Presenilin is necessary for the function of CBP in the adult Drosophila CNS

Randy S. Boyles

University of Nevada, Las Vegas
THE ROLE OF NOTCH, PRESENILIN, AND CBP IN THE ADULT DROSOPHILA
CENTRAL NERVOUS SYSTEM

by

Randy S. Boyles

Bachelor of Science
University of Nevada, Las Vegas, Las Vegas, Nevada
2005

A dissertation submitted in partial fulfillment of
the requirements for the

Doctor of Philosophy in Biological Sciences
School of Life Sciences
College of Science

Graduate College
University of Nevada, Las Vegas
August 2010
THE GRADUATE COLLEGE

We recommend that the dissertation prepared under our supervision by

Randy S. Boyles

entitled

**Presenilin is Necessary for the Function of CBP in the Adult**
*Drosophila CNS*

be accepted in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in Biological Sciences**
School of Life Sciences

Andrew J. Andres, Committee Chair

Steven deBelle, Committee Member

Frank van Breukelen, Committee Member

Allen Gibbs, Outside Committee Member

Bryan Spangelo, Graduate Faculty Representative

Ronald Smith, Ph. D., Vice President for Research and Graduate Studies and Dean of the Graduate College
ABSTRACT

The Role of Notch, Presenilin, and CBP in the Adult Drosophila Central Nervous System

By

Randy S. Boyles

Dr. Andrew Andres, Examination Committee Chair  
Associate Professor of Biological Sciences  
University of Nevada, Las Vegas

Dominant mutations in Presenilin (Psn) have been correlated with the formation of Aß-containing plaques in patients with inherited forms of Alzheimer’s disease (AD). However, a clear mechanism directly linking amyloid plaques to the pathology of familial or sporadic forms of AD has remained elusive. Thus, recent discoveries of several new substrates for Psn protease activity have sparked alternative hypotheses to explain the preclinical symptoms of AD. CBP (CREB-binding protein) is a haplo-insufficient transcriptional co-activator with histone acetyltransferase (HAT) activity that has been proposed to be a downstream target for Psn signaling. Individuals with reduced CBP levels have cognitive deficits that have been linked to several neurological disorders. However, there are contradictory reports in the vertebrate literature regarding the relationship between Psn activity and CBP levels. This dissertation using Drosophila melanogaster, provides evidence for the first time that Psn is required for normal CBP levels and for maintaining global acetylations of the central nervous system of the adult fly. This work also demonstrates that adult flies conditionally compromised for CBP display an altered geotaxis response to gravity that likely reflects a neurological defect.

The association between Psn and CBP is most likely not direct, but through a signaling molecule released via Psn-mediated substrate processing. One possible
candidate for this intermediate molecule is the transmembrane receptor Notch. Notch is attractive because it has been shown previously to be required for long-term memory, and I have provided evidence here that suggests Notch is required for neurite outgrowth through the culturing of primary neurons of the mushroom body (a region known to be important for learning and memory in flies). In addition I have located putative DNA binding sites for the Notch transcription factor Su(H) in the CBP enhancer, which suggests a regulatory role for Notch in CBP transcription. Although this is an attractive model, my data do not support it. Therefore it is likely that Notch and Psn/CBP control functions of the adult CNS through independent signaling molecules or pathways. If the model proves correct for vertebrates, it will have a significant impact on the development of new therapies and pharmaceuticals agents for the treatment of Alzheimer’s disease.
ACKNOWLEDGMENTS

I would like to thank, first and foremost, my wife Heather Boyles. Without her support and understanding I would have never became a scientist. Through her personal sacrifice I was provided with the means to seek out and accomplish my goals. For this I owe her a debt that can never be repaid.

I also owe a debt of gratitude to my parents for allowing me to follow my own path. They never stifled my dreams no matter how poorly they were designed, nor how rapidly they changed. I was always in an encouraging environment and provided with rich cultural nourishment that allowed me to become a well-rounded person.

Finally, I would like to thank the person who had the most influence on my education, Andy Andres. I started working in his lab as an undergraduate and stayed there, for better or worse, to finish my graduate degree. Andy gave me a dissertation project with a clearly defined goal, of which I was allowed to design its path. This of course led to a completely different goal. My journey however, provided me with several obstacles to overcome, which ultimately forced me to become a scientist. I will always remember what he said to me one frustrating day when I could get nothing to work, “If all of your experiments work, you will never learn anything.” Well, hardly any of them worked at their inception, so I must have learned a lot. Thank you Andy.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................................. iii

ACKNOWLEDGEMENTS .................................................................................................................................. v

LIST OF FIGURES ......................................................................................................................................... ix

CHAPTER 1 INTRODUCTION .......................................................................................................................... 1
  1.1 The Structure and Function of Presenilin ................................................................................................. 2
  1.2 Alzheimer’s Disease – The “Amyloid” vs. “Presenilin” Hypotheses ....................................................... 7
  1.3 Notch and other Presenilin Substrates that may Influence CBP Expression ....................................... 10
  1.4 The Regulation and Function of CREB-Binding Protein and Its Association with Neurological Disorders ........................................................................................................................................... 15
  1.5 The Function of Histone Acetylation and Its Role in Memory and Neurological Disorders .............. 17

CHAPTER 2 MATERIALS AND METHODS .................................................................................................... 20
  2.1 Drosophila Stocks and Culture .................................................................................................................. 21
  2.2 Generation of Transgenic UAS-Psni Fly Lines ....................................................................................... 21
  2.3 Selecting an effective UAS-Psni Line ........................................................................................................ 22
  2.4 Western Blotting ...................................................................................................................................... 23
  2.5 Immunohistochemistry ............................................................................................................................ 24
  2.6 Quantitative Real-Time (qRT) PCR ......................................................................................................... 25
  2.7 Primary Neuron Culture ......................................................................................................................... 26
  2.8 Heat Shock Treatments ......................................................................................................................... 27
  2.9 Geotaxis Response Assay ...................................................................................................................... 28
  2.10 Accession Numbers ............................................................................................................................... 28

CHAPTER 3 PRESENILIN CONTROLS CBP FUNCTION IN DROSOPHILA ................................................. 30
  3.1 Introduction ........................................................................................................................................... 31
  3.2 Results .................................................................................................................................................. 34
    3.2.a Phenotypic-Based Analysis of Transgenic RNAi Lines ................................................................. 34
    3.2.b Presenilin Regulates CBP in a Notch-Independent Manner ......................................................... 41
    3.2.c Presenilin is Necessary for CBP Expression .................................................................................... 49
    3.2.d Global Acetylation Levels of H4 are Reduced in the Brain when the HAT Domain of CBP is Disrupted ........................................................................................................................................... 54
    3.2.e Presenilin-Compromised Brains Also Exhibit Reductions in Global Acetylation ................. 59
    3.2.f CBP-Compromised Flies Exhibit an Altered Geotactic Response .................................................. 62
  3.3 Discussion ............................................................................................................................................. 72
  3.4 Supplemental data not submitted for publication .................................................................................. 76

CHAPTER 4 NOTCH DYSFUNCTION RESULTS IN REDUCED NEURITE OUTGROWTH IN CULTURED PRIMARY NEURONS ................................................................. 88
4.1 Introduction ........................................................................................................     89
4.2 Results ............................................................................................................. 91
4.3 Discussion ....................................................................................................... 99

CHAPTER 5 CONCLUDING REMARKS ................................................................. 101

BIBLIOGRAPHY ..................................................................................................... 105

VITA ....................................................................................................................... 112
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Molecular Functions of Presenilin .................................................. 6</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Design of UAS-Psni ............................................................... 38</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Developmental phenotypes of flies compromised for Notch, Psn or CBP using transgenic RNAi ............................................................... 40</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Putative Su(H) DNA Binding Sites in the CBP Enhancer ....................... 44</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Notch is not required for CBP expression ........................................ 46</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Notch Dysfunction Brains .............................................................. 48</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Psn is necessary for maintaining proper CBP protein levels in the adult Drosophila CNS ................................................................. 51</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Dissected Adult Brains Stained for CBP in a Psn-Reduced Background ...... 53</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Expression of UAS-CBP-FLAD in developing tissues results in a consistent phenotype ........................................................... 56</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>The HAT domain of CBP is required for acetylation of histone 4 at Lysine 8 in the adult Drosophila CNS .............................................. 58</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>Psn affects global acetylation levels in the adult brain ....................... 61</td>
</tr>
<tr>
<td>Figure 3.11</td>
<td>Assaying geotaxis using a countercurrent device ................................ 65</td>
</tr>
<tr>
<td>Figure 3.12</td>
<td>Western analysis determines the number of heat shocks required to effectively reduce CBP through RNA interference ..................... 67</td>
</tr>
<tr>
<td>Figure 3.13</td>
<td>Flies compromised for CBP exhibit poor geotaxis ................................ 69</td>
</tr>
<tr>
<td>Figure 3.14</td>
<td>Geotaxis performance scores ............................................................. 71</td>
</tr>
<tr>
<td>Figure S.1</td>
<td>Phenotypic Examples Derived from the RNAi Screen ........................... 80</td>
</tr>
<tr>
<td>Figure S.2</td>
<td>RNAi Screen for Psn, N, and CBP ....................................................... 83</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Example of Neuron Analysis Using Neuronmetrics™ ............................. 94</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Notch-Reduced Neurons Exhibit Reduced Neurite Outgrowth ................. 96</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Statistical Analysis Quantifies Notch-Reduced Phenotype .................. 98</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1: The Structure and Function of Presenilin.

The *Presenilin* (*Psn*) gene encodes a nine-pass transmembrane protein that is inserted into the membrane as a heterodimer after being processed in the Golgi. Functional Psn contains an aspartyl protease domain and is the catalytic subunit of the $\gamma$-secretase complex (Wolfe et al. 1999). This complex is made up of four proteins that include: Psn, nicastrin, Presenilin enhancer 2 (Pen2), and anterior pharynx-defective 1 (APH1). It is suggested that the $\gamma$-secretase complex is a major contributor of regulated intramembrane proteolysis (RIP), which is a fairly new model that focuses on external signals coupled to internal ones mediated through a proteolytic event within the transmembrane (Brown et al. 2000).

The participation of Psn in RIP is represented by the increasing data that implicates (to date) roughly 55 substrates that are processed by Psn (Beel and Sanders 2008). The majority of these substrates are single pass type I transmembrane receptors (Fig. 1) composed of a large external domain (ECD), an intermembraneous region, and an intracellular domain (ICD); the most notable examples are Notch and amyloid precursor protein (APP) (De Strooper 1998) (Wolfe and Guenette 2007). One known exception is the glutamate receptor subunit 3 (GluR3), which is a multipass glutamate receptor (Meyer et al. 2003). Also, Psn associates, non-catalytically, with over 40 other proteins. Figure 1 (steps 7 and 8) presents two such examples using glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) and $N$-methyl-$D$-aspartate receptor (NMDAR) (Parks and Curtis 2007).
Prior to Psn processing, the ECD undergoes “shedding” mediated by either a Disintegrin And Metalloproteinase (ADAM) (a metalloprotease) or β-Site of APP Cleaving Enzyme (BACE) (an aspartyl protease) proteolysis (Fig. 1, step 1) (Kopan and Ilagan 2004). This event is prompted externally usually through ligand binding. One exception to this model is APP, which apparently undergoes unregulated shedding (Ho et al. 2004). This shedding of the ECD prompts the remaining portion of the receptor to be cleaved by Psn to free the ICD (Fig. 1, steps 2-3). The ICD acts as a signaling molecule and translocates to the nucleus to affect gene transcription, usually as a coactivator (Fig. 1, step 4). Therefore, these gene products represent the internal consequence resulting from an external cue. ADAM and BACE processing likely are the regulating step in this process, where Psn, depending on availability, will process these primed substrates.

