An epigenetic role for Tip60 in APP mediated neuronal processes

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Abstract
An epigenetic role for Tip60 in APP mediated neuronal processes
Sheila K. Pirooznia

Dynamic epigenetic regulation of neurons is emerging as a fundamental mechanism by which neurons adapt their transcriptional responses to specific developmental and environmental cues. Recent studies bolster the concept that age associated aberrant changes to the epigenetic modification code within the genome of the brain, specifically histone acetylation, cause gene misregulation that drive cognitive decline. Tip60 is a histone acetyltransferase (HATs) enzyme that is involved in the transcriptional regulation of genes enriched for neuronal function and control of synaptic plasticity. Accordingly, Tip60 has been implicated in the neurodegenerative disorder Alzheimer's disease (AD) via transcriptional regulatory complex formation with the AD linked amyloid precursor protein (APP) intracellular domain (AICD). As such, misregulation of neuronal target genes by the Tip60/AICD complex is thought to lead to neurotoxicity associated with AD. However, a direct and causative epigenetic based role for Tip60 HAT activity in mediating such gene expression changes *in vivo* remains unclear and is thus the subject of this study. **Chapter 1** reviews the role of specific HATs including Tip60 in mediating neuronal survival and high order brain functions like learning and memory. The pros and cons of using HDACi as therapeutic strategies and the beneficial effects of modulating the function of specific HATs in neurodegenerative diseases is also discussed. **Chapter 2** demonstrates a functional interaction between Tip60 and APP during neurogenesis using a transgenic AD fly model that was uniquely adapted to induce varying levels of Tip60 HAT activity and describes the neuroprotective role Tip60 HAT activity exhibits towards AD neurodegenerative pathology. **Chapter 3** demonstrates that Tip60 HAT activity in conjunction with APP mediates axonal

growth of the *Drosophila* pacemaker cells, the sLNvs, and their production of the neuropeptide PDF to stabilize *Drosophila* sleep–wake cycles. The study provides novel insight into epigenetic-based regulation of sleep disturbances observed in neurodegenerative diseases like AD. **Chapter 4** describes the effects Tip60 plays in mediating gene expression changes that underlie memory formation in *Drosophila* via the mushroom body encompassing neural circuit. Together, these studies add dTip60 to the growing list of HAT chromatin regulators critical for nervous system function.

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

The human genome encodes approximately 30,000 genes - but can this relatively fixed genome explain who we are or how we act? A wealth of accumulating evidence suggests that there is much more to the human genome than its linear sequence of 3 billion basepairs. In fact, an additional level of "instructive" information superimposed on the DNA double helix in the form of a nucleoprotein entity termed 'chromatin' defines the three dimensional structure of the genome in the cell nucleus. The core unit of chromatin is the nucleosome, which consists of 147 bp of DNA folded around histone octomers consisting two each of the histone proteins H2A, H2B, H3 and H4 [1]. Changes in the chromatin structure allow (or forbid) specific transcriptional regulator complexes to access DNA sequences and subsequently lead to enduring effects on gene expression and cellular function [2]. Such changes in chromatin structure are mediated by stable and heritable modifications of both the DNA and its associated histone proteins that are independent of the underlying DNA sequence and together constitute the 'epigenome' ('epi' – derived from Greek for 'over' or 'above'). Only a few years ago, the epigenome was primarily viewed in the context of cell division and early development wherein it serves to choreograph the myriad cellular and molecular events that distinguish the various cell types that share a genome within an individual. At first glance, this seemed to bear little relevance to the adult brain that is composed of a large proportion of post-mitotic and highly differentiated cells [3]. However, recent explorations of the brain epigenome are providing unprecedented insights into the importance of specific epigenetic modification patterns in controlling gene expression not only in early brain development, but in adult brain functions as well, calling into place a 'reprogramming

process' that allows for plasticity at many levels of the neural circuitry in response to environmental cues [4]. Together with reports implicating disordered chromatin organization and function in several neurodegenerative diseases, this has in turn ignited enormous interest in examining how the course of normal maturation and aging affect the brain epigenome. While age related accumulation of somatic mutations and structural changes to the DNA are likely irreversible, most if not all of the epigenetic modification marks studied to date are in fact reversible. Thus targeting the neural epigenome appears to be a promising strategy for neuroprotection and/or neuroregeneration both early in development as well as during the aging process [3]. This chapter will summarize the recent progress in research linking epigenetic mechanisms, specifically histone acetylation to pathogenesis associated with age related neurodegenerative disorders. I will also discuss how this knowledge could be translated into suitable therapeutic strategies to treat these devastating conditions.

Epigenetic mechanisms in the brain

Epigenetics is historically defined as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence". This definition, however, is not particularly well suited for the nervous system where there is overall absence of mitosis. Thus, a more recent definition for epigenetics would be "the structural adaptation of chromosomal regions that allows to register, signal, or perpetuate altered activity states" [5]. Such effects are primarily mediated *via* three major levels of epigenetic changes: 1) chemical modifications at the level of nucleotides that include DNA methylation and RNA interference (RNAi); 2) post-translational modifications (PTMs) of histone proteins and incorporation of histone

variants; and 3) nucleosome remodeling, referring to ATP dependent processes that regulate the accessibility of nucleosomal DNA [4]. Histones are covalently modified at their amino terminal tails that extend beyond the globular core and undergo numerous PTMs which include in addition to the well studied acetylation and methylation, phosphorylation, ADP-ribosylation, sumoylation, ubiquitination and proline isomerization [6]. Remarkable progress has been made in characterizing the regulatory molecules that elicit such PTMs on the histone tails. Conceptually, these include the 1) Writers, enzymes that modify specific substrates by adding functional moieties like phosphate, acetyl or methyl groups; 2) Readers, regulatory proteins that share unique domains implicated in recognizing acetyl or methyl groups; 3) Erasers, enzymes that directly remove PTMs [4]. Most PTMs target specific amino acid residues in the histone tails. For instance, phosphorylation is directed to serine and theronine residues, and methylation to arginines. However, lysines are targets for most modifications including acetylation and methylation. Covalent histone modifications, histone variants, or chromatin remodeling complexes work together to alter the chromatin fiber, causing subtle but meaningful differences in chromatin compaction that correlate with "euchromatin" (open) versus "heterochromatin" (closed) states [7,8]. These states often, but not always align with "active" versus "inactive" states of gene expression, respectively [1]. Moreover, covalently modified histones alone or in combination generate distinct docking sites and orchestrate the recruitment of multiprotein nuclear protein complexes that mediate cell- and promoter-specific gene expression. Histones are often concurrently modified on several residues and there is also a dynamic interplay between histone modifications and DNA modifications (such as DNA methylation), thus

creating staggering combinatorial possibilities for gene regulation [9]. For example, the combination of histone H4 Lys8 acetylation, histone H3 Lys14 acetylation, and histone H3 Ser10 phosphorylation is often associated with transcriptional activation. On the other hand, tri-methylation of histone H3 Lys9 and the lack of histone H3 and H4 acetylation is associated with transcriptional repression [6]. These findings suggest that the controlled addition and removal of specific PTMs result in unique combinations that correspond to distinct physiological states and genomic functions.

Decoding the epigenetic language

Recent high resolution genomic profiling studies reveal that ‘epigenomes’ are highly organized and strikingly nonrandom with respect to histone and DNA modifications [10]. For example, high levels of H3 and H4 acetylation and H3 Lys4 methylation are generally present in promoter regions of active genes [11]. In contrast, elevated levels of H3 Lys27 methylation correlate with polycomb protein mediated gene repression [12]. Interestingly, such epigenetic patterns vary in different cell types or during different stages of development [12-14]. More recently, specific chromatin signatures were also found at gene promoters, enhancers [15] and even exons [16-19]. Moreover, individual PTMs can favor or inhibit consequent modifications on nearby residues of the same tail and examples of PTMs that influence modifications on different tails have also been reported [20,21]. For instance, H3 Lys4 methylation facilitates subsequent H3 and H4 acetylation [22], whereas histone deacetylation and methylation of H3 Lys9 represses transcription [23]. Another example is phosphorylation of Ser-10 on H3, a positive signal for subsequent acetylation at K14 on the same tail [24]. As lysines can be modified in various manners, it is the competition between various PTMs for the

same residue that may determine functional outcomes [25]. For example, the lysine 9 residue of the H3 tail can be acetylated or methylated ; while acetylation of this residue is associated with transcriptional activation, methylation is associated with silencing [26]. In addition, the same modification may be linked to totally opposite functions: histone H3 and H4 lysine or arginine methylation can promote both transcriptional activation and repression [27]. Since distinct histone PTMs correlate with specific transcriptional states, it is conceivable that distinct histone modifications patterns on one or more tails are likely read like a molecular bar code to recruit chromatin remodeling complexes that drive gene expression profiles required for particular cellular events, a paradigm referred to as the 'histone-code hypothesis' [8,28,29].

Accumulating evidence also indicates that there also exists a 'histone code' that regulates gene expression profiles for distinct brain functions. For instance, histone H3 phosphorylation is regulated in the hippocampus during induction and consolidation of contextual fear conditioning memory [30]. In this paradigm, a peak in H3 phosphorylation, acetylation and phosphor-acetylation occurs an hour after training, corresponding to the period when rapid hippocampal gene induction occurs [31]. Thus, it seems like these epigenetic marks may serve as part of a histone code that is subsequently interpreted as a pattern of gene expression specific to this form of long-term 'fear' memory. Recent studies have also implicated DNA methylation, once thought to be a static process after cellular differentiation, to dynamically regulate hippocampal memory formation in conjunction with H3 acetylation [32,33]. But, how can such combinatorial histone modifications affect memory formation? [9] proposed that histone modifications may gate a burst of transcription for a specific set of plasticity effector and regulator

genes that then change the response properties of individual neurons in a network. Histone modifications may also mediate persistent changes in the expression of key plasticity effector or regulator genes required for maintenance of changes in neuronal behavior. It is likely that transient histone modifications may act downstream of signaling cascades to integrate multiple signals and ensure that a cascade of gene expression is activated only after a particular stimulus pattern (either spatially or temporally) is generated [34]. Under such conditions, histone modifications may act to integrate information about the activation and regulate recruitment of process specific transcription factors. Thus, specific histone modification patterns not only serve to alter the chromatin structure but also provide an interaction interface for transcriptional co-activators or co-repressors that bind modified histone tails to regulate specific transcription events [9]. However, studies aimed at deciphering the "epigenetic indexing code" specific for high-order brain functions like memory formation are still in their infancy. An increased understanding of chromatin function and epigenetic tagging may further help delineate the role of particular epigenetic mechanisms in brain functions in more molecular detail.

Epigenetics based plasticity in brain function

Phenotype is the net result of continued gene – environment interactions. Environmentally regulated intracellular signals 'program' regulated expression of very specific gene sets that are required for the development and function of specific cell lineages [19]. In the nervous system, the mechanisms by which extracellular signals regulate gene expression have just begun to be characterized. Indeed, epigenetic modifications such as DNA methylation and PTMs of histone proteins are emerging as fundamental mechanisms by which neurons adapt their transcriptional response to

developmental and environmental cues. The implicit hypothesis is that environmental signals alter such chromatin modifications, allowing for the transcriptional 'plasticity' that in turn mediates sustained variation in neural function [35]. For instance, [36] reported that spontaneous synaptic transmission in hippocampal neurons is regulated by alterations in DNA methylation that occur in response to synaptic activity. Sensory experiences in the form of neuronal activity also have differential effects on synaptic plasticity at excitatory or inhibitory synapses, leading to either long term potentiation (LTP) or long term depression (LTD), whereby the efficacy of synaptic transmission is up- or downregulated, respectively [4]. Certain forms of LTP and LTD require long-lasting changes in gene expression and a growing body of evidence suggests that histone PTMs may be involved in these processes. In an elegant study using sensory motor neurons of *Aplysia*, [37] showed that an increase and decrease of acetylated histones might constitute the switch between LTP and LTD at the same synapses. In the hippocampus, various signaling pathways involving dopaminergic, acteylcholinergic, and glutamatergic signaling have been implicated in synaptic plasticity. Stimulation of each of these pathways is accompanied by increased H3S10 phosphorylation and H3K14 acetylation. The mitogen-activated protein kinase (MAPK) pathway is yet another signaling cascade implicated in different forms of memory and synaptic plasticity. Interestingly, changes in H3 phosphorylation patterns parallel those of a member of the MAPK pathway, namely, extracellular regulated kinase (ERK) [30,38-40]. Activation of ERK through the protein kinases PKC and PKA also increases H3K14 acetylation; while activation of N-methyl D-aspartate (NMDA) receptors results in a similar increase of acetylated H3, an effect that could be blocked by inhibition of the ERK signaling [15].

These studies provided the first evidence that intracellular signaling pathways interact with the epigenetic machinery to modulate synaptic plasticity. With identification of nuclear enzymes that regulate histone PTMs (like acetylation, lysine/argine methylation, phosphorylation, deamination, ubiquitination), it is conceivable that most, if not all, chromatin modifying enzymes are targeted by signaling pathways that directly link environmental cues to gene expression [9]. Nevertheless, the complete repertoire of extracellular signals and corresponding intracellular pathways that mediate dynamic regulation of histone modifications in neurons remains poorly understood.

Alterations to the brain epigenome as part of aging and in neurodegenerative diseases

An increasing body of evidence indicates that substantial reorganization of the brain epigenome occurs during aging and such age related epigenetic drift could further exacerbate an individual's vulnerability to aging related cognitive decline [11]. This notion that aging is associated with epigenetic changes in the brain is not unsubstantiated, with studies reporting widespread age-related changes in gene expression in the cerebral cortex, including downregulation of many neuronal genes [41,42]. Recent studies have also reported global loss of DNA methylation in aging, or the hypermethylation of regulatory regions (promoters) of genes associated with accelerated aging [43-45]. In addition, dynamic changes to the epigenetic landscapes of PTMs can also occur and are characterized by loss of markings associated with active gene expression, such as monomethylation of H4 Lys20 and trimethylation of H3 Lys36, in conjunction with robust increase in the repressive mark H3 Lys27me3 [46]. Likewise, in the hippocampi of 16 month old wild type mice, genomic regions associated with actively expressed genes

shows a decline in acetylated H4Lys12, a PTM linked to transcription elongation (Peleg et al., 2010). It is likely that such age-related drifts in brain epigenomes negatively affect neuronal and oligodendroglial transcriptomes, thereby leading to a decline in signaling capacity of nerve cells [47-49]. With regards to specific neurophysiological processes, it is well-established that memory and synaptic plasticity processes in the cognitively healthy adult require transcription of immediate-early genes (IEGs), including *Arc* (activity-regulated cytoskeletal gene), *zif268* (also known as nerve growth factor inducible-A), and *bdnf* (brain-derived neurotrophic factor) [50-52]. While blocking the expression of these genes in adult animals prevents the consolidation of memory [50,53], decreased IEG expression is also prevalent in many models of memory disorders [54-56] and as a result of the normal aging process [57-59]. Accumulating evidence indicates that epigenetic mechanisms play a key role in dynamically regulating memory associated gene transcription in the adult CNS and are thus integral to long term memory formation [40,60]. In light of studies reporting a decline in the transcription of key memory-promoting genes during aging [45,48,57] it has been hypothesized that such changes could be mediated by dysregulation of epigenetic control mechanisms over the lifespan of an individual. Consequently, accumulation of aberrant epigenetic marks within brain regions vulnerable to the aging process may result in age-related cognitive deficits and are also manifested in the form of neurodegenerative diseases [61].

Aging-related neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and others are multifactorial illnesses in which many as yet poorly understood pathways are affected serially and in parallel resulting in pathologic phenotypes like cognitive decline. Recent studies have

linked phenotypic as well as mechanistic features common to many neurodegenerative diseases to epigenetic defects [62]. Although both familial and sporadic forms of AD, PD and ALS are known to occur, familial forms represent only a minority of the cases and the vast majority of cases occur as sporadic forms that are likely to result from complex interactions between genetic and environmental factors that superimpose on the slow, sustained neuronal dysfunction due to aging [63]. In fact, 'synucleopathies' such as Parkinson's disease and dementia with Lewy bodies are associated with dysregulation of DNA methylation at the promoters of several disease-associated genes [64]. Histone modifying enzymes have also been implicated in neurodegenerative diseases. For example, the pathological sequestration of transcription factors vital for neuronal health, such as the cAMP response element-binding protein CREB and its binding partner CBP, a histone acetyltransferase (HAT), has been linked to the beta amyloid plaques seen in the brains of individuals with Alzheimer's disease (AD) [65-67] and the polyglutamine aggregates and nuclear inclusions in Huntington's chorea [68]. Likewise, misregulation of the HAT, Tip60 that transcriptionally regulates neuronal genes has been implicated in AD pathogenesis [69].The interaction of Tip60 with ataxin 1 protein has also been reported to contribute to cerebellar degeneration associated with Spinocerebellar ataxia (SCA1), a neurodegenerative disease caused by polyglutamine tract expansion [70]. Furthermore, excessive H3Lys9 methylation [71] and increased expression of macro H2A1, a variant histone broadly associated with repressive chromatin [72], have been observed in blood and brain tissues from individuals with Huntington's disease in brains regions like the striatum and cerebral cortex which are heavily affected by the disease associated neurodegenerative process [3]. Despite the growing number of studies, much

of the evidence linking histone modifying enzymes to neurodegenerative diseases has been correlative and thus warrants further investigation. Nevertheless, these studies highlight the fact that epigenetic mechanisms may be crucial to advancing our understanding of how individual differences modulate susceptibility to neurodegenerative diseases. Originally thought to be stable and irreversible, epigenetic mechanisms have been demonstrated by several recent studies to be dynamic and reversible even in fully differentiated brain cells. This reversibility further confers on epigenetic mechanisms the potential of being targeted by pharmacological interventions to alleviate or reverse the symptoms resulting from their dysfunctions [73].

Histone acetylation: a key epigenetic modification for neuronal survival and function

HAT: HDAC imbalance in the etiology of neurodegenerative diseases

In neurons, histone acetyltransferases (HATs) and histone deacetylases (HDACs) are among the best characterized chromatin modifying enzymes and represent distinct classes that, respectively, catalyze forward and reverse reactions of lysine residue acetylation in specific histone substrates. HATs function enzymatically by transferring an acetyl group from acetyl-coenzyme A to the ϵ-amino groups of histone lysine residues thereby creating an appropriate ‘histone code’ for chromatin modification and enhanced DNA accessibility of transcription factors. Contrarily, HDACs attenuate the transcription process at particular sites by deacetylating such lysine targets [74]. Under normal conditions, HAT and HDAC levels are maintained in a highly harmonized state of

balance. Such equilibrium is crucial for neuronal homeostasis and is also responsible for regulated gene expression essential for normal neurophysiological outputs like long-term potentiation, learning and memory [75]. Treatment of neurons with HDAC inhibitors like trichostatin A (TSA) in normal conditions induces neuronal apoptosis [76]. Similarly, overexpression of CBP in resting cerebellar granule neurons under prosurvival conditions leads to chromatin condensation and cell death [77]. Neuronal overexpression of Tip60 also leads to increased apoptosis and lethality in *Drosophila* [78]. Such lethal effects are likely mediated by skewing the HAT/HDAC balance towards increased acetylation that in turn brings about alterations in the chromatin structure that leads to activation/de-repression of genes that are quiescent under basal conditions. On the other hand, *in vitro* models of neuronal apoptosis such as cultured cerebellar neurons subjected to neurotrophic deprivation exhibit H3 and H4 deacetylation that precedes neuronal death and is also accompanied by loss of CBP [77]. Together, these studies support the maintenance of optimal HAT/HDAC balance for neuronal survival, notably in differentiated adult neurons that have to maintain their functional status and homeostasis throughout their lifetime.

Remarkably, altered levels of histone acetylation have also been observed in several models of neurodegenerative diseases. For instance, toxic accumulation of α-synuclein in the nucleus of dopaminergic neurons induces neurotoxicity by promoting H3 deacetylation through direct association with histones thereby shielding residues targeted for acetylation [79]. A similar histone ‘masking’ mechanism is thought to be involved in transcriptional repression mediated by the polyglutamine disease protein ataxin-3 [80]. Expression of the polyglutamine-containing domain of the pathogenic Huntington (htt)

protein in cultured cells (PC12) also leads to H3 and H4 deacetylation [81]. In cultured cortical neurons, modulation of APP dependent calcium/calmodulin protein kinase-IV signaling pathway results in reduced histone acetylation [82]. In an ALS mouse model (SOD1 G86R), H3 hypoacetylation has been observed in cholinergic motor neurons from the lumbar spinal cord [77](Rouaux et al., 2003).While these studies identify histone deacetylation as a common feature of neurotoxicity under pathological conditions, as mentioned above, histone hyperacetylation can also be fatal to neurons. In a study by [83], it was reported that Dieldrin, a neurotoxic peptide implicated in the etiopathogenesis of PD, induces a time dependent accumulation of CBP, resulting in increased H3 and H4 acetylation in dopaminergic neurons. Together, this series of studies strongly point towards a loss of neuronal acetylation homeostasis during neurodegeneration. But how can impairment of acetylation homeostasis lead to neuronal loss? The clue to this question revolves around the theory of 'transcriptional dysfunction' that attributes the degenerative fate of neurons to altered transcription profiles resulting from complex changes in the chromatin landscape that differs sharply from activity dependent normal transcription patterns. As a result, expression of survival-associated genes is likely attenuated by altered acetylation and expression of pro-apoptotic genes is stimulated, consequently leading to neuronal cell death, a major pathological hallmark of many neurodegenerative diseases [75]. However, neuronal cell death and activation of apoptotic pathways associated with loss of neurons is a late event in the disease associated pathogenesis [84]. In fact, accumulating evidence indicate that the clinical symptoms associated with neurodegenerative diseases are the result of neuronal dysfunction that precedes cell demise and manifested through loss of synaptic

connectivity. For instance, in AD, synaptic degeneration appears to be an early event in pathogenesis with synapse loss evident in patients with early AD and mild cognitive impairment [85,86]. Accordingly, it has been proposed that synapse loss underlies memory impairment evident in the early phase of AD [87]. Recent studies propose that changes in histone acetylation levels may be involved in the altered synaptic function and memory associated with AD [88,89]. Consistent with this hypothesis, pre-clinical studies in APP/PS1 mouse model of AD have reported differences in histone acetylation levels during associative memory formation wherein levels of hippocampal acetylated histone H4 in APP/PS1 mice were about 50% lower than in wild-type littermates after fear conditioning training [90]. Likewise, in HD, there is now considerable evidence that early cognitive deficits appear in patients before the onset of the characteristic motor disturbances [91]. Early impairment of long-term spatial and recognition memory in heterozygous HD knock-in mutant mice (HdhQ7/Q111) is also associated with reduced hippocampal expression of CBP and diminished levels of histone H3 acetylation with concomitant reduction in expression of memory related genes [92].These studies further suggest that disruption of acetylation homeostasis can lead to early widespread synaptic dysfunction that in turn impairs neuronal connectivity. Such progressive damage of the neural network is likely followed by eventual neuronal apoptosis. However, whether histone acetylation changes are a cause or consequence of neuronal dysfunction requires further investigation.

HAT: HDAC interplay in memory formation

A number of recent studies have identified histone acetylation as an essential mechanism for formation of long-term memories [40]. Associative learning in rats induces a transient increase in hippocampal acetylation of histone H3 but not H4 [31], suggesting that this type of memory formation leads to very specific re-organization of the chromatin structure. Importantly, these changes in histone acetylation are transient and observed 1hr but not 24hrs after training. Such transient changes in histone acetylation have also been observed in other hippocampus-dependent learning paradigms (reviewed in [5]). Subsequent studies in mice have shown that memory formation also leads to transient increase in acetylation of various lysine residues within histones H2A, H3 and H4 [93,94]. Intriguingly, altered histone acetylation in the hippocampus has been associated with aging associated memory disturbances. A recent study using a hippocampus dependent associative learning paradigm, reported that in aged mice, the onset of memory disturbances correlated with a lack of learning-induced acetylation of histone H4 at lysine12 (H4K12), while there was no effect on other histone modifications [94]. Furthermore, the specific lack of H4K12 acetylation correlated with a severely impaired hippocampal gene expression program required for memory formation. By analyzing the distribution of H4K12 acetylation in young and aged mice during learning, this study found that decreased H4K12 acetylation was selectively associated with the coding regions of genes that are normally upregulated during learning. Together, these studies provide convincing evidence in favor of a casual role for histone acetylation in mediating gene expression changes associated with memory consolidation as well as age-associated memory impairment.

Recent studies have also identified specific HATs and HDACs that are required for memory formation and deregulation of such enzymes have also been linked to age-associated memory impairment [49]. To this end, several genetic studies have identified the HAT CBP as a major contributor to memory formation [95]. Mice haploinsufficient for CBP (*cbp*+/−) exhibit reduced acetylation, defects in hippocampal late long-term potentiation (L-LTP), and some forms of long-term memory (LTM) deficits [96]. Importantly, the HAT activity of CBP was shown to be required for these processes [97]. In addition, other HATs like the E1A-binding protein p300 (p300) and p300/CBP-associated factor (PCAF), have also been implicated in memory processes [98,99]. PCAF homozygous knock-out mice are viable and display short term memory impairments at adolescent age (2 months) and gradually increasing learning and memory deficits with progressive age (6 and 12 months) [99]. In addition, learning induced upregulation of CBP, p300 and PCAF has also been associated with elevated H2B and H4 acetylation during spatial memory consolidation [100]. This is consistent with previous studies that showed that learning increases hippocampal H2B and H4 acetylation [93,94]. Together, these studies suggest that HATs exhibit certain substrate specificity during memory formation in the adult brain and mediate dynamic acetylation of such substrates. Interestingly, histone acetylation seems to occur in a sequential manner. For instance, the vast majority of H4K16 sites appear to be acetylated at basal levels while other sites like H4K5 and H4K12 are acetylated at low levels [101]. Yeast Gcn5 bromodomain has been shown to bind to acetylated H4K16 in vitro which leads to subsequent acetylation of other nearby lysine residues [102]. Interestingly, H4K16 is the only histone modification that is not regulated during memory consolidation, while exposure of mice to associative

learning increases hippocampal H4K5, H4K8 and H4K12 acetylation [94]. Thus, H4K16 is likely at the base of the pyramid of H4 acetylation and in turn mediates acetylation of nearby lysine substrates in a process specific manner [103]. A recent study reported that HATs like CBP, p300 and PCAF that all harbor a bromodomain are upregulated during spatial memory formation while the HAT Tip60 that lacks a bromodomain was not upregulated [100]. Similarly, in a recent gene array study, the bromodomain containing HATs, Taf1/Kat4, Gcn5/Kat2a were found to be upregulated one hour after a fear conditioning stimulus [94]. Together, these findings suggest a model wherein a stimulus driven upregulation of bromodomain containing HATs induce histone acetylation that is required for transcription of plasticity-related genes [103].

