fragment between the S1 and S2 cleavage sites could shed light on this matter. Trans-endocytosis of that peptide fragment would be strong evidence that S2 cleavage occurs in parallel or prior to any mechanical strain;

alternatively, shedding of the small peptide into the extracellular space or internalization into the signal-receiving cell would be consistent with S2 cleavage following NECD trans-endocytosis.

Unlike its mammalian homologue, *Drosophila* Notch exists on the cell surface predominantly in the uncleaved, full-length form, and appears to lack a consensus motif for S1 cleavage (Kidd and Lieber, 2002). It follows that S2, rather than S1, cleavage must be necessary for NECD trans-endocytosis in flies, unless there is a smaller population of heterodimeric Notch that constitutes the active form of the receptor. There is experimental evidence for the presence of heterodimeric Notch in *Drosophila*, most notably the finding that divalent cation-chelation leads to the release of NECD even when added in the presence of protease inhibitors (Rand et al., 2000). Because the structural integrity of the LNR domain is dependent on millimolar levels of calcium (Aster et al., 1999), this result is interpreted to mean that calcium chelation disrupts interactions between the LNR and HD domains, leading to release of the S1-cleaved NECD. Importantly, though, it is unclear whether the protease inhibitor cocktail used in these experiments was effective against ADAM metalloproteases and/or γ-secretase. This leaves open the possibility that calcium chelation merely relieves the structural auto-inhibition by LNR domains and leads to ligand-independent S2 or S3 cleavage (Rand et al., 2000). More experiments are needed to unequivocally determine whether the active form of *Drosophila* Notch undergoes S1 cleavage and heterodimerization. For example, immunopurification and sequencing of trans-endocytosed NECD would demonstrate whether the peptide fragment ends at a putative furin (S1) or metalloprotease (S2) cleavage site.

One caveat of the mechanical strain model with respect to signaling in *Drosophila* is borne out of this controversy surrounding Notch S1 pre-processing: if the functional Notch molecule is not expressed as a heterodimer, the pulling force would not separate but instead unfold and expose a cleavage site for processing, but this appears to be insufficient for Notch signaling in other model systems. In mammals, S1 cleavage and heterodimerization must precede S2 cleavage. Mammalian S1 cleavage-incompetent Notch is only observed to transendocytose as full-length protein and no signal activation is detected (implying S2 and S3 processing are absent) (Nichols et al., 2007). If the ligand-expressing cell does pull on the ligand-receptor complex, 'stretching out' of the Notch LNR autoinhibitory domains would still be predicted to occur in these mutants, which would expose the S2 cleavage site for processing and then cause NICD liberation. Perhaps this reflects differences between mammalian and *Drosophila* processing machinery (Kidd and Lieber, 2002). It may be that mammalian Notch is obliged to separate to entirely expose cleavage sites or allow for ADAM/metalloprotease activity whereas the homologous *Drosophila* S2 cleavage machinery has access to, or can act on the unprocessed, full-length receptor. Expression and analysis of mammalian notch — either wild-type forms in the presence of potent furin inhibitors or S1 cleavage-incompetent mutants — in *Drosophila* cell culture could address this discrepancy.

The most difficult experimental observation to reconcile with the mechanical strain model is the discovery of some soluble Notch ligands that have signaling capability. Under this model, soluble ligands that are unable to provide traction are predicted to be antagonists of Notch signaling, and some reports bear this prediction out (Sun and Artavanis-Tsakonas, 1997; Qi et al., 1999; Hicks et al., 2002). However, there are also examples of soluble DSL ligands that activate Notch signaling in a myriad of contexts (Fitzgerald and Greenwald, 1995; Varnum-Finney et al., 1998; Han et al., 2000; Hicks et al., 2002;

Chen and Greenwald, 2004). Most soluble ligands, including the one known endogenous example (DSL-1 in *Caenorhabditis elegans*), occur as multimers (DSL-1 is a dimer) or are bound to artificial substrates. This configuration may somehow lead to another form of mechanical strain sufficient for Notch activation. It is equally plausible that these soluble signaling events occur via non-canonical Notch pathways. A recent study of human hematopoietic stem cells suggests soluble and membrane-bound Delta4 have differential effects on cell proliferation via Notch signaling and that soluble ligand signaling can only be partially accounted for by canonical, S3/γ-secretase cleavage (Lahmar et al., 2008).