There are emerging data that suggest that Psn also has some γ-secretase-independent functions. One example is the association between Psn and the Wnt-signaling component β-Catenin. Psn phosphorylates β-Catenin thereby destabilizing and targeting it for ubiquination. This data was acquired using a Psn mutant lacking proteolysis capabilities in the presence of γ-secretase inhibitors which resulted in similar β-Catenin turnover rates (McCarthy et al. 2005).

Psn is conserved in Metazoans, including the Drosophila Psn which encodes a 59 kD protein with three known splice variants. It is characterized best in this system for its role in Notch processing, because its mutant phenotype is often similar to Notch mutants, specifically in the development of the nervous system during bilateral cell-fate decisions (Artevanis-Tsakonas et al. 1999). There are two human Presenilin genes PS1 and PS2 that appear to be expressed in similar quantities and tissues (Sherrington et al. 1995).
In embryonic mice, PS double conditional knockouts (cKO), in which both PSI and PS2 genes are absent, show a reduction in neural progenitor cells and the Notch target genes HES1 and HES5, which positively influence the cell cycle. As adults, these mice have morphologically smaller brains that exhibit signs of hemorrhaging (Kim & Shen 2008). Furthermore, PS double cKO show signs of age-dependent neurodegeneration and have deficits in long-term memory (LTM), whereas STM remains intact (Saura et al. 2004). Also, these mice show reduced NMDAR activity, a requirement for synaptic plasticity, and reduced CREB-regulated gene expression, which is necessary for LTM maintenance (Saura et al. 2004)(Bourtchuladze et al. 1994).

Psn was identified in 1995 (Rogaev et al. 1995) during a genetic screen involving the familial form of Alzheimer’s disease (FAD). Its implications in AD have led to a plethora of knowledge surrounding the importance of Psn, specifically with the multitude of substrates that are rapidly being discovered. Because of this, the role of Psn as a signal mediator is emerging where its requirement for the proper function in both the developing and established nervous system suggests a necessity for neuronal survival.
Figure 1.1: Molecular functions of Presenilin.

This image depicts the functions of Psn based on the most current research. Psn is shown by itself in the membrane for simplicity; but it should be noted that Psn is one of four subunits in the γ-secretase complex.

Step 1 shows the shedding of the external cellular domain (ECD), induced by ligand binding, of a generic type I transmembrane receptor mediated through either ADAM or BACE proteases. Step 2 is Psn cleavage of the remaining fragment, thereby freeing the intracellular domain (ICD), where it translocates to the nucleus (3). The ICD usually binds a transcription factor and co-activates gene expression (4).

In step 5, the type I receptor APP is processed by a mutated form of Psn, which gives rise to Aβ fragments 40 and 42 amino acids in length. These fragments, mainly Aβ-42, aggregate into plaques (6), which is a hallmark of AD.

There are several proteins that associate with Psn, including Glycogen Synthase Kinase 3β (GSK3β), which has been shown to phosphorylate Psn (7). Also, Psn assists in localizing the subunits of the N-Methyl-D-Aspartate Receptor (8).
Figure 1.1

The familial or inherited form of Alzheimer’s disease (FAD) accounts for about 5% of clinical cases of AD; whereas the sporadic form accounts for the rest (Hutton & Hardy 1997). This inherited form is thought to derive mainly from mutations in either Psn or APP, which is a substrate of Psn. The mutations in Psn that give rise to AD are dominantly inherited and individuals with these alleles have a 100% penetrant. Roughly 90% of FAD cases can be attributed to mutations in Psn, and about 10% have a mutation in APP (Hutton & Hardy 1997).

The clinical signs of AD are loss of memory, progressive neurodegeneration, amyloid plaque formation, and neurofibrillary tangles. Amyloid plaques, considered a gain of function, are considered the classical hallmark of AD and have been the focus of AD research for several decades. Researchers investigating the idea that these plaques are the main contributor to the mechanism of AD manifestation follow what has been termed the “Amyloid hypothesis” (C. A. McLean et al. 2001). This hypothesis suggests that mutated forms of Psn process APP in a fashion that enhances the release of a fragment containing 42 amino acids called Aβ42. These apparently functionless fragments are not cleared and aggregate into Aβ plaques over time. This phenomenon also occurs with certain mutations in APP. These plaques are often regionally associated with inflammation and increased presence of microglia and astrocytes, which are common AD-related symptoms (Griffin 2006). Perhaps the most compelling evidence for this hypothesis though, is the link to Down’s syndrome. Individuals with trisomy for chromosome 21 have 3 copies of APP and are usually afflicted with this disease (Rubinsztein et al. 1999).
Although there has been an exhaustive investigation into the role of Aβ42 in AD, there remain many unanswered questions. The biggest problem with the Amyloid hypothesis is that Aβ plaques have never been correlated directly to the neurodegeneration associated with AD. In fact it is not uncommon for plaques and neurodegeneration to occur in regionally different parts of the brain (Terry & Masliah 1991). Additionally, there are several instances in which individuals show AD-associated dementia, but have no Aβ plaques. Also, there are many reported cases of plaque formation with no signs of dementia (Snowdon 1997).

These accumulating gaps in Aβ research have given rise to alternative hypotheses, the most notable being the Presenilin hypothesis (Shen & Kelleher 2007). This hypothesis suggests that the mutated forms of Psn associated with AD not only misactivate APP, but also many of its other substrates resulting in a loss of signal and downstream target gene expression. Such signal losses have been reported with Ncadherin, Notch, Wnt, and reduced CRE-regulated gene expression. The idea is that any one of these, or in a combination, could have downstream targets that exhibit reduced expression and become a contributing factor to AD manifestation (Marambaud et al. 2003)(Schroeter et al. 2003)(Moehlmann et al. 2002)(Saura et al. 2004).

Furthermore, virtually all γ-secretase inhibitors, once thought of as a possible treatment for AD, result in reduced Psn substrate signaling and an unanticipated increased Aβ42 release, phenocopies with mutations in Psn. Most, if not all, of the Psn substrates require modification, usually to a portion extending into the extracellular matrix, prior to Psn processing. This modification occurs due to a developmental or external cue, such as the binding of a ligand to a receptor. Interestingly, this is not a
requirement for APP, because the shedding of its extra-cellular domain is thought to be constitutively processed in an unregulated manner.

The catalytic domain of Psn is thought to cleave in a rather non-specific manner. Based on the diversity in amino acid sequence of known cleavage sites among substrates, Struhl et al. (2000) suggests that the size of the extra-cellular domain of the transmembrane receptor determines Psn processing. Psn passes through the membrane nine times, where its conformation is very important and may be controlled through allosteric measures, which suggests that mutations in Psn could easily alter and influence a fairly non-specific catalytic domain, thereby reducing Psn cleavage-induced signaling from its substrates.

Saura et al. (2004) provide evidence that suggests Psn conditional knockdown mice have progressive age-dependent neurodegeneration, reduced synaptic plasticity, and hyperphosphorylated tau (concomitant with neurofibrillary tangles). Expression of the human mutant form of APP gave rise to an abundance of Aβ plaques in mice and resulted in little to no neurodegeneration. This further suggests that it is not a gain of function, but most likely a loss of function mediated through Psn that results in neurodegeneration.

Until recently, Aβ could not be attributed to any clearly defined physiological role. Recent research however, reported in Soscia et al. (2010), suggests that Aβ acts as an antimicrobial protein (AMP), that is a component of the innate immune response. The acquired immune response is excluded from the brain, and antibodies cannot pass the blood/brain barrier. Interestingly, Aβ can shuttle through this barrier (Zlokovic et al. 2000). Soscia et al. observe that Aβ shares similar qualities of AMPs, most importantly its ability to aggregate into soluble oligomers. This group conducted a screen comparing
Aβ oligomers to that of a very well characterized human AMP (LL-37) in their ability to inhibit growth of known pathogenic microbes. Seven of twelve in the screen showed equal or increased antimicrobial function for Aβ. Furthermore, temporal lobe homogenates from AD brains inhibited growth of *Candida albicans* with much more efficacy than non-AD brains. This is interesting because AD brains are usually inflamed, which is a condition induced by the innate response.

Based on their results, this group suggests that perhaps low levels of Aβ are released in the brain as a natural component of a healthy immune system. Alternatively, progressively increased processing of APP over several years leads to levels that accumulate into plaques in AD brains. Whether or not these plaques function as a neurodegenerative agent or are a uselessly accumulated AMP still remains to be determined. These data do however provide an interesting insight into a putative physiological role for Aβ.

There still is a large body of information that remains to be elucidated about the functions of these Psn-processed substrates, specifically with regards to their role in the nervous system and relation to each other; whether it is several independent pathways or one or more large networks where complex interactions can occur. As more of their roles are being defined more accurate insight can be attained into the putative loss of function mechanism(s) that contribute to AD.

1.3: Notch and other Presenilin Substrates that may Influence CBP Expression.

*CBP* expression was thought to be constitutive, but recent evidence suggests that *Psn* dKO mice result in a reduction of *CBP* transcript and protein levels. Intuitively, The role for Psn in this pathway probably exists in its substrate processing of one or more of the...
transmembrane receptors, culminating into a transduction of an extracellular signal to an internal one. The question remains, which transmembrane receptor(s) are responsible for taking this signal from the membrane to the nucleus to affect CBP transcription?

The Notch receptor is an appealing candidate based on the following observations: Notch and CBP-reduced model systems exhibit similar phenotypes in LTM. They also exhibit developmental phenotypes when they are reduced in a tissue-specific manner (compare wing, eye, and thorax images from both of these groups in chapter 3). Finally, Suara et al. (2004) found putative DNA binding sites for Notch co-activator Su(H) in the mouse CBP enhancer, as did we in the Drosophila CBP enhancer, which suggests possible regulation by the NICD.

Notch is a type I single-pass transmembrane receptor which is very well characterized in the development of the nervous system. The gene is haploinsufficient and was named for its “notching” phenotype that appeared in the wings of heterozygous null Drosophila. During development Notch is necessary for binary cell fate decisions that result in either an epidermal or neuronal cell (reviewed in Artevanis-Tsakonas et al. 1999).

The canonical Notch signaling pathway, conserved among all metazoans, starts with the cleavage of the extra-cellular segment of the Notch receptor induced by its association with a ligand, usually Delta or Serrate, presented from an adjacent cell. The remaining intra-membranous and intra-cellular portion of the receptor prompt a second cleavage event facilitated by Psn. This frees the Notch intra-cellular domain (NICD), which translocates to the nucleus to co-activate transcription (see Fig. 1)(Artavanis-Tsakonas et al. 1999).
Once in the nucleus, the NICD binds and alters the transcription factor Suppressor of Hairless Su(H) from a repressor to an activator, which induces transcription form target gene expression. In the developing nervous system Notch is responsible for activating the \textit{Enhancer of Split E(Sp)} complex of genes, which ultimately suppress pro-neural genes resulting in a process called lateral inhibition (Heitzler et al. 1996).

Although the role of \textit{Notch} in development has been well described, its role in the post-mitotic adult system, until recently, has remained lacking and fairly unexplored. \textit{Notch} is vital during nervous system development, any perturbation in its signaling results in early lethality, thereby limiting investigations into the function of \textit{Notch} in the adult animal. Recently, with the advent of inducible transgenes via \textit{Gal4/UAS} system, we can now inhibit or enhance gene expression temporally (Brand & Perrimon 1993). For example, where various \textit{Notch} mutants may live only to a certain point in development, expression of an inducible \textit{Notch-RNAi} transgene can remain inactive throughout development ensuring a complete and functioning nervous system; activation can then be initiated at a post-mitotic point, which will allow for the analysis of a Notch-impaired adult.

Prior to the use of transgenic reagents, \textit{Notch} was implicated in synaptic plasticity, structural plasticity, and a regulator of neurite outgrowth (Berezovska, P. McLean et al. 1999)(Sestan et al. 1999). Also, several cognitive diseases attribute disrupted Notch signaling, or at least in part, to their cause including CADASIL, Schizophrenia, and Alagille syndrome (Joutel et al. 1996)(Wassink et al. 2003) (L. Li et al. 1997).