Histone acetylation is mediated by the concerted actions of HATs and HDACs [74]. The mammalian genome encodes 11 HDAC proteins consisting of the class I (HDACs 1, 2, 3 and 8), class II (HDACs 4, 5, 6, 7, 9 and 10), class III sirtuins (SIRT 1, 2, 3, 4, 5, 6 and 7) and class IV (HDAC 11) HDACs [104]. With regards to memory formation, HDAC2 was recently shown to be associated with promoters of genes implicated in synaptic plasticity including *Egr1* (also known as *zif*268), *Bdnf*, *Fos*, and *Creb*. Accordingly, neuron-specific overexpression of HDAC2, but not that of HDAC1, decreased dendritic spine density, synaptic plasticity and memory formation, indicating that HDAC2 negatively regulates memory formation [105]. Conversely, HDAC2 knock-out mice exhibit enhanced memory formation that correlated with elevated H4K12 acetylation which as mentioned above has been implicated in gene expression programs required for memory formation. Similar to HDAC2, specific deletion of HDAC3 in the dorsal hippocampus of mice leads to enhanced long term memory and elevated

expression of Nr4a2, a gene associated with long term memory formation [106]. However, not all HDACs have memory related functions [105]. For instance, deletion of HDAC1 in mice has no obvious phenotype. However, HDAC1 activity seems to be rather neuroprotective [107]. Likewise, loss of HDAC7 in a HD mouse model has no detectable phenotype either [108]. This series of studies identifying the role of specific HATs and HDACs in memory formation highlight the crucial dependency of long term memory formation on these key epigenetic players.

Targeting histone acetylation: epigenetic strategy for neurodegenerative diseases

The above studies identifying a critical role for histone acetylation in promoting cell survival and memory formation have in turn led to the discovery that deregulated histone acetylation might be involved in the pathogenesis of various neurodegenerative diseases [103]. In light of these studies, the use of histone deacetylase inhibitors (HDACi) as a therapeutic tool for neurodegenerative disorders has been examined with great interest in the last decade [109]. This section will review some of the recent data linking dysregulation of specific HATs and HDACs to neurodegenerative diseases as well as the promising effects observed with HDACi in preventing cell death and alleviating disease associated pathological symptoms.

Huntington's disease

Huntington's disease (HD) is an inherited genetic disorder, caused by an abnormally expanded and unstable CAG repeat (polyglutamine or polyQ expansion) within the coding region of the gene encoding the huntington (htt) protein. One of the models for mutant huntington protein induced toxicity is based on the finding that

abnormal htt directly binds the acetyltransferase domains of CBP and PCAF. This appears to sequester these acetyltransferases, resulting in globally reduced H3 and H4 acetylation levels, and altered gene expression [81]. Overexpression of the expanded HD constructs has been shown in different cellular models to cause redistribution of CBP in nuclear or cytoplasmic inclusions. This phenomenon is accompanied by inhibition of HAT activity of CBP, further leading to global deacetylation and cell death [110]. Mutated polyQ-expanded htt has also been shown to selectively enhance ubiquitylation and degradation of CBP [111,112]. Further supporting the notion that HD is a disease of aberrantly reduced histone acetylation are observations that treatment with HDAC inhibitors (HDACi) rescue histone acetylation levels and improve neurodegeneration and pathological symptoms in cellular, *Drosophila* and mouse models of HD [81,113,114]. Administration of the pan-HDACi, suberoylanilide hydroxamic acid (SAHA) has been shown to increase histone acetylation and improve motor impairment in the R6/2 transgenic HD mouse model [115]. In the same model, presymptomatic intraperitoneal administration of another pan-HDACi, sodium butyrate extended survival and prevented striatal neuronal atrophy with resultant improvement in motor performance [116]. A novel pimelic diphenylamide HDACi, 4b, has also shown beneficial effects on disease phenotype and transcriptional abnormalities in an HD mouse model [117]. Recent studies also highlight a novel therapeutic approach for HD using a combination of class I and class III HDAC inhibitors [113]. While these studies point to deregulation of histone acetylation in HD associated pathogenesis, the precise molecular mechanisms that mediate these effects remain to be investigated.

Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by degeneration and death of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNc) of the ventral midbrain [118]. The initial link between PD and deregulation of histone acetylation came from observations that the PD linked presynaptic protein, α-synuclein, binds histones to inactivate HATs like CBP, p300 and PCAF, causing apoptosis in human neuroblastoma cells [79]. More recently, the ability of Valproic acid to increase histone acetylation in a rat model of Parkinson's disease was associated with prevention of neuronal death in the substantia nigra, decrease of the PD marker, α-synuclein and an increase in tyrosine hydroxylase in both the substantia nigra and striatum [119]. Class III HDACs (sirtuins) are known to participate in an array of cellular functions related to aging. [120] reported that inhibition of sirtuin 2 (SIRT2) in a cellular model of PD abates α-synuclein toxicity, altered inclusion morphology, and protects against DA cell death. In addition, genetic inhibition of SIRT2 via small interfering RNA (siRNA) also reverses α-synuclein toxicity. Together, these studies provide a link between α-synuclein activity, histone deacetylation, neurodegeneration and aging as well as identify HDACi as potential targets for therapeutic intervention in PD.

Amylotrophic lateral sclerosis (ALS)

The efficacy of restoring histone acetylation levels has also been investigated in Amylotrophic lateral sclerosis (ALS) using HDACi treatments as transcriptional dysregulation is thought to play a role in the disease pathophysiology. ALS an adult-onset neurodegenerative disease characterized by progressive loss of motor neurons in the

brain, brain stem, and spinal cord, resulting in generalized weakness, muscle atrophy, paralysis, and eventual mortality [121,122]. ALS has been attributed to gain-of-function mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1) [123]. In a SOD1 point mutation mouse model of ALS, ALS symptoms were molecularly accompanied by reduced CBP levels in motorneurons [77]. Treatment of SOD1 mutant mice with HDACi like VPA and TSA has been shown to restore the resulting histone acetylation deficits together with the motor deficits back to baseline [124]. Similarly, treating SOD1 mutant mice with 4-phenylbutyrate starting before or shortly after onset of symptoms extends survival and improves pathological phenotypes [125]. This study also found that 4-phenylbutyrate treatment ameliorated hypoacetylation, upregulated Bcl-2, NF-κB, p50 and phospho-IκB, and downregulated cytochrome c caspases in the spinal tissues of treated mice. Additionally, Rouaux and colleagues found that, in SOD1 mutant mice, VPA treatment maintained normal levels of histone acetylation, restored the loss of CBP and significantly suppressed the death of motor neurons, although it did not prolong survival [124]. Further evidence for a deregulation of histone acetylation in ALS comes from a recent human post mortem. Comparing the protein expression levels of all class I, II, and IV HDACs in the ALS brain and spinal cord, this study found that HDAC2 and HDAC11 were up- and downregulated, respectively [126]. The functional consequences in terms of histone acetylation changes and resulting gene expression changes however, remain unclear.

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of neurodegenerative disorder and dementia in the elderly. AD arises on the pathological background of

amyloid beta (Aβ) plaques, neurofibrillary tangles (NFTs) resulting from intraneuronal aggregates of the microtubule-associated protein, *tau*, and neuronal cell death. Aβ plaques consist of extracellular aggregates of small Aβ peptides, which are generated from sequential endo-proteolytic cleavage of the type 1 trans-membrane glycoprotein, β-amyloid precursor protein APP via the action of β secretase- and γ-secretase [127]. β-site APP cleaving enzyme 1 (BACE1) is the β-secretase in vivo and γ-secretase is a membrane-protein aspartic protease composed of at least four subunits—presenilin (PS1 or PS2), nicastrin (Nct), APH-1 (APH-1aL, APH-1aS, or APH-1b), and PEN-2 (Mattson, 2004). In AD patients, increased propensity towards generation of the more toxic Aβ42 peptide is observed [128]. The precise mechanism by which Aβ eventually contributes to synaptic dysfunction and neuronal loss is still an area of intense research. The current view is that during AD pathogenesis, Aβ peptides start to form aggregates that affect neuronal integrity through multiple mechanisms [128-130]. Similar to Aβ pathology, current data suggest that soluble forms of *tau* protein rather than the insoluble NFTs, at least initially, lead to cognitive decline [131,132]. Accumulating evidence also suggests that Aβ and *tau* pathology are linked and that the presence of *tau* is critical for Aβ pathology [132]. While promising progress has been made at identifying therapeutic approaches targeting either the amyloid cascade or tau pathology, an effective therapy is still not available.

Growing evidence suggests that the cognitive impairment in AD as well as signaling between neurons is interrupted at early stages of the disease [133]. Recent studies point to dysregulation of epigenetic control mechanisms and the resultant aberrant epigenetic marks as contributing factors to such cognitive dysfunction [15,134]. A

number of different epigenetic abnormalities including histone acetylation have also been reported in AD [134]. Further evidence linking histone acetylation and cognitive decline in AD stems from the observation that histone acetylation declines in mouse models for AD. For example, decreased acetylation of H4 but not H3 has been observed in tg2576 mice, a model for amyloid pathology [135]. Interestingly, administration of the pan-HDACi phenylbutyrate has been reported to reinstate associative memory and synaptic plasticity in 6- and 16- month old tg2576 mice [136]. Similarly, administration of various pan-HDACi also reinstates associative memory in APP/PS1Δ9 mice, also a mouse model for amyloid pathology [137]. The pan-HDACi TSA has also been reported to restore associative memory function in hippocampal LTP in another mouse model for AD-like amyloid pathology (APP/PS1) that exhibit impaired H4 acetylation upon exposure to a learning stimulus [90].

Recent studies have also implicated specific HATs and HDACs in AD associated pathophysiology. [138] showed that over-expression of SIRT1, the NAD^+-dependent deacetylase in a mouse model of AD reduces the production of Aβ and formation of plaques via activation of transcription of the gene encoding α-secretase, ADAM10 that cleaves APP at a site with the Aβ domain and thus preempts the formation of Aβ . Presenelin 1 (PS1) which is part of the γ-secretase complex has been reported to have an inhibitory role on the HAT CBP through proteasomal degradation, and mutations in PS1 found in hereditary AD result in aberrantly high CBP activity [90,139]. Additionally, p25/Cdk5, a kinase complex implicated in AD and other neurodegenerative disorders inhibits HDAC1, rendering neurons susceptible to DNA damage, cell cycle reentry, and ultimately cell death [140]. Remarkably, overexpression of HDAC1 rescues such

p25/Cdk5-mediated DNA damage and neurotoxicity [107]. While these findings suggest that AD could be a disease of aberrantly increased histone acetylation, a substantial body of evidence also supports the notion that inhibition of HDACs can be protective and beneficial in AD. In fact, APP overexpression in cultured cortical neurons leads to H3 and H4 hypoacetylation, and is paralleled by decreased CBP levels (Rouaux et al., 2003). Loss of function mutations in genes coding for PS1 and PS2 has been shown to reduce expression of CBP and CBP/CREB target genes such as *c-fos* and BDNF with negative effects on synaptic plasticity, spatial and contextual memory [141]. Moreover, in the p25/Cdk5 model of neurodegeneration, treatment with the broad HDACi sodium butyrate not only increased H3 and H4 acetylation levels, but also resulted in the reestablishment of learning abilities, as well as access to long-term memories that had been ablated by prior hyperactivation of p25/Cdk5 [142]. Similarly, both general and class I-selective HDAC inhibitors have been shown to ameliorate cognitive defects in transgenic AD mouse harboring hereditary AD mutation [136,137].

The sequential processing of APP by β- and γ-secretases generates an intracellular fragment, the APP intracellular domain (AICD) that is released into the cytosol [143]. AICD has been shown to interact with the HAT Tip60 *via* the scaffolding protein Fe65 [144]. It has been demonstrated that this complex is recruited to the promoters of certain target genes where it acts to acetylate select histone proteins to epigenetically regulate gene transcription [144-146]. Importantly, aberrant expression of some of these genes like LRP1, GSK-3B, KAI-1 has been linked to AD pathophysiology [147-149]. Based on these findings, it has been proposed that the inappropriate AICD/Tip60 complex formation and/or recruitment may contribute or lead to AD pathology via misregulation

of target genes required for neuronal functions. The APP intracellular domain was recently shown to lower the sensitivity of neuronal cells to toxic stimuli and transcriptionally activate genes involved in signaling pathways that are not active under basal conditions [150]. We recently reported that co-expression of APP with HAT activity deficient Tip60 leads to misregulation of a number of pro-apoptotic genes in a *Drosophila* AD model with a resultant increase in neuronal apoptotic cell death. Contrarily, expressing HAT competent wild type Tip60 in conjunction with APP led to induction of pro-survival genes like *Drosophila* Bcl-2 with a concomitant reduction in neuronal apoptosis. These findings point to the fact that Tip60 may play a neuroprotective role during disease progression via its histone acetylase function. By complexing with the AICD region of APP, Tip60 may epigenetically regulate transcription of genes essential for tipping the cell fate control balance from apoptotic cell death towards cell survival under APP induced neurodegenerative conditions [78]. Together, these studies suggest that the overall misregulation of histone acetylation characteristic of AD is complex. While the beneficial effects observed with general or partially selective HDACi are promising, it is essential to identify the specific HATs and HDACs that can be targeted for therapeutic interventions.

Perspectives on use of HDAC inhibitors for treatment of neurodegenerative diseases

As described above, the promising effects observed with the use of small molecule HDAC inhibitors has ignited enormous interest in their therapeutic potential for various neurodegenerative conditions. However, most HDAC inhibitors that have been tested in the context of neurodegenerative diseases are non-selective, inhibit multiple HDAC proteins, and the observed therapeutic effects likely result from increased

"global" acetylation levels and potentially HDAC inhibitor dependent genetic programs [151]. These issues have in turn raised widespread speculation about the target specificity of HDAC inhibitors [152]. Recent targeted gene deletion studies indicate that HDACs serve very distinct functions within the adult brain. Cellular localization and tissue-specific expression for different HDACs also vary [49]. [153] recently reported that under native conditions, all HDACs are expressed in the adult rodent brain. However, expression level of HDAC10 is very low under native conditions and can be detected only in the hippocampal formation. In some instances, interactions between different HDAC classes are required to activate their deacetylase function. For example, HDACs 4, 5 and 7 (class II HDACs) lack the ability to deacetylate histones independently and require interaction with HDAC 3 (class I) to be active [154]. Contrarily, while class I HDACs 1 and 2 form complexes with each other and are often found in the same protein complexes, they appear to serve distinct functions. Global loss of HDAC1 leads to early lethality, suggesting that HDAC2 cannot compensate for the absence of HDAC1 [155]. In neurons, however, deletion of HDAC1 or HDAC2 individually has no apparent effect on neuronal development while loss of both HDAC 1 and 2 leads to loss of neuronal differentiation. Thus, in addition to their distinct roles in the adult brain, HDAC 1 and 2 appear to have important redundant functions during neuronal development [156]. Distinct as well as complementary roles for HDAC 1 and 2 have also been observed with regards to synapse development. In immature hippocampal neurons, a targeted knockdown of HDAC 1 and 2 increased synaptic activity and synapse numbers. However, in mature neurons, the knockdown of HDAC2 alone decreased synaptic activity, whereas the loss of HDAC1 had no effect [157]. Thus, inhibition of HDAC1 and

2 during development, and HDAC2 in mature brain, may have potential unexpected neurological side effects. HDAC2 knockout in mice has also been shown to enhance learning and memory and synaptic plasticity (Guan et al., 2009). Although targeting specific class of HDACs has been perceived as a suitable therapeutic avenue for some neurodegenerative diseases, it can lead to very different and potentially opposing clinical implications. For example, activation and/or overexpression of HDACs 2 and 3 is associated with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and neural cell toxicity [126,158], while inhibition of HDAC 1 has been found to lead to neurodegeneration [107,159]. Moreover, subcellular localization of HDACs and thus, their ability to repress gene targets is controlled by synaptic activity in neurons. For instance, localization of class II HDACs 4 and 5 is dynamic and signal-regulated in cultured hippocampal neurons wherein nuclear export of HDAC4 is initiated by spontaneous electrical activity and HDAC5 translocation to nucleus is induced by stimulation of calcium flux through synaptic NMDA receptors [160]. Such activity dependent regulation of HDAC function further necessitates a clearer understanding of specific activating stimuli if pharmacological interventions targeting these HDACs are to be developed. Thus, in light of these studies demonstrating opposing as well as redundant functions of members of class I HDACs and their requirement for activation of other HDACs suggest that targeting specific HDACs might be more beneficial than class specific modulation of HDAC activity.

Another issue to consider in terms of HDAC based therapeutic efficacy is that although HDAC inhibitors are generally considered to promote neuronal growth and differentiation, they also exhibit toxicity in various cell types of the central nervous

system. For instance, there is evidence that they could have potentially detrimental effects on the orderly maturation of astrocytes and oligodendrocytes [161-163]. There is also evidence that neuroprotection can result from non-enzymatic activity of HDACs, as was demonstrated in the case of a mutated inactive form of SIRT1 that prevents apoptosis when overexpressed in cerebellar granule neurons (CGNs) [164]. Moreover, like their counterparts, the HATs – class I, II and III of HDACs also regulate lysine acetylation of non-histone proteins that exert neuroprotective effects [165] adding a further layer of complexity to the interpretation of therapeutic potentials of currently available broad spectrum or even class specific HDAC inhibitors for neurodegenerative diseases. Thus, the specificity and side-effect profiles of inhibitors of HDACs require additional investigation to fully gauge their neuroprotective abilities. Further exploration of isoform-selective HDAC inhibitors that are also region-specific may provide a therapeutic advantage in targeting specific cell and tissue functions under pathological conditions.

Modulating HAT function: a promising therapeutic option for neurodegenerative diseases?

It has become increasingly clear that chromatin acetylation status can be impaired during the lifetime of neurons through loss of function of specific HATs with deleterious consequences on neuronal function [152]. Once the acetylation balance is disturbed by the loss of HAT dose, the HAT: HDAC ratio tilts in favor of HDACs in terms of availability and enzymatic functionality, a fact highlighted by amelioration of several neurodegenerative conditions by various HDAC inhibitors [130]. In fact, a clue to explain the net deacetylation observed during neurodegeneration came with the finding that dying

neurons exhibit progressive loss of HAT activity and/or expression, particularly that of CREB binding protein (CBP) and to a lesser extent p300. Notably, overexpression of CBP under apoptotic conditions delays neuronal cell death, an event that was dependent on the HAT function of CBP [123,166]. We recently reported that neuronal misregulation of Tip60's HAT function in *Drosophila* via overexpression of wild type Tip60 or the HAT defective mutant Tip60 leads to apoptotic cell death, an effect predominantly mediated through transcriptional dysregulation of pro-apoptotic genes as well as genes required for normal development [78]. Specific HATs are also emerging as regulators that gate access to genes regulating specific neuronal processes that are essential for maintaining neuronal health and for mediating higher order brain functions. Notably, such processes are also affected in neurodegenerative conditions and significantly contribute to pathological consequences. For instance, CBP has been shown to mediate specific forms of hippocampal long term potentiation, a form of synaptic plasticity thought to underlie memory storage [167]. In contrast, the HAT p300 has been shown to constrain synaptic plasticity in the prefrontal cortex and reduced function of this HAT is required for formation of fear extinction memory [168]. Importantly, overexpression of p300 but not HDAC inhibition has been shown to promote axonal regeneration in mature retinal ganglion cells following optic nerve injury, an effect mediated by p300 induced hyperacetylation of histone H3 and p53 that consequently leads to increased expression of selected pro-axonal outgrowth genes [169]. Overexpression of Tip60 under APP induced neurodegenerative conditions also induces intrinsic axonal arborization of the *Drosophila* small ventrolateral neurons, a well characterized model system for studying axonal growth [170].

It is important to note that modulation of specific HAT levels and/or activity may alter the expression of many genes or "cassettes" of specific genes that act together produce a neuroprotective effect. In fact, in the case of Tip60, overexpression of wild type Tip60 but not the HAT defective mutant increases survival in a *Drosophila* AD model, an effect that is mediated *via* enhanced repression of a "cassette" of pro-apoptotic genes and induction of pro-survival factors like Bcl-2 [78]. With regards to non-chromatin associated cellular processes, the acetyltransferase Elp3 known to acetylate microtubules has been shown to be involved in the regulation of synaptic bouton expansion during neurogenesis [171] and recent studies suggest that regulation of microtubule acetylation by the ELP3 might be commonly affected in neurological diseases making it a potential target for acetylation modulator based therapies (reviewed in [172]). Tip60 has also been recently shown to play a causative role in synaptic plasticity partly through acetylation of microtubules [173]. Together, these studies raise the possibility that modulation of expression levels and/or activity of specific HATs such as Tip60 could be an alternative therapeutic option for neurological conditions. Importantly, targeting HATs rather than HDACs can also be beneficial because unlike HDACs, HATs have non-redundant functions under physiological conditions and thus the presence of these specific modulators can have more direct effects. In a study by [174], it was reported that the total protein amount and activity of various HDACs is not altered by mutant huntington protein expression in primary cortical neurons. Thus, the neurodegeneration associated tilt in HAT: HDAC does not appear to include augmentation of HDAC protein level. Therefore, activation of specific HATs may restore acetylation balance in addition to activating specific gene expression programs that

consequently have neuroprotective effects. In fact, a number of recent studies conclude that HDAC inhibitor induced hyperacetylation alone may not be sufficient to produce beneficial effects. In a study by [175], it was reported that HDAC inhibition mediated enhancement of synaptic plasticity and hippocampus dependent memory formation requires the presence of at least one wild type allele of *cbp* highlighting the requirement of HATs like CBP for site specific acetylation and the recruitment of the basal transcriptional machinery. However, increasing neuronal dosage of specific HATs to reinstate acetylation homeostasis calls for the same concern as does the utilization of HDAC inhibitors. Non-specific enhancement of HAT levels and/or activity may lead to further complications by skewing the acetylation balance in the neighboring cell population towards hyperacetylation. Therefore, in order to reap the full potential of specific HAT activators, it is also essential to quantify HAT-HDAC dose in specific cell populations that are vulnerable to different degenerative etiologies [75].

Conclusion

In summary, histone acetylation is now recognized as one of the key mechanisms that regulate gene expression programs critical for high-order brain functions like learning and memory. While dynamic yet controlled regulation of histone acetylation and deacetylation is crucial for these functions, deregulation of the system may lead to complex changes in the epigenetic landscape that impairs cognitive functions. Chronic deregulation of the acetylation machinery can ultimately lead to neuronal death and brain atrophy as manifested in neurodegenerative diseases. Clearly, more research is required to fully understand the precise mechanism(s) by which this system impacts neuronal survival and mediates memory functions. This knowledge can then be translated to novel

HAT/HDAC based therapeutic strategies for the treatment of neurodegenerative diseases. However, a major challenge with utilization of modifiers of cellular acetylation levels is the identification of *bona fide* targets of HATs and HDACs and the integration of histone and transcription factor acetylation into a broader context of neuronal, and importantly, cellular homeostasis [38]. Although still in its infancy, the neuroprotective effects displayed by HATs like CBP, p300 and Tip60 and specificity of these effects for particular neuronal processes appears more promising than currently available non-selective HDAC inhibitors. However, determining the genes or “cassettes” of genes that are regulated by such HATs and characterizing the survival or degenerative effects such genes have would subsequently facilitate the development of novel drugs and specific therapeutic strategies with lower adverse side effects than those currently available.

CHAPTER 2: TIP60 HAT ACTIVITY MEDIATES APP INDUCED LETHALITY AND APOPTOTIC CELL DEATH IN THE CNS OF A DROSOPHILA ALZHEIMER'S DISEASE MODEL

ABSTRACT

Histone acetylation of chromatin promotes dynamic transcriptional responses in neurons that influence neuroplasticity critical for cognitive ability. It has been demonstrated that Tip60 histone acetyltransferase (HAT) activity is involved in the transcriptional regulation of genes enriched for neuronal function as well as the control of synaptic plasticity. Accordingly, Tip60 has been implicated in the neurodegenerative disorder Alzheimer's disease (AD) *via* transcriptional regulatory complex formation with the AD linked amyloid precursor protein (APP) intracellular domain (AICD). As such, inappropriate complex formation may contribute to AD-linked neurodegeneration by misregulation of target genes involved in neurogenesis; however, a direct and causative epigenetic based role for Tip60 HAT activity in this process during neuronal development *in vivo* remains unclear. Here, we demonstrate that nervous system specific loss of Tip60 HAT activity enhances APP mediated lethality and neuronal apoptotic cell death in the central nervous system (CNS) of a transgenic AD fly model while remarkably, overexpression of Tip60 diminishes these defects. Notably, all of these effects are dependent upon the C-terminus of APP that is required for transcriptional regulatory complex formation with Tip60. Importantly, we show that the expression of certain AD linked Tip60 gene targets critical for regulating apoptotic pathways are modified in the presence of APP. Our results are the first to demonstrate a functional interaction between Tip60 and APP in mediating nervous system development

and apoptotic neuronal cell death in the CNS of an AD fly model *in vivo*, and support a novel neuroprotective role for Tip60 HAT activity in AD neurodegenerative pathology.