Receptor endocytosis: required for γ-secretase cleavage of Notch?

Genetic and cell biological studies have identified an important role for the endocytosis of not only the DSL ligands, but also the Notch receptor in generating an active signal. Endocytosis by the signal-receiving cell has been proposed to be required for S3 γ-secretase cleavage of Notch (Fig. 2C), although this remains controversial. Consistent with this possibility, γ-secretase is more enzymatically active at a lower pH characteristic of endosomes and lysosomes, and is found primarily in intracellular compartments, although a small fraction can be detected at the plasma membrane as well (Pasternak et al., 2003). Studies in vertebrates and flies, however, offer conflicting evidence for whether or not γ-secretase cleavage requires endocytosis of Notch. Further, if receptor endocytosis is necessary, how it influences NICD cleavage and stability is also an area of interest. Evidence against and in favor of a role for endocytosis by the Notch receptor-presenting cell in canonical signal activation is presented in this section.

Genetic studies in *Drosophila* suggest that S3 γ-secretase cleavage occurs at the plasma membrane, rather than intracellularly, and does not require receptor

internalization. One such study showed that whereas γ-secretase dependent cleavage of full-length Notch does not occur in *shibire* mutant embryos, which are defective in endocytosis due to loss of Dynamin activity, γ-secretase dependent cleavage of a truncated form of Notch lacking most of the extracellular domain (and mimicking S2-cleaved Notch) proceeds normally in these embryos (Struhl and Adachi, 1998). This suggests that endocytosis is required for S2 cleavage (perhaps through models 1 and/or 2 discussed above), but *not* for S3 cleavage. A second study examined embryos mutant for Nicastrin, an essential component of the γ-secretase complex. Nicastrin is required for S3 cleavage of Notch and, thus, in cells defective for this protein Notch processing stalls following S2 ADAM/TACE-mediated ectodomain shedding. If γ-secretase cleavage occurs intracellularly, one would predict that the S2 cleaved form of Notch would undergo endocytosis and accumulate in γ-secretase containing intracellular vesicles in Nicastrin mutant cells, but instead it is detected primarily at the plasma membrane (Lopez-Schier and St Johnston, 2002).

More recent studies in mammalian cell lines support the model that endosomal entry is essential for γ-secretase mediated activation of Notch. In cultured cells, dynamin-dependent endocytosis was shown to be essential for γ-secretase mediated cleavage of a truncated form of murine Notch that mimics the S2-cleaved form (Gupta-Rossi et al., 2004). These same studies showed that Notch is monoubiquitinated on a juxtamembrane lysine residue, by an as yet unidentified ubiquitin-ligase, and that this is critical for generation and nuclear accumulation of the NICD. Careful analysis of endocytic uptake and subcellular localization of full-length Notch mutants that cannot be monoubiquitinated revealed that these are retained at the plasma membrane, whereas mutant forms that lack the γ-secretase cleavage motif are internalized, but not cleaved or transported to the nucleus. Collectively, these studies suggest that monoubiquitination

takes place at the membrane, and is essential for S2-cleaved Notch to reach internal compartments within the cell where S3 γ-secretase cleavage takes place.

Only recently has a requirement for internalization of endogenous Notch for signaling been examined in detail in *Drosophila*. Vaccari et al (2008) looked at Notch endocytosis, cleavage and pathway activation in a panel of *Drosophila* mutant cells that are defective in various stages of endocytosis. This study revealed that there is a sharp increase in γ-secretase cleavage and pathway activation upon entry into endosomes, indicating that endosomal entry of Notch is required for efficient signaling. Curiously, signaling was not completely abolished in mutants with severely restricted endosomal entry, and this residual activity was shown to be due to an alternate, dynamin-independent internalization route. These alternate pathways may be sufficient for activation of Notch when it is highly overexpressed, which could account for the seemingly conflicting data showing that truncated Notch can still signal in flies that are defective for dynamin-dependent endocytosis (Struhl and Adachi, 1998). Alternatively, it is possible that there is a low level of γ-secretase activity at the plasma membrane that can generate enough activated Notch to trigger signaling when the receptor is overexpressed, but not when it is present at endogenous levels.

