An Epigenetic role for Tip60 in synaptic plasticity and neurodegenerative diseases

A Thesis

Submitted to the Faculty

of

Drexel University

by

Jessica B. Sarthi

in partial fulfillment of the

requirements for the degree

of

Doctor of Philosophy

May 2013

© Copyright 2013

Jessica B. Sarthi. All Rights Reserved.

Dedications

Dedicated to my parents for their love and blessings and my husband; my best friend and support through the years.

Acknowledgements

I would like to take this opportunity to thank everyone who has been part of my doctoral studies. First and foremost, I thank my mentor and advisor; Dr. Felice Elefant immensely for her guidance, support and constructive criticism of this work that has helped shape my project and helped me become a better scientist. She has been extremely supportive of my project ideas and has always encouraged me to grow as a scientist and improve my technical and communication skills. She has been an amazing mentor. I also thank my committee members including my committee chair Dr. Joseph Bentz, Dr. Wen-Jun Gao, Dr. Daniel Marenda and Dr. Elias Spiliotis for their support, scientific discussion and constructive critiques. I thank my past labmates Neetu Singh and Meridith Lorbeck for training me in fly techniques and for helpful discussions. I thank Dr. Daniela Zarnescu and Patty Estes for training me in *Drosophila* larval NMJ techniques. I would like to thank my present labmates, Ashley Johnson for her helpful suggestions, Songjun Xu for useful discussions and Priya Panikker for her invaluable help with the final experiments and timely completion of this project. I also thank Sarah Puller for being a great undergrad and Billy Reube for his useful tips and tricks in formatting this thesis. I would like to thank the Department of Biology, faculty, staff and graduate students for a memorable graduate experience. I wish to thank my parents Basu and Nancy Sarthi for their support, blessings and encouragement in pursuing my scientific goals and career. Above all, I would like to thank my husband, Aurelien Brentraus for his undying love, his immense support, his faith in me and for his relentless motivation to aim higher.

Table of Contents

List of Tables vi

List of Figures..vii

Abstract ix

Chapter 1: Background and Significance 1

Chromatin packaging and epigenetic modifications.....................................1

The Histone Acetyltransferase Tip60 10

Epigenetic modifications in neuronal processes 19

Epigenetics of cognitive and neurodegenerative diseases 31

Neuronal Pathways in Alzheimer's Disease 35

Conclusions 39

Chapter 2: dTip60 HAT Activity Controls Synaptic Bouton Expansion at the *Drosophila* Neuromuscular Junction 42

Abstract 42

Introduction 43

Materials and Methods 45

Results 47

Discussion 59

Chapter 3: An epigenetic role for Tip60 in gene control that regulates synaptic plasticity at the *Drosophila* Neuromuscular Junction …………………………...68

Abstract …………………………………………………………………………. 68

Introduction ……………………………………………………………………... 69

Materials and Methods ………………………………………………………….. 72

Results …………………………………………………………………………… 76

Discussion ……………………………………………………………………….. 87

Chapter 4: Conclusions and Future Directions ………………………………… 96

Chapter 5: Tables and Figures …………………………………………………..103

Appendix A: ChIP enrichment of axonal transport genes in Tip60 HAT deficient mutants and Ms-275 mediated rescue of the acetylation levels………………...131

Appendix A: Figures……………………………………………………………133

List of References ………………………………………………………………135

List of Tables

Chapter 3

1. RPIseq analysis of putative Tip60 chromodomain mRNA targets. ____________103

List of Figures

Chapter 2

1. dTip60 is localized at the pre and postsynaptic sides of larval NMJ. __________104

2. Presynaptic reduction of dTip60 in the nervous system leads to an expansion of synaptic boutons at larval NMJs. __________ 106

3. Postsynaptic reduction of dTip60 in the nervous system leads to a decrease of synaptic boutons at larval NMJs __________107

4. Presynaptic vesicle protein distribution is not altered in dTip60 mutants _____ 109

5. Presynaptic reduction of dTip60 in the nervous system leads to suppression of active zone marker bruchpilot at larval NMJs. __________110

6. The number of presynatpic futsch-positive loops are increased at synapses in mutant dTip60 larvae. __________112

7. Loss of presynaptic dTip60 in the nervous system results in a reduction of acetylated microtubules in axons extending into terminal boutons.__________ 114

Chapter 3

1. Post-synaptic loss of Tip60 HAT activity affects DLG localization. __________115

2. Post-synaptic loss of Tip60 HAT activity leads to decrease in the number of presynatpic futsch-positive loops. __________116

3. Post-synaptic loss of Tip60 HAT activity leads to decrease in GluRIIB and GluRIIC clusters.__________117

4. Pre- and post-synaptic loss of Tip60 HAT activity leads to misexpression of synaptic plasticity genes. __________ 118

5. APP and Tip60 functionally interact pre-synaptically at the NMJ. __________ 119

6. Presynaptic expression of APP and HAT deficient dTip60 in the nervous system leads to suppression of active zone marker bruchpilot at larval NMJs. __________ 121

7. Post synaptic expression of APP and HAT deficient Tip60 causes decrease in muscle size. __________122

8. Postsynaptic expression of APP and HAT deficient dTip60 affects presynaptic bouton formation. __________ 123

9. Post-synaptic loss of Tip60 HAT activity and APP overexpression leads to decrease in GluRIIA, GluRIIB and GluRIIC clusters. __________ 125

10. ChIP enrichment of target genes by Tip60 and acetylated H3K9, H4K12 and H4K16 antibodies. ______ 126

11. Amino acid sequence alignment of yeast Esa1, drosophila Tip60 and human Tip60 and HP1 chromodomains. ______ 127

12. Model for role of Tip60 in synaptic plasticity and wg pathway. ______ 128

Chapter 4

1. Endogenous levels of H3K27ac in rat brain and liver tissues. ______ 130

Appendix A

1. ChIP enrichment of target genes by Tip60 and pan-acetylated histone H4 antibodies. ______ 133

Abstract

An Epigenetic role for Tip60 in synaptic plasticity and neurodegenerative diseases

Jessica B. Sarthi

Felice Elefant, Ph.D.

Age-associated cognitive decline and neurodegenerative disorders such as Alzheimer's disease (AD) are associated with misregulation of synaptic plasticity linked genes; however the mechanisms underlying decline of such gene control during aging are unknown. Histone acetylation of chromatin promotes dynamic transcriptional responses in neurons that influence neuroplasticity critical for cognitive ability. Accordingly, aberrant changes to histone acetylation patterns in the aging brain epigenome are linked to memory loss. It is therefore critical to identify and study the histone acetyltransferases (HAT) that create such marks. One such promising candidate is Tip60, a HAT important for various cellular processes and also implicated in AD and shown by our laboratory to be critical in regulating neuronal processes linked to cognition. To explore a direct role for Tip60 in synaptic plasticity, here we explore the consequences of misregulating Tip60 HAT activity in the Drosophila neuromuscular junction (NMJ). The Drosophila NMJ is an extremely well characterized, highly tractable and valuable tool to study synaptic plasticity. In addition, many of the pathways present at the Drosophila NMJ are well conserved and homologous to the mammalian CNS. We show that the HAT dTip60 is concentrated both pre- and post-synaptically within the NMJ. Presynaptic targeted reduction of dTip60 HAT activity significantly increases synaptic bouton number that specifically affects type Is boutons while postsynaptic reduction results in significant

loss of these boutons. The excess boutons demonstrate defects in neurotransmission function. Analysis using immunohistochemical staining to the MAP, futsch reveals a significant increase in the rearrangement of microtubule loop architecture that is required for bouton division. Our results are the first to demonstrate a causative role for the HAT dTip60 in the control of synaptic plasticity that is achieved, at least in part, via regulation of the synaptic microtubule cytoskeleton. We also show its post-synaptic role in the muscles and its function in retrograde signaling in addition to anterograde mechanisms. We show that postsynaptic loss of Tip60 HAT activity affects DLG localization, leads to decrease in GluRIIC subunit localization thus suggesting roles in activity dependent mechanisms. We also demonstrate its role in regulating genes involved in activity dependent synaptic plasticity and wingless pathway. Our ChIP-qPCR data suggests regulation of these genes via acetylation of learning and memory marks, H3K9, H4K12 and H4K16. We also report the functional interaction between HAT deficient Tip60 and hAPP at the NMJ, pre- and post-synaptically via the intracellular domain of APP (AICD), the molecule implicated in AD. Pre-synaptic expression of APP/Tip60 double mutants cause drastic increases in bouton numbers, and decrease in active zone synaptic function marker bruchpilot suggesting defects in neurotransmission. Conversely, post-synaptic expression of the APP/Tip60 double mutants leads to marked decrease in bouton numbers and absence of GluRIIC and decrease in GluRIIB and GluRIIA receptor subunits suggesting defects in signaling mechanisms. These findings have implications for dTip60 HAT dependant epigenetic mechanisms in neurodevelopment and neurodenerative diseases.

Chapter 1: Background and Significance

Chromatin Packaging and epigenetic modifications

Chromatin Packaging and regulation

All living cells are pre-programmed to differentiate into specific cell types and have distinct functions that are tightly regulated. This regulation is highly coordinated and occurs at various levels. However, all the cells in a eukaryotic organism consist of the same genetic material and yet retain the ability to differentiate into cell-type specific tissues and carry out distinct functions (Brownell & Allis, 1996; Jenuwein & Allis, 2001). For the cells to be able to maintain their cellular identity and successfully function as part of various pathways the inherent 'cellular memory' plays an important role. It is this cellular memory that allows for specific gene expression patterns to be established early during development yet allowing them to be highly 'plastic' and thus giving rise to 'chromatin plasticity' much like neuronal plasticity. The greek philosopher Aristotle coined the term 'epigenesis', (epi- coined from the greek word for 'over' or 'in addition to') to explain the interplay between nature and nurture. Modern day epigenetics explains it as a highly dynamic process that regulates gene expression and consists of changes to the chromatin packaging via post-translational histone and histone variant modifications and structural modifications to the DNA that creates a link between gene control and the environment thus conforming to the nature vs nuture theory. Epigenetic mechanisms play crucial roles in various cellular processes such as DNA repair (Peterson and Cote 2004; Mellor 2005), transcription, and now are gaining more attention for their role in

neuronal processes and pathways implicating them in various disease pathogenesis including cognition and cognition related disorders (Graff & Mansuy, 2008).

Within the eukaryotic nucleus the chromatin undergoes a series of hierarchically compaction and packaging that includes the histone and non-histone proteins and the DNA. 147 base paris of DNA is wrapped around a histone octomeric core consisting of two copies each of histones H2A, H2B, H3 and H4 forming a nucleosome (Mizzen & Allis, 1998). The nucleosomes are joined to the neighboring nucleosomes at regular intervals via a linker histone proteins H1, H^{o} and H5 that help further package the chromatin into a highly compact structure from a 10nm fibre to a 30nm solenoid into higher order structures until it is finally condensed enough to form distinct chromosomes (Bottomley, 2004; Godde & Ura, 2008; C. Wood, Snijders *et al.*, 2009). It is the chromatin remodeling complexes, histone modifying enzymes and DNA methylation events that help in the process of compaction and organization of the chromatin while maintaining genome integrity and function (Grewal & Jia, 2007; Jenuwein & Allis, 2001). This compaction is extremely important for precise regulation of gene transcription. When the chromatin is packaged loosely it facilitates transcriptional activation and becomes more accessible to the transcriptional machinery whereas tighter packaging is thought to be associated with transcriptional repression preventing the transcriptional machinery from accessing the gene promoters and enhancers (A. Johnson, Li *et al.*, 2009; Reddy & Jia, 2008). Histone and DNA modifying enzymes aid this compaction-mediated transcription by altering and changing the post-translational modifications on the N-terminal tails of the histone proteins as well as modifying various methylation marks

on the CpG islands along the DNA (Cao & Jacobsen, 2002; Fuks, 2005). Histone modifying enzymes act by altering the post-translational modifications on the histone N-terminal tails extending out from the nucleosomal core. These modifications are present on highly conserved amino acid residues which can be methylated, acetylated, phosphorylated, sumoylated as well as ubquitinilated in various combinations to allow formation of patterns unique for each gene that can act locally or globally over large chromatin regions altering global gene expression (Gelato & Fischle, 2008; Vermaak & Wolffe, 1998).

Specific histone marks assign unique transcriptional states to genes such as activation and repression. Activation is usually associated with the more 'open' chromatin called euchromatin and these regions have a characteristic histone profile or 'histone code' as put forth by David Allis. Regions of the euchromatin are mostly associated with H4 acetylation and also methylation of H3K4 (Cam, Sugiyama *et al.*, 2005; Rice & Allis, 2001) whereas heterochromtic regions are associated with H3K9 methylation and decreased acetylation (Cam, Sugiyama *et al.*, 2005; Grunstein, 1998; Nakayama, Rice *et al.*, 2001; Rice & Allis, 2001). Thus the histone modifications and DNA methylation allow for lasting and robust changes to the cell required to maintain their identity through generations as well as allow for dynamic and flexible changes in gene expression patterns as a response to environmental cues and stimuli. For example, neurons are constantly bombarded with environmental cues that are relayed and interpreted over great distances. These environmental cues are interpreted and executed with the help of flexible and dynamic changes in the epigenome that allow for specific neuronal genes to transcribed to be able to complete the circuit from

environment stimuli to the correct synaptic output (Levenson & Sweatt, 2005; T. L. Roth, Roth *et al.*, 2010).

Post-Translational Modifications of Histones

The 'histone code' consists of various different histone modifications working together to write a script on each unique gene and include enzymes that can 'edit' the code as needed. These enzymes are broadly classified as 'writers', 'erasers' and 'readers' based on their functional and structural domains (Borrelli, Nestler *et al.*, 2008; Ruthenburg, Allis *et al.*, 2007). 'Writers' as the name suggests 'write' or post-translationally modify specific histone amino acid residues. These marks include methylation, acetylation, phosphorylation, sumoylation, ubiquitinylation and ADP-ribosylation usually in various combinations allowing for transient or lasting changes (Day & Sweatt, 2011; Li, Samanta *et al.*, 2007; Strahl & Allis, 2000). These marks may serve as docking sites for the next group of enzymes called 'readers'. 'Readers' as the name suggests are a group of proteins that contain domains that can identify the marks written by the writer enzymes and in effect can recruit additional chromatin remodeling complexes. 'Presenter' readers are the ones that structurally and spatially hold the modified histone tail in a position that would be favorable for additional enzymes to dock and modify. 'Effector' readers also help hold the histone tail but at the same time also contain a catalytic domain to modify the site themselves (Marmorstein & Berger, 2001). As these modifications are reversible and dynamic there are a final group of enzymes that can reverse the marks in yet another level of gene regulation. These enzymes called 'erasers' such as deacetylses, demethylases

and dephosphorylases remove the modification marks written by the writer enzymes by either physically removing the mark or by masking the site. This group of 'writers', 'readers' and 'erasers' more often than not are part of unique complexes that are recruited to specific sites on the histone tails of specific genes. The proteins and enzymes involved in these complexes may or may not be dedicated to the complex and can form transient complexes with other proteins thus allowing for flexibility and dynamic control of the epigenome.

Histone Acetyltransferases (writers)

Histone acetylation is widely associated with trascriptional activation. Histone acetyltransferases (HATs) are one of the most extensively characterized writer enzymes that modify the N-terminal histone tails by acetylation. HATs transfer acetyl group from acetyl CoA to highly conserved lysine (K) residues on the N-terminal histone tails (Carrozza, Utley *et al.*, 2003; Smith & Denu, 2009; Struhl, 1998). This acetylation of the K residues results in the neutralization of the positive charge on the ε-amino group thus weakening the electrostatic attraction between the histone tail and the negatively charged DNA backbone and thus opens up the chromatin structure (Brownell & Allis, 1996; Cheung, Allis *et al.*, 2000). This open chromatin structure can now allow other proteins such as readers to recruit chromatin remodeling and transcriptional regulatory enzyme complexes therefore associated with transcriptional activation (Nightingale & Becker, 1998; S. Y. Roth, Denu *et al.*, 2001; Vettese-Dadey, Grant *et al.*, 1996)

HATs function predominantly in the nucleus and can be broadly classified into five distinct families based on the structural similarities: the MYST family

(MOZ, Ybf2/Sas3, Sas2, Tip60) (Borrow, Stanton *et al.*, 1996; Reifsnyder, Lowell *et al.*, 1996), GNAT family (GCN5-related N-acetyltransferases) (Neuwald & Landsman, 1997), p300/CBP HATs, nuclear hormone-related HATs and general transcription factor HATs (Marmorstein & Berger, 2001).The HATs in the GNAT family are essential for cell growth and development and play essential roles in DNA repair and transcriptional activation (Carrozza, Utley *et al.*, 2003).The GNAT family HATs apart from their HAT activity also have a 'bromodomain' which confers them the additional 'reader' activity (Pandey, Muller *et al.*, 2002).The MYST family HATs play essential roles in a number of cellular processes. The MYST domain contains a smaller highly conserved HAT domain which in turn contains a zinc finger domain that confers it its HAT function (Utley & Cote, 2003). Unlike the GNAT family HATs that contain a bromodomain that reads acetyl lysine marks, the MYST family HATs contain a domain with putative reader activity for methyl marks called the 'chromodomain'(Utley & Cote, 2003). The p300 and CBP HAT families have also been characterized as well as the MYST and GNAT family HATs (Eckner, 1996; Lundblad, Kwok *et al.*, 1995; Shikama, Lee *et al.*, 1999). These HATs also contain the acetyl lysine reader bromodomain as well as three zinc finger domains (Shikama, Lee *et al.*, 1999). There is more evidence that shows that these HATs are indeed similar to the GNAT family HATs (S. Y. Roth, Denu *et al.*, 2001).

HATs are generally associated with transcriptional activation however, more recently they were found to be involved in repression of specific targets (Deckert & Struhl, 2001; Suka, Suka *et al.*, 2001; Vogelauer, Wu *et al.*, 2000). However, they are not directly involved in the transcriptional repression of target genes via acetylation.

Some HATs however, are thought to specifically acetylate sites that prime them for repression and gene silencing such as SAS2 and SAS3 (Carrozza, Utley *et al.*, 2003; Reifsnyder, Lowell *et al.*, 1996).

Histone Deacetylases (Erasers)

Histone modifications are highly dynamic and undergo constant editing in response to environmental stimuli. As the marks are written they also need to be constantly erased or masked in response to various cues. Histone deacetylases (HDACs) are a special class of enzymes that catalyze the removal of acetylation marks laid down by HATs (Haberland, Montgomery *et al.*, 2009; Shahbazian & Grunstein, 2007). Histone deacetylation is thought to assist in transcriptional repression by unmasking the histone lysine residue, allowing condensation of chromatin and thus reducing the affinity for TF binding and hence histone deacetylation is more often then not associated with heterochromatin (Grewal & Jia, 2007; Shahbazian & Grunstein, 2007).

Similar to HATs, HDACs can be grouped into 4 classes based on sequence and functional similarities. Class I HDACs are predominantly nuclear in localization whereas Class II HDACs shuttle between the nucleus and the cytoplasm. Class I HDACs are HDAC1, HDAC2, HDAC3, HDAC4 and HDAC8 (Dokmanovic & Marks, 2005). Class IIA consist of HDACs 5, 7 and 9 which are nuclear and cytoplasmic in localization. HDACs 6 and 10 belong to the class IIB. HDAC6 is predominantly cytoplasmic and is involved in deacetylation of the lysine residue on α-tubulin of microtubule cytoskeleton (Fischle, Dequiedt *et al.*, 2002; Zhang, Li *et al.*, 2003). Class IV HDAC consists of HDAC11 whose localization is still not known

(Woan, Sahakian *et al.*, 2012). There is yet another class of histone deacetylases known as Sirtuins (SIRT1-SIRT7) that are not classified as HDACs and are structurally distinct form Class I and II but are involved in NAD^+ -dependent histone deacetylation grouped into Class III (Blander & Guarente, 2004). The most extensively studied SIRT1 is the closest homolog of the yeast Sir2. Apart from histones, HDACs and sirtuins also deacetylate other non-histone proteins including p53, NFκB, PPARγ, PTEN (Dai & Faller, 2008; Ikenoue, Inoki *et al.*, 2008).

Histone Methyltrasnferases (writers)

Histones can also undergo methylation on specific target lysine (lys) and arginine (Arg) residues. Unlike acetylation, histone tails can be methylated to different degrees such as mono-methylation, di-methylation and tri-methylation on the lysine residues catalyzed by histone methyltransferases (HMTs) (Cloos, Christensen *et al.*, 2008). Lysine methylation takes place on 5 sites on H3 (K4, K9, K27, K36 and K79) and 1 site on H4 (K20) (D. Y. Lee, Teyssier *et al.*, 2005; Sims, Nishioka *et al.*, 2003). This differential methylation allows for recruitment of different 'reader' complexes that recognize unique methyl marks (Cloos, Christensen *et al.*, 2008).

DNA Methyltransferases (writers)

Epigenetic modifications not only include histone modifications but also modifications to the DNA. Histone and DNA modifications work together to regulate gene expression. Methylated H3K9 serves a docking site for effector complexes that aid the DNA methyltransferases to methylate CpG islands that further serve as

landing sites for HDACs to help condense the DNA and thus repress gene expression (Cao & Jacobsen, 2002; Rea, Eisenhaber *et al.*, 2000). DNA methylation is catalyzed by DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. Dnmts transfer methyl groups to deoxycytosine bases to form deoxymethylcytosine and can either directly interfere with TF binding or recruit HDACs (Fan & Hutnick, 2005; Miranda & Jones, 2007).

Histone Demathylases (Erasers)

Until recently histone methylation was thought to be irreversible however, studies involving lysine specific histone demethylase (LSD1) shed light on methylation being reversible as well (Shi, Lan *et al.*, 2004; Wynder, Hakimi *et al.*, 2005). LSD demethylases are one family of demethylases that include LSD1 and LSD2 which demethylate H3K4me1, H3K4me2, H3K9me1 and H3K9me2 as well as non-histone substrates including p53, E2F1 and DNMT1. Another class of demethylases are JMJC demethylases and consist of 29 different demethylases based on the JMJC domain (Kooistra & Helin, 2012)

Acetyl-Lysine and Methyl-Lysine Readers

Once the marks are laid down by HATs and HMTs, they are read by readers that allow to either recruit effector complexes to the sites or could also themselves catalyse and modify the site further to recruit complexes (Marmorstein & Berger, 2001). The proteins that read acetyl lysine histone modifications are termed bromodomains whereas the ones that read methyl-lysine residues are termed chromodomains. The chromodomain is a 40-50 amino acid structural domain present

in proteins with reader activity. Chromodomain is a highly conserved domain involved in chromatin remodeling and gene regulation and found in HATs, HMTs as well as ATP-dependent remodelers (Eissenberg, 2001; D. O. Jones, Cowell *et al.*, 2000). The HAT Tip60, HAT of interest in this study has a chromodomain that binds specifically to H4K9me3 and is also essential for HAT activity (Sun, Jiang *et al.*, 2009). Chromodomains not only bind to histone marks but also bind to RNAs thus involved in another level of gene regulation serving as protein–RNA interaction modules (Akhtar, Zink *et al.*, 2000; Ishida, Shimojo *et al.*, 2012). Bromodomains are also found in association with HATs, HMTs and ATP-dependent chromatin remodelers (Winston & Allis, 1999). The HAT CBP has a bromodomain and recognizes acetyl lysines on histones as well as non-histone proteins (Yang, 2004). Thus, all the enzymes are involved in specific and coordinated functions and recognize specific set of marks unique to each gene that allows them to regulate gene expression and chromatin structure.

The Histone Acetyltransferase Tip60

Tip60 belongs to the MYST family of histone acetyltransferases and was originally identified as part of a complex with the HIV-1 tat protein (Kamine, Elangovan *et al.*, 1996). Tip60 exhibits high ubiquitous expression and also demonstrates cell type specificity (Hlubek, Lohberg *et al.*, 2001). Human Tip60 (hTip60) exhibits high homology with the ones from various species such as *M. musculus*, *G. gallus* and invertebrates including *D. melanogaster (dTip60)*, *C. elegans*

and *S. cerevisiae (esa1)* (Ceol & Horvitz, 2004; Doyon & Cote, 2004; Kusch, Florens *et al.*, 2004; McAllister, Merlo *et al.*, 2002; Zhu, Singh *et al.*, 2007). Tip60 is predominantly nuclear in its localization (Cao & Sudhof, 2001; Gavaravarapu & Kamine, 2000; Yamamoto & Horikoshi, 1997) as it is a transcriptional activator where it acetylates histones H4, H2A and H3 and also non histone proteins including transcription activators, kinases and membrane bound receptors (Sapountzi, Logan *et al.*, 2006). Tip60 has been shown to acetylate specific marks on these histones including H4K5, H4K8, H4K12, H4K16, H2AK5, H3K14 and H3K9 (Karmodiya, Krebs *et al.*, 2012; Kimura & Horikoshi, 1998; K. K. Lee & Workman, 2007).

Tip60 consists of two functional domains, the C-terminal MYST domain and the N-terminal chromodomain. The MYST domain contains the catalytic HAT domain that catalyses the acetylation of histone and other non-histone proteins in addition to the zinc finger necessary for its HAT activity (Nordentoft & Jorgensen, 2003; Xiao, Chung *et al.*, 2003). Recent studies have shed some new light on the function of chromodomain containing proteins such as HP1, that binds to methylated lysine residues associated with epigenetic silencing and is therefore important in heterochromatin maintenance (Jacobs & Khorasanizadeh, 2002). In addition to binding to histones, chromodomains have also been found to associate with RNA molecules for example, HAT MOF binds roX2 RNA, a non coding RNA via its chromodomain *in vivo* (Akhtar, Zink *et al.*, 2000). Tip60 has also been demonstrated to bind H3K9me3 via its chromodomain that is necessary for its HAT activity in DSB repair (Sun, Jiang *et al.*, 2009).

Tip60 is usually found as part of a multiprotein complex, Tip60/NuA4 complex that includes 18 subunits (Ikura, Ogryzko *et al.*, 2000) including DNA helicase, ATPase, and other subunits required for chromatin remodeling (Ikura, Ogryzko *et al.*, 2000). The p400/Domino subunit has the ATPase activity as well as the chromatin remodeling activity. In addition, RuvBL1 and RuvBL2 subunits also play roles in chromatin remodeling. BAF53, is an actin-related protein that recruits chromatin remodeling enzymes at sites of DNA damage thus pointing to a role for Tip60 in DNA repair. Another subunit involved in DNA damage response is the inhibitor of growth 3 protein (ING3), which has been shown to be important in transcription of p53. p53 is a tumor suppressor gene and is known to play roles in DNA damage induced apoptosis, thus supporting a role for Tip60 in apoptosis (Sapountzi, Logan *et al.*, 2006). Indeed recent data has shown the role of Tip60 in neuronal apoptosis as well where it is involved in the regulation of key neuronal apoptotic genes (Pirooznia, Sarthi *et al.*, 2012). Tip60 is also important in cell cycle regulation where it interacts with mortality factor 4 related gene 15 (Mrg15) and mortality factor 4 related gene X (MrgX) (Sapountzi, Logan *et al.*, 2006). Mrg15, MrgX and Tip60 itself contain chromodomains suggesting a role for Tip60 not only as a HAT but also as chromatin modifier (Ikura, Ogryzko *et al.*, 2000). The Tip60 complex also contains DNA methyltransferase associated protein 1 (DMAP1), the histone tail binding protein involved in DNA replication (Cai, Jin *et al.*, 2003; Doyon, Selleck *et al.*, 2004). The ING3 subunit contains the PHD finger, important in chromatin remodeling (Doyon & Cote, 2004). The *Drosophila* Tip60 complex contains histones H2A.v (histone variant) and H2B and is involved in DNA repair

mechanisms (Kusch, Florens *et al.*, 2004). However, Tip60 is thought to form transient complexes as well both in the nucleus and the cytoplasm where it utilizes its HAT function. For example, Tip60 forms a complex with the intracellular domain of APP (AICD), the protein implicated in Alzheimer's Disease (AD) and Fe65, an adaptor protein and helps regulate target gene expression. Similarly, it forms many more transient complexes and is recruited to different promoters for transcriptional regulation (von Rotz, Kohli *et al.*, 2004).

Functions of Tip60

Cell cycle

Tip60 plays a role in numerous cellular processes and as such is extremely important in many developmental and regulatory processes. Tip60 has been widely studied in cell cycle progression and DNA repair. Tip60 plays a role in cell cycle as part of the multisubunit complex where it is recruited to promoters of cell cycle promoting target gene expression including proliferating cell nuclear antigen (PCNA), pocket protein p107 and minichromosome maintenance 3/4 (MCM3 and MCM4) via E2F1. Indeed, loss of Tip60 HAT activity is important for cell cycle progression and cells that lack Tip60 HAT activity show cell cycle defects (Ikura, Ogryzko *et al.*, 2000). Tip60 HAT activity is required to acetylate key residues on histone H4 thus activating cell cycle progression (Taubert, Gorrini *et al.*, 2004).

DNA Repair

Tip60 plays essential roles in maintaining genomic stability and as such is involved in double strand break (DSB) repair. DSB require the coordination of many proteins and as such perturbation of any of the components leads to inaccurate repair mechanisms thus resulting in genomic instability and cancer as well as many neurodegenerative disorders characterized by an increase in reactive oxygen species leading to neuronal apoptosis (Kastan, 2008; van Attikum & Gasser, 2009). The cell therefore has highly regulated mechanisms to detect DNA damage and repair the damage before the cell enters the cell cycle and undergoes division. DSB repair defects in HeLa cells expressing the dominant negative Tip60 were the first studies that implicated Tip60 in DNA repair (Ikura, Ogryzko *et al.*, 2000). Subsequent studies showed that Tip60 plays important roles in DNA damage sensing and repair (Squatrito, Gorrini *et al.*, 2006). Inactivation of Tip60 leads to increased sensitivity to ionizing radiations and in turn results in increased genomic instability suggesting a key role for Tip60 in maintaining genomic integrity (Sun, Jiang *et al.*, 2005; Sun, Jiang *et al.*, 2009).

At DSBs, Tip60 has been shown to bring about the exchange of the histone variant H2AX and H4 via acetylation (Jha, Shibata *et al.*, 2008). Upon double strand break, the histone variant H2AX is rapidly phosphorylated at Ser 139 leading to formation of γH2AX (Kusch, Florens *et al.*, 2004; Rogakou, Pilch *et al.*, 1998). γH2AX then serves as a docking site for DNA damage break repair factors such as BRCA1, MRN and 53BP1 (Bassing & Alt, 2004; Stucki & Jackson, 2006). The

phosphorylation of γH2AX causes Tip60 to be recruited to the site of DNA damage where Tip60 acetylates γH2AX (H2AK5) (Ikura, Tashiro *et al.*, 2007) followed by ubiquitylation required for its removal following repair (Ikura, Tashiro *et al.*, 2007; Yuan, Adamski *et al.*, 2010). Phosphorylated γH2AX is then replaced with unmodified H2A in a reaction catalysed by p400/Domino of the Tip60 complex that exchanges H2Av-H2B dimmer (Kusch, Florens *et al.*, 2004; Yuan, Adamski *et al.*, 2010).

In addition to histone variant exchange mediated DNA break repair, Tip60 is also important in ATM activation (Sun, Jiang *et al.*, 2005). Upon sensing DNA break by ionizing radiation, Tip60 is recruited to the break site along with ATM kinase by the MRN (MRE11-RAD50-NBS1) complex. ATM kinase is coded by the *ataxia telangiectasia* gene and cells lacking ATM function show defects in DSB repair and loss of cell-cycle checkpoints (Lavin, 2008; Lavin, Birrell *et al.*, 2005). Tip60 and ATM form a complex wherein Tip60 associates with the highly conserved FATC domain of ATM (Jiang, Sun *et al.*, 2006). Tip60 then acetylates lysine 3016 residue of ATM following binding of Tip60 chromodomain to the H3K9me3 marks in the damaged DNA site (Jiang, Sun *et al.*, 2006; Sun, Jiang *et al.*, 2005). Acetylation of ATM activates it thus autophosphorylating itself, which then phosphorylates other damage response factors such as p53, chk2, SMC1, brca1 and H2AX (Jackson & Bartek, 2009; Lavin, Birrell *et al.*, 2005; Lavin & Gueven, 2006). H3K9me3 marks are associated with heterochromatin which are normally masked by another chromodomain containing protein HP1, thus suggesting Tip60 is crucial for DNA

repair in the heterochromatin region via its chromodomain and HAT function (Jacobs & Khorasanizadeh, 2002).

Apoptosis

Tip60 is involved in apoptotic pathways and has been shown to be critical in the p53 pathway. p53 and Tip60 share functional similarities in that, both the proteins are regulated by Mdm2, which marks them for proteosomal degradation. Tip60 and p53 both rapidly accumulate after DNA damage and are important in the repair mechanisms that follow DNA damage (Logan, Sapountzi *et al.*, 2004; Sapountzi, Logan *et al.*, 2006). The tipping point for the cell towards apoptosis from cell cycle arrest and DNA repair upon DNA damage depends on p53-mediated activation of specific pathways (Levine, 1997; Michael & Oren, 2002; Sengupta & Harris, 2005; Vousden, 2002). ING3, a component of the Tip60 complex is also part of the p53 pathway. Tip60 was initially identified as a component of the p53 pathway in an RNAi screen in human cells where it was found to be essential for p53-dependent G1/S arrest in response to DNA damage. It was also found that Tip60 HAT activity was required to upregulate p21, growth arrest and DNA damage inducible genes 45 (GADD45) and MDM2 in response to DNA damage (Doyon & Cote, 2004). Cells expressing HAT-deficient Tip60 demonstrate defects in apoptosis following ionizing irradiation (Ikura, Ogryzko *et al.*, 2000).

In response to cellular stress p53 levels upregulate and it is further activated via numerous post-translational modifications including acetylation. This acetylation of p53 in response to stress is catalyzed by CBP/p300 (Avantaggiati, Ogryzko *et al.*,

1997; W. Gu, Shi *et al.*, 1997), PCAF (L. Liu, Scolnick *et al.*, 1999) and Tip60 (Legube, Linares *et al.*, 2004; Tang, Luo *et al.*, 2006). Tip60, specifically its chromodomain identifies the methyl mark on p53 laid down by Set7/9 at K369 followed by acetylation of p53 at K120 by Tip60 HAT domain (Tang, Luo *et al.*, 2006). It is this acetylation of p53 K120 that acts as a switch that can either induce apoptosis or cell cycle arrest upon DNA damage and stress (Legube, Linares *et al.*, 2004; Tang, Luo *et al.*, 2006). Indeed, cancer cells exhibit mutations in K120 (Deissler, Kafka *et al.*, 2004; Leitao, Soslow *et al.*, 2004) suggesting acetylation of K120 by Tip60 is essential for p53 function the lack of which would allow cells to grow without sensing and repairing the damage and lead to cancer (Meyers, Chi *et al.*, 1993; Sykes, Mellert *et al.*, 2006). Another protein found to associate with Tip60 important in apoptosis is programmed cell death 5 (PDCD5) that stabilizes Tip60 and acts as a co-activator with Tip60 in p53-mediated activation of pro-apoptotic target genes (Xu, Chen *et al.*, 2009).

Tip60 also plays a role in neuronal apoptosis. Neuroglioma cells that undergo apoptosis do so via AICD induced mechanism that involves HAT Tip60 (Kinoshita, Whelan *et al.*, 2002). Recent studies from our lab have also pointed to a role for Tip60 HAT activity in *Drosophila* overexpressing the human APP as well as HAT deficient Tip60. These flies show increased levels of apoptotic cells in the larval brain suggesting a role for Tip60 in neurodegenration (Pirooznia, Sarthi *et al.*, 2012).

Transcriptional regulation

One of the first transcriptional roles of Tip60 was discovered as a nuclear receptor (NR) regulator involved in NR signaling. The C-terminus domain of Tip60 binds to androgen receptor and other NRs via the Leu-X-X-Leu-Leu motif in the NR-box and mediates gene expression (Brady, Ozanne *et al.*, 1999; Gaughan, Brady *et al.*, 2001). Tip60 acetylates AR by forming a complex with HDAC1 and AR and competes with HDAC1 to acetylate AR (Gaughan, Logan *et al.*, 2002) thus suggesting a role for Tip60 in prostate cancer. Tip60 is also involved in stabilizing c-myc, a transcription factor important tin G1/S phase progression by acetylating it (Patel, Du *et al.*, 2004). The nuclear factor kappa light chain gene enhancer in B cells (NF-κB) that plays a role in immunity, cell proliferation, apoptosis and inflammatory processes can regulate its target genes for example KAI1, via Tip60 as a co-activator. Tip60 coactivating complex containing TRRAP derepresses KAI1 promoter. Conversely, the AICD, Tip60 and Fe65 complex can also directly displace the NCoR complex that represses KAI1 thus activating its transcription (Baek, Ohgi *et al.*, 2002; J. H. Kim, Kim *et al.*, 2005).

Tip60 apart from its role as a coactivator has also been reported to corepress gene expression. As part of its repressive function Tip60 recruits complexes such as deacetylases. For example, Tip60 is reported to corepress cAMP response element binding protein (CREB) and STAT3 (Gavaravarapu & Kamine, 2000; Xiao, Chung *et al.*, 2003). Tip60 recruits HDAC7 in response to IL-9R signaling thereby repressing STAT3 promoters in cytokine signaling (Xiao, Chung *et al.*, 2003). Another protein that associates with Tip60 is the Zinc finger E box (ZEB) binding protein whose activity is stimulated by Tip60. Tip60 also interacts with Translocation E26

transforming-specific leukemia (TEL) gene and corepresses TEL dependent genes by stimulating interaction of TEL with corepressor complexes such as Switch independent 3 (Sin3) and Silencing mediator for Retinoid and Thyroid receptor (SMRT/NCoR) (Nordentoft & Jorgensen, 2003).

Epigenetic modifications in neuronal Processes

Until a few years ago epigenetic mechanisms were thought to play a role only in ‘cellular memory’ and were thought to be important in cell division and cell fate specification. However, recent studies in neuronal pathways and processes have expanded the scope of epigenetics to a much broader view encompassing the role of epigenomic regulatory mechanisms involved in neuronal processes in response to external environmental stimuli thus translating ‘chromatin plasticity’ to ‘neural plasticity’(Borrelli, Nestler *et al.*, 2008). Neural stem cell (NSC) populations give rise to three major cell types: neurons, astrocytes and oligodentrocytes that maintain the ability to self renew (Gage, 2000). As put forth by Waddington in 1957, cells differentiate by maneuvering through specific ‘epigenetic landscape’ that shapes their final physiological cell type and function (Slack, 2002). Neural differentiation involves inactivation of neuronal genes in the non-neuronal tissues by recruitment of repressors via the neuron-restrictive silencer element (NRSE) to the promoters of neuronal genes (Mori, Schoenherr *et al.*, 1992). The neuronal gene silencing via NRSE is achieved via recruitment of RE1-silencing transcription factor (REST) wherein loss of REST during embryonic development caused reversal of repressive

state of neuronal genes in non-neuronal cell types leading to embryonic lethality (Z. F. Chen, Paquette *et al.*, 1998; Chong, Tapia-Ramirez *et al.*, 1995).

Studies involving NRSE/REST complexes later found that NRSE also recruits several corepressors. Methyl-CpG-binding protein 2 (MeCP2) and DNA methylation were found to be present on the promoters of numerous neuronal genes such as brain derived neurotrophic factor (BDNF), calbindin and sodium channel type II involved in transcriptional silencing (Ballas, Grunseich *et al.*, 2005; Graff, Kim *et al.*, 2011; Lunyak, Burgess *et al.*, 2002). HDACs type I and II have also been shown to form complexes with CoRest another corepressor that binds to NRSE suggesting changes in both histone acetylation as well as DNA methylation in developing non-neuronal cell thus pointing to epigenetic modes of regulation (Andres, Burger *et al.*, 1999; Ballas, Battaglioli *et al.*, 2001; Huang, Myers *et al.*, 1999). Histone acetylation levels are elevated in neuron specific genes allowing for regulated neural differentiation. Certain histone methylation marks such as H3K4 associated with transcriptional activity were also found to be increased in the promoter region of opiod receptors wheras H3K9me3 levels were reduced (Y. L. Chen, Law *et al.*, 2008; S. W. Park, He *et al.*, 2008). Thus contrary to the notion that DNA methylation levels remain static in adult brain it is now superseded by the idea that these are indeed reversible modifications and infact take place at a higher rate in the adult brain compared to other tissues (Tawa, Ono *et al.*, 1990; Wilson, Smith *et al.*, 1987). Similarly histone acetylation also seems to play important roles in neural differentiation (Jenuwein & Allis, 2001).