INTRODUCTION

Epigenetic regulation of chromatin structure *via* histone acetylation promotes coordinated and dynamic transcriptional responses in neurons that influence the neuroplasticity critical for cognitive ability [73]. Tip60 is a cellular acetyltransferase protein that was originally identified by its interaction with the HIV-1 transactivator protein Tat [176]. As such, a role for Tip60 in transcription regulation has been investigated intensively with accumulating data linking Tip60 to diverse processes including cell signaling, DNA damage repair, cell cycle and checkpoint control and apoptosis [177]. Recent work from our laboratory demonstrates that the HAT activity of Tip60 is required for the transcriptional regulation of genes enriched for neuronal function [69] as well as the regulation of synaptic plasticity [173]. Consistent with these findings, Tip60 has been implicated in the neurodegenerative disorder Alzheimer's disease (AD) *via* its formation of a transcriptional regulatory complex with the AD linked amyloid precursor protein (APP) intracellular domain (AICD). It has been demonstrated that this complex is recruited to the promoters of certain target genes where it acts to acetylate select histone proteins to epigenetically regulate gene transcription [144-146,149]. Importantly, aberrant expression of some of these genes has been linked to AD pathophysiology [143,147,148]. Based on these findings, it has been proposed that inappropriate complex formation and/or recruitment may contribute or lead to AD

pathology *via* misregulation of target genes required for neurogenesis. Growing evidence suggests that the cognitive impairment in AD as well as signaling between neurons is interrupted at early stages of the disease [133]. It has also been hypothesized that dysregulation of epigenetic control mechanisms and the resultant aberrant epigenetic marks may contribute to such cognitive dysfunction [61]. However, a direct and causative epigenetic based role for Tip60 HAT activity misregulation in disrupting APP mediated neuronal processes linked to AD during nervous system development *in vivo* remains to be tested.

Apoptosis or programmed cell death is crucial in guiding the physiological development of individual cells and organs and is particularly important for CNS development [77]. Misregulation of this process leads to inappropriate induction of neuronal specific apoptotic cell death that has been shown to be a hallmark of certain progressive neurodegenerative diseases, one of which is AD. Importantly, Tip60 and AICD have each been shown to play separate and critical roles in the induction of apoptosis. For example, Tip60 plays a central role as a primary cell cycle mediator by modulating the direction of p53-dependent cell fate towards either cell cycle arrest or apoptotic induction. Tip60 carries out this role by first sensing the level of irrepairable DNA damage, and then inducing the appropriate p53-dependant response pathway via its HAT activity [178]. Interestingly, the Tip60 interacting γ-secretase derived APP intracellular C-terminal domain (AICD) fragment has also been shown to trigger p53-dependent cell death by increasing p53 expression and activity in human brain and neuronal cell models [179]. Additionally, ectopic expression of AICD in H4 neuroglioma cells leads to dramatic nuclear localization and apoptosis [180]. Moreover,

mutations in the presenilin proteins of the AICD generating γ-secretase complex are also linked to neurodegeneration and AD progression [181-184]. However, despite the convincing evidence that Tip60 and APP are each separately involved in promoting neuronal apoptotic induction, a functional interaction between Tip60 and APP in the control of this process remains to be explored, and an *in vivo* model to test this hypothesis has yet to be generated.

In this report, we test the hypothesis that Tip60 HAT activity mediates APP induced lethality and apoptotic neuronal cell death in the central nervous system (CNS) using a transgenic AD fly model that we uniquely adapted to express varying levels of Tip60 HAT activity. We demonstrate that nervous system specific loss of Tip60 HAT activity enhances APP mediated lethality and neuronal apoptotic cell death in the developing central nervous system (CNS) of these transgenic flies while remarkably, overexpression of Tip60 counteracts these defects. Notably, all of these effects are dependent upon the APP C-terminal domain that is required for transcriptional regulatory complex formation with Tip60. Importantly, we show that the expression of certain AD linked Tip60 gene targets critical for regulating apoptotic pathways are modified in the presence of APP. Our findings are the first to show a functional interaction between Tip60 HAT activity and APP in mediating both nervous system development and apoptosis linked neuronal cell death in the CNS of an AD fly model *in vivo*, and point to a novel neuroprotective role for Tip60 HAT activity in AD neurodegenerative pathology.

MATERIALS AND METHODS

Drosophila Genetics

Drosophila stocks were maintained at 25°C on standard cornmeal/agar/molasses medium supplemented with yeast. The w^{1118} line served as the genetic background control. The generation and characterization of the dominant negative HAT mutant dTIP60^{E431Q} lines A and B is described in [69]. Transgenic UAS lines carrying human APP 695 isoform (UAS-APP) and APP lacking the C-terminus (UAS-APP dCT) were obtained from *Drosophila* Stock Center (Bloomington, IN, USA). Stocks carrying dTIP60^{E431Q} lines A or B were introduced into UAS-APP and UAS-APP dCT backgrounds using standard genetic techniques. As previously described [69], transgenic UAS fly lines that would allow for expression of varying levels of wild type *Drosophila* Tip60 (dTip60^{WT}) were generated and crossed into both UAS-APP and UAS-APP dCT backgrounds using standard genetic techniques. The ubiquitously expressed 337-Gal4 driver and the nervous system specific 179y-Gal4 driver were obtained from *Drosophila* Stock Center (Bloomington, IN, USA). Viability analysis was performed using newly eclosed age matched virgin females. For ubiquitous expression of the different transgenic lines, ten virgin females from each of the lines were crossed to seven 337-Gal4 males. The crosses were maintained at 25°C and transferred to fresh food every 24 hrs for 3 days. Each transfer was counted as day 1. The crosses were monitored daily and the developmental stage at which lethality (if any) occurred was recorded. The number of flies that eclosed were counted daily starting on day 10 for a period of ten days at which point all the F1 progeny had either eclosed or died as pupae. The average number of flies

for the three days was calculated. For each transgenic line, three replicate crosses were done as described above and the developmental stage at which lethality occurs as well as average number of eclosed flies were reported. The same was repeated for nervous system specific expression of the different transgenes using ten newly eclosed age matched 179y-Gal4 females and seven males from each of the transgenic lines.

Quantitative Real Time RT-PCR

Quantification of RNA transcript levels of dTip60^{E431Q} or dTip60^{WT} in the different double transgenic lines was done by crossing the respective fly lines to 337-Gal4 driver at 25°C as described earlier. As a control, W^{1118} flies were crossed to 337-Gal4 flies. Staged F1 second instar larvae that resulted from the cross were used for RNA extraction. Total RNA was isolated using Trizol (Invitrogen Corporation, Carlsbad, CA, USA) and treated twice with DNase II (Ambion, Austin, TX) to remove DNA. Complementary DNA (cDNA) was synthesized from 1ug total RNA and oligo-dT primers using Superscript II Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA,USA). Real-time quantitative PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Poster City, CA, USA) using the Power SYBR Green PCR master mix (Applied Bioystems, Poster City, CA, USA). Real time RT-PCR reactions were carried out in triplicate in 20ul reaction volumes containing 1ng cDNA template and 1.5uM each of forward and reverse primer. Transgene induced expression of exogenous dTip60^{E431Q} or dTip60^{WT} for each line was determined as described in Lorbeck *et al* (2010) by amplifying total dTip60 mRNA using primers designed to amplify a non-conserved region within both the endogenous dTip60 and exogenous transgene induced dTip60, and comparing the relative fold change in mRNA expression levels

to just the endogenous dTip60 mRNA level that was determined using primers that amplify the endogenous 5'UTR dTip60 region that is lacking in the exogenously expressed dTip60. Forward and reverse primer sets designed to amplify a 97 bp nonconserved region of dTIP60 were 5'GACGGCTCACAAACAGGC 3'and 5'GGTGTTGCGGTGATGTAGG 3', respectively. Forward and reverse primers designed to amplify a 105 bp region within the 5'UTR region of endogenous dTIP60 were 5'CAGTTGTGGTT CACAATTACCC 3' and 5'GTGCGCAGAAAGTTATACAGC 3', respectively. PCR was carried out by 40 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min with plate readings recorded after each cycle. Threshold cycle (Ct) values were obtained, and the ΔΔCT method [185] was used to calculate the fold change in transcript level of the sample relative to the control. RP49 which encodes the *Drosophila* ribosomal protein L32 was used as an internal standard and reference gene using forward and reverse primer pairs 5'CTGCTCATGCAGAACCGCGT 3'and 5'GGACCGACAGCTGCTTGGCG 3', respectively.

Semi-quantitative RT-PCR analysis

The presence of UAS-APP or UAS-APP dCT constructs in the double transgenic lines was verified by semi-quantitative RT-PCR. Total RNA and cDNA preparation from staged second instar larvae was done as before. PCR amplification was done in 20ul reactions using forward and reverse primer pairs 5'-GCCGTGGCATTCTTTTGGGGC-3' and 5'-GTGGTCAGTCCTCGGTCGGC-3', respectively that amplify a 100 bp region in the APP N-terminus region. The PCR reaction mixture contained reaction buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 3mM MgCl2 and 0.01% Triton X-100), 200 uM dNTPs, 1.5uM of each primer, 1.25U DNA polymerase (Qiagen, Hilden), and cDNA template. Thermal cycling

conditions consisted of an initial melting step at 95°C for 1 min, followed by 39 cycles of melting at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 60 s. PCR products were visualized by agarose gel (2%) electrophoresis containing ethidium bromide.

TUNEL Staining for Apoptosis

Third instar larval brains were carefully dissected and fixed in 4% Paraformaldehyde. Brains were washed 3 times in 1X PBST (0.1% Triton X) for 15 minutes and incubated for 15 minutes in block solution (5% normal goat serum, 0.1% Triton X). Detection of apoptotic neuronal cells was performed using the Fluorescein Cell Death Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The reaction mixture was made using enzyme solution and label solution (1:9) and brains were incubated for 90 minutes at 37°C. Samples were then washed three times in 1X PBST and mounted in Vectashield anti-fade mounting medium. Confocal microscopy was performed using Olympus Microscope with fluoview software. For each genotype including the wild type control, the replicate samples were dissected, fixed and stained on the same day using aliquots of enzyme reaction mixtures prepared from the same buffer/enzyme stock. The samples were protected from light and were also imaged within 24 hrs of preparing the slides to avoid loss of signal. Confocal imaging of whole-CNS was done by maintaining PMT voltage, offset, and laser power settings the same for the replicate samples in each case. Larval brain images were displayed as projections of 1uM serial Z sections and represent whole compressed Z-stacks of the larval central nervous system.

Microarray experiment

The experimental condition that was compared in the microarray experiment was wild type (WT) versus dTip60 ^{E431Q} B. As described previously (Lorbeck et al., 2011), respective flies were crossed to 337-Gal4 driver to allow for ubiquitous expression of the transgene. In each case, two samples of thirty-five staged three day old whole larvae progeny were used for RNA extraction and probing two separate microarray chips on the GeneChip Drosophila 2.0 Array (Affymetrix, Santa Clara, CA) following a standard Affymetrix protocol.

Microarray data analysis

GeneChip CEL files were generated using the Affymetrix GeneChip operating system (GCOS). The CEL files are available at NCBI GEO (GEO Acc num. GSE25635). The open source packages in R and bioconductor were used for data analysis. The data were imported into R and after a series of pre-processing analysis (background correction and mean scaling), the data was normalized. The RMA normalization [186] which has been shown to have high efficiency for Affymetrix data normalization was chosen to minimize the systematic variation in the experiment. Limma (Linear Models for Microarray Data) package was used for detection of differentially expressed genes by fitting a linear model to the expression data for each gene. This package fully models the systematic part of the data and creates a design matrix. Each row of the design matrix corresponds to an array in the experiment and each column corresponds to a coefficient. In Affymetrix analysis, the linear modeling implemented by Limma is much

the same as ordinary ANOVA or multiple regression except that a model is fitted for every gene. A list of the top genes which show evidence of differential expression between the dTip60 ^{E431Q} B and WT was then generated by estimating the fold change of dTip60 ^{E431Q} B over WT. The results of the linear model were then summarized, and the p-values for multiple testing adjusted using a FDR (Benjamini and Hochberg's method) threshold of 0.05. The genes whose *P*-value of the log ratio are over 95% were categorized as 'no-change' in gene expression and the genes with expression levels that have a significant difference between the dTip60 ^{E431Q} B and WT (P<0.05) are either 'up or down-regulated'. Thus genes which have positive log ratios of dTip60 ^{E431Q} B/WT are up-regulated in dTip60 ^{E431Q} B while genes with negative log ratios are down-regulated in dTip60 ^{E431Q} B. The misregulated genes were analyzed using Gene Ontology (www.geneontology.com) and the panther protein classification system (www.pantherdb.org) to identify apoptosis related genes that were significantly enriched in the microarray dataset.

Quantitative RT-PCR analysis of microarray targets

Apoptosis related genes that were found to be significantly misregulated in response to loss of Tip60 HAT activity in the microarray analysis were further validated by quantitative RT-PCR in the following transgenic fly lines: dTip60^{E431Q}, dTip60^{WT}, APP; dTip60^{E431Q}, APP; dTip60^{WT}. In each case, F1 second instar larvae resulting from a cross between each of these transgenic fly line and 337-Gal4 driver were used for cDNA preparation. Wild type w^{1118} flies crossed to 337-Gal4 driver were used as control. Primer sets were designed using NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-

blast/). Primer sequences are available upon request. Fold change of the respective transcript level in the sample was calculated relative to the control by the ΔΔCT method using RP49 as internal control.

RESULTS

Tip60 and APP functionally interact to mediate both general and nervous system specific development.

To create an *in vivo* multicellular model system suitable for investigating a functional link between Tip60 HAT activity and APP in neuronal function *in vivo,* we generated transgenic flies expressing either our previously characterized HAT-defective dominant negative Tip60 transgene (dTIP60^{E431Q}) or additional copies of wild-type Tip60 transgene (dTip60^{WT}) in a well characterized AD fly model [187,188] that overexpresses either full-length human APP (APP) or human APP lacking the Tip60-interacting C-terminal domain (APP dCT) under the control of the UAS promoter. Double transgenic lines were generated for two independent dTip60^{E431Q} lines expressing low and high levels of the HAT activity defective mutant dTip60 (dTip60^{E431Q} A and dTip60^{E431Q} B, respectively) (Table 1). Similarly, double transgenic lines for three independent dTip60^{WT} lines expressing varying levels of wild type dTip60 (dTip60^{WT} A, dTip60^{WT} B, and dTip60^{WT} C, respectively) were generated (Table 1). Expression levels for the exogenously expressed dTip60^{E431Q} or dTip60^{WT} from each of these transgenic lines were quantitatively assessed using quantitative RT-PCR to allow for selection of lines that had comparable levels of exogenous Tip60^{E431Q} and Tip60^{WT} expression for

further analysis (Figure 1A and Figure 2A). Comparable levels of APP and APP dCT transgene expression were previously characterized [188] and presence of each of these transgenes in the APP; dTip60 fly lines was confirmed using semi-quantitative PCR (Figure 1B and Figure 2B).

To determine whether Tip60 and APP functionally interact during general *Drosophila* development, we first expressed each of the transgenes ($dTip60^{E431Q}$, $dTip60^{WT}$, APP, APP-dCT) separately at the normal physiological temperature of 25°C using GAL4 driver line 337, that induces robust and ubiquitous GAL4 expression beginning during late embryogenesis and continuing into adulthood. The crosses were monitored daily to examine if the expression of the different transgenes affects development. In cases where the transgene expression induced lethality, the developmental stage at which lethality occurred was recorded (Table 2). In cases where the F1 progeny progressed through normal development and eclosed, the number of flies that eclosed over a ten day period were counted (Figure 3). The w^{1118} fly line crossed to 337-GAL4 served as a control. As we previously reported, induction of $Tip60^{E431Q}$ for both independent lines A and B reduced fly viability to 0%, with the majority of lethality occurring during the late third instar larval stage. Moreover, ubiquitous induction of APP resulted in 60% lethality that occurred in the pupal stage, with the remaining 40% of progeny surviving only 2-5 days after eclosion (Table 2, Figure 3). Co-expression of $dTip60^{E431Q}$ and APP using both APP; $dTip60^{E431Q}$ line A and APP; $dTip60^{E431Q}$ line B resulted in 0% viability, with lethality occurring during the early second instar larval stage (Table 2). Additionally, hatching of 100% of these larvae was delayed by 24-48 hours. Thus, co-expression of both APP and $Tip60^{E431Q}$ resulted in a much more severe

developmental phenotype as it induced lethality approximately 3 days earlier in development than when compared to either APP or dTip60^{E431Q} expressed alone. The genetic enhancement of lethal effects observed in the double mutants compared to when either APP or dTip60^{E431Q} is expressed alone is indicative of a synergistic interaction between Tip60 and APP.

To determine whether the genetic enhancement we observed between APP and dTip60 was dependent upon the C-terminal domain of APP that is required for interaction with dTip60, we co-expressed the dTip60^{E431Q} transgene with APP dCT, a version of APP lacking the C-terminal domain. Ubiquitous expression of APP dCT alone with the 337-GAL4 driver at 25°C did not cause any observable developmental phenotype although there was a non-significant decrease in the number of F1 progeny that eclosed (Table 2, Figure 3). However, unlike the APP eclosed flies that survived only 2-5 days (Table 2), the eclosed APP dCT adult progeny in this case did not exhibit any early lethality. This finding indicates that the decrease in viability in response to APP overexpression is dependent upon the C-terminus domain of APP. Moreover, co-expression of dTip60^{E431Q} with APP dCT using both APP dCT; dTip60^{E431Q} line A and APP dCT; dTip60^{E431Q} line B resulted in a phenotype identical to that of dTip60^{E431Q} alone (Table 2). These results indicate that the synergistic interaction between dTip60^{E431Q} and APP is dependent upon the Tip60 interacting C-terminal domain of APP.

We also examined the effect of overexpressing varying levels of wild type dTip60 using dTip60^{WT} lines A, B and C using the ubiquitous 337-Gal4 driver. As shown in Table 2, ubiquitous expression of each of these transgenes did not affect development *per*

se but the number of F1 progeny that eclosed in each case was significantly less than the wild type control (Figure 3). Although these dTip60 ^{WT} lines express varying levels of the wild type dTip60, there was no significant difference in the number of surviving F1 progeny between dTip60 ^{WT} lines A, B and C indicating that the observed effect is not dose dependent. In contrast, co-expression of dTip60^{WT} with APP using lines APP; dTip60^{WT} A, B and C rescued the APP induced loss of viability in a dose dependent fashion, as indicated by the increase in the number of surviving F1 progeny in the double mutants compared to flies expressing APP alone (Figure 3). However, the number of F1 progeny was still less than the wild type control in all three cases indicating only a partial rescue of the APP induced lethality. Notably, with APP; dTip60^{WT} line C that co-expresses APP with the highest level of wild type Tip60, the number of F1 progeny that eclosed was significantly more than that observed in the respective single mutant dTip60^{WT} lines (Figure 3). Thus, co-expression of APP with additional levels of Tip60 not only counteracts the lethal effects induced by APP but also alleviates the effect that overexpression of Tip60 has on viability. Lack of similar effects in the APP dCT; dTip60^{WT} C flies (Figure 3) suggest that the observed rescue phenotype was mediated through interaction of Tip60 with the APP C-terminal domain. Together, our findings indicate that while loss of Tip60 HAT activity enhances the APP induced lethal effects, additional levels of Tip60 suppress such lethal effects, further supporting a synergistic interaction between Tip60 and APP.

APP and Tip60 are each neuronally expressed and are both required for nervous system function [4, 27]. Thus, the phenotypic enhancement we observed between APP and Tip60 during general development prompted us to ask whether this interaction was

also specific for nervous system development and function. To investigate whether Tip60 and APP genetically interact in the nervous system, we carried out the same crosses as above, this time using the pan-neuronal 179y- GAL4 driver line which induces robust pan- neuronal GAL4 expression at 25°C (Table 2). Again, we observed the same pattern of lethality as for general development for the stronger fly line APP; Tip60^{E431Q} B in that lethality caused by APP overexpression was enhanced by reduction of Tip60 HAT activity, supporting the specificity of the Tip60 and APP genetic interaction (Table 2) in nervous system development. As before, this nervous system specific interaction was dependent upon the Tip60 interacting C-terminal domain of APP (Table 3). In contrast, when Tip60^{E431Q} A was expressed in the nervous system in combination with APP or APP dCT, it resulted in partial lethality wherein only a fraction of the F1 progeny in each of these cases died as second and third instars, respectively similar to that seen in APP; Tip60^{E431Q} B and APP dCT; Tip60^{E431Q} B flies. However, the majority of F1 progeny did not have any lethal developmental effect (Table 2). This milder effect observed with Tip60^{E431Q} A expressing flies is likely due to the low level of dTip60 HAT mutant that is expressed in these flies. Similar to the effects we observed with ubiquitous expression, pan neuronal expression of dTip60^{WT} with APP suppressed the APP induced lethality in a dose dependent fashion (Figure 3). Furthermore, with APP; dTip60^{WT} line C, the number of F1 progeny that eclosed were significantly more than that observed in the respective single mutant dTip60^{WT} lines (Figure 3). Taken together, our results demonstrate that Tip60 and APP functionally interact to mediate both general and nervous system specific development and that this interaction is dependent upon the

Tip60 interacting C-terminal domain of APP. These data further support an epigenetic based role for Tip60 HAT activity in mediating APP induced developmental effects.

Tip60 HAT activity is required for the transcriptional regulation of genes linked to a variety of distinct apoptotic pathways.

The above findings indicating a functional interaction between APP and Tip60 in mediating general and nervous system specific lethality prompted us to ask whether a potential mechanistic basis for this lethal phenotype was *via* induction of an apoptotic response in these flies. We recently reported a microarray analysis comparing global changes in gene expression in response to ubiquitous induction of Tip60^{E431Q} in the fly [69]. While this study reported misregulation of genes linked to diverse neuronal functions, the identity of genes that function in specific neuronal processes was not explored. To address this and to examine the causative mechanism that mediates the Tip60/APP induced lethal phenotype, we wanted to further analyze our previously published microarray gene expression data with specific focus on genes that are known to function in apoptosis related pathways. Towards this end, we performed pathway analysis by first identifying canonical apoptotic pathways and their respective genes from online databases like Gene Ontology and the PANTHER classification system. The dTip60^{E431Q} microarray data set was then examined to see if genes linked to such apoptotic pathways were misregulated in response to loss of Tip60's HAT activity. Our analysis identified 53 such unique genes that are involved in 17 different apoptotic pathways to be misregulated in the dTip60^{E431Q} data set (Table 3). Intriguingly, the identified pathways included those that are associated with Alzheimer's, Parkinson's and

Huntington's diseases, all neurodegenerative disorders in which massive neuronal death due to apoptosis is a common characteristic. Importantly, the p53 mediated pathway and Wnt signaling pathway were among the most highly represented pathways, consistent with previous reports implicating Tip60 in a p53 mediated apoptotic response. To validate our microarray results, we carried out quantitative RT-PCR analysis of nine genes that encoded protein products with known functions involved in inducing an apoptotic response (Figure 4A and 4B) and were representative of a particular pathway (Table 3). Of the genes that were upregulated in response to Tip60 HAT loss was Calpain, a calcium dependent enzyme that mediates proteolytic cleavage of proteins like APP and tau. Abnormal activation of Calpain has also been reported to initiate degradation of proteins essential for neuronal survival [133]. Among the other confirmed targets that were upregulated were genes with established roles in the induction of the p53 mediated apoptotic pathway such as TRAF4 and CG9418 (High mobility group protein 1/2). The wingless protein (wg) and Frizzled (Fz), a transmembrane protein that functions as Wg receptor were two confirmed upregulated targets critical in the Wnt signaling pathway involved in regulating apoptosis. Also upregulated in the microarray data was ALiX (apoptosis linked gene 2 interacting X), a calcium dependent ubiquitously expressed protein involved in neuronal cell death. Consistent with this finding, upregulation of endogenous ALiX has also been reported to correlate with cell death *in vivo* [189,190]. Myc proteins are essential regulators of cellular growth and proliferation during normal development. Recently, the ability of overexpressed Myc to induce cell-autonomous apoptosis has been shown to be evolutionarily conserved in *Drosophila* Myc [191]. Interestingly, we too found Myc to be upregulated in response to loss of Tip60

HAT activity. Our identification of these target genes that are affected by loss of Tip60 HAT activity further support an as yet unidentified putative role for Tip60 in the respective cellular pathways in which such targets function. Among the genes downregulated in response to loss of Tip60 HAT activity was the apoptosis related protein, Programmed Cell Death 5 (PDCD5) that has also been reported to interact with Tip60 to mediate DNA damage induced apoptosis [192]. In summary, our identification of misregulated apoptosis related pathways and their respective genes in response to Tip60 HAT loss further support a regulatory role for Tip60 in multiple pathways linked to apoptotic control.

In order to examine if expression of these genes that are misregulated in dTip60^{E431Q} are also altered due to overexpression of wild type dTip60, we performed qPCR analysis of the above mentioned nine genes in dTip60^{WT} second instar larvae, as this was the developmental stage used for dTip60^{E431Q} microarray analysis (Table 4). While loss of Tip60 HAT activity induced expression of genes like Frizzled, Wingless and dMyc, Tip60 overexpression had the converse effect resulting in marked downregulation of these genes. Significant differential regulation was also observed between dTip60^{E431Q} and dTip60^{WT} flies for PDCD5 expression. Similar to that observed in the Tip60 HAT mutants, expression of genes like Buffy, ALiX, CalpA, TRAF4 was also induced under Tip60 overexpressing conditions (Table 4).