Presente et al. (2004), Costa et al. (2003), and Ge et al (2004) showed that Notch is required for long-term memory (LTM) in mouse and fly systems, where learning and
short-term memory were unaffected in the absence of Notch, likely because de novo protein synthesis is required for LTM.

Although there is much interest in correlating Notch with memory at the molecular level, any clear insight remains elusive. Intuitively one would expect that Notch mediates gene expression through its canonical pathway via Su(H), where these gene products ultimately participate in the maintenance of LTM. Also, although less widely known, Notch has some Su(H)-independent activity involving the regulation of Armadillo/β-Catenin, which is required in Wnt signaling (Hayward et al. 2006). Whether or not this regulation occurs at the DNA or protein level remains to be elucidated.

Although it is probable that Notch influences CBP expression, which has been suggested in several reviews, other Psn substrates are being discovered at an increasing rate (Koo & Kopan 2004)(Wines-Samuelson & Shen 2005)(Parks & Curtis 2007)(Shen & Kelleher 2007). The current number is 55, with an additional ~40 known proteins that associate with Psn. Therefore, these interactions should be included in this investigation (see supplemental data).

Two intriguing Psn substrates that are known to associate with CBP are Ncadherin (Ncad) and CD44 (Marambaud et al. 2003)(Lammich et al. 2002). Ncadherin is a type I receptor with its internal domain tethered to the transcription factor β-Catenin. Upon Psn processing, the ICD is freed and dissociates with β-Catenin (Noll et al. 2000). β-Catenin is either targeted for degradation via the Glycogen Synthase Kinase 3β (GSK3β) degradation complex, or it translocates to the nucleus where and binds with T-cell factor (TCF) and CBP to affect Wnt target gene expression. Marambaud et al. (2003) showed, using mouse embryo cells, that the Ncad ICD associates with CBP in the cytoplasm and
targets it for degradation, therefore, in the absence of Psn, CBP protein levels actually increase. This is interesting because this data is in direct contrast to Saura et al. (2004) and the data reported in chapter 3 of this dissertation. CD44, another Type I receptor, is a transcription factor for certain neural genes and has nuclear associations with CBP, whether this has any implications with CBP expression requires further investigation (Koo & Kopan 2004).

Also, there are interesting Psn-associating proteins as well. GSK3β, or Shaggy in Drosophila, as mentioned above, not only may be involved in CBP degradation, but also is reported to phosphorylate Psn in certain circumstances (Twomey & McCarthy 2006). Additionally, N-methyl-D-aspartate receptor (NMDAR) requires Psn for localizing its subunits prior to formation (Saura et al. 2004). This receptor induces long-term potentiation (LTP), a neuronal state in LTM, and also is required for maintaining synaptic plasticity.

These are just a few examples of Psn substrates or associating proteins that not only may influence CBP levels, but also appear to be components in overlapping pathways involving the nervous system. Therefore it is possible that the regulation of CBP may rely on several factors drawn from a complex signaling network perhaps mediated by Psn.

1.4: The Regulation and Function of CREB-Binding Protein and Its Association with Neurological Disorders.

CREB-binding protein (CBP) is a conserved gene among the more complex metazoans including C. elegans. This gene encodes a multifunctional protein known most for its varying roles in transcriptional regulation, most notably with CREB
(reviewed in Goodman & Smolik 2000). Currently CBP has been reported to associate with over 300 different proteins, where roughly 200 derive from genes that are considered essential in the mouse genome (Kasper et al. 2006)(Barrios-Rodiles et al. 2005).

CBP has a histone acetyltransferase (HAT) domain, which facilitates acetylation of N-terminal lysines on histone tails of H2B, H3 and H4. Alternatively, CBP can perform factor acetylation (FAT), where acetylation occurs directly to coactivators or transcriptional factors (Sterner & Berger 2000)(Yang 2004). Thus, the majority of its protein associations are with transcription factors, in which CBP usually acts as a scaffolding protein to facilitate acetylation. This appears to be a level of transcriptional regulation based on the knowledge that CBP is tightly controlled at the protein level and is usually the limiting factor in signaling pathways. Also, CBP regulates DNA replication and repair proteins like Flap endonuclease I and DNA polymerase β (Kasper et al. 2006).

CBP is a large protein, over 300 kD in Drosophila, and has several domains including: nuclear hormone receptor binding domain, zinc finger, kix (CREB-binding), bromodomain (binds lysines), HAT, and a polyglutamine stretch that aids in transcription (Kumar et al. 2004). CBP appears to be expressed in all tissues and is maternally loaded. Two functional copies of the CBP gene are necessary because haploinsufficient null humans develop cognitive disorders, and Drosophila exhibit broad and severe developmental phenotypes (Goodman & Smolik 2000). CBP expression was originally thought to be constitutive, but recently a report from Saura et al. (2004) shows that Presenilin conditional knockdown mice exhibit a 40% reduction in CBP expression
based on northern blot data, which suggests that Presenilin is somewhere upstream in pathway involved in the transcriptional regulation of CBP.

CBP has been implicated in several diseases including Huntington’s disease, where expanded tracts of glutamines (polyQ) are translated from the mutated form of the Huntingtin gene. These extra Q residues cause the protein to aggregate (Zoghbi & Orr 2000). These aggregations sequester CBP, most likely due to its polyQ region, and inhibit function (McCampbell et al. 2000). Also, certain chromosomal translocation events link the histone methyltransferase-coding MLL gene or the histone acetyltransferase-coding gene MOZ gene to CBP, which results in a fused protein, are commonly seen in those afflicted with acute myeloid leukemia (AML) (Sobulo et al. 1997)(Taki et al. 1997). Additionally, individuals with only one functional copy of CBP develop a form of mental retardation, among other physical deformities, called Rubenstein Taybi syndrome (Rubenstein and Taybi 1963).

The multiple functions and associations which require CBP suggest it functions as a “pathway integrator” involving several signaling cascades that have broad biological implications. Alterations in CBP protein levels can culminate into catastrophic results, especially with regards to gene transcription. Several diseases and disorders can be attributed to CBP, specifically the HAT function, which is why this has become an increasingly successful target for therapeutic treatments.

1.5: The Function of Histone Acetylation and Its Role in Memory and Neurological Disorders.

There are four different histone proteins; H2A, H2B, H3, and H4 that form a nucloesome complex. The functional complex is an octomer assembled from two of each
histone type and wrapped with roughly 150 base pairs of double-stranded DNA in a tight configuration. This condensed state of chromatin is concomitant with reduced levels of expression generically resulting from limited access to the transcriptional machinery.

Chromatin remodeling is usually necessary for transcription and the four most common methods are: acetylation, phosphorylation, methylation, and ubiquination (reviewed in Berger 2007), where acetylation is probably the best understood.

Acetylation occurs through the enzymatic transfer of acetyl groups to Lysine residues located on histone tails. This is accomplished by proteins that contain histone acetyltransferase (HAT) domains, and their removal occurs via histone deacetylase proteins (HDACs). Acetylation reduces the electronegative charge of the Lysine, thereby making the DNA less attractive to the histone resulting in a more relaxed state, which ultimately allows for increased transcription.

The state of chromatin is highly dynamic and complex. The expressive state relies on several things including type, number, and arrangement of these combinatorial modifications. These modifications impart a very influential level of transcriptional regulation that can occur epigenetically or through an external signal. With regards to acetylation, the ratio between HATs and HDACs directly correlates to the modified state of the chromatin. For example, the addition of HDAC inhibitors to a system results in increased acetylation, whereas reduced levels of specific HAT proteins result in decreased acetylation levels.

HAT acetylation can occur globally, encompassing large distances of chromatin, or locally, at specific promoter sites (Cheng et al. 2008). HATs are evolutionarily
conserved from Yeast to humans and fall into three groups based on their acetylating domain, and they are: CBP/p300, GNAT, and MYST (Lee and Workman 2007).

CBP is perhaps the best characterized HAT based on its role in CREB coactivation at CRE-containing gene regulatory regions. Phosphorylated CREB recruits CBP to these enhancers where histone acetylation occurs locally prior to transcription. The importance of CREB regulation is seen through its emerging cognitive role, specifically with regards to long-term memory (LTM) (reviewed in Yin and Tully 1996). Also, CBP recently has been implicated in memory. Alarcon et al. (2004) and Korzus et al. (2004) show that CBP mutant mice have LTM deficits that can be ameliorated through the use of HDAC inhibitors, which suggests that the LTM phenotype derives from reduced levels of histone acetylation.

The use of HDACs to modulate memory is a common practice, but Stefanko et al. (2009) show that HDAC inhibitors can actually increase wildtype memory in mice. LTM retention remained far longer in HDAC-inhibited mice compared to mice with normal memory. Also, they show that a training regimen designed for retention to last only in the short-term (STM), persists into LTM in HDAC-inhibited mice.

Acetylated states are clearly a regulatory mechanism governing transcription; therefore, any disruption in this process can contribute to diseases ranging from mental retardation, schizophrenia, Huntington’s and Alzheimer’s disease, Fragile X, and certain types of cancers. These are compelling reasons to shift research focus to histone acetylation as a therapeutic target in an attempt to arrest or ameliorate the affects of these diseases.
2.1 Drosophila Stocks and Culture.

All flies were raised on standard cornmeal-molasses medium supplemented with live baker's yeast using the recipe recommended by the Bloomington Stock Center (Bloomington, Indiana, United States) (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm).

The stocks listed below were obtained from Bloomington: \( w^{1118} \) (FBst0307124), \( hs-CBP+ \) (FBst0003730), \( UAS-Psn+ \) (FBst0008309), \( UAS-Psni \) [selected as described below (FBst0008317)], \( GMR-Gal4 \) (FBst0001104), \( pnr-Gal4 \) (FBst0003039), and \( UAS-Ni \) (FBst0007078).

The following stocks were obtained as gifts: \( hs-N+ \) and \( c96-Gal4 \) (Rebay et al. 1993) from S. Artevanis-Tsakonas; \( UAS-CBP+ \) and \( UAS-CBPi \) (Kumar et al. 2004) from J. Kumar; \( UAS-CBP-FLAD \) (Ludlam et al. 2002) from S. Smolik; and \( hsGal4 \) (Brand & Perrimon 1993) from R. Holmgren; \( 247-Gal4 \) (Zars et al 2000) from J. Steven deBelle.

The following lines were used in RNAi screen, but were not used further in this report: \( UAS-Psni-43082 \), Vienna Drosophila RNAi Center (Dietzl et al. 2007), \( UAS-Psni \) (FBst0008318 and FB0008316).

2.2: Generation of Transgenic UAS-Psni Fly Lines.

Two separate DNA fragments of 262 base pairs were amplified from the ninth exon of the \( Drosophila Psn \) gene using two separate primer sets (Integrated DNA Technologies Coralville, Iowa, United States). The first primer set (GGGAATTCCGGCATATAAGCTTTGGCCTC and
GGCTCGAGTATAAACACCTGCTTGGC) produced a fragment that was digested with
EcoRI and XhoI and directionally cloned into the corresponding sites of the pWIZ vector
downstream of UAS sequences (Takemaru et al. 2003). The second primer set
(GGGCTAGCTATAAACACCTGCTTGGC and
GGTCTAGAGGCATAAAGCTTGGCCTC) amplified the same piece of DNA.
However, it was digested with NheI and XbaI and directionally cloned into pWIZ to
produce an inverted repeat of the first fragment. The completed pWIZ-Psni construct was
send to the Holmgren Lab (Northwestern University, Evanston, Illinois, United States)
for the production of transgenic flies using standard techniques. Eight transgenic lines
were produced: UAS-Psni-J1, UAS-Psni-J2, UAS-Psni-J3, UAS-Psni-J5, UAS-Psni-J7,
UAS-Psni-2a, UAS-Psni-2b, and UAS-Psni-15.