Post translational histone modifications in synaptic plasticity

Neurons communicate with countless other synaptic connections via chemical signals transmitted through neurotransmitters. These signals could be long lasting or short activity dependent bursts. Synaptic plasticity is the strengthening and weakening of these connections with neighboring synapses as a result of environmental cues leading to activity dependent changes. The signals involved in maintaining synaptic plasticity are transient and fast acting and require quick response to incoming stimuli. These dynamic changes to synaptic connections are brought about by post-translational histone modifications that remodel chromatin and activate or repress genes that would be required during the activity dependent phase. DNA methylation and PTMs such as histone acetylation and methylation have been shown to play roles in long term plasticity that involve changes in synaptic function by regulating expression of neuron specific genes. Long-term synaptic plasticity is the basis of memory formation and long-term memory storage in the brain and recent studies show epigenetic mechanisms central to gene regulation in both vertebrates and invertebrates.

Histone acetylation in synaptic plasticity

Two forms of long-term synaptic plasticity have been identified in *Aplysia* : long-term facilitation (LTF) characterized by persistent increase and long-term depression (LTD) characterized by persistent decrease in synaptic transmission (Kandel, 2001; Pittenger & Kandel, 2003). Studies in Aplysia have shown that global changes in acetylation modify synaptic plasticity in invertebrates. In these studies

increase in H3K14 acetylation and H4K8 acetylation was demonstrated via recruitment of CBP HAT to the promoter region of C/EBP (CCAAT/enhancer binding protein). This binding of CBP HAT and increase in acetylation was also accompanied by LTF. Conversely, LTD led to decrease in acetylation by recruiting HDAC5 that was reversed by administering TSA thus suggesting an epigenetic switch mechanism to maintain and modify synaptic plasticity (Z. Guan, Giustetto *et al.*, 2002). Studies from our lab in *Drosophila* larvae expressing HAT defective Tip60 show decrease in global H4 acetylation levels one of the well-characterized target histones of HAT Tip60 (Lorbeck, Pirooznia *et al.*, 2011b). These HAT defective mutants also exhibit defects in synaptic bouton formation and function at the glutamatergic fly neuromuscular junction (Sarthi & Elefant, 2011)

Vertebrates also exhibit two forms of plasticity: long-term potentiation (LTP) and long-term depression (LTD) that refer to increase and decrease in synaptic transmission efficiency (Bliss & Lomo, 1973). Synaptic plasticity in mammalian systems like their invertebrate counterparts involve complex pathways thought to mediate post-translational modifications of histone proteins resulting in LTP and LTD induction. MAPK pathway has been implicated in memory and synaptic plasticity and activation of this cascade leads to increase in H3K14 acetylation levels during memory formation (Levenson, O'Riordan *et al.*, 2004; Sweatt, 2001). These epigenetic modifications have important functional readout and are not just correlative in nature. For example, mice that lack CBP HAT activity demonstrate decrease in global H2B acetylation and have impaired transcription-dependent late phase LTP. Conversely, administration of HDAC inhibitor suberoylanilide

hydroxamic acid (SAHA) restores the late phase LTP impairment. Similar results have been seen in rats treated with TSA and Na-butyrate that enhance LTP in the hippocampus, the center for learning and memory (Alarcon, Malleret *et al.*, 2004; Levenson, O'Riordan *et al.*, 2004). Work from our lab has shown that larvae expressing the HAT defective Tip60 demonstrate decrease in global H4 acetylation levels whereas treatment with Ms275 a potential HDAC1 inhibitor significantly increases acH4 levels (A. A. Johnson, Sarthi *et al.*, 2013).

Histone methylation in synaptic plasticity

Posttranslational modifications such as histone acetylation and phosphorylation are important in early induction of synaptic plasticity and formation of long term memory. However, recent studies have also implicated yet another histone modification: histone methylation in synaptic plasticity and memory formation. Studies have shown that contextual fear conditioning in mice leads to an upregulation of H3K4me3 in the hippocampus. Mice lacking the H3K4 specific HMT, *Mll,* demonstrated defects in contextual fear conditioning thus suggesting role for histone methylation in long-term memory consolidation (Gupta, Kim *et al.*, 2010). Unlike long-term plasticity where histone modifications have been shown to be essential components, short-term plasticity involves spontaneous activity. Thus, it does not entirely depend on gene expression changes, however growing evidence points to a baseline neurotransmission maintained by epigenetic signaling. It is interesting to see that DNA methylation plays a role in spontaneous miniature postsynaptic currents (Nelson, Kavalali *et al.*, 2008).

DNA methylation in synaptic plasticity

In addition to posttranslational histone modifications, DNA methylation also plays important roles in synaptic plasticity (Levenson, Roth *et al.*, 2006). DNA methylation is associated with transcriptional repression. Memory suppressor gene protein phosphatase 1(PP1) mediated transcriptional silencing thought to play a role in memory and LTP, is induced by methylation after contextual fear conditioning. This response induces decrease in DNA methylation, DNMT and demethylase activity of synaptic plasticity related reelin gene promoter that induces its expression (Dong, Guidotti *et al.*, 2007; Miller & Sweatt, 2007).

It has been reported that DNA methylation can also affect histone acetylation levels (A. L. Collins, Levenson *et al.*, 2004; Cross, Meehan *et al.*, 1997; P. L. Jones, Veenstra *et al.*, 1998). Indeed, studies show rescue in memory formation induced by DNMT inhibitors by elevating histone acetylation by administering HDAC inhibitors. Thus, DNA methylation and histone acetylation interaction plays important roles in memory consolidation and synaptic plasticity possible involving bidirectional mechanisms (Miller, Campbell *et al.*, 2008).

Learning and Memory

Francis Crick in 1984 first proposed the idea that epigenetic mechanisms play a role in memory and cognition by stating that “memory might be coded in alterations to particular stretches of chromosomal DNA” (Crick, 1984). One of the first studies that indicated the role for epigenetic mechanisms in memory came from studies in the insular cortex. Insular cortex is a brain region for novel taste learning and

administration of TSA to the region after novel taste learning demonstrated increased H2A and H4 acetylation. These studies suggest that taste learning can induce a set of PTMs to be modified and thus are specific (Vecsey, Hawk *et al.*, 2007). There is growing evidence that histone modifications are elemental in memory formation and consolidation. Indeed environmental cues that bring about appropriate changes in synaptic output do so via histone modifications and epigenetic regulation of specific genes (A. Fischer, Sananbenesi *et al.*, 2007; Gupta, Kim *et al.*, 2010; Levenson, O'Riordan *et al.*, 2004; Peleg, Sananbenesi *et al.*, 2010). In support of these findings it is reported that contextual fear conditioning in mice lead to increase in H3K9 dimethylation and H3K4 trimethylation. Contextual fear conditioning is a hippocampus-dependent form of memory (Gupta, Kim *et al.*, 2010). An increase in histone H3K14 acetylation is induced upon activation of extracellular regulated kinase (ERK) and the mitogen-activated protein kinase (MAPK) signaling pathway (English & Sweatt, 1997; Levenson, O'Riordan *et al.*, 2004). This LTP induction and the resulting acetylation levels increase with experience dependent plasticity in the hippocampus and hence LTP is has been defined as a mode of memory storage (Bliss & Gardner-Medwin, 1973).

Multiple neurons communicate with neighboring neuronal cells simultaneously and over great biological distances. However, each neuron is able to interpret results form each incoming stimulus and alter the synaptic output precisely in response to environmental cues, thus allowing for short-term synaptic plasticity, which is a local phenomenon (Allen & Barres, 2005; Spruston, 2008). After an action potential threshold reaches an axon, a likelihood of a spiking event depends on the

post synaptic responses to the presynaptic activity that can persist for hours as activity dependent changes that gives rise to long-term synaptic plasticity, a cellular correlate for long-term memory. Associative memory is a form of long-term memory linked to hippocampus and cortex regions of the brain and is thought to be essential in memory consolidation and storage. The HAT CBP has been shown to be essential for long-term memory and lack of CBP impairs long-term memory in fear conditioning and novel object recognition tasks (Alarcon, Malleret *et al.*, 2004). Histone acetylation is important in both threshold and strength for long-term memory. HDAC inhibition rescues memory deficits in mice expressing either CBP HAT deleted mutant or a CBP with a mutated CREB-binding domain. CREB is a transcription factor in neuronal function (Josselyn & Nguyen, 2005; Stevens, 1994). The cortex on the other hand regulates short-term working memory. Mice with deleted p300 exhibit impaired short-term working memory, which may be attributed to its HAT function (Maurice, Duclot *et al.*, 2008). In addition to histone modifications, DNA methylation also plays essential roles in learning and memory. Inhibition of DNA methyltrasferase DNMT results in aberrant transcription of protein phosphatase 1 (PP1), a memory suppressor gene in addition to blocking memory consolidation. DNMT mediated inhibition of gene methylation prevents induction of LTP and memory consolidation thus suggesting DNA methylation influences memory via chromatin structure modulation (Levenson, Roth *et al.*, 2006; Miller & Sweatt, 2007).

Experiences shape the DNA, as Waddington clearly suggested that ‘epigenetic landscapes’ allow a cell to differentiate that shapes their final physiological cell type and function (Slack, 2002). The epigenetic landscape is not permanent and allows

flexibility and plasticity to be able to change and adapt based on experiences. Early exposure to experiences during development can be stored within the epigenome as 'behavioral memory'. This memory can also be passed on to the next generation and is thus heritable. For example, rats show differential maternal grooming behavior towards their pups, where high levels of maternal grooming induce histone acetylation and DNA methylation at the glucocorticoid receptor (GR) that regulates hypothalamic pituitary adrenal response to stress in the hippocampus (Weaver, Cervoni *et al.*, 2004). This activation further affects the synaptic response by increasing the uptake of serotonin and thus reducing the stress response in pups. These gene expression changes and the response that the pups experienced early on are stored and maintained throughout their lifetime. Interestingly it is the grooming mother that causes this stress response and not the mother that gives birth to the puppies (D. Liu, Diorio *et al.*, 1997) again pointing to the experience dependent changes in epigenome and thus the nervous system and not just its heritable nature. Drug-related behaviors also indicate aberrant epigenetic modifications. Exposure to drugs of abuse produce long-term changes in the structure and function of the brain reward circuits (LaPlant, Vialou *et al.*, 2010; Nestler, 2001; Robinson & Kolb, 1997). Studies have found that HDAC inhibitor treatment prior to drug exposure causes an increase in locomotor response associated with robust preference for places of drug delivery (Kumar, Choi *et al.*, 2005; Renthal, Maze *et al.*, 2007; Sanchis-Segura, Lopez-Atalaya *et al.*, 2009). Whereas, over expression of HDAC4 decreased the motivation to seek the drug of abuse (Kumar, Choi *et al.*, 2005; Wang, Lv *et al.*, 2010). These results suggested that histone acetylation plays an essential role in

conditioned place preference (CPP) for drugs of abuse. These data suggest that epigenetic modifications shaping gene expression patterns are crucial to long-term responses to different stressful and rewarding experiences. These modifications could have evolved as a way of avoiding or associating with different environmental stimuli.

Aging and Cognition

In recent years a number of studies have linked aging to cognitive defects. Cognition is a complex process involving many brain regions and functions. Many of the studies to understand cognition were geared towards understanding cognitive defects in clinical studies of patients that exhibited these defects. Gene expression profile studies in young and aged mice before and after contextual fear conditioning revealed a number of genes that were misregulated in the aged mice (Peleg, Sananbenesi *et al.*, 2010). However it was the lack of targets that showed differential expression patterns in the aged mice compared to the young mice that seemed unusual thus indicating defects in memory formation in aged mice as these mice were unable to exhibit changes in gene expression patterns and induce facilitated memory formation as seen in young mice.

Specific histone modifications have been found to play roles in memory formation and storage. Of these modifications, histone H4 lysine 12 acetylation (H4K12ac) marks have been linked to learning and memory. In the study carried out by Peleg *et al*, H4K12ac was found to be associated with the upregulation of the induced target genes while it was absent in the aged mice (Peleg, Sananbenesi *et al.*,

2010). These studies suggested that increase in this mark was a response to fear conditioning and thus was specific to learning and memory conditions. The same study also focused on the level of HATs belonging to the MYST and GNAT (MYST4 and GCN512) families as well as HDACs (HDAC2 and HDAC4) where it was observed that following fear conditioning there was an increase in their levels while the aged mice did not show such elevated levels of these modifiers. Thus H4K12ac plays a crucial role in activating specific target genes necessary for formation and maintenance of long-term memory.

Early life experiences can have a significant impact on the epigenetic patterns laid down during development that affect long-term memories (Alarcon, Malleret *et al.*, 2004; Z. Guan, Giustetto *et al.*, 2002; Korzus, Rosenfeld *et al.*, 2004; Levenson, O'Riordan *et al.*, 2004; Levenson & Sweatt, 2005; M. A. Wood, Kaplan *et al.*, 2005). Aging can permanently alter these gene expression patterns thus by changing the epigenetic landscape and can cause detrimental effects to the cellular processes including long-term memory (Borrelli, Nestler *et al.*, 2008). Epigenetic modifications affect neuronal differentiation, learning and memory and studies aimed at understanding this interplay between epigenetic mechanisms and cognition can now explain how memories are formed and maintained and how they could be lost due to aging related perturbation of the epigenome (Crosio, Heitz *et al.*, 2003; Levenson & Sweatt, 2005; Tsankova, Kumar *et al.*, 2004). Aging affects many cellular pathways many of which are interconnected and involve changes in neuronal functions that affect cognition that has been shown to decline with age (Burke & Barnes, 2006).

Many of the age-related cognitive defects arise from loss of synaptic connections that affect how information is processed and can also stop processing in certain regions of the brain completely (Andrews-Hanna, Snyder *et al.*, 2007),as well as changes in gene expression that play important roles in synaptic function and neuronal pathways (Blalock, Chen *et al.*, 2003; Crosio, Heitz *et al.*, 2003; Erraji-Benchekroun, Underwood *et al.*, 2005; Fraser, Khaitovich *et al.*, 2005; Loerch, Lu *et al.*, 2008), as well as inefficient organization and recruitment of neuronal networks in response to environmental stimuli and tasks (Cabeza, Anderson *et al.*, 2002; D. C. Park & Reuter-Lorenz, 2009). Changes in early-gene expression associated with aging have been important in understanding learning and memory and these changes have been detected in the aging brain hippocampus (Blalock, Chen *et al.*, 2003; Desjardins, Mayo *et al.*, 1997; Small, Chawla *et al.*, 2004). LTP and LTD play crucial roles in long-term memory formation and these processes are believed to play important roles in neural circuits giving rise to lasting memories (Lynch, 2004; Morris, 2003) and the aged brain requires higher stimulation thresholds for LTP than LTD thus allowing for constant LTD facilitation leading to decrease in synaptic connectivity as the brain ages. Studies aimed at effects of aging on learning demonstrate that aged rodents are able to learn spatial discrimination in water maze tasks and are able to maintain the information over short periods of time however this spatial discrimination decreases after an interval of few days thus suggesting that its not the learning ability but the ability to retain over longer periods that is affected in these aged rodents compared to the younger animals (Mabry, McCarty *et al.*, 1996; Norris & Foster, 1999; Peleg, Sananbenesi *et al.*, 2010). However, the aged rodents

exhibited slower learning than their younger counterparts which can be attributed to decreased LTP induction (Barnes & McNaughton, 1980; Mabry, McCarty *et al.*, 1996; Norris & Foster, 1999; Oler & Markus, 1998; Solomon, Wood *et al.*, 1995). Thus a tilt towards LTD from LTP that would otherwise normally occur in the opposite direction in a younger brain may underlie the age-related cognitive decline that would be useful in understanding mechanisms perturbed in age-related neurodegenerative diseases.

Epigenetics of cognitive and neurodegenerative diseases

Several studies have implicated disruption of epigenetic mechanisms in cognitive disorders. There are several cognitive disorders that link cognitive dysfunction to perturbed epigenetic modifications on the genome.

Rett Syndrome

Rett syndrome is an X-linked debilitating neurodevelopmental disorder characterized by communication dysfunction, cognitive impairment, stereotypic movements and pervasive growth failure and has been attributed to mutations in the MeCP2 gene (Wan, Lee *et al.*, 1999). Overexpression of MeCP2 in animal models increased long-term memory formation and induced hippocampal LTP (Amir, Van den Veyver *et al.*, 1999; A. L. Collins, Levenson *et al.*, 2004; Ellaway & Christodoulou, 2001). Deletion of the gene also leads to deficits in short-term plasticity involving increased vesicle release probability thus pointing to epigenetic

mechanisms in presynaptic function (Asaka, Jugloff *et al.*, 2006; Sultan & Day, 2011). MeCP2 and DNA methylation are present on the promoters of numerous neuronal genes such as brain derived neurotrophic factor (BDNF), calbindin and sodium channel type II involved in transcriptional silencing (Ballas, Grunseich *et al.*, 2005; Graff, Kim *et al.*, 2011; Lunyak, Burgess *et al.*, 2002).

Fragile-X Syndrome

FMR1 and FMR2 are two genes implicated in Fragile X syndrome (C. T. Ashley, Sutcliffe *et al.*, 1993; Turner, Webb *et al.*, 1996). Fragile X syndrome is also X-linked and is characterized by intellectual disability and behavioral defects. It is caused due to abnormal trinucleotide repeat expansion in the FMR1 or FMR2 genes. FMR1 and FMR2 genes contain polymorphic trinucleotide repeat (CGG and CCG respectively) in their 5' UTR region (Gecz, Gedeon *et al.*, 1996; Y. Gu, Shen *et al.*, 1996). This expansion leads to hypermethylation of regions as well as the flanking CpG islands thus causing transcriptional silencing (Levenson & Sweatt, 2005).

Rubinstein-Tyabi Syndrome

Rubinstein-Tyabi Syndrome (RTS) is a rare congenital disorder cause by autosomal mutations in the gene encoding the HAT CBP wherein many of the mutations result in a HAT deficient CBP thus causing loss of its HAT activity (Kalkhoven, Roelfsema *et al.*, 2003; Murata, Kurokawa *et al.*, 2001; Roelfsema, White *et al.*, 2005). As seen earlier, CBP is a co-activator of CREB-dependent genes and thus mutations in CBP lead to misregulation and aberrant transcription of the targets genes. Animal models studies with the CBP HAT mutation exhibit deficits in

contextual, spatial and object memory thus providing a causal relationship between histone acetylation and the disease (Alarcon, Malleret *et al.*, 2004; Korzus, Rosenfeld *et al.*, 2004).

Early epigenetic transcriptional regulatory mechanisms involving histone acetylation are critical in neurogenesis and as such, their misregulation may contribute to early pathophysiological mechanisms underlying progressive cognitive disorders (Levenson, O'Riordan *et al.*, 2004; Peleg, Sananbenesi *et al.*, 2010). In recent years epigenetic mechanism have thought to play essential roles in progressive neurodegenerative diseases such as Parkinson's Disease, Alzheimer's Disease (AD) and Huntington's Disease. Aberrant DNA methylation and histone modifications have been reported to lead to shift the balance towards the disease pathology.

Parkinson's Disease

Parkinson's Disease (PD), a common neurodegenerative disorder characterized by resting tremor, bradykinesia, rigidity, and postural instability including cognitive impairment and sleep disorders. One of the genes implicated in PD, *SCNA* is epigenetically modified in PD brains including some recently identified PD-related genes (Jowaed, Schmitt *et al.*, 2010; Matsumoto, Takuma *et al.*, 2010), *SCNA* gene in patients heterozygous for A53T mutation showed monoallelic expression of the gene and link it to histone modification (Voutsinas, Stavrou *et al.*, 2010). In addition various studies showed that DNA methylation of *SCNA* intron 1 was key in the pathogenesis of PD (Jowaed, Schmitt *et al.*, 2010). Studies have revealed that α-synuclein can inhibit histone acetylation and administration of HDAC

inhibitors rescues α-synuclein mediated toxicity (Kidd & Schneider, 2010; Kontopoulos, Parvin *et al.*, 2006; Monti, Gatta *et al.*, 2010; Outeiro, Kontopoulos *et al.*, 2007).

Huntington's Disease

Huntington's Disease (HD) is another progressive neurodegenerative disorder characterized by emotional dysfunctions, uncontrolled movements and well as cognitive defects. The gene that has been implicated in HD is Huntington (*htt)* and causes increased CAG repeats producing polyQ extensions of the protein (Bates, 2005). Interestingly, binding of these extensions to the CBP HAT and p300/CBP-associated factor (PCAF) genes inhibits their activity thus leading to transcriptional dysregulation (Steffan, Bodai *et al.*, 2001). In fly models of HD, reduction of global H3 and H4 acetylation has been reported which was revered by administering SAHA and sodium butyrate (Steffan, Bodai *et al.*, 2001). Similar studies in mouse models using HDAC inhibitors rescued motor defects (Ferrante, Kubilus *et al.*, 2003; Hockly, Richon *et al.*, 2003) accompanied by increase in histone acetylation yet again pointing to a role for histone acetylation in HD.

Alzherimer's Disease

Late onset Alzheimer's Disease (AD) is the most common form of age-related dementia. AD is characterized by formation of Aβ plaques and neurofibrillary tangles that affect synaptic connections and lead to decrease in cognition (Cummings, 2004). The protein implicated in AD is APP, which when cleaved via two different secretases (β and α secretase) produces products that can be either toxic β-amyloid

peptide or non-toxic p3 peptide fragment respectively. One other cleavage product produced by γ-secretase is APP intracellular domain (AICD). AICD forms a complex with Fe65, an adaptor protein and HAT Tip60 and acts as a transcriptional activator (Cao & Sudhof, 2001). This interaction of AICD with the HAT Tip60 suggests an epigenetic mechanism in AD etiology. In addition to HAT Tip60, HAT CBP has also been reported to increase acetylation due to the sustained HAT activity caused by the AD-related presenilin1 (PS1) gene coding for the catalytic subunit of the γ-secretase complex (Rouaux, Jokic *et al.*, 2003). Apart from histone acetylation, DNA methylation has also been implicated in AD etiology. PS1 enzymatic activity increases and in turn rises β-amyloid production caused due to hypomethylation of the *PS1* promoter region (Scarpa, Fuso *et al.*, 2003).

Neuronal Pathways in Alzhiemer's Disease

Alzheimer's Disease

Alzheimer's Disease is a progressive neurodegenerative disease characterized by dementia, loss of neurons and synaptic connections, and one of the hallmarks of AD, the presence of β-amyloid plaques and neurofibrillary tangles. The protein involved in the pathophysiology of the disease is amyloid precursor protein (APP) (Hardy & Selkoe, 2002; Selkoe, 1999). APP is a single pass transmembrane protein that can be cleaved via two distinct routes one of which produces the cytotoxic peptide Aβ. In the two non-amyloidogenic and amyloidogenic pathways APP is first cleaved by α and β sectretases that cleave the APP in the ectodomain. Another set of

γ-sectretases also cleaves the APP protein in the intramembranous region releasing the Aβ fragment and the intracellular domain called AICD (De Strooper, 2003; Passer, Pellegrini *et al.*, 2000; Sisodia & St George-Hyslop, 2002; Walter, Kaether *et al.*, 2001). Since the discovery of AICD, APP processing has been correlated to Notch receptor signaling thus suggesting similar signaling mechanisms via AICD (De Strooper, 2003; Schroeter, Kisslinger *et al.*, 1998). Studies have reported the formation of a notch like complex wherein AICD forms a complex with Fe65, and adaptor protein and HAT Tip60 and is important in transcriptional activation (Duilio, Zambrano *et al.*, 1991; Minopoli, de Candia *et al.*, 2001; Sterner & Berger, 2000). These experiments were the first to suggest a role for AICD in nuclear signaling mechanisms mediated by HAT Tip60. In addition to AICD mediated nuclear signaling there are various neuronal pathways that can feed into or interact with the APP signaling pathway and lead to perturbation and imbalance of the APP signaling components and others important in maintaining neuronal growth and function.

The Notch Signalling pathway

Notch is a cell surface receptor, evolutionarily conserved type I membrane protein (Wharton, Johansen *et al.*, 1985). It is important in cell fate determination in both invertebrates and vertebrates (Lewis, 1998), cell differentiation, proliferation and apoptosis as well as neurodegeneration (Artavanis-Tsakonas, Rand *et al.*, 1999; Osborne & Miele, 1999). Notch signalling is initiated when the receptor binds its potential ligand (Delta or Serrate) on the neighboring cells. Notch undergoes a series of proteolytic cleavage events like APP and in the intracellular region is cleaved by γ-

sectretase complex to release the Notch-intracellular Domain (NICD). NICD then translocates to the nucleus where it is involved in regulation of target gene expression (Harper, Yuan *et al.*, 2003; Radtke & Raj, 2003). Presenilins (PSs) have been known to play critical roles in Notch signaling for a while before they were found to be key players in AD. Presenilins play a role in apoptosis and animals with PS1 and PS2 genes show defects in synaptic function, learning and memory and neuronal cell death providing a link between APP signaling and Notch signaling via PSs (Saura, Choi *et al.*, 2004). Furthermore, PS1 regulates the activity of γ-sectretase, the same enzyme that cleaves APP to generate Aβ and AICD. Notch plays key roles in many neuronal processes and its dysregulation is evident in many neuronal diseases such as Schizophrenia, CADASIL syndrome, cortical dysplasia as well as brain tumors (Joutel, Corpechot *et al.*, 1996; Wei & Hemmings, 2000). These studies suggest common mechanisms for Notch and APP signalling involved in similar neuronal processes and suggest a putative interaction between the two at the level of PS1.

Wingless/Wnt Signalling Pathway

Like Notch, Wnt also plays crucial roles in cell fate determination and cell adhesion and perturbation of this pathway leads to defects in developmental processes and oncogenesis (Bullions & Levine, 1998; Dale, 1998; Wodarz & Nusse, 1998). Wnt (human) or wingless, the *Drosophila* homolog encode secreted glycoproteins, 350-400 amino acids in length. Wnt binds to frizzled (fz), a membrane receptor and activates Dvl (Dsh in drosophila) thus inhibiting GSK-3β (sgg in drosophila) (Cook, Fry *et al.*, 1996). In the absence of Wnt signal, GSK-3β phosphorylates β-catenin

targeting it to ubiquitination and leading to degradation by the 26S proteasome. On the other hand, activation of Wnt inhibits GSK-3β mediated phosphorylation of β-catenin, thus increasing its cytoplasmic levels, stabilizing it and allowing β-catenin translocation to the nucleus inturn activating target genes (Dale, 1998). Indeed, GSK-3β has also been reported be a target of APP in HEK293 cells and rat cortical neurons via AICD dependent transcription of this gene (H. S. Kim, Kim *et al.*, 2003) Studies in *Drosophila* have further demonstrated that sgg, the *Drosophila* homolog of GSK-3β plays a role in the canonical wg pathway at the NMJ and it is here that it is involved in phosphorylating futsch, the MAP1B protein homolog in *Drosophila* (Franco, Bogdanik *et al.*, 2004; Miech, Pauer *et al.*, 2008). β-catenin and PS1 have been found to bind each other forming a complex with a few other members, one of them being GSK-3β (Levesque, Yu *et al.*, 1999; Murayama, Tanaka *et al.*, 1998; Zhou, Liyanage *et al.*, 1997). These data suggest another possible interaction between APP signaling and Wnt/wigless signaling pathway.

Interaction of APP, Notch and the Wnt/Wg pathway

Based on studies, the common component of the Notch and Wnt pathway is PS1. However, Notch and Wnt could also interact at the level of Dishevelled (Dvl) as it has been reported that the intracellular domain of Notch (NICD) can also form a complex with Dvl (Axelrod, Matsuno *et al.*, 1996). In summary, mutantions in presenilins lead to production of Aβ42 that leads to Aβ plaque formation. Since PS1s also interact with GSK-3β and β-catenin it could lead to altered regulation of β-catenin target genes while increasing the levels of GSK-3β as a result further altering

Notch signaling to Dvl. Thus based on these data, it seems plausible that dysregulation of components of the Notch and Wnt signaling pathways could lead to the hallmark amyloid and tau pathologies associated with AD (Anderton, Dayanandan *et al.*, 2000). However, the effects of PS1, Dvl and GSK-3β are contextual and depend of the system being studied as Notch and wingless signaling could be interactive, combinatorial or even antagonistic.

Conclusions

There are numerous mechanisms involved from the time when an external stimulus is received by a single neuron to the appropriate synaptic response executed, all of which are coordinated and highly regulated mechanisms. External cues, environmental signals and activity dependent stimuli such as learning and experience shape the synaptic structure and regulate synaptic plasticity and allow for strengthening and weakening of synaptic connection in response to the stimuli received. As such, these stimuli and cues can result in long-term potentiation (LTP) or long-term depression (LTD) that play important roles in cognition and long-term memory. Synapses undergo pruning, shaping and stabilization during post-natal development and the synapses are then subject to various regulatory mechanisms that help maintaining them and reinforcing these connections important in experience based memory formation and consolidation.

Activity dependent gene expression has been studied for quite some time now and the focus has been on the epigenetic modifications that play a role in execute these activity-based stimuli received. The effect of epigenetic modifications and the

resulting gene expression on synaptic plasticity begins when specific synaptic receptors are activated (NMDA) leading to the influx of Ca-dependent signaling molecules as well as activation of CAMKII. These second messengers can in turn activate histone modifying and chromatin remodeling enzymes that modify the target gene and allow for expression of genes involved in immediate response as well as effectors that function to modify synapses. These changes in gene expression could be short-lived or long-lasting resulting in short-term plasticity and long-term plasticity respectively.

Numerous cognitive disorders have been found to be associated with changes in histone modifications levels such as histone acetylation, histone methylation as well as DNA methylation. It as now widely believed that as one ages the epigenetic marks laid down early on during development begin to undergo changes and the aging brain hippocampus accumulates these aberrant marks that finally lead to learning and memory defects (Levenson & Sweatt, 2005). These defects though a natural part of aging have been shown to be exacerbated in neurodegenerative diseases suggesting perturbation of theses epigenetic mechanisms plays a role in the neurodegenerative disease pathology such as Alzheimer's Disease. Recent findings have bolstered the concept that HATs involved in neuronal processes play critical roles in regulating these epigenetic modifications and thus are important in neurodegenerative diseases. Histone acetylation altered by CBP in age related cognitive defects and dementia has garnered much attention and suggests other HATs that could also be key players in neuronal gene regulation.

One such HAT that is thought to play a role in AD is Tip60. Tip60 has been shown to bind to the C-terminus domain of APP (AICD), the molecule implicated in AD. However, studies based on its role in specific neuronal processes are limited. Tip60 is a HAT identified as a genetic hub gene involved in numerous cellular processes such as DNA repair, cell cycle progression, apoptosis and transcriptional regulation as well as in many human diseases. In its association with AICD, Tip60 is believed to be important in regulating AICD target genes important in synaptic activity and function. Thus Tip60 could play a role in synaptic plasticity, which is a strong correlate of learning and memory. Investigating the role for Tip60 in synaptic plasticity as well as its role in neurodevelopmental pathways involved in synaptic plasticity such as wingless/Wnt that in turn signal to the APP signaling pathway will allow us to better elucidate the neuronal function of Tip60 in both neurodevelopment and neurodegeneration.

Chapter 2: dTip60 HAT Activity Controls Synaptic Bouton Expansion at the *Drosophila* Neuromuscular Junction

Abstract

Background: Histone acetylation of chromatin plays a key role in promoting the dynamic transcriptional responses in neurons that influence the neuroplasticity linked to cognitive ability, yet the specific histone acetyltransferases (HATs) that create such epigenetic marks remain to be elucidated.

Methods and Findings: Here we use the *Drosophila* neuromuscular junction (NMJ) as a well characterized synapse model to identify HATs that control synaptic remodeling and structure. We show that the HAT dTip60 is concentrated both pre and post-synaptically within the NMJ. Presynaptic targeted reduction of dTip60 HAT activity causes a significant increase in synaptic bouton number that specifically affects type Is boutons. The excess boutons show a suppression of the active zone synaptic function marker bruchpilot, suggesting defects in neurotransmission function. Analysis of microtubule organization within these excess boutons using immunohistochemical staining to the microtubule associated protein futsch reveals a significant increase in the rearrangement of microtubule loop architecture that is required for bouton division. Moreover, α-tubulin acetylation levels of microtubules specifically extending into the terminal synaptic boutons are reduced in response to dTip60 HAT reduction.

Conclusions: Our results are the first to demonstrate a causative role for the HAT dTip60 in the control of synaptic plasticity that is achieved, at least in part, *via* regulation of the synaptic microtubule cytoskeleton. These findings have implications for dTip60 HAT dependant epigenetic mechanisms underlying cognitive function.

Introduction

Synaptic plasticity, or activity dependent changes in synaptic strength, is the mechanism by which information is processed, and stored as short or long term memory in the central nervous system (CNS) (Benfenati, 2007; Martin, Barad *et al.*, 2000; Muller, 2002). Epigenetic regulation of chromatin structure plays a key role in providing a coordinated system of gene control critical for promoting the distinct phases of neuronal plasticity that are subsequently converted to short and long term memory formation (Alarcon, Malleret *et al.*, 2004; Riccio, 2010). One such epigenetic modification is histone acetylation, that serves to epigenetically mark DNA associated histone proteins within chromatin at distinct sites and patterns over time to drive gene expression profiles in the brain (Elefant, Su *et al.*, 2000; Feng, Fouse *et al.*, 2007; Ho, Elefant *et al.*, 2006; Hsieh & Gage, 2005; Levenson, O'Riordan *et al.*, 2004). Recent studies support the concept that aberrant changes to the histone acetylation code within the genome of the aging brain cause gene misregulation that drives cognitive decline (Borrelli, Nestler *et al.*, 2008; A. Fischer, Sananbenesi *et al.*, 2007; Levenson & Sweatt, 2006; Peleg, Sananbenesi *et al.*, 2010; Sweatt, 2010). As individuals age, the accumulation of inappropriate changes in these epigenetic marks is thought to alter transcription of synaptic plasticity genes with

subsequent negative consequences on cognitive function (Mattson, 2003; Sweatt, 2009, 2010). Although the histone acetyltransferase activity of CREB binding protein (CBP) has been implicated in synaptic plasticity linked gene regulation, additional specific histone acetyltransferases (HATs) important in these processes remain to be elucidated (Borrelli, Nestler *et al.*, 2008; Riccio, 2010; Singh, Lorbeck *et al.*, 2011).

Work from our laboratory support a role for the HAT Tip60 in nervous system function (Lorbeck, Pirooznia *et al.*, 2011a) The Tat-interactive protein-60 kDa (Tip60) is a member of the MYST family of histone acetyltransferases (HATs) (Utley & Cote, 2003). Tip60 plays essential roles in many cellular processes in large part, by its function in regulating gene expression profiles *via* histone acetylation (Baek, Ohgi *et al.*, 2002; Sapountzi, Logan *et al.*, 2006). We previously demonstrated that the *Drosophila* homolog of mammalian Tip60 (dTip60) is produced robustly in the anterior brain neuroblast population regions of the central nervous system (CNS) in *Drosophila*, *in vivo*, suggesting a role for dTip60 in synapse formation in the brain. Moreover, our microarray analysis of flies depleted in dTip60 HAT activity reveal that dTip60 regulates genes enriched for neuronal functions that include neuronal development and synaptic function (Lorbeck, Pirooznia *et al.*, 2011a). These findings prompted us to investigate a causative role for dTip60 in synaptic plasticity, with the rationale that such findings may have implications for this HAT in cognitive ability.

In this report, we ask whether the HAT Tip60 plays a causative role in the control of synaptic plasticity and structure using the *Drosophila* neuromuscular junction (NMJ) as a well characterized neuroplasticity model (Budnik & Ruiz-Canada, 2006). By analyzing the effects of dTip60 HAT activity misregulation on synaptic growth, we causatively link dTip60 HAT activity in negatively controlling synaptic bouton formation *via* regulation of the synaptic microtubule cytoskeleton. Our results are the first to demonstrate a novel role for dTip60 in the control of synaptic plasticity at the *Drosophila* NMJ.

Materials and Methods

Drosophila Stocks:

All fly lines used were raised under standard conditions at 25^{0}C on standard yeasted *Drosophila* media (Applied Scientific Jazz Mix *Drosophila* Food, Thermo Fisher Scientific, Waltham, MA, USA). The pan-neuronal driver $elav^{c155}$ was obtained from Bloomington Stock Center (Bloomington, IN). *P(pUAST)/P(pUAST)* fly line containing $dTip60^{WT}$ (independent lines A and B), $dTip60^{E431Q}$ (independent lines A and B) and $dTip60^{RNAi}$ (line A) were created as described (Zhu, Singh *et al.*, 2007). Muscle specific GAL4 drivers MEF-2 and TWI;MEF2 was a generous gift from Xianmin Zhu. As the $elav^{c155}$ transgene is located on the X chromosome, homozygous female $elav^{c155}$ flies were crossed to homozygous male $dTip60^{WT}$, $dTip60^{E431Q}$ and $dTip60^{RNAi}$ fly lines for all studies. All experimental crosses were carried out at 25°C.

Immunohistochemistry:

3^{rd}instar larvae were filleted in HL-3 saline, pH7.2 and pinned out on Sylgard dishes with guts removed. Larvae progeny of either sex were used from $elav^{c155}$ lines crossed to $dTip60^{WT}$ and $dTip60^{E431Q}$ lines. For the $dTip60^{RNAi}$ line, as this transgene is located on the X chromosome, only female larvae progeny were selected for analysis. Fillet preparations were fixed in 3.5% paraformaldehyde for 30 minutes, followed by 3 washes in PBS and one with 1X PBS with 0.1% Triton. The larvae were then incubated overnight in primary antibody overnight at 4°C. After 6 washes in PBS-T (1x phosphate buffered saline +0.1% Triton), fillets were incubated in 2° antibody for 1 hour, washed twice in 1x PBS-T, once in 1x PBS, then mounted onto slides in Vectashield antifade mounting media (Vector laboratories, Burlingame CA). Confocal microscopy was performed using Olympus Microscope with fluoview software.

Antibodies:

The primary antibodies, anti-DCSP-3 (1G12), anti-DLG (4F3), anti-Futsch (22C10) and anti-nc82 antibodies were all obtained from Developmental Studies Hybridoma Bank, DSHB (University of Iowa, Iowa City, IA). Each of these antibodies was diluted in block (2%BSA, 5%NGS in 1XPBS-T) and used at a dilution of 1:10. *Anti-Syt* (*Littleton et al*., 1993) was a generous gift from Hugo Bellen and was used at 1:5000 dilution. The primary mouse monoclonal anti-acetylated tubulin antibody (clone 6-11B-1) was obtained from Sigma-Aldrich (St. Louis, MO) and used at a dilution of 1:1000 in block. Polyclonal rabbit anti-dTip60 antibody (Lorbeck et

al., 2011) was used at a dilution of 1:100. The HRP Antibody was obtained from Jackson Immunoresearch Laboratories Inc. and used at a dilution of 1:25**.**

Imaging and Analysis:

Larval NMJ's were imaged on a Olympus (Germany) FV1000 laser scanning confocal microscope. All images were captured using same constant confocal gain settings. Images were acquired as a Z-stack and then rendered as a maximum projection. Individual entire muscle 6/7 NMJ synapses were optically sectioned (0.2 um; series of 15 to 20 sections per synapse). A 2D projection was generated that projected the pixel of maximal intensity found within the series of sections. Quantitative analysis of fluorescence intensities was done using FV1000 software. For comparison between genotypes, all samples were processed simultaneously and imaged using identical microscopic acquisitions parameters. All images were also corrected for any background before any intensity measurements. Student's T-test was used for all statistical analysis.

Results

dTip60 is localized at the pre and postsynaptic sides of *Drosophila* NMJ.