To complicate matters, recent studies suggest the rate of endocytosis in a given cell type may influence the site of S3 cleavage and potency of the resultant NICD fragment. Biochemical analysis in a mammalian system has revealed that γ-secretase cleaves Notch at more than one site, generating ligands that differ in their stability, and thus signaling potency. The choice of cleavage site varies according to subcellular location (Tagami et al., 2008). At the plasma membrane, γ-secretase is more likely to generate a relatively stable NICD fragment containing valine at the amino (N)-terminus, whereas in

endosomes, cleavage is likely to occur at a more C-terminal site that generates a less stable fragment containing a serine or threonine residue at the N-terminus. The serine-ended NICD is not only more easily degraded, but also shows reduced activation of Notch signaling. These findings demonstrate that, at least in cell culture, g-secretase cleavage occurs both at the cell surface and in intracellular compartments. Since Notch is important in many tissues for a variety of functions, this complex activation scheme may allow for nuanced regulation of Notch for its diverse functions. It remains to be examined, however, whether there are *in vivo* circumstances in which the rate of endocytosis modulates Notch signaling, much less the mechanism for such a hypothetical shift.

Receptor endocytosis: a mechanism to restrict ligand-independent Notch activation?

Endocytosis has long been described as a means of signal termination for various plasma membrane signaling events. Likewise, Notch endocytosis appears to terminate signaling, and to prevent inappropriate ligand-independent activity. Indeed, HECT-type E3-ubiquitin ligases of the Nedd4 family have been implicated in the targeting of full-length Notch from the plasma membrane to endocytic and then lysosomal vesicles as a part of its natural turnover (Cornell et al., 1999; Qiu et al., 2000; Sakata et al., 2004; Wilkin et al., 2004). However, if endocytosis by the receptor-presenting cell promotes ligand-dependant Notch activation (as discussed in the previous section), could the same set of machinery go awry and lead to ligand-independent Notch activation? This section examines this possibility and discusses research that indicates endocytic function serves a critical role in tamping down spontaneous Notch activation.

Analogous to mutations in the Notch receptor that have shed light on auto-inhibition

(Sanchez-Irizarry et al., 2004), examination of proteins involved in endosomal trafficking, sorting, or both have revealed circumstances in which their dysregulation may lead to 'accidental' Notch signaling in the absence of ligand-receptor interaction. One protein that has been identified as being necessary for proper endosomal trafficking of Notch is lethal giant disks (Lgd). Lgd is a conserved cytosolic protein containing a lipid-interacting, C2 motif. In *lgd* mutant cells, Notch accumulates in early endosomal vesicles marked by expression of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and ectopic pathway activation occurs in a γ-secretase dependent but ligand independent fashion (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Interestingly, overexpression of wild-type Lgd leads to the same ligand-independent activation of canonical Notch signaling, suggesting that Lgd is titrating out one or more other requisite trafficking proteins that are required to target Notch for lysosomal degradation (Klein, 2003). A similar accumulation of Notch in early endosomal vesicles is observed in cells mutant for either *hrs*, which recruits monoubiquitinated plasma membrane proteins to endosomes, or for components of the Endosomal Sorting Complexes Required for Transport (ESCRT) complex that is necessary for maturation of endosomes into multivesicular bodies. Whereas ligand-independent activation of Notch is observed in cells mutant for ESCRT components, for *lgd* or for both, it is not observed in *hrs* or in *hrs/lgd* double mutant cells. This indicates that Lgd functions downstream of Hrs, and that constitutive activation of Notch in *lgd* mutant cells requires transit through an early endosomal compartment. It should be noted that although mutations in *hrs* rescue ligand-independent Notch signaling in *lgd* mutant cells, Hrs is not required for endogenous ligand-induced signaling. Recently, a thorough study of how blockade of endocytosis at discrete stages affects levels of endogenous Notch signaling was performed. Obstruction of Notch trafficking after formation of Hrs-positive early endosomes, but before maturation into lysosomes, resulted in ectopic Notch activation

similar to that observed in *lgd* mutants (Vaccari et al., 2008). Taken together, these findings suggest that Lgd normally functions to traffic Notch to the lysosome, and that blockade of endocytic maturation at late stages enables Notch to either enter or accumulate in an endosomal compartment that promotes excessive g-secretase cleavage and signal activation (Fig. 2D).