2.3: Selecting an effective UAS-Psni Line.

While we were generating the eight UAS-Psni transgenic flies described above, four
independently produced lines became available from the Drosophila community. All of
these contained inverted repeats of Psn sequences under UAS control. Three lines came
from Bloomington: FBti0040721, FBti0040747, FBti0040722; and one line from the
Vienna Drosophila RNAi Center (UAS-Psni-43082) (Dietzl et al. 2007).

We tested all 12 lines by expressing them in the developing wing, thorax, and eye
(Fig. 2C,G,K) and selected the one line that produced the most severe phenotypes.

2.4: Western Blotting.

Protein extracts using Drosophila were prepared as described in (Vecsey et al. 2007).
They were removed from anesthetized flies of the appropriate genotype with a razor
blade and homogenized in a lysis buffer (1% NP-40, 0.5% deoxycholic acid, 0.1%
Triton-X-100, 100 mM NaCl, 0.1 mM CaCl$_2$, 2 mM MgCl$_2$) supplemented with freshly prepared protease inhibitor cocktail (Sigma-Aldrich Inc. St. Louis, Missouri, United States) to a final concentration of 1%. Cell debris was pelleted in a microfuge (1000 g for 1 minute) and the supernatant was transferred to an equal volume of 2x Laemmli buffer (2x=4% SDS, 20% glycerol, 120 mM Tris pH 6.8, 10% β-mercaptoethanol, 0.002% bromophenol blue). The equivalent of 7.5 heads for Notch detection and 2.5 heads for CBP detection was boiled for 5 minutes and loaded on a 7.5% polyacrylamide gel.

Gels were blotted and treated with antibodies using a modified protocol of (Vaskova et al. 2000). After electrophoresis, the gel was electroblotted onto an Immobilon-P membrane (Millipore, Bedford, Massachusetts, United States) for 2.5 hours and blocked overnight in PBST (1.4M NaCl, 26.8mM KCl, 101.4 mM Na$_2$PO$_4$, 17.6mM KH$_2$PO$_4$, and 1% Tween 2) plus 5% dry milk (Nestle USA, Inc., Solon, Ohio, United States) at 4°C. The following primary antibodies were used: rabbit polyclonal anti-dCBP (a gift from Alexander Mazo) diluted 1:1000, mouse monoclonal anti-Notch C17.9c6 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, United States) diluted 1:2000, and mouse monoclonal anti-α-tubulin (Sigma-Aldrich) diluted 1:15000. Primary antibody incubation was done in PBST and 5% milk overnight at 4°C. Goat-anti-mouse-HRP diluted 1:25,000 and Goat-anti-rabbit-HRP diluted at 1:40000 (Jackson Immuno Research, West Grove, Pennsylvania, United States) were used as secondary antibodies. An ECL Plus Detection System (GE Healthcare, Piscataway, New Jersey, United States) was used for protein detection and visualized on a Typhoon 8600 Variable Mode Phosphorimager (GE Healthcare).
2.5: Immunohistochemistry.

Flies of the appropriate genotype were selected from progeny that were raised at 18°C and collected 3-5 days post eclosion. They were subjected to a heat shock regimen to induce the proper transgene by either being moved to 29°C for the duration of the experiment or by being immersed in their culture tubes in a 37°C water bath as described below. Brains dissected in PBS (1.9 mM NaH₂PO₄, 8.4 mM Na₂HPO₄, 175 mM NaCl), were fixed in 4% paraformaldehyde (in PBS), and treated with primary and secondary antibody (Wulbeck & Helfrich-Forster 2007). The following primary antibodies were used: rabbit polyclonal anti-CBP (Alexander Mazo) diluted 1:350 in PBSBT (PBS, 0.2% Triton-X100, 0.25% BSA) and rabbit polyclonal anti-Ac-H4-K8 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA United States) diluted 1:350 in PBSBT. Incubation in primary antibody occurred overnight with gentle rocking at 4°C. After washing 3-5 times in PBT (PBS, 0.2% Triton-X100) the brains were incubated with Rhodamine-conjugated goat-anti-rabbit secondary antibody (Jackson Immuno Research), diluted 1:1000 in PBSBT for 4 hours at room temperature. The final washes (3 x 5 minutes followed by 3 x 20 minutes) were done in PBT. The tissues were mounted in Fluoro-Gel (Electron Microscopy Sciences, Hattfield, Pennsylvania, United States) and imaged on a LSM 510 Axioplan confocal microscope (Carl Zeiss SMT, Peabody, Massachusetts, United States) equipped with LSM 510 image-analysis software (Carl Zeiss).

2.6: Quantitative Real-Time (qRT) PCR.

RNA was extracted from 100 adult Drosophila heads using the RNeasy Mini Kit (Qiagen Valencia, California, United States) and treated with Turbo DNA-free (Ambion Austin, Texas, United States) using their respective protocols. cDNAs were synthesized
using SuperScript First-Strand (Invitrogen, Carlsbad, California, United States) according to manufacturer’s instructions. GAGCAGGCGGTAATCTTCAG and TTGCTGGGGAAGAAGTTATGG primers (Integrated DNA Technologies) and PerfeCTa SYBR Green SuperMix (Quant Biosciences, Gaithersburg, Maryland, United States) were used to amplify and detect CBP sequences during the reactions. Each reaction was performed in triplicate on a Bio-Rad iCycleriQ Real Time PCRSys system (Bio-Rad Laboratories, Hercules, CA United States), and experimental Ct values were normalized to Beta-Actin using TCTACGAGGGTTATGCCCTT and GCACAGVTTCTCCTTGATGT primers. The data were subjected to ΔΔct statistical analysis.

2.7: Primary Neuron Culture.

Brains dissected from wandering third instar were used for primary neuron cultures, where transgenic expression was confined to the mushroom body via the 247-gal4 diver. These neurons were identified with green fluorescent protein (GFP) using the following groups: \textit{UAS-Ni;UAS-GFP/247-gal4} (experimental) and \textit{UAS-GFP/247-gal4} (control). The larvae were initially sterilized in 95% ethanol for 1 minute, washed 3 times in water, and transferred to a well containing Schneider’s Insect Media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Logan, UT), and 50 μg/ml of insulin (Sigma, St. Louis, MO). Brains were incubated at room temperature for 1 hour in a dissociation media containing 1X Liberase (Roche) and Rinaldini’s saline (137mM NaCl, 2.68 mM KCl, 0.36 mM NaH₂PO₄, 11.9 mM NaHCO₃, and 5.55 mM glucose). For a complete dissociation, the brains were pipetted through a P-200 tip for 40 repetitions for two rounds in 200μl of media. The final volume was adjusted to 500μl, where 100μl aliquots
were distributed among 5 culture dishes featuring a small circular well containing 167µg/ml of Concanavilin A (Sigma) and 1.67µg/ml laminin (BD Biosciences, Bedford, MA). The cells were given two hours to attach to this surface before flooding the dish to a final volume of 1ml. The dishes were sealed with two strips of parafilm and incubated at 25°C for 3 days for neurite growth prior to analysis.

The cultured primary neurons were analyzed on a free access semi-automated computer program called Neuronmetrics (http://www.ibridgenetwork.org/arizona/UA07-56-Neuronmetrics). The neurites were evaluated for the following characteristics: total neurite length, branchiness, number of primary neurites, and territory occupied by neuron. These measurements were statistically analyzed by a Mann-Whitney rank sum test using GraphPad Prism software.

2.8: Heat Shock Treatments.

For experiments that involved the conditional induction of transgenes, flies were raised at 18°C until 3-5 days post-eclosion. They were then either moved to 29°C (for experiments using UAS-CBP-FLAD) or subjected to heat shocks in a 37°C waterbath (for experiments involving UAS-Ni, UAS-Psni, UAS-CBPi, UAS-N+, UAS-Psn+, UAS-CBP+). Prior to administering the heat shocks, flies were transferred to 9.5x2.5 cm polypropylene vials containing 8 ml of cornmeal-molasses medium covered with a thin layer of rayon to protect them from dehydration and getting stuck in the food. Buzz plugs (Genesee Scientific, San Diego, California, United States) were used to create a chamber that was submerged in the water bath. Conditions for multiple heat shocks were determined empirically for each genotype to maximize target-gene silencing (determined by Western blot) without causing lethality.
The following heat shock regimens were used: 7 one-hour treatments (~12-14 hours apart) for CBPi (hsGal4; UAS-CBPi) and controls; 9 one-hour treatments (~12-14 hours apart) for Ni (hsGal4; UAS-Ni), P斌i (hsGal4; UAS-P斌i), and their control groups; 7 one-hour treatments (~12-14 hours apart) for P斌+ (hsGal4; UAS-P斌+) and controls; and 2 one-hour treatments (~12-14 hours apart) for N+ (hsN+), CBP+ (hsCBP+ and hsGal4; UAS-CBP+), and controls.

Flies recovered between heat shocks at 25°C and were given a one-hour rest period at 25°C after the last treatment before their brains were dissected.

2.9: Geotaxis Response Assay.

All geotaxis experiments were performed in a countercurrent apparatus as described in Benzer (1967). The apparatus consists of 6 sets of polypropylene tubes (9 x 1.5 cm) horizontally opposed. The opposing tubes are held in a plexiglass frame that allows the tubes to move in unison relative to each other. The experiments were conducted without light in an environmentally controlled chamber at 25°C with 65% relative humidity, and all fly lines were outcrossed for 7 generations to reduce the occurrence of modifiers.

Roughly 25 flies of mixed sex were used per round. Flies are given a score based on how far they advanced by the conclusion of the round. The percentage of the cohort in tube 6 was multiplied by 1, those in tube 5 by 0.8, those in tube 4 by 0.6, those in tube 3 by 0.4, those in tube 2 by 0.2 and those in tube 1 by 0. These scores were totaled for each round and the mean and standard error were calculated based on a total of twelve different rounds. All groups were compared for significance using a one-way ANOVA using GraphPad Prism software (La Jolla, CA United States).
2.10: **Accession Numbers.**

The FlyBase (http://flybase.bio.indiana.edu/search/) identification numbers are used in this work to describe genes, gene products, vectors, and *Drosophila* stocks.
CHAPTER 3

PRESENILIN CONTROLS CBP FUNCTION IN DROSOPHILA

The following chapter was the submitted to journal PLoS One for publication with the following author list: Randy S. Boyles, Katheryn M. Lantz, Steven Poertner, and Stephanie J. Georges, and Andrew J. Andres.

My contribution to this work is as follows: Designed every experiment, performed all of the molecular biological experiments, performed or directed all of the cell biological experiments. I also performed all qRT-PCR experiments and bioinformatic analyses. My undergraduate assistants participated in immunohistochemistry assays involving acetylation levels, and also geotactic experiments, and transgenetic outcrossing. All of the fly lines were either collected or made by me except hs-N, hs-gal4, and UAS-Ni.

3.1: Introduction.

In humans, functional Psn, the catalytic subunit of the γ-secretase complex, is a nine-pass transmembrane protein that contains an aspartyl protease domain. Psn is best characterized for its role in early onset familial forms of Alzheimer’s disease (FAD). Patients with FAD often have single gain-of-function mutations in Psn that are thought to process amyloid precursor protein (APP) in a manner that releases amyloid (Aβ) fragments into the extracellular matrix. These fragments, usually 40 and 42 amino acids in length, aggregate into the amyloid plaques that are often considered a hallmark of Alzheimer’s Disease (AD) (Tanzi & Bertram 2005).

Psn is known to cleave 55 substrates (Beel & Sanders 2008), the majority of which are single-pass Type-I Transmembrane Receptors that include Notch and APP (Parks & Curtis 2007). Prior to Psn processing, ligand binding usually activates a metalloprotease
that processes the extracellular domain (ECD) of the receptor. The truncated receptor then becomes a substrate for Psn, which processes it further to release the intracellular domain (ICD) from the membrane. The ICD normally translocates to the nucleus to co-activate gene transcription. Thus, this regulated intramembrane proteolysis (RIP) activity of Psn (Hass et al. 2009) couples external signals to changes in the transcription pattern of target genes.