Since Tip60 forms a transcriptionally active complex with the APP C-terminal domain, we also wished to examine how these gene expression changes are modified by APP in the dTip60^{E431Q} or dTip60^{WT} background. We therefore performed qPCR

analysis of these nine genes in APP; dTip60^{E431Q} and APP; dTip60^{WT} double mutant lines to identify genes that are differentially regulated between these lines and their respective single mutants (Table 4). Notably, while Tip60 HAT loss in dTip60^{E431Q} fly lines induced expression of the genes Buffy, CalpA, TRAF4, Frizzled, Wingless, dMyc, co-expression of APP with dTip60^{E431Q} had a repressive effect on each of these genes. Similar differential regulation was observed with PDCD5 wherein presence of APP with dTip60^{E431Q} relieved the repressive effect on PDCD5 that expression of dTip60^{E431Q} alone had. With respect to APP; dTip60^{WT} flies, CalpA, TRAF4 and Dmel\CG9418 each exhibited differential regulation in comparison to flies expressing dTip60^{WT} alone. While CalpA and TRAF4 were upregulated in dTip60^{WT} flies, they were downregulated in APP; dTip60^{WT} flies. Although Dmel\CG9418 was upregulated in dTip60^{WT} flies, its fold increase was much higher in APP; dTip60^{WT} flies. Finally, Buffy was significantly upregulated in the APP;dTip60^{WT} flies when compared to flies expressing dTip60^{WT} alone (Table 4). Taken together, these results indicate that Tip60 target gene expression profiles can be modified in the presence of APP.

TIP60 and APP functionally interact to mediate apoptotic cell death in the *Drosophila* CNS.

Our finding that Tip60 and APP genetically interact to specifically mediate nervous system development prompted us to ask what specific neuronal processes might be regulated by this interaction. Targeted overexpression of APP in the *Drosophila* nervous system was previously shown to induce neuronal apoptosis in the CNS at 29°C,

[187], however whether this phenotype can be induced at normal physiological temperature as well as the mechanism underlying such apoptotic induction remain to be elucidated. Moreover, and in agreement with previous reports, here we show that Tip60 HAT activity controls apoptotic pathways *via* the transcriptional regulation of apoptosis linked genes. These findings prompted us to ask whether dTip60 and APP genetically interact to mediate apoptotic neuronal cell death in the *Drosophila* CNS.

To first determine whether misregulation of dTip60 levels causes neuronal specific apoptosis, Tip60^{E431Q} and Tip60 ^{WT} fly lines were crossed to the 179y-GAL4 pan-neuronal driver flies at 25°C. The w^{1118} fly line crossed to 179y-GAL4 served as a control. Third instar larval brains were dissected from the progeny of these crosses and tested for apoptosis using dUTP nick end labeling (TUNEL) staining. As seen in Figure 5B and 5C, moderate levels of apoptotic induction were observed in larval brains of transgenic lines expressing either dTip60^{E431Q} A or dTip60^{E431Q} B while higher levels of apoptotic death were found for flies expressing comparable levels of Tip60^{WT} (Figure 5 compare B, C and D; Figure 5K). These results indicated that appropriate regulation of Tip60 levels play a critical role in controlling the balance of neuronal apoptotic cell death in the larval brain and that overexpression of Tip60 may be more detrimental than Tip60 HAT loss in this process. TUNEL staining of third instar larval brains from APP and APP dCT flies crossed to 179y-GAL4 at 25°C were also assessed to determine whether APP overexpression induces neuronal apoptosis at physiological temperature and whether APP induced cell death is dependent upon its C-terminal domain, respectively. As shown in Figure 5E, moderate levels of apoptotic death were observed for APP overexpression at 25°C while no apoptosis was detected for flies expressing equivalent

levels of APP dCT (Figure 5F). Furthermore, the extent of apoptosis induced by APP overexpression was comparable to that observed in both dTip60^{E431Q} A and dTip60^{E431Q} B flies (Figure 5K). These results indicated that APP overexpression induces neuronal apoptosis at physiological temperature, and that this phenotype is dependent upon its C-terminal domain, consistent with previous findings [187].

Given that Tip60 and APP each separately induced neuronal apoptosis in the *Drosophila* CNS, and that APP induced cell death was dependent upon its Tip60 interacting C-terminal domain, we predicted that Tip60 and APP might functionally interact to induce apoptosis mediated neurodegeneration when misregulated. To test this possibility, we first performed TUNEL assays in larval brains co-expressing either Tip60^{E431Q} and APP or Tip60^{E431Q} and APP dCT under the control of the pan-neuronal 179y-GAL4 driver. For these studies, we used our lower expressing APP; Tip60^{E431Q} line A and APP dCT; Tip60^{E431Q} line A fly lines (Figure 1A), as co-expression of higher expressing Tip60^{E431Q} line B and APP induced lethality at the second instar larvae stage which was too early to assess by TUNEL stain. Indeed, as shown in Figure 5G, co-expression of Tip60^{E431Q} and APP resulted in a marked induction of apoptosis that was more robust than either Tip60^{E431Q} or APP alone (Figure 5K), indicative of a synergistic interaction between Tip60 and APP in neuronal apoptotic induction. Importantly, and as we predicted, this interaction was dependent upon the C-terminus of APP that interacts with Tip60 (Cau and Sudhoff, 2001) as co-expression of Tip60^{E431Q} and APP dCT resulted in only a moderate level of neuronal apoptosis induction that was approximately equivalent to that observed for Tip60^{E431Q} alone (Figure 5H and 5K). To determine whether additional Tip60 levels would suppress the APP induced neuronal apoptotic

phenotype as well as to confirm the specificity of the interaction, we performed TUNEL assays in larval brains co-expressing Tip60^{WT} with APP using APP; Tip60^{WT} line C. This line was selected because line Tip60^{WT} C expressed the highest levels of wild type dTip60 for all of our dTip60^{WT} lines (Figure 2A) and also displayed the highest level of rescue for APP induced lethality (Figure 3). Remarkably, we found that additional levels of Tip60 partially rescued APP induced apoptotic cell death as evidenced by a visible reduction of the presence of TUNEL-positive cells in these brains when compared to APP alone (Figure 5I, compare 5E and 5I; Figure 5K). Co-expression of dTip60^{WT} and APP also appeared to suppress neuronal apoptosis induced by Tip60 overexpression alone, as we observed less TUNEL-positive cells in brains co-expressing dTip60^{WT} and APP when compared with brains expressing equivalent levels of Tip60^{WT} alone (Figure 5, compare D and I). Interestingly, rescue of cell death appeared more prominent in the proximal central brain of APP; dTip60^{WT} flies, as we consistently observed virtually no apoptotic cell death in this area (Figure 5I), where vital structures like the *Drosophila* learning and memory center, mushroom body are located**.** Importantly, and as we predicted, partial rescue of APP induced neuronal apoptosis by Tip60 was dependent upon the Tip60 interacting C-terminus of APP, as brains co-expressing both Tip60^{WT} and APP dCT showed no rescue as shown by the equivalent number of TUNEL positive cells in these brains compared to those expressing Tip60^{WT} alone (Figure 5J and 5K). Taken together, our results demonstrate that Tip60 and APP functionally interact to regulate neuronal apoptotic cell death in the *Drosophila* CNS and that this interaction is dependent upon the C-terminus of APP.

DISCUSSION

In this study, we have generated a unique transgenic *Drosophila* model system suitable for investigating a functional link between Tip60 HAT activity and APP in neuronal development, *in vivo*. We demonstrate that Tip60 and APP functionally interact in both general and nervous system development in *Drosophila*, *in vivo* and that this interaction specifically mediates apoptotic neuronal cell death in the CNS, a process that when misregulated is linked to AD pathology [193]. Remarkably, Tip60 appears to display a neuroprotective function in that Tip60 overexpression can rescue both loss of viability and neuronal apoptosis induction in a *Drosophila* AD model. While a number of *in vitro* studies supporting the transcription regulatory role of the Tip60/AICD complex in gene control have been reported, our work is the first to demonstrate a functional interaction between Tip60 HAT activity and APP in nervous system development *in vivo*.

Here we show that misexpression of Tip60 induces neuronal apoptotic cell death in the *Drosophila* CNS, and that this process is mediated *via* a functional interaction between Tip60 and APP C-terminal domain. Since disruption of Tip60 HAT activity induced neuronal cell death, we examined whether there was specific misregulation of apoptosis linked genes due to loss of Tip60 HAT activity. Pathway analysis of our previously reported microarray data set of genome wide changes in gene expression induced in the fly in response to Tip60 HAT loss [69] revealed genes functioning in 17 different apoptotic pathways to be enriched, many of which were associated with the p53 apoptotic pathway. Our findings are consistent with previous studies demonstrating a role for Tip60 as a p53 co-activator in p53 mediated apoptotic pathways [194]. Recent

studies have found Tip60 to be required for activation of proapoptotic genes through acetylation of p53 DNA binding domain [178,194]. TRAF4, one such p53 regulated pro-apoptotic gene [195] that responds to cellular stress was one of the genes that we found to be significantly upregulated in response to Tip60 HAT loss. The Myc family of transcription factors presents another instance of proteins involved in inducing apoptosis that are directly acetylated and stabilized by Tip60 [191] and accordingly, *Drosophila* dMyc was found to be significantly upregulated in response to Tip60 HAT loss. Thus it is possible that the pro-apoptotic genes enriched in our dataset may represent both direct targets regulated by Tip60 epigenetic function as well as indirect targets of apoptosis regulators such as p53 that are controlled *via* their acetylation by Tip60. Misregulation of these pro-apoptotic genes in response to disruption of Tip60 HAT activity is also consistent with our observation that nervous system specific expression of dTip60^{E431Q} induces apoptotic cell death in the CNS of dTi60^{E431Q} larvae. This finding is in contrast to previous studies wherein cells expressing mutated Tip60 lacking HAT activity were reported to be resistant to apoptosis. However, these studies examined a role for Tip60 in DNA damage repair following cellular stress using the H4 neuroglioma cells *in vitro*. WhileTip60 HAT activity is vital for DNA repair competency as well as for the ability to signal the presence of damaged DNA to the apoptotic machinery [196], how Tip60 HAT activity regulates differential gene expression profiles to prevent unwanted neuronal cell death during organismal development remains unclear. A number of mammalian studies have indicated that Tip60 can function not only as a coactivator, but also as a corepressor [197,198] and as such, Tip60 has been shown to repress a vast array of developmental genes during ESC differentiation to maintain ESC identity [199]. Consistent with these

findings, the majority of pro-apoptotic genes we identified that were misregulated in response to disruption of Tip60 HAT activity were upregulated, highlighting the crucial role Tip60 HAT activity plays in repression of apoptotic genes during neurogenesis that when misregulated, likely contribute to dTip60^{E431Q} induced apoptosis.

Interestingly, we find that overexpression of wild type Tip60 in the nervous system also induced apoptosis in the CNS. Furthermore, overexpressing Tip60 was found to induce expression of pro-apoptotic genes such as ALiX and CalpA while downregulating others like Wingless, Frizzled and dMyc that have multiple essential functions during *Drosophila* development. These bidirectional gene expression changes suggest that increasing Tip60 mediated acetylation can also lead to complex changes in the chromatin landscape resulting in inappropriate activation and/or repression of apoptosis competent genes as well as those crucial for development. Accumulating evidence shows that hyperacetylation can be fatal to neurons. Under normal conditions, increasing hyperacetylation by treating neurons with a general HDAC inhibitor like trichostatin A has been found to induce neuronal apoptosis [200,201]. Similarly, increasing acetylation levels by overexpressing the HAT CBP in resting neurons has been reported to enhance chromatin condensation and neuronal death [77]. In order to maintain cellular homeostasis, HAT/HDAC equilibrium and therefore histone acetylation is strictly regulated as it is essential to maintain the functional status of neurons [152]. Based on these findings, we can speculate that overexpression of Tip60 disrupts the acetylation balance, thus skewing the neuronal survival pathway towards apoptosis and ultimately cell death. In support of this concept, altered levels of global histone

acetylation have been observed in many *in vivo* models of neurodegenerative diseases [166,202].

Another striking feature of our apoptotic microarray gene enrichment search was our identification of apoptosis linked pathways associated with neurodegenerative diseases like Parkinson's, Huntington's and Alzheimer's disease. These diseases are also characterized by neuronal cell death that increases over time and underlies an array of symptoms that depend on the function of the lost neuronal population [152]. It has been proposed that in AD, in addition to the deposition of toxic β-amyloid plaques in the brain, neurodegeneration may also be caused *via* γ-secretase cleavage of APP that generates AICD carboxy terminal fragments that are toxic to neurons [180]. Accordingly, ectopic expression of AICD in rat pheocytoma cells and cortical neurons [203] and H4 neuroglioma cells [180] has been shown to induce apoptosis upon nuclear translocation. Consistent with these reports, we too observe induction of apoptosis when APP is expressed in the nervous system of *Drosophila in vivo* at physiological temperatures and that this phenotype is dependent upon the C-terminal domain of APP. Interestingly, APP C-terminal domain induced apoptosis has previously been reported to be mediated *via* Tip60 HAT activity *in vitro*, such that induction of apoptosis in neuroglioma cells transfected with APP C-terminal domain is enhanced by co-transfection of wild type Tip60 and decreased by a dominant negative version of Tip60 lacking HAT activity [180]. In contrast, here we demonstrate that nervous system specific co-expression of APP and HAT defective mutant Tip60 increases apoptosis while overexpression of wild-type Tip60 with APP counteracts this effect and that these phenotypes are dependent upon the Tip60 interacting C-terminus of APP. Such differences may be accounted for

by the fact that we are carrying out our studies in a developmental model system, *in vivo*. However, the effects we show on neuronal apoptosis are also consistent with the effects we observed in the viability assay wherein lethality caused by neuronal overexpression of APP was enhanced by reduction of Tip60 HAT activity and suppressed by additional Tip60 levels. Importantly, this finding, in conjunction with our previously published reports supporting a causative role for Tip60 in the control of synaptic plasticity [5] and the transcriptional regulation of genes enriched for neuronal function [69], support the concept that misregulation of Tip60 HAT activity can lead to aberrant gene expression within the nervous system that contributes to the AD associated neurodegenerative process.

Tip60 has been implicated in AD *via* its transcriptional complex formation with AICD [144,149]. Thus, we carried out experiments to determine whether the expression of specific genes that are misregulated by $dTip60^{E431Q}$ or $dTip60^{WT}$ are modified by the presence of APP. Intriguingly, we found a number of these genes to be differentially regulated under APP expressing conditions. Two such genes, Wingless and Frizzled, which are upregulated in $dTip60^{E431Q}$ flies and repressed in $dTip60^{WT}$ flies are particularly interesting. Wingless, the *Drosophila* segment polarity gene and its membrane receptor Frizzled are known to be required for specification and formation of various neurons in the CNS [204] and belong to the Wnt signaling pathway. In addition to Wingless and Frizzled being important for the disease process, they are also crucial for normal growth and development. Intriguingly, we find that co-expressing APP with either the Tip60 HAT mutant or in the Tip60 overexpressing background has a repressive effect on these essential genes. Recent evidence supports a neuroprotective role for the

Wnt signaling pathway [205,206] and a sustained loss of Wnt signaling function is thought to be involved in aβ induced neurodegeneration [207]. *Drosophila* Myc is a regulator of rRNA synthesis and is necessary for ribosome biogenesis during larval development [208] and is another instance of a vital gene that exhibited reduced expression under APP expressing conditions. Thus misregulation of such developmentally required genes in conjunction with the other pro-apoptotic genes in our data set likely contributed to the observed enhanced apoptotic cell death in the CNS of APP;dTip60^{E431Q} larvae. In contrast, we find the *Drosophila* homolog of Bcl-2 protein, Buffy to be repressed in the APP; dTip60^{E431Q} flies that displayed an increase in apoptosis. Consistent with our findings, recent studies have reported that Buffy has anti-apoptotic functions *in vivo* [209] and intriguingly, we find its expression to be significantly induced in the APP; dTip60^{WT} flies that also exhibited a marked reduction in apoptosis induced cell death when compared to flies expressing dTip60^{WT} alone. These findings suggest that induction of such pro-survival factors could mediate the dTip60 induced rescue of APP mediated defects that we observe in these flies. We observe differential regulation of the microarray targets between flies that express dTip60^{E431Q} alone and in conjunction with APP, in that the majority of genes we tested are repressed in the APP;dTip60^{E431Q} double mutants and activated in dTip60^{E431Q} flies. These results indicate that the presence of APP can modulate the transcriptional regulatory potential of Tip60. The APP intracellular domain was recently shown to lower the sensitivity of neuronal cells to toxic stimuli and transcriptionally activate genes involved in signaling pathways that are not active under basal conditions [150]. APP could mediate such effects either by sequestering Tip60 away from its typical target

promoters or by displacing another factor in the complex that is also required for regulating transcription. Additionally, Tip60 has been shown to function as a negative regulator of gene expression. In fact, overexpression of Tip60 but not its HAT deficient mutant has been reported to function as co-repressor for gene repression mediated by transcription factors like STAT3 and FOX3, an effect that is mediated through association with specific histone deacetylases [210,211]. This could partly account for the repressive effects that we observe due to overexpression of wild type Tip60 either alone or in conjunction with APP. Tip60 can also function as a co-activator of gene transcription *via* displacement of co-repressors on the promoters of specific genes. For instance, in a study by Baek *et al* [147], it was reported that following IL-1 stimulation, recruitment of a wild type Tip60 containing co-activator complex leads to activation of p50 target genes like KAI1/CD82 through displacement of a specific NCoR co-repressor complex. Intriguingly, the Tip60-FE65-AICD containing complex was shown to similarly displace the NCoR complex and derepress such targets, suggesting a potential transcription activation strategy that underlies the gene expression changes we observe under APP overexpressing conditions. Since loss of Tip60 HAT activity enhances APP induced lethal effects in the nervous system and overexpression of wild type Tip60 diminishes these defects, we hypothesize that the Tip60-AICD containing complex may mediate these rescue effects either *via* regulation of a subset of gene targets different from those targeted by either APP or Tip60 alone or by differentially regulating the same gene pool such as that seen in the case of the anti-apoptotic gene Buffy. Thus, although the repertoire of genes that we tested include both mediators as well as inhibitors of apoptosis, taken together our data support a model by which Tip60 HAT activity plays a

neuroprotective role in disease progression by complexing with the AICD region of APP to epigenetically regulate transcription of genes essential for tipping the cell fate control balance from apoptotic cell death towards cell survival under neurodegenerative conditions such as excess APP. We therefore propose a neuroprotective role for Tip60 in AD linked induction of apoptotic cell death. Future investigation into the mechanism by which Tip60 regulates these processes may provide insight into the utility of specific HAT activators as therapeutic strategies for neurodegenerative disorders.

CHAPTER 3: EPIGENETIC REGULATION OF AXONAL GROWTH OF DROSOPHILA PACEMAKER CELLS BY HISTONE ACETYLTRANSFERASE TIP60 CONTROLS SLEEP

ABSTRACT

Tip60 is a histone acetyltransferase (HAT) enzyme that epigenetically regulates genes enriched for neuronal functions through interaction with the amyloid precursor protein (APP) intracellular domain. However, whether Tip60 mediated epigenetic dysregulation affects specific neuronal processes *in vivo* and contributes to neurodegeneration remains unclear. Here, we show that Tip60 HAT activity mediates axonal growth of the *Drosophila* pacemaker cells, termed small ventrolateral neurons (sLNvs), and their production of the neuropeptide pigment dispersing factor (PDF) that functions to stabilize *Drosophila* sleep-wake cycles. Using genetic approaches, we show that loss of Tip60 HAT activity in the presence of the Alzheimer's disease (AD) associated amyloid precursor protein (APP) affects PDF expression and causes retraction of the sLNv synaptic arbor required for presynaptic release of PDF. Functional consequence of these effects is evidenced by disruption of sleep-wake cycle in these flies. Notably, overexpression of Tip60 in conjunction with APP rescues these sleep-wake disturbances by inducing overelaboration of the sLNv synaptic terminals and increasing PDF levels, supporting a neuroprotective role for dTip60 on sLNv growth and function under APP induced neurodegenerative conditions. Our findings reveal a novel mechanism for Tip60 mediated sleep-wake regulation *via* control of axonal growth and PDF levels within the sLNv encompassing neural network and provide insight into

epigenetic based regulation of sleep disturbances observed in neurodegenerative diseases like Alzheimer's disease.

INTRODUCTION

Chromatin remodeling through histone-tail acetylation is critical for epigenetic regulation of transcription and has been recently identified as an essential mechanism for normal cognitive function [142]. Altered levels of global histone acetylation have been observed in several *in vivo* models of neurodegenerative diseases and are thought to be involved in the pathogenesis of various memory related disorders [103] . Chromatin acetylation status can become impaired during the lifetime of neurons through loss of function of specific histone acetyltransferases (HATs) with negative consequences on neuronal function [152]. In this regard, the HAT Tip60 is a multifunctional enzyme involved in a variety of chromatin-mediated processes that include transcriptional regulation, apoptosis and cell-cycle control, with recently reported roles in nervous system function [177,212]. Work from our laboratory demonstrated that Tip60 HAT activity is required for nervous system development *via* the transcriptional control of genes enriched for neuronal function [69]. We have also shown that Tip60 HAT activity controls synaptic plasticity and growth [173] as well as apoptosis in the developing *Drosophila* central nervous system (CNS) [78]. Consistent with our findings, studies have implicated Tip60 in pathogenesis associated with different neurodegenerative diseases. The interaction of Tip60 with ataxin 1 protein has been reported to contribute to cerebellar degeneration associated with Spinocerebellar ataxia (SCA1), a neurodegenerative disease caused by polyglutamine tract expansion [70]. Tip60 is also

implicated in Alzheimer's disease (AD) *via* its formation of a transcriptionally active complex with the AD associated amyloid precursor protein (APP) intracellular domain (AICD) [144,149]. This complex increases histone acetylation [213] and co-activates gene promoters linked to apoptosis and neurotoxicity associated with AD [180]. Additionally, misregulation of certain putative target genes of the Tip60/AICD complex has been linked to AD related pathology [147,214]. These findings support the concept that inappropriate Tip60/AICD complex formation and/or recruitment early in development may contribute or lead to AD pathology *via* epigenetic misregulation of target genes that have critical neuronal functions. In support of this concept, we recently reported that Tip60 HAT activity exhibits neuroprotective functions in a *Drosophila* model for AD by repressing AD linked pro-apoptotic genes while loss of Tip60 HAT activity exacerbates AD linked neurodegeneration [78]. However, whether misregulation of Tip60 HAT activity directly disrupts selective neuronal processes that are also affected by APP *in vivo* and the nature of such processes remains to be elucidated.

In *Drosophila*, the small and large ventrolateral neurons (henceforth referred to as sLNv and lLNv, respectively) are part of the well characterized fly circadian circuitry [215]. Recent studied have implicated the l-and s-LNvs as part of the "core" sleep circuitry in the fly, an effect that is predominantly coordinated *via* the neuropeptide pigment dispersing factor (PDF) [216,217] that serves as the clock output, mediating coordination of downstream neurons [218,219]. PDF is thought to be the fly equivalent of the mammalian neurotransmitter orexin/hypocretin because of its role in promoting wakefulness and thus stabilizing sleep-wake cycles in the fly [220]. Within this circuit,

the sLNvs are a key subset of clock neurons that exhibit a simple and stereotypical axonal pattern that allows high resolution studies of axonal phenotypes using specific expression of an axonally transported reporter gene controlled by the Pdf-Gal4 driver or by immunostaining for the Pdf neuropeptide that is distributed throughout the sLNv axons [221]. These features make the sLNvs an excellent and highly characterized model neural circuit to study as they are amenable to cell type specific manipulation of gene activity to gain molecular insight into factors and mechanisms involved in CNS axonal regeneration as well as those that mediate behavioral outputs like sleep-wake cycle. Importantly, the *Drosophila* ventrolateral neurons (LNvs) have been previously used as a well characterized axonal growth model system to demonstrate that the AD linked amyloid precursor protein (APP) functions in mediating the axonal arborization outgrowth pattern of the sLNv [221]. Based on these results, and our previous studies reporting that Tip60 HAT activity itself is required for neural function [69,173] and mediates APP induced lethality and CNS neurodegeneration in an AD fly model [78], we hypothesized that APP and Tip60 are both required to mediate selective neuronal processes such as sLNv morphology and function that when misregulated, are linked to AD pathology.

In the present study, we test this hypothesis by utilizing the sLNvs as a model system to examine whether Tip60 mediated epigenetic dysregulation under neurodegenerative conditions such as that induced by APP overexpression leads to axonal outgrowth defects and if there is a corresponding effect on sLNv function in sleep regulation, a process that is also affected in neurodegenerative diseases like AD.

In this report, we show that Tip60 is endogenously expressed in both the sLNv and lLNvs. Specific loss of Tip60 or its HAT activity causes reduction of PDF expression selectively in the sLNvs and not the lLNv and shortening of the sLNv distal synaptic arbors which are essential for the pre-synaptic release of PDF from these cells. The functional consequence of these effects is evidenced by the disruption of the normal sleep-wake cycle in these flies, possibly through disruption of PDF mediated signaling to downstream neurons. By using transgenic fly lines that co-express full length APP or APP lacking the Tip60 interacting C-terminus with a dominant negative HAT defective version of Tip60, we demonstrate that the APP C-terminus enhances the susceptibility of the sLNvs and exacerbates the deleterious effects that the loss of Tip60 HAT activity has on axon outgrowth and PDF expression. Importantly, our studies identify the neuropeptide PDF as a novel target of Tip60 and APP, that when misregulated results in sleep disturbances reminiscent to those observed in AD. Remarkably, overexpression of wild type Tip60 with APP rescues these sleep defects by increasing PDF expression and inducing overelaboration of the sLNv synaptic arbor area. Taken together, our findings support a neuroprotective role for Tip60 on sLNv growth and function under APP induced neurodegenerative conditions. Our data also reveal a novel mechanism for PDF control *via* Tip60 and APP that provide insight into understanding aspects of sleep dependent mechanisms that contribute to early pathophysiology of AD.