The most obvious difference between endogenous Notch activation and the ectopic signaling observed in *lgd* mutants is the dependence of the former, but not the latter, on ligand interaction. This raises the question of whether activation occurs independent of S2 and/or S3 cleavage in *lgd* mutants. As previously discussed, binding of DSL ligands at the cell surface is believed to relieve auto-inhibition mediated by Notch's LNR domains, such that the S2 cleavage event can occur. Given the new appreciation for the structural basis of auto-inhibition (Gordon et al., 2007), it is reasonable to hypothesize that the progressively lower pH found in endocytic vesicles distorts H-bonding networks and electrostatic interactions, thus relieving steric hindrance to allow for S2-, and subsequent S3-cleavage in the absence of ligand. In fact, there is evidence that a sequence of cleavages similar or identical to the canonical ligand-induced signaling is required for the endocytic ligand-independent signal in *lgd* mutants. First, S2 cleavage of Notch has recently been found to depend on post-translational O-linked-glycosylation of the extracellular EGF-repeats of Notch by the *O*-glucosyltransferase Rumi (Acar et al., 2008). Endogenous Notch signaling is lost in *rumi* mutant flies, and loss of *rumi* suppresses ectopic activation of Notch in *lgd* mutant cells. Thus, Rumi function and, by inference, S2 cleavage of Notch are required for both ligand-dependant and ligand-independent Notch signaling. Analysis of flies mutant for both *lgd* and *kuzbanian* (the ADAM protease involved in S2 Notch processing) would clarify the necessity of S2 cleavage in endocytic ligand-independent signaling. The evidence that S3 cleavage is

required for endocytic, ligand-independent signaling is more direct since decreasing the dose of presenilin (the enzymatic component of the γ-secretase complex) by half attenuates Notch activation in *lgd* mutant flies (Jaekel and Klein, 2006). Given that γ-secretase may target different cleavage sites within Notch depending on cellular compartmentalization (Tagami et al., 2008), it would be interesting to compare cleavage site usage in wild type tissue with that in *lgd*, or other endosomal mutants in which Notch is ectopically activated. To this end, western blot and/or proteomic analysis of Notch fragments may shed light on the nature of endosomal, ligand-independent Notch cleavage.

Conclusions

While Notch signaling is, to date, unique in its requirement for endocytosis of both the ligand and the receptor for full pathway activation, it is becoming increasing apparent that cell signaling and endocytic membrane trafficking are intimately connected for many different signaling pathways. For example, there is evidence that presenilins, the catalytic component of the γ-secretase complex, modulate diverse biological processes independent of conventional γ-secretase protease activity — including regulation of protein trafficking (Hass et al., 2009). With this in mind, we cannot rule out the possibility that presenilins participate in directing Notch for internalization via dynamin-independent pathways.

Overlap between Notch signaling components and those of other signaling pathways imply that some of the models described here may have broader influence. For instance, there is a growing list of substrates for which γ-secretase is involved in their regulated intramembrane proteolysis (Beel and Sanders, 2008). One such target for γ-secretase cleavage is amyloid precursor protein (APP), which has been extensively studied for its

role in neurodegenerative diseases such as Alzheimer's Disease. It is entirely possible that greater similarities between these two pathways may emerge when the ligand for APP is revealed (Ma et al., 2008). In conclusion, mechanisms for how endocytosis and membrane trafficking activate and restrain the Notch signaling pathway will likely extend beyond the specific proteins we have highlighted in this review.