Although extensive investigations have attempted to demonstrate the causative action of amyloid plaques in the manifestation of Alzheimer’s disease (AD), no clear mechanism has been demonstrated for how these plaques cause the overall neurological disease (reviewed in Shen & Kelleher 2007), (Wines-Samuelson & Shen 2005), and (Hass et al. 2009)). Thus, elucidating the signaling pathways controlled by Psn remains an important area of basic research that could impact future treatments of AD.

One interesting candidate gene that may be regulated through Psn processing encodes CBP [Cyclic-AMP-Response Element-Binding (CREB)-Binding Protein]. CBP is promiscuous and known to interact with over 300 different substrates, 200 of which are categorized as essential in the mouse genome (Kasper et al. 2006), (Barrios-Rodiles et al. 2005). It is a multi-functional protein that can co-activate transcription factors, bridge enhancer-binding complexes, and acetylate histones (reviewed in (Goodman & Smolik 2000)). Thus, it is often the limiting factor or signal integrator in many important pathways that control nervous system activities. Null mutations in CBP are homozygous lethal, but hypomorphs and haploinsufficiencies have been linked to human cognitive disorders including Huntington’s disease and Rubenstein Taybi disease, a developmental
defect characterized by reduced mental functions (McCampbell et al. 2000), (Rubinstein and Taybi 1963).

The association between Psn and CBP is unlikely to be direct, but mediated through one or more of the several Psn-processed substrates. Previously we have shown a requirement for Notch in the Long-Term-Memory (LTM) of flies (Presente et al. 2004), and we are testing a molecular model whereby Notch signaling acts as an intermediate between Psn and CBP. In this report we examine the conflicting relationship between Psn, Notch, and CBP using the conditional genetic reagents of the *Drosophila* model system (Saura et al. 2004), (Marambaud et al. 2003). Because CBP is needed for many higher order cognitive functions in flies and vertebrates, we are confident that important aspects of the signaling mechanism will be conserved between the two systems. However, although our data suggest that Psn is necessary for CBP expression and global chromatin acetylation in the adult fly brain, these activities are not dependent on a simple mechanism whereby Psn processes Notch into a transcriptional co-activator that regulates CBP expression.
3.2: Results.

3.2.a: Phenotypic-Based Analysis of Transgenic RNAi Lines.

Psn is emerging as an important signal integrator in which external cues are transmitted internally through signaling cascades that affect many cellular processes including changes in gene expression. Because Psn contains an aspartyl protease domain, its primary role in this process is thought to occur through proteolytic processing of Type I Transmembrane Receptors. Furthermore, the link between mutation in Psn and inherited forms of AD has placed a premium on trying to understand the intricate signaling network mediated by this complex. The powerful genetic tools unique to Drosophila are particularly valuable in trying to understand this signaling mechanism because the Gal4/UAS binary expression system (Brand & Perrimon 1993) can be used to over-express and/or silence virtually any gene in the organism in a temporally and spatially specific manner.

We began this analysis by first trying to understand what happens to flies when protein levels of Psn, Notch, and CBP are reduced in the adult nervous system. Our approach was to use an inducible-RNAi strategy that allows these genes to be fully functional during embryonic and larval development, but to silence them in selected adult tissues.

We have previously described the generation and characterization of several transgenic lines of flies that placed an inverted repeat of Notch sequence under control of GAL4 transcription factor binding sites (UAS elements). We produced 24 lines and we showed that all could selectively silence the Notch gene to some degree. However, we demonstrated that one line, UAS-Ni-14E (hereafter referred to as UAS-Ni) effectively
reduced the accumulation of Notch protein (as judged by Western analysis) and eliminated Notch signaling when expressed in target tissues (Presente et al. 2002). For example, Notch signaling has been shown by mutant analysis to be required for the proper specification of the wing margin (De Celis et al. 1996). Thus, when UAS-Ni is expressed in that tissue using the c96-Gal4 driver, the wing margin is dramatically scalloped and almost devoid of trichomes (Fig. 3.2B). Notch is also required for proper patterning of macrochete and microchete sensory bristles on the thorax (Heitzler & Simpson 1993), and when UAS-Ni is expressed in that tissue using a pnr-Gal4 driver, a disruption of bristle pattern and balding is apparent (Fig 3.2F). Finally, it has been demonstrated that Notch is needed for R8 photoreceptor specification in the developing eye disc (Baker & Yu 1998), and when it is silenced in developing eye disc with the GMR-Gal4 driver, the eye appears disorganized and roughened (Fig. 3.2J).

Because Psn is known to process Notch into a signaling molecule, we used the same logic to judge the effectiveness of twelve UAS-Psni lines generated in our lab or collected from the Drosophila community (Fig. 3.1). The most efficacious line produced wrinkles and notches in the wing blade (Fig. 3.2C), bristle fusion and elimination on the thorax (Fig. 3.2G), and roughening of the eye (Fig. 3.2K).

Finally, because we expected Psn, Notch, and CBP to be involved in a related molecular pathway (see below), we examined the phenotypes produced when CBP was silenced with RNAi. We obtained a UAS-CBPi line that was shown to be an effective reagent for silencing the endogenous CBP gene (Kumar et al. 2004). Interestingly, when we expressed the UAS-CBPi construct in the same three tissues using the same three drivers, we observed similar phenotypes to those seen when Notch and Psn were
silenced: wrinkles and notches in the wing blade (Fig. 3.2D), machrochete fusion (Fig. 3.2H, arrows and insert), and a roughened appearance of the eye Fig. 3.2L).
**Figure 3.1: Design of UAS-Psni.**

A 262-base sequence was amplified by polymerase chain reaction (PCR) from the ninth exon of *Psn* from genomic DNA. Two primer sets were used for a sense and antisense PCR product with the appropriate restriction enzyme sequences added. Both the sense and antisense products were directionally cloned into a pWIZ vector at the multiple cloning site (MCS). The finished vector was injected into fly embryos to be taken up into their own genomic DNA.

There are upstream activating sequences (UAS) located upstream of the MCS which allow for conditional expression in the presence of Gal4 protein. Upon expression the *Psni* transcript, which contains complimentary sequences, will flip back on itself and a double-stranded RNA fragment will result. This will prompt the RNA-interference cascade, which will ultimately limit the number of endogenous RNA transcripts for translation and subsequently reduce protein levels.
Figure 3.2: Developmental phenotypes of flies compromised for Notch, Psn or CBP using transgenic RNAi.

(A) represents a wildtype wing and (B-D) are examples of the UAS-RNAi lines crossed to c96-Gal4. Silencing either gene with this driver results in irregular or missing wing margins and wrinkling and notching of the wing blade. (E) is a wildtype thorax and (F-H) are representative samples of the UAS-RNAi lines crossed to pnr-Gal4. Silencing the genes with this driver leads in improper sensory organ development that gives rise to balding and/or supernumerary machrochete on the thorax and notum. (I) is a wildtype eye and (J-L) represent UAS-RNAi lines crossed to GMR-Gal4, which causes blistered and fused ommatidia, and with the Psn knockdowns in particular (J), sporadic elongated sensory hairs.
Figure 3.2
3.2.b: Presenilin Regulates CBP in a Notch-Independent Manner.

We and others have demonstrated that Notch is needed for long-term memory (LTM) in flies and mice (Presente et al. 2004), (Ge et al. 2004), and (Costa et al. 2003). Others have also shown that the CREB transcription factor is important in the same process in the same organisms (Bourtchuladze et al. 1994), (Mantamadiotis et al. 2002), and that a key co-activator of CREB-mediated transcription is CBP. CBP is also required for LTM (Lonze & Ginty 2002), and has been hypothesized to be a target gene for Notch signaling (Saura et al. 2004). Thus, we began testing a model in which the Psn-dependent release of the Notch ICD transcriptionally controls the expression level of CBP in the adult nervous system of flies.

To test this hypothesis, we first examined the genomic DNA surrounding the CBP transcription unit and its flanking regions for Su(H)/CBF1/RBP-Jκ (a transcription factor co-activated by Notch) binding sites. We identified four sites that matched the reported consensus sequences (Nellesen et al. 1999). These occurred upstream and downstream (within the first intron) relative to the start of transcription (Fig. 3.3), and the presence of these elements strengthened the model that CBP is transcriptionally regulated by the Notch ICD.

To test this possibility further, we analyzed CBP protein levels in adult flies in which Notch had been silenced with RNAi. The Western analysis in Fig. 3.4 shows that when Notch is compromised (hsGal4; UAS-Ni, + heat shocks), CBP protein levels are similar to those detected in the controls [(no Gal4; UAS-Ni; + heat shocks) and (hsGal4; UAS-Ni; no heat shocks)]. Similar levels of CBP protein were also observed when control and Ni adult brains were compared using immunohistochemistry (Fig. 3.5). We further analyzed
the effects of Notch on CBP expression using qRT-PCR to measure CBP RNA levels from animals in which we either silenced Notch (with UAS-Ni) or overexpressed it (with hsN+) (see supplemental data). The results indicated that there was not a significant difference in CBP transcripts when these samples were compared to normalized wildtype levels.

Taken together, these results suggest that the impaired LTM phenotype associated with Notch loss-of-function in the adult central nervous system cannot be easily explained by an appreciable effect on the expression of the CBP gene.
Figure 3.3: Putative Su(H) DNA binding sites in the CBP enhancer.

The CBP locus, including 2.5kb upstream of the transcription start site, reveals three occurrences of the putative Su(H) binding sites RTGRGAR and YGTGRGAA and one in the first intron. From the start of transcription, the upstream site locations are -388, -2064, and -2112, whereas the downstream site is at +353.
Figure 3.3
Figure 3.4: Notch is not required for CBP expression.

This image represents a Western analysis of Notch and CBP protein levels under conditions in which Notch is silenced with RNAi by applying multiple heat shocks (see materials and methods). Two blots were made from each protein sample. One blot was incubated with Notch antibody (top row) and the other was incubated with CBP antibody (3rd row). Both blots were cut and incubated separately with tubulin antibody to serve as a control for protein loading and transfer (2nd and 4th rows).
Figure 3.4

Heat Shocks: - - + +
hsGal4; UAS-Ni No Gal4; UAS-Ni hsGal4; UAS-Ni

Proteins:
- Notch
- Tubulin
- CBP
- Tubulin
Figure 3.5: Notch dysfunction brains.

Both (A and B) are confocal images of dissected brains from control (no Gal4, UAS-Ni) and Notch-silenced experimental animals (hsGal4; UAS-Ni). Both groups were heat-shocked using the same regimen as B, and both were stained with CBP antibody and imaged using the same conditions and microscope settings.
Figure 3.5
3.2.c: Presenilin is Necessary for CBP Expression.

Although we failed to establish a link between Notch and the expression of CBP, there are reports in the literature describing a connection between Psn and CBP (Saura et al. 2004), (Marambaud et al. 2003). Thus, we used this transgenic RNAi strategy to ascertain whether CBP would be affected when Psn was silenced in adult flies.

As shown in the Western-blot analysis of Fig. 3.6, there is a ~90% reduction in CBP protein in the Psn-reduced lane (hsGal4; UAS-Psni; + heat shocks) compared to the control (no Gal4; UAS-Psni; + heat shocks). In addition, a 20-fold increase in CBP transcript was observed by qRT-PCR when a wildtype construct of Psn was overexpressed with a heat shock driver (see supplemental data). Finally, in order to investigate whether Psn affects CBP accumulation in specific regions of the adult central nervous system, we performed antibody staining on dissected brains. Shown are representative examples of confocal Z-stack images of whole mounts from control [no Gal4; UAS-Psni; + heat shocks (Fig. 3.7B) and experimental [hsGal4; UAS-Psni; + heat shocks (Fig. 3.7A)] groups. Clearly, there is a dramatic reduction in the amount of CBP protein detected when Psn is silenced. These data suggest that CBP is regulated by Psn in Drosophila, and given that Psn functions as membrane-bound catalytic subunit of the g-secretase, the mechanism probably requires intermediate signaling molecules.
Figure 3.6: Psn is necessary for maintaining proper CBP protein levels in the adult Drosophila CNS.