Our previous microarray analysis of mutant flies specifically depleted for dTip60 HAT activity identified misregulated genes enriched for diverse neuronal processes, many of which were linked to synaptic function (Lorbeck, Pirooznia *et al.*, 2011a). These findings prompted us to ask whether Tip60 was localized at the synapses of the

Drosophila NMJ. The *Drosophila* NMJ is a dynamic structure that constantly changes in response to activity and body size, making it a particularly suitable model to investigate proteins involved in synaptic plasticity (Broadie & Bate, 1993; C. A. Collins & DiAntonio, 2007; Koh, Gramates *et al.*, 2000). Moreover, it provides relevance for understanding brain function in that it shares central features with major excitatory synapses of the mammalian brain including ionotropic glutamate receptors and many other proteins also found in mammalian central synapses (Budnik, 1996; C. A. Collins & DiAntonio, 2007; Wollmuth & Sobolevsky, 2004). To visualize proteins localized in the NMJ, we performed immunohistochemistry using antibodies to dTip60 and antibodies against HRP, a commonly used marker that specifically labels the entire presynaptic membrane (Franco, Bogdanik *et al.*, 2004; Snow, Patel *et al.*, 1987) (Figure 1, A-C). These studies revealed enrichment of dTip60 that was concentrated at the synapses of the NMJ and overlapped with preysnaptic marker HRP, indicating that dTip60 was presynpatically localized. Co-localization studies using antibodies to Tip60 and the well characterized and commonly used postsynaptic density marker protein Discs Large (Dlg) (K. Chen & Featherstone, 2005; Franco, Bogdanik *et al.*, 2004) revealed co-localization of dTip60 with Dlg (Figure 1, D-F). Importantly, these images also showed dTip60 immunoreactivity within the presynaptic boutons in a pattern identical to presynaptic HRP staining (Figure 1F), supporting a pre and postsynaptic localization of dTip60 in the NMJ boutons. To confirm pre and postsynaptic localization of dTip60 and the specificity of the dTip60 Ab at the NMJ, we knocked down dTip60 levels specifically in the presynaptic NMJ using the GAL4/UAS targeted gene expression system with well characterized fly

lines we previously created that carry a UAS responsive transgene for dTip60^{RNAi} (Franco, Bogdanik *et al.*, 2004; Snow, Patel *et al.*, 1987). This fly line was crossed to the pan-neuronal presynaptic driver GAL4 driver elav^{C155} and localization and levels of dTip60 were assessed using immunohistochemistry with antibodies against dTip60 and another presynaptic marker cysteine string protein (csp) (Figure 1 G-I). These studies revealed the presence of postsynaptic dTip60 and importantly, the absence of presynaptic dTip60 localization due to RNAi presynaptic knockdown, demonstrating specificity of the dTip60 Ab at the NMJ and confirming dTip60 pre and postsynaptic localization. Post-synaptic knockdown of dTip60 using GAL4 driver MEF-2 showed reduction of dTip60 postsynaptic localization, further confirming pre and postsynaptic localization of dTip60 and the efficacy of RNAi induced dTip60 knockdown (Figure 3 E-F, Supplemental Figure S1). Taken together, these results demonstrate that dTip60 is localized at both the pre and postsynaptic sides of the *Drosophila* NMJ, suggesting a role for dTip60 in synaptic function.

Pre and postsynaptic dTip60 HAT activity controls synaptic bouton formation in the developing NMJ.

The enrichment of dTip60 in both pre and postsynaptic sides of the NMJ prompted us to investigate a potential function for dTip60 in bouton development by examining the effects resulting from depletion or overexpression of dTip60 specifically in the nervous system. Misregulation of dTip60 was achieved for all experiments by utilizing the GAL4/UAS targeted gene expression system with flies carrying UAS responsive transgenes for either dTip60^{RNAi}, a dominant negative HAT

defective version of dTip60 (dTip60^{E431Q}), or wild-type dTip60 (dTip60^{WT}), all well characterized transgenic fly lines created in our laboratory (Lorbeck, Pirooznia *et al.*, 2011a; Zhu, Singh *et al.*, 2007). Independent fly line dTip60^{RNAi}A was chosen for this study as it is our strongest dTip60^{RNAi} line (Zhu, Singh *et al.*, 2007) and showed robust knockdown of dTip60 pre and post-synaptically (Figure 1G-I and Figure 3 E-F). Independent fly lines dTip60^{E431Q} line B and dTip60^{WT} line B were selected for this study as these lines show equivalent and robust expression of transgene expression as assessed by qPCR (Lorbeck, Pirooznia *et al.*, 2011a) as well as equivalent Gal4 driven expression at the NMJ as assessed by immunostaining using dTip60 Abs (Supplemental Figure S1). Of note, Tip60^{RNAi} and Tip60^{E431Q} induced lethality can be fully rescued by specifically increasing dTip60 levels (Lorbeck, Pirooznia *et al.*, 2011a; Zhu, Singh *et al.*, 2007), demonstrating that the phenotypic effects we observe in our Tip60 mutants are indeed specifically caused by disruption of Tip60 HAT function.

To investigate a presynaptic role for dTip60 in bouton formation, fly lines dTip60^{RNAi}, dTip60^{E431Q}, dTip60^{WT}, and w^{1118} control flies were each crossed to the presynaptic pan-neuronal driver GAL4 driver elav^{C155}. Induction of dTip60^{RNAi} and dTip60^{E431Q} resulted in pupal lethality while dTip60^{WT} and w^{1118} control flies showed no observable negative phenotypic effects. To examine basic synaptic morphology, boutons at muscles 6 and 7 at abdominal segment A4 were stained with anti-HRP antibodies that label and allow for visualization of the entire presynaptic membrane, and Phalloidin, a toxin that stains F-actin within the muscles, to identify and measure the surface area of the appropriate muscle groups and abdominal segments (data not

shown). Changes in NMJ development were assessed by counting the number of synaptic boutons for each genetic strain. This analysis revealed that reduction of dTip60 had clear consequences on the expansion of the boutons, while muscle surface area remained unchanged. Remarkably, there was a significant increase of the total number of synaptic boutons in the $dTip60^{RNAi}$ and $dTip60^{E431Q}$ larvae (56.45 ± 2.54 for w^{1118}, 84.26 ± 6.06 for $Tip60^{E431Q}$ and 82.73 ± 3.97 for $Tip60^{RNAi}$) when compared with the wild type control (Figure 2A-D; all error bars are standard error of mean). Of note, there are two types of neurons that innervate the larval NMJ at muscles 6/7 giving rise to two sets of boutons. These boutons are classified as type I small (Is) and type I big (Ib) based on their size. Type Is boutons have larger stimulation thresholds and excitatory junctional currents of larger amplitude while type Ib boutons exhibit more pronounced short-term facilitation (Koh, Gramates *et al.*, 2000; Song, Ranjan *et al.*, 2002). Intriguingly, although the total number of boutons in the $dTip60^{RNAi}$ and $dTip60^{E431Q}$ lines were significantly increased when compared to the wild-type control, there was a substantially larger expansion of type-Is boutons when directly compared to Ib (26.05 ± 1.7 type Is and 28.8 ± 1.4 type Ib for w^{1118} , 50.52 ± 2.2 type Is boutons and 28.26 ± 1.8 type Ib boutons for $dTip60^{RNAi}$, 47.31 ± 4.2 type Is and 33.31 ± 2.4 type Ib for $dTip60^{E431Q}$). Thus, in comparison to the w^{1118} control, there was a 92.82% increase in type Is and 0% increase in type Ib for $dTip60^{RNAi}$, and a 80.59% increase in type Is and a 15.65% increase in type Ib for $dTip60^{E431Q}$. Additionally, "satellite" bouton budding, a process that involves the budding of bouton(s) from one central parent bouton on the main branch to form smaller "satellites boutons", was also affected in response to dTip60 reduction. We

observed a significant increase in the number of satellite boutons in the dTip60^{RNAi}and dTip60^{E431Q} when compared to the w^{1118} (2.45% ± 0.64 for w^{1118}, 4.62% ±0.96 for Tip60^{RNAi}, 4.21% ± 0.87% for Tip60^{E431Q}, Figure 2I). Finally, we observed no significant changes in bouton number in dTip60^{WT} flies when compared to the control flies, consistent with our observation that induction of dTip60^{WT} in the nervous system led to no observable phenotypic effects and no significant effects on gene expression in our prior microarray analysis (Lorbeck, Pirooznia *et al.*, 2011a). Of note, bouton counts for UAS-RNAi dTip60 control flies that contain a non-inverted RNAi target sequence (Zhu, Singh *et al.*, 2007), all UAS lines in the absence of the GAL4 driver, and GAL4 driver in the absence of the UAS transgene showed no significant changes with respect to the w^{1118} control flies. Moreover, bouton counts for additional independent fly lines dTip60^{WT} line A and dTip60^{E431Q} line A crossed to the GAL4 driver elav^{c155} showed similar bouton counts to dTip60^{WT} line B and dTip60^{E431Q} line B, respectively. These additional controls, as well as our observation that both dTip60^{E431Q} and dTip60^{RNAi} showed similar changes in bouton number, confirmed that changes in bouton number were indeed due to disruption of dTip60 at the NMJ (Supplementary Figure S2 A and B).

Our observation that dTip60 is localized both pre and post-synaptically, prompted us to ask whether dTip60 also plays a postsynaptic role in regulating bouton formation at the NMJ. For this analysis, fly lines dTip60^{RNAi}, dTip60^{E431Q} , dTip60^{WT}, and w^{1118} control flies were each crossed to the postsynaptic muscles specific GAL4 driver MEF-2 and quantitation of bouton number at the NMJ was carried out as described for our presynaptic analysis. Similar to presynaptic

knockdown of dTip60, postsynaptic induction of dTip60^{RNAi} and dTip60^{E431Q} resulted in pupal lethality while dTip60^{WT} and w^{1118} control flies showed no observable negative effects on viability. However, quantitative bouton analysis revealed that in contrast to the increased number of boutons we observed in response to presynaptic dTip60 loss (Figure 2), here we observed a significant reduction in bouton number in both dTip60^{RNAi} and dTip60^{E321Q} larvae that was specific for 1s boutons (total number of boutons were 54.33 ± 1.14 for w^{1118}, 39.08 ± 2.17 for dTip60^{E431Q}, 45.92 ± 1.83 for dTip60^{RNAi}, 1s bouton numbers were 27.8 ± 1.11 for w^{1118}, 17.58 ± 1.4 for dTip60^{E431Q}, 19.92 ± 1.49 for dTip60^{RNAi}) as well as the absence of satellite boutons (Figure 3). Of note, no significant changes in bouton number were observed in dTip60^{WT} larvae when compared to the w^{1118} control larvae. Analysis using a second muscle specific driver GAL4 twi;mef2 showed similar results (data not shown). Taken together, our results indicate that dTip60 plays both pre and postsynaptic roles in controlling the degree of synaptic bouton number, and displays at least some presynaptic specificity in preferentially controlling type Is bouton development.

Presynaptic reduction of dTip60 in the nervous system leads to suppression of active zone marker bruchpilot at larval NMJs.

The effects of dTip60 reduction on NMJ expansion led us to ask whether the additional synaptic boutons that were formed in response to presynaptic dTip60 reduction impacted the production of synaptic machinery. To address this question, we analyzed the presence and distribution of two presynaptic vesicle associated proteins essential for synaptic function: the cysteine string protein (csp) that regulates

the activity of presynaptic Ca^{2+} channels to control exocytosis, and synaptotagmin (syt), a protein that promotes both synaptic vesicle fusion and endocytosis mediated vesicle recycling (Bronk, Nie *et al.*, 2005; Evans & Morgan, 2002; Zinsmaier, Eberle *et al.*, 1994), thus functionally "marking" both sides of the vesicle cycle. Immunostainings against these proteins revealed that type Is, Ib and satellite boutons in $dTip60^{RNAi}$ and $dTip60^{E431Q}$ flies were immunoreactive for these markers (Figure 4 A-F shows csp stain; data not shown for syt stain), and that the intensity and distribution of these proteins was identical as compared to wild-type w^{1118} control flies. These results suggested normal morphology and functionality for these particular vesicle proteins in synaptic transmission.

Bouton number within the fly NMJ is constantly changing to correlate with changes in muscle size as development proceeds. Bouton number changes are accompanied by compensatory changes in the number of synapses, so that synaptic functionality is also maintained throughout development (Franco, Bogdanik *et al.*, 2004; Lnenicka & Keshishian, 2000). Thus, it is important to ask whether the expansion in bouton number that we observed in response to dTip60 loss is also accompanied by compensatory changes in synapse number. To address this question, we carried out immunostaining with antibody nc82, a commonly used marker that recognizes the *Drosophila* active zone protein component Bruchpilot (BRP) (C. A. Collins & DiAntonio, 2007; Wagh, Rasse *et al.*, 2006). Active zones are presynaptic specializations where synaptic vesicles accumulate and fuse to the plasma membrane in response to an action potential, and thus nc82 is a commonly used marker for both synapse number and functionality in synaptic transmission.

Both dTip60^{RNAi} and dTip60^{E431Q} larvea showed presence of nc82 staining, confirming the presence of active zones in these dTip60 mutants (Figure 5 A-F). To determine if dTip60 has an impact on the number of active zones within the boutons, we measured the mean fluorescence intensity of nc82 staining which should be proportional to the number of active zones and is a correlate of synaptic function (M. Fischer, Raabe *et al.*, 2009). The percentage maximum intensities for both dTip60^{RNAi} and dTip60^{E431Q} flies were found to be significantly lower when compared to the w^{1118} control line (Figure 5 G,H). Of note, nc82 staining patterns were normal for all UAS fly lines in the absence of the GAL4 transgene. Thus, although there was an increase in bouton number that resulted from dTip60 loss, the boutons show a decrease in the abundance in the active zone synaptic function marker bruchpilot, suggesting potential defects in their neurotransmission function. Taken together, these results demonstrate that Tip60 is required to regulate both bouton number and appropriate production of synaptic transmission machinery during NMJ development.

dTip60 controls bouton expansion by affecting microtubule cytoskeleton dynamics

Regulated microtubule dynamics and architecture is an essential element in the control of synapse division. The formation of synaptic boutons at axon terminals is achieved *via* microtubules that invade and promote bouton budding at the plasma membrane. (C. A. Collins & DiAntonio, 2007; Colon-Ramos, 2009) Hairpin microtubule loop formation is associated with stable synaptic boutons within the *Drosophila* NMJ, while the opening or splaying of these loops is associated with

boutons undergoing division or sprouting. As such, many synaptic bouton NMJ overgrowth phenotypes, particularly those with excess satellite boutons, are also associated with an excess of microtubule loops (Franco, Bogdanik *et al.*, 2004; Roos, Hummel *et al.*, 2000). Thus, we asked whether the increase in normal and satellite boutons we observed in response to dTip60 reduction was related to alterations in microtubule cytoskeleton dynamics. To address this question, we carried out immunostaining against the *Drosophila* MAP1B homolog Futsch, a microtubule associated protein shown to be essential for the stabilization of microtubule hairpin loop formation that promotes synaptic bouton formation (Miech, Pauer *et al.*, 2008; Roos, Hummel *et al.*, 2000). Accordingly, Futsch mutants lacking Futsch protein show a decrease in bouton numbers and Futsch has been shown to completely colocalize with microtubules only at proximal boutons undergoing division, and is not found at terminal boutons as these boutons have typically completed division (Miech, Pauer *et al.*, 2008; Roos, Hummel *et al.*, 2000; Ruiz-Canada, Ashley *et al.*, 2004). Because of these findings, Futsch is a commonly used marker to depict whether Futsch associated microtubule loops have formed that in turn, promote bouton formation and therefore represent sites of active bouton division (Roos, Hummel *et al.*, 2000; Sapountzi, Logan *et al.*, 2006).

Fly lines dTip60^{RNAi}, dTip60^{E431Q} were expressed presynaptically in the motorneurons using the pan-neuronal driver GAL4 driver elav^{C155}, and the number of Futsch immunostained microtubule loops was quantified for each genotype. Fly line w^{1118} was used for a control. Presynaptic overexpression of Tip60^{E431Q} showed a drastic and significant increase in the number of futsch loops as compared to the w^{1118}

control (Figure 6, A-D). Quantification of the number of loops confirmed this increase (15 ± 0.3 for dTip60^{E431Q}, 6 ± 0.4for w^{1118}) (Figure 6 G), consistent with the increased number of boutons we observe (Figure 2). Intriguingly, presynaptic RNAi reduction of dTip60 also revealed a significant increase in futsch positive loops when compared to the control (11 ± 0.5 for Tip60^{RNAi}) (Figure 6 E-F), however the increase was less than that of the dTip60^{E431Q} mutants (Figure 6 C-D). Moreover, in contrast to dTip60^{E431Q} flies, the dTip60^{RNAi} mutants showed increased reorganization and splaying of futsch-associated microtubules shown to precede loop formation in newly divided or dividing boutons (Figure 6 G-H). The differences in Futsch associated microtubule reorganization between dTip60^{RNAi} versus dTip60^{E431Q} line may reflect the different mechanisms of dTip60 knockdown between the two lines. For example, dTip60^{E431Q} effects are directly reliant on the HAT activity of dTip60 and its competition with wild-type dTip60, while dTip60 RNAi induced knockdown may also interfere with additional dTip60 processes, such as dTip60 complex formation with additional proteins that carry out various functions (Utley, Lacoste *et al.*, 2005). Normalization of bouton number to bouton loops in w^{1118} control, dTip60^{RNAi} and dTip60^{E431Q} larvae revealed that there was also a significant increase in this number in the mutant dTip60 larvea when compared to w^{1118} control (Supplementary Figure S3). Of note, Futsch staining patterns were similar to that of w^{1118} control larvae for all UAS fly lines in the absence of the GAL4 transgene, indicating that changes in futsch associated microtubule dynamics resulted from loss of dTip60 in the nervous system. Thus, changes in dTip60 levels via RNAi knockdown or dTip60 HAT activity by dominant negative dTip60^{E431Q} production at the presynaptic level both

affect the organization of microtubule architecture, albeit at different levels, *via* Futsch associated mechanisms, with consequences on synaptic bouton division and formation.

Loss of presynaptic dTip60 in the nervous system results in a reduction of acetylated microtubules in axons extending into terminal boutons.

The increase in bouton number in $dTip60^{RNAi}$ and $dTip60^{E431Q}$ flies was accompanied by an excess of futsch-associated microtubule looping and splaying, suggesting that the boutons are undergoing rapid expansion as a result of being unstable. Microtubule dynamics play an important role in assisting bouton expansion and retraction, with acetylation of α-tubulin associated with microtubule stability and deacetylation associated with destabilization (Baas & Sudo, 2010). Though not important for cell survival, acetylation of microtubules has been shown to affect motor-dependant trafficking throughout neurons (Hammond, Cai *et al.*, 2008). Recently, the histone acetyltransferase Elp3 was shown to directly acetylate microtubules in cortical neurons that appears to contribute to their migration and differentiation (Creppe, Malinouskaya *et al.*, 2009), and control bouton expansion at the *Drosophila* NMJ (Singh, Lorbeck *et al.*, 2011), however additional HATs that may also be involved in this process have not been identified. Thus, we asked whether acetylation of microtubules was reduced in response to dTip60 loss. To address this question, we performed immunohistochemical staining of NMJ boutons with anti-acetylated tubulin, which specifically recognizes α-tubulin in the acetylated state. In w^{1118} control NMJs, an acetylated microtubule network extended into the

terminal boutons and was also present along the entire length of the axon (Figure 7 A and B). In contrast, examination of the NMJs in the dTip60^{E431Q} larvae revealed that acetylated microtubule staining as a whole was decreased (Figure 7 C and D). Closer observation revealed that acetylated microtubule staining was also consistently decreased in the axons extending into the terminal boutons, tapering off towards the end and particularly in those regions showing increased branching and bouton division, suggesting possible loss of stability due to reduced acetylation (Figure 7 C and D.) Of note, dTip60^{RNAi} expression did not show an observable effect on α-tubulin acetylation in the larvae, possibly due to the different mechanisms of dTip60 knockdown between the dTip60^{RNAi} versus dTip60^{E431Q} flies (Lorbeck, Pirooznia *et al.*, 2011a). Additionally, overexpression of dTip60^{WT} also did not show an observable effect in tubulin acetylation level, bouton number or microtubule organization, possibly due to the fact that dTip60 has already reached its maximum function at the NMJ. These findings suggest that the dTip60^{E431Q} induced NMJ expansion phenotype may arise at least in part, from alteration in microtubule networks *via* loss of acetylation, either directly or indirectly by dTip60.

Discussion:

In this report, we investigate a role for dTip60 in synapse development and function using the highly characterized *Drosophila* neuromuscular junction as a model system. We show that dTip60 is highly concentrated at larval motor neuron synaptic boutons and is localized both pre and post synaptically, suggesting that

dTip60 plays a role on both pre and postsynaptic sides of the NMJ in the differentiation of these neurons. In support of this possibility, we show that presynaptic depletion of dTip60 in the nervous system using GAL4 inducible fly lines $dTip60^{RNAi}$ and HAT defective $dTip60^{E421Q}$ results in a significant expansion of synaptic bouton number, indicating that this HAT negatively controls synaptic bouton formation and differentiation at the presynaptic side of the NMJ during the third instar larval stage. Interestingly, only type Is and not type Ib bouton numbers undergo significant expansion in response to dTip60 loss, supporting partial specificity in dTip60 function in certain bouton types. In addition to an increase in type Is boutons, we also observed an increase in the production of satellite boutons in response to dTip60 loss. Satellite bouton production has been observed for certain *Drosophila* proteins that affect synaptic plasticity, including overexpression of the *Drosophila* amyloid precursor protein APPL, a pan-neuronal protein implicated in Alzheimer's disease (Torroja, Packard *et al.*, 1999) and Shaggy, the *Drosophila* homolog of the glycogen synthase kinase 3 (GSK3β), a kinase that negatively controls NMJ growth via microtubule cytoskeleton dynamics (Franco, Bogdanik *et al.*, 2004). It is thought that satellite bouton formation is caused by an increased rate of sprouting and subsequent abnormal bouton differentiation (Budnik & Ruiz-Canada, 2006; Torroja, Packard *et al.*, 1999), thus implicating dTip60 in these processes. Remarkably, we find that there is an opposite effect on synaptic bouton number at the NMJ in response to postsynaptic Tip60 knockdown using both $dTip60^{RNAi}$ and $dTip60^{E431Q}$ HAT defective lines when compared to presynaptic knockdown, in that there is a significant reduction in bouton number, with virtually

no formation of satellite boutons. Consistent with our finding, there are a number of NMJ proteins that contribute both pre and post-synaptically in the control of synaptic plasticity. For example, Discs Large (Dlg) is localized to type-1 glutamatergic synaptic terminals pre and post synaptically where it serves as a major scaffolding component at the larval NMJ (K. Chen & Featherstone, 2005; Packard, Koo *et al.*, 2002). Spastin is another protein expressed at the NMJ that is concentrated both pre and post-synaptically with inappropriate localization having effects on microtubule stability that affect synaptic growth (Sherwood, Sun *et al.*, 2004; Trotta, Orso *et al.*, 2004). The translational repressor Nanos also influences bouton number at the *Drosophila* NMJ from both the pre and postsynaptic sides (Menon, Andrews *et al.*, 2009). Importantly, anterograde and retrograde modes of signaling are required at sites of synaptic contact and these signaling pathways play critical roles in the formation, differentiation and plasticity of synaptic connections (Zweifel, Kuruvilla *et al.*, 2005). For example, synaptic development at the *Drosophila* NMJ is dependent upon the bidirectional influence of wingless signaling on both pre and postsynaptic structures *via* distinct intracellular pathways (Miech, Pauer *et al.*, 2008). Moreover, specific pre and postsynaptic levels of the cell adhesion molecule Fasciclin II are required to regulate appropriate synaptic growth *via* signaling through the fly homolog of amyloid precursor protein APPL (J. Ashley, Packard *et al.*, 2005). Intriguingly, dTip60 has also been shown to be involved in APP signaling pathways *via* its complex formation with the C-terminal fragment of the amyloid precursor protein (APP) known as the APP intracellular domain (AICD) and linker protein Fe65 (Baek, Ohgi *et al.*, 2002; Cao & Sudhof, 2001; Schettini, Govoni *et al.*, 2010; von

Rotz, Kohli *et al.*, 2004). Thus, based on the pre and postsynaptic staining pattern of dTip60, and the opposite phenotypic consequences on bouton number that results from its pre versus postsynaptic reduction, it is tempting to speculate that dTip60 may also be required for bi-directional signaling at the NMJ to regulate synapse formation and function.

Active zones are presynaptic specializations where synaptic vesicles accumulate and fuse to the plasma membrane in response to an action potential. Importantly, they are located in perfect opposition to the glutamate receptors on the postsynaptic side of the NMJ, and thus are a commonly used marker for both synapse number and functionality in synaptic transmission. Electron-dense cytoplasmic projections termed T-bars are often observed that extend from the active zone into the presynaptic cytoplasm and are believed to facilitate vesicle movement to this site to mediate neurotransmitter release (Fouquet, Owald *et al.*, 2009; E. L. Johnson, 3rd, Fetter *et al.*, 2009). Bruchpilot (brp) is a scaffolding protein found in *Drosophila* at the active zones where it localizes to T-bars and is essential for synaptic transmission (Kittel, Wichmann *et al.*, 2006; Wagh, Rasse *et al.*, 2006). Here, we show that although there was an increase in bouton number that resulted from presynaptic dTip60 loss, the boutons show a significant reduction in the abundance in the active zone synaptic function marker bruchpilot, suggesting a decrease in their neurotransmitter function and linking dTip60 HAT activity to this process. As postsynaptic reduction of dTip60 results in a significant reduction of 1s boutons at the NMJ, it will be important for future studies to investigate whether these remaining boutons are also negatively impacted, in order to determine whether there is

postsynaptic contribution of dTip60 on Bruchpilot expression and bouton function. Intriguingly, the precise regulation of BRP mediated neurotransmission process has been shown to be critical for higher order nervous system function that includes learning, memory and cognition (Knapek, Sigrist *et al.*, 2011) . Thus, it is tempting to speculate that dTip60 HAT activity plays a critical role in the control of neurotransmission important for these processes.

Tip60 HAT activity has been implicated in the age-related neurodegenerative disorder Alzheimer's disease (AD) *via* its HAT dependent complex formation with the C-terminal fragment of the amyloid precursor protein (APP) known as the APP intracellular domain (AICD) and linker protein Fe65 (Baek, Ohgi *et al.*, 2002; Cao & Sudhof, 2001; Schettini, Govoni *et al.*, 2010; von Rotz, Kohli *et al.*, 2004). The association of these proteins, termed the AFT complex, epigenetically control the transcriptional regulation of target genes involved in neuronal function (Baek, Ohgi *et al.*, 2002; Schettini, Govoni *et al.*, 2010). Interestingly, the *Drosophila* NMJ phenotype that results from presynaptic inhibition of Shaggy kinase activity (Franco, Bogdanik *et al.*, 2004) is very similar to our dTip60 HAT presynaptic depletion mutant NMJ phenotype, which includes expansion of synaptic bouton number, and an increase in satellite bouton formation that is accompanied by an excess of Futsch stained microtubule loops. Shaggy is thought to function in the negative regulation of bouton expansion *via* phosphorylation of the MAP1B microtubule binding protein Futsch, thus inhibiting its action in promoting formation of the microtubule loops that are associated with stable bouton formation (Miech, Pauer *et al.*, 2008; Roos, Hummel *et al.*, 2000). Intriguingly, Gsk3β (the mammalian shaggy homolog) has

been shown to be a direct transcriptional target of the AFT complex, where Tip60 HAT activity is required for the epigenetic control of Gsk3β transcriptional activation (von Rotz, Kohli *et al.*, 2004). Based on these findings, the NMJ overgrowth phenotype shared by Tip60, Shaggy and APPL (*Drosophila* APP homolog) mutants may suggest that these proteins are involved in overlapping transcriptional regulatory pathways that could potentially be involved in the synaptic defects observed in early AD progression. Additionally, as we observed a decrease in bruchopilot immunostaining and an increase of Futsch stained microtubule loops in our dTip60 mutant larvae, it will be important in future studies to determine whether these neuronal marker genes, as well as *shaggy* are direct transcriptional targets of dTip60.

Alternatively, dTip60 may also directly regulate synaptic microtubule architecture independent of epigenetic based transcriptional mechanisms. For example, we found that α-tubulin acetylation levels of microtubules specifically extending into the terminal synaptic boutons are reduced in response to dominant negative HAT defective $dTip60^{E431Q}$ overexpression, but not $dTip60^{RNAi}$ induced knockdown. One possible explanation for this observation is that in the $dTip60^{E431Q}$ mutant, there is competition for acetylation of tubulin between the wild-type dTip60 and $dTip60^{E431Q}$ protein, whereas in the RNAi induced knockdown, there is still enough residual endogenous dTip60 protein available for acetylation function. Consistent with this interpretation, the decrease in tubulin acetylation levels we observe in the $dTip60^{E431Q}$ larvae is subtle, possibly indicative of the presence of endogenous dTip60 protein and/or other endogenous factors that can still acetylate tubulin. One such endogenous factor may be the HAT Elp3, an acetylase shown to

directly acetylate microtubules in cortical neurons that contributes to their migration and differentiation (Creppe, Malinouskaya *et al.*, 2009). These studies demonstrate that loss of Elp3 in cultured projection neuronal cells leads to severe defects in axonal branching (Creppe, Malinouskaya *et al.*, 2009; Gardiner, Barton *et al.*, 2007; Wynshaw-Boris, 2009). Intriguingly, our previous studies investigating a role for Elp3 in synaptic plasticity demonstrate a very similar phenotype to our dTip60 mutants, in that bouton expansion is significantly increased in response to presynaptic RNAi induced Elp3 loss (Creppe, Malinouskaya *et al.*, 2009). Thus, it will be important to determine whether expression, localization and activity of Elp3 is affected at the NMJ in our dTip60 mutant lines. We do note that the link we observe between dTip60 loss and reduction of α-tubulin acetylation levels of microtubules at the NMJ is correlative in nature. Therefore, it will also be important to decipher whether dTip60 acts indirectly or directly to acetylate tubulin on specific residues and whether this acetylation directly impacts specific biological processes such as microtubule architecture and/or influences interaction with microtubule binding proteins such as Futsch. Interestingly, here we observe that although acetylation of tubulin is not significantly decreased in response to $dTip60^{RNAi}$ induced knockdown, pupal lethality still occurs, suggesting that the acetylation of tubulin cannot be specifically linked to nervous specific dTip60 induced lethality. These results are consistent with studies on HDAC6 knockout mice, a class II HDAC known to target K40 acetylation of α-tubulin, demonstrating that although these mice display a significant increase in tubulin acetylation in the brain, this phenomenon does not

rescue disease progression in a mouse model of Huntington's disease (Bobrowska, Paganetti *et al.*; J. S. Guan, Haggarty *et al.*, 2009)

The emerging hypothesis that age-related aberrant changes of specific acetylation marks in chromatin in the adult brain lead to gene misregulation that drives cognitive decline and specifically, memory impairment in the elderly, underscores the crucial role HATs play in cognitive ability (Peleg, Sananbenesi *et al.*, 2010; Sweatt, 2010). A role for HAT activity in learning and memory is not unprecedented, with the HAT CBP implicated as a critical component of memory consolidation via the site specific histone acetylation of chromatin in the brain that in turn, regulates long-term transcriptional changes associated with long-lasting forms of neuronal plasticity (Korzus, Rosenfeld *et al.*, 2004). Importantly, recent studies show that synaptic activity can influence such CBP associated histone acetylation marks, thus providing a mechanism for how external environmental stimuli can influence behavioral dependant synaptic plasticity that is linked to memory. In this model of synaptic activity-dependent epigenetic regulation, synaptic input and depolarizing stimuli cause an increase in Ca2+ intracellular levels via specific Ca2+ channels, thus activating certain kinases to phosphorylate CBP which is thought to promote its recruitment to chromatin (Hardingham, Chawla *et al.*, 1999; Riccio, 2010). CBP mediated specific histone acetylation marks cooperate with additional epigenetic modifications to induce chromatin structural changes that regulate gene expression of synaptic activity-dependant genes. In support of this model, genome-wide screens of mouse cortical neurons using ChIP-seq have shown that membrane depolarization markedly enhances CBP recruitment to such enhancers (T. K. Kim,

Hemberg *et al.*, 2010). Intriguingly, neural activity can also modulate chromatin acetylation by regulating the shuttle of class II HDACs in and out of the nucleus in hippocampal neurons (de Ruijter, van Gennip *et al.*, 2003). As dTip60 has also been shown to shuttle between nuclear and cytoplasmic cellular compartments, with misregulation of this process associated with prostate cancer (Halkidou, 2003; H. J. Lee, Chun *et al.*, 2001), it is tempting to speculate that cellular localization of Tip60 influences synaptic plasticity in a manner similar to certain HDACs. Our findings causatively linking Tip60 HAT activity to the control of synaptic bouton formation and function, in conjunction with future studies deciphering the mechanisms of how Tip60 controls such processes, should further our understanding of epigenetic mechanisms underlying synaptic plasticity and memory formation in neurodevelopment, age-related cognitive decline and neurodegenerative disorders.

Chapter 3: An epigenetic role for Tip60 in gene control that regulates synaptic plasticity at the *Drosophila* Neuromuscular Junction

Abstract:

Age related cognitive decline and neurodegenerative disorders such as Alzheimer's Disease (AD) are associated with misregulation of genes involved in a variety of neuronal process including synaptic plasticity. Histone acetylation promotes dynamic transcriptional responses in the epigenome of neurons in turn influencing activity dependent neuronal activity that plays critical roles in cognitive function.

We have previously shown the importance of Tip60 in regulation of pre-synaptic bouton growth and function. Here we report that Tip60 not only controls synaptic plasticity when misregulated post-synaptically but also affects the localization of post-synaptic components such as DLG, GluRIIB and GluRIIC. We demonstrate via real time PCR that loss of HAT activity of Tip60 at the NMJ leads to misregulation of genes important in activity dependent synaptic plasticity and components of the wingless pathway. ChIP-qPCR analysis of the genes misregualted presynaptically further reveals changes in enrichment levels of epigenetic modifications H4K12ac and H4K16ac, marks linked to learning and memory in the Tip60 HAT defective mutants suggesting a role for Tip60 in the wg pathway via epigenetic modifications of the genes. We also report the functional interaction between HAT deficient Tip60 and hAPP at the NMJ, pre- and post-synaptically via

the intracellular domain of APP (AICD), the molecule implicated in AD. Pre-synaptic expression of APP/Tip60 double mutants cause drastic increases in bouton numbers, and decrease in active zone synaptic function marker bruchpilot suggesting defects in neurotrasmission. Conversely, post-synaptic expression of the APP/Tip60 double mutants leads to marked decrease in bouton numbers and absence of GluRIIC and GluRIIB receptor subunits.

Our results are the first to demonstrate the role of Tip60 HAT activity in regulating components of the wingless pathway via acetylation of H4K12 and H4K16 marks in the coding region of these genes. We also demonstrate the functional interaction of Tip60 and APP at the NMJ and thus implicating Tip60 HAT activity in neurodevelopmental and neurodegenerative processes.

Introduction

Synaptic plasticity is a highly regulated process requiring anterograde and retrograde modes of signaling pathways that play a critical role in synapse formation, differentiation and maintenance of plasticity (Ginty & Segal, 2002; Tao & Poo, 2001; Zweifel, Kuruvilla *et al.*, 2005). Synaptic connections initially formed during development are further modified to form adult patterns brought about by eliminating weak and incorrect synaptic connections and reinforcing appropriate connections. This activity-dependent process is brought about by coordinated and regulated pre- and postsynaptic activities that induce long-term potentiation (LTP) or long-term depression (LTD) during development (Artola & Singer, 1990; Tao & Poo, 2001;

Zhang & Wu, 2000). Numerous pre and post-synaptic components at the NMJ have been shown to interact to bring about this pre- and post synaptic communication.

Epigenetic mechanisms have been shown to play important roles in many human diseases such as prostate cancer, neurodegeneration namely Alzheimer's Disease (AD) and Parkinson's (PD), neurodevelopmental disorders such as ASD, Fragile X Syndrome where changes in DNA methylation and histone acetylation have been shown to be altered (Levenson, O'Riordan *et al.*, 2004; Levenson & Sweatt, 2005; Sweatt, 2010). Recent studies in neurodegenerative diseases such as Alzheimer's and Huntington's have brought to light the role of HATs in particular, as important epigenetic code modifiers essential for regulating neuronal gene expression and shed light on activity dependent mechanisms in neurons (Ferrante, Kubilus *et al.*, 2003; Marambaud, Wen *et al.*, 2003; Saura, Choi *et al.*, 2004; Steffan, Bodai *et al.*, 2001). Histone modifications have been shown to be important in regulating neural-activity dependent transcription. Studies have shown that acetylation by histone acetyltransferases (HATs) play an important role in regulating gene transcription required in LTP (Crosio, Heitz *et al.*, 2003). Of note, CBP was the only HAT to be shown to be conclusively involved in synaptic plasticity wherein inactivation of HAT CBP characterized by decrease in H2B acetylation caused impaired induction of transcription-dependent late-phase LTP that can be ameliorated by treating the mice with HDAC inhibitors (SAHA) thus restoring acetylation levels (Alarcon, Malleret *et al.*, 2004). Recent work from our lab has shown that Tip60 is another HAT to be involved in a variety of neuronal processes such as synaptic plasticity, axonal outgrowth, axonal transport and sleep (A. A. Johnson, Sarthi *et al.*, 2013; Pirooznia,

Sarthi *et al.*, 2012; Sarthi & Elefant, 2011). Tip60 is present at the *Drosophila* NMJ both pre and post-synaptically and increases bouton formation when misregulated presynaptically in part due to disrupted MT organization (Sarthi & Elefant, 2011). In this study, we show that Tip60 affects various components of the wg pathway either directly or indirectly when misregulated pre-synatically thus shedding light on its role in anterograde signaling. However, due to its post-synaptic localization we believe it could be involved in retrograde signaling as well and thus be part of retrograde signaling pathways at the NMJ.

Here we show that post-synaptic Tip60 is also important in synapse formation and regulation and loss of Tip60 HAT activity affects localization of postsynaptic components such as DLG, GluRIIB and GluRIIC and also has a pre-synaptic output where it affects bouton numbers and branching thus suggesting a retrograde signaling mechanism involving Tip60. We also show a number of synaptic function genes misregulated in the Tip60 HAT defective mutants suggesting transcriptional regulation of these genes by Tip60 HAT activity. Furthermore, we also show that Tip60 is not just important during neurodevelopment but also plays an important role in neurodegeneration. Here we show that, in flies over-expressing hAPP and the HAT deficient dTip60 the effects seen are more pronounced both pre- and post-synaptically when compared to just dTip60 alone. These defects are partially rescued in flies expressing a C-terminal deficient hAPP and dTip60. This suggests a functional interaction between Tip60 and APP at the NMJ and suggests a role for Tip60 HAT function in APP mediated neurodegenerative process. In conclusion we show through ChIP studies that the misregulated synaptic plasticity genes show changes in various

acetylation marks associated with learning and memory, thus demonstrating that Tip60 HAT function is essential in cognition process important in both neurodevelopment and neurodegeneration.

Materials and Methods:

Drosophila Stocks:

All fly lines used were raised under standard conditions at 22°C on standard yeasted *Drosophila* media (Applied Scientific Jazz Mix *Drosophila* Food, Thermo Fisher Scientific). The pan-neuronal driver $elav^{c155}$ were obtained from Bloomington Stock Center (Bloomington, IN). Muscle specific GAL4 driver Twi;Mef-2 was a generous gift from Xianmin Zhu. Independent transgenic fly line $dTip60^{WT}$ (line C), $dTip60^{E431Q}$ (independent lines A and B), were created as previously described (Lorbeck, Pirooznia *et al.*, 2011b; Zhu, Singh *et al.*, 2007). APP; $dTip60^{E431Q}$ (independent lines A and B), APP dCT;$dTip60^{E431Q}$ (independent lines A and B); APP;$dTip60^{WT}$ (line C), and APP dCT; $dTip60^{WT}$ (line C) were created as previously described by standard genetic crosses (Pirooznia et al., 2012b). All experimental crosses were performed at normal physiological temperature of 25°C.

Immunohistochemistry:

Third instar larvae were filleted in HL-3 saline, pH7.2 and pinned out on Sylgard dishes with guts removed. Fillet preparations were fixed in 3.5% paraformaldehyde for 30 minutes (for glutamate receptor staining experiments

Bouin's Fixative was used to fix larval fillet preparations for 25 mins), followed by 3 washes in PBS and one with 1X PBS with 0.1% Triton.). The larvae were then incubated overnight in primary antibody overnight at 4°C. After 6 washes in PBS-T (1x phosphate buffered saline +0.1% Triton), fillets were incubated in 2° antibody for 1 hour, washed twice in 1x PBS-T, once in 1x PBS, then mounted onto slides in Vectashield antifade mounting media (Vector laboratories, Burlingame CA). Confocal microscopy was performed using Olympus Microscope with fluoview software.