MATERIALS AND METHODS

Drosophila Stocks

The generation and characterization of fly lines carrying the GAL4 responsive dTip60^{RNAi} or the dTip60$^{RNAi\ Control}$ construct is described in [222]. Fly lines carrying the dominant negative HAT mutant dTip60^{E431Q} (UAS- dTip60^{E431Q}, line B) or wild type dTip60^{WT} (UAS- dTip60^{WT}, line C) or dTip60^{Rescue} (UAS-dTip60^{Rescue}, line B) construct are described in [69]. Fly lines expressing dTip60^{E431Q} or dTip60^{WT} with UAS-APP (UAS-APP; dTip60^{E431Q}, UAS-APP; dTip60^{WT} , line C in both cases) or UAS-APP dCT (UAS-APP dCT; dTip60^{E431Q} , UAS-APP dCT; dTip60^{WT}, line C in both cases) are described in [78]. Transgenic UAS lines carrying human APP 695 isoform (UAS-APP) and APP 695 lacking the C-terminus (UAS-APP dCT) were obtained from *Drosophila* Stock Center (Bloomington, IN, USA). Stocks carrying both Pdf-Gal4 and UAS-mCD8-GFP were obtained from B Hassan (University of Leuven, Belgium). R6-Gal4, Mai 179-Gal4 and UAS-Pdfrnai lines were obtained from O Shafer (University of Michigan, US). The w^{1118} line served as the genetic background control. Experimental crosses were carried out at the normal physiological temperature of 25°C as higher temperature changes have been reported to induce non-specific physiological and developmental alterations [223].

Immunohistochemistry

Third instar larvae or adult brains were dissected in PBS, fixed in 4% paraformaldehyde in PBS, washed thrice in PBS containing 0.1% Triton X-100, blocked

for 1 hr at RT in PBT containing 5% normal goat serum, and incubated with primary anti-GFP (Millipore, CA), anti-Tip60 (Open Biosystems, Rockford, IL) and anti-PDF (Developmental studies hybridoma bank, University of Iowa, IA) antibodies in blocking solution overnight at 4 C. Samples were washed thrice in PBT at RT, and secondary antibodies (Jackson Immunoresearch, PA) were applied in blocking solution for 2 hr at RT. After washing thrice in PBS, samples were mounted in Vectashield (Vector Laboratories, CA).

Imaging and quantifications

Larval and adult brain preparations were imaged using GFP, Tip60 or PDF antibodies. Anti-GFP immunostaining was visualized using Alexa-Fluor 488. Alexa-Fluor 568 and Alexa-Fluor 647 was used for anti-PDF and anti-Tip60, respectively. Imaging experiments were performed at Drexel University's Cell Imaging Center. Confocal microscopy was performed using Olympus Microscope with fluoview acquisition software (Olympus, Center Valley, PA). Images were displayed as projections of 1uM serial Z- sections. Quantitative analysis of sLNv axon length was performed using NIH ImageJ software by measuring axon length from the base of the cell body to the distal tip of the axon in the different genotypes. Quantification of the two dimensional area of the sLNv terminal axonal arbor was done as described in [221] using NIH ImageJ software. Briefly, the sLNv axon stem on either half of the brain hemisphere was marked by a straight vertical line followed by a horizontal line between the points that mark the sLNv axon. The outline of the axonal processes dorsal to the horizontal line was traced, and the area inside was measured. The distance between the vertical lines was used as a

measure for brain size (Figure S2). Area measurements were normalized for brain size by scaling the distance between the dorsal projections to the median of the distance as a correction factor. The resulting corrected area is represented in the graphs. Student's t-tests were used to calculate the significance in difference between the mean axon lengths and arbor areas, as indicated by the P-values in the graphs.

Confocal imaging of whole-CNS PDF expression was done by determining PMT voltage, offset, and laser power settings for the control line and maintaining the same for the experimental genotype, making sure that there was no saturation effect in either the s- or l-LNvs. Gain was always maintained at 1.0x. The mean pixel intensity of cytoplasmic PDF was quantified using Fluoview software (Olympus, Center Valley, PA), with the cytoplasmic region of interest determined by GFP expression. Sequential scans were used to avoid bleed-through. Mean background pixel intensity was also measured in a region surrounding each neuron and this value was subtracted from each cytoplasmic value. To compare somatic PDF expression in the different genotypes, the average, background subtracted mean pixel intensity of PDF immuno-reactivity (IR) was calculated from 10-15 brains for each genotype, with four l- and s-LNvs measured in each brain.

Behavioral recording and analysis

Activity assay:

Locomotor activity of individual flies was recorded at 25°C using the *Drosophila* Activity Monitoring (DAM) system (Trikinetics, Waltham, MA) as per manufacturer's instructions. Briefly, individual F1 female progeny in each case were collected upon eclosion and allowed to acclimate to 12 : 12 h light/dark cycle at 25°C for 4 days after

eclosion. The significant difference observed between the control and each of the different experimental groups was determined using a Student's t-tests for each time point (n = 24).

Digital video monitoring:

Individual F1 female progeny (n=28) in each case were collected upon eclosion and allowed to acclimate to 12 : 12 h light/dark cycle at 25°C for 4 days after eclosion. Video recording of sleep in these flies was done on day 5 (after eclosion). On day 4, individual flies were anesthetized and transferred to Corning Pyrex Glass tubes (65 mm length, 5 mm diameter) containing *Drosophila* media at one end and capped with a cotton plug at the other end. Movements were monitored at 25°C and recorded every 5 secs by use of digital video recording. Total sleep, sleep bout number and mean sleep bout duration were calculated from video data using custom software as previously described [224].

RESULTS

Tip60 immunolocalization in the *Drosophila* ventrolateral neurons (LNvs)

Immunostaining using anti-Tip60 antisera was used to determine whether Tip60 is endogenously expressed in the *Drosophila* Tip60 ventrolateral neurons (LNvs) and to examine the pattern of dTip60 expression in these cells in the third instar larval and adult brains. In the adult brains, the large and small subset of LNvs revealed different patterns of dTip60 localization. While strong dTip60 immunoreactivity was observed in the lLNv, relatively weaker expression was observed in the sLNvs (Figure 1A'). Tip60 expression was not detected in the larval sLNvs, the only LNv subgroup found in larvae. Experiments examining Tip60 levels at Zeitgeber time (ZT) 2 and 14, corresponding to two and 14 hours after lights on respectively, suggest that the protein levels do not undergo circadian oscillation (data not shown).

HAT defective Tip60 negatively affects axonal growth of sLNv in the *Drosophila* brain

To determine whether Tip60 has an effect on sLNv axon growth and morphology, we specifically knocked down Tip60 in these cells by utilizing the GAL4/UAS targeted gene expression system. Flies carrying the LNv specific Pdf-Gal4 driver were crossed to our previously characterized UAS-dTip60^{RNAi} lines [222] to induce the RNAi response. LNv specific knock-down of Tip60 was confirmed by lack of Tip60 expression in the lLNv and sLNv as assessed by Tip60 immunostaining (Figure 1D and 1E). The effects on sLNv axonal outgrowth were then examined by confocal microscopy using specific

expression of the UAS-mCD8-GFP membrane marker transgene. Structurally, the sLNv axons display a well characterized and stereotypical migration pattern. During larval and pupal development, the axon stem that projects from the sLNv cell body grows dorsally, bends towards the center of the brain and sprouts into branches forming the terminal synaptic arbor in the adult animals (Figure 2A and 2A'). Induction of the Tip60 RNAi response in the LNv did not have any effect on the early development of the axonal pattern of these cells as evident from the intact axonal pattern seen in the third instar larva (Figure 2B). In contrast, in the adult brains, expression of dTip60^{RNAi} results in shortening of the sLNv terminal synaptic arbor, evident in the complete lack of the medially projecting axonal branches (Figure 2B'). As a control for the Tip60 RNAi experiments, we used a corresponding UAS-dTip60$^{RNAi\ control}$ construct [222]. As expected, LNv specific expression of dTip60$^{RNAi\ control}$ did not affect Tip60 levels in the lLNv and sLNvs (Figure 1D and 1E) and had no effect on sLNv axon morphology in the third instar larval or adult brains (Figure 2C and 2C'), confirming the specificity of the dTip60^{RNAi} induction.

We next wanted to examine if the observed effects on sLNv axon growth due to loss of Tip60 were specifically mediated by Tip60 HAT activity. To determine whether Tip60 HAT activity affects sLNv axonal growth, we misregulated *Drosophila* Tip60 (dTip60) in these cells by utilizing well characterized transgenic flies [69] that carry Gal4 responsive transgenes for either a dominant negative HAT defective version of dTip60 (dTip60^{E431Q}), or wild-type dTip60 (dTip60^{WT}). Quantification of Tip60 levels in flies overexpressing either dTip60^{WT} or dTip60^{E431Q} in the LNvs revealed a significant increase in Tip60 compared to the control flies (Pdf-Gal4/ UAS-mCD8-GFP /+) (Figure

1A', 1B', 1C', 1D and 1E). Tip60 levels in the lLNv were significantly higher than the sLNv (Figure 1D and 1E), likely due to the higher levels of endogenous dTip60 expressed in the lLNvs compared to the sLNvs. However and importantly, comparison of Tip60 levels in the lLNv and sLNv between dTip60^{WT} and dTip60^{E431Q} flies revealed equivalent levels of exogenous dTip60 in the respective neurons (Figure 1D and 1E). Similar to the effects we observed in the dTip60^{RNAi} flies, targeted expression of dTip60 ^{E431Q} in the LNv leads to shortening of the outward projecting sLNv axon terminals in the adult brains without a marked effect on the larval sLNv axon morphology (Figure 2D, 2D' and 2G). In contrast, overexpression of wild type dTip60 (dTip60^{WT}) showed no significant effect on either the larval or adult sLNv axonal architecture compared to the control flies (Figure 2E and 2E'). To confirm these results, we measured the sLNv axon length and also quantified the synaptic arbor area as described in [221] (Figure S2, Figure 2G and 2H). Since the effects we observed in the dTip60^{E431Q} flies indicate that the HAT activity of Tip60 is crucial for establishing the normal sLNv axon morphology, we wanted to examine if additional levels of HAT competent Tip60 could rescue dTip60^{E431Q} mediated effects on sLNv axonal growth to confirm that such defects were specifically caused by loss of dTip60 function. For this purpose, we utilized our previously characterized UAS-dTip60^{Rescue} line [69] that allows overexpression of equivalent levels of wild type dTip60 in the dTip60^{E431Q} background. GFP analysis revealed normal sLNv axon morphology in the dTip60^{Rescue} flies similar to the control flies (Pdf-Gal4/ UAS-mCD8-GFP / +) (Figure 2F and 2F'), indicating that the axonal defects induced by the mutant dTip60^{E431Q} can be counteracted by the presence of additional levels of HAT competent Tip60. Taken together, these results further

demonstrate that dTip60 HAT activity is crucial for establishing appropriate sLNv axon morphology.

Co-expression of dTip60 modulates APP mediated effect on sLNv axonal growth that is dependent on the APP C-terminus

Expression of the neuronal isoform of human APP (APP695) in the sLNv at 28°C using the LNv specific Pdf-Gal4 driver has been shown to induce increased axonal extension and extensive arborization of the sLNv axon terminals [221]. Our observation that the dTip60 HAT mutant affects sLNv axon growth prompted us to examine how depletion of Tip60 HAT activity affects sLNv morphology under APP overexpressing conditions. Although expression of human APP (APP) in the sLNv at 25°C (Figure 3B, 3B', 3H and 3I) did not have the drastic effect that has been reported at 28°C, co-expression of APP along with the Tip60 mutant construct (APP; dTip60 ^{E431Q}) at 25°C was found to exacerbate the negative effect that dTip60 ^{E431Q} alone had on the sLNv axonal growth, resulting in drastic shortening of the axons (Figure 3D, 3D'and 3H). Since the APP C-terminus is required for interaction with Tip60, we also examined the effects of expressing a truncated version of APP lacking the C-terminus alone (APP dCT) as well as with the HAT defective Tip60 mutant (APP dCT; dTip60 ^{E431Q}). Expression of APP dCT alone did not affect the sLNv axon morphology and was not different from that seen in the control (Pdf-Gal4/ UAS-mCD8-GFP/ +) (Figure 3C, 3C', 3H and 3I). In contrast, expression of APP dCT; dTip60 ^{E431Q} in the sLNv exhibited a less severe effect than that induced by APP; dTip60 ^{E431Q} in that the axon length was almost identical to

that seen when dTip60 ^{E431Q} was expressed alone (compare Figure 2D' and Figure 3E'). Since co-expression of APP with dTip60^{E431Q} resulted in a phenotypic enhancement of the dTip60^{E431Q} induced shortening of the sLNv axon, we examined the effect of overexpressing wild type dTip60 along with APP or APP lacking the C-terminus to gain insight into the nature of the functional interaction under these conditions. Targeted overexpression of dTip60 along with APP (APP; dTip60^{WT}) in the LNv resulted in a large increase in the area of the sLNv axonal arbor in the adult flies although there was no significant effect on the early development of sLNv axonal pattern (Figure 3F, 3F', 3H and 3I). Most of the sLNv axons grew along the right path, but further extended and arborized over a larger area than those seen in control flies or in flies that overexpressed dTip60 alone (compare Figure 2E' and Figure 3F'). In contrast, overexpression of dTip60^{WT} along with APP lacking C-terminus (APP dCT; dTip60^{WT}) did not have any significant effect and resulted in the normal axonal pattern seen in control flies (Figure 3G, 3G', 3H and 3I). Thus, co-expression of APP with dTip60 enhances the normal sLNv axonal arborization phenotype observed for overexpression of dTip60 alone, further supporting a synergistic interaction between APP and dTip60 that is dependent on the APP C-terminus.

LNv specific expression of dTip60 or APP leads to selective decrease in pigment dispersing factor (PDF) immunoreactivity in small LNv, but not in the large LNv

The LNv specific neuropeptide PDF is required for circadian behavioral rhythmicity and is expressed in both the large LNv and small LNv subset of cells.

Typically, there are 4-5 PDF positive lLNvs and four sLNvs in wild type flies. PDF is also periodically released from the lLNv varicosities and the sLNv terminal synaptic arbor in the dorsal brain [225,226] . Our observation that loss of dTip60 or expression of the HAT defective dTip60 mutant abolished the formation of these sLNv axon terminals prompted us to examine whether PDF expression and/or transport along the axons was also affected. Anti-PDF immunocytochemical analysis was performed on whole brains dissected from 4-7 day old flies resulting from a cross between Pdf-Gal4 driver and either w^{1118} (Pdf-Gal4/ UAS-mCD8-GFP/ +), dTip60^{RNAi} (Pdf-Gal4/ UAS-mCD8-GFP/ UAS-dTip60^{RNAi}) or dTip60 ^{E431Q} (Pdf-Gal4/ UAS-mCD8-GFP/ UAS-dTip60 ^{E431Q}) flies that were maintained under standard light/dark (LD) conditions. GFP expression was used as a marker to locate the l- and s- LNvs. Examination of the l- and s-LNv soma for PDF imunoreactivity (IR) in flies expressing either dTip60^{RNAi} or dTip60 ^{E431Q} revealed a partial loss of PDF IR in the sLNv of adult flies (Figure 4B' and 4D') although PDF could still be detected in the soma as well as along the axons indicating that dTip60 specifically affects PDF expression although its transport along the axons is unaffected. Quantification of PDF intensity also revealed a significant reduction of PDF IR in the sLNv in the dTip60^{RNAi} and dTip60^{E431Q} flies (Figure 4I). However, PDF expression was unaffected in the larval sLNvs in both cases. PDF expression in the lLNv soma and varicosities was also unaffected (Figure 4J). GFP expression in both cell types was unaffected indicating that the observed effects are specifically on PDF expression (Figure 4B and 4D). The persistence of similar effects due to loss of dTip60 protein and expression of the HAT defective dTip60^{E431Q} indicate that the observed effects on PDF are primarily mediated by Tip60's HAT activity. On the other hand, while targeted

overexpression of dTip60^{WT} in the LNvs did not affect PDF IR in the lLNv (Figure 4E'and 4J), it resulted in significant increase ($P < 0.05$) in PDF IR in the sLNv, compared to the control flies (Pdf-Gal4/ UAS-mCD8-GFP / +) (Figure 4E' and 4I). Since dTip60^{E431Q} leads to a decrease in sLNv PDF and overexpression of wild type Tip60 had the converse effect and increased PDF levels in the sLNv, we hypothesized that co-expression of the HAT competent Tip60 with dTip60^{E431Q} in the dTip60^{Rescuse} flies would counteract the effects of the latter. Consistent with our hypothesis, PDF levels in the sLNv but not the lLNv of dTip60^{Rescue} flies was significantly greater ($P < 0.05$) than that observed in the dTip60^{E431Q} flies as well as the control flies (Figure 4F', 4I and 4J). However, the sLNv PDF level in this case was much less than that observed in flies overexpressing wild type dTip60 alone ($P < 0.001$) (Figure 4I). This indicates that when co-expressed in equivalent amounts, dTip60^{E431Q} and dTip60^{WT} counteract their respective effect on PDF expression.

Finally, we examined whether LNv directed expression of APP had any effect on PDF expression. Similar to the dTip60^{RNAi} and dTip60 ^{E431Q} flies, APP expression did not have any observable effect on PDF IR in the lLNv (Figure 4J) but specifically affected PDF IR in the sLNv, resulting in partial reduction of PDF IR in sLNv soma (Figure 4G' and 4I). In contrast, APP lacking C-terminus (APP dCT) did not have any significant effect on PDF IR in the sLNv or the lLNv (Figure 4H', 4I and 4J). These observations suggest that dTip60 and APP selectively affect PDF expression in the sLNvs, and that the effects are dependent upon APP C-terminus. Moreover, the observed effect on PDF levels due to dTip60^{E431Q} or APP and the lack of any significant effect with

APP lacking C-terminus which is required for interaction with Tip60 suggests that PDF is a potential target of the Tip60/APP containing complex.

dTip60 and APP functionally interact to regulate PDF expression in sLNv

Our observation that expression of either dTip60 ^{E431Q} or APP each affected PDF IR in the sLNv prompted us to ask whether dTip60 and APP functionally interact to mediate the sLNv specific effect on PDF expression. We therefore measured PDF IR in the sLNv soma in flies co-expressing APP or APP dCT with either Tip60^{WT} or HAT defective mutant Tip60^{E431Q}. Although PDF IR in the lLNv remained largely unaffected (Figure 5G), significant effects on sLNv PDF IR were observed in all fly lines. APP;Tip60^{E431Q} expressing flies exhibited the most drastic effect, with APP expression exacerbating the effects of Tip60^{E431Q} expression alone, thereby resulting in complete loss of PDF IR in the sLNv (Figure 5B' and 5F). Although PDF IR was absent in the sLNv soma, these cells could still be located using GFP expression (Figure 4B). Importantly, the observed effect on sLNv PDF in the APP; dTip60^{E431Q} flies is similar to the phenotypic enhancement we observed on the sLNv axon growth in these flies. We therefore examined if the degenerative effects on sLNv axon and PDF expression in the APP; dTip60^{E431Q} flies was due to induction of an apoptotic response. To address this, we performed TUNEL assays using whole mount brains of 4-7 day old adult flies that resulted from a cross between the Pdf-Gal4 driver and w^{1118} or APP; dTip60^{E431Q} flies. However, we did not detect any TUNEL specific signal in the sLNvs or in other regions of the adult brain for this age group in either the APP; dTip60^{E431Q} or the control flies

(data not shown). This indicates that co-expression of dTip60^{E431Q} with APP leads to neuronal dysfunction, likely *via* a mechanism distinct from apoptosis.

In contrast to the above, flies that expressed APP lacking the C-terminus with dTip60^{E431Q} (APP dCT; dTip60^{E431Q}) resulted in only a partial loss of PDF IR in the sLNv, identical to that observed when dTip60^{E431Q} was expressed alone (Figure 5C' and 5F), indicating that the APP C-terminus is required for the Tip60^{E431Q}/APP mediated negative effects on PDF expression. Remarkably, overexpression of wild type dTip60 with APP appeared to rescue the APP mediated negative effects on PDF expression as APP; Tip60^{WT} expressing flies had significantly increased PDF IR in the sLNv in comparison to control flies (Pdf-Gal4; UAS-mCD8-GFP / +) (Figure 5D' and 5F). Quantification of PDF IR however, revealed a small but significant decrease in PDF in sLNv in the APP; dTip60^{WT} flies compared to flies expressing dTip60^{WT} alone ($p < 0.05$) (Figure 4I and 5F). Similar to dTip60^{WT} flies, an increase in PDF levels was also observed in the sLNvs of flies co-expressing both Tip60^{WT} and APP lacking the C-terminus (Figure 5E' and 5F), suggesting that the increase in PDF expression is predominantly mediated by Tip60. Together these findings suggest that Tip60 and APP functionally interact to regulate PDF expression in the sLNvs. However, the effect on PDF expression seems to be critically dependent upon the HAT activity of Tip60.

dTip60 ^{E431Q} flies exhibit night time sleep deficits with an increase in day time sleep

The PDF neuropeptide is implicated as the principal transmitter of the LNv group, as flies lacking Pdf function exhibit phenotypes similar to ablation of the PDF positive

LNv. These phenotypes include loss of morning anticipatory behavior and advanced evening behavior in LD and locomotor arhythmicity in DD [227]. Our observation that LNv targeted expression of dTip60^{E431Q} and APP results in selective disruption of PDF levels specifically in the sLNv prompted us to ask whether biphasic locomotor rhythm in these flies was also affected. Towards this end, we first examined locomotor behavior in Pdf-Gal4/ UAS-mCD8-GFP/ dTip60^{E431Q} flies using the *Drosophila* Activity Monitor (DAM) in standard LD condition for 2 days followed by constant darkness for 5 days. Pdf-Gal4/ UAS-mCD8-GFP/ + and UAS-dTip60^{E431Q} /+ flies were used as controls for the DAM assay. Inspection of averaged locomotor activity of control and experimental dTip60 ^{E431Q} flies showed similar gradual increases in activity in anticipation of morning and evening, coinciding with lights-on and lights-off in standard 12 h:12 h LD cycles (Figure 6A). The dTip60^{E431Q} flies also maintained rhythmicity in constant darkness similar to the control flies (Figure 6B). However, dTip60 ^{E431Q} flies exhibited significantly less locomotor activity during the day compared to the controls with a concomitant increase in night time activity, both under LD and DD conditions (Figure 6A and 6B), suggestive of sleep defects in these flies.

Recent studies have demonstrated that PDF expressing LNvs are the target of GABA (γ-aminobutyric acid)-ergic sleep-promoting cells and that their activation promotes arousal through release of the neuropeptide PDF [216,225,228]. Flies mutant for pdf or its receptor are hypersomnolent, exhibit more daytime sleep (Parisky et al., 2008) as well as reduced sleep consolidation at night [228]. Our observation that loss of dTip60 HAT activity in the LNvs reduced sLNv PDF expression prompted us to

examine whether there was also a corresponding effect on sleep in these flies. Although the DAMS assay is widely used to assess both circadian and sleep behavior [229], it has certain limitations for specifically studying sleep wherein it is insensitive to small fly movements which occur outside of the path of the infrared beam and thus affects the identification of actual quiescent sleep behavior [224]. We therefore used digital video analysis to monitor if LNv specific expression of dTip60 ^{E431Q} leads to sleep disturbances using single staged, 4-7 day old female dTip60 ^{E431Q} flies (Pdf-Gal4/ UAS-GFP/ dTip60^{E431Q}). Behavioral recording of fly sleep were carried out for 3 days at 25°C in standard LD condition. LNv specific expression of dTip60 ^{E431Q} was found to specifically disrupt nocturnal sleep without a marked variation in total sleep within a LD cycle compared to control flies (Pdf-Gal4/ UAS-mCD8-GFP / + and UAS-dTip60^{E431Q}/+) (Figure 6B). Similar sleep defects were observed in the dTip60^{RNAi} flies that exhibited a partial reduction in sLNv PDF IR (Figure 6B). Interestingly, the night time sleep in both cases was characterized by increased sleep bout number and decreased duration of sleep bout (Figure 6B' and 6B"). The number and duration of sleep bout are used to assess consolidation of sleep [230]. Changes in these sleep parameters with the dTip60^{RNAi} and dTip60 ^{E431Q} flies indicate that sleep becomes highly fragmented during the night. Additionally, the dTip60^{RNAi} and dTip60 ^{E431Q} expressing flies slept more during the day (Figure 6C) with an increase in both sleep bout number and duration of sleep bout (Figure 6C' and 6C"). Taken together, these sleep data indicate that flies expressing dTip60^{RNAi} or dTip60 ^{E431Q} exhibit night time sleep disruption and fragmentation as well as daytime sleepiness, reminiscent of sundown syndrome exhibited by human AD patients. PDF has also been reported to have a wake promoting effect in the fly and as a

result is thought to function as a stabilizer of sleep-wake cycle [220]. Since overexpression of wild type Tip60 in the LNvs increased PDF expression in the sLNv, we wanted to examine how sleep is affected under these conditions. In contrast to $dTip60^{E431Q}$ and $dTip60^{RNAi}$ expressing flies, the $dTip60^{WT}$ flies did not exhibit any significant effect on daytime sleep (Figure 6C), likely due to increased expression of PDF in the sLNv. However, these flies exhibited reduced consolidation of sleep during the night resulting in a significant decrease in night sleep (Figure 6B and 6B"). We also examined the sleep pattern in the $dTip60^{Rescue}$ flies as the effect on sLNv PDF expression in these flies was different from either the $dTip60^{E431Q}$ or $dTip60^{WT}$ flies. Intriguingly, there was no observable effect on sleep in the $dTip60^{Rescue}$ flies even though these flies exhibited a moderate increase in sLNv PDF level compared to the control flies (Figure 6B and 6C). However, the lack of any effect on sleep in the $dTip60^{Rescue}$ flies indicate that the observed sleep defects in the $dTip60^{E431Q}$ and $dTip60^{WT}$ flies are mediated through misregulation of Tip60's HAT function.