(A) shows a Western analysis from fly heads in which CBP protein levels are reduced >90% (hsGal4; UAS-Psn; + heat shocks) compared to the controls (heat shocked animals without a driver or an RNAi responder) using Tubulin as an internal standard for protein loading and transfer.
Figure 3.6

Heat Shock

CBP

α-Tubulin
Figure 3.7: Dissected adult brains stained for CBP in a Psn-reduced background.

The following images are dissected brains stained with anti-CBP: Figure (A) *hsGal4; UAS-Psn*, and (B) *no Gal4; UAS-Psn* brain. Both groups were given the heat shock treatment.
Figure 3.7
3.2.d: Global Acetylation Levels of H4 are Reduced in the Brain when the HAT Domain of CBP is Disrupted.

Because the previous data suggest that Psn is required for CBP expression, we expect that phenotypes associated with mutations in Psn should overlap with mutations in CBP. Thus, one function of CBP that is critical to an active cell is the histone acetyl-transferase (HAT) function that modifies nucleosomes, presumably so that they can be more easily displaced by the transcription machinery. Therefore the HAT activity of CBP could influence the acetylation of a few nucleosomes at key gene targets such as those that contain CRE elements for CREB-regulated transcription (Vecsey et al. 2007), or it might have global effects on chromatin structure throughout the genome (Cheng et al. 2008).

To distinguish between these two possibilities, we used a dominant-negative variant of CBP in which the HAT domain is disrupted (UAS-CBP-FLAD) (see (Ludlam et al. 2002) for a complete description). When expressed in developing tissues, UAS-CBP-FLAD produces a roughened eye (Fig. 3.8A) and a notched wing (Fig. 3.8B, arrowhead), similar to the phenotypes observed when UAS-CBPi is expressed in the same tissues (Fig. 3.2D,L). We next compared whole-mount brains of control and UAS-CBP-FLAD adults for global acetylation levels by staining them with a well-characterized antibody that specifically recognizes acetylated Lysines (at position 8) of Histone 4 (AC-H4-K8). As shown in Figure 3.9, a dramatic reduction in AC-H4-K8 staining was observed when control brains (Fig. 3.9A) were compared to those expressing the UAS-CBP-FLAD construct (Fig. 3.9B).
Figure 3.8: Expression of UAS-CBP-FLAD in developing tissues results in a consistent phenotype.

(B) depicts a typical roughened eye phenotype produced with $UAS$-$CBP$-$FLAD$ is expressed with the $GMR$-$Gal4$ driver during development. (D) displays a wing phenotype with a prominent notch (arrowhead) that is typically observed with $UAS$-$CBP$-$FLAD$ is expressed with the $c96$-$Gal4$ driver (A and C are WT). Both phenotypes are similar to those detected with $UAS$-$CBP_i$ is expressed with the same drivers (Fig. 3.2).
Figure 3.8
Figure 3.9: The HAT domain of CBP is required for acetylation of histone 4 at Lysine 8 in the adult *Drosophila* CNS.

(A) is a control brain (no *Gal4; UAS-CBP-FLAD*; + heat shocks) stained for acetylated Histone 4, and (B) is an experimental brain (*hsGal4; UAS-CBP-FLAD*; + heat shocks) stained with the same antibody under the same conditions. Note the dramatic reduction in staining intensity when the HAT-defective form of CBP is expressed.
Figure 3.9
3.2.e: Presenilin-Compromised Brains Also Exhibit Reductions in Global Acetylation.

To investigate whether Psn affects the overall levels of acetylation of H4 in the adult brain (as expected if Psn affects CBP function), we compared the staining patterns of dissected brains in which CBP (Fig. 3.10A-C), Psn (Fig. 3.10D-F), and Notch (Fig. 3.10G-I) had been silenced with RNAi. For each gene, two control groups were analyzed [(hsGal4; UAS-RNAi; no heat shocks) (Fig. 3.10A, D, G)] and [(no Driver; UAS-RNAi; + heat shocks) (Fig. 3.10B, E, H)]. It is clear from the data presented that H4 acetylation levels are drastically reduced in brains expressing UAS-CBP (Fig. 3.10C), moderately reduced in brains expressing UAS-Psn (Fig. 3.10D), and unaffected in brains expressing UAS-N (Fig. 3.10I).

These results demonstrate that CBP is needed for global acetylation in the adult brain, and they are consistent with our model that Psn controls the expression of CBP. They also suggest, once again, that Notch has little or no effect on the function of CBP.
Figure 3.10: Psn affects global acetylation levels in the adult brain.

The above images are whole-mount adult fly brains stained for AC-H4-K8 to assay global acetylation levels. (A,D,G) represent one control group (hsGal4; UAS-RNAi; no heat shocks), (B,E,H) depict a second control group (no Gal4; UAS-RNAi; + heat shocks), and (C,F,I) represent the experimental group (hsGal4; UAS-RNAi; + heat shocks). Note that when CBP is silenced there is a dramatic effect on global acetylation levels (compare C with the A and B controls); when Psn is silenced there is a significant but less dramatic effect (F); and when Notch is silenced (I) there is not noticeable effect. All brains were dissected, stained, and photographed using the same conditions and microscope settings.
Figure 3.10
3.2.f: CBP-Compromised Flies Exhibit an Altered Geotactic Response.

Having demonstrated that CBP is needed for global levels of acetylation in the adult brain, we next wanted to investigate what effect, if any, these reductions had on the biology of the fly. In outward appearance, *Drosophila* compromised for CBP functions post development, appear normal. Both males and females are fertile and their lifespan is not dramatically affected. Thus in an effort to ascertain if they display any subtle behavioral phenotypes that might underlie neurological defects, we tested them for a normal geotactic response.

Some of the earliest discoveries that revealed a genetic basis for complex behavior were made by analyzing geotaxis—the movement of an organism in response to gravity (Benzer 1967), (Erlenmeyer-Kimling & Hirsch 1961). *Drosophila* exhibit strong negative geotaxis behavior because when placed in a tube, they prefer to climb toward the top. In addition, mutant studies have identified several genes that are required for this complex behavior (Toma et al. 2002).

Thus, to investigate whether CBP-compromised flies display an altered geotaxis, we built a countercurrent apparatus using specifications originally described by Benzer (1967). This apparatus monitors progressive geotactic performance as described in the Materials and Methods chapter. The number of heat shock treatments needed to effectively reduce CBP protein was determined by Western blot analysis shown in Figure 3.12. The following control groups exhibit normal CBP protein levels: (*hsGal4; UAS-CBPi* no heat shocks), and (*no Gal4; UAS-CBPi* with 7 heat shocks). The first experimental group (*hsGal4; UAS-CBPi* with 5 heat shocks) shows no quantifiable reduction in CBP protein compared to the controls, whereas the experimental group
(hsGal4; UAS-CBPi with 7 heat shocks) exhibited a ~75% reduction in CBP protein.

Based on these results we used 7 heat shock treatments to reduce CBP levels for the data presented in Figure 13 and 14. Based on these western blot results CBP protein reduction, via RANi, is not progressive over the course of the heat shock regimen. The short time where CBP is reduced occurs around the 7th heat shock, which may only be a 12-24 hour period. Because of this short time, it is not expected that any observed behavioral phenotypes acquired through reduced CBP, are age-dependent, nor is neurodegeneration anticipated.

The mean tube distribution for four different cohorts of flies — three controls (without driver, without responder, without heat shocks) and one experimental—are presented by a best-fit line in Figure 3.13. Clearly most of the flies in the experimental group (hsGal4; UAS-CBPi; +heat shocks) were impaired in their ability to progress beyond the first tube. In addition, an ANOVA analysis of the data indicates that the performance of the experimental group is significantly impaired ($F_{(3,53)}=21.02; P<0.0001$) compared to the performance of each control group (Fig. 14). These results suggest that CBP is required in the adult animal for the maintenance of some normal behaviors, and the fact that CBP compromised flies display this behavior phenotype may be indicative of some time of neurological defect associated with its dysfunction.
Figure 3.11: Assaying geotaxis using a countercurrent device.

Pictured is the countercurrent device we built for assaying geotaxis behavior. This apparatus was modeled directly from a similar design created by Seymour Benzer (1967). Each experiment starts as flies are placed in tube set 1 and given 3 sharp taps on a rubber mat to knock them to the bottom. Flies are then given 10 seconds to climb into the upper tube, after which time it is moved over to the bottom of set 2. They are again tapped to the bottom, allowed to climb for 10 seconds, and moved to the bottom of set 3. The procedure continues until set 6 is reached. At the end of the experiment, the percentage of the fly cohort in each tube is calculated. The experiment is calibrated in such a way that most of the wildtype flies (exhibiting normal negative geotaxis) accumulate in tube number six.
Figure 3.11
Figure 3.12: Western analysis determines the number of heat shocks required to effectively reduce CBP through RNA interference.

The following western blot reveals that 7 heat shocks were needed to reduce CBP protein levels effectively. For the heat shock regimen see the Material and Methods chapter. The first two lanes and the last lane are control lanes \((hsGal4; UAS-CBPi \text{ no hs})\), \((no \text{ Gal4}; UAS-CBPi [7] \text{ hs})\), and \((hs-CBP [2] \text{ hs})\). The second and third lanes are experimental \((hsGal4; UAS-CBPi [5] \text{ hs})\) and \((hsGal4; UAS-CBPi [7] \text{ hs})\). Alpha tubulin was used as a loading control. The histogram shows a \(~70\%\) reduction in CBP protein levels compared to the non-heat shock control group in the first lane.
Figure 3.12
Figure 3.13: Flies compromised for CBP exhibit poor geotaxis.

The following graph represents the mean distribution of flies within each tube after 5 successive trials. Tube distribution for each group is presented by a best-fit line, which is based on a linear regression. The slope of the experimental group (hs-Gal4; UAS-CBPi) indicates that the majority of these cohorts remained in the first sets of tubes, whereas the control group accumulated in the last sets of tubes.
Figure 3.13
Figure 3.14: Geotaxis performance scores.

Mean performance scores for each group are shown (see Material and Methods chapter for a description). This graph represents the mean score for each group with a significant difference between the experimental group (hsGal4; UAS-CBPi; +heat shocks) and all three control groups ($F_{(3,53)}=11.8; P<0.0001$). There were no significant differences between genders or control groups. A (+) indicates a heat shock treatment and (-) indicate no heat shock.
Figure 3.14

Mean Score

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Gal4 UAS-CBPi +hs</td>
<td>0.55</td>
</tr>
<tr>
<td>hsGal4 No UAS +hs</td>
<td>0.60</td>
</tr>
<tr>
<td>CBP1 hsGal4 -hs</td>
<td>0.80</td>
</tr>
<tr>
<td>CBP1 hsGal4 +hs</td>
<td>0.30</td>
</tr>
</tbody>
</table>

**Note:** The bar with the label “CBP1 hsGal4 +hs” is significantly different from the others (***p < 0.001**).
3.3: Discussion.

Based on the data presented in this report, we have provided clear evidence to support the conclusion that CBP is required to maintain global levels of Histone H4 acetylation in the adult *Drosophila* CNS. We base our conclusions on the powerful combination of using both loss-of-function (expression of RNAi against CBP) and dominant-negative (expression of CBP-FLAD) genetic reagents (Fig. 3.6; Fig. 3.9). We also show that when CBP expression is uncoupled from development and conditionally silenced only in adults, we detect subtle changes in the behavior of the fly than can be easily quantified in a geotaxis assay (Fig. 3.14). Because CBP is a multifunctional protein that co-activates so many genes and because CBP deficiencies have drastic developmental effects, our results are surprising in the sense that the phenotype described in these adult flies is not more severe. There are at least two possible explanations for this observation.