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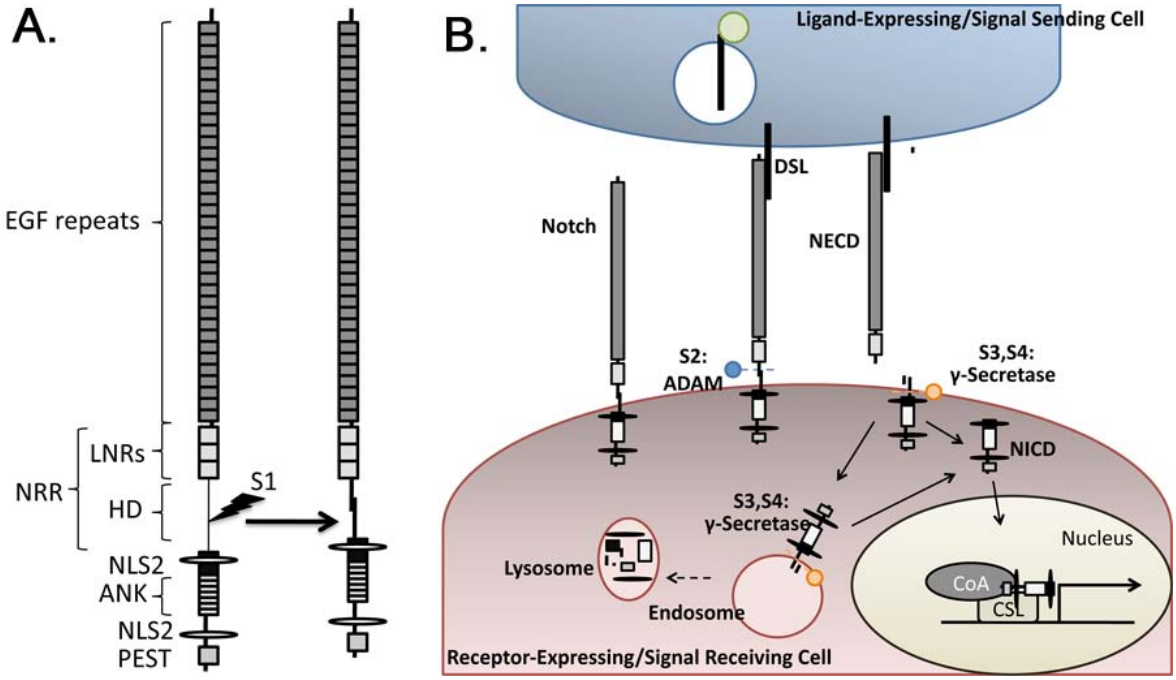

Figure 1. Schematic illustration of Notch structure and pathway activation. (**A**) Notch receptors have an extracellular domain composed of reiterated Epidermal Growth Factor (EGF)-like repeats and a conserved negative regulatory region (NRR) consisting of Lin12/Notch repeats (LNRs) and a heterodimerization (HD) domain. The intracellular portion of Notch contains repeated ankyrin (ANK) repeats, nuclear localization signals (NLS) and a PEST domain that controls receptor half life. Vertebrate Notch undergoes S1 cleavage within the secretory pathway to generate the heterodimeric receptor that is found on the cell surface. (**B**) Notch is activated by binding to ligands of the Delta/Serrate/Lag-2 (DSL) family. The ligands are ubiquitinated (green circle) and internalized into signal sending cells before and/or after receptor activation. Activated Notch undergoes sequential cleavage, initially at the S2 site by members of the ADAM family of metalloproteases (blue ball), and then at the S3 and S4 sites by γ-secretase (orange circle). S2 cleavage occurs at the cell surface and releases the Notch extracellular domain (NECD) from the heterodimer. γ-secretase mediated cleavages take place on the plasma membrane and/or in endosomes. These cleavages release the Notch intracellular domain (NICD), which translocates to the nucleus where it interacts with members of the CBF1/Su(H)/Lag-1 (CSL) family of transcription factors, and recruits co-activators (CoA) to activate transcription of Notch target genes. NICD signaling is terminated by lysosomal degradation.