Knockdown of PDF in the sLNv replicates $dTip60^{E431Q}$ mediated effects on sleep

Our observations that $dTip60^{E431Q}$ induced selective disruption of PDF expression in the sLNv soma as well as features of sleep interference similar to Pdf null mutants prompted us to ask whether it was the lack of PDF that contributed to these sleep phenotypes. Towards this end, we used a Pdf-RNAi approach to knockdown PDF specifically in the sLNv and monitored how this affects sleep. PDF knockdown was carried out using Mai179-Gal4 and R6-Gal4 drivers, well characterized drivers that

predominantly express Gal4 in the sLNv [231,232]. Mai179-Gal4 mediated knockdown of PDF in the sLNv reduced sleep during the night with a concomitant increase in daytime sleep (Figure 7A and 7B, respectively) similar to that observed with dTip60 ^{E431Q} flies. The night time sleep was also highly fragmented as evident from increase in bout number and decreased duration of sleep bout (Figure 7A' and 7A"). Similar effects were observed with R6-Gal4 driven knockdown of PDF (Figure 8A and 8B, respectively). Taken together, these data demonstrate that knockdown of PDF in the sLNv affects sleep consolidation and suggests that reduction of PDF is responsible for the sleep disturbances observed in the dTip60^{E431Q} flies.

Tip60 and APP functionally interact to mediate PDF expression and sleep-wake cycles in the fly.

Since the selective reduction of PDF IR in the sLNv by dTip60^{E431Q} was accompanied by sleep defects reminiscent of those seen in AD, we wished to examine how APP expression in the LNv affects sleep as sLNv PDF expression was affected in these flies as well. Similar to dTip60 ^{E431Q} flies, APP expression significantly decreased night time sleep (Figure 9A) with concomitant increase in daytime sleep (Figure 9B). Expression of APP dCT in the LNv did not have a significant effect on sleep (Figure 9A and 9B), consistent with its lack of effect on sLNv PDF expression, indicating that the C-terminus of APP mediates the sleep effects seen in the APP flies. Our observation that dTip60 and APP functionally interact to regulate PDF expression in the sLNv, prompted us to ask whether this interaction also mediates the effects we observed on day and night

time sleep when either of these constructs were expressed alone. Using video monitoring assessment, we found that the APP; dTip60^{E431Q} and APP dCT; dTip60^{E431Q} flies that displayed complete and partial loss of PDF in the sLNv respectively, also exhibited significant decrease in night time sleep with a concomitant increase in day sleep (Figure 9A and 9B). Taken together, these data suggest that the sleep defects are primarily due to dTip60^{E431Q} and APP mediated effects on PDF expression in the sLNv.

Overexpression of wild type dTip60 rescues APP induced sleep deficits.

The increase in PDF expression in sLNv due to dTip60^{WT} overexpression prompted us to examine how sleep was affected in APP; dTip60^{WT} and APP dCT; dTip60^{WT} flies as these flies exhibited a similar increase in sLNv PDF. Although overexpression of wild type dTip60 (dTip60^{WT}) decreased night time sleep (Figure 8A), co- expression of dTip60^{WT} with APP rescued the nighttime sleep defects we observed for dTip60^{WT} or APP alone (Figure 9A). Co-expression of Tip60^{WT} with APP lacking the Tip60 interacting C-terminal domain did not rescue Tip60^{WT} induced decrease in night sleep, indicating that an interaction between Tip60 and APP is required for the rescue of the night sleep deficits (Figure 9A). In addition, neither of these fly lines exhibited any significant effects on the day sleep, and their sleep pattern was similar to wild type controls (Figure 9B). Taken together, these data indicate that dTip60 and APP functionally interact to mediate sleep in the fly and that the sleep phenotypes we observe are dependent upon the APP C-terminus. Moreover, overexpression of Tip60 appears to rescue both day and night sleep defects that are induced by APP alone, indicating that

under APP expressing conditions, Tip60 HAT activity alleviates the sleep deficits that are reminiscent of sundown syndrome seen in AD patients.

DISCUSSION

Selective vulnerability of specific neuronal populations to degeneration even before disease symptoms are seen is a characteristic feature of many neurodegenerative diseases. Consistent with these studies, here we show that when induction of the dTip60 RNAi response or expression of the dTip60 HAT mutant was directed to both the small and large LNvs, only the sLNvs were susceptible to the mutant effects induced under these conditions while the lLNvs were spared. The lack of any morphological effect on the lLNvs in the dTip60^{E431Q} flies could stem from the fact that compared to the sLNvs, these neurons express higher levels of endogenous Tip60 that counteracts the mutant dTip60^{E431Q} protein. However, induction of the RNAi response causes complete loss of Tip60 expression in both the lLNv and sLNv (Figure 1), and yet only the sLNvs are affected while the lLNv are spared, similar to our findings with dTip60^{E431Q} expression. This suggests that the sLNvs may be more susceptible to misregulation of Tip60 or its HAT activity. Of note, the dTip60^{WT} flies did not have any marked effect on the lLNv either, likely because these neurons are not susceptible to the moderate increase in Tip60 levels in the lLNvs induced under these conditions compared to the sLNvs. Developmentally, the sLNvs are known to differentiate much earlier than the large cells [233] and this developmental difference may also in part account for the selective vulnerability of the sLNvs. In many neurodegenerative diseases, axon

degeneration is known to involve protracted gradual 'dying-back' of distal synapses and axons that can precede neuron cell body loss and contribute to the disease symptoms [234,235]. Importantly, loss of synapses and dying back of axons are also considered as early events in brain degeneration in AD [236]. While APP overexpression in the LNvs did not have any observable effect on the sLNv axon growth at normal physiological temperatures, co-expression of the dTip60 HAT mutant with APP C-terminus appears to cause the sLNv axons in the adult animals to retract. The lack of any effect on the sLNv axon in the third instar larva in this case indicates that the axons grow to their full potential in the larval stage, but undergo degeneration post-mitotically in a process similar to 'dying-back'.

A functional interaction between Tip60 and the amyloid precursor protein (APP) intracellular domain (AICD) has been shown by us and others to epigenetically regulate genes essential for neurogenesis [78,147,180]. Such an effect is thought to be mediated by recruitment of the Tip60/AICD containing complex to certain gene promoters in the nervous system that are then epigenetically modified by Tip60 *via* site specific acetylation and accordingly activated or repressed. While the E431Q mutation in our dominant negative HAT defective version of Tip60 ($dTip60^{E431Q}$) reduces Tip60 HAT activity, it should not interfere with its ability to assemble into a protein complex [69,237]. Thus, $dTip60^{E431Q}$ likely exerts its dominant negative action over endogenous wild-type Tip60 *via* competition with the endogenous wild-type Tip60 protein for access to the Tip60/AICD complex and/or additional Tip60 complexes, with subsequent negative consequences on chromatin histone acetylation and gene regulation critical for

nervous system function. Here, we show that co-expression of HAT defective Tip60 (dTip60^{E431Q}) with APP in the APP; dTip60^{E431Q} flies exacerbates the mutant effects that either of these interacting partners has on the sLNv axon growth and Pdf expression when expressed alone. In contrast, co-expression of additional dTip60^{WT} with APP alleviates these effects and this rescue is dependent upon the presence of the AICD region of APP. Thus, Tip60 HAT activity appears to display a neuroprotective effect on axonal outgrowth, Pdf expression, with concomitant alleviation of sleep defects under APP expressing neurodegenerative conditions. We propose that Tip60 might exert this neuroprotective function either by itself or by complexing with other peptides such as AICD for its recruitment and site specific acetylation of specific neuronal gene promoters to redirect their expression and function in selective neuronal processes such as sLNv morphology and function. Such a neuroprotective role for Tip60 is consistent with our previous work demonstrating that excess dTip60^{WT} production under APP expressing neurodegenerative conditions in the fly rescues APP induced lethality and CNS neurodegeneration and that dTip60 regulation of genes linked to AD is altered in the presence of excess APP [78]. We speculate that the degenerative effects we observe in the APP; dTip60^{E431Q} flies may result from formation of Tip60^{E431Q}/AICD complexes that ultimately cause activation or de-repression of factors that promote axonal degeneration while excess Tip60/AICD complex formation in the APP;dTip60^{WT} expressing flies promote gene regulation conducive for sLNv outgrowth and Pdf expression.

Sleep or wake promoting neurons in the hypothalamus or brainstem are known to undergo degeneration in a number of neurodegenerative diseases resulting in sleep dysregulation [238]. In AD, such sleep disturbances are characterized by excessive daytime sleepiness and disruption of sleep during the night. These features resemble the symptoms of narcolepsy, a sleep disorder caused by general loss of the neurotransmitter hypocretin/orexin [239]. Hypocretin is involved in consolidation of both nocturnal sleep and diurnal wake [240] and loss of hypocretin levels have been correlated with sleep disturbances observed in AD [241]. While the neuropathological changes in AD may contribute to hypocretin disturbances, a direct and causative role for APP in regulating hypocretin expression is not yet known. The LNv specific neuropeptide PDF is postulated to be the fly equivalent of hypocretin [220] and has been shown to promote wakefulness in the fly. Consistent with these reports, our data demonstrating somnolence during the light phase due to knock-down of PDF in the sLNv further supports a wake-promoting role for PDF. Accordingly, we observed that overexpression of APP in the LNvs results in reduction of sLNv PDF expression as well as sleep disturbances that intriguingly, have been associated with AD pathology. The presence of similar effects on PDF and sleep due to loss of dTip60 HAT activity supports a role for both APP and Tip60 in controlling the PDF mediated sleep-wake regulation pathway. Previous studies have reported that the circadian modulators CLOCK and CYCLE regulate PDF expression in the sLNvs but not in the lLNvs [242]. We also observe a similar sLNv specific regulation of PDF by dTip60 in the adult flies. However, there was no effect on PDF expression in sLNvs in the larvae when Tip60 levels are undetectable. This is also consistent with the sLNv axonal defects that persist only in the adult flies. This suggests

that the sLNvs may be subject to differential regulation during development as well as a temporal requirement for Tip60 in these cells in the adult flies. A recent study reported persistence of morning anticipation and morning startle response in LD in the absence of functional sLNv that were ablated due to expression of the pathogenic Huntington protein with poly glutamine repeats (Q128) [243]. Consistent with this study, we did not observe any marked effect on the morning and evening anticipatory behavior in LD in the $dTip60^{E431Q}$ flies that exhibit a partial reduction in sLNv PDF. However, while the Q128 expressing flies were arrhythmic under constant darkness, $dTip60^{E431Q}$ flies maintain rhythmicity in DD indicating that the sLNvs are still functional in these flies. The remarkable cell specificity of PDF regulation indicates the presence of additional as yet unidentified clock relevant elements or developmental events that distinguish between the two cell types.

Recent evidence indicates that LNvs are light responsive and that their activation promotes arousal through release of PDF. Furthermore, PDF signaling to PDF receptor (PDFR) expressing neurons outside the clock, such as those found in the ellipsoid body that directly control activity, is thought to be important in translating such arousal signals into wakefulness [216]. Since PDF is released from the sLNv axon terminals, the retraction of the sLNv axon terminals induced by the Tip60 HAT mutant can interfere with PDF mediated interaction of the sLNvs with downstream circuits. In the case of APP overexpression, while sLNv axon structure is unaffected, PDF expression is reduced; we speculate that the decrease in PDF under these conditions is responsible for the abnormal sleep phenotype observed. In support of this theory, we find that

expression of APP lacking the C-terminus that also has no observable effect on the sLNv axon growth or PDF expression did not have any effect on sleep behavior. Thus our results indicate that the degenerative effect on the sLNv axons and/or the effect on PDF expression could both contribute to the observed sleep disturbances. Likewise, co-expression of the dTip60 HAT mutant with full length APP or APP lacking the C-terminus affected both the sLNv axon growth and PDF expression and consequently resulted in similar sleep disturbances.

In addition to the wake promoting role, the LNvs also express $GABA_A$ receptors [216,228] and are thus subject to inhibition by sleep promoting GABAergic inputs, analogous to those from the mammalian basal forebrain that regulate hypocretin neurons [244]. The current consensus view is that sleep regulation is mediated by mutually inhibitory interactions between sleep and arousal promoting centers in the brain [245,246]. The normal release of PDF from LNvs is part of the arousal circuitry in the fly and determines the duration of the morning and evening activity peaks [215,219] while inhibition of these neurons and thus reduction in PDF release is necessary for normal sleep [228]. Current models of sleep regulation suggest that the drive to sleep has two components, the first component is driven by the circadian clock and the second component is homeostatic in nature and the strength of this drive is based upon the amount of time previously awake [247]. PDF release from sLNvs axon terminals exhibits diurnal variation [242] and its release increases the probability of wakefulness by activating arousal promoting centers [216]. However, the homeostatic drive for sleep that accumulates during the wake period eventually inhibits such arousal centers to promote

sleep [248]. Consistent with these reports, the reduction of PDF we observe due to either dTip60^{E431Q} expression alone or co-expression of dTip60^{E431Q} with APP that leads to flies sleeping more during the day may also lead to a decrease in their homoeostatic drive for sleep, thus resulting in the less consolidated sleep patterns we observe for these flies during the night. Conversely, we found that overexpression of sLNv PDF due to dTip60 overexpression induces wakefulness and arousal. Additionally, these flies exhibit impaired ability to maintain sleep at night that may be mediated through inappropriate activation of arousal circuits due to PDF overexpression. Similar effects have been reported in a Zebrafish model due to hypocretin overexpression that results in hyperarousal and dramatic reduction in ability to initiate and maintain a sleep-like state at night [249]. Despite the moderate increase in sLNv PDF levels in the dTip60^{Rescue} flies, we did not observe a marked effect on sleep-wake cycle in these flies. Extracellular levels of PDF and its signaling at synapses is thought to be regulated by neuropeptidases like neprilysin. In fact, neprilysin mediated cleavage of PDF has been shown to generate metabolites that have greatly reduced receptor mediated signaling [250]. Thus, we speculate that the lack of any corresponding effect on sleep in the Tip60^{Rescue} flies could be because such small increases in PDF might be regulated by endopeptidases like neprilysin.

Although overexpression of wild type dTip60 with full length APP increased PDF expression in the sLNv compared to the normal levels that persist in the control flies, it did not result in the sleep defects that were observed when Tip60^{WT} was overexpressed alone or with APP lacking the C-terminus. The absence of any observable effects on

sleep under these conditions suggests the presence of other sleep promoting compensatory mechanisms that counteract the sleep defects mediated by PDF overexpression. Intriguingly, significant exacerbation of axonal arborization was only observed as a result of co-expression of APP and $Tip60^{WT}$, and not when $Tip60^{WT}$ was expressed alone or with APP lacking the C-terminus and this may account for the differences in sleep phenotypes between these two genotypes. Consistent with this notion, recent electron microscopy studies indicate the presence of sparsely distributed input synapses at the sLNv axon terminals in addition to the PDF positive output synapses. This indicates that the sLNvs may also receive additional neural inputs directly through such synaptic connections in the dorsal protocerebrum [251]. The sLNv axon terminals have also been reported to express post-synaptic $GABA_B$ receptors and thus receive slow inhibitory GABAergic input through the dorsal terminals. Incidentally, GABAergic neurons have also been observed in the vicinity of the sLNv axon terminals in the adult CNS [252]. These observations suggest that the sLNv can also integrate signals from GABAergic or other sleep promoting neurons *via* their axon terminals. Indeed, the firing rate of sLNv is thought to be dependent on a finely balanced interaction of cholinergic, GABAergic and glutamate signaling [253]. Based on these studies, we propose a model by which the overelaborated sLNv synaptic arbors observed in flies co-expressing $Tip60^{WT}$ and APP may provide additional input sites for signals from sleep promoting neurons in the vicinity that counteract the arousing effect of PDF overexpression on nocturnal sleep (Figure 10).

Light mediated release of PDF from the lLNvs has been reported to modulate arousal and wakeful behavior as well as sleep stability. Thus, it has been suggested that the lLNvs may be part of an arousal circuit that is physiologically activated by light and borders with, but is distinct from the sLNvs and downstream sleep circuits [217]. However, other studies have suggested that both LNv sub-groups promote wakeful behavior and that the lLNv act upstream of the sLNv [216,254]. Our observation of sLNv directed effects on PDF expression and the persistence of sleep-wake disturbances suggest that the sLNvs may be part of the neural circuitry that regulates sleep downstream of the lLNvs *via* a PDF dependent mechanism. In this regard, the sLNvs may participate in the communication between the lLNvs and other brain regions to promote light mediated arousal. It has been proposed by [254] that the lLNvs may promote neural activity of the Ellipsoid body (EB) in the central complex (CC), a higher center for locomotor behavior that expresses the PDF receptor [219]. However, we observe disruption of sleep-wake cycles even in the absence of any marked effect on the lLNv morphology or PDF expression. While no direct projections from the lLNvs to the EB have been detected, the sLNv axonal projections are relatively closer to the CC and thus may promote PDF receptor mediated signaling in such regions that control activity. Sleep disturbances, while prominent in many neurodegenerative diseases are also thought to further exacerbate the effects of a fundamental process leading to neurodegeneration [255]. For these reasons, optimization of sleep-wake pattern could help alleviate the disease symptoms and slow the disease progression. In this regard, the modulatory effects that Tip60 HAT activity (dTip60^{E431Q} versus dTip60^{WT}) has on the sLNvs, the fly counterpart of the mammalian pacemaker cells, under APP overexpressing conditions,

may provide novel mechanistic insights into epigenetic regulation of neural circuits that underlie behavioral symptoms like the "sundowners syndrome" in AD. Future investigation into the downstream mechanism by which Tip60 regulates the sleep-wake cycle may further provide insight into the utility of specific HAT activators as therapeutic strategies for sleep disturbances observed in AD.

CHAPTER 4: TIP60 MEDIATED NEURONAL GENE EXPRESSION CHANGES UNDERLIE MEMORY FORMATION IN DROSOPHILA

ABSTRACT

Epigenetic mechanisms are not only essential for stable molecular changes required for establishment of cellular identity, but also for dynamic intracellular processes that translate environmental stimuli into modifications in gene expression. Recent studies also highlight the importance of epigenetic mechanisms in the CNS in regulating transcriptional programs linked to synaptic plasticity and cognition. Histone acetylation is one of the best characterized epigenetic mechanisms essential for regulating neuronal gene expression related to learning and memory, although specific HATs and HDACs that mediate these effects are yet to be fully characterized. In this study, we have investigated a role for the HAT Tip60 in memory formation in *Drosophila*. We show that misregulation of Tip60 HAT activity in the *Drosophila* mushroom body (MB) leads to defects in immediate recall memory. Furthermore, disruption of Tip60 HAT activity leads to abnormal development of the axonal lobes in the MB, resulting in thinner and shorter lobes. We also show that Tip60 is endogenously expressed in the Kenyon cells, the intrinsic neurons of the MB as well as in the MB lobes. Together, our studies identify essential roles for Tip60 in establishing the stereotypical MB structure during development as well as a transcription regulatory function in mediating gene expression changes that underlie memory formation via the MB encompassing neural circuit.

INTRODUCTION

Epigenetic marking of chromatin in the brain is emerging as a pivotal molecular mechanism underlying certain forms of synaptic plasticity and cognition [256]. While conferring nerve cells the ability to establish and maintain their identity, epigenetic modifications of chromatin also allow nerve cells to respond to environmental stimuli and modulate their gene expression profiles [257]. The best-studied form of chromatin modification in the learning and memory field is histone acetylation [258], which is regulated by the antagonistic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [74]. Blocking histone acetylation has been reported to impair both long lasting synaptic plasticity as well as behavioral performance [97]. Notably, inhibition of histone deacetylase activity rescues these deficits and improves memory formation [31,97], thus highlighting the importance of histone acetylation for memory formation.

Cognitive decline is also a debilitating feature of most neurodegenerative diseases of the central nervous system including Alzheimer's disease (AD). Cognitive capacities in the neurodegenerating brain are thought to be constrained by an epigenetic blockade of gene transcription that is potentially reversible [259]. Several recent studies have reported sporadic cases of reduced histone acetylation in animal models of neurodegeneration that are characterized by cognitive decline, including models of AD. Accordingly, pharmacological treatments aimed at increasing histone acetylation levels have shown promising effects in reversing cognitive deficits in such models [151]. However, little is known about HATs that modify the neural epigenome by laying down specific epigenetic marks required for proper cognition and thus, likely serve as causative agents of memory

impairing histone acetylation changes. A promising candidate is the HAT Tip60, that has been implicated in Alzheimer's disease (AD) owing to its role in epigenetically regulating gene expression via complex formation with the amyloid precursor protein (APP) intracellular domain (AICD) [144,260].

Tip60 (Tat interactive protein, 60KDa) is a multifunctional HAT that has been shown by us and others to epigenetically regulate genes essential for neurogenesis [69,78]. Such an effect is thought to be mediated through recruitment of Tip60 containing protein complexes to target gene promoters in the nervous system that are then epigenetically modified via site-specific acetylation and accordingly activated or repressed. We have recently reported that the histone acetylase function of Tip60 promotes neuronal and organismal survival in a *Drosophila* model of AD by activating pro-survival factors while concomitantly repressing activators of cell death [78]. Overexpression of Tip60 also promotes axonal growth of the *Drosophila* circadian neurons, the small ventrolateral neurons (sLNvs) under APP overexpressing conditions [170]. While these effects support a neuroprotective role for Tip60 under degenerative conditions such as those induced by neuronal overexpression of APP, an epigenetic role for Tip60 in mediating gene expression changes that underlie memory formation remains to be elucidated.

Drosophila is an attractive model for studies aimed at molecular dissection of components of memory formation due to the availability of reproducible memory assays and genetic tools that enable restricting gene expression to specific subregions of the brain for instance, by using appropriate GAL4 drivers [261]. In this study, we focused on the *Drosophila* mushroom body (MB) to investigate a role for Tip60's epigenetic HAT

function in memory formation. The *Drosophila* MB is deemed as the learning and memory center, analogous to the mammalian hippocampus as it is known to regulate a range of behavioral and physiological functions that range from olfactory learning, courtship conditioning to decision making under uncertain conditions [262]. Courtship conditioning in *Drosophila* is a complex behavioral learning paradigm that requires multimodal sensory input, involving chemosensory, mechanosensory, visual and olfactory pathways and is thus well suited to study experience dependent synaptic plasticity [263]. In this report, we show that misregulation of Tip60 HAT activity in the MB leads to courtship memory deficits suggesting potential Tip60 mediated gene expression changes that underlie these memory defects. Immunohistochemical analysis of brains from adult flies expressing a dominant negative mutant form of Tip60 defective in its HAT activity in the MB revealed dramatic effects on the axonal fields of the MB lobes, suggesting a possible anatomical mechanism for the observed behavioral defects.

METHODS

Drosophila Stocks

Flies were reared on standard medium (cornmeal/sugar/yeast) at 25 degrees with a 12-h light/dark cycle. Canton S flies were used as wildtype controls. OK107-GAL4 and UAS-GFP stocks were obtained from the Bloomington *Drosophila* stock center (Indiana University). The generation and characterization of UAS-dTip60^{E431Q} and UAS-dTip60^{WT} flies are described in [69] and [262], respectively. Double transgenic lines carrying the UAS-GFP and either UAS-dTip60^{E431Q} or UAS-dTip60^{WT} constructs were generated according to standard procedures.

Courtship Suppression Assay

Assays were performed as described in [264]. Briefly, virgin males of the appropriate genotype were collected within 6 hr of eclosion, and reared in individual food vials at 25°C in 12:12 LD for 5 days prior to behavioral training and testing. Virgin wild type Canton S females were collected and kept in groups in food vials. Mated Canton S females used for training were 5 days old and observed to have mated with a Canton S male the evening prior to training. Virgin Canton S females used for testing were 5 days old. All experiments were conducted during light phase. All behavior was digitally recorded using a Sony DCR-SR47 Handycam with Carl Zeiss optics. The total time that a male performed courtship activity was subsequently measured and scored. The courtship index was calculated as the total time observed performing courting behavior divided by the total time assayed.

On the day of training (day 5), male flies were assigned to random groups, and the assay set up with the experimenter blind to the genotype of the test males. Male flies were transferred without anesthesia to one half of a partitioned mating chambers from Aktogen (http://www.aktogen.com) that contained a previously mated Canton S female in the other partitioned half. Males were allowed to acclimate for 1 min, then the partition between the male and female was removed. Male flies were then trained for 60 min. After 60 min, male flies were transferred within 2 min without anesthesia to one half of a clean partitioned mating chamber that contained a virgin Canton S female in the other partitioned half. The partition was removed and behavior of the flies was recorded for 10 min. During the testing phase, untrained males of the appropriate genotype were assayed alongside the trained males to serve as controls. To determine the significance between

different measures of the same genotype, a two-tailed paired Student's t-test was performed. Significance was determined at the 95% confidence interval.

Immunohistochemistry and antibodies

Third instar larvae or adult brains were dissected in PBS, fixed in 4% paraformaldehyde in PBS, washed thrice in PBS containing 0.1%Triton X-100, blocked for 1 hr at RT in PBT containing 5% normal goat serum, and incubated with primary antibodies in blocking solution overnight at 4 C. Anti-Tip60 (1:400) was generated by Open Biosystems (Rockford, IL), Anti-Fasciclin (mAb1D4; 1:10), anti-Trio(mAb9.4A; 1:4), anti-ELAV (1:400) were obtained from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa, IA). Anti-GFP (1:100) was obtained from Millipore (CA). Samples were washed thrice in PBT at RT, and secondary antibodies (Jackson Immunoresearch, PA) were applied in blocking solution for 2 hr at RT. After washing thrice in PBS, samples were mounted in Vectashield (Vector Laboratories, CA).