The first concerns the limitations of the inducible RNAi strategy, and the fact that after multiple rounds of heat shocks *CBP* is not completely silenced (Fig. 3.12). Thus, the remaining low levels of CBP may supply enough vital functions for the fly. The second possibility speaks to the function of CBP in acetylating histones, and the fact that we monitored the K8 residue on Histone 4. In this model, the adult nervous system is supplied with wildtype levels of CBP during development, but as levels drop during our experimental manipulations of aging adults, histone modifications required for more open chromatin configurations are slowly being eroded. Thus, subtle nervous system activities that control behavioral functions, such as negative geotaxis, would likely be affected first, followed by more vital functions. Based on our observations with *Notch* loss-of-function manipulations in adults (Presente et al. 2004), we favor the later explanation, and we are
currently testing this model further by examining more complex behaviors such as learning and memory under conditions in which Psn and CBP are compromised in aging animals. Finally if this model is correct, understanding the role of CBP (and molecules that affect its activity such as Psn) in an aging nervous system, might have important implications for the study of human neurodegenerative diseases. For example, subtle genetic mutations in CBP may be amplified if a person has a specific environmental exposure to an agent that modifies chromatin structure.

A second significant conclusion from this report is that we clearly established relationship between Psn and CBP protein accumulation and function in the fly. We show that when Psn is silenced with RNAi, we see dramatically reduced levels of CBP Protein (Fig. 3.6) and similar effects on global acetylation levels of K8 on Histone 4 (Fig. 3.9). In addition, our results are interesting because reports examining the link between Psn and CBP in mice are contradictory. One group reported that the Psn-processed N-cadherin ICD targets CBP for degradation, and that PS1 FAD-linked mutant mouse embryonic fibroblasts showed increased levels of CBP protein (Marambaud et al. 2003). However, another reported that conditional knockdowns of both Psn homologues (PS1/PS2) showed a reduction in CBP protein and mRNA levels in the mouse hippocampus (Saura et al. 2004). Perhaps the fly system can be used in combination with the mouse system to clear up the discrepancy and/or speak to the divergent pathways that can explain these results in vertebrates. In either case the relationship between Psn activity and CBP expression is probably not direct given our understanding of Psn as a transmembrane protein that processes type I receptors.
We and others have clearly demonstrated the when Notch is compromised in the nervous system of adult animals (flies or mice) LTM is specifically impaired (Presente et al. 2004), (Ge et al. 2004), and (Costa et al. 2003). Thus, we began this analysis with the expectation that Notch, Psn, and CBP would be linked in a common pathway in which a Psn-processed form of Notch would co-activate target genes that regulated global acetylation levels. The model was attractive because it had the potential to connect a molecular “memory” of histone acetylation and placement with an organic explanation of how an organism’s long-term memory is either established or maintained. We were encouraged to pursue this model when we were able to identify four putative Su(H) binding sites within 2.1 kB of the start of transcription of CBP (Fig. 3.3), but we were unable to show that compromising Notch had any effect on CBP RNA levels, CBP protein levels, or K8 Histone 4 acetylation (Fig. 3.4, Fig. 3.101). Also, mouse brains compromised for Psn show reduced CREB activity, where early hypotheses suggested that Psn influences CRE-regulated genes through Notch signaling-induced CBP coactivation (Saura et al. 2004). However, a recent report shows that Notch signaling does not directly affect CREB activity (Watanabe et al. 2009). Nevertheless it still remains a formal possibility that in key neurons of the fly brain that control LTM, a large reduction in Notch could have a subtle reduction in CBP, and that this subtle reduction is responsible for the LTM phenotype that is observed.

We are still unsure how Psn and CBP are connected at the molecular level. One possibility is that Psn processing of another Type I receptor leads to a transcriptional control of CBP. We attempted to address this possibility by collecting and testing several Type I receptor lines from the Vienna Drosophila RNAi Center in which an inverted
repeat of sequence unique for each of 17 Type I receptors was cloned under UAS control. Unfortunately we were unable to detect any effects on CBP mRNA levels as judged by qRT-PCR (table 2). A second possibility is that the activity of some of the protein targets that interact non-catalytically with Psn may become altered when Psn is reduced, and their dysfunction might affect CBP expression or stability. However, a third possibility exists that Psn regulates CBP through a mechanism that is currently unknown.

Regardless of mechanism, our data clearly link Psn and CBP in a pathway that is required for normal acetylation levels and for normal behavioral activities. In the vertebrate literature, Psn is linked to AD, and CBP is associated with specific mental disorders including Rubenstein-Taybi and Huntington’s disease.

Although there has been an extensive investigation into the role of Aβ42 in AD, there remain many unanswered questions. The biggest hurdle associated with the “Amyloid hypothesis” is that Aβ plaques have never been correlated directly to the neurodegeneration associated with AD. These gaps in Aβ research have given rise to alternative hypotheses, the most notable being the “Presenilin hypothesis” (reviewed by (Shen & Kelleher 2007)). This model suggests that the mutated forms of Psn associated with AD not only mis elevate APP, but also mis elevate other substrates that result in a loss of signal. Such signal disruptions have been reported with Ncadherin, Notch, and NMDAR activity, where any one of these (or their combinations) could reduce downstream target expression manifesting the symptoms of AD (Marambaud et al. 2003), (Schroeter et al. 2003), (Moehlmann et al. 2002), and (Saura et al. 2004). Therefore, it is possible that Aβ plaque formation is more of a terminal phenotype with regards to the age of the disease, and that the onset of pathology occurs much earlier through subtle
losses in these signaling pathways. One such model for this is presented in this report where reduced Psn leads to CBP reduction and dysfunction resulting in decreased global histone acetylation.

The rapidly increasing list of Psn substrates and associating proteins not only suggests a broader role for Psn, but also reveals new therapeutic targets in AD research. The use of HDAC inhibitors may prove to be useful as a treatment designed to arrest the neurodegeneration associated with AD, but the cause for these certain forms of AD may reside in the increasingly complex networks integrated through Psn, and therefore more research is needed to elucidate their functions.

3.5: Supplemental data not submitted for publication.

Presenilin (Psn) is the catalytic subunit of the γ-secretase complex located in the plasma transmembrane. Recent focus has been directed towards this protein because certain mutated forms have been implicated in the inherited form of Alzheimer’s disease (AD). Psn processes about 30 known substrates, usually type I transmembrane receptor, where the freed intracellular domain (ICD) translocates to the nucleus to affect gene transcription. There have been several reports revealing that these mutated forms of Psn result with incorrect substrate processing that could disrupt receptor signaling or produce harmful byproducts. The most notorious example is the Aβ fragment, which is released through improper processing of Amyloid Precursor Protein (APP). These fragments aggregate and forms plaques on AD-afflicted brains. In an attempt to uncover possible downstream genes that may be affected by this disruption in receptor signaling we found that the level of a multifunctional protein called CREB Binding Protein (CBP) is severely reduced in a Psn-silenced background. This is interesting because certain mammalian
systems with reduced CBP show cognitive problems including mental retardation in humans. Therefore, it is possible that a reduction in CBP could be a contributing factor in AD. It is probable that Psn does not directly influence CBP levels, but through an intermediate signaling molecule, most likely one of its processed substrates. Identifying this intermediate molecule is important because it could ultimately be a therapeutic target in AD treatment. To that purpose, we have performed an RNAi-based screen designed to reveal a pool of possible intermediate signaling molecules that may ultimately influence CBP levels.

This screen consists of 18 RNAi lines that target the transcripts of Psn substrates and 3 additional lines that target the Psn non-catalytic associating protein transcripts. The goal of the screen was to reveal the possible intermediate signaling molecule(s) that may be in the same pathway as Psn and CBP. Each RNAi line was crossed to a wing, eye, and thorax Gal4 driver (c96-Gal4, GMR-Gal4, pnr-Gal4), which are the same used in an earlier screen (see chapter 3). The progeny from this cross were then scored on the severity of the RNAi-induced phenotype (Table 1). qRT-PCR analysis was used to monitor the transcription of CBP mRNA relative to a control for four of these RNAi lines (Table 2).
Table S.1

<table>
<thead>
<tr>
<th>Presenilin Substrates</th>
<th>c96-Gal4</th>
<th>GMR-Gal4</th>
<th>pnr-Gal4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian Name</td>
<td>Drosophila Name</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoE R2</td>
<td>LpR1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DCC</td>
<td>Sidekick</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DCC</td>
<td>Frazzled</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ephrin</td>
<td>D-Ephrin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Black Cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dachsous</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>App</td>
<td>App1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nectin 1a</td>
<td>Kirre</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LRP</td>
<td>Arrow</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>γ-Protocadherin</td>
<td>Cad99C</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CSF1</td>
<td>PvR</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CD44</td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Cadherin N</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Jagged</td>
<td>Serrate</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Delta</td>
<td>Delta</td>
<td>0</td>
<td>Lethal</td>
</tr>
<tr>
<td>GHR</td>
<td>Sallimus</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Shotgun</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>P75</td>
<td>Srp54</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Presenilin Associating Proteins</th>
<th>c96-Gal4</th>
<th>GMR-Gal4</th>
<th>pnr-Gal4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian Name</td>
<td>Drosophila Name</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gsk3B</td>
<td>Shaggy</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>NMDA-R</td>
<td>NMDA-R</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>B-catenin</td>
<td>Armadillo</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

*The numerical scores used in this table are subjective and describe phenotypic severity where the least severe is 0 and the most severe is 5.*
Figure S.1: Phenotypic Examples Derived from the RNAi Screen
<table>
<thead>
<tr>
<th>Genotype</th>
<th>qRT-PCR Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>1.0</td>
</tr>
<tr>
<td>hs-gal4/+</td>
<td>2.1</td>
</tr>
<tr>
<td>UAS-Ni/hs-gal4</td>
<td>1.0</td>
</tr>
<tr>
<td>hs-N</td>
<td>1.9</td>
</tr>
<tr>
<td>hs-Gal4/UAS-Ncadi</td>
<td>1.7</td>
</tr>
<tr>
<td>hs-Gal4/UAS-Ecadi</td>
<td>1.7</td>
</tr>
<tr>
<td>hs-Gal4/UAS-Armi</td>
<td>0.5</td>
</tr>
<tr>
<td>hs-Gal4/UAS-Arm+</td>
<td>2.2</td>
</tr>
<tr>
<td>hs-Gal4/UAS-NMDARi</td>
<td>1.7</td>
</tr>
<tr>
<td>UAS-Psn-8309/hs-gal4</td>
<td>23.8</td>
</tr>
<tr>
<td>hs-CBP</td>
<td>69.3</td>
</tr>
</tbody>
</table>

*The above table represents qRT-PCR results representing the relative quantity of CBP mRNA transcribed by each genotype. The number corresponding to each genotype was normalized to wildtype CBP transcription.*
Figure S.2: Phenotypic Screen for the most effective RNAi lines.

The following images are the results of a screen used to determine the most effective RNAi line based on the severity of three developmental phenotypes in the eye, thorax, and wing. The same Gal4 drivers were used as in Supplemental Data A. Transgenic RNAi lines that target specific genes usually vary in their effectiveness at silencing its targeted gene. This is usually due to where the transgenic element inserts into the genome i.e., high expressing regions vs. low expressing regions, and the mRNA sequence targeted by the RNAi molecule. Therefore it is common to analyze multiple lines using a screen similar to this one, where not only can an observable phenotype be easily detected, but also variances in that phenotype can be used to gauge each line within its respective group.
CHAPTER 4

NOTCH DYSFUNCTION RESULTS IN REDUCED NEURITE OUTGROWTH IN CULTURED PRIMARY NEURONS

The following chapter is part of a manuscript being prepared for publication.