Antibodies:

The primary antibodies, anti-DLG (4F3), anti-Futsch (22C10), anti-nc82, and anti-GluRIIA antibodies were all obtained from Developmental Studies Hybridoma Bank, DSHB (University of Iowa, Iowa City, IA). Each of these antibodies was diluted in block (2%BSA, 5%NGS in 1XPBS-T) and used at a dilution of 1:10. *Anti-Syt* (*Littleton et al*., 1993) was a generous gift from Hugo Bellen and was used at 1:5000 dilution. Anti-GluRIIB and anti-GluRIIC were generous gifts from Aaron DiAntonio and were used at 1:2500 and 1:5000 dilutions respectively. The HRP Antibody was obtained from Jackson Immunoresearch Laboratories Inc. and used at a dilution of 1:25**.**

Imaging and Analysis:

Larval NMJ's were imaged on a Olympus (Germany) FV1000 laser scanning confocal microscope. All images were captured using same constant confocal gain settings. Images were acquired as a Z-stack and then rendered as a maximum

projection. Individual entire muscle 6/7 NMJ synapses were optically sectioned (0.2 um; series of 15 to 20 sections per synapse). A 2D projection was generated that projected the pixel of maximal intensity found within the series of sections. Quantitative analysis of fluorescence intensities for nc82 quantitation was done using FV1000 software. Image J was used for all other quantitation and cluster size anaylsis was done using the Image edge detection plugin. For comparison between genotypes, all samples were processed simultaneously and imaged using identical microscopic acquisitions parameters. All images were also corrected for any background before any intensity measurements. Student's T-test was used for all statistical analysis.

Real-time PCR analysis:

Total RNA was isolated from wandering third instar dTip60^{E431Q}B and wild-type larvae using the RNeasy Plus Mini Kit (QIAGEN). cDNA was prepared using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions with 1 μg total RNA and 0.2 μg/ml random hexamer primers (Roche Applied Science). PCRs were performed in a 20 μl reaction volume containing cDNA, 2 X Power SYBR Green PCR Master Mix (Applied Bio- systems), and 10 μM both forward and reverse primers (primer pairs available upon request). PCR was performed using an ABI 7500 Real-Time PCR system (Applied Biosystems) following the manufacturer's instructions. Fold change in mRNA expression was determined by the $\Delta\Delta$Ct method (Livak & Schmittgen, 2001).

Chromatin immunoprecipitation (ChIP) and qPCR analysis:.

Chromatin from third instar larval heads was prepared as follows. Approximately 300 third instar larval heads for each genotype were homogenized first with a pestle and then using a potter homogenizer (5 strokes) containing homogenization buffer (60 mM KCl, 15 mM NaCl, 4 mM MgCl, 15 mM HEPES, pH 7.6, 0.5% Triton X-100, 0.5 mM DTT, protease and phosphatase inhibitors mixture; Roche). Cross-linking was performed for 15 min by adding formaldehyde to a final concentration of 1.8%. Cross-linking reaction was stopped by adding glycine solution (final concentration of 225 mM) followed by incubation on ice for 5 min. Fixed tissue homogenate was then washed 3 homogenization buffer and 1X in lysis buffer (140 mM NaCl, 15 mM HEPES, pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, protease and phosphatase inhibitors [Roche], 1 mM PMSF, and 10 mM sodium butyrate). Next, lysis buffer containing SDS (0.1%) and sodium lauryl sar- cosine (0.5%) was added to the homogenate and incubated for 10 min at 4°C. After incubation, the cells were disrupted by 8 pulses for 30 s each on ice using a tip sonicator (Fisher Scientific Sonic Dismembrator 50 Ultrasonic Homogenizer), yielding sheared chromatin fragments ranging from 200 to 500 bp. The sheared chromatin was then incubated at 4°C for 10 min and centrifuged twice, yielding the final chromatin extract for ChIP.

Chromatin precipitation assays were performed using ChIP-IT Ex- press Kit (Active Motif), following the manufacturer's instructions. Briefly, ChIP was performed with 20 μg sheared chromatin for acetylated H3K9, H4K12 and H4K16 antibodies (0.5 μl) (Active Motif) and 30 μg sheared chromatin for Tip60 antibody (2

μg) (Abcam). A mock reaction containing all reagents, except the antibody, was performed simultaneously for each sample as a control. The chromatin was immunoprecipitated using the ChIP IT Express kit (Active Motif) exactly following the manufacturer's specifications. The eluted material from the immunoprecipitation was then purified using QIAquick PCR purification kit (QIAGEN) and was directly used for real-time PCR. PCRs were performed in triplicate a 20 μl reaction volume containing 10 μl of SYBR Green PCR master mix (Applied Biosystems), 1 μl of DNA template, and 0.5 M each of forward and reverse primer for each gene sequence (primer pairs available upon request). Quantitative PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions. For each primer set, fold enrichment was calculated using the slope of a standard curve according to the manufacturer's instructions in the ChIP-IT Express kit. Briefly, a standard curve was generated from serial 10-fold dilutions of the Input DNA. The Ct values were used to estimate DNA quantity of the ChIP and no antibody mock control samples. Fold enrichment was calculated as a ratio of the DNA quantity in the ChIP and no antibody mock control.

Results:

Postsynaptic knockdown of Tip60 HAT activity affects localization of DLG.

Discs Large 1 (DLG) is a member of the MAGUK family of proteins. DLG is expressed presynaptically prior to its postsynaptic localization and is required for normal synaptic function (B. Guan, Hartmann *et al.*, 1996; Lahey, Gorczyca *et al.*, 1994). There are at least three locations involved in postsynaptic trafficking of DLG,

the intracellular subcortical network, the plasma membrane and the synaptic membrane. DLG immunoreactivity shifts continuously throughout development from extrasynaptic sites to synaptic regions. By the third instar stage DLG shows exclusive localization at synapses with minimal DLG detected at the surface and subcortical regions (Thomas, Ebitsch *et al.*, 2000).To identify the factors affected by loss of boutons caused by postsynaptic knockdown of Tip60 HAT activity we carried out immunostaining experiments with DLG and syt, a presynaptic transmembrane protein present in synaptic veiscles. We observed that unlike the control larvae that showed sharp and exclusive localization at synapses, the mutants showed diffused and disorganized DLG pattern around the synapses in the dTip60^{E431Q}B and dTip60^{E431Q}A mutants (Fig. 1C-F). This type of diffused DLG pattern has been seen during synapse dismantling process that takes place during the pupal stages of development starting from 6h APF (Z. Liu, Chen *et al.*, 2010). This diffused staining of DLG is followed by loss of boutons thus contributing to the dismantling process. The syt vesicle staining however was unaffected (data not shown). Of note, presynaptic reduction of Tip60 HAT activity did not lead to any misregulation of DLG localization suggesting distinct mechanisms and pathways Tip60 could be involved in at the NMJ with respect to DLG localization. In summary, when we misregulate Tip60 HAT in the muscles the decrease in bouton numbers could be at least in part due to the postsynaptic disorganization of DLG and possibly other postsynaptic components.

Postsynaptic knockdown of Tip60 HAT activity affects futsch loops.

New bouton formation at synapses is achieved by microtubules that invade and promote bouton budding at the plasma membrane. Many NMJ overgrowth phenotypes are associated with increase in the microtubule loops (Franco, Bogdanik *et al.*, 2004; Roos, Hummel *et al.*, 2000). We have previously shown that the increase in bouton numbers due to the presynaptic reduction of Tip60 HAT activity leading to increased bouton numbers was in part due to increased microtubule loops structures as seen by immunostaining the *Drosophila* MAP1B homolog Futsch. Futsch is a microtubule-associated protein essential in promoting bouton formation by stabilization of MT hairpin loop (Miech, Pauer *et al.*, 2008; Roos, Hummel *et al.*, 2000). Futsch knockdown mutants show decreased bouton formation characterized by decrease in number of futsch loops (Roos, Hummel *et al.*, 2000; Ruiz-Canada, Ashley *et al.*, 2004). Thus, we asked whether the decrease in bouton numbers and satellite boutons we observed in response to postsynaptic dTip60 reduction was due to altered microtubule cytoskeleton dynamics.

Fly lines dTip60^{E431Q}A, dTip60^{E431Q}B were expressed postsynaptically in the muscles using the muscle GAL4 driver Twi;Mef2 and the number of futsch immunostained microtubule loops was quantified for each genotype. w^{1118} was used as a control. Postsynaptic expression of dTip60^{E431Q}A and dTip60^{E431Q}B mutants showed significant decrease in number of futsch loops compared to w^{1118} control (Fig. 2C-F). The total number of loops was quantified and was consistent with the decrease in bouton numbers we observe (7.22 ± 0.49 for w^{1118}, 4.85 ± 0.57 for Tip60 ^{E431Q}B, 5.55 ± 0.64 for Tip60^{E431Q}A and 7 ± 1.06 for Tip60^{WT}). Thus, these data support our hypothesis that post-synaptic dTip60 is also important in maintaining

normal bouton numbers and structure at the NMJ in part via organization of the microtubule architecture.

Tip60 mutants show defects in glutamate receptors at the NMJ

The decrease in active zone abundance in the Tip60 HAT defective mutants when misregulated pre-synaptically prompted us to take a closer look at the glutamate receptors that are present opposite to the active zones post-synaptically. The *Drosophila* NMJ is glutamatergic consisting of non-NMDA receptors homologous to the vertebrate non-NMDA receptors (DiAntonio, Petersen *et al.*, 1999; Marrus, Portman *et al.*, 2004; Qin, Schwarz *et al.*, 2005; Schuster, Ultsch *et al.*, 1991). There are five different ionotropic receptors in *Drosophila* GluRIIA, GluRIIB, GluRIIC, GluRIID and GluRIIE of which GluRIIC, GluRIID and GluRIIE are the essential subunits present in two types of receptors namely, Type A and Type B each consisting of GluRIIA and GluRIIB respectively (Mayer & Armstrong, 2004; Sun, Olson *et al.*, 2002). To investigate the presence of glutamate receptors $Tip60^{E431Q}A$, $Tip60^{E431Q}B$ and $Tip60^{WT}$ were expressed post-synaptically using the Twi;Mef2-GAL4 drivers. We chose to look at the presence of GluRIIA and GluRIIB as they form the two types of subunits and GluRIIC as it is one of the essential subunits. We observe presence of both GluRIIA and GluRIIB in the mutants compared to the w^{1118} controls, however there was significant decrease in the number of GluRIIC clusters in the $Tip60^{E431Q}A$ and $Tip60^{E431Q}B$ mutants compared to w^{1118} control larvae (Fig. 3C-F). As demonstrated earlier, $Tip60^{WT}$ flies showed no significant difference in number of clusters when compared to control flies (Fig 3.G,H). Overall, these data

suggest that Tip60 HAT activity affects the localization of GluRIIC subunits at the NMJ.

Genes associated with synaptic function are misregulated in dTip60^{E431Q} mutants pre- and post-synaptically

Our results thus far indicate that dTip60 is required for normal bouton growth, division and maintenance and growth of both the pre- and post synaptic sides of the NMJ (Sarthi & Elefant, 2011). Tip60 is a HAT and is predominantly involved in gene regulation and transcriptional activation. Recent data from our lab shows its role in regulating genes involved in apoptosis and axonal transport (A. A. Johnson, Sarthi *et al.*, 2013; Pirooznia, Sarthi *et al.*, 2012). Based on previous data and data presented here we show that dTip60 is important for proper distribution and function of various proteins present at the NMJ such as Futsch, Brp, Dlg and GluR. Numerous *in vivo* and *in vitro* studies have shown that AICD is involved in the regulation of GSK-3β, the human homolog of *Drosophila* shaggy (sgg) (Baek, Ohgi *et al.*, 2002; H. S. Kim, Kim *et al.*, 2003; von Rotz, Kohli *et al.*, 2004). Furthermore, Tip60 has been demonstrated to interact with the intracellular domain of APP (AICD) and bring about transcriotonal activation of target genes (Duilio, Zambrano *et al.*, 1991; Minopoli, de Candia *et al.*, 2001; Sterner & Berger, 2000). The non-canonical wg pathway involved in synaptic bouton growth has been shown to inactivate sgg and thus activate futsch thus suggesting a possible role for Tip60 in the wg pathway. Microarray data from our lab also indicate misregulation of synaptic plasticity genes in the dTip60 HAT defective mutants (Lorbeck, Pirooznia *et al.*, 2011b). Pathway

analysis using the Panther classification of the microarray targets also identified genes linked to synaptic plasticity (Supp.Fig. 1). We therefore performed quantitative real-time PCR using staged third instar dTip60^{E431Q}B brains and NMJs under the control of elav-GAL4 and Twi;Mef2-GAL4 drivers respectively and quantified the relative mRNA expression levels of candidate synaptic genes identified (Fig.4). Since we observe two opposing effects when dTip60 is misregulated pre- and post-synaptically we performed qPCR experiments with two sets of genes predominantly present on each side and checked for their expression levels. Wg (wingless) and sgg (Shaggy) and dsh (disheveled), three genes involved in the wg pathway were found to be significantly upregulated in response to Tip60 HAT loss pre-synaptically using the elav-GAL4 driver. The genes involved in the wg pathway have been shown to be important in maintenance of synaptic plasticity (Cook, Fry *et al.*, 1996; Miech, Pauer *et al.*, 2008; Packard, Koo *et al.*, 2002). Additional genes that were upregulated presynaptically were genes encoding the Sh (Shaker) channel, an ion channel involved in activity dependent synaptic plasticity and Hdac6, a confirmed HDAC shown to specifically deacetylate α-tubulin subunit of microtubules (Budnik, Zhong *et al.*, 1990; Zhang, Li *et al.*, 2003). This result is consistent with our previous finding at the NMJ showing decrease in α-tubulin acetylation levels in the terminal boutons (Sarthi and Elefant, 2011). Futsch, which was one of the targets identified showed no significant change despite being overexpressed pre-synaptically in the boutons (Sarthi & Elefant, 2011) (Fig 4A). On the other hand, Tip60^{E431Q}B mutants expressed in the muscles using Twi;Mef2/GAL4 driver demonstrate down-regulation of key genes involved in postsynaptic activity dependent retrograde signaling components such as

wg, GluRIIA, GluRIIB, GluRIIC as well as the post synaptic scaffold protein DLG (Fig 4B). In summary, identification of genes involved in the wg pathway and activity dependent synaptic plasticity pre- and post-synaptically in response to loss of Tip60 HAT activity supports a role for Tip60 in transcriptional regulation of genes involved in synaptic plasticity as well as activity dependent control of NMJ growth and maintenance.

APP-mediated effects of Tip60 on synaptic bouton growth and function, role in neurodegeneration:

APP and Tip60 functionally interact pre-synaptically

Tip60 is a HAT and is known to be important in numerous cellular processes such as DNA repair, apoptosis and cell-cycle checkpoints as well as interacts with a number of cytoplasmic receptors such as androgen receptor (AR), endothelin receptor thus activating many downstream pathways (Sapountzi, Logan *et al.*, 2006). As part of its cytoplasmic function it has been shown to interact with the C-terminus of APP (AICD), a ubiquitous transmembrane protein involved in the pathophysiology of Alzheimer’s disease. This interaction is mediated by Fe65 *in vivo* and is involved in downstream signaling to the nucleus where Tip60/AICD target genes are modified (Baek, Ohgi *et al.*, 2002; Cao & Sudhof, 2001). Microarray data from our lab has uncovered numerous dTip60 target genes that are important in neuronal processes such as olfaction, synaptic plasticity and learning and memory (Lorbeck, Pirooznia *et al.*, 2011b). Since Tip60 interacts with APP, which is the molecule involved in AD,

we further investigated the role of Tip60 in AD, a neurodegenerative disease involving the loss of synaptic connections leading to cognitive decline.

Fly lines dTip60^{E431Q}A, APP, APP$^{\Delta CT}$, APP/dTip60^{E431Q}A, APP$^{\Delta CT}$/Tip60^{E431Q}A, Tip60^{WT}, APP/Tip60^{WT}, APP$^{\Delta CT}$/Tip60^{WT} and w^{1118} were expressed pre-synaptically in the nervous system using the pan-neuronal elav-GAL4 driver. Quantitation of total number of boutons for each of the genotypes revealed drastic increase in the APP and APP/Tip60^{E431Q}A mutants compared to the w^{1118} (total bouton numbers were 56.45 ± 1.45 for w^{1118}, 120.58 ± 3.68 for APP, 122 ± 4.61 for APP/Tip60^{E431Q}A) (Fig. 5G). On further investigation, though the APP and APP/Tip60^{E431Q}A lines showed similar increase, the APP/Tip60^{E431Q}A boutons were noticeably smaller in size when compared to the APP lines thus suggesting a more pronounced physiological/morphological change when APP and dTip60 were both misregulated instead of APP alone (Fig. 5C, F). The mutants with the deleted C-terminus (APP$^{\Delta CT}$) did not show any increase in total bouton numbers and were very similar to the w^{1118} control larvae (55.9 ± 2.41 for APP$^{\Delta CT}$.) (Fig. 5A,D,G). However, APP$^{\Delta CT}$/Tip60^{E431Q}A mutants showed numbers similar to Tip60^{E431Q}A alone (76.45 ± 2.24 for Tip60^{E431Q}A and 66 ± 1.98 for APP$^{\Delta CT}$/Tip60^{E431Q}A) (Fig. 5B,E,G).

We next investigated the abundance of the active zones in the mutant lines by immunostaining with nc82 a commonly used marker that recognizes Bruchpilot (Brp), the *Drosophila* active zone (AZ) protein component (C. A. Collins & DiAntonio, 2007; Wagh, Rasse *et al.*, 2006). Active zones are presynaptic sites for synaptic vesicle accumulation, fusion and release in response to an action potential.

Boutons stained for nc82 were observed in all the mutant fly lines thus confirming the presence of active zones. Previous data from our lab has demonstrated reduction in active zone abundance in $Tip60^{E431Q}B$ mutant lines compared to the w^{1118} control larvae (Sarthi & Elefant, 2011). To investigate AZ abundance in APP/Tip60 double mutants we quantified the number of active zones by measuring the mean fluorescence intensity, which is proportional to the active zones and is a correlate of synaptic function in the APP mutants (M. Fischer, Raabe *et al.*, 2009). The percentage maximum intensities were markedly reduced in the APP, $APP/Tip60^{E431Q}A$, $APP^{\Delta CT}/Tip60^{E431Q}A$ and $Tip60^{E431Q}A$ lines when compared to the w^{1118} control lines (Fig. 6E-L, M). However, there was no significant difference between the nc82 intensities for $APP^{\Delta CT}$ and the w^{1118} control (Fig. 6A-D, M). The pattern for the active zone abundance are consistent to the bouton number increase observed earlier, thus suggesting a functional interaction between APP and Tip60 coherent with previous data from our lab showing Tip60 and APP interaction in apoptotic cell death, nervous system development and axonal transport (A. A. Johnson, Sarthi *et al.*, 2013; Pirooznia, Sarthi *et al.*, 2012).

APP and Tip60 functionally interact post-synaptically

Studies using primary rat hippocampal neurons and mouse brain lysates have shown that APP co-localizes with postsynaptic markers and co-precipitates with PSD proteins in the brain (Hoe et al., 2009). APP has been suggested to form homodimers on the opposite side of the synaptic cleft (Soba *et al.*, 2005). Conversely, Tip60 is required for both pre- and post-synaptic regulation of NMJ growth. We therefore

investigated the function of Tip60 in the APP mutants when misregulated post-synaptically. Fly lines dTip60^{E431Q}A, APP, APP$^{\Delta CT}$, APP/dTip60^{E431Q}A, APP$^{\Delta CT}$/Tip60^{E431Q}A, Tip60^{WT}, APP/Tip60^{WT}, APP$^{\Delta CT}$/Tip60^{WT} and w^{1118} were expressed post-synaptically in the muscles using the muscle GAL4 driver Twi;Mef2 and bouton numbers were quantified. Quantification of total number of boutons for each of the genotypes revealed drastic decrease in the APP and APP/Tip60^{E431Q}A mutants compared to the w^{1118} (total bouton numbers were 52.6 ± 2.52 for w^{1118}, 33 ± 1 for APP, 14.5 ± 1.1 for APP/Tip60^{E431Q}A) (Fig8. C, E,G). As observed earlier with pan-neuronal expression, the mutants with the deleted C-terminus (APP$^{\Delta CT}$) did not show any decrease in total bouton numbers and were very similar to the w^{1118} control animals (45 ± 2.6 for APP$^{\Delta CT}$) (Fig8.A,D,G). As expected APP$^{\Delta CT}$/Tip60^{E431Q}A mutants showed similar decrease in bouton numbers as Tip60^{E431Q}A (42.1 ± 1.4 for Tip60^{E431Q}A and 40.6 ± 1.1 for APP$^{\Delta CT}$/Tip60^{E431Q}A) (Fig 8. B,F,G).

The decrease in active zone abundance in APP, APP/Tip60^{E431Q}A, APP$^{\Delta CT}$/Tip60^{E431Q}A and Tip60^{E431Q}A when misregulated pre-synaptically as well as the decrease in GluRIIC clusters in the Tip60^{E431Q}B and Tip60^{E431Q}A mutants prompted us to take a closer look at the glutamate receptors that are present opposite to the active zones post-synaptically in the APP/Tip60 double mutants. We therefore investigated the presence of GluR clusters in the double mutants by immunostaining for GluRIIA, GluRIIB and GluRIIC. GluRIIA and GluRIIB were present in APP, APP$^{\Delta CT}$, APP$^{\Delta CT}$/Tip60^{E431Q}A, however were not absent at the NMJ in the APP/Tip60^{E431Q}A double mutants. Quantitation of cluster size revealed that though there were GluRIIB clusters present in the APP mutants, the cluster size was

significantly smaller than the w^{1118} control, $APP^{\Delta CT}$ (Fig. 9). These data are consistent with immunostaining experiments we carried out with DLG in the double mutants that demonstrate disorganized DLG pattern around the boutons in the APP and APP/$Tip60^{E431Q}$A mutants (data not shown). In summary, our data suggest that Tip60 interacts with the C-terminus of APP to control organization and function of components of the post-synapse

ChIP analysis of changes in specific lysine marks associated with learning and memory of Tip60 targets:

The seven genes analyzed for their pre-synaptic expression levels in the $dTip60^{E431Q}$B mutants were further analyzed to examine transcriptional regulatory mechanisms. We first asked whether these genes were indeed Tip60 targets in the wild-type control flies (w^{1118}) and whether the recruitment of Tip60 to these gene loci was hindered in the dominant negative Tip60 HAT mutant flies ($dTip60^{E431Q}$B). Chromatin was isolated from the head regions of wandering 3rd instar larvae from each of the genotypes and ChIP-qPCR was performed to detect binding of Tip60 to the candidate gene loci *in vivo*. We demonstrate that Tip60 is significantly enriched at 5 of the 7 gene loci in the w^{1118} control flies (Sgg, Sh, Dlg, Futsch, Dsh) (Fig. 10A). In contrast, $dTip60^{E431Q}$B larvae did not show significant Tip60 binding at any of the candidate gene loci (Fig. 10A).

Histone acetylation has garnered much attention and is important in memory and cognitive function and thus we hypothesize that acetylation of key residues via Tip60 HAT activity could shed light on the transcriptional regulation of the target

genes. Tip60 has been shown to acetylate number of lysine specific histone residues three of which; H3K9, H4K12 and H4K16 play important roles in learning and memory (A. Fischer, Sananbenesi *et al.*, 2007). We therefore hypothesize that acetylation levels of H3K9, H4K12 and H4K16 would be enhanced in the w^{1118} control larvae at the target gene loci compared to the $dTip60^{E431Q}B$ larvae where Tip60 is not recruited to the sites. To test this, we carried out ChIP-qPCR to assess enrichment levels of H3K9ac, H4K12ac and H4K16ac at each of the target gene loci in w^{1118} and $dTip60^{E431Q}B$. We found that all the genes were enriched for the three specific marks in the w^{1118} except on the wg loci where the H3K9ac and H4K12ac enrichment levels were higher in the $Tip60^{E431Q}B$ as well as the Sh gene loci where enrichment levels for H4K12ac was higher in the $Tip60^{E431Q}B$ mutants thus suggesting distinctive mechanisms involved in transcriptional regulation via Tip60 HAT activity at these gene loci. Also, our results show that wg is not a direct target of Tip60 suggesting Tip60 could be involved in repressive of the wg gene (Fig 10 B-D).

<u>Discussion:</u>

Tip60 affects components of the postsynaptic muscle membrane:

In this report, we further investigate the role for dTip60 in synapse development and function at the *Drosophila* NMJ. Previous data from our lab has shown that Tip60 is present both pre- and post synaptically, suggesting a role for Tip60 in anterograde as well as retrograde signaling (Sarthi *et al.*, 2011). We have shown that presynaptic misregulation of dTip60 HAT activity affects bouton numbers and their function and increases futsch loops thus suggesting that the overgrowth

phenotype we see as with many other genes that control NMJ growth, maybe partially attributed to misregulated microtubule (MT) organization (Franco *et al*., 2004). In this study, we further explore the role for dTip60 function on the postsynaptic side of the NMJ. There is increasing evidence that supports bidirectional signaling at the NMJ that involve a variety of retrograde signals provided by the postsynaptic cell to the presynaptic neuron (Tao *et al*., 2001). Importantly, anterograde and retrograde modes of signaling are required at sites of synaptic contact and these signaling pathways play critical roles in the formation, differentiation and plasticity of synaptic connections (Zweifel *et al*., 2005). Wingless (wg) is one example of a secreted ligand involved in such bidirectional signaling that operates at the *Drosophila* NMJ on both pre- and postsynaptic sides involved in synapse development (Miech *et al*., 2008, Ataman *et al*., 2008). There are also a number of NMJ proteins such as Discs large (DLG) that contribute to both pre- and postsynaptic control of synaptic plasticity (Chen *et al*., 2005, Lahey *et al*., 1994). Our results indicate that DLG is disorganized in the dTip60^{E431Q} mutants compared to the control. DLG is a scaffold protein that is also involved in synapse dismantling process. Synapse remodeling is a fundamental process that is required for development and function of neural circuitry in response to a developing larva and responses to environmental cues. DLG has been shown to play an important role during the synapse dismantling process of pupae formation and undergoes changes in organization as synapse elimination proceeds during normal larval development into pupae (Liu *et al*., 2010). Synapse elimination also occurs in response to injury and pathological diseases (Luo *et al*., 2005). During larval development the synapses undergo pruning and around 7hrs APF DLG starts to look

disorganized and is a marker for the dismantling process (Liu *et al*., 2010). It could be this disorganization of DLG in the dTip60 mutants that leads to the consequent loss of boutons when Tip60 is misregulated in the muscles. We also see this consistently in the APP/Tip60 double mutants expressed in the muscles that also show severe reduction in bouton numbers. Studies in humans and rats have shown that APP present at neuromuscular junctions and skeletal muscles is one of the hallmarks of Alzheimer's Disease (Schubert et al., 1991, Kuo *et al*., 2000, Kim *et al*., 2011). Studies in flies expressing hAPP in flight muscles show age related climbing and flying defects but no morphological defects in muscle structure (Kim *et al*., 2011). Here we report that over-expression of hAPP and Tip60^{E431Q}A have severe defects in the muscles. We also notice 100% lethality at the pupal stage in these double mutants show abnormalities in the muscle architechture at the 3rd instar larval stages which in turn are abnormally thin and small compared to the wild type controls as well as the APPΔCT/Tip60^{E431Q}A, Tip60^{E431Q}A, and APPΔCT mutant lines. Our results demonstrate severe muscular defects and drastic reduction in pre-synaptic neuron branching and bouton formation at the NMJ in APP as well as APP/Tip60 double mutants consistent with studies documenting loss of lean muscle mass in early AD as well as build up of Aβ in skeletal muscles and its uptake by the brain by crossing the blood brain barrier. Loss of lean muscle has been attributed to defects in cognitive performance and brain atrophy in AD (Kuo *et al*., 2000, Burns et al., 2010). This suggests a role for Tip60 not only during development of the postsynaptic density/SSR but also a role in a neurodegenerative model suggesting defects in the

SSR and postsynaptic components at the NMJ could be one of the processes affected early on in AD pathology.

Role for Tip60 in activity dependent retrograde signaling

The *Drosophila* NMJ is a glutamatergic synapse and has been studied extensively to understand the mechanisms of excitatory synaptic transmission (Bellen, 1998; Gramates and Budnik, 1999; Featherstone and Broadie, 2000; Koh *et al*., 2000). The synapse expresses glutamate receptors that are homologous to non-NMDA receptors found in mammalian nervous system (Schuster *et al*., 1991; DiAntonio *et al*., 1999; Marrus *et al*., 2004). There are five types of GluR subunits namely; GluRIIA, GluRIIB, GluRIIC, GluRIID and GluRIIE found at the *Drosophila* NMJ and are significantly homologous to vertebrate AMPA- and kainate-type GluR subunits (Sigrist *et al*., 2012). GluRIIC, GluRIID and GluRIIE are the essential subunits found in both A and B subtypes composed of GluRIIA and GluRIIB subunits respectively. Loss of even one of the essential subunits causes defects in GluR formation at the NMJ and leads to paralysis and late embryonic lethality (DiAntonio *et al.,* 1999; Marrus *et al.,* 2004; Featherstone *et al.,* 2005; Qin *et al.*, 2005). Here we show decreased transcript levels of GluRIIA, GluRIIB and GluRIIC genes in Tip60^{E431Q}B mutants using the larval bodywall muscles. We also show that NMJs in the Tip60^{E431Q}A mutants immunostained for GluRIIC show decerease in the subunit cluster size at the synapses. On closer inspection we found that there was absence of GluRIIA , GluRIIB and GluRIIC receptor subunits at the synapses of the

NMJ in the APP/Tip60^{E431Q}A mutants, which explains the paralysis induced failure to eclose and thus lethality in pupal stages. DLG has been shown to be required to regulate subunit composition by proper stabilization of GluRIIB containing receptors at the NMJ (Chen and Featherstone, 2005). This explains the absence of GluRIIB and GluRIIC at the NMJ concomitant with the disorganized DLG localization at these synapses. However, absence/decrease in GluRIIA subunits in the APP/Tip60^{E431Q}A mutants at the NMJ suggest different/parallel mechanisms playing a role at the postsynapse. Coracle a homolog of mammalian 4.1 proteins has been found to directly interact with and stabilize GluRIIA by anchoring them to spectrin-actin cytoskleteton on the postsynaptic side (Chen *et al.,* 2005). Bidirectional signaling at the NMJ involves a variety of retrograde signals provided by the postsynaptic cell to the presynaptic neuron (Tao *et al*., 2001). GluRs have also been implicated in activity dependent retrograde signaling and thus our results point to a role for Tip60 in retrograde signaling via regulation of the postsynaptic components DLG, GluRIIB and GluRIIC. It will be interesting to see how activity dependent changes in GluR, DLG, sgg and other synaptic components using hyperexcitable mutants like *eag Sh* and *Hk eag* and hypo excitable mutants such as *para* and *mle* are affected in the Tip60 and APP mutants thus shedding more light on the role of Tip60 in activity dependent synaptic plasticity (Olsen and Keshishian, 2013).

Tip60 HAT activity regulates genes in involved in the Wingless pathway and activity dependent synaptic plasticity

The wingless pathway in *Drosophila* is homologous to the Wnt pathway in humans. In *Drosophila* it is involved in pattern formation and cell fate as well as

synaptic growth and differentiation (Ruel et al., 1993, Packard *et al*., 2002,). Wingless is involved in bidirectional signaling as part of distinct pathways on the pre- and post-synaptic sides of the NMJ (Miech *et al*., 2008). The canonical wg pathway operates at the pre-synaptic terminal and involves sgg (*shaggy*) a homolog of mammalian GSK3β, a protein kinase shown to function in neuronal development (Lucas and Salinas, 1997; Hall *et al*., 2000). Over-expression of Wg and inhibition of Shaggy kinase activity or sgg hypomorph show similar overgrowth phenotypes at the NMJ analogous to our data shown previously where HAT defective dominant negative form of Tip60 as well as RNAi induced Tip60 knockdown mutants show increase in bouton numbers (Liebl *et al*., 2007, Miech *et al*., 2008, Sarthi *et al*., 2011). Over-expression of $Tip60^{WT}$ pre-synaptically however, does not have any significant effect on the bouton numbers suggesting HAT activity of Tip60 is required and that Tip60 could be a part of the canonical wg pathway either directly or indirectly. Our data suggests that indeed Tip60 is involved in transcriptional regulation of the genes involved in the canonical wg pathway, namely; *wg, sgg*, *dsh and futsch.*

We have demonstrated earlier that Futsch, the downstream target of Sgg in the canonical wg pathway leads to the increased loop formation in the Tip60 HAT mutants (Sarthi et al., 2011). However, though we did not see a transcriptional misregulation of futsch in the Tip60 HAT mutants, we do demonstrate that futsch is indeed a direct target of Tip60 where it is involved in H4K12 and H4K16 acetylation suggesting alternate ways by which Tip60 could control some components of the wg pathway. Tip60 encodes for a protein that contains a C-terminus MYST domain and N-terminus chromodomain (Sapountzi, Logan *et al.*, 2006; Zhu, Singh *et al.*, 2007).

The MYST domain is the catalytic domain and has the HAT function whereas the chromodomain of Tip60 still needs to be characterized however studies have shown the putative interaction of the chromodomain containing proteins with mRNA as seen with HP1 protein (Akhtar, Zink *et al.*, 2000; Jacobs & Khorasanizadeh, 2002). Synaptic processes are present at great biological distances from their cell bodies that require presence of vital synaptic proteins in response to a stimulus/action potential and have the ability to synthesize these proteins via local translation. Pre- and post-synaptic local translation are now found to be present and play important roles in long term potentiation (LTP) and long term depression (LTD) both of which are prolonged periods of synaptic activity (Steward and Levy, 1982; Sutton and Schuman, 2006; Bramham and Wells, 2007, Eberwine *et al.,* 2001). We therefore hypothesize that a possible indirect interaction between the chromodomain of Tip60 and dormant mRNAs present in the pre-synaptic terminal could lead to local translation important in activity dependent synaptic plasticity. The candidate mRNA targets we found including futsch could give us clues to cytoplasmic local translational regulation of synaptic plasticity by HAT Tip60 (Fig. 11).

Histone modifications are highly dynamic and occur in combination with various different histone marks such as acetylation, phosphorylation, sumoylation as well as DNA methylation (Grant, 2001; Wu & Grunstein, 2000). Thus it is not just one specific type of post-translational modification but a multivariate coordination of 'epimarks' that regulate gene transcription. We show that wg and sgg are differentially regulated and the enrichment for wg and sgg is opposite for H3K9ac and H4K12ac marks. Unlike wg, Sgg was also acetylated at lysine 16 (H4K16ac).

Furthermore, wg is not a direct target of Tip60 suggesting an indirect mode of regulation for wg and also a repressive role for Tip60 in wg regulation. In the w^{1118} larvae wg gene loci could be in a repressive state brought about by recruiting Tip60 by chromatin modulators, which could further recruit HDACs to repress the wg gene loci. With the loss of HAT activity in the mutants, Tip60 is not recruited to the wg gene loci thus unable to recruit HDACs resulting in acetyaltion levels to remain and thus activating wg. In addition, all of the 5 targets of Tip60 (sgg, sh, futsch, dlg and dsh) are acetylated at K12 and K16 residues on histone H4. Disregulation of H4K12 acetylation is associated with age-associated memory impairment (Peleg *et al*., 2010). Mice that overexpress HDAC2 show reduced levels of H4K12ac suggesting similar mechanism in the Tip60 HAT defective mutant leading to reduction of H4K12ac. Similarly, H4K16 acetylation is involved in diverse processes such as DNA repair, transcriptional activation, genomic stability and has also been shown to be important in aging and age related diseases (Shahbazian and Grunstein, 2007, Kouzarides *et al*., 2007, Dion *et al*., 2005, Kind *et al*., 2008, Krishnan *et al*., 2011).

In conclusion, our data suggest strong roles for the proposed Tip60 target genes in learning and memory and age associated neurodegeneration.

Model for neurodevelopmental role of Tip60:

Based on the data presented here, we suggest Tip60 is important in both neurodevelopmental and neurodegenerative synaptic plasticity mechanisms (Fig. 12). We hypothesize a model in which Tip60, predominantly acts via its nuclear localization and targets genes that are part of the wingless pathway (wg, dsh, sgg and futsch), genes involved in activity dependent synaptic plasticity (GluRs, Sh) and

scaffold protein Dlg. On the presynaptic side Tip60 functions via the canonical wg pathway where in it allows for regulation of the wg pathway genes all the way to activation of futsch thus allowing for bouton budding and synaptic growth. On the post-synaptic side in the muscle, Tip60 again acts via the non-canonical wg pathway by regulating wg and possibly other downstream targets. Concurrently, Tip60 also regulates Dlg organization and GluRIIC localization. Tip60 present in the muscle cell nuclei could be important as one of the upstream targets in the cascade leading to GluR mRNA expression in the muscle cytoplasm (Ganesan, *et al*., 2011). We hypothesize another level of regulation via Tip60 that takes place in the cytoplasm via interaction of the chromodomain of Tip60 with dormant mRNA's which are important in activity dependent synaptic plasticity. Futsch, one of the putative mRNA targets identified in this study could interact with the chromodomain of Tip60 and allow Tip60 to activate local translation of futsch by recruiting the translational machinery and thus allowing for local synthesis of the protein required for microtubule looping and promoting bouton budding. On the postsynaptic side, the chromodomain-mediated regulation could be involved in regulation of the dormant mRNAs present postsynaptically, including the GluR mRNA's to regulate the local translation.

In summary, multiple levels of coordinated regulation of synaptic plasticity via HAT Tip60 are present at the NMJ and it is when this coordination is perturbed by improper HAT function affecting epigenetic modification of genes necessary to maintain proper synaptic growth and maintenance as well as function that it leads to a neurodegenerative disease such as in this case Alzheimer's disease.

Chapter 4: Conclusions and Future Directions

Tip60 HAT activity has been extensively studied in various cellular processes including DNA repair, apoptosis, cell cycle progression as well as its role in diseases such as cancer and tumorigenesis (Sapountzi, Logan *et al.*, 2006). However, recent microarray data form our lab identified its role in numerous neuronal processes such as olfaction, synaptic plasticity and learning and memory. Tip60 has been identified as one of the genes in the genetic hub and as such is suggested to be linked to various human diseases. Identification of its role in neuronal processes therefore suggests its possible role in diseases of the nervous system as well.

In this study, we have used the neuromuscular junction (NMJ) of *Drosophila melanogaster* 3rd instar larvae to investigate the role of Tip60 HAT activity in synaptic plasticity. Microarray studies from our lab have identified a role for Tip60 in learning and memory. Synaptic plasticity is a strong correlate for learning and memory as it depends on the strengthening and weakening of synaptic connections and forming new ones allowing memory formation and storage. Furthermore, studies demonstrating the interaction of Tip60 with the AICD domain of APP, the protein implicated in AD suggest its role in neurodegeneration.

To investigate the role of Tip60 HAT function in synaptic plasticity we characterized its localization at the NMJ. We show that Tip60 is present pre- and post-synaptically at the NMJ. Further investigation on the presynaptic side showed its

importance in synapse formation via its effect on microtubules as well as genes involved in the wingless pathway. The canonical wg pathway at the *Drosophila* NMJ consists of wingless (wg) ligand binding to its receptor thus activating disheveled (dsh) which in turn inactivates shaggy (sgg). Thus the *Drosophila* MAP1B homolog, Futsch can now bind to the microtubules (MT) allowing MT looping and promoting bouton budding and division. We also show transcript upregulation of the wg pathway components in the larval brains including HDAC6. HDAC6 is a cytoplasmic histone deacetylase specifically involved in deacetylating α-tubulin subunit of MTs. This correlates with the loss of acetylated tubulin in the terminal boutons that we observe in the Tip60 HAT deficient mutants thus suggesting a more plausible role for Tip60 in MT acetylation similar to another HAT Elp3. However, the effects seen are subtle in the Tip60 HAT mutant NMJs. A future approach to this would be to investigate loss of acetylated α-tubulin in specific neuronal cell lines by carrying out immunostaining and immunoprecipitation experiments. Trichostatin A (TSA) has been shown to inhibit HDAC6, thus further drug studies could be carried out to investigate rescue of acetylated α-tubulin loss seen due loss of Tip60 HAT activity.

We also investigated the role of Tip60 post-synaptically in the muscles and its role in localization of post-synaptic components such as DLG, glutamate receptors as well as wg. However, misregulation of Tip60 HAT activity on the post-synaptic side resulted in a phenotype opposite to the misregulation of HAT activity pre-synaptically. Loss of Tip60 HAT function on the post-synaptic side also had effects on the presynaptic bouton growth and maintenance thus suggesting its role not only in anterograde but also retrograde signaling. We show that Tip60 is important for NMJ

growth and maintenance and plays a crucial role in the canonical wg pathway on the presynaptic side as well as regulation of post-synaptic components involved in activity dependent synaptic plasticity. The data presented in this study brings to light a novel role for Tip60 in synaptic plasticity as well as its epigenetic function in activity dependent signaling. It would be interesting to investigate Tip60 function in NMJ growth and maintenance when its HAT function is misregulated both pre- and post synaptically simultaneously. It might be expected to see a 'tip' towards the stronger phenotype depending on genes expression on both sides including wg that appears to be regulated differentially on either side. It might also result in complete lethality in the flies at an earlier stage expressing Tip60 HAT in the nervous system as well as the muscles simultaneously compared to when expressed separately.