Imaging and quantification

Larval and adult brain preparations were imaged using the appropriate secondary antibodies. Anti-GFP and anti-Tip60 immunostaining were visualized using Alexa-Fluor 488 and Alexa-Fluor 647, respectively. Anti-Elav, anti-Fasciclin, anti-Trio were visualized using Alexa-Fluor 568. Confocal microscopy was performed using Olympus Microscope with fluoview acquisition software (Olympus, Center Valley, PA). Images were displayed as projections of 1uM serial Z- sections. Area of the mushroom body lobes in the different genotypes was measured using NIH ImageJ software,

RESULTS

Tip60 is expressed throughout the adult fly brain including the mushroom body

Tip60 expression in the adult fly brain was characterized by immunohistochemistry on whole mount Canton S adult brains with an anti-*Drosophila* Tip60 antibody. We found that Tip60 was widely expressed throughout the adult brain with an expression pattern similar to the pan-neuronal ELAV protein including the mushroom body (MB) lobes (Figure 1, A-C). In order to examine Tip60 expression in the MB, immunohistochemistry for Tip60 was performed on brains expressing mCD8-GFP under control of OK107-GAL4. In the MB neurons, called Kenyon cells, mCD8-GFP expression was observed in the cytoplasm surrounding the Tip60 positive nuclei and Tip60 was detected in all cells that expressed mCD8-GFP (Figure 1, E-F).

During development, the Kenyon cells of the MB undergo an ordered differentiation process into three types of neurons, namely, the α/α' neurons, β/β' neurons and γ neurons [265]. Each neuron projects dendrites that contribute to a large dendritic field in the calyx and an axon that travels anteroventrally, forming a tightly bundled peduncle before branching dorsally to form the α/α' lobes and medially to form the β/β' and γ lobes. In addition to the Kenyon cells, Tip60 was also detected in the α/α', β/β' and γ lobes (Figure 2A and 2D). Specific MB lobes were unambiguously identified immunohistochemically by co-staining with markers specific for each of the lobes. Fasciclin II (Fas II) is a cell adhesion molecule that participates in axonal pathfinding [266] and is expressed strongly in the α/ β lobes (Figure 2B) [267]. *Drosophila* Trio is a Dbl family protein that participates in patterning of axons by regulating their directional

extension and is expressed strongly in the α'/β' lobes (Figure 2E) [268]. Both markers are expressed weakly in the γ lobe as well (Figure 2B and 2E) [269]. Tip60 expression in the α/ β and α'/β' lobes followed the expression pattern of Fas-II and Trio, respectively (Figure 2C and 2F).

Tip60 HAT activity is required for immediate recall memory

Since Tip60 is endogenously expressed in the adult MB, we wanted to examine if Tip60 epigenetically regulates memory formation using the conditioned courtship suppression assay [270]. This assay is an associative conditioning procedure that measures both learning and memory in individual flies [271]. The conditioning aspect of the assay is based on the observation that male courtship behavior is modified by exposure to a previously mated female that is unreceptive to courting [270,272]. Thus, after a one hour training session with a mated female, wild type males suppress their courtship behavior even towards subsequent receptive virgin females, an effect that decays after 1-3 hrs [273].

In order to examine the effect of Tip60 HAT function on learning and memory, we misregulated *Drosophila* Tip60 in the mushroom body by utilizing our previously reported transgenic lines that carry GAL4 responsive transgenes for either a dominant negative HAT defective version of dTip60 ($dTip60^{E431Q}$), or wild-type dTip60 ($dTip60^{WT}$) [69,270]. Expression of the respective transgenes was achieved continuously during development using the GAL4 driver, OK107. This driver is expressed in discrete neuronal populations in the adult fly brain that includes high expression in the Kenyon cells, the intrinsic neurons of the MB as well as in the pars intercerebralis,

suboesophageal ganglion and optic lobes [274].To determine the effects on learning, male flies were placed in a courtship chamber with a previously mated (unreceptive) wild-type female for 60 min. The amount of time the male spent performing courtship behavior was assessed during the initial 10 min of this training and compared with the final 10 min of the training period. Male control flies (OK107-GAL4/+) show a significant drop in courtship behavior in the final 10 min of training when compared with the initial 10 min (Figure 3A), indicative of an appropriate learning response. Similar effect was observed in the UAS background control flies (UAS-dTip60^{E431Q}/+ and UAS-dTip60^{WT}/+) and in the wild type Canton S flies (Figure 3A). Male flies expressing either the Tip60 HAT mutant (dTip60^{E431Q}) or additional copies of wild type Tip60 (dTip60^{WT}) also showed a significant decrease in courtship behavior in the final 10 min of the training period compared with the initial 10 (Figure 3A). This indicates that misregulation of Tip60 HAT activity in the MB does not interfere with the successful perception and interpretation of sensory stimuli required in this assay and that these flies are capable of altering their behavior appropriately (learn) in response to this training.

Different phases of memory have been defined in *Drosophila* and include immediate recall (0–2 min post-training), short-term memory (up to 1 h post-training), medium-term memory (up to 6 h), anesthesia-resistant memory (up to 2 days) and long-term memory (up to 9 days) [275,276]. In order to test for the earliest phase of memory first, we assayed male flies expressing either the Tip60 HAT mutant (dTip60^{E431Q}) or wildtype Tip60 (dTip60^{WT}) by transferring the respective trained males to clean mating chambers and pairing with a receptive virgin female within two mins of training, following which, their courtship behavior was monitored for 10 mins. Trained male

control flies (OK107-GAL4/+) showed a marked decrease in their courtship activity compared to untrained male flies (Sham) that were assayed in parallel (Figure 3B). Similar effect was observed in the UAS background control flies (UAS-dTip60^{E431Q}/+ and UAS-dTip60^{WT}/+) and in the wild type Canton S flies (Figure 3B). This indicates a change in behavior in these flies that is consistent with normal immediate recall memory of training. However, such a decrease in courtship behavior was not observed in flies expressing either the Tip60 HAT mutant (dTip60^{E431Q}) or additional copies of the HAT competent wild type Tip60 (dTip60^{WT}) (Figure 3B). Since these flies were capable of normal sensory perception and were also able to alter their behavior in response to their experience during the learning component of the assay, their inability to effectively suppress courtship behavior during the second component of the assay indicates that these flies are defective in immediate recall memory of this form of learning.

Tip60 is required for formation of normal mushroom body structure in adult brains

Development of precise axonal connectivity and plasticity in their connectivity are required for maintenance of functional neural circuits that facilitate learning and memory [277]. Accordingly, degeneration of neural circuits essential for learning and memory may lead to impaired behavioral plasticity. We have recently reported that Tip60’s HAT function promotes axonal growth of the *Drosophila* small ventrolateral neurons (sLNv), a well characterized model system for axonal growth [170]. We therefore wanted to examine if the observed memory deficits in the Tip60 mutant flies were accompanied by axonal growth defects in the MB. In order to examine the Tip60

mediated anatomical effects in the MB, we generated GAL4 responsive transgenic fly lines carrying a membrane bound mCD8-GFP construct with either the dominant negative Tip60 HAT mutant (UAS-mCD8-GFP; UAS-dTip60^{E431Q}) or wild type Tip60 (UAS-mCD8-GFP; UAS-dTip60^{WT}). Expression of the respective transgenes was directed by the OK107-GAL4 driver. MB structural phenotypes under the different conditions were identified by immunostaining for GFP in whole brains dissected from adult animals.

In the third instar larvae and adult control flies (OK107-GAL4/US-GFP), confocal microscopy revealed GFP immunolabeling of α/β neurons along the peduncles as well as distally as their axons bifurcate and project dorsally into the α/α' lobes and medially into the β/β' and γ lobes (Figure 4A and 4A'). The stereotyped morphology of the MB lobes was detected in third instar brain of flies expressing either the HAT mutant dTip60^{E431Q} or the wild type dTip60^{WT} (Figure 4B and 4C). However, GFP staining of adult brains from the dTip60^{E431Q} mutants revealed dramatic reduction of the MB axonal fields resulting in α/α' lobes that were much thinner than those in the control flies (Figure 4B' and 4D). Additionally, severe reduction in the area of the β/β' and γ lobes was also observed in these flies (Figure 4B' and 4D). Thinner α and β lobes were observed in both sides of the brain in the dTip60^{E431Q} mutants, indicating that the axonal defects are common to both the brain hemispheres. Developing axons of α/β neurons normally bifurcate at the base of the lobes, and the resulting sister branches subsequently extend in diverging directions: one dorsally to the α lobe and the other medially to the β lobe. Similarly, α'/β' neurons also develop dorsal (α') and medial (β') lobes. In order to examine which particular lobe(s) were specifically affected in the dTip60^{E431Q} flies, area

measurements of the different MB lobes were carried out by co-staining with anti-Fas II or anti-Trio antibodies that exhibit weak expression in the γ-lobe while strongly labeling α/β and α’/β’ lobes, respectively. Fas II staining (Figure 5B and 5B’) was used for quantification of α/β and γ-lobes while Trio staining (Figure 6B and 6B’) was used for quantification of area of α’/β’ lobes. Quantification using these lobe specific markers revealed a marked decrease in the area of all the MB lobes in the dTip60^{E431Q} flies compared to the control flies (OK107-GAL4; UAS-GFP) (Figure 4D). On the contrary, adult brains from the dTip60^{WT} flies did not exhibit any significant effect on α/β, α’/β’ and γ lobes on either side of the brain as revealed by GFP (Figure 4C’ and 4D), Fas II (Figure 5C’) and Trio labeling (Figure 6C’). Thus, the expression of Tip60 within the MB lobes and the axonal growth defects observed due to disruption of Tip60’s HAT function together suggest that Tip60 HAT activity may play essential roles in MB axonal development.

DISCUSSION

Transcription of genes involved in synaptic plasticity is a highly regulated process and it is becoming increasingly clear that HATs and HDACs are key regulators in this process [278,279]. Here, we provide evidence that the HAT Tip60 plays an integral role in memory formation in *Drosophila*. Inducing expression of dominant negative mutant form of Tip60 using the OK107-GAL4 driver that drives expression in all the lobes of the MB results in defects in immediate recall memory while there is no effect on learning. These memory defects are also accompanied by axonal growth defects that are evident in

dorsal α/ α’ and medial β/ β’ and γ lobes of the adult MB in these flies with no marked effect on the larval MB structure. We have recently reported a similar effect in the axons of *Drosophila* small ventrolateral neurons (sLNv), a well characterized model system for studying axonal growth, wherein disruption of Tip60’s HAT function affects sLNv axon growth in the adult flies although there was no effect in the third instar larva [170].

The α, β, and γ neurons composing the mushroom body undergo considerable structural reorganization during embryonic, larval and pupal development. The γ neurons are the earliest born and develop during first instar larval stages while development of α’/β’ axons and α/β axons takes place during the third instar larval and pupal stages, respectively [280]. Although the α/β lobes appear much later in development than the α’/β’ lobes, the dramatic effects we observe on both these lobes in the dTip60^{E431Q} adult flies indicate that Tip60’s HAT activity may be crucial for development of α/β lobes as well as for maintaining branch stability in the larval born α’/β’ lobes as development proceeds. During metamorphosis, the γ neurons undergo a stereotypical process of axon elimination wherein the dorsal and medial segments of its axon are pruned back [281,282]. The γ axons subsequently re-extend medially during pupal remodeling. Tip60’s HAT activity likely mediates regeneration of γ axons as well during pupal development as evidenced by the severe reduction of these axons in the adult flies that express the HAT defective dTip60^{E431Q} mutant. While these effects suggest that Tip60 HAT activity is required for mediating MB axonal growth metamorphic development, the precise developmental window of Tip60 requirement needs further investigation.

Outgrowth and stabilization of axons during development of the nervous system and reorganization of axonal connections in the adult are based on the dynamic

rearrangement of the cytoskeleton [283,284]. Axon growth and elongation depends, among other factors, on microtubules polymerization [285] and acetylation of α-tubulin has been reported to stabilize microtubules and promote polymerization [286]. Tip60 has also been reported to partially acetylate microtubules in the larval neuromuscular junction, an effect that was dependent on its HAT function [173]. Our analysis also reveals that Tip60 is localized to all the lobes of the mushroom body which raises the possibility that Tip60 may promote axonal growth by modulating cytoskeletal dynamics in the MB through direct binding and acetylation of cytoskeletal proteins that function to promote and stabilize axon growth. In addition, localization of Tip60 in the Kenyon cell nuclei also suggests a transcriptional regulatory function that is dependent on its HAT activity. The E431Q mutation in the HAT-defective version of Tip60 ($dTip60^{E431Q}$) while reducing Tip60 HAT activity, does not interfere with its ability to assemble into a protein complex [69,237]. Thus, the mutant $dTip60^{E431Q}$ protein likely exerts its dominant negative action over endogenous wild-type Tip60 via competition with the wild-type Tip60 protein for access to native protein complexes, with subsequent negative consequences on histone acetylation and gene regulation critical for mediating axonal growth and memory formation.

While considerable evidence supports a crucial role for MBs in immediate recall memory (i.e. 0-2 mins memory) in the odor avoidance paradigm [287-289], MBs have been reported to be dispensable for immediate recall memory pertaining to courtship conditioning [290]. However, besides the MBs, several other brain regions have been identified to be important for courtship and courtship learning. In fact, basic courtship involves communication between the projection neurons from the antennal glomeruli

with higher centers in the lateral protocerebrum and mushroom bodies [263]. Recent studies using cobalt labeling and ectopic expression of the ATP receptor P2X2 in the MB Kenyon cells also suggest the existence of functional feedback from MBs to the antennal lobes, a process crucial for sensory processing [291,292]. Furthermore, such functional feedbacks from the Kenyon cells are thought to be mediated by the β and γ lobes [292] which are also severely affected in the $dTip60^{E431Q}$ flies. Changes in neuronal connectivity in the central nervous system are also thought to contribute to behavioral defects in several *Drosophila* learning mutants that alter cAMP signaling [293]. Thus, we speculate that the axonal growth defects we observe in the $dTip60^{E431Q}$ flies may result in disruption of synaptic connectivity between the MB and neural circuits in the protocerebrum essential for sensory processing, subsequently leading to the observed memory impairment. Intriguingly, although overexpression of wild type Tip60 ($dTip60^{WT}$) did not have any marked effect on the MB structure per se, the $dTip60^{WT}$ expressing flies exhibit defects in immediate recall memory similar to the $dTip60^{E431Q}$ flies. Overexpression of wild type Tip60 in the *Drosophila* nervous system has been reported to induce bidirectional changes in expression genes that are enriched for neuronal functions [69,285]. Thus, it is likely that increasing Tip60 mediated acetylation in the MB can also lead to complex changes in the chromatin landscape resulting in misregulation of genes that are induced following patterned synaptic stimulation, such as behavioral experiences and play a critical role in transformation of activity in neural circuits into accessible memories in the brain.

Together, these data support an epigenetic role for Tip60 in the development of all three subtypes of MB neurons by regulating axon extension and branching in the later

stages of brain development during pupal metamorphosis. *Drosophila* MBs manifest not only developmental reorganization [265,294], but also experience-dependent plasticity [295-297]. The memory effects that we observe due to misregulation of Tip60's HAT activity in the MB, a highly plastic brain region that is also well known for its role in multimodal sensory integration and associative learning further identifies a transcription regulatory function for Tip60 in promoting expression of genes that are essential for experience dependent associative memory. Identification of the repertoire of genes regulated by Tip60 and mapping the exact mechanism by which Tip60 mediates the observed effects on the MB structure and function will be the subject of future studies.

WORK IN PROGRESS

Genome-wide profiling of target genes regulated by Tip60 using ChIP-Seq

Chromatin immunoprecipitation (ChIP) has become an important assay for the genome-wide study of protein-DNA interactions and gene regulation [298]. A typical ChIP experiment involves treating cells with a cross-linking agent like formaldehyde to preserve protein-DNA interactions. The DNA is then sheared into small, relatively uniform fragments using either sonication or enzymatic digestion, and specific protein-DNA complexes are immunoprecipitated with an antibody that targets the DNA binding protein of interest. Following immunoprecipitation, the cross-linking is reversed, proteins digested and DNA is recovered. The DNA can then be analyzed by a number of different methods to determine which DNA fragments were in complex with the protein of interest. Standard PCR methods are often employed to measure the relative abundance of a particular DNA sequence enriched by immunoprecipitation using the protein of interest versus an immunoprecipitation with a non-specific antibody control or no antibody control. Alternatively, genome wide analysis of protein binding sites can be acheived by hybridization of the DNA pool to a tiling array (ChIP-chip) or by end sequencing the precipitated DNA (ChIP-Seq) [299].

Chromatin immunoprecipitation followed by sequencing (ChIP-Seq) is emerging as a powerful technique to generate genome wide read-out of the protein binding sites and is achieved by end sequencing millions of different DNA fragments [300]. Owing to the tremendous progress in next-generation sequencing technology, ChIP–seq offers the feasibility of generating higher resolution genome-wide profiling of DNA-binding

proteins with less background noise and greater coverage and has thus become an indispensable tool for studying gene regulation and epigenetic mechanisms. Nearly all ChIP–seq data to-date have been generated through the Illumina Genome Analyzer, although other platforms, such as Applied biosystems' SOLiD and the Helicos platform, are now available [301]. ChIP-Seq data analyses typically generate regions of high sequencing read density, referred to as "peaks" that evoke the visual impression of many reads mapping to a specific region. In other words, peaks are genomic regions that are enriched by the antibody of interest in comparison to fewer reads mapping to the observed non-enriched genomic background.

In order to generate high resolution whole genome profiles of genes regulated by Tip60, chromatin immunoprecipitation (ChIP) assays were carried out using *Drosophila* Schneider 2 (S2) cells, the details of which are described below.

Cell culture

Drosophila S2 cells (Invitrogen, Carlsbad, CA) were grown at 22°C in Schneider's Drosophila Medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated Fetal Bovine serum (SAFC Biosciences, Lenexa, KS) and Penicillin-Streptomycin (Invitrogen, Carlsbad, CA).

Chromatin immunoprecipitation

Chromatin precipitation assays were performed using ChIP-IT Express Kit (Active Motif, Carlsbad, CA), following the manufacturer's protocol. Briefly, protein from 1-5 x 10^7 cells was cross-linked to DNA using 1% formaldehyde for 10 mins at

room temperature. Cross-linking was quenched by adding 2.5M glycine to a final concentration of 0.125M. Quenching was performed at room temperature for 10 minutes with constant agitation. The cells were the pelleted by centrifugation for 10 mins at 2500 rpm at 4°C. The cells were washed with 1 mL of 1X PBS, pelleted by centrifugation for 10 mins at 2500 rpm at 4°C. The pellet was then resuspended in 1 mL of Cell Lysis Buffer supplemented with 5uL each of protease inhibitor cocktail (PIC) and PMSF. The cells were transferred to an ice cold douncer and dounced on ice to aid in release of nuclei. Lysed cells were transferred to a 1.7ml centrifuge tube and centrifuged for 10 min at 5000 rpm at 4°C to pellet the nuclei. The supernatant was removed and the pelleted nuclei were resuspended in 350 ul of Shearing Buffer supplemented with 1.75ul each of PIC and PMSF. The nuclei were sonicated at 30% output using Sonic dismembrator (Fischer Scientific, Pittsburg, PA) on ice for 40 seconds. Sonication was carried out for a total of 3 times with 2 min intervals on ice. The sheared chromatin was centrifuged at 15000 rpm for 10 mins at 4°C. The supernatant containing the sheared chromatin was transferred to a fresh 1.7 ul centrifuge tube. In order to check the shearing efficiency, a 50 ul aliquot of the sheared chromatin was reverse cross-linked by incubating at 65°C overnight. Thereafter, the sheared chromatin sample was treated with 10 ul of proteinase K by incubating at 37°C for 15 mins and DNA was precipitated using phenol:chloroform. 10 ul of the sheared chromatin was loaded on a 1% agarose gel and electrophoresed at 100V for 45 mins. Optimal sonication shearing resulted in a 150 bp – 1500 bp smear (Figure 1).

Chromatin immunoprecipitation (ChIP) was carried out with 50ug of sheared chromatin using three different antibodies: A) 10 ug of RNA Pol II antibody (Abcam,

Cambridge, MA); B) 10 ug of Tip60 antibody that targets residues 450-513 in the C-terminus of Tip60 (Abcam, Cambridge, MA); C) 10 ug of Tip60 antibody that targets residues 500-513 in the C-terminus of Tip60 (Open Biosystem, Huntsville, AL). Each ChIP reaction was set up in 1.7 ul centrifuge tubes by adding 25 ul of protein G magnetic beads, 20 uL ChIP buffer I, 2 ul of PIC to 50 ug of chromatin and 10 ug of the respective antibody in a total reaction volume of 200 ul. A mock reaction containing all reagents except the antibody was also set up as a control. The tubes were incubated at $4^{o}C$ overnight on end-to-end rotator. Following this incubation, the beads were washed once with ChIP buffer I and twice with ChIP buffer II. The washed beads were then resuspended in 50 ul Elution Buffer AM2 and incubated at room temperature on an end-to-end rotator. 50ul of Reverse Cross-linking Buffer was added to the eluted chromatin and mixed by pipetting up and down. The beads were then allowed to pellet and the supernatant containing precipitated DNA (ChIP'd) was transferred to a fresh centrifuge tube. A small aliquot (usually 10 ul) of the sheared chromatin was also processed to serve as "Input" DNA. To 10 ul of the Input DNA sample, 88 ul of ChIP Buffer II and 2 ul of 5M NaCl were added. The ChIP'd DNA and Input DNA samples were incubated at $95^{o}C$ for 15 mins to reverse cross-linking and the treated with 2 ul proteinase K by incubating at $37^{o}C$ for 1.5 hrs. Proteinase K digestion was stopped by adding 2 ul of Stop Buffer at room temperature.

Quantitative PCR analysis

Following the final elution, cross-link reversal and proteinase K digestion of the immunoprecipitated chromatin, the ChIP'd DNA and Input DNA samples were analyzed by quantitative PCR analysis. Prior to PCR, the Input DNA was diluted a 100 fold in TE.

PCR reactions were carried out in triplicate in 20 ul reaction volumes containing 10 ul of SYBR green PCR master mix (Applied Bioystems, Poster City, CA, USA), 2 ul of DNA template and 1.5 uM each of forward and reverse primer. Quantitative PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Poster City, CA, USA). PCR was carried out by 40 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min with plate readings recorded after each cycle. *Drosophila* primer sets that amplify each of the following genes were used for the PCR analysis: GAPDH1 (Active Motif, Carlsbad, CA), Tip60 and LRP1. For each primer, fold enrichment was calculated using the slope of a standard curve generated from serial 10 fold dilutions of the Input DNA. First, the Ct values were used to estimate DNA quantity of the ChIP and No antibody control samples. Fold enrichment was then calculated as a ratio of the DNA quantity in the ChIP and No antibody control. Figure 2 illustrates significant fold enrichment of each of the above mentioned genes in DNA samples that were immunoprecipitated with the RNA Pol II or Tip60 antibodies.

Sequencing ChIP samples

ChIP-DNA library generation and sequencing

Sequencing of ChIP DNA samples will be performed using the Illumina HiSeq2000 platform. In order to allow massive parallel sequencing, ChIP samples have to be first converted into DNA libraries using Illumina recommendations. DNA library preparation and sequencing will be carried out at the DRC/IDOM Functional Genomics Core, University of Pennsylvania. Briefly, Illumina library generation involves introducing oligonucleotide adapters at the ends of the ChIP DNA fragments that were

bound by the protein of interest. These adapters allow hybridization of the sample to a flowcell containing a lawn of primers which is used for subsequent cluster generation and sequencing-by-synthesis.

During Illumina library preparation, the sheared ChIP DNA is end repaired. A single adenosine base ("A") is added to the 3'end of both strands, preparing them for ligation to the sequencing adapters. This is followed by annealing and ligation to the double stranded adapter containing a "T" overhang. A short PCR amplification (15-17 cycles) with primers annealing to the adapter sequence is performed to generate a population of adapter-ChIP DNA fragments termed the library. In order to enable sequencing of multiple sample libraries, barcoded Illumina libraries will be generated by replacing the above adapter sequences with "indexed" adapter sequences that contain adapter sequences followed by a nucleotide tag of at least two bases (called the barcode or index) and terminated by a "T" for annealing and ligation to the end repaired DNA containing an "A" overhang. Size selection on a 2% agarose gel allows isolation of the amplified DNA library between 150 and 350 bp. This is the optimal range of fragment size for hybridization to the flowcell and cluster generation (according to Illumina's recommendations).

ChIP-Seq data analysis plan

Genome Alignment

Following sequencing on the Illumina HiSeq2000 platform, Illumina Analysis Pipeline will be used for primary data acquisition, determining base calls and confidence scores from the fluorescent signals on the Genome Analyzer. Reads (sequence tags) that

are 35 bases long and have less than 5 ambiguous bases will be collected along with their corresponding quality tracks from the "Bustard" base calling module of Illumina Analysis Pipeline [302]. The reads will then be transformed into FASTQ format [303]. Input reads will be iteratively mapped to the *Drosophila melanogaster* genome (BDGP Release 5) using Bowtie alignment program [304] with default mismatches and indels allowance settings and the best genomic mapping site(s) will be reported.

Identification of enriched regions

After sequenced reads are aligned to the genome, the next step is to identify regions that are enriched in the ChIP sample relative to the control with statistical significance [301]. This can be achieved by 'peak callers' that scan along the genome to identify the enriched regions that are then visually represented as a 'peak' in corresponding regions of the genome. For the current ChIP-Seq data analysis, enriched regions in the target data will be identified using CisGenome, an integrated software system for analyzing ChIP-seq data [305,306]. CisGenome's peak calling algorithm uses a sliding window approach to scan the genome. For each window, the number of reads in the ChIP and the control sample will be counted. A binomial distribution will then be estimated from the data to calculate the probability of finding reads by chance within a genomic region. Using this estimation, a false discovery rate (FDR) for each window will be determined. Windows with a FDR smaller than a specified cutoff will be reported as 'peak call' and thus represent significant binding regions. For each ChIP-Seq dataset, the final ChIP-Seq read output will be recorded into files in wiggle track format (WIG) and browser extensible format (BED) for viewing the data in the UCSC Genome Browser.