My contribution to this work is as follows: I made all the genetic crosses, cultured the primary neurons, measured the neurites, and performed all of the statistical analyses. Our collaborators Linda Restifo and Robert Kraft provided us with the use of reagents and the equipment used to grow the neurons.

4.1: Introduction.

Subtle perturbations in the complex network of neurons and their ultrastructure are oftentimes difficult to detect (Kraft et al. 2006). This is especially true at the level of the synapse, where function relies in part on morphological design (Rohrbough et al. 2003). The plasticity at the synapse does not remain static, and external cues can direct the dynamic changes necessary for learning, memory, and the maintenance of a properly functioning nervous system (Dubnau et al. 2003). These networks rely on several intricate signaling pathways where their loss of signal through gene mutation or dysfunction is seemingly more difficult to identify because they regularly do not leave a footprint as do the majority of gain of function mutations, which may appear obvious, i.e. Aβ plaques on Alzheimer diseased brains (Kraft et al. 2006)(Shen & Kelleher 2007). Therefore it is sometimes necessary to induce specific gene dysfunction in vitro, thereby isolating these neurons from their normal highly supportive social environment, in an attempt to tease out these subtle phenotypes that would normally go unnoticed.
Our lab has previously shown that when Notch is conditionally silenced in adult *Drosophila* they display long-term memory impairments (Presente et al. 2004). However, we observed no discernable physiological phenotype when we analyzed these Notch-deficient flies for brain morphology and bouton number of innervated larval body wall muscles. Therefore we required a more powerful assay in order to test the model whereby the Notch-induced behavioral impairments can be connected to a gross physiological phenotype.

The technique of culturing primary neurons has proven highly effective at identifying previously unseen phenotypes for several different model systems, which have provided for new insights into possible mechanisms for neurological disease pathologies (Kraft et al. 1998)(Romijn et al. 1981).

To that end we have analyzed primary cultured neurons that are silenced for *Notch* in specific lobes of an established larval central nervous system. These neurons do exhibit severely reduced neurite outgrowth and showed significant differences compared to the control, when examined for four specific parameters using the semi-automated statistical software called Neuronmetrics™ (Narro et al. 2007).
4.2: Results.

In an attempt to link LTM impairments derived from conditionally Notch-silenced *Drosophila* to an observable structural phenotype in the central nervous system, we employed the well-established technique for culturing the primary neurons of wandering third instar larvae. This is a method for revealing phenotypes that normally are concealed by the complexities of an intricate nervous system (Kraft et al. 2006).

The larval CNS is removed through dissection and the cells are dissociated and cultured in media for three days. The transgenic neurons are identified through Gal4-directed GFP expression in a field of non-GFP-expressing neurons (Fig. 4.1 B). Roughly 60 images of each group are taken and analyzed by Neuronmetrics software for four categories (Fig. 4.1 A).

Our goal was to determine if induced Notch dysfunction would result in quantifiable neurite differences among control (247-Gal4; UAS-GFP) and experimental (247-GFP; UAS-Ni; UAS-GFP) groups. As presented in figure 4.2 the example of a Notch-reduced neuron (bottom image) shows an obvious reduction in all for analyzed categories including: number of primary neurons, branchiness, neurite length, and total area covered, when compared to the extensive arborization of the control neuron (top image).

The images for each group were quantified by Neuronmetrics (Fig. 4.3). Three of the four categories exhibited highly significant differences (p<0.0001). The category for the number of primary extensions was slightly less significant (p<0.024) based on a Mann-Whitney rank sum test. Therefore based on these results, Notch is required for proper neurite regrowth, which may result in subtler phenotypes at the synapse.
Figure 4.1: Example of Neuron Analysis Using Neuronmetrics™.

Neuronmetrics™ is a semi-automated computer program that is used to measure neurites (Fig. A) in the following categories: 1) number of primary processes (neurites that directly extend from the cell body), 2) branchiness, 3) total neurite length, and 4) territory occupied by the neuron (area calculated by surrounding the neuron with a best-fit polygon).

Figure B is the mushroom body from a third instar larvae (247-gal4; UAS-GFP), identified with green fluorescent protein (GFP).
Figure 4.1
Figure 4.2:  Notch-Reduced Neurons Exhibit Reduced Neurite Outgrowth.

Figures A and B are example images of neurons from the control group 247-gal4; UAS-GFP and the Notch-reduced group 247-gal4; UAS-Ni; UAS-GFP respectively. Notice the size and complex arborization in the control group (A) compared to the “stunted” linear arrangement in the Notch-reduced group (B).
Figure 4.2

247-Gal4,
UAS-GFP

247-Gal4,
UAS-GFP;
UAS-Ni
Figure 4.3: Statistical Analysis Quantifies Notch-Reduced Phenotype.

A Mann-Whitney rank sum test was used to determine any statistical differences, where the range of the box is from the 25th percentile to the 75th percentile, the arrowhead indicates the median, and the bars indicate the 10th and 90th percentile. Sixty neurons were measured in each group and identified in culture as mushroom body neurons by GFP expression. A gray box represents data for W1118; 247-gal4; UAS-GFP neurons, and 247-gal4; UAS-Ni; UAS-GFP neuron data are represented by the red box.

The number of primary processes is significantly reduced in the Ni group (p=0.0024). More compelling significant differences, between Ni and the control, were seen in the remaining three measurements including, branchiness, total neurite length, and territory occupied by neuron, where the p-value for these groups was P<0.0001.
Figure 4.3
4.3: Discussion.

Notch is very well characterized for its role in binary cell fate decisions during nervous system development. Although Notch is highly expressed during this developmental period, observations in Drosophila, mouse, and human systems show that these levels drop considerably in postmitotic adults where Notch is expressed at low levels (Presente et al. 2002). Because Notch is present in adults and the apparent LTM impairments associated with Notch dysfunction suggest a role for Notch beyond development.

Interestingly, there are reports that show transfection of Notch 1 in mouse hippocampal primary neurons results in reduced neurite outgrowth, which suggests an inhibitory role for Notch in neurites (Berezovska, P. McLean et al. 1999a)(Berezovska, Frosch et al. 1999b). These results are strikingly similar to ours when we silence Notch in larval primary MB neurons. There are several possibilities for these contradictory results. The first could simply be subtle differences between these model organisms. The second however, could be that the mouse primary neurons used were embryonic. Although these neurons are post mitotic, the organism is still in the womb and at the very early stages of development, whereas the larval MB neurons used in this report derived from a fully intact CNS, and furthermore, larvae directly interact with their environment and are maternally independent. Although these larvae will eventually undergo metamorphosis, the MB lobes that house these neurons either remain consistent through metamorphosis, or are remodeled for higher order structures. Finally, a third possibility is that Notch within specific contexts, may have varying functions that could provide for these conflicting results.
Several reports have shown that memory is impaired in the absence of Notch (Presente et al. 2004)(Costa et al. 2003)(Ge et al. 2004). One report however, tested the cognitive effects of overexpression of Notch adult *Drosophila* and found that LTM was enhanced (Ge et al. 2004). This is consistent with our results which suggest that the requirement for Notch in the post-mitotic CNS is not inhibitory, but necessary for maintaining a properly functioning nervous system.

Notch loss of signal has implications in disease (see Introduction chapter). Losses in Notch signaling have been reported in those afflicted with the familial form of Alzheimer’s disease (Selkoe 2001). The majority of these cases are thought to be caused by mutations in the Presenilin gene (*Psn*). *Psn* is intramembraneous and thought to process Notch in a manner that frees its signaling domain. It is possible that these mutated forms of *Psn* are not processing Notch efficiently resulting in both loss of signal and target gene expression. Based on our data and others, this could lead to memory impairment, neuronal dysfunction, and even neurodegeneration, which are commonly observed in AD patients. Therefore if reductions in Notch contribute to AD manifestation, perhaps this could lead to new therapeutic targets designed to arrest AD pathology.
CHAPTER 5

CONCLUDING REMARKS

This study demonstrates that CBP is a downstream target of Psn in the adult Drosophila CNS. The cell and molecular data presented here clearly show that diminished CBP levels and function occur in the absence of Psn. These results should help clarify the controversial reports that discuss the interactions of these two proteins. Additionally, these are the first data that suggest Notch does not regulate CBP expression even though the mouse and Drosophia CBP enhancer contain putative Su(H)/CBF1/RBP-Jκ binding sequences.

Although these data suggest that Notch does not influence CBP expression, the methods that we used to identify this putative association were more of a global approach, i.e., Western blot using whole fly heads, or CBP antibody staining in the entire CNS. It is possible that Notch does affect CBP expression in a subset of neurons that would go undetected using our techniques. The mechanism for which Notch is required in memory is still not known, therefore further analysis may be required in, perhaps, more specific nervous system tissue to completely rule out an association between Notch and CBP expression.

The memory and neurite phenotypes derived from Notch dysfunction are comparable to the cognitive and neurodegenerative conditions associated with AD, which suggests that Notch dysfunction could possibly contribute to the pathology of this disease. Although Notch signaling does not fit into our Psn/CBP model, the possibility that it could be a causative agent in AD manifestation, via an alternative or redundant pathway, should not be overlooked.
The association between Psn and CBP is probably not direct, but through an intermediate signaling molecule most likely mediated through Psn processing. The identification of this intermediate is important because it could be an effective therapeutic target. Its detection however, may prove difficult based on the rapidly accumulating number of Psn substrates and associating proteins. The RNAi screen presented in the supplemental data in chapter 3 focused on 17 Psn substrates and 3 association proteins. Although a few of these RNAi lines produced phenotypes consistent with those observed in Psn and CBP-silenced developmental tissues, qRT-PCR data demonstrated that when these genes are silenced, CBP transcription is relatively unaffected. This suggests a few things; the first is that we could simply have not used the correct RNAi line yet, but could with further investigation; alternatively, the substrate may not have been discovered yet; and finally the link between Psn and CBP may be a network of several of these signaling molecules. Therefore, it could take a while before the components in this pathway are identified.

We have shown that a complex behavior (geotaxis) is impaired when CBP is conditionally reduced in adult flies. Other studies using the mouse system have shown that induced CBP dysfunction in mice resulted in not only LTM, but also a STM memory impairment (Lonze & Ginty 2002)(personal communication with Jie Shen). It would be interesting to see if Drosophila also exhibit both a short and long-term memory phenotype. The effects of conditionally-silenced Psn in adult Drosophila should also be analyzed for both of these phases of memory. If flies dysfunctional for Psn exhibit memory impairments, an attempt to rescue this phenotype should be made by ectopically expressing CBP.
Finally, we have shown that both \textit{Psn} and \textit{CBP}-silenced flies have global reductions in histone acetylation. This type of chromatin remodeling is normally associated with gene activation. Reductions in acetylation could result in the silencing of genes required for maintaining a properly functioning nervous system. This could be mechanism for memory impairments associated with the knockdown of these genes in mice and a possible contributing factor to FAD that directly results from mutant forms of \textit{Psn}. If a link between AD and histone acetylation can be demonstrated, the use of HDAC inhibitors may prove useful in ameliorating the effects of this disease.


Kraft, R. et al., 2006. Phenotypes of Drosophila brain neurons in primary culture reveal a role for fascin in neurite shape and trajectory. *Journal of Neuroscience*, 26(34),


Vita
Graduate College
University of Nevada, Las Vegas
Randy S. Boyles

Degrees:
  Bachelor of Science, Biological Sciences 2004
  University of Nevada, Las Vegas

Publications:
  Notch is required for long-term Memory in Drosophila. 2004 Presente, A. et al.
  PNAS: 101(6), 1764.

Dissertation Title:
  Presenilin is Necessary for the Function of CBP in the Adult *Drosophila* CNS.

Dissertation Examination Committee:
  Chairperson, Andrew J. Andres, Ph. D.
  Committee Member, J. Steven deBelle, Ph. D.
  Committee Member, Frank van Bruckelen, Ph. D.
  Committee Member, Allen Gibbs, Ph. D.
  Graduate Faculty Representative, Bryan Spangelo, Ph. D.