Here we have shown the role of Tip60 in synaptic plasticity and also shed light on its role in bidirectional signaling at the NMJ. Bidirectional signaling plays a crucial role in synaptic plasticity and increasing evidence supports importance of retrograde signaling to the presynaptic neuron (Tao & Poo, 2001). We present evidence to support this role of Tip60 in this study and also point to its role in activity dependent signaling. Data presented in this study demonstrate the role of Tip60 in regulation of DLG, GluRIIB and GluRIIC postsynaptic components. GluRs have been implicated in activity dependent retrograde signaling that has effects on the pre-synaptic side affecting synaptic plasticity. Glutamate released by the presynaptic terminal binds to NMDA and AMPA receptors causing a calcium influx that depolarizes the postsynaptic cell. This in turn activates mGluRs that are involved in signaling mechanisms that allow local mRNA translation important in long-lasting

synaptic plasticity. The mGluRs are also coupled to G protein G_q and together activate PLCβ, which in turn converts PIP_2 to DAG, which is then converted to endocannabinoid 2AG. 2AG then activates presynaptic CB1Rs which regulate the probability of release in the presynaptic side (Regehr, Carey *et al.*, 2009). In *Drosophila*, postsynaptic calcium $^{2+}$ion (Ca^{2+}) influx through glutamate receptors leads to subsequent postsynaptic vesicle fusion that in turn triggers a robust induction of presynaptic miniature release at *Drosophila* neuromuscular junctions. GluRIIA has been reported to be involved in activity dependent retrograde signaling where decrease in postsynaptic receptors leads to increase in presynaptic transmitter release indicating a role for glutamate receptors in activity dependent retrograde signaling. However, a more activity-based study will help in further understanding the role of Tip60 in activity dependent plasticity. There are various ways synaptic connections can be manipulated which can further aid in studying the role of a certain protein in activity dependent plasticity. One of the ways this can be achieved is by using mutants that affect either the expression or function of ion channels. The effect of these mutations can be regulated by using temperature sensitive (ts) mutants that allow us to manipulate the phenotype of these ion channel mutants. Loss-of-function ion channel mutants elevate membrane excitability, such as K^+ channel mutations *Sh* and *ether-a-gogo (eag)* which are often used in combination (*eag Sh*) (Olsen & Keshishian, 2012). Hyperexcitability is known to cause expansion of synapse including increase in synaptic boutons as well as neurotransmitter release (Budnik, Zhong *et al.*, 1990; Schuster, Davis *et al.*, 1996). Interestingly, we demonstrate

upregulation of Sh K^+ channels when Tip60 is misregulated presynaptically suggesting its role in activity dependent synaptic plasticity.

Here we also report changes in acetylation of histone marks associated with learning and memory. H3K9, H4K12 and H4K16 acetylation marks have been shown to be associated with learning and memory and in this study we investigated their levels at the target gene loci (wg, sgg, Sh, futsch, dlg, dsh) in the Tip60 HAT deficient mutants. This study suggests defects in acetylation of H4K12 and H4K16 at the gene loci of the target genes that are also Tip60 targets thus supporting their role in learning and memory process. It will be interesting to understand how these changes in the learning and memory marks at the specific gene loci are perturbed in a neurodegenerative background such as the APP/Tip60 double mutants. It will also be interesting to investigate the enrichment levels of these marks in higher animals such as mice and rats. As a prelude to this experiment we performed western blot analysis of select histone acetylation marks using rat brains (Fig. 13). We have been able to harvest young and aged rat brain hippocampal tissues that will be tested for levels of specific histone marks. Rats aged at 1.5 months (puberty) and 24 months will be used, as these ages are equivalent to 12.5 and 60 human years, respectively. Future work to be carried out by Priya Panikker would involve ChIP-qPCR analysis of target genes identified through the recent ChIP-Seq data as well as ChIP-qPCR data presented in this study for enrichment levels of H4K12 and H4K16 acetylation marks at the candidate gene loci in the harvested young and aged rat hippocampal tissues. This experiment will further allow us to investigate the role of these genes and their regulation in an aging brain thus supporting a function for the target genes in age-

associated cognitive disorders thereby shedding light on the role of Tip60 in the aging brain.

In addition to the HAT domain, Tip60 also consists of the chromodomain that has not been completely characterized yet. Chromodomains have been shown to play important roles in chromatin binding and recruiting coactivators and other modifiers to the promoters of target genes. Tip60 has also been demonstrated to bind H3K9me3 via its chromodomain that is necessary for its HAT activity in DSB repair (Sun, Jiang *et al.*, 2009). However, chromodomains have been demonstrated to bind to RNA as well serving as protein–RNA interactions (Ishida, Shimojo *et al.*, 2012). Local protein synthesis is a common phenomenon that occurs at synapses to be able to modulate synaptic function and connections in an activity dependent manner. Here we have shown putative mRNA targets of Tip60 that could bind to the chromodomain of Tip60 and could thus be involved in local translation important in synaptic plasticity. In this study we show that Tip60 is present not only in the nucleus but also in the cytoplasm at the NMJ and its localization thus points to a possible role in regulating synaptic plasticity via its cytoplasmic localization as well. Further work involving the localization of Tip60 with mRNA targets identified in the synapses would shed more light on this unique role of Tip60 chromodomain. RiboGreen is a well-known fluorescent marker that specifically marks nucleic acid molecules. Colocalization experiment using RiboGreen that stains mRNA present in the presynaptic terminal and antibody to Tip60 will allow us to further investigate the role of Tip60 in synaptic plasticity via regulation of local translation. Further work can be carried out by performing an RNA-IP that will allow us to pull down specific RNA targets

associated with Tip60 either directly or indirectly. Mutations in the chromodomain region of Tip60 can be further carried out and the mutant can be utilized and analyzed to study its effects on RNA binding as well as the HAT activity of Tip60. ChIP-qPCR for the same targets identified in this study can be performed using the chromodomain mutant to see if it affects the enrichment levels of the targets.

Investigating the chromodomain of Tip60 will give us more insight into the mechanism of Tip60 regulation of the target genes as well as provide answers to its localization in the cytoplasm and alternate ways in which Tip60, a HAT could play a role in synaptic plasticity. Overall, our results indicate an epigenetic role for Tip60 in synaptic plasticity via regulation of components of the wg pathway and its function in neurodegeneration, specifically Alzheimer's Disease.

Chapter 5: Tables and Figures

Chapter 3 Table

RNA target	Chromodomain		Whole protein	
	RF Classifier	SVM classifier	RF Classifier	SVM classifier
Futsch	0.9	0.71	0.85	0.9
CASK	0.7	0.81	0.7	0.98
synapsin	0.7	0.93	0.8	0.99
GluRIIC	0.8	0.94	0.85	0.97
GluRIIA	0.8	0.95	0.9	0.99
GluRIIB	0.8	0.93	0.85	0.99
armadillo (β catenin)	0.7	0.9	0.75	0.95
neuroligin 2	0.75	0.93	0.8	0.97
bruchpilot	0.55	0.87	0.7	0.97
Shibire	0.8	0.92	0.9	0.98
Shaker	0.75	0.8	0.65	0.97

Table 1. RPIseq analysis of putative Tip60 chromodomain mRNA targets.

Sequence analysis of the putative mRNA targets identified key mRNA involved in synaptic plasticity. RPIseq algorithm was used to analyze the probability of the mRNA targets binding to the chromodomain of Tip60 and the entire sequence of Tip60. A probability of higher than 0.5 suggests a strong possibility of the mRNA candidates being a target of the Tip60 chromodomain or the protein Tip60.

Chapter 2 Figures

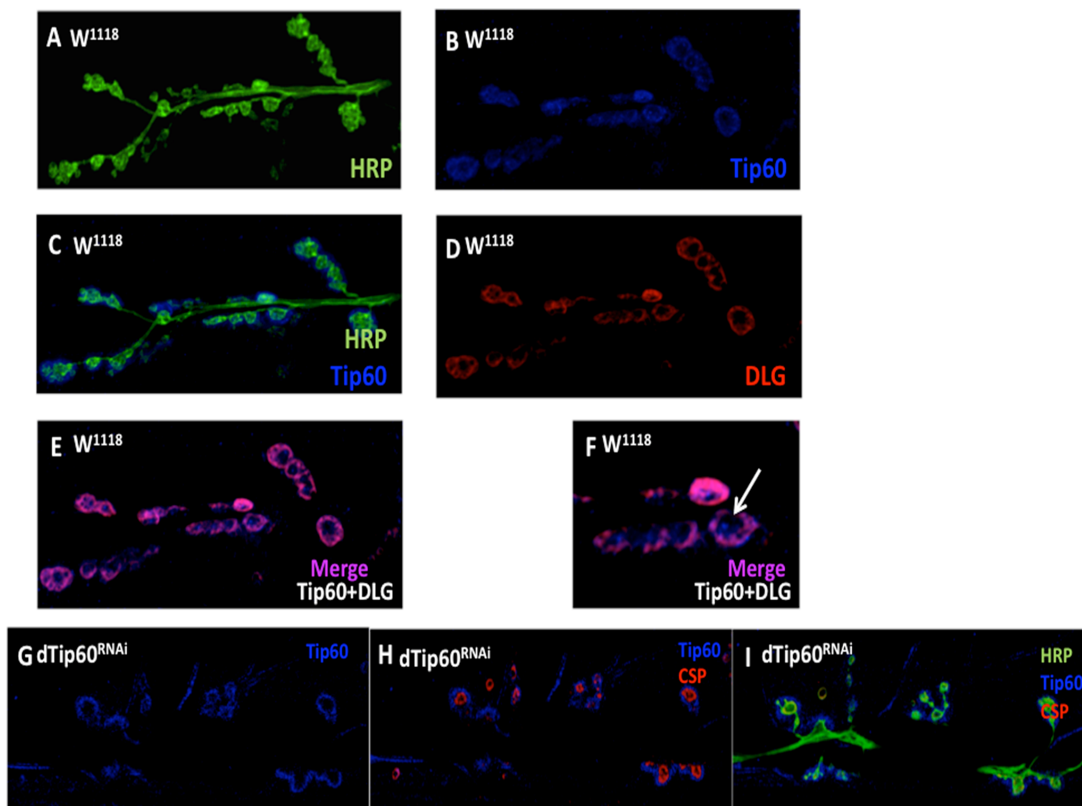


Figure 1. dTip60 is localized at the pre and postsynaptic sides of larval NMJ. Figures (**A–E**) represent confocal imaging analysis of control w^{1118} larval boutons on muscles 6/7 at abdominal segment A4 immunohistochemically triple stained with (**A**) HRP antibody (green) that labels the entire presynaptic membrane (**B**) dTip60 antibody (blue) and (**C**) HRP and dTip60 merged (green and blue) (**D**) Dlg antibody stain (red) that labels post-synaptic membrane. (**E**) dTip60 and Dlg merged (purple) image showing dTip60 co-localization with post-synaptic labeled Dlg-membrane and dTip60 immunoreactivity within the presynaptic bouton. (**F**) Enlargement of Figure (**E**) where arrow depicts dTip60 immunoreactivity within the presynaptic bouton. Figures (**G–I**) represent confocal imaging analysis of presynaptic RNAi knockdown of dTip60 in larval boutons on muscles 6/7 at abdominal segment A4 immunohistochemically triple stained with pre-synaptic markers HRP, cysteine string protein (csp) antibody (red), a presynaptic vesicle protein that controls vesicle exocytosis and Tip60 (blue). (**G**) dTip60 (blue) showing the presence of postsynaptic

Figure 1 (continued)

dTip60 and absence of presynaptic dTip60 localization due to RNAi presynaptic knockdown demonstrating specificity of dTip60 Ab and confirming dTip60 pre and postsynaptic localization (**H**) dTip60 and Csp merged (blue and red) and (**I**) dTip60, Csp and HRP merged (blue, green and red). In the analyses, wild-type control w^{1118} genotype and test $dTip60^{RNAi}$ genotypes are each represented by 25 larval preparations (n = 25). Scale bar 10 um.

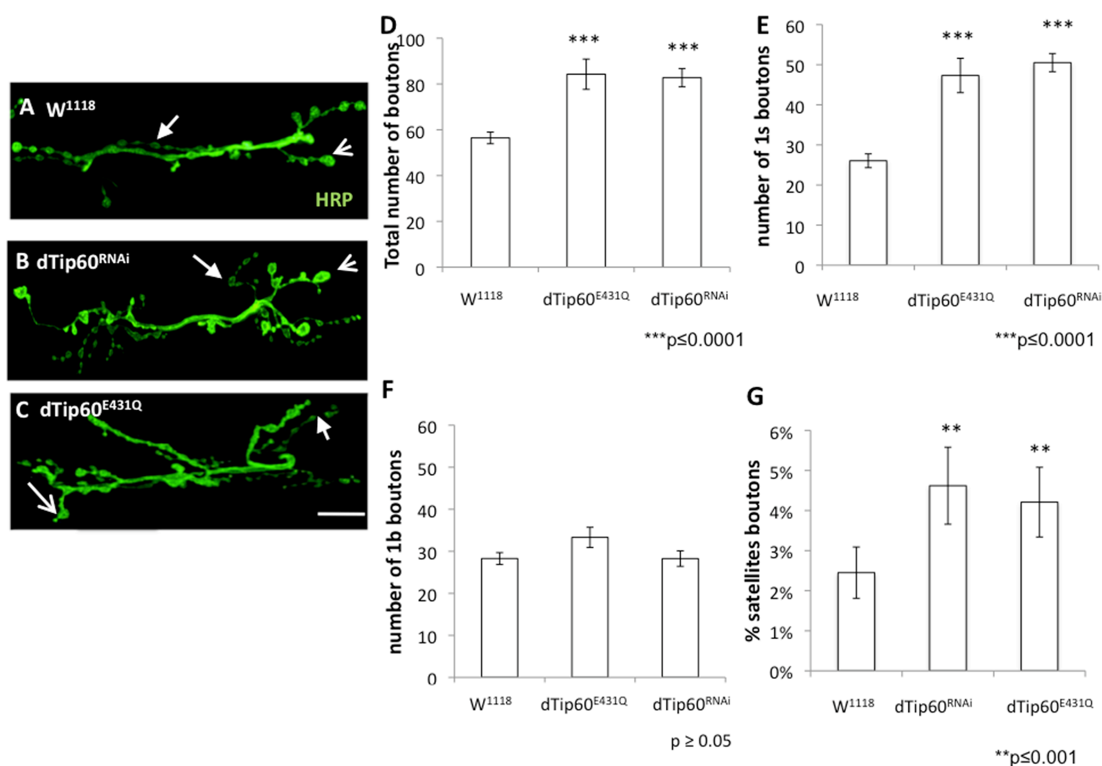


Figure 2. Presynaptic reduction of dTip60 in the nervous system leads to an expansion of synaptic boutons at larval NMJs.

Flies homozygous for either dTip60^{E431Q}, dTip60^{RNAi} or control w^{1118} were crossed to flies homozygous for the nervous system elav^{C155} pan-neuronal GAL4 driver, and staged third instar progeny larvae were collected. Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment A4 immunohistochemically stained with anti-HRP (green) that labels the entire presynaptic membrane. (**A**) control w^{1118} larvae (**B**) larvae expressing dTip60^{RNAi} (**C**) larvae expressing dTip60^{E431Q}. Scale bar 10 um. Line arrow depicts Ib bouton, thick arrow depicts Is bouton. Histogram depicts quantitative analysis of bouton number on muscles 6 and 7 at abdominal segment 4 where (**D**) represents total bouton number (**E**) number of Is boutons (**F**) number of Ib boutons and (**G**) percentage of satellite boutons. Small arrow depicts type Is bouton, line arrow depicts type Ib bouton. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), dTip60^{RNAi} (n = 19) and dTip60^{E431Q} (n = 19). Asterisks (*) indicates statistically significant difference in relation to control where single asterisks indicate p≤0.001 and double asterisks indicate p≤0.0001. All error bars depict standard error of the mean.

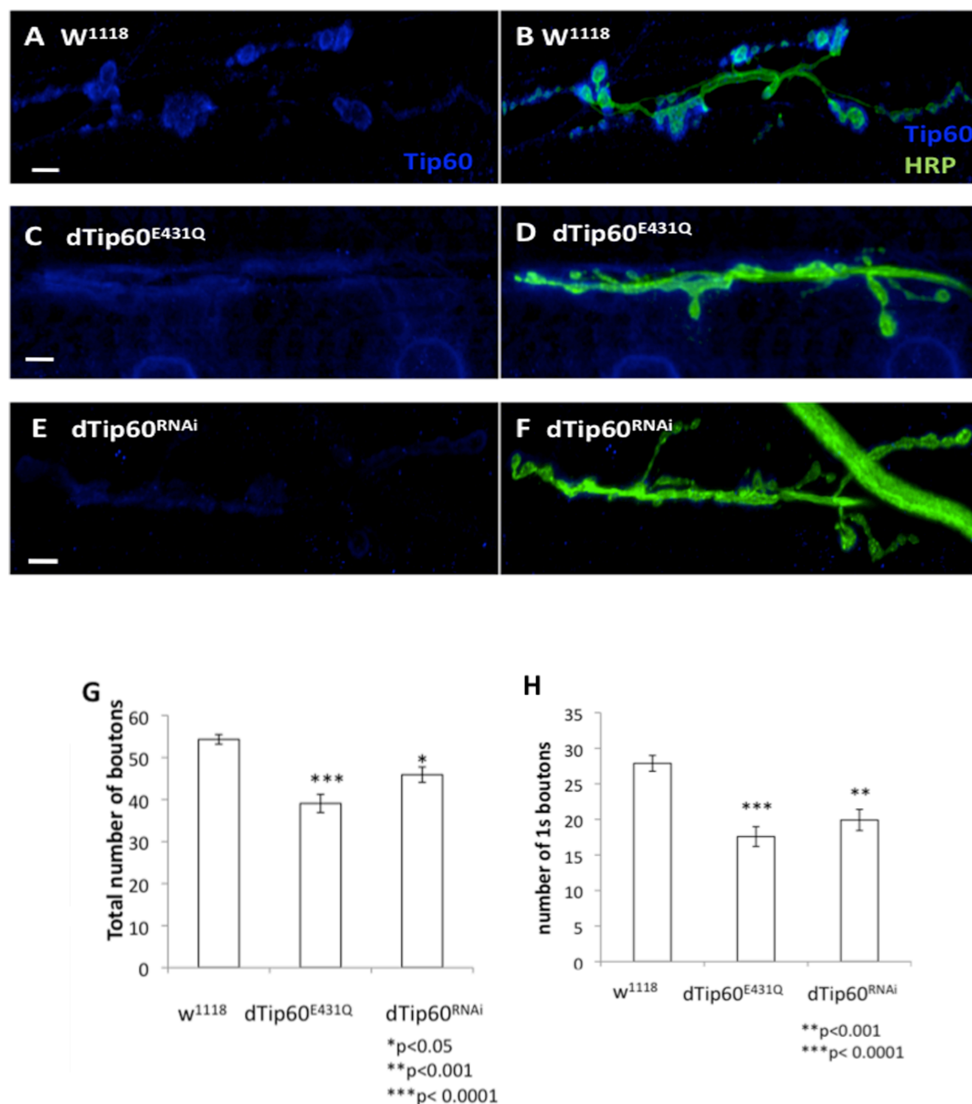


Figure 3. Postsynaptic reduction of dTip60 in the nervous system leads to a decrease of synaptic boutons at larval NMJs.

Flies homozygous for either dTip60^{E431Q}, dTip60^{RNAi} or control w^{1118} were crossed to flies homozygous for the muscle specific GAL4 driver MEF-2, and staged third instar progeny larvae were collected. Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment A4 double labeled with anti-HRP (green) that labels the entire presynaptic membrane and anti-dTip60 antibody (blue). (**A and B**) control w^{1118} larvae (**C and D**) larvae expressing dTip60^{E431Q} (**E and F**) larvae expressing dTip60^{RNAi}. Scale bar 10 um. Line arrow depicts Ib bouton, thick arrow depicts Is bouton. Histogram depicts quantitative analysis of bouton number on muscles 6 and 7 at abdominal segment 4 where (**G**) represents total bouton number

Figure 3 (continued)

(**H**) number of Is boutons. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), $dTip60^{RNAi}$ (n = 19) and $dTip60^{E431Q}$ (n = 19). Asterisks (*) indicates statistically significant difference in relation to control where single asterisks indicate $p \leq 0.001$ and double asterisks indicate $p \leq 0.0001$. All error bars depict standard error of the mean.

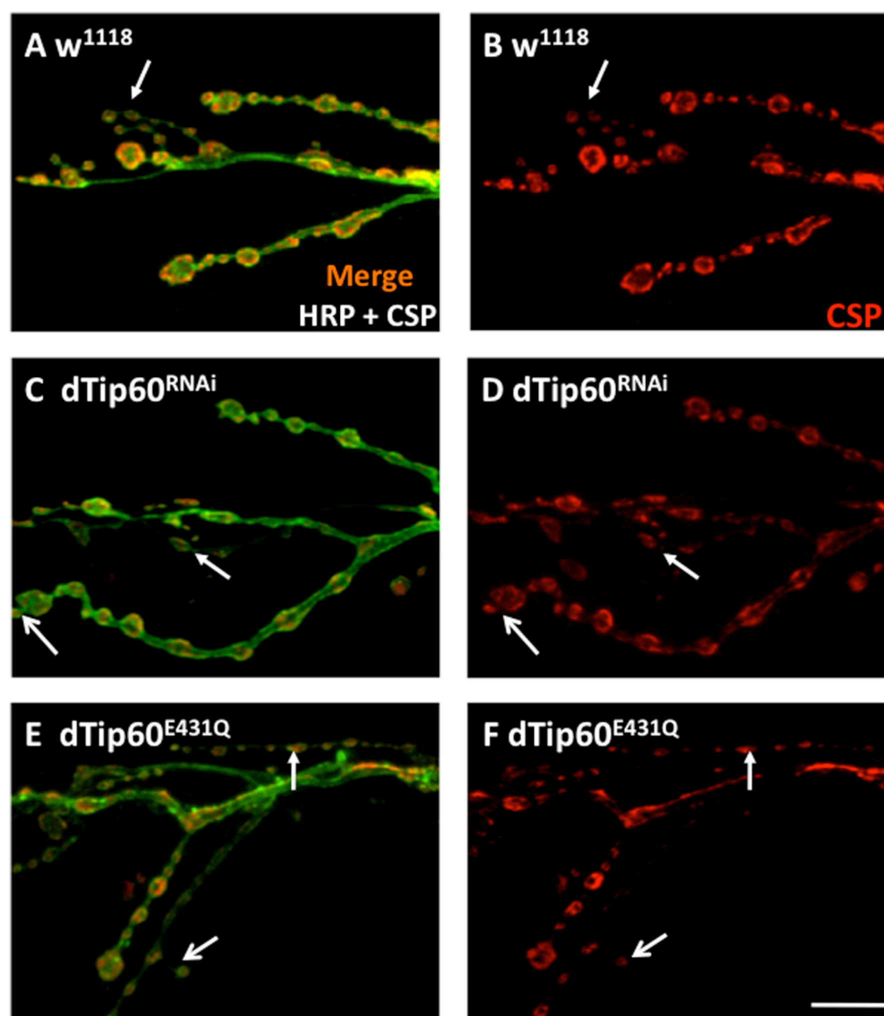


Figure 4. Presynaptic vesicle protein distribution is not altered in dTip60 mutants.

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment A4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and cysteine string protein (csp) antibody (red), a presnynaptic vesicle protein that controls vesicle exoctyosis. (**A,B**) wild-type control w^{1118} (**C,D**) $dTip60^{RNAi}$ (**E,F**) $dTip60^{E431Q}$. Immunostaining with csp antibodies indicate that these vesicle proteins are present in mutant dTip60 NMJs with a distribution similar to wild-type. In the analyses, w^{1118} genotype is represented by 21 larval preparations (n = 21), $dTip60^{RNAi}$ (n = 19) and $dTip60^{E431Q}$ (n = 20). Line arrow depicts Is bouton, small thick arrow depicts satellite bouton. Scale bar is 10 um.

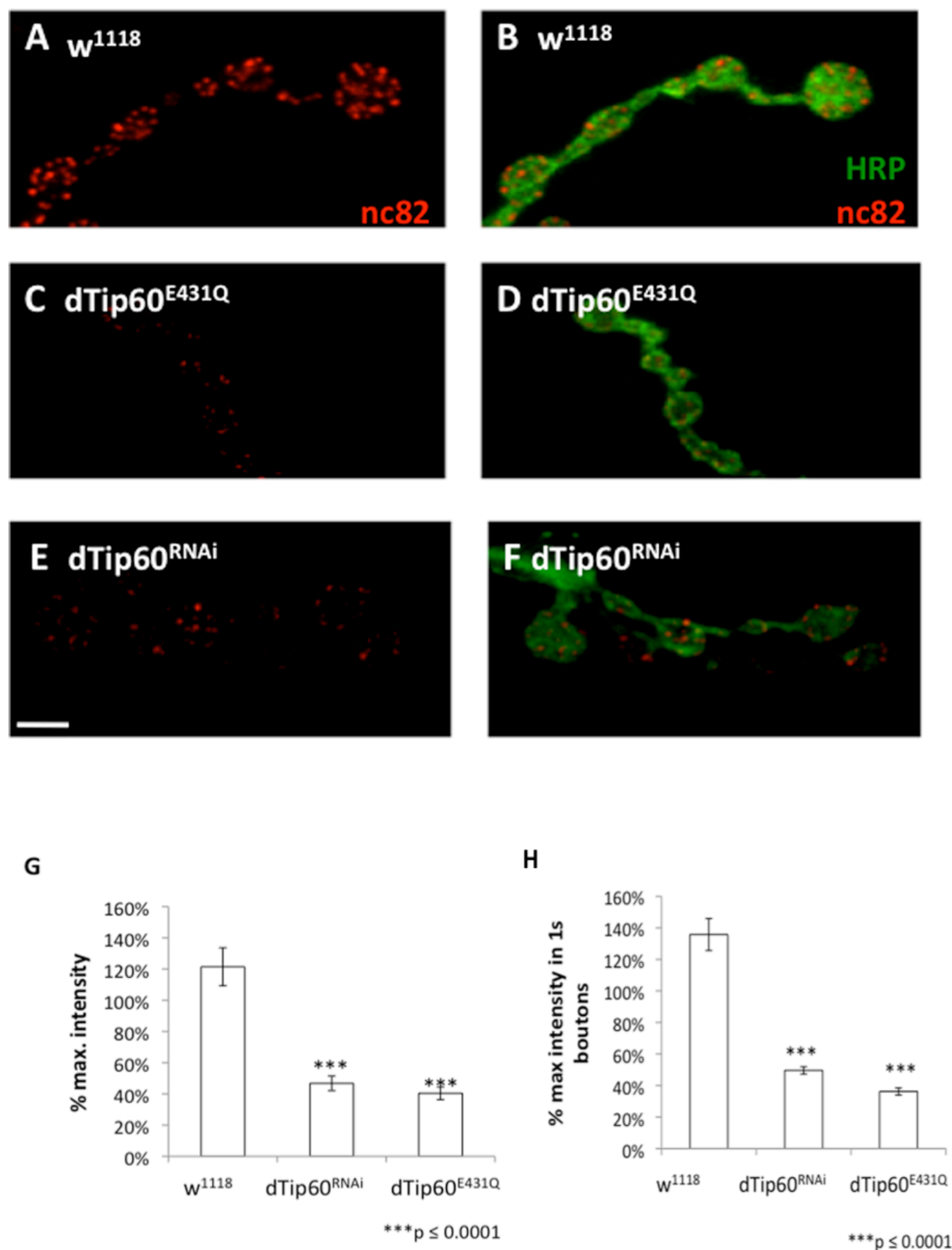
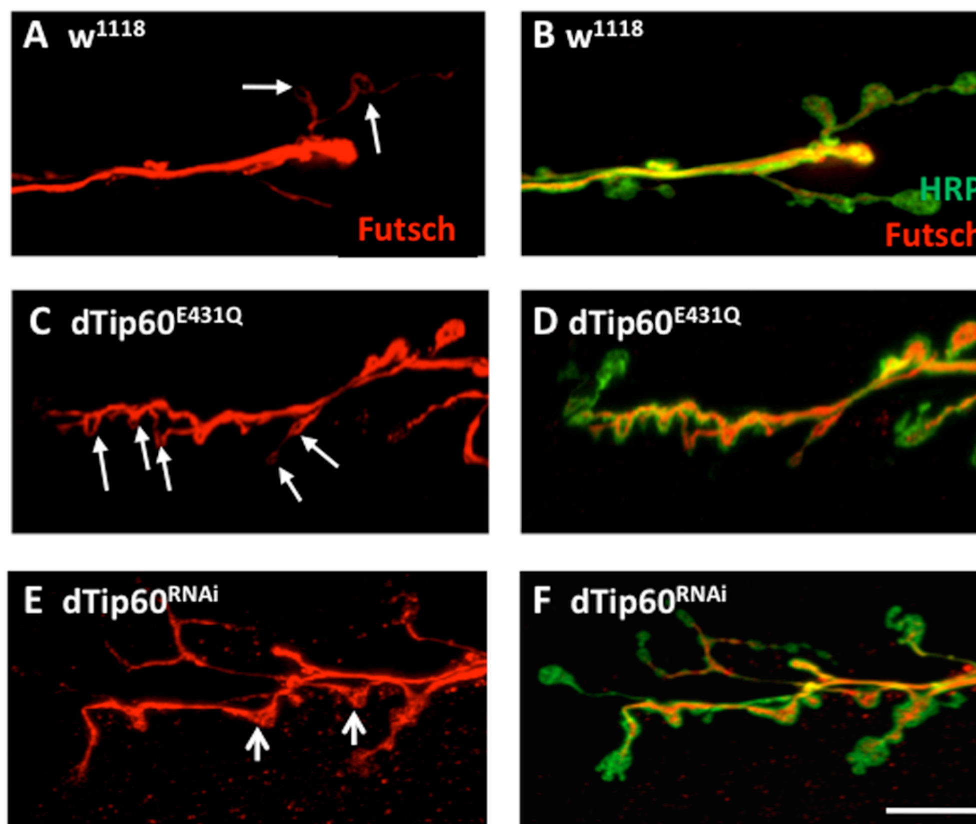


Figure 5. Presynaptic reduction of dTip60 in the nervous system leads to suppression of active zone marker bruchpilot at larval NMJs.

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and nc82 antibody (red) that recognizes bruchpilot, a protein

Figure 4 (continued)

associated with functional active zones and neurotransmission. (**A,B**) is w^{1118} (**C,D**) is dTip60^{E431Q} (**E,F**) is dTip60^{RNAi}. In the analyses, w^{1118} genotype is represented by 20 larval preparations (n = 20), dTip60^{RNAi} (n = 18) and dTip60^{E431Q} (n = 21). Scale bar is 5 um. Quantification of the fluorescence intensity as a measure of nc82 abundance at the NMJ muscle 6/7 of segment A4. The relative fluorescence intensities were measured as number of pixels per measured area and represented as % maximum intensities. Fluorescence intensity was determined by first quantitatively measuring the intensity profiles of all the nc82 punctae in a given marked area of the boutons and then calculating the average intensity of the brightest punctae in all of the samples as given by their intensity values. The nc82 intensities were normalized against HRP fluorescence intensities and corrected for background before all intensity measurements. Histogram depicts quantitative analysis of antibody fluorescence intensity where (**G**) is percentage maximum intensity of antibody stain in all boutons (**H**) is percentage maximum intensity of antibody stain in Is boutons. Asterisks (*) indicates statistically significant difference in relation to control where double asterisks indicate $p \leq 0.001$ and three asterisks indicate $p \leq 0.0001$. All error bars depict standard error of the mean.



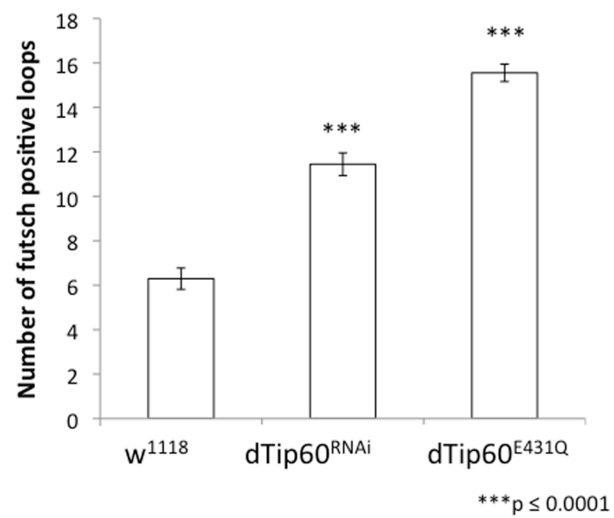


Figure 6. The number of presynatpic futsch-positive loops are increased at synapses in mutant dTip60 larvae.

Figure 6 (continued)

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and Futsch antibody (red) that labels Futsch stained microtubule loops. (**A,B**) is w^{1118} (**C,D**) is dTip60^{E431Q} (**E,F**) is dTip60^{RNAi}. Histogram depicts quantitative analysis of Futsch-positive loop number on muscles 6 and 7 at abdominal segment A4 where (**G**) is total number of loops. In the analyses, w^{1118} genotype is represented by 30 larval preparations (n = 30), dTip60^{RNAi} (n = 25) and dTip60^{E431Q} (n = 27). Small thick arrows depict Futsch stained microtubule loops (**C**), line arrows (**E**) depict Futsch stained microtubule rearrangement within loops. Scale bar is 10 um. Asterisks (\) indicates statistically significant difference in relation to control where double asterisks indicate p≤0.001 and three asterisks indicate p≤0.0001. All error bars depict standard error of the mean.

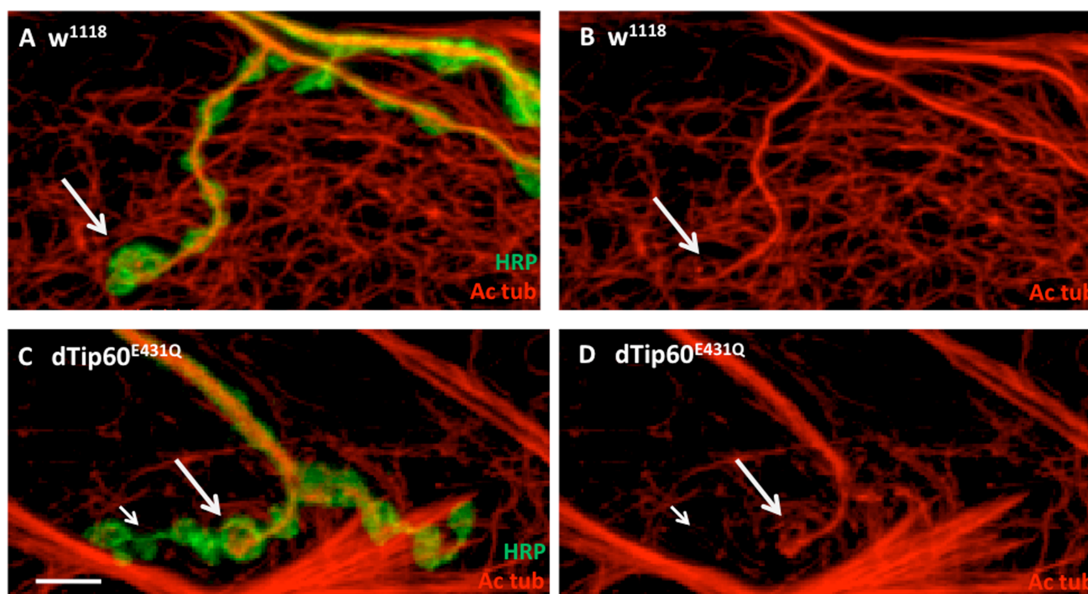


Figure 7. Loss of presynaptic dTip60 in the nervous system results in a reduction of acetylated microtubules in axons extending into terminal boutons.

Confocal imaging analysis of boutons on muscles 6/7 at abdominal segment A4 immunohistochemically double labeled with HRP (green) and anti-acetylated α-tubulin antibody (red) that labels acetylated microtubules. (**A**) Merged image of HRP and acetylated α-tubulin antibody double labeled control w^{1118} larvae (**B**) acetylated α-tubulin antibody labeled control w^{1118} larvae (**C**) Merged image of HRP and acetylated α-tubulin antibody double labeled $dTip60^{E431Q}$ larvae (D) acetylated α-tubulin antibody labeled $dTip60^{E431Q}$ larvae. Of note, acetylated microtubule staining as a whole was decreased in $dTip60^{E431Q}$ larvae (Figure 7 C and D). Large arrows represent acetylated microtubule staining in axons extending into boutons and small arrow represents their absence in more terminal boutons of $dTip60^{E431Q}$ mutant larvae. In the analyses, w^{1118} genotype is represented by 31 larval preparations (n = 31) and $dTip60^{E431Q}$ (n = 34). Scale bar is 5 um. Arrows show region of axon terminals where reduction of acetylated α-tubulin is consistently observed.

Chapter 3 Figures

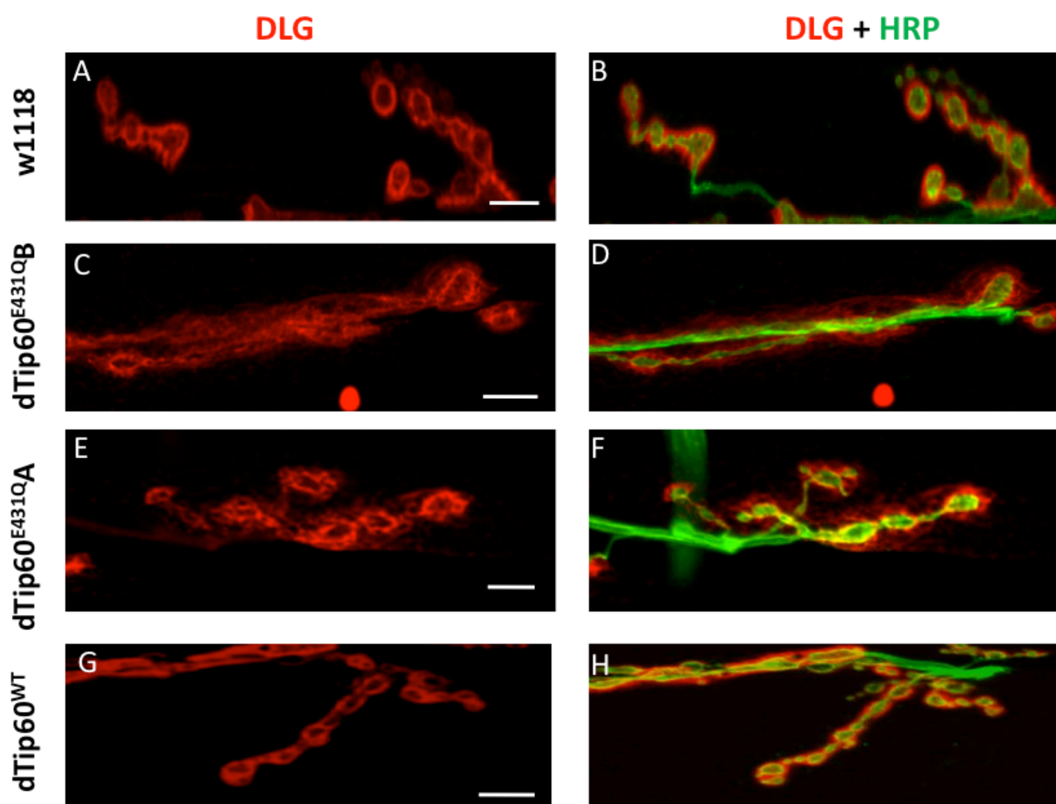


Figure 1. Post-synaptic loss of Tip60 HAT activity affects DLG localization.

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and Dlg antibody (red) that labels Discs large, a postsynaptic scaffold protein. (**A,B**) is w^{1118} (**C,D**) is $dTip60^{E431Q}B$ (**E,F**) is $dTip60^{E431Q}A$ (**G,H**) is $Tip60^{WT}$. DLG staining appears diffused in the $Tip60^{E431Q}B$ and $Tip60^{E431Q}A$ mutants suggesting defects in localization of DLG in the mutants. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), $dTip60^{E431Q}B$ (n = 18), $dTip60^{E431Q}A$ (n = 17) and $dTip60^{WT}$ (n = 16). Scale bar is 10 um.