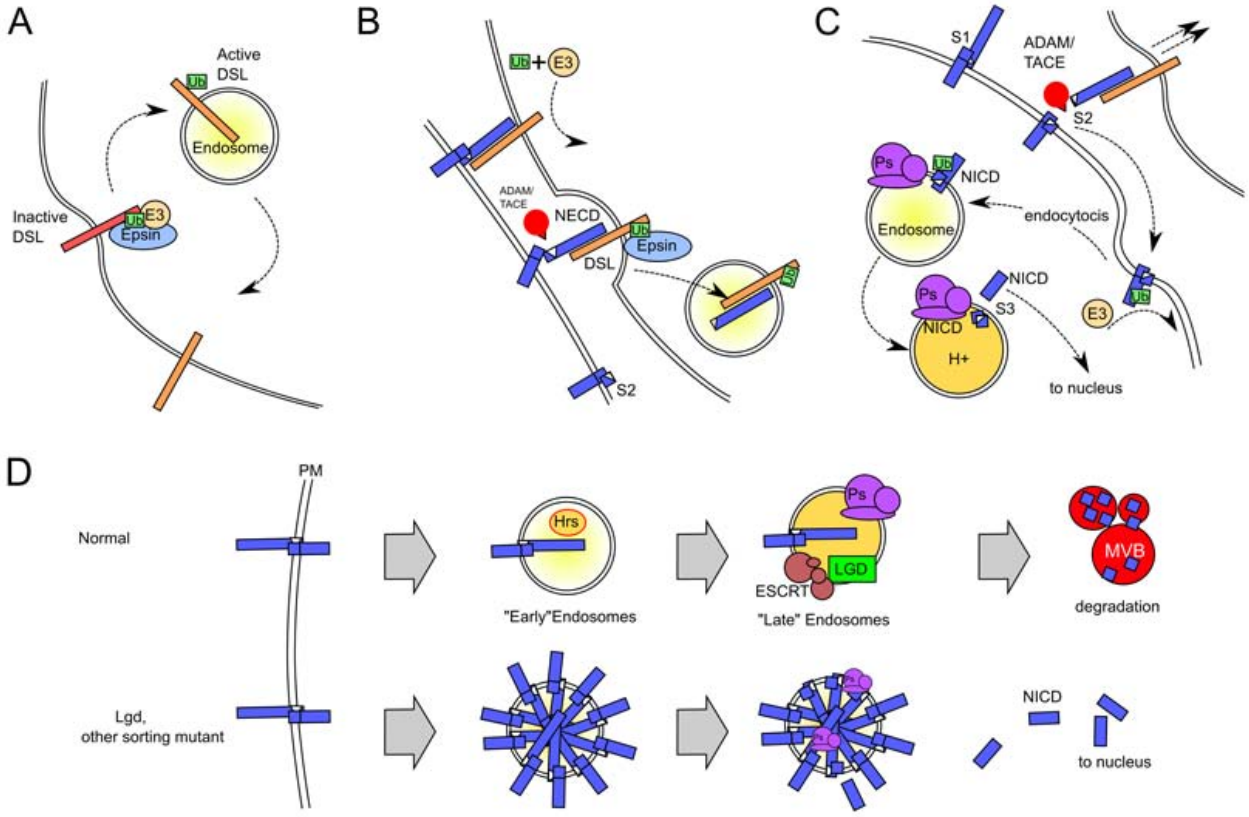

Figure 2. Models for how endocytosis activates Notch signaling. (**A**) Endocytosis as a means of generating active DSL ligand. In this model, DSL ligand is synthesized, and reaches the plasma membrane in an inactive (red) form. E3 ubiquitin ligases monoubiquitinate (Ub) the cytoplasmic tail of DSL, leading to epsin-dependent endocytosis. The ligand is converted to its active form (orange) in an intracellular compartment and then returned to the plasma membrane. The nature of the postranslational modification that activates the ligand is unknown. (**B**) Ligand endocytosis as a means of generating mechanical strain. In this model, endocytosis of DSL ligand (orange) bound to the Notch heterodimer (blue) on adjacent cells generates mechanical strain that unmasks the S2 cleavage site on Notch, enabling it to be cleaved by ADAM/TACE metalloproteases, thereby generating the remaining transmembrane fragment that is the substrate for the γ-secretase complex in the signal receiving cell. The completion of endocytosis results in trans-endocytosis of NECD and DSL into the signal sending cell. **C.** Notch endocytosis is required for S3 cleavage. Following ligand binding and S2 cleavage, ubiquitination of the cytoplasmic tail of the remaining transmembrane fragment of Notch triggers its endocytosis. The more acidic environment of the endosomes is required for the proteolytic activity of presenilin (Ps), the component of the γ-secretase complex (purple) that cleaves the S3 site to generate active NICD. **D.** Endocytosis as a means of preventing ligand-independent activation of the Notch receptor. A fraction of full length Notch undergoes ligand independent internalization from the plasma membrane and traffics to endocytic and lysosomal vesicles as part of its natural turnover. Mutations in components of the intracellular trafficking machinery, such as lethal giant discs (Lgd), that obstruct endosomal trafficking at a step after the formation of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)-positive endosomes but before maturation into lysosomes, enable Notch to either enter or accumulate in an early endosomal compartment that allows for excessive, ligand independent γ-secretase cleavage of the S3 site to generate the active NICD fragment.

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