Downstream Analysis

In order to gain insight into the biological implications of the ChIP-Seq data, the following follow-up analyses will be performed.

Motif discovery:

In order to identify potential regulatory sequences in the genome that are bound by Tip60 containing complexes, the ChIP-Seq data will be further analyzed to identify binding sequence motifs. This will be done by employing the recently published software tool, Peak-Motifs [307]. Peak-Motifs uses peak sequences generated from ChIP-Seq experiments to identify key binding motifs. It further compares the identified motifs with transcription factor binding motifs from various databases, predicts the location of binding sites within the peaks and exports them in a format suitable for visualization in the UCSC Genome Browser

Relationship to gene structure:

Another basic analysis that can be performed with the Tip60 ChIP-Seq data is to annotate the location of the peaks on the genome in relation to known genomic features, such as the transcriptional start site (TSS), exon-intron boundaries and the 3' end of genes. This will be done as described by [308]. Briefly, genomic co-ordinates of TSS, transcription end points, exon-intron junctions, intron-exon junctions will be collected from Drosophila melanogaster annotation database R5.5 (ftp://ftp. flybase.net/releases). Genes identified in the different experimental groups will be annotated by aligning them in the same direction at the TSSs, at the mid-points of gene bodies and transcription end points, respectively.

In vivo gene expression analysis of target genes identified from ChIP-Seq

A) Functional annotation of target genes

Following identification of target genes that exhibit significant enrichment in the Tip60 ChIP-Seq data, functional annotation of the genes will be carried out using DAVID annotation tool [309]. A subset of genes that map to synaptic plasticity, learning and memory related pathways will be further analyzed as below.

B) Quantitative RT-PCR analysis

In order to gain insight into the mechanism underlying the learning and memory defects observed in the Tip60 mutant flies, the ChIP-Seq target genes that map to synaptic plasticity and/or learning/memory pathways will be examined by quantitative RT-PCR analysis to determine whether misregulation of such genes contribute to the observed memory defects. This will be done by preparing cDNA from whole adult flies that ubiquitously express either the HAT defective Tip60 mutant (dTip60^{E431Q}) or additional copies of the wild type Tip60 (dTip60^{WT}). Expression of the respective transgenes will be driven using the pan-neuronal ELAV-GAL4 driver. Canton S flies will serve as controls for this analysis. Gene expression changes will then be analyzed by quantitative RT-PCR analysis using gene specific primers.

CHAPTER 5: PROSPECTS FOR FUTURE RESEARCH

The field of neuroepigenetics is evolving at a rapid pace. It is also becoming an enticing area of research as increasing number of studies bolster the concept that epigenetic dysregulation of gene expression and chromatin architecture could play a prominent role in the pathophysiology of age related memory disorders. Recent evidence indicates that chromatin remodeling via histone acetylation plays a crucial role in regulating synaptic and cognitive function [40] [310]. However, further studies are needed to identify specific HATs that mediate these effects and to determine the precise sites of histone acetylation alterations, key genes affected, and associated signaling changes involved in this regulatory mechanism. Understanding how specific HATs impacts gene expression changes in vivo has been greatly aided by chromatin immunoprecipitation studies coupled with high throughput sequencing technologies. While the current Tip60 based ChIP-Seq studies will aid in mapping protein binding sites across the genome and provide spatial and temporal resolution of protein-DNA binding events, further genetic and experimental manipulation studies are required to place the ChIP data in a meaningful biological context. A starting point in this regard would be to examine how specific gene targets identified by the Tip60 ChIP-Seq studies are affected by misregulation of Tip60's HAT function, for instance in flies expressing $dTip60^{E431Q}$ or $dTip60^{WT}$. Furthermore, ChIP using specific histone antibodies followed by qPCR using gene specific primers will help identify specific histone proteins and lysine residues that are targeted by Tip60 for gene specific transcriptional regulation and provide mechanistic insight into how such target genes are affected by misregulation of Tip60's HAT activity.

Histone acetylation has been reported to play a role in synaptic plasticity, experience-dependent neural plasticity, learning and memory and neuroprotection [311,312]. A number of recent studies have also demonstrated a cognitive role for sleep in various animal models including humans [313]. While some researchers believe that sleep promotes global synaptic downscaling [314,315], others propose that sleep also triggers experience-dependent synaptic upscaling able to consolidate recently acquired memories [316,317]. Consistent with this notion, flies increase sleep both after exposure to an enriched social environment and after courtship conditioning that induce long-term memory [318]. Increased sleep after social enrichment has also been reported to be dependent upon genes that are required for learning and memory, including genes that alter cyclic adenosine monophosphate signaling [319]. In order to examine if Tip60 plays a role in experience-dependent gene expression changes that underlie neural plasticity, specific Tip60 regulated genes (identified by ChIP-Seq studies) that have memory related functions can be examined in flies raised in isolation versus flies raised in groups (socially enriched). Furthermore, the effect of sleep deprivation on regulation of such gene targets and on memory formation in the $dTip60^{E431Q}$ and $dTip60^{WT}$ flies can be examined to gain insight into whether Tip60 also plays a role in sleep dependent mechanisms of neural plasticity that underlie memory formation. While our studies thus far have identified a neuroprotective role for Tip60 under neurodegenerative conditions, Tip60 seemingly operates in a highly complex and multifunctional manner at a large number of genomic loci as evidenced by the multitude of genes bidirectionally regulated by Tip60 [69]. Therefore, it will be important to examine and clarify precisely which of these genomic loci impacted have degenerative effects when misregulated by Tip60 as

well as identify Tip60 regulated neuroprotective factors. Such studies will help pinpoint the specific roles such gene targets play in a particular neuronal process and may thus serve as better targets for epigenetic based therapeutic interventions.

It is becoming increasingly clear that in response to signaling events, the three dimensional organization of the genome influences recruitment of *cis*-acting regulatory elements and chromatin modifying enzymes to transcriptional hotspots [320]. For instance, recent three dimensional reconstruction analyses show that the nuclei of hippocampal neurons undergo infolding and changes in chromatin organization in response to short bursts of synaptic activity. Such nuclear infolding events were initiated by intrasynaptic NMDA receptors that lead to transcriptional activation mediated by Ca^{2+} and ERK-MAPK signaling [321]. However, for many known post-translational modifications that recruit chromatin regulatory enzymes, the signaling mechanisms that link them to environmental cues remains obscure [2]. With regards to Tip60, identification of signaling pathways that modulate its activity will greatly enhance our understanding of how Tip60 mediated neuronal transcriptional programs respond to environmental changes. It is also vital to understand if and how Tip60 interplays with other chromatin modifiers to regulate gene expression.

Given the essential roles Tip60 plays during development as well as in maintaining the functional status of differentiated neurons in the adult brain, it is essential to distinguish such roles. For instance, the morphological effects observed due to misregulation of Tip60 in the mushroom body could be induced either by developmental and/or degenerative defects in the axons. Developmental abnormalities in axonal growth, branching, or guidance or degeneration leading to axon loss could result in the shortening

and thinning of MB lobes observed in the dTip60^{E431Q} mutants. The MB γ-axons are the earliest born and develop as early as in the first instar larva while the α'/β' and α/β axons are third instar larval and pupal born, respectively. Although no obvious phenotype was observed in the dTip60^{E431Q} third instar larva, to differentiate between developmental and degenerative effects, wild type control and mutant brains can be analyzed during the pupal stage when the α/β axons are still developing. Wild type, control and mutant brains can be examined one day after puparium and four days after puparium. If the MB phenotype is observed in the early pupal brains, persistence of the same throughout pupal development without exacerbation in the adult flies would indicate that the observed MB phenotype in the dTip60^{E431Q} flies is the consequence of developmental defects rather than age related retraction of these axons.

Additionally, to examine if the observed MB phenotypes are induced post-developmentally, the temporal and regional gene expression targeting (TARGET) system [322] can be utilized. In the TARGET system, the conventional GAL4-UAS system is combined with a temperature sensitive GAL80 molecule (GAL80ts), which represses GAL4 transcriptional activity at permissible temperatures, thus providing temporal control over GAL4 activity. The system can be induced at any time during development or adulthood. One to several copies of the P[tubP-GAL80ts] (GAL80ts fused to tubulin promoter and thus expressed ubiquitously) construct can be introduced into a fly line carrying the OK107- GAL4 driver. Double transgenic lines carrying OK107-GAL4 and P[tubP-GAL80ts] can then be crossed to either the UAS-GFP; UAS-dTip60^{E431Q} or UAS-GFP; UAS-dTip60^{WT} flies. F1 progeny resulting from each of these crosses can be raised to adulthood at lower temperatures (18°C) to repress GAL4 mediated expression of the

respective transgenes. The GAL4-UAS system can then be induced by exposing the F1 progeny after eclosion to elevated temperatures (e.g. 30°C) and MB phenotypes examined using GFP expression. The presence of MB axonal growth defects in the adult flies would then indicate that the observed MB phenotypes are a consequence of degenerative effects induced post-developmentally by misregulation of Tip60's HAT activity.

If the observed MB phenotypes in the dTip60^{E431Q} and dTip60^{WT} flies are verified by the above experiments to be developmental defects rather than post-developmental effects, it is likely that such anatomical effects contributed to the memory defects observed in these flies or could be induced independently by impacting specific memory related neural circuits. This can be examined by utilizing the TARGET system described above. F1 progeny resulting from a cross between P[tubP-GAL80ts]; OK107-GAL4 and either UAS-dTip60^{E431Q} or UAS-dTip60^{WT} can be raised at 18°C to adulthood and then transferred to 30°C to induce GAL4 mediated transgene expression specifically in the adult flies. Memory defects can then be assessed with the *Drosophila* courtship suppression assay using F1 male progeny expressing either dTip60^{E431Q} or dTip60^{WT}. The presence of memory defects in these flies similar to the ones observed when the transgenes are expressed throughout development would indicate that the memory defects are induced post-developmentally independent of the MB phenotypes.

Taken together, our studies thus far demonstrate yet another example of the importance of HAT function during nervous system development, regulation of certain neuronal genes associated with various forms of behavioral outputs like sleep, learning, memory and synaptic function and add dTip60 to the growing list of HAT chromatin

regulators critical for nervous system function. Future investigation into the molecular mechanisms underlying Tip60 HAT function in specific neuronal processes in the fly, particularly those associated with learning and memory, should enhance our understanding into the link between acetylation, cognitive aging and age-related neurodegenerative disorders and may further provide insight into the utility of specific HAT activators as therapeutic strategies.

CHAPTER 6: TABLES AND FIGURES

Chapter 2 Tables

Table 1. Transgenic fly lines used for this study.

Transgenic fly lines [a]	Source [b]
UAS-dTip60^{E431Q} A	Lorbeck et al., 2011
UAS-APP; dTip60^{E431Q} A	This study
UAS-APP dCT: dTip60^{E431Q} A	
UAS-dTip60^{E431Q} B	Lorbeck et al., 2011
UAS-APP; dTip60^{E431Q} B	This study
UAS-APP dCT; dTip60^{E431Q} B	
UAS-dTip60^{WT} A	
UAS-dTip60^{WT} B	
UAS-dTip60^{WT} C	
UAS-APP; dTip60^{WT} A	
UAS-APP; dTip60^{WT} B	
UAS-APP; dTip60^{WT} C	
UAS-APP dCT; dTip60^{WT} A	
UAS-APP dCT; dTip60^{WT} B	
UAS-APP dCT; dTip60^{WT} C	

[a] The Tip60 P-element insertion is located on chromosome 3 and the APP P-element insertion is located on chromosome 2.
[b] Indicates where the transgenic fly lines were generated.

Table 2. Developmental stage at which expression of the different transgenes induces lethality.

Transgenic fly lines [a]	Developmental Stage of Lethality [b]	
	Ubiquitous expression [c]	Pan-neuronal expression [d]
Wild type (*w*^{1118})	Not lethal	Not lethal
APP	Pupae / Adult	Pupae / Adult
APP dCT	Not lethal	Not lethal
dTip60^{E431Q} A	Late 3rd instar	Late 3rd instar*
APP; dTip60^{E431Q} A	Early 2nd instar (hatching delayed by 24 - 48 hrs)	Early 2nd instar*
APP dCT; dTip60^{E431Q} A	Late 3rd instar	Late 3rd instar*
dTip60^{E431Q} B	Late 3rd instar	Late 3rd instar
APP; dTip60^{E431Q} B	Early 2nd instar (hatching delayed by 24 - 48 hrs)	Early 2nd instar
APP dCT; dTip60^{E431Q} B	Late 3rd instar	Late 3rd instar
dTip60^{WT} lines A, B, C	Partially lethal	Partially lethal
APP; dTip60^{WT} lines A, B, C	Partially lethal	Partially lethal
APP dCT; dTip60^{WT} lines A, B, C	Partially lethal	Partially lethal

[a] Ten female virgin flies homozygous for the indicated transgene or control *w*^{1118} were crossed to seven males homozygous for the Gal4 driver. All crosses were carried out in triplicate at 25° C.

[b] The crosses were monitored daily and the developmental stage at which lethality occurred was scored.

[C] The 337-Gal4 was used to drive ubiquitous expression of transgenes.

[d] The 179-Gal4 driver located on the X-chromosome was used to drive pan-neuronal expression of transgenes. * Neuronal expression of low expressing independent fly line dTip60 HAT mutant (dTip60^{E431Q} A) alone or in conjunction with APP/APP dCT induced lethality in a fraction of the respective F1 progeny at the indicated developmental stage while the remainder of F1 progeny did not exhibit any lethal effect.

Table 3. Apoptosis pathways significantly misregulated in response to dTip60 HAT loss.

Apoptosis related pathway	Number of genes
Alzheimer disease – presenilin pathway	11
Angiogenesis	28
Apoptosis signaling pathway	19
ATP synthesis	3
Denovo purine biosynthesis	15
Denovo pyrimidine deoxyribonucleotide biosynthesis	7
Denovo pyrimidine ribonucleotide biosynthesis	6
EGF receptor signaling pathway	26
FAS signaling pathway	8
FGF signaling pathway	28
Huntington disease	37
Integrin signaling pathway	32
Notch signaling pathway	4
Oxidative stress response	11
P53 pathway	33
Parkinson disease	15
Wnt signaling pathway	46

Table 4: Gene expression changes of dTip60^{E431Q} misregulated target genes in the different transgenic lines

Gene Name [a]	Transgenic Fly Line (Relative Fold Change) [b]			
	dTip60^{E431Q}	dTip60^{WT}	APP; dTip60^{E431Q}	APP; dTip60^{WT}
Buffy §, ǂ	1.4	1.6	-1.5	3.5
ALiX	1.4	2.1	1.5	2.3
CalpA §, ǂ	1.5	3.5	-2.1	-1.8
TRAF4 §, ǂ	1.7	3.9	-1.5	-1.7
Frizzled §	2.3	-1.5	-2.1	-1.5
Wingless §	2.4	-1.7	-1.9	-1.5
dMyc §	3.8	-3.2	-2.5	-2.3
PDCD5 §	-4.7	2	1	1.8
Dmel\CG9418 ǂ	1193.4	14.8139954	824.094897	2472.348951

[a] Quantitative RT-PCR analysis was performed for the indicated target genes

[b] Staged second instar larvae ubiquitously expressing the indicated transgene(s) were used for cDNA preparation. Quantitative RT-PCR reactions were carried out in triplicate and the relative fold change was calculated using the 2-ΔΔCT method using RP49 as control. § Genes that were differentially regulated between flies expressing the Tip60 HAT mutant dTip60^{E431Q} alone and in conjunction with APP. ǂ Gene that were differentially regulated between flies overexpressing dTip60^{WT} alone or together with APP.

Chapter 2 Figures

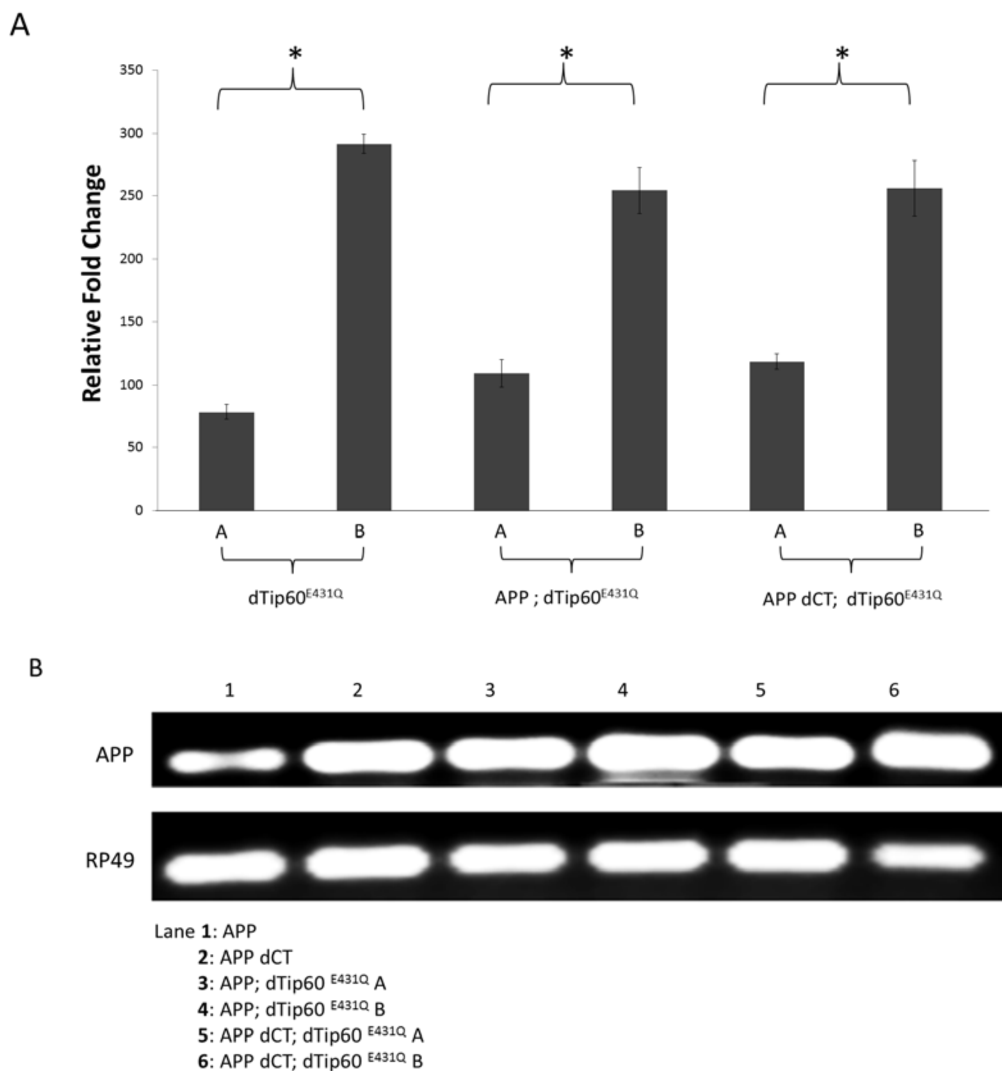


Figure 1. Generation and characterization of dTip60^{E431Q} containing APP or APP-dCT double transgenic flies. The dominant negative HAT defective lines dTip60^{E431Q} A or dTip60^{E431Q} B were introduced into an APP or APP dCT background using standard genetic techniques. **(A)** Histogram depicting qPCR analysis of exogenous levels of dTip60^{E431Q} in staged F1 second instar larval progeny resulting from a cross between the ubiquitous driver 337 and either dTip60^{E431Q} (lines A and B), APP; dTip60^{E431Q} (lines A and B) or APP dCT; dTip60^{E431Q} (lines A and B). 337-Gal4 crossed to w^{1118} served as a control. Quantification of the exogenously expressed dTip60^{E431Q} mRNA levels relative to endogenously expressed dTip60 mRNA was done using the comparative CT method with RP49 as internal control as described in (Lorbeck *et al,* 2011). Asterisks (*)

Figure 1 (Continued)

indicate significant fold change between the lines A and B for each genotype with values of $p<0.05$; n = 3. Error bars represent standard error of the mean. **(B)** Semiquantitative RT-PCR analysis of APP or APP dCT expression in the different transgenic lines to confirm APP transgene presence. cDNA was prepared as before from staged second instar larvae ubiquitously expressing $dTip60^{E431Q}$ with APP or APP dCT (lines A or B in each case) and PCR amplified using primers that flank a 100 bp region in the N-terminal portion of APP. PCR products were visualized using 2% agarose gel containing ethidium bromide. Staged second instar larvae ubiquitously expressing APP or APP dCT were used as controls.

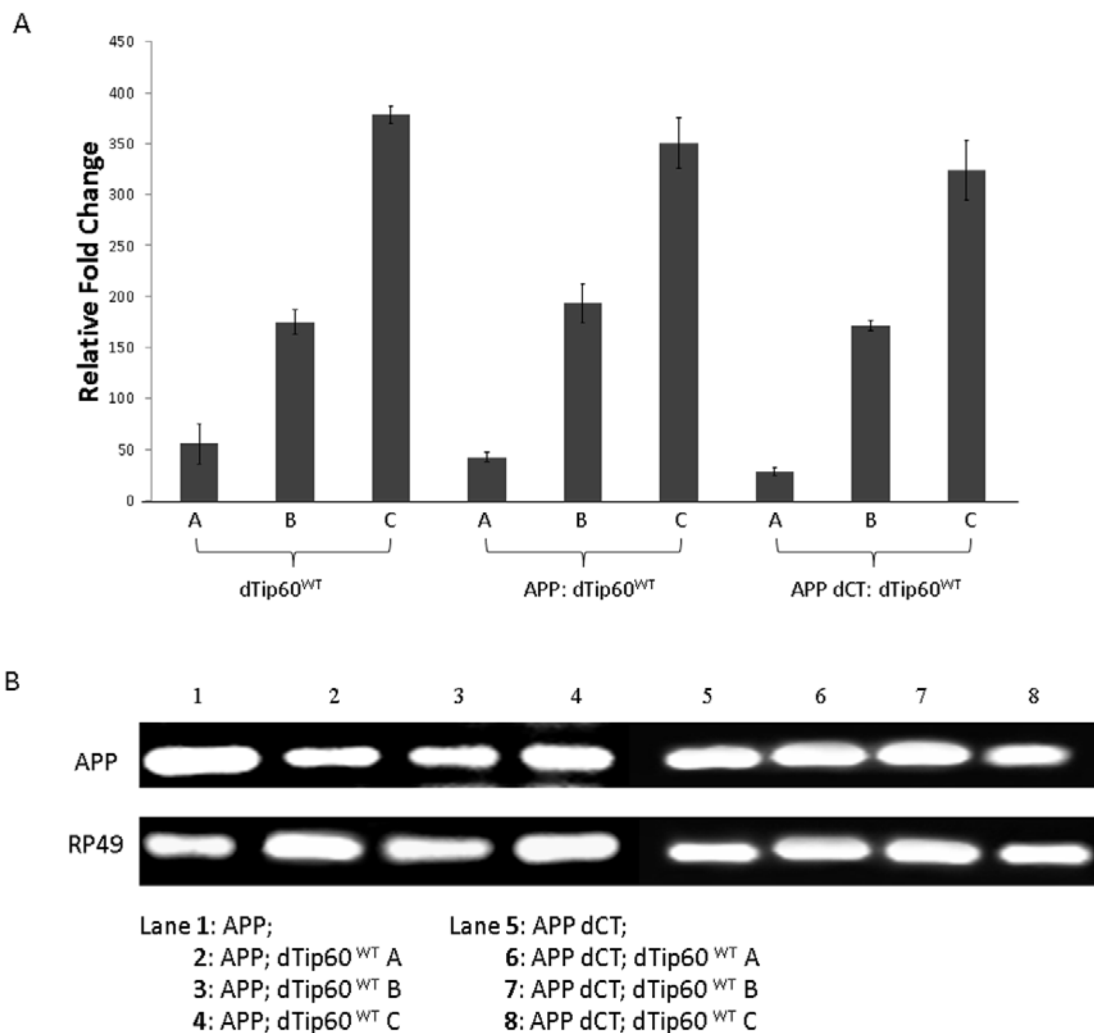


Figure 2. Generation and characterization of dTip60^{WT} containing APP or APP-dCT double transgenic flies. Flies expressing varying levels of wild type dTip60 (low, medium and high) were generated and then each introduced into APP or APP dCT background using standard genetic techniques. **(A)** The amount of wild type dTip60 that is exogenously induced relative to endogenous dTip60 was quantified by RT-PCR analysis of staged F1 second instar larvae resulting from the a cross between the ubiquitous driver 337 and either dTip60^{WT} (lines A, B and C), APP; dTip60^{WT} (lines A, B and C) or APP dCT; dTip60^{WT} (lines A, B and C). 337-Gal4 crossed to w^{1118} was used as control. The relative fold change in mRNA expression levels between exogenous and

Figure 2 (Continued)

endogenous dTip60 was measured as described before using the comparative CT method with RP49 as the internal control, and these results are summarized in the histogram. The amount of exogenously induced wild type dTip60 levels is significantly different between lines A, B and C in each case with values of $p<0.05$; $n = 3$. Error bars represent standard error of the mean. **(B)** Semi-quantitative RT-PCR analysis of APP or APP dCT expression in the different dTip60^{WT} containing transgenic lines to confirm APP transgene presence. cDNA was prepared as before from staged second instar larvae ubiquitously expressing dTip60^{WT} with APP or APP dCT (lines A, B or C in each case) and PCR amplified using primers that flank a 100 bp region in the N-terminal portion of APP. PCR products were visualized using 2% agarose gel containing ethidium bromide. Staged second instar larvae ubiquitously expressing APP or APP dCT were used as controls.