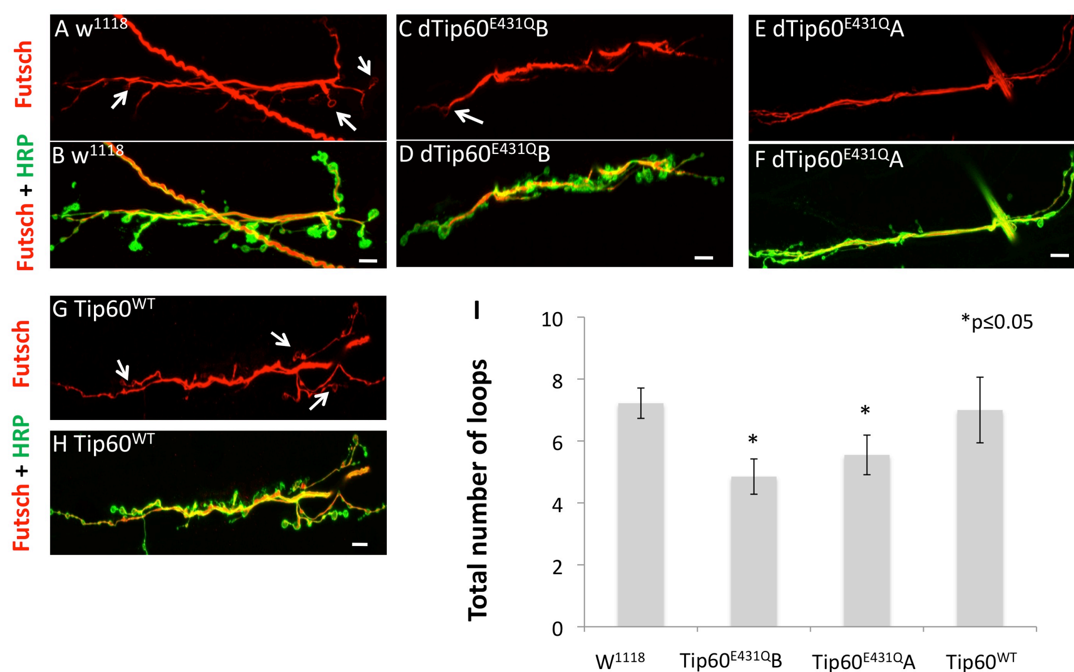


Figure 2. Post-synaptic loss of Tip60 HAT activity leads to decrease in the number of presynatpic futsch-positive loops

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and Futsch antibody (red) that labels Futsch stained microtubule loops. (**A,B**) is w^{1118} (**C,D**) is $dTip60^{E431Q}B$ (**E,F**) is $dTip60^{E431Q}A$ (**G,H**) $Tip60^{WT}$. Histogram depicts quantitative analysis of Futsch-positive loop number on muscles 6 and 7 at abdominal segment A4 where (**I**) is total number of loops. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), $dTip60^{E431Q}B$ (n = 16) and $dTip60^{E431Q}A$ (n = 17) and $Tip60^{WT}$ (n=15). Small thick arrows depict Futsch stained microtubule loops. Scale bar is 10 um. Asterisks (*) indicates statistically significant difference in relation to control where single asterisk indicates $p \leq 0.05$. All error bars depict ± SEM.

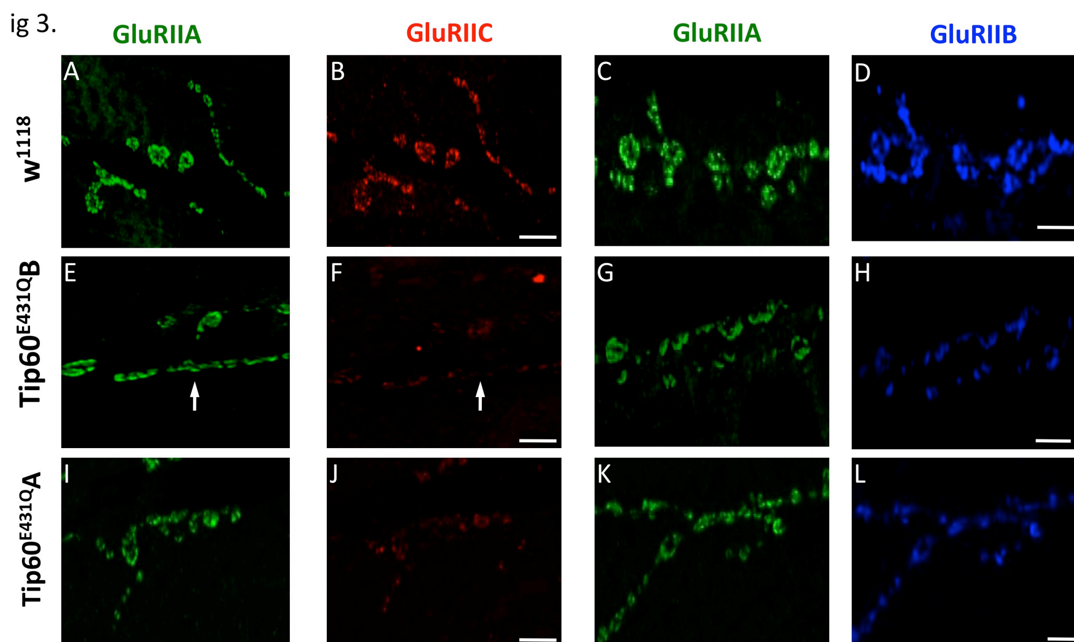


Figure 3. Post-synaptic loss of Tip60 HAT activity leads to decrease in GluRIIB and GluRIIC clusters.

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemically labeled with GluRIIA antibody (green) that labels the GluRIIA subunit, GluRIIC antibody (red) that labels GluRIIC subunit and GluRIIB antibody (blue) that labels GluRIIB subunit. (**A-D**) is w^{1118} (**E-H**) is $dTip60^{E431Q}B$ (**I-L**) is $dTip60^{E431Q}A$. Arrow heads indicate loss of GluRIIC in $Tip60^{E431Q}A$ and $Tip60^{E431Q}B$ mutants. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), $dTip60^{E431Q}B$ (n = 19), $Tip60^{E431Q}A$ (n = 17). Scale bar = 10μm.

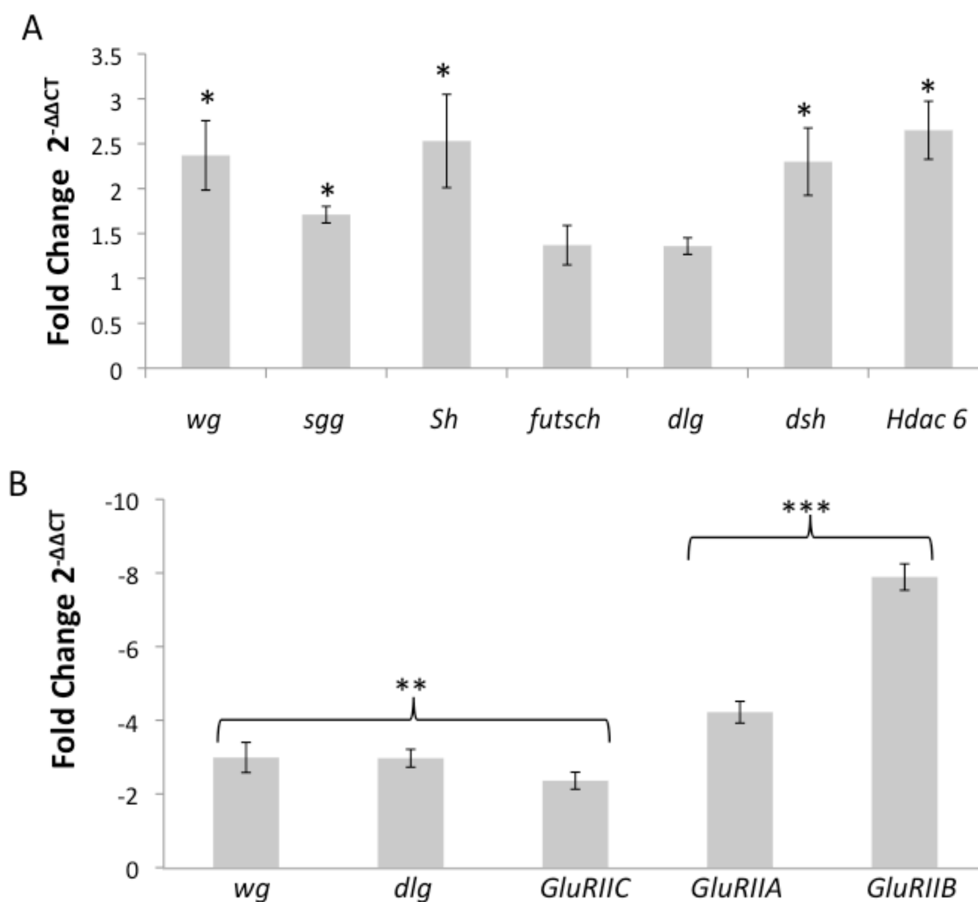
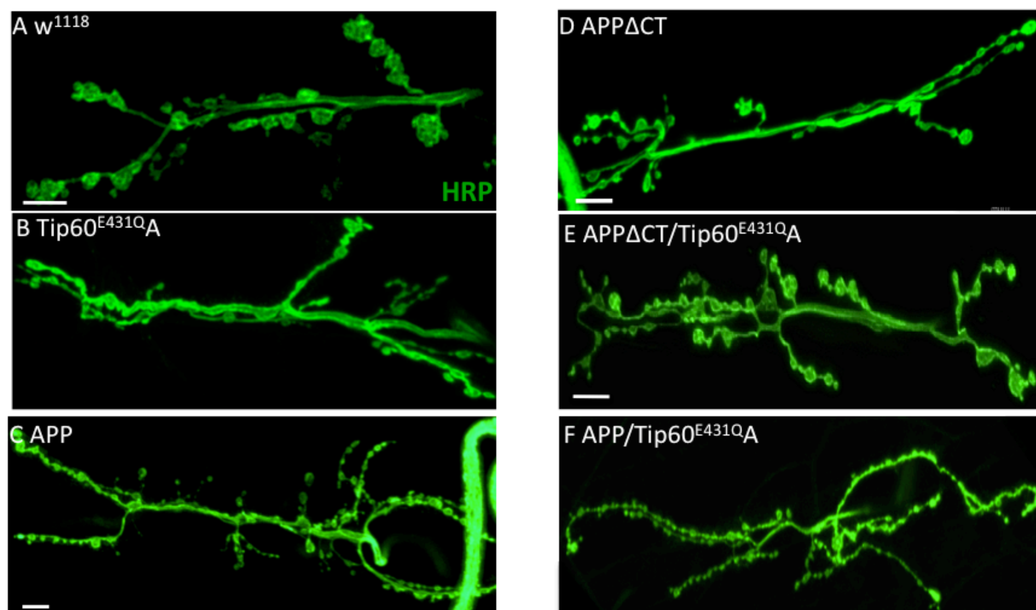


Figure 4. Pre- and post-synaptic loss of Tip60 HAT activity leads to misexpression of synaptic plasticity genes.

Loss of Tip60 HAT activity pre- and post-synaptically causes misregulation of synaptic plasticity genes. ***A***. Real-time PCR was performed on cDNA isolated from staged third instar larval dissected brain tissue from larvae expressing $dTip60^{E431Q}B$ using the pan-neuronal elav/GAL4 driver. Each histogram bar represents the fold change in expression level of synaptic plasticity target genes in brains of larvae expressing $dTip60^{E431Q}B$ relative to w^{1118} control flies. ***B***. Real-time PCR was performed on cDNA isolated from staged third instar larval dissected bodywall tissue from larvae expressing $dTip60^{E431Q}B$ using the muscle specific Twi;Mef2/GAL4 driver. Each histogram bar represents the fold change in expression level of synaptic plasticity target genes in brains of larvae expressing $dTip60^{E431Q}B$ relative to w^{1118} control flies. Real-time PCRs were performed in triplicate, and the fold change was calculated using the ΔΔCT method using RP49 as control. Statistical significance was calculated using an unpaired Student's *t* test: **p* <0.05, ***p* <0.001, ****p* <0.0001.



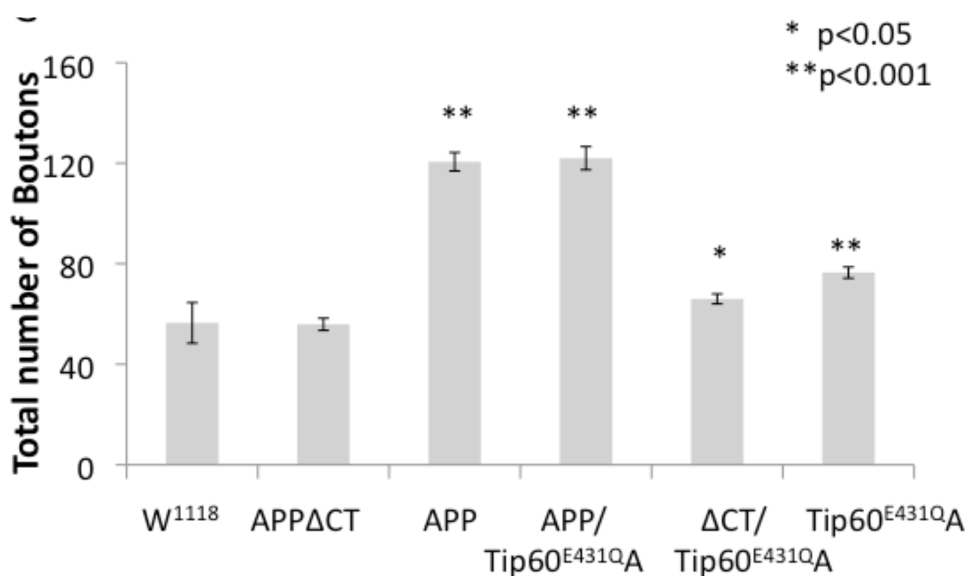


Figure 5. APP and Tip60 functionally interact pre-synaptically at the NMJ
Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment A4 immunohistochemically stained with anti-HRP (green) that labels the entire presynaptic membrane. (**A**) control w^{1118} larvae (**B**) larvae expressing $dTip60^{E431Q}A$ (**C**) larvae expressing APP (**D**) larvae expressing $APP^{\Delta CT}$ (**E**) $APP^{\Delta CT}/Tip60^{E431Q}A$ (**F**) $APP/Tip60^{E431Q}A$. Scale bar 10 um. Histogram depicts quantitative analysis of

Figure 5 (continued)

bouton number on muscles 6 and 7 at abdominal segment 4 where (**G**) represents total bouton number. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), dTip60^{E431Q}A (n = 19), APP (n = 19) APP$^{\Delta CT}$ (n = 17), APP$^{\Delta CT}$/Tip60^{E431Q}A (n = 19), APP/Tip60^{E431Q}A (n = 18). Asterisks (*) indicates statistically significant difference in relation to control where single asterisks indicate $p \leq 0.05$ and double asterisks indicate $p \leq 0.001$. All error bars depict ± SEM.

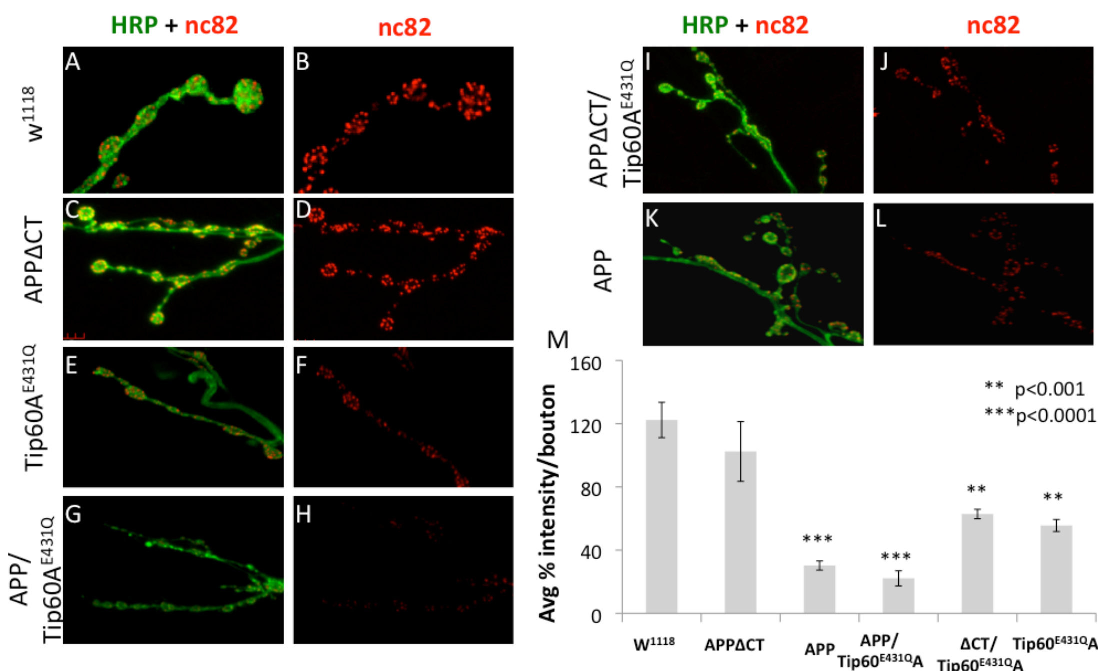


Figure 6. Presynaptic expression of APP and HAT deficient dTip60 in the nervous system leads to suppression of active zone marker bruchpilot at larval NMJs.

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and nc82 antibody (red) that recognizes bruchpilot, a protein associated with functional active zones and neurotransmission. (**A,B**) is w^{1118} (**C,D**) is $APP^{\Delta CT}$(**E,F**) is $dTip60^{E431Q}A$ (**G,H**) $APP/Tip60^{E431Q}A$ (**I,J**) $APP^{\Delta CT}/Tip60^{E431Q}A$ (**K,L**) APP . In the analyses, w^{1118} genotype is represented by 20 larval preparations (n = 20), $APP^{\Delta CT}$ (n = 17), $dTip60^{E431Q}A$ (n = 18), $APP/Tip60^{E431Q}A$ (n = 16), $APP^{\Delta CT}/Tip60^{E431Q}A$ (n = 18) and APP (n = 18). Scale bar is 5 um. Quantification of the fluorescence intensity as a measure of nc82 abundance at the NMJ muscle 6/7 of segment A4. The relative fluorescence intensities were measured as number of pixels per measured area and represented as % maximum intensities. Fluorescence intensity was determined by first quantitatively measuring the intensity profiles of all the nc82 punctae in a given marked area of the boutons and then calculating the average intensity of the brightest punctae in all of the samples as given by their intensity values. The nc82 intensities were normalized against HRP fluorescence intensities and corrected for background before all intensity measurements. Histogram depicts quantitative analysis of antibody fluorescence intensity where (**M**) is average percentage intensity per bouton. Asterisks (*) indicates statistically significant difference in relation to control where double asterisks indicate $p \leq 0.001$ and three asterisks indicate $p \leq 0.0001$. All error bars depict ± SEM.

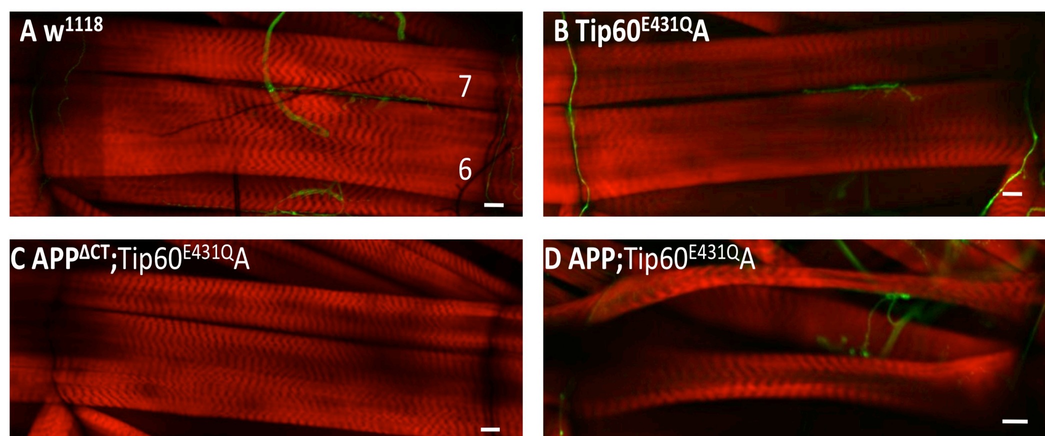


Figure 7. Post synaptic expression of APP and HAT deficient Tip60 causes decrease in muscle size

Confocal imaging analysis of larval muscles 6/7 at abdominal segment 4 immunohistochemical double labeled with HRP antibody (green) that labels the entire presynaptic membrane and phalloidin (red) that stains the muscle. We observe severe reduction in muscle size in the (**D**) APP/Tip60^{E431Q}A mutants when compared to (**A**) w^{1118}, (**B**) Tip60^{E431Q}A and (**C**) APP$^{\Delta CT}$/Tip60^{E431Q}A. In the analyses, w^{1118} genotype is represented by 17 larval preparations (n = 17), dTip60^{E431Q}A (n = 19), APP$^{\Delta CT}$/Tip60^{E431Q}A (n = 18) and APP/Tip60^{E431Q}A (n = 17) . Scale bar =20μm.

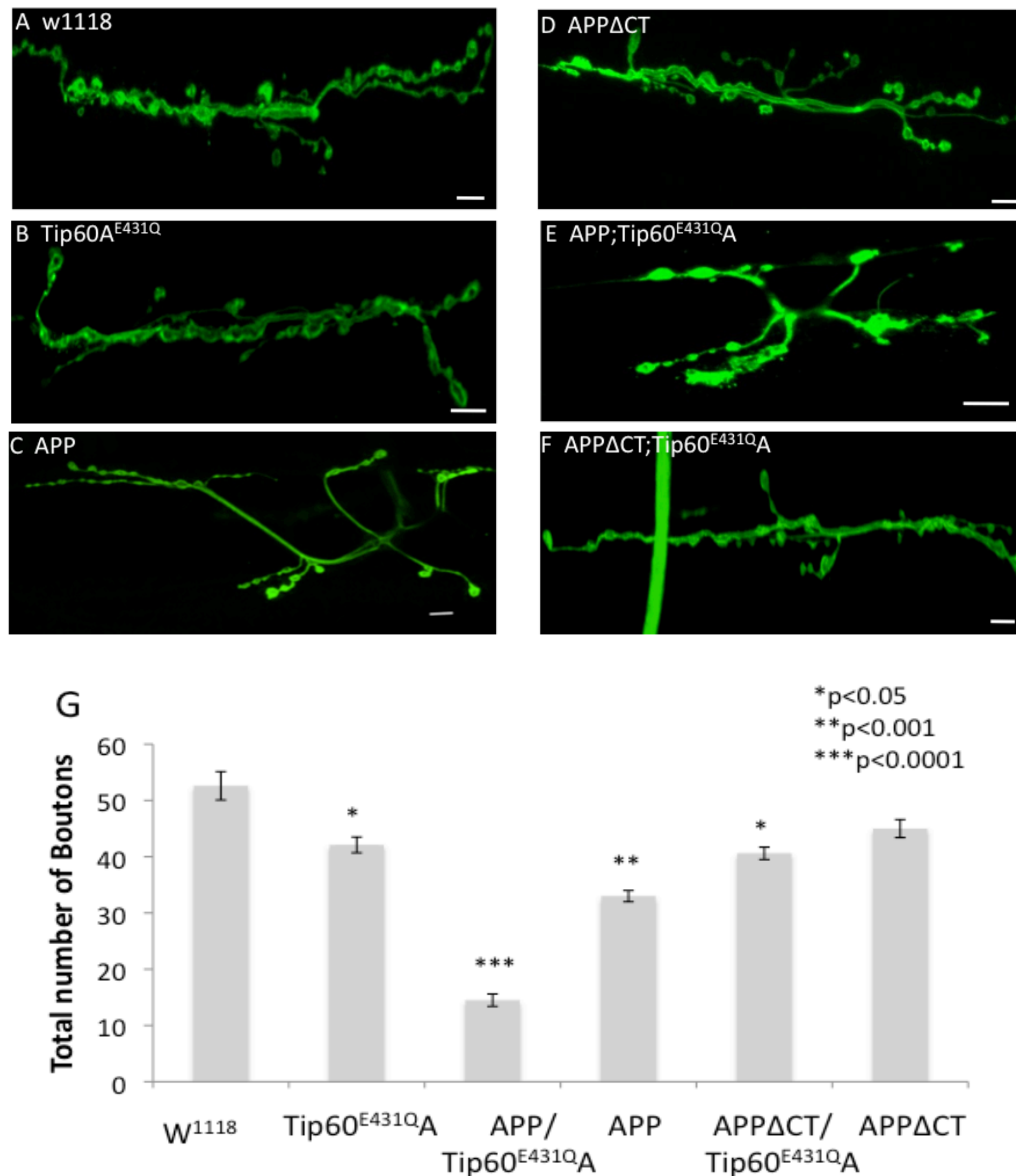


Figure 8. Postsynaptic expression of APP and HAT deficient dTip60 affects presynaptic bouton formation

Flies homozygous for either dTip60^{E431Q}A, APP, APP$^{\Delta CT}$, APP/Tip60^{E431Q}A, APP$^{\Delta CT}$/Tip60^{E431Q}A or control w^{1118} were crossed to flies homozygous for the muscle specific GAL4 driver Twi;Mef-2, and staged third instar progeny larvae were collected. Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal

Figure 8. (continued)

segment A4 double labeled with anti-HRP (green) that labels the entire presynaptic membrane. (**A**) control w^{1118} larvae (**B**) larvae expressing $dTip60^{E431Q}A$ (**C**) larvae expressing APP (**D**) larvae expressing $APP^{\Delta CT}$ (**E**) larvae expressing $APP/Tip60^{E431Q}A$ and (**F**) $APP^{\Delta CT}/Tip60^{E431Q}A$. Scale bar 10 um. Histogram depicts quantitative analysis of bouton number on muscles 6 and 7 at abdominal segment 4 where (**G**) represents total bouton number. In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), $dTip60^{E431Q}A$ (n = 19), APP (n = 16), $APP^{\Delta CT}$ (n = 18), $APP/Tip60^{E431Q}A$ (n = 17) and $APP^{\Delta CT}/Tip60^{E431Q}A$ (n = 18). Asterisks (*) indicates statistically significant difference in relation to control where single asterisks indicate $p \leq 0.05$, double asterisks indicate $p \leq 0.001$ and three asterisks indicate $p \leq 0.0001$. All error bars depict ± SEM. Scale bar = 10μm.

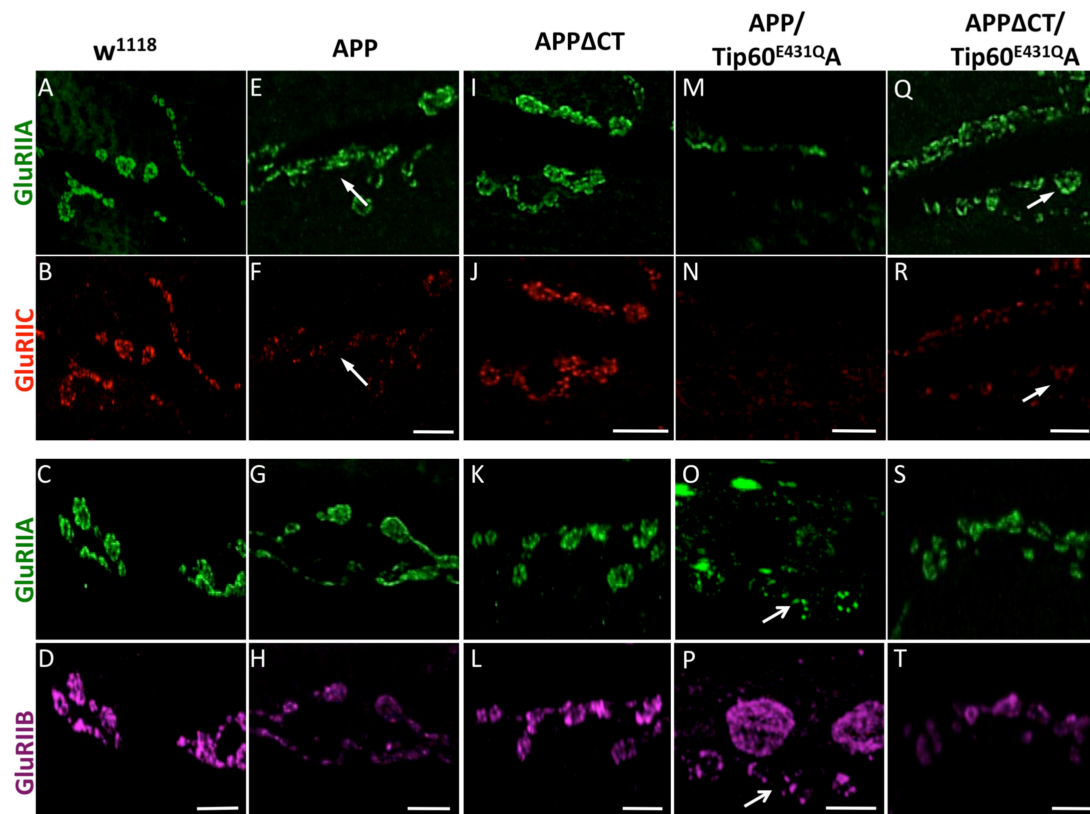


Figure 9. Post-synaptic loss of Tip60 HAT activity and APP overexpression leads to decrease in GluRIIA, GluRIIB and GluRIIC clusters.

Confocal imaging analysis of larval boutons on muscles 6/7 at abdominal segment 4 immunohistochemically labeled with GluRIIA antibody (green) that labels the GluRIIA subunit, GluRIIC antibody (red) that labels GluRIIC subunit and GluRIIB antibody (magenta) that labels GluRIIB subunit. (**A-D)** is w^{1118} (**E-H**) is APP (**I-L**) is $APP^{\Delta CT}$ (**M-P**) $APP/Tip60^{E431Q}A$ **(Q-T**) $APP^{\Delta CT}/Tip60^{E431Q}A$. $APP/Tip60^{E431Q}A$ shows decrease in all the three receptor subunits where GluRIIA and GluRIIB do not colocalize (arrow) and IIC is completely absent at the synapses. APP and $APP^{\Delta CT}/Tip60^{E431Q}A$ also show decrease in GluRIIC receptor subunits at the synapses compared to w^{1118} (arrow heads). In the analyses, w^{1118} genotype is represented by 18 larval preparations (n = 18), APP (n = 17), $APP^{\Delta CT}$ (n = 18), $APP/Tip60^{E431Q}A$ (n = 17) and $APP^{\Delta CT}/Tip60^{E431Q}A$ (n = 18). Scale bar = 10μm.

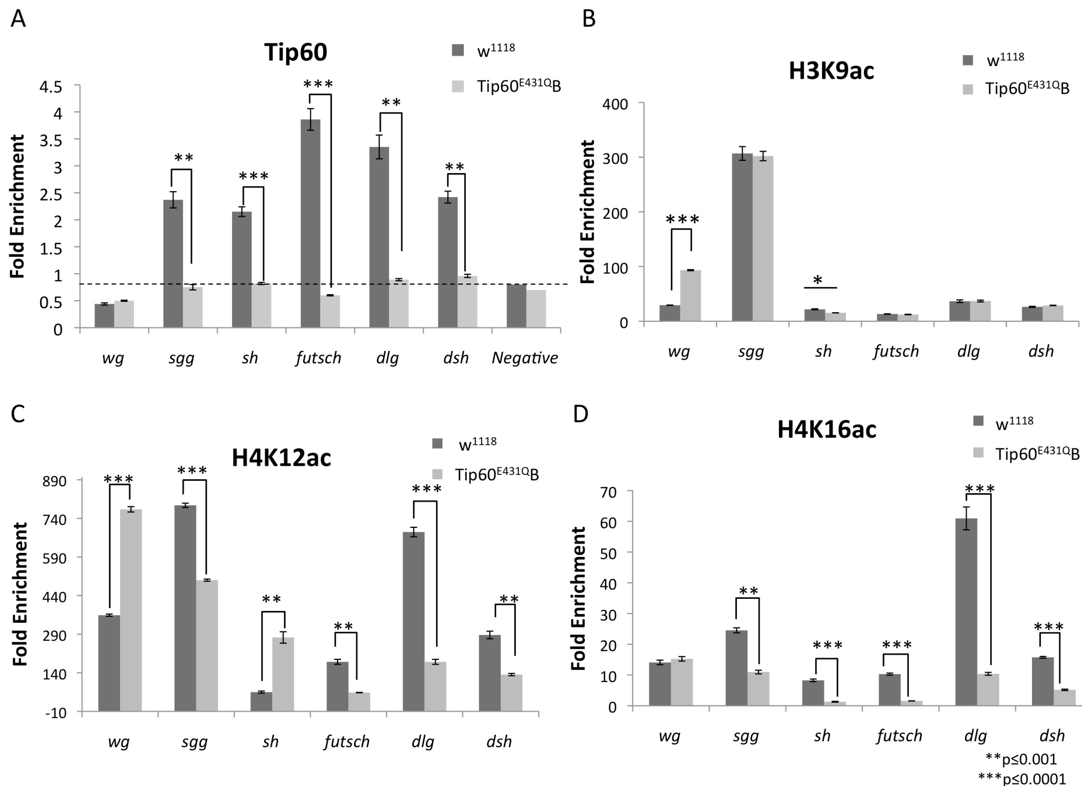


Figure 10. ChIP enrichment of target genes by Tip60 and acetylated H3K9, H4K12 and H4K16 antibodies. ***A***, Chromatin was isolated from 300 pooled larvae heads for each of the indicated genotypes. ChIP was performed using ChIP-IT Express Kit (Active Motif) using Tip60 antibodies and (***B***) acetylated histone H3K9, (***C***) H4K12 and (***D***) H4K16 antibodies. For each ChIP experiment, a mock reaction containing no antibody was performed simultaneously as a negative control. Real-time PCR was performed on DNA purified from each of the ChIP reactions using primer pairs specific for each gene loci. Fold enrichment of the respective genes was calculated relative to the mock no antibody control using the standard curve method as described in the ChIP-IT Express Kit manual. ***A***. Dotted line represents baseline enrichment level set by using negative control primers that amplify a fragment within a gene desert within Drosophila chromosome 3L. All data are from three independent qPCR experiments for each genotype. Statistical significance for all experiments was calculated using an unpaired Student's t test: *$p \leq 0.05$; **$p \leq 0.001$; $p \leq 0.0001$. Values are mean ± SEM

```
Esa1      35  ERLAEILSINTRKAPPKFYVHYVNYNKRLDEWITTDRINLDKEVLYPKLK---------  93
dTip60    42  WPLAEIVSIKELDGRRQFYVHYVDFNKRLDEWVNEEDLYTRK-VQFPRRDGSQTGTSTG  100
hTip60    59  WPLAEILSVKDISGRKLFYVHYIDFNKRLDEWVTHERLDLKK-IQFPKKE-AKTPTKNG  117
                **** *         *****   *******          *    *

dHp1      24  YAVEKIIDRRVRKGKVEYYLKWKGYPETENTWEPENNLDCQDLIQQYEASR-KDEEKSA  81
                   *      #    *             *     #      #
```

chromodomain

Figure 11. Amino acid sequence alignment of yeast Esa1, drosophila Tip60 and human Tip60 and HP1 chromodomains.

The chromodomain homology regions are indicated. Asterix show conserved sequence residues. Alignment was carried out using Clustal W (1.83) sequence alignment program.

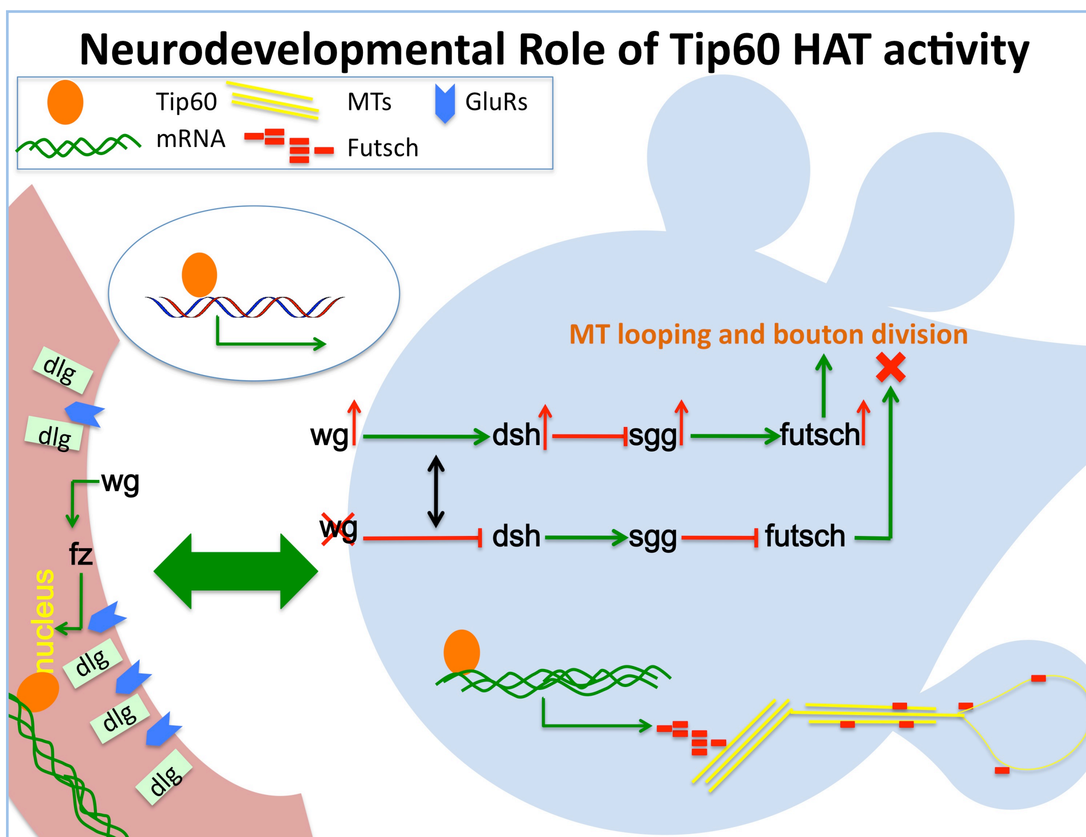


Figure 12. Model for role of Tip60 in synaptic plasticity and wg pathway.

Tip60 is important in both neurodevelopmental and neurodegenerative synaptic plasticity mechanisms. We hypothesize a model in which Tip60, predominantly acts via its nuclear localization and targets genes that are part of the wingless pathway (wg, dsh, sgg and futsch), genes involved in activity dependent synaptic plasticity (GluRs, Sh) and scaffold protein Dlg. On the presynaptic side, Tip60 functions via the canonical wg pathway where in, it allows for regulation of the wg pathway genes all the way to activation of futsch thus allowing for bouton budding and synaptic growth. On the post-synaptic side in the muscle, Tip60 again acts via the non-canonical wg pathway by regulating wg and possibly other downstream targets. Concurrently, Tip60 also regulates Dlg organization and GluRIIC localization. We hypothesize another level of regulation via Tip60 that takes place in the cytoplasm via interaction of the chromodomain of Tip60 with dormant mRNA's, which are important in activity dependent synaptic plasticity. For example, Futsch could interact with the chromodomain of Tip60 and allow Tip60 to activate local translation of futsch by recruiting the translational machinery and thus allowing for local synthesis of the protein required for microtubule looping and promoting bouton budding. On the post-synaptic side, the chromodomain-mediated regulation could be involved in regulation

Figure 12 (continued)

of the dormant mRNAs present postsynaptically, including the GluR mRNA's to regulate the local translation.

Chapter 4 Figures

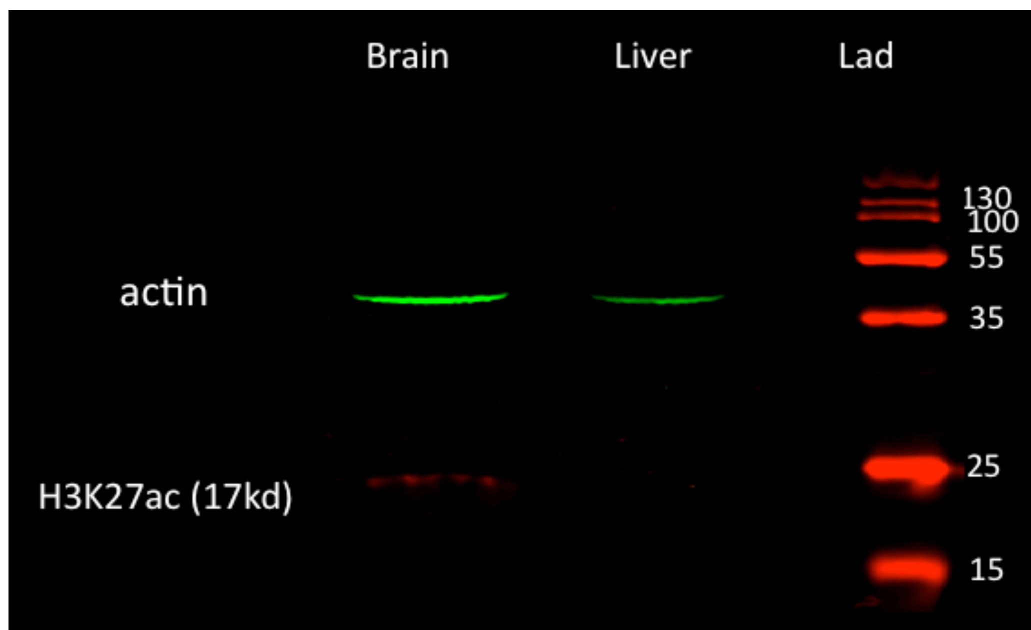


Figure 13. Western blot carried out using lysates from homogenized rat brain and liver tissues.

Core histones isolated from rat brain and liver tissues resolved by 18% polyacrylamide gel electrophoresis, Western-blotted, and immunostained with antibody that recognizes acetylated lysine residues (K27) of histone H3.

Appendix A: ChIP enrichment of axonal transport genes in Tip60 HAT deficient mutants and Ms-275 mediated rescue of the acetylation levels.

We have recently shown that loss of Tip60 HAT activity leads to decrease in locomotor function in Tip60^{E431Q}A and Tip60^{E431Q}B mutants compared to the w^{1118} controls (Johnson, Sarthi, *et al*., 2013). These defects in locomotor function are coupled with misregulation of axonal transport genes. The six genes (Khc, Klp64D, Dhc64C, p150^{Glued}, Appl and syt1) that showed significant misregulation were then treated with an HDACi Ms-275 that resulted in rescue of the phenotype as well as mRNA transcript levels. The rescue of these defects by using and HDACi led us to believe that the acetylation levels of these six genes could have been restored that led to the rescue.

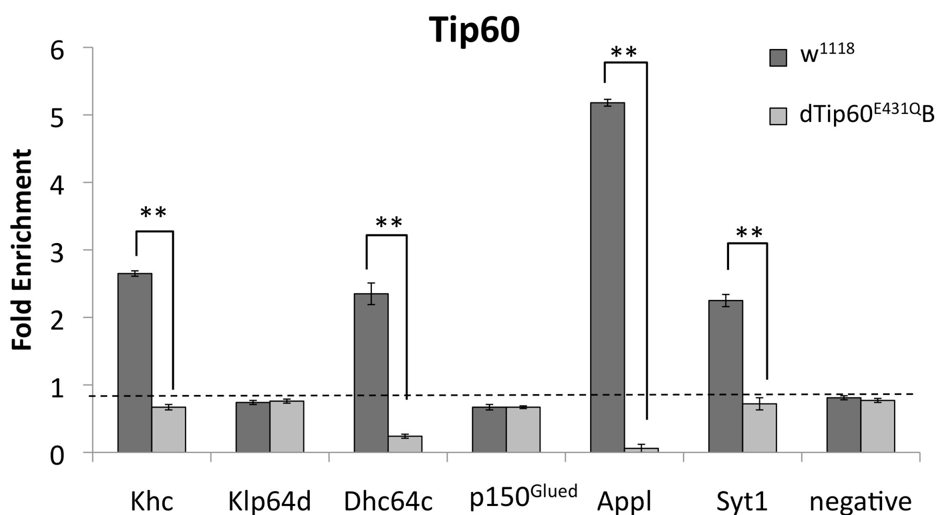
The six genes were then analyzed to investigate the mechanism of transcriptional regulation in Tip60^{E431Q}B and Tip60^{E431Q}B-Ms-275, Ms-275 treated HAT defective mutants. To answer this question we made use of Chromatin Immunoprecipitation (ChIP) to pull down genes that are bound by either Tip60 or acetylated H4 proteins. We first investigated if the axonal transport genes identified were indeed direct targets of Tip60 in the w^{1118} control flies and if the recruitment of Tip60 to these genes would be affected in the Tip60 HAT mutants. To address this, chromatin was isolated from larval posterior head regions from each of these genotypes and ChIP-qPCR analysis was used to detect *in vivo* binding of Tip60 at each of these gene loci. Significant enrichment was observed for Tip60 binding *in vivo* at four of the six gene loci tested in w^{1118} larvae, thus suggesting these genes (Appl, Dhc64C, Khc, and Syt1) as novel direct targets of Tip60 and Glued and

Klp64D as possible indirect targets of Tip60 (Fig. 1A). Conversely, Tip60^{E431Q}B larvae showed no enrichment for Tip60 binding at any of these gene loci suggesting disruption of Tip60 binding to these loci due to loss of HAT activity.

As Tip60 is a HAT and regulates genes by primarily affecting their acetylation levels we hypothesized that loss of Tip60 HAT activity could lead to decrease in acetylation levels of histone at these gene loci. Furthermore, since ms-275 is an HDACi we also hypothesize that treatment of Tip60 HAT defective mutants could lead to restoration of the acetylation levels at the gene loci. To test this, we assessed enrichment levels of pan-acetylated histone H4 in chromatin isolated from larval heads, which is the preferential histone target of Tip60, at each gene loci in w^{1118}, Tip60^{E431Q}B and ms-275-treated Tip60^{E431Q}B larvae. All the previously identified Tip60 target genes showed enhanced H4 acetylation levels in the w^{1118} flies and significantly reduced levels in Tip60^{E431Q}B flies (Fig. 1B). As expected, treatment of Tip60^{E431Q}B larvae with ms-275 partially rescues acetylation loss at all but one (Appl) of the genes (Fig. 6*C*). Of note, we did observe enrichment of H4 acetylation at Tip60 indirect targets (Glued, Klp64D) relative to that found in Tip60^{E431Q}B larvae, suggesting that perhaps Tip60 may indirectly lead to increased H4 acetylation or non enrichment for Tip60 site-specific acetylation at these sites is masked by the global pan-acetylation H4 antibodies used in these studies. In summary, these results demonstrate that reduction of Tip60 HAT activity leads to reduced histone H4 acetylation levels at Tip60 gene targets that can be partially rescued by treatment with the ms-275 HDACi.

Appendix A: Figures

A



B

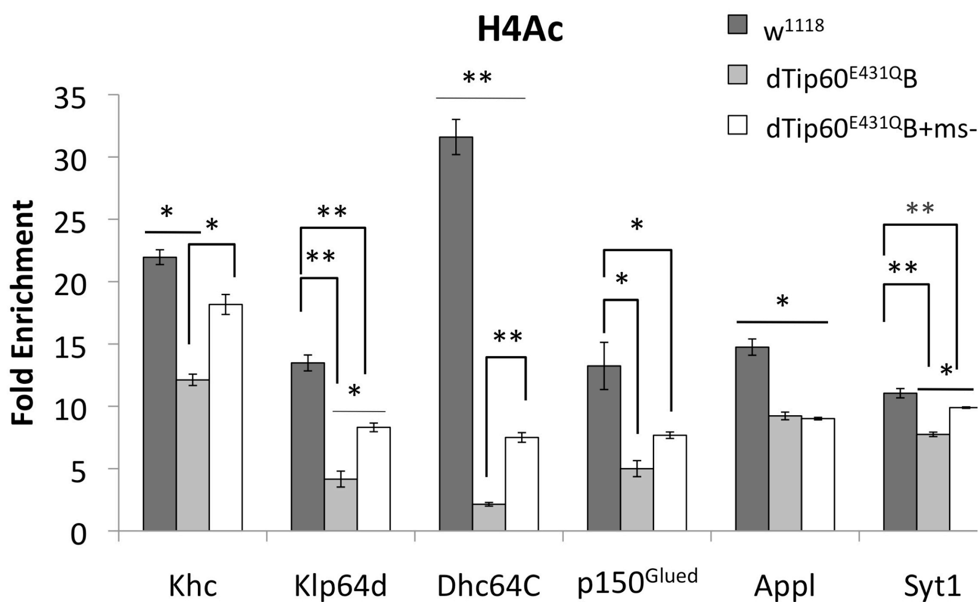


Figure 1. ChIP enrichment of target genes by Tip60 and pan-acetylated histone H4 antibodies. Chromatin was isolated from 300 pooled larvae heads for each of the indicated genotypes. ChIP was performed using ChIP-IT Express Kit (Active Motif)

Figure 1 (continued)

using Tip60 antibodies (***A***) and (***B***) pan-acetylated histone H4 antibodies. For each ChIP experiment, a mock reaction containing no antibody was performed simultaneously as a negative control. Real-time PCR was performed on DNA purified from each of the ChIP reactions using primer pairs specific for each gene loci. Fold enrichment of the respective genes was calculated relative to the mock no antibody control using the standard curve method as described in the ChIP-IT Express Kit manual. ***A***, Dotted line represents baseline enrichment level set by using negative control primers that amplify a fragment within a gene desert within *Drosophila* chromosome 3L. All data are from three independent qPCR experiments for each genotype. Statistical significance for all experiments was calculated using an unpaired Student's *t* test: $*p \leq 0.05$; $**p \leq 0.01$. Values are mean ± SEM.

List of References

Akhtar, A., Zink, D., & Becker, P. B. (2000). Chromodomains are protein-RNA interaction modules. *Nature, 407*(6802), 405-409. doi: 10.1038/35030169

Alarcon, J. M., Malleret, G., Touzani, K., Vronskaya, S., Ishii, S., Kandel, E. R., & Barco, A. (2004). Chromatin acetylation, memory, and LTP are impaired in CBP+/- mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. *Neuron, 42*(6), 947-959. doi: 10.1016/j.neuron.2004.05.021

Allen, N. J., & Barres, B. A. (2005). Signaling between glia and neurons: focus on synaptic plasticity. *Curr Opin Neurobiol, 15*(5), 542-548. doi: 10.1016/j.conb.2005.08.006

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., & Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet, 23*(2), 185-188. doi: 10.1038/13810

Anderton, B. H., Dayanandan, R., Killick, R., & Lovestone, S. (2000). Does dysregulation of the Notch and wingless/Wnt pathways underlie the pathogenesis of Alzheimer's disease? *Mol Med Today, 6*(2), 54-59.

Andres, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., . . . Mandel, G. (1999). CoREST: a functional corepressor required for regulation of neural-specific gene expression. *Proc Natl Acad Sci U S A, 96*(17), 9873-9878.

Andrews-Hanna, J. R., Snyder, A. Z., Vincent, J. L., Lustig, C., Head, D., Raichle, M. E., & Buckner, R. L. (2007). Disruption of large-scale brain systems in advanced aging. *Neuron, 56*(5), 924-935. doi: 10.1016/j.neuron.2007.10.038

Artavanis-Tsakonas, S., Rand, M. D., & Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science, 284*(5415), 770-776.

Artola, A., & Singer, W. (1990). The Involvement of N-Methyl-D-Aspartate Receptors in Induction and Maintenance of Long-Term Potentiation in Rat Visual Cortex. *Eur J Neurosci, 2*(3), 254-269.

Asaka, Y., Jugloff, D. G., Zhang, L., Eubanks, J. H., & Fitzsimonds, R. M. (2006). Hippocampal synaptic plasticity is impaired in the Mecp2-null mouse model of Rett syndrome. *Neurobiol Dis, 21*(1), 217-227. doi: 10.1016/j.nbd.2005.07.005

Ashley, C. T., Sutcliffe, J. S., Kunst, C. B., Leiner, H. A., Eichler, E. E., Nelson, D. L., & Warren, S. T. (1993). Human and murine FMR-1: alternative splicing and translational initiation downstream of the CGG-repeat. *Nat Genet, 4*(3), 244-251. doi: 10.1038/ng0793-244

Ashley, J., Packard, M., Ataman, B., & Budnik, V. (2005). Fasciclin II signals new synapse formation through amyloid precursor protein and the scaffolding protein dX11/Mint. *J Neurosci, 25*(25), 5943-5955. doi: 25/25/5943 [pii]

10.1523/JNEUROSCI.1144-05.2005

Avantaggiati, M. L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A. S., & Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. *Cell, 89*(7), 1175-1184.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S., & Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by dishevelled. *Science, 271*(5257), 1826-1832.

Baas, P. W., & Sudo, H. (2010). More microtubule severing proteins: More microtubules. *Cell Cycle, 9*(12). doi: 12026 [pii]

Baek, S. H., Ohgi, K. A., Rose, D. W., Koo, E. H., Glass, C. K., & Rosenfeld, M. G. (2002). Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. *Cell, 110*(1), 55-67.

Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., . . . Mandel, G. (2001). Regulation of neuronal traits by a novel transcriptional complex. *Neuron, 31*(3), 353-365.

Ballas, N., Grunseich, C., Lu, D. D., Speh, J. C., & Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell, 121*(4), 645-657. doi: 10.1016/j.cell.2005.03.013

Barnes, C. A., & McNaughton, B. L. (1980). Physiological compensation for loss of afferent synapses in rat hippocampal granule cells during senescence. *J Physiol, 309*, 473-485.

Bassing, C. H., & Alt, F. W. (2004). H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity. *Cell Cycle, 3*(2), 149-153.

Bates, G. P. (2005). History of genetic disease: the molecular genetics of Huntington disease - a history. *Nat Rev Genet, 6*(10), 766-773. doi: 10.1038/nrg1686

Benfenati, F. (2007). Synaptic plasticity and the neurobiology of learning and memory. *Acta Biomed, 78 Suppl 1*, 58-66.

Blalock, E. M., Chen, K. C., Sharrow, K., Herman, J. P., Porter, N. M., Foster, T. C., & Landfield, P. W. (2003). Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci, 23*(9), 3807-3819.

Blander, G., & Guarente, L. (2004). The Sir2 family of protein deacetylases. *Annu Rev Biochem, 73*, 417-435. doi: 10.1146/annurev.biochem.73.011303.073651

Bliss, T. V., & Gardner-Medwin, A. R. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaestetized rabbit following stimulation of the perforant path. *J Physiol, 232*(2), 357-374.

Bliss, T. V., & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol, 232*(2), 331-356.

Bobrowska, A., Paganetti, P., Matthias, P., & Bates, G. P. Hdac6 knock-out increases tubulin acetylation but does not modify disease progression in the R6/2 mouse model of Huntington's disease. *PLoS One, 6*(6), e20696. doi: 10.1371/journal.pone.0020696

PONE-D-11-02335 [pii]

Borrelli, E., Nestler, E. J., Allis, C. D., & Sassone-Corsi, P. (2008). Decoding the epigenetic language of neuronal plasticity. *Neuron, 60*(6), 961-974. doi: 10.1016/j.neuron.2008.10.012

Borrow, J., Stanton, V. P., Jr., Andresen, J. M., Becher, R., Behm, F. G., Chaganti, R. S., . . . Housman, D. E. (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat Genet, 14*(1), 33-41. doi: 10.1038/ng0996-33

Bottomley, M. J. (2004). Structures of protein domains that create or recognize histone modifications. *EMBO Rep, 5*(5), 464-469. doi: 10.1038/sj.embor.7400146

Brady, M. E., Ozanne, D. M., Gaughan, L., Waite, I., Cook, S., Neal, D. E., & Robson, C. N. (1999). Tip60 is a nuclear hormone receptor coactivator. *J Biol Chem, 274*(25), 17599-17604.

Broadie, K. S., & Bate, M. (1993). Development of the embryonic neuromuscular synapse of *Drosophila* melanogaster. *J Neurosci, 13*(1), 144-166.

Bronk, P., Nie, Z., Klose, M. K., Dawson-Scully, K., Zhang, J., Robertson, R. M., . . . Zinsmaier, K. E. (2005). The multiple functions of cysteine-string protein analyzed at *Drosophila* nerve terminals. *J Neurosci, 25*(9), 2204-2214. doi: 25/9/2204 [pii]

10.1523/JNEUROSCI.3610-04.2005

Brownell, J. E., & Allis, C. D. (1996). Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr Opin Genet Dev, 6*(2), 176-184.

Budnik, V. (1996). Synapse maturation and structural plasticity at *Drosophila* neuromuscular junctions. *Curr Opin Neurobiol, 6*(6), 858-867. doi: S0959-4388(96)80038-9 [pii]

Budnik, V., & Ruiz-Canada, C. (2006). *The fly neuromuscular junction:structure and function* (second ed.): Elsevier.

Budnik, V., Zhong, Y., & Wu, C. F. (1990). Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J Neurosci, 10*(11), 3754-3768.

Bullions, L. C., & Levine, A. J. (1998). The role of beta-catenin in cell adhesion, signal transduction, and cancer. *Curr Opin Oncol, 10*(1), 81-87.

Burke, S. N., & Barnes, C. A. (2006). Neural plasticity in the ageing brain. *Nat Rev Neurosci, 7*(1), 30-40. doi: 10.1038/nrn1809

Cabeza, R., Anderson, N. D., Locantore, J. K., & McIntosh, A. R. (2002). Aging gracefully: compensatory brain activity in high-performing older adults. *Neuroimage, 17*(3), 1394-1402.

Cai, Y., Jin, J., Tomomori-Sato, C., Sato, S., Sorokina, I., Parmely, T. J., . . . Conaway, J. W. (2003). Identification of new subunits of the multiprotein mammalian TRRAP/TIP60-containing histone acetyltransferase complex. *J Biol Chem, 278*(44), 42733-42736. doi: 10.1074/jbc.C300389200

Cam, H. P., Sugiyama, T., Chen, E. S., Chen, X., FitzGerald, P. C., & Grewal, S. I. (2005). Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. *Nat Genet, 37*(8), 809-819. doi: 10.1038/ng1602

Cao, X., & Jacobsen, S. E. (2002). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. *Proc Natl Acad Sci U S A, 99 Suppl 4*, 16491-16498. doi: 10.1073/pnas.162371599

Cao, X., & Sudhof, T. C. (2001). A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science, 293*(5527), 115-120. doi: 10.1126/science.1058783

Carrozza, M. J., Utley, R. T., Workman, J. L., & Cote, J. (2003). The diverse functions of histone acetyltransferase complexes. *Trends Genet, 19*(6), 321-329. doi: 10.1016/S0168-9525(03)00115-X

Ceol, C. J., & Horvitz, H. R. (2004). A new class of C. elegans synMuv genes implicates a Tip60/NuA4-like HAT complex as a negative regulator of Ras signaling. *Dev Cell, 6*(4), 563-576.

Chen, K., & Featherstone, D. E. (2005). Discs-large (DLG) is clustered by presynaptic innervation and regulates postsynaptic glutamate receptor subunit composition in *Drosophila*. *BMC Biol, 3*, 1. doi: 1741-7007-3-1 [pii]

10.1186/1741-7007-3-1

Chen, Y. L., Law, P. Y., & Loh, H. H. (2008). NGF/PI3K signaling-mediated epigenetic regulation of delta opioid receptor gene expression. *Biochem Biophys Res Commun, 368*(3), 755-760. doi: 10.1016/j.bbrc.2008.01.164

Chen, Z. F., Paquette, A. J., & Anderson, D. J. (1998). NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat Genet, 20*(2), 136-142. doi: 10.1038/2431

Cheung, P., Allis, C. D., & Sassone-Corsi, P. (2000). Signaling to chromatin through histone modifications. *Cell, 103*(2), 263-271.

Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., . . . Mandel, G. (1995). REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell, 80*(6), 949-957.

Cloos, P. A., Christensen, J., Agger, K., & Helin, K. (2008). Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev, 22*(9), 1115-1140. doi: 10.1101/gad.1652908

Collins, A. L., Levenson, J. M., Vilaythong, A. P., Richman, R., Armstrong, D. L., Noebels, J. L., . . . Zoghbi, H. Y. (2004). Mild overexpression of MeCP2 causes a progressive neurological disorder in mice. *Hum Mol Genet, 13*(21), 2679-2689. doi: 10.1093/hmg/ddh282

Collins, C. A., & DiAntonio, A. (2007). Synaptic development: insights from *Drosophila*. *Curr Opin Neurobiol, 17*(1), 35-42. doi: S0959-4388(07)00003-7 [pii]

10.1016/j.conb.2007.01.001

Colon-Ramos, D. A. (2009). Synapse formation in developing neural circuits. *Curr Top Dev Biol, 87*, 53-79. doi: S0070-2153(09)01202-2 [pii]

10.1016/S0070-2153(09)01202-2

Cook, D., Fry, M. J., Hughes, K., Sumathipala, R., Woodgett, J. R., & Dale, T. C. (1996). Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J, 15*(17), 4526-4536.

Creppe, C., Malinouskaya, L., Volvert, M. L., Gillard, M., Close, P., Malaise, O., . . . Nguyen, L. (2009). Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin. *Cell, 136*(3), 551-564. doi: 10.1016/j.cell.2008.11.043

Crick, F. (1984). Memory and molecular turnover. *Nature, 312*(5990), 101.

Crosio, C., Heitz, E., Allis, C. D., Borrelli, E., & Sassone-Corsi, P. (2003). Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons. *J Cell Sci, 116*(Pt 24), 4905-4914. doi: 10.1242/jcs.00804

Cross, S. H., Meehan, R. R., Nan, X., & Bird, A. (1997). A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. *Nat Genet, 16*(3), 256-259. doi: 10.1038/ng0797-256

Cummings, J. L. (2004). Alzheimer's disease. *N Engl J Med, 351*(1), 56-67. doi: 10.1056/NEJMra040223

Dai, Y., & Faller, D. V. (2008). Transcription Regulation by Class III Histone Deacetylases (HDACs)-Sirtuins. *Transl Oncogenomics, 3*, 53-65.

Dale, T. C. (1998). Signal transduction by the Wnt family of ligands. *Biochem J, 329 (Pt 2)*, 209-223.

Day, J. J., & Sweatt, J. D. (2011). Epigenetic mechanisms in cognition. *Neuron, 70*(5), 813-829. doi: 10.1016/j.neuron.2011.05.019

de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S., & van Kuilenburg, A. B. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J, 370*(Pt 3), 737-749. doi: 10.1042/BJ20021321

BJ20021321 [pii]

De Strooper, B. (2003). Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex. *Neuron, 38*(1), 9-12.

Deckert, J., & Struhl, K. (2001). Histone acetylation at promoters is differentially affected by specific activators and repressors. *Mol Cell Biol, 21*(8), 2726-2735. doi: 10.1128/MCB.21.8.2726-2735.2001

Deissler, H., Kafka, A., Schuster, E., Sauer, G., Kreienberg, R., & Zeillinger, R. (2004). Spectrum of p53 mutations in biopsies from breast cancer patients selected for preoperative chemotherapy analysed by the functional yeast assay to predict therapeutic response. *Oncol Rep, 11*(6), 1281-1286.

Desjardins, S., Mayo, W., Vallee, M., Hancock, D., Le Moal, M., Simon, H., & Abrous, D. N. (1997). Effect of aging on the basal expression of c-Fos, c-Jun, and Egr-1 proteins in the hippocampus. *Neurobiol Aging, 18*(1), 37-44.

DiAntonio, A., Petersen, S. A., Heckmann, M., & Goodman, C. S. (1999). Glutamate receptor expression regulates quantal size and quantal content at the *Drosophila* neuromuscular junction. *J Neurosci, 19*(8), 3023-3032.

Dokmanovic, M., & Marks, P. A. (2005). Prospects: histone deacetylase inhibitors. *J Cell Biochem, 96*(2), 293-304. doi: 10.1002/jcb.20532

Dong, E., Guidotti, A., Grayson, D. R., & Costa, E. (2007). Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. *Proc Natl Acad Sci U S A, 104*(11), 4676-4681. doi: 10.1073/pnas.0700529104

Doyon, Y., & Cote, J. (2004). The highly conserved and multifunctional NuA4 HAT complex. *Curr Opin Genet Dev, 14*(2), 147-154. doi: 10.1016/j.gde.2004.02.009

Doyon, Y., Selleck, W., Lane, W. S., Tan, S., & Cote, J. (2004). Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. *Mol Cell Biol, 24*(5), 1884-1896.

Duilio, A., Zambrano, N., Mogavero, A. R., Ammendola, R., Cimino, F., & Russo, T. (1991). A rat brain mRNA encoding a transcriptional activator homologous to

the DNA binding domain of retroviral integrases. *Nucleic Acids Res, 19*(19), 5269-5274.

Eckner, R. (1996). p300 and CBP as transcriptional regulators and targets of oncogenic events. *Biol Chem, 377*(11), 685-688.

Eissenberg, J. C. (2001). Molecular biology of the chromo domain: an ancient chromatin module comes of age. *Gene, 275*(1), 19-29.

Elefant, F., Su, Y., Liebhaber, S. A., & Cooke, N. E. (2000). Patterns of histone acetylation suggest dual pathways for gene activation by a bifunctional locus control region. *Embo J, 19*(24), 6814-6822.

Ellaway, C., & Christodoulou, J. (2001). Rett syndrome: clinical characteristics and recent genetic advances. *Disabil Rehabil, 23*(3-4), 98-106.

English, J. D., & Sweatt, J. D. (1997). A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J Biol Chem, 272*(31), 19103-19106.

Erraji-Benchekroun, L., Underwood, M. D., Arango, V., Galfalvy, H., Pavlidis, P., Smyrniotopoulos, P., . . . Sibille, E. (2005). Molecular aging in human prefrontal cortex is selective and continuous throughout adult life. *Biol Psychiatry, 57*(5), 549-558. doi: 10.1016/j.biopsych.2004.10.034

Evans, G. J., & Morgan, A. (2002). Phosphorylation-dependent interaction of the synaptic vesicle proteins cysteine string protein and synaptotagmin I. *Biochem J, 364*(Pt 2), 343-347. doi: 10.1042/BJ20020123

BJ20020123 [pii]

Fan, G., & Hutnick, L. (2005). Methyl-CpG binding proteins in the nervous system. *Cell Res, 15*(4), 255-261. doi: 10.1038/sj.cr.7290294

Feng, J., Fouse, S., & Fan, G. (2007). Epigenetic regulation of neural gene expression and neuronal function. *Pediatr Res, 61*(5 Pt 2), 58R-63R. doi: 10.1203/pdr.0b013e3180457635

Ferrante, R. J., Kubilus, J. K., Lee, J., Ryu, H., Beesen, A., Zucker, B., . . . Hersch, S. M. (2003). Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J Neurosci, 23*(28), 9418-9427.

Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M., & Tsai, L. H. (2007). Recovery of learning and memory is associated with chromatin remodelling. *Nature, 447*(7141), 178-182. doi: 10.1038/nature05772

Fischer, M., Raabe, T., Heisenberg, M., & Sendtner, M. (2009). *Drosophila* RSK negatively regulates bouton number at the neuromuscular junction. *Dev Neurobiol, 69*(4), 212-220. doi: 10.1002/dneu.20700

Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., & Verdin, E. (2002). Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. *Mol Cell, 9*(1), 45-57.

Fouquet, W., Owald, D., Wichmann, C., Mertel, S., Depner, H., Dyba, M., . . . Sigrist, S. J. (2009). Maturation of active zone assembly by *Drosophila* Bruchpilot. *J Cell Biol, 186*(1), 129-145. doi: jcb.200812150 [pii]

10.1083/jcb.200812150

Franco, B., Bogdanik, L., Bobinnec, Y., Debec, A., Bockaert, J., Parmentier, M. L., & Grau, Y. (2004). Shaggy, the homolog of glycogen synthase kinase 3, controls neuromuscular junction growth in *Drosophila*. *J Neurosci, 24*(29), 6573-6577. doi: 10.1523/JNEUROSCI.1580-04.2004

Fraser, H. B., Khaitovich, P., Plotkin, J. B., Paabo, S., & Eisen, M. B. (2005). Aging and gene expression in the primate brain. *PLoS Biol, 3*(9), e274. doi: 10.1371/journal.pbio.0030274

Fuks, F. (2005). DNA methylation and histone modifications: teaming up to silence genes. *Curr Opin Genet Dev, 15*(5), 490-495. doi: 10.1016/j.gde.2005.08.002

Gage, F. H. (2000). Mammalian neural stem cells. *Science, 287*(5457), 1433-1438.

Gardiner, J., Barton, D., Marc, J., & Overall, R. (2007). Potential role of tubulin acetylation and microtubule-based protein trafficking in familial dysautonomia. *Traffic, 8*(9), 1145-1149. doi: TRA605 [pii]

10.1111/j.1600-0854.2007.00605.x

Gaughan, L., Brady, M. E., Cook, S., Neal, D. E., & Robson, C. N. (2001). Tip60 is a co-activator specific for class I nuclear hormone receptors. *J Biol Chem, 276*(50), 46841-46848. doi: 10.1074/jbc.M103710200

Gaughan, L., Logan, I. R., Cook, S., Neal, D. E., & Robson, C. N. (2002). Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. *J Biol Chem, 277*(29), 25904-25913. doi: 10.1074/jbc.M203423200

Gavaravarapu, S., & Kamine, J. (2000). Tip60 inhibits activation of CREB protein by protein kinase A. *Biochem Biophys Res Commun, 269*(3), 758-766. doi: 10.1006/bbrc.2000.2358

Gecz, J., Gedeon, A. K., Sutherland, G. R., & Mulley, J. C. (1996). Identification of the gene FMR2, associated with FRAXE mental retardation. *Nat Genet, 13*(1), 105-108. doi: 10.1038/ng0596-105

Gelato, K. A., & Fischle, W. (2008). Role of histone modifications in defining chromatin structure and function. *Biol Chem, 389*(4), 353-363. doi: 10.1515/BC.2008.048

Ginty, D. D., & Segal, R. A. (2002). Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr Opin Neurobiol, 12*(3), 268-274.

Godde, J. S., & Ura, K. (2008). Cracking the enigmatic linker histone code. *J Biochem, 143*(3), 287-293. doi: 10.1093/jb/mvn013

Graff, J., Kim, D., Dobbin, M. M., & Tsai, L. H. (2011). Epigenetic regulation of gene expression in physiological and pathological brain processes. *Physiol Rev, 91*(2), 603-649. doi: 10.1152/physrev.00012.2010

Graff, J., & Mansuy, I. M. (2008). Epigenetic codes in cognition and behaviour. *Behav Brain Res, 192*(1), 70-87. doi: 10.1016/j.bbr.2008.01.021

Grant, P. A. (2001). A tale of histone modifications. *Genome Biol, 2*(4), REVIEWS0003.

Grewal, S. I., & Jia, S. (2007). Heterochromatin revisited. *Nat Rev Genet, 8*(1), 35-46. doi: 10.1038/nrg2008

Grunstein, M. (1998). Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell, 93*(3), 325-328.

Gu, W., Shi, X. L., & Roeder, R. G. (1997). Synergistic activation of transcription by CBP and p53. *Nature, 387*(6635), 819-823. doi: 10.1038/42972

Gu, Y., Shen, Y., Gibbs, R. A., & Nelson, D. L. (1996). Identification of FMR2, a novel gene associated with the FRAXE CCG repeat and CpG island. *Nat Genet, 13*(1), 109-113. doi: 10.1038/ng0596-109

Guan, B., Hartmann, B., Kho, Y. H., Gorczyca, M., & Budnik, V. (1996). The *Drosophila* tumor suppressor gene, dlg, is involved in structural plasticity at a glutamatergic synapse. *Curr Biol, 6*(6), 695-706.

Guan, J. S., Haggarty, S. J., Giacometti, E., Dannenberg, J. H., Joseph, N., Gao, J., . . . Tsai, L. H. (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature, 459*(7243), 55-60. doi: 10.1038/nature07925

Guan, Z., Giustetto, M., Lomvardas, S., Kim, J. H., Miniaci, M. C., Schwartz, J. H., . . . Kandel, E. R. (2002). Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell, 111*(4), 483-493.

Gupta, S., Kim, S. Y., Artis, S., Molfese, D. L., Schumacher, A., Sweatt, J. D., . . . Lubin, F. D. (2010). Histone methylation regulates memory formation. *J Neurosci, 30*(10), 3589-3599. doi: 10.1523/JNEUROSCI.3732-09.2010

Haberland, M., Montgomery, R. L., & Olson, E. N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet, 10*(1), 32-42. doi: 10.1038/nrg2485

Halkidou, K., Gnanapragasam, V.J., Mehta, P.B., Logan, I.R., Brady, M.E., Cook, D., Leung, H.Y., Neal, D.E., Robson, C.N. (2003). Expression of Tip60 an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene, 22*, 2466-2477.

Hammond, J. W., Cai, D., & Verhey, K. J. (2008). Tubulin modifications and their cellular functions. *Curr Opin Cell Biol, 20*(1), 71-76. doi: S0955-0674(07)00178-0 [pii]

10.1016/j.ceb.2007.11.010

Hardingham, G. E., Chawla, S., Cruzalegui, F. H., & Bading, H. (1999). Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. *Neuron, 22*(4), 789-798. doi: S0896-6273(00)80737-0 [pii]

Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science, 297*(5580), 353-356. doi: 10.1126/science.1072994

Harper, J. A., Yuan, J. S., Tan, J. B., Visan, I., & Guidos, C. J. (2003). Notch signaling in development and disease. *Clin Genet, 64*(6), 461-472.

Hlubek, F., Lohberg, C., Meiler, J., Jung, A., Kirchner, T., & Brabletz, T. (2001). Tip60 is a cell-type-specific transcriptional regulator. *J Biochem, 129*(4), 635-641.

Ho, Y., Elefant, F., Liebhaber, S. A., & Cooke, N. E. (2006). Locus control region transcription plays an active role in long-range gene activation. *Mol Cell, 23*(3), 365-375.

Hockly, E., Richon, V. M., Woodman, B., Smith, D. L., Zhou, X., Rosa, E., . . . Bates, G. P. (2003). Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc Natl Acad Sci U S A, 100*(4), 2041-2046. doi: 10.1073/pnas.0437870100

Hsieh, J., & Gage, F. H. (2005). Chromatin remodeling in neural development and plasticity. *Curr Opin Cell Biol, 17*(6), 664-671.

Huang, Y., Myers, S. J., & Dingledine, R. (1999). Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. *Nat Neurosci, 2*(10), 867-872. doi: 10.1038/13165

Ikenoue, T., Inoki, K., Zhao, B., & Guan, K. L. (2008). PTEN acetylation modulates its interaction with PDZ domain. *Cancer Res, 68*(17), 6908-6912. doi: 10.1158/0008-5472.CAN-08-1107

Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., . . . Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell, 102*(4), 463-473.

Ikura, T., Tashiro, S., Kakino, A., Shima, H., Jacob, N., Amunugama, R., . . . Kamiya, K. (2007). DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. *Mol Cell Biol, 27*(20), 7028-7040. doi: 10.1128/MCB.00579-07

Ishida, M., Shimojo, H., Hayashi, A., Kawaguchi, R., Ohtani, Y., Uegaki, K., . . . Nakayama, J. (2012). Intrinsic nucleic acid-binding activity of Chp1 chromodomain is required for heterochromatic gene silencing. *Mol Cell, 47*(2), 228-241. doi: 10.1016/j.molcel.2012.05.017

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature, 461*(7267), 1071-1078. doi: 10.1038/nature08467

Jacobs, S. A., & Khorasanizadeh, S. (2002). Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science, 295*(5562), 2080-2083. doi: 10.1126/science.1069473

Jenuwein, T., & Allis, C. D. (2001). Translating the histone code. *Science, 293*(5532), 1074-1080. doi: 10.1126/science.1063127

Jha, S., Shibata, E., & Dutta, A. (2008). Human Rvb1/Tip49 is required for the histone acetyltransferase activity of Tip60/NuA4 and for the downregulation of phosphorylation on H2AX after DNA damage. *Mol Cell Biol, 28*(8), 2690-2700. doi: 10.1128/MCB.01983-07

Jiang, X., Sun, Y., Chen, S., Roy, K., & Price, B. D. (2006). The FATC domains of PIKK proteins are functionally equivalent and participate in the Tip60-

dependent activation of DNA-PKcs and ATM. *J Biol Chem, 281*(23), 15741-15746. doi: 10.1074/jbc.M513172200

Johnson, A. A., Sarthi, J., Pirooznia, S. K., Reube, W., & Elefant, F. (2013). Increasing Tip60 HAT Levels Rescues Axonal Transport Defects and Associated Behavioral Phenotypes in a *Drosophila* Alzheimer's Disease Model. *J Neurosci, 33*(17), 7535-7547. doi: 10.1523/JNEUROSCI.3739-12.2013

Johnson, A., Li, G., Sikorski, T. W., Buratowski, S., Woodcock, C. L., & Moazed, D. (2009). Reconstitution of heterochromatin-dependent transcriptional gene silencing. *Mol Cell, 35*(6), 769-781. doi: 10.1016/j.molcel.2009.07.030

Johnson, E. L., 3rd, Fetter, R. D., & Davis, G. W. (2009). Negative regulation of active zone assembly by a newly identified SR protein kinase. *PLoS Biol, 7*(9), e1000193. doi: 10.1371/journal.pbio.1000193

Jones, D. O., Cowell, I. G., & Singh, P. B. (2000). Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays, 22*(2), 124-137. doi: 10.1002/(SICI)1521-1878(200002)22:2<124::AID-BIES4>3.0.CO;2-E

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., . . . Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet, 19*(2), 187-191. doi: 10.1038/561

Josselyn, S. A., & Nguyen, P. V. (2005). CREB, synapses and memory disorders: past progress and future challenges. *Curr Drug Targets CNS Neurol Disord, 4*(5), 481-497.

Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., . . . Tournier-Lasserve, E. (1996). Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature, 383*(6602), 707-710. doi: 10.1038/383707a0

Jowaed, A., Schmitt, I., Kaut, O., & Wullner, U. (2010). Methylation regulates alpha-synuclein expression and is decreased in Parkinson's disease patients' brains. *J Neurosci, 30*(18), 6355-6359. doi: 10.1523/JNEUROSCI.6119-09.2010

Kalkhoven, E., Roelfsema, J. H., Teunissen, H., den Boer, A., Ariyurek, Y., Zantema, A., . . . Peters, D. J. (2003). Loss of CBP acetyltransferase activity by PHD finger mutations in Rubinstein-Taybi syndrome. *Hum Mol Genet, 12*(4), 441-450.

Kamine, J., Elangovan, B., Subramanian, T., Coleman, D., & Chinnadurai, G. (1996). *Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator.* (216 Research Support, U.S. Gov't, Non-P.H.S.

Research Support, U.S. Gov't, P.H.S.). Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8607265 (2)

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science, 294*(5544), 1030-1038. doi: 10.1126/science.1067020

Karmodiya, K., Krebs, A. R., Oulad-Abdelghani, M., Kimura, H., & Tora, L. (2012). H3K9 and H3K14 acetylation co-occur at many gene regulatory elements, while H3K14ac marks a subset of inactive inducible promoters in mouse embryonic stem cells. *BMC Genomics, 13*, 424. doi: 10.1186/1471-2164-13-424

Kastan, M. B. (2008). DNA damage responses: mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture. *Mol Cancer Res, 6*(4), 517-524. doi: 10.1158/1541-7786.MCR-08-0020

Kidd, S. K., & Schneider, J. S. (2010). Protection of dopaminergic cells from MPP+-mediated toxicity by histone deacetylase inhibition. *Brain Res, 1354*, 172-178. doi: 10.1016/j.brainres.2010.07.041

Kim, H. S., Kim, E. M., Lee, J. P., Park, C. H., Kim, S., Seo, J. H., . . . Suh, Y. H. (2003). C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. *FASEB J, 17*(13), 1951-1953. doi: 10.1096/fj.03-0106fje

Kim, J. H., Kim, B., Cai, L., Choi, H. J., Ohgi, K. A., Tran, C., . . . Baek, S. H. (2005). Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes. *Nature, 434*(7035), 921-926. doi: 10.1038/nature03452

Kim, T. K., Hemberg, M., Gray, J. M., Costa, A. M., Bear, D. M., Wu, J., . . . Greenberg, M. E. (2010). Widespread transcription at neuronal activity-regulated enhancers. *Nature, 465*(7295), 182-187. doi: nature09033 [pii]

10.1038/nature09033

Kimura, A., & Horikoshi, M. (1998). Tip60 acetylates six lysines of a specific class in core histones in vitro. *Genes Cells, 3*(12), 789-800.

Kinoshita, A., Whelan, C. M., Berezovska, O., & Hyman, B. T. (2002). The gamma secretase-generated carboxyl-terminal domain of the amyloid precursor protein induces apoptosis via Tip60 in H4 cells. *J Biol Chem, 277*(32), 28530-28536. doi: 10.1074/jbc.M203372200

Kittel, R. J., Wichmann, C., Rasse, T. M., Fouquet, W., Schmidt, M., Schmid, A., . . . Sigrist, S. J. (2006). Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. *Science, 312*(5776), 1051-1054. doi: 1126308 [pii]

10.1126/science.1126308

Knapek, S., Sigrist, S., & Tanimoto, H. (2011). Bruchpilot, a synaptic active zone protein for anesthesia-resistant memory. *J Neurosci, 31*(9), 3453-3458. doi: 31/9/3453 [pii]

10.1523/JNEUROSCI.2585-10.2011

Koh, Y. H., Gramates, L. S., & Budnik, V. (2000). *Drosophila* larval neuromuscular junction: molecular components and mechanisms underlying synaptic plasticity. *Microsc Res Tech, 49*(1), 14-25. doi: 10.1002/(SICI)1097-0029(20000401)49:1<14::AID-JEMT3>3.0.CO;2-G [pii]

10.1002/(SICI)1097-0029(20000401)49:1<14::AID-JEMT3>3.0.CO;2-G

Kontopoulos, E., Parvin, J. D., & Feany, M. B. (2006). Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Hum Mol Genet, 15*(20), 3012-3023. doi: 10.1093/hmg/ddl243

Kooistra, S. M., & Helin, K. (2012). Molecular mechanisms and potential functions of histone demethylases. *Nat Rev Mol Cell Biol, 13*(5), 297-311. doi: 10.1038/nrm3327

Korzus, E., Rosenfeld, M. G., & Mayford, M. (2004). CBP histone acetyltransferase activity is a critical component of memory consolidation. *Neuron, 42*(6), 961-972. doi: 10.1016/j.neuron.2004.06.002

Kumar, A., Choi, K. H., Renthal, W., Tsankova, N. M., Theobald, D. E., Truong, H. T., . . . Nestler, E. J. (2005). Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron, 48*(2), 303-314. doi: 10.1016/j.neuron.2005.09.023

Kusch, T., Florens, L., Macdonald, W. H., Swanson, S. K., Glaser, R. L., Yates, J. R., 3rd, . . . Workman, J. L. (2004). Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. *Science, 306*(5704), 2084-2087. doi: 10.1126/science.1103455

Lahey, T., Gorczyca, M., Jia, X. X., & Budnik, V. (1994). The *Drosophila* tumor suppressor gene dlg is required for normal synaptic bouton structure. *Neuron, 13*(4), 823-835.

LaPlant, Q., Vialou, V., Covington, H. E., 3rd, Dumitriu, D., Feng, J., Warren, B. L., . . . Nestler, E. J. (2010). Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat Neurosci, 13*(9), 1137-1143. doi: 10.1038/nn.2619

Lavin, M. F. (2008). Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. *Nat Rev Mol Cell Biol, 9*(10), 759-769. doi: 10.1038/nrm2514

Lavin, M. F., Birrell, G., Chen, P., Kozlov, S., Scott, S., & Gueven, N. (2005). ATM signaling and genomic stability in response to DNA damage. *Mutat Res, 569*(1-2), 123-132. doi: 10.1016/j.mrfmmm.2004.04.020

Lavin, M. F., & Gueven, N. (2006). The complexity of p53 stabilization and activation. *Cell Death Differ, 13*(6), 941-950. doi: 10.1038/sj.cdd.4401925

Lee, D. Y., Teyssier, C., Strahl, B. D., & Stallcup, M. R. (2005). Role of protein methylation in regulation of transcription. *Endocr Rev, 26*(2), 147-170. doi: 10.1210/er.2004-0008

Lee, H. J., Chun, M., & Kandror, K. V. (2001). Tip60 and HDAC7 interact with the endothelin receptor a and may be involved in downstream signaling. *J Biol Chem, 276*(20), 16597-16600.

Lee, K. K., & Workman, J. L. (2007). Histone acetyltransferase complexes: one size doesn't fit all. *Nat Rev Mol Cell Biol, 8*(4), 284-295. doi: 10.1038/nrm2145

Legube, G., Linares, L. K., Tyteca, S., Caron, C., Scheffner, M., Chevillard-Briet, M., & Trouche, D. (2004). Role of the histone acetyl transferase Tip60 in the p53 pathway. *J Biol Chem, 279*(43), 44825-44833. doi: 10.1074/jbc.M407478200

Leitao, M. M., Soslow, R. A., Baergen, R. N., Olvera, N., Arroyo, C., & Boyd, J. (2004). Mutation and expression of the TP53 gene in early stage epithelial ovarian carcinoma. *Gynecol Oncol, 93*(2), 301-306. doi: 10.1016/j.ygyno.2004.01.043

Levenson, J. M., O'Riordan, K. J., Brown, K. D., Trinh, M. A., Molfese, D. L., & Sweatt, J. D. (2004). Regulation of histone acetylation during memory formation in the hippocampus. *J Biol Chem, 279*(39), 40545-40559. doi: 10.1074/jbc.M402229200

M402229200 [pii]

Levenson, J. M., Roth, T. L., Lubin, F. D., Miller, C. A., Huang, I. C., Desai, P., . . . Sweatt, J. D. (2006). Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. *J Biol Chem, 281*(23), 15763-15773. doi: 10.1074/jbc.M511767200

Levenson, J. M., & Sweatt, J. D. (2005). Epigenetic mechanisms in memory formation. *Nat Rev Neurosci, 6*(2), 108-118. doi: 10.1038/nrn1604

Levenson, J. M., & Sweatt, J. D. (2006). Epigenetic mechanisms: a common theme in vertebrate and invertebrate memory formation. *Cell Mol Life Sci, 63*(9), 1009-1016. doi: 10.1007/s00018-006-6026-6

Levesque, G., Yu, G., Nishimura, M., Zhang, D. M., Levesque, L., Yu, H., . . . St George-Hyslop, P. (1999). Presenilins interact with armadillo proteins including neural-specific plakophilin-related protein and beta-catenin. *J Neurochem, 72*(3), 999-1008.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell, 88*(3), 323-331.

Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol, 9*(6), 583-589. doi: 10.1006/scdb.1998.0266

Li, B., Samanta, A., Song, X., Iacono, K. T., Bembas, K., Tao, R., . . . Greene, M. I. (2007). FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc Natl Acad Sci U S A, 104*(11), 4571-4576. doi: 10.1073/pnas.0700298104

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., . . . Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science, 277*(5332), 1659-1662.

Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., & Berger, S. L. (1999). p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Mol Cell Biol, 19*(2), 1202-1209.

Liu, Z., Chen, Y., Wang, D., Wang, S., & Zhang, Y. Q. (2010). Distinct presynaptic and postsynaptic dismantling processes of *Drosophila* neuromuscular junctions during metamorphosis. *J Neurosci, 30*(35), 11624-11634. doi: 10.1523/JNEUROSCI.0410-10.2010

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408. doi: 10.1006/meth.2001.1262

Lnenicka, G. A., & Keshishian, H. (2000). Identified motor terminals in *Drosophila* larvae show distinct differences in morphology and physiology. *J Neurobiol, 43*(2), 186-197. doi: 10.1002/(SICI)1097-4695(200005)43:2<186::AID-NEU8>3.0.CO;2-N [pii]

Loerch, P. M., Lu, T., Dakin, K. A., Vann, J. M., Isaacs, A., Geula, C., . . . Yankner, B. A. (2008). Evolution of the aging brain transcriptome and synaptic regulation. *PLoS One, 3*(10), e3329. doi: 10.1371/journal.pone.0003329

Logan, I. R., Sapountzi, V., Gaughan, L., Neal, D. E., & Robson, C. N. (2004). Control of human PIRH2 protein stability: involvement of TIP60 and the proteosome. *J Biol Chem, 279*(12), 11696-11704. doi: 10.1074/jbc.M312712200

Lorbeck, M., Pirooznia, K., Sarthi, J., Zhu, X. , & Elefant, F. (2011a). Microarray Analysis Uncovers a Role for Tip60 in Nervous System Function and General Metabolism. *PLoS One, 6*(4), 14.

Lorbeck, M., Pirooznia, K., Sarthi, J., Zhu, X., & Elefant, F. (2011b). Microarray analysis uncovers a role for Tip60 in nervous system function and general metabolism. *PLoS One, 6*(4), e18412. doi: 10.1371/journal.pone.0018412

Lundblad, J. R., Kwok, R. P., Laurance, M. E., Harter, M. L., & Goodman, R. H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature, 374*(6517), 85-88. doi: 10.1038/374085a0

Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., . . . Rosenfeld, M. G. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science, 298*(5599), 1747-1752. doi: 10.1126/science.1076469

Lynch, M. A. (2004). Long-term potentiation and memory. *Physiol Rev, 84*(1), 87-136. doi: 10.1152/physrev.00014.2003

Mabry, T. R., McCarty, R., Gold, P. E., & Foster, T. C. (1996). Age and stress history effects on spatial performance in a swim task in Fischer-344 rats. *Neurobiol Learn Mem, 66*(1), 1-10. doi: 10.1006/nlme.1996.0038

Marambaud, P., Wen, P. H., Dutt, A., Shioi, J., Takashima, A., Siman, R., & Robakis, N. K. (2003). A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell, 114*(5), 635-645.

Marmorstein, R., & Berger, S. L. (2001). Structure and function of bromodomains in chromatin-regulating complexes. *Gene, 272*(1-2), 1-9.

Marrus, S. B., Portman, S. L., Allen, M. J., Moffat, K. G., & DiAntonio, A. (2004). Differential localization of glutamate receptor subunits at the *Drosophila* neuromuscular junction. *J Neurosci, 24*(6), 1406-1415. doi: 10.1523/JNEUROSCI.1575-03.2004

Martin, K. C., Barad, M., & Kandel, E. R. (2000). Local protein synthesis and its role in synapse-specific plasticity. *Curr Opin Neurobiol, 10*(5), 587-592. doi: S0959-4388(00)00128-8 [pii]

Matsumoto, L., Takuma, H., Tamaoka, A., Kurisaki, H., Date, H., Tsuji, S., & Iwata, A. (2010). CpG demethylation enhances alpha-synuclein expression and affects the pathogenesis of Parkinson's disease. *PLoS One, 5*(11), e15522. doi: 10.1371/journal.pone.0015522

Mattson, M. P. (2003). Methylation and acetylation in nervous system development and neurodegenerative disorders. *Ageing Res Rev, 2*(3), 329-342. doi: S1568163703000138 [pii]

Maurice, T., Duclot, F., Meunier, J., Naert, G., Givalois, L., Meffre, J., . . . Gongora, C. (2008). Altered memory capacities and response to stress in p300/CBP-associated factor (PCAF) histone acetylase knockout mice. *Neuropsychopharmacology, 33*(7), 1584-1602. doi: 10.1038/sj.npp.1301551

Mayer, M. L., & Armstrong, N. (2004). Structure and function of glutamate receptor ion channels. *Annu Rev Physiol, 66*, 161-181. doi: 10.1146/annurev.physiol.66.050802.084104

McAllister, D., Merlo, X., & Lough, J. (2002). Characterization and expression of the mouse tat interactive protein 60 kD (TIP60) gene. *Gene, 289*(1-2), 169-176.

Menon, K. P., Andrews, S., Murthy, M., Gavis, E. R., & Zinn, K. (2009). The translational repressors Nanos and Pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition. *J Neurosci, 29*(17), 5558-5572. doi: 29/17/5558 [pii]

10.1523/JNEUROSCI.0520-09.2009

Meyers, F. J., Chi, S. G., Fishman, J. R., deVere White, R. W., & Gumerlock, P. H. (1993). p53 mutations in benign prostatic hyperplasia. *J Natl Cancer Inst, 85*(22), 1856-1858.

Michael, D., & Oren, M. (2002). The p53 and Mdm2 families in cancer. *Curr Opin Genet Dev, 12*(1), 53-59.

Miech, C., Pauer, H. U., He, X., & Schwarz, T. L. (2008). Presynaptic local signaling by a canonical wingless pathway regulates development of the *Drosophila* neuromuscular junction. *J Neurosci, 28*(43), 10875-10884. doi: 10.1523/JNEUROSCI.0164-08.2008

Miller, C. A., Campbell, S. L., & Sweatt, J. D. (2008). DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiol Learn Mem, 89*(4), 599-603. doi: 10.1016/j.nlm.2007.07.016

Miller, C. A., & Sweatt, J. D. (2007). Covalent modification of DNA regulates memory formation. *Neuron, 53*(6), 857-869. doi: 10.1016/j.neuron.2007.02.022

Minopoli, G., de Candia, P., Bonetti, A., Faraonio, R., Zambrano, N., & Russo, T. (2001). The beta-amyloid precursor protein functions as a cytosolic anchoring site that prevents Fe65 nuclear translocation. *J Biol Chem, 276*(9), 6545-6550. doi: 10.1074/jbc.M007340200

Miranda, T. B., & Jones, P. A. (2007). DNA methylation: the nuts and bolts of repression. *J Cell Physiol, 213*(2), 384-390. doi: 10.1002/jcp.21224

Mizzen, C. A., & Allis, C. D. (1998). Linking histone acetylation to transcriptional regulation. *Cell Mol Life Sci, 54*(1), 6-20.

Monti, B., Gatta, V., Piretti, F., Raffaelli, S. S., Virgili, M., & Contestabile, A. (2010). Valproic acid is neuroprotective in the rotenone rat model of Parkinson's disease: involvement of alpha-synuclein. *Neurotox Res, 17*(2), 130-141. doi: 10.1007/s12640-009-9090-5

Mori, N., Schoenherr, C., Vandenbergh, D. J., & Anderson, D. J. (1992). A common silencer element in the SCG10 and type II Na+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron, 9*(1), 45-54.

Morris, R. G. (2003). Long-term potentiation and memory. *Philos Trans R Soc Lond B Biol Sci, 358*(1432), 643-647. doi: 10.1098/rstb.2002.1230

Muller, U. (2002). Learning in honeybees: from molecules to behaviour. *Zoology (Jena), 105*(4), 313-320. doi: S0944-2006(04)70070-7 [pii]

10.1078/0944-2006-00075

Murata, T., Kurokawa, R., Krones, A., Tatsumi, K., Ishii, M., Taki, T., . . . Hayashi, Y. (2001). Defect of histone acetyltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein-Taybi syndrome. *Hum Mol Genet, 10*(10), 1071-1076.

Murayama, M., Tanaka, S., Palacino, J., Murayama, O., Honda, T., Sun, X., . . . Takashima, A. (1998). Direct association of presenilin-1 with beta-catenin. *FEBS Lett, 433*(1-2), 73-77.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., & Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science, 292*(5514), 110-113. doi: 10.1126/science.1060118

Nelson, E. D., Kavalali, E. T., & Monteggia, L. M. (2008). Activity-dependent suppression of miniature neurotransmission through the regulation of DNA methylation. *J Neurosci, 28*(2), 395-406. doi: 10.1523/JNEUROSCI.3796-07.2008

Nestler, E. J. (2001). Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci, 2*(2), 119-128. doi: 10.1038/35053570

Neuwald, A. F., & Landsman, D. (1997). GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem Sci, 22*(5), 154-155.

Nightingale, K. P., & Becker, P. B. (1998). Structural and functional analysis of chromatin assembled from defined histones. *Methods, 15*(4), 343-353. doi: 10.1006/meth.1998.0638

Nordentoft, I., & Jorgensen, P. (2003). The acetyltransferase 60 kDa trans-acting regulatory protein of HIV type 1-interacting protein (Tip60) interacts with the translocation E26 transforming-specific leukaemia gene (TEL) and functions as a transcriptional co-repressor. *Biochem J, 374*(Pt 1), 165-173. doi: 10.1042/BJ20030087

Norris, C. M., & Foster, T. C. (1999). MK-801 improves retention in aged rats: implications for altered neural plasticity in age-related memory deficits. *Neurobiol Learn Mem, 71*(2), 194-206. doi: 10.1006/nlme.1998.3864

Oler, J. A., & Markus, E. J. (1998). Age-related deficits on the radial maze and in fear conditioning: hippocampal processing and consolidation. *Hippocampus, 8*(4), 402-415. doi: 10.1002/(SICI)1098-1063(1998)8:4<402::AID-HIPO8>3.0.CO;2-I

Olsen, D. P., & Keshishian, H. (2012). Experimental methods for examining synaptic plasticity in *Drosophila*. *Cold Spring Harb Protoc, 2012*(2), 162-173. doi: 10.1101/pdb.top067785

Osborne, B., & Miele, L. (1999). Notch and the immune system. *Immunity, 11*(6), 653-663.

Outeiro, T. F., Kontopoulos, E., Altmann, S. M., Kufareva, I., Strathearn, K. E., Amore, A. M., . . . Kazantsev, A. G. (2007). Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science, 317*(5837), 516-519. doi: 10.1126/science.1143780

Packard, M., Koo, E. S., Gorczyca, M., Sharpe, J., Cumberledge, S., & Budnik, V. (2002). The *Drosophila* Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell, 111*(3), 319-330. doi: S0092867402010474 [pii]

Pandey, R., Muller, A., Napoli, C. A., Selinger, D. A., Pikaard, C. S., Richards, E. J., . . . Jorgensen, R. A. (2002). Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res, 30*(23), 5036-5055.

Park, D. C., & Reuter-Lorenz, P. (2009). The adaptive brain: aging and neurocognitive scaffolding. *Annu Rev Psychol, 60*, 173-196. doi: 10.1146/annurev.psych.59.103006.093656

Park, S. W., He, Y., Ha, S. G., Loh, H. H., & Wei, L. N. (2008). Epigenetic regulation of kappa opioid receptor gene in neuronal differentiation. *Neuroscience, 151*(4), 1034-1041. doi: 10.1016/j.neuroscience.2007.12.015

Passer, B., Pellegrini, L., Russo, C., Siegel, R. M., Lenardo, M. J., Schettini, G., . . . D'Adamio, L. (2000). Generation of an apoptotic intracellular peptide by gamma-secretase cleavage of Alzheimer's amyloid beta protein precursor. *J Alzheimers Dis, 2*(3-4), 289-301.

Patel, J. H., Du, Y., Ard, P. G., Phillips, C., Carella, B., Chen, C. J., . . . McMahon, S. B. (2004). The c-MYC oncoprotein is a substrate of the acetyltransferases hGCN5/PCAF and TIP60. *Mol Cell Biol, 24*(24), 10826-10834. doi: 10.1128/MCB.24.24.10826-10834.2004

Peleg, S., Sananbenesi, F., Zovoilis, A., Burkhardt, S., Bahari-Javan, S., Agis-Balboa, R. C., . . . Fischer, A. (2010). Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science, 328*(5979), 753-756. doi: 10.1126/science.1186088

Pirooznia, S. K., Sarthi, J., Johnson, A. A., Toth, M. S., Chiu, K., Koduri, S., & Elefant, F. (2012). Tip60 HAT activity mediates APP induced lethality and apoptotic cell death in the CNS of a *Drosophila* Alzheimer's disease model. *PLoS One, 7*(7), e41776. doi: 10.1371/journal.pone.0041776

Pittenger, C., & Kandel, E. R. (2003). In search of general mechanisms for long-lasting plasticity: Aplysia and the hippocampus. *Philos Trans R Soc Lond B Biol Sci, 358*(1432), 757-763. doi: 10.1098/rstb.2002.1247

Qin, G., Schwarz, T., Kittel, R. J., Schmid, A., Rasse, T. M., Kappei, D., . . . Sigrist, S. J. (2005). Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of *Drosophila*. *J Neurosci, 25*(12), 3209-3218. doi: 10.1523/JNEUROSCI.4194-04.2005

Radtke, F., & Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer, 3*(10), 756-767. doi: 10.1038/nrc1186

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., . . . Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature, 406*(6796), 593-599. doi: 10.1038/35020506

Reddy, B. D., & Jia, S. (2008). Heterochromatin: lost in transcription? *Cell Cycle, 7*(22), 3479-3480.

Regehr, W. G., Carey, M. R., & Best, A. R. (2009). Activity-dependent regulation of synapses by retrograde messengers. *Neuron, 63*(2), 154-170. doi: 10.1016/j.neuron.2009.06.021

Reifsnyder, C., Lowell, J., Clarke, A., & Pillus, L. (1996). Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. *Nat Genet, 14*(1), 42-49. doi: 10.1038/ng0996-42

Renthal, W., Maze, I., Krishnan, V., Covington, H. E., 3rd, Xiao, G., Kumar, A., . . . Nestler, E. J. (2007). Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron, 56*(3), 517-529. doi: 10.1016/j.neuron.2007.09.032

Riccio, A. (2010). Dynamic epigenetic regulation in neurons: enzymes, stimuli and signaling pathways. *Nat Neurosci, 13*(11), 1330-1337. doi: 10.1038/nn.2671

Rice, J. C., & Allis, C. D. (2001). Histone methylation versus histone acetylation: new insights into epigenetic regulation. *Curr Opin Cell Biol, 13*(3), 263-273.

Robinson, T. E., & Kolb, B. (1997). Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *J Neurosci, 17*(21), 8491-8497.

Roelfsema, J. H., White, S. J., Ariyurek, Y., Bartholdi, D., Niedrist, D., Papadia, F., . . . Peters, D. J. (2005). Genetic heterogeneity in Rubinstein-Taybi syndrome: mutations in both the CBP and EP300 genes cause disease. *Am J Hum Genet, 76*(4), 572-580. doi: 10.1086/429130

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., & Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem, 273*(10), 5858-5868.

Roos, J., Hummel, T., Ng, N., Klambt, C., & Davis, G. W. (2000). *Drosophila* Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. *Neuron, 26*(2), 371-382. doi: S0896-6273(00)81170-8 [pii]

Roth, S. Y., Denu, J. M., & Allis, C. D. (2001). Histone acetyltransferases. *Annu Rev Biochem, 70*, 81-120. doi: 10.1146/annurev.biochem.70.1.81

Roth, T. L., Roth, E. D., & Sweatt, J. D. (2010). Epigenetic regulation of genes in learning and memory. *Essays Biochem, 48*(1), 263-274. doi: 10.1042/bse0480263

Rouaux, C., Jokic, N., Mbebi, C., Boutillier, S., Loeffler, J. P., & Boutillier, A. L. (2003). Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. *EMBO J, 22*(24), 6537-6549. doi: 10.1093/emboj/cdg615

Ruiz-Canada, C., Ashley, J., Moeckel-Cole, S., Drier, E., Yin, J., & Budnik, V. (2004). New synaptic bouton formation is disrupted by misregulation of microtubule stability in aPKC mutants. *Neuron, 42*(4), 567-580. doi: S0896627304002557 [pii]

Ruthenburg, A. J., Allis, C. D., & Wysocka, J. (2007). Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol Cell, 25*(1), 15-30. doi: 10.1016/j.molcel.2006.12.014

Sanchis-Segura, C., Lopez-Atalaya, J. P., & Barco, A. (2009). Selective boosting of transcriptional and behavioral responses to drugs of abuse by histone deacetylase inhibition. *Neuropsychopharmacology, 34*(13), 2642-2654. doi: 10.1038/npp.2009.125

Sapountzi, V., Logan, I. R., & Robson, C. N. (2006). Cellular functions of TIP60. *Int J Biochem Cell Biol, 38*(9), 1496-1509.

Sarthi, J., & Elefant, F. (2011). dTip60 HAT activity controls synaptic bouton expansion at the *Drosophila* neuromuscular junction. *PLoS One, 6*(10), e26202. doi: 10.1371/journal.pone.0026202

Saura, C. A., Choi, S. Y., Beglopoulos, V., Malkani, S., Zhang, D., Shankaranarayana Rao, B. S., . . . Shen, J. (2004). Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. *Neuron, 42*(1), 23-36.

Scarpa, S., Fuso, A., D'Anselmi, F., & Cavallaro, R. A. (2003). Presenilin 1 gene silencing by S-adenosylmethionine: a treatment for Alzheimer disease? *FEBS Lett, 541*(1-3), 145-148.

Schettini, G., Govoni, S., Racchi, M., & Rodriguez, G. (2010). Phosphorylation of APP-CTF-AICD domains and interaction with adaptor proteins: signal transduction and/or transcriptional role--relevance for Alzheimer pathology. *J Neurochem, 115*(6), 1299-1308. doi: 10.1111/j.1471-4159.2010.07044.x

Schroeter, E. H., Kisslinger, J. A., & Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature, 393*(6683), 382-386. doi: 10.1038/30756

Schuster, C. M., Davis, G. W., Fetter, R. D., & Goodman, C. S. (1996). Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. *Neuron, 17*(4), 641-654.

Schuster, C. M., Ultsch, A., Schloss, P., Cox, J. A., Schmitt, B., & Betz, H. (1991). Molecular cloning of an invertebrate glutamate receptor subunit expressed in *Drosophila* muscle. *Science, 254*(5028), 112-114.

Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature, 399*(6738 Suppl), A23-31.

Sengupta, S., & Harris, C. C. (2005). p53: traffic cop at the crossroads of DNA repair and recombination. *Nat Rev Mol Cell Biol, 6*(1), 44-55. doi: 10.1038/nrm1546

Shahbazian, M. D., & Grunstein, M. (2007). Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem, 76*, 75-100. doi: 10.1146/annurev.biochem.76.052705.162114

Sherwood, N. T., Sun, Q., Xue, M., Zhang, B., & Zinn, K. (2004). *Drosophila* spastin regulates synaptic microtubule networks and is required for normal motor function. *PLoS Biol, 2*(12), e429. doi: 10.1371/journal.pbio.0020429

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., . . . Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell, 119*(7), 941-953. doi: 10.1016/j.cell.2004.12.012

Shikama, N., Lee, C. W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M., & La Thangue, N. B. (1999). A novel cofactor for p300 that regulates the p53 response. *Mol Cell, 4*(3), 365-376.

Sims, R. J., 3rd, Nishioka, K., & Reinberg, D. (2003). Histone lysine methylation: a signature for chromatin function. *Trends Genet, 19*(11), 629-639. doi: 10.1016/j.tig.2003.09.007

Singh, N., Lorbeck, M. T., Zervos, A., Zimmerman, J., & Elefant, F. (2011). The histone acetyltransferase Elp3 plays in active role in the control of synaptic bouton expansion and sleep in *Drosophila*. *J Neurochem, 115*(2), 493-504. doi: JNC6892 [pii]

10.1111/j.1471-4159.2010.06892.x

Sisodia, S. S., & St George-Hyslop, P. H. (2002). gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci, 3*(4), 281-290. doi: 10.1038/nrn785

Slack, J. M. (2002). Conrad Hal Waddington: the last Renaissance biologist? *Nat Rev Genet, 3*(11), 889-895. doi: 10.1038/nrg933

Small, S. A., Chawla, M. K., Buonocore, M., Rapp, P. R., & Barnes, C. A. (2004). Imaging correlates of brain function in monkeys and rats isolates a hippocampal subregion differentially vulnerable to aging. *Proc Natl Acad Sci U S A, 101*(18), 7181-7186. doi: 10.1073/pnas.0400285101

Smith, B. C., & Denu, J. M. (2009). Chemical mechanisms of histone lysine and arginine modifications. *Biochim Biophys Acta, 1789*(1), 45-57. doi: 10.1016/j.bbagrm.2008.06.005

Snow, P. M., Patel, N. H., Harrelson, A. L., & Goodman, C. S. (1987). Neural-specific carbohydrate moiety shared by many surface glycoproteins in *Drosophila* and grasshopper embryos. *J Neurosci, 7*(12), 4137-4144.

Solomon, P. R., Wood, M. S., Groccia-Ellison, M. E., Yang, B. Y., Fanelli, R. J., & Mervis, R. F. (1995). Nimodipine facilitates retention of the classically conditioned nictitating membrane response in aged rabbits over long retention intervals. *Neurobiol Aging, 16*(5), 791-796.

Song, W., Ranjan, R., Dawson-Scully, K., Bronk, P., Marin, L., Seroude, L., . . . Zinsmaier, K. E. (2002). Presynaptic regulation of neurotransmission in *Drosophila* by the g protein-coupled receptor methuselah. *Neuron, 36*(1), 105-119. doi: S0896627302009327 [pii]

Spruston, N. (2008). Neuroscience: strength in numbers. *Nature, 452*(7186), 420-421. doi: 10.1038/452420a

Squatrito, M., Gorrini, C., & Amati, B. (2006). Tip60 in DNA damage response and growth control: many tricks in one HAT. *Trends Cell Biol, 16*(9), 433-442. doi: 10.1016/j.tcb.2006.07.007

Steffan, J. S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B. L., . . . Thompson, L. M. (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature, 413*(6857), 739-743. doi: 10.1038/35099568

Sterner, D. E., & Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev, 64*(2), 435-459.

Stevens, C. F. (1994). CREB and memory consolidation. *Neuron, 13*(4), 769-770.

Strahl, B. D., & Allis, C. D. (2000). The language of covalent histone modifications. *Nature, 403*(6765), 41-45. doi: 10.1038/47412

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev, 12*(5), 599-606.

Stucki, M., & Jackson, S. P. (2006). gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst), 5*(5), 534-543. doi: 10.1016/j.dnarep.2006.01.012

Suka, N., Suka, Y., Carmen, A. A., Wu, J., & Grunstein, M. (2001). Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol Cell, 8*(2), 473-479.

Sultan, F. A., & Day, J. J. (2011). Epigenetic mechanisms in memory and synaptic function. *Epigenomics, 3*(2), 157-181. doi: 10.2217/epi.11.6

Sun, Y., Jiang, X., Chen, S., Fernandes, N., & Price, B. D. (2005). A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. *Proc Natl Acad Sci U S A, 102*(37), 13182-13187. doi: 10.1073/pnas.0504211102

Sun, Y., Jiang, X., Xu, Y., Ayrapetov, M. K., Moreau, L. A., Whetstine, J. R., & Price, B. D. (2009). Histone H3 methylation links DNA damage detection to activation of the tumour suppressor Tip60. *Nat Cell Biol, 11*(11), 1376-1382. doi: 10.1038/ncb1982

Sun, Y., Olson, R., Horning, M., Armstrong, N., Mayer, M., & Gouaux, E. (2002). Mechanism of glutamate receptor desensitization. *Nature, 417*(6886), 245-253. doi: 10.1038/417245a

Sweatt, J. D. (2001). The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem, 76*(1), 1-10.

Sweatt, J. D. (2009). Experience-dependent epigenetic modifications in the central nervous system. *Biol Psychiatry, 65*(3), 191-197. doi: 10.1016/j.biopsych.2008.09.002

Sweatt, J. D. (2010). Neuroscience. Epigenetics and cognitive aging. *Science, 328*(5979), 701-702. doi: 10.1126/science.1189968

Sykes, S. M., Mellert, H. S., Holbert, M. A., Li, K., Marmorstein, R., Lane, W. S., & McMahon, S. B. (2006). Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell, 24*(6), 841-851. doi: 10.1016/j.molcel.2006.11.026

Tang, Y., Luo, J., Zhang, W., & Gu, W. (2006). Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol Cell, 24*(6), 827-839. doi: 10.1016/j.molcel.2006.11.021

Tao, H. W., & Poo, M. (2001). Retrograde signaling at central synapses. *Proc Natl Acad Sci U S A, 98*(20), 11009-11015. doi: 10.1073/pnas.191351698

Taubert, S., Gorrini, C., Frank, S. R., Parisi, T., Fuchs, M., Chan, H. M., . . . Amati, B. (2004). E2F-dependent histone acetylation and recruitment of the Tip60 acetyltransferase complex to chromatin in late G1. *Mol Cell Biol, 24*(10), 4546-4556.

Tawa, R., Ono, T., Kurishita, A., Okada, S., & Hirose, S. (1990). Changes of DNA methylation level during pre- and postnatal periods in mice. *Differentiation, 45*(1), 44-48.

Thomas, U., Ebitsch, S., Gorczyca, M., Koh, Y. H., Hough, C. D., Woods, D., . . . Budnik, V. (2000). Synaptic targeting and localization of discs-large is a stepwise process controlled by different domains of the protein. *Curr Biol, 10*(18), 1108-1117.

Torroja, L., Packard, M., Gorczyca, M., White, K., & Budnik, V. (1999). The *Drosophila* beta-amyloid precursor protein homolog promotes synapse differentiation at the neuromuscular junction. *J Neurosci, 19*(18), 7793-7803.

Trotta, N., Orso, G., Rossetto, M. G., Daga, A., & Broadie, K. (2004). The hereditary spastic paraplegia gene, spastin, regulates microtubule stability to modulate synaptic structure and function. *Curr Biol, 14*(13), 1135-1147. doi: 10.1016/j.cub.2004.06.058

S0960982204004634 [pii]

Tsankova, N. M., Kumar, A., & Nestler, E. J. (2004). Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. *J Neurosci, 24*(24), 5603-5610. doi: 10.1523/JNEUROSCI.0589-04.2004

Turner, G., Webb, T., Wake, S., & Robinson, H. (1996). Prevalence of fragile X syndrome. *Am J Med Genet, 64*(1), 196-197. doi: 10.1002/(SICI)1096-8628(19960712)64:1<196::AID-AJMG35>3.0.CO;2-G

Utley, R. T., & Cote, J. (2003). The MYST family of histone acetyltransferases. *Curr Top Microbiol Immunol, 274*, 203-236.

Utley, R. T., Lacoste, N., Jobin-Robitaille, O., Allard, S., & Cote, J. (2005). Regulation of NuA4 histone acetyltransferase activity in transcription and DNA repair by phosphorylation of histone H4. *Mol Cell Biol, 25*(18), 8179-8190.

van Attikum, H., & Gasser, S. M. (2009). Crosstalk between histone modifications during the DNA damage response. *Trends Cell Biol, 19*(5), 207-217. doi: 10.1016/j.tcb.2009.03.001

Vecsey, C. G., Hawk, J. D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., . . . Wood, M. A. (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J Neurosci, 27*(23), 6128-6140. doi: 10.1523/JNEUROSCI.0296-07.2007

Vermaak, D., & Wolffe, A. P. (1998). Chromatin and chromosomal controls in development. *Dev Genet, 22*(1), 1-6. doi: 10.1002/(SICI)1520-6408(1998)22:1<1::AID-DVG1>3.0.CO;2-A

Vettese-Dadey, M., Grant, P. A., Hebbes, T. R., Crane- Robinson, C., Allis, C. D., & Workman, J. L. (1996). Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA in vitro. *EMBO J, 15*(10), 2508-2518.

Vogelauer, M., Wu, J., Suka, N., & Grunstein, M. (2000). Global histone acetylation and deacetylation in yeast. *Nature, 408*(6811), 495-498. doi: 10.1038/35044127

von Rotz, R. C., Kohli, B. M., Bosset, J., Meier, M., Suzuki, T., Nitsch, R. M., & Konietzko, U. (2004). The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. *J Cell Sci, 117*(Pt 19), 4435-4448. doi: 10.1242/jcs.01323

Vousden, K. H. (2002). Activation of the p53 tumor suppressor protein. *Biochim Biophys Acta, 1602*(1), 47-59.

Voutsinas, G. E., Stavrou, E. F., Karousos, G., Dasoula, A., Papachatzopoulou, A., Syrrou, M., . . . Athanassiadou, A. (2010). Allelic imbalance of expression and epigenetic regulation within the alpha-synuclein wild-type and p.Ala53Thr alleles in Parkinson disease. *Hum Mutat, 31*(6), 685-691. doi: 10.1002/humu.21248

Wagh, D. A., Rasse, T. M., Asan, E., Hofbauer, A., Schwenkert, I., Durrbeck, H., . . . Buchner, E. (2006). Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron, 49*(6), 833-844. doi: S0896-6273(06)00124-3 [pii]

10.1016/j.neuron.2006.02.008

Walter, J., Kaether, C., Steiner, H., & Haass, C. (2001). The cell biology of Alzheimer's disease: uncovering the secrets of secretases. *Curr Opin Neurobiol, 11*(5), 585-590.

Wan, M., Lee, S. S., Zhang, X., Houwink-Manville, I., Song, H. R., Amir, R. E., . . . Francke, U. (1999). Rett syndrome and beyond: recurrent spontaneous and familial MECP2 mutations at CpG hotspots. *Am J Hum Genet, 65*(6), 1520-1529. doi: 10.1086/302690

Wang, L., Lv, Z., Hu, Z., Sheng, J., Hui, B., Sun, J., & Ma, L. (2010). Chronic cocaine-induced H3 acetylation and transcriptional activation of CaMKIIalpha in the nucleus accumbens is critical for motivation for drug reinforcement. *Neuropsychopharmacology, 35*(4), 913-928. doi: 10.1038/npp.2009.193

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., . . . Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nat Neurosci, 7*(8), 847-854. doi: 10.1038/nn1276

Wei, J., & Hemmings, G. P. (2000). The NOTCH4 locus is associated with susceptibility to schizophrenia. *Nat Genet, 25*(4), 376-377. doi: 10.1038/78044

Wharton, K. A., Johansen, K. M., Xu, T., & Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell, 43*(3 Pt 2), 567-581.

Wilson, V. L., Smith, R. A., Ma, S., & Cutler, R. G. (1987). Genomic 5-methyldeoxycytidine decreases with age. *J Biol Chem, 262*(21), 9948-9951.

Winston, F., & Allis, C. D. (1999). The bromodomain: a chromatin-targeting module? *Nat Struct Biol, 6*(7), 601-604. doi: 10.1038/10640

Woan, K. V., Sahakian, E., Sotomayor, E. M., Seto, E., & Villagra, A. (2012). Modulation of antigen-presenting cells by HDAC inhibitors: implications in autoimmunity and cancer. *Immunol Cell Biol, 90*(1), 55-65. doi: 10.1038/icb.2011.96

Wodarz, A., & Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol, 14*, 59-88. doi: 10.1146/annurev.cellbio.14.1.59

Wollmuth, L. P., & Sobolevsky, A. I. (2004). Structure and gating of the glutamate receptor ion channel. *Trends Neurosci, 27*(6), 321-328. doi: 10.1016/j.tins.2004.04.005

S0166223604001250 [pii]

Wood, C., Snijders, A., Williamson, J., Reynolds, C., Baldwin, J., & Dickman, M. (2009). Post-translational modifications of the linker histone variants and their association with cell mechanisms. *FEBS J, 276*(14), 3685-3697. doi: 10.1111/j.1742-4658.2009.07079.x

Wood, M. A., Kaplan, M. P., Park, A., Blanchard, E. J., Oliveira, A. M., Lombardi, T. L., & Abel, T. (2005). Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage. *Learn Mem, 12*(2), 111-119. doi: 10.1101/lm.86605

Wu, J., & Grunstein, M. (2000). 25 years after the nucleosome model: chromatin modifications. *Trends Biochem Sci, 25*(12), 619-623.

Wynder, C., Hakimi, M. A., Epstein, J. A., Shilatifard, A., & Shiekhattar, R. (2005). Recruitment of MLL by HMG-domain protein iBRAF promotes neural differentiation. *Nat Cell Biol, 7*(11), 1113-1117. doi: 10.1038/ncb1312

Wynshaw-Boris, A. (2009). Elongator bridges tubulin acetylation and neuronal migration. *Cell, 136*(3), 393-394. doi: S0092-8674(09)00072-5 [pii]10.1016/j.cell.2009.01.024

Xiao, H., Chung, J., Kao, H. Y., & Yang, Y. C. (2003). Tip60 is a co-repressor for STAT3. *J Biol Chem, 278*(13), 11197-11204. doi: 10.1074/jbc.M210816200

Xu, L., Chen, Y., Song, Q., Xu, D., Wang, Y., & Ma, D. (2009). PDCD5 interacts with Tip60 and functions as a cooperator in acetyltransferase activity and DNA damage-induced apoptosis. *Neoplasia, 11*(4), 345-354.

Yamamoto, T., & Horikoshi, M. (1997). Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60. *J Biol Chem, 272*(49), 30595-30598.

Yang, X. J. (2004). Lysine acetylation and the bromodomain: a new partnership for signaling. *Bioessays, 26*(10), 1076-1087. doi: 10.1002/bies.20104

Yuan, J., Adamski, R., & Chen, J. (2010). Focus on histone variant H2AX: to be or not to be. *FEBS Lett, 584*(17), 3717-3724. doi: 10.1016/j.febslet.2010.05.021

Zhang, Y., Li, N., Caron, C., Matthias, G., Hess, D., Khochbin, S., & Matthias, P. (2003). HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J, 22*(5), 1168-1179. doi: 10.1093/emboj/cdg115

Zhang, Y., & Wu, S. H. (2000). Long-term potentiation in the inferior colliculus studied in rat brain slice. *Hear Res, 147*(1-2), 92-103.

Zhou, J., Liyanage, U., Medina, M., Ho, C., Simmons, A. D., Lovett, M., & Kosik, K. S. (1997). Presenilin 1 interaction in the brain with a novel member of the Armadillo family. *Neuroreport, 8*(8), 2085-2090.

Zhu, X., Singh, N., Donnelly, C., Boimel, P., & Elefant, F. (2007). The cloning and characterization of the histone acetyltransferase human homolog Dmel\TIP60 in *Drosophila* melanogaster: Dmel\TIP60 is essential for multicellular development. *Genetics, 175*(3), 1229-1240. doi: 10.1534/genetics.106.063685

Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N., & Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of *Drosophila*. *Science, 263*(5149), 977-980.

Zweifel, L. S., Kuruvilla, R., & Ginty, D. D. (2005). Functions and mechanisms of retrograde neurotrophin signalling. *Nat Rev Neurosci, 6*(8), 615-625. doi: nrn1727 [pii]10.1038/nrn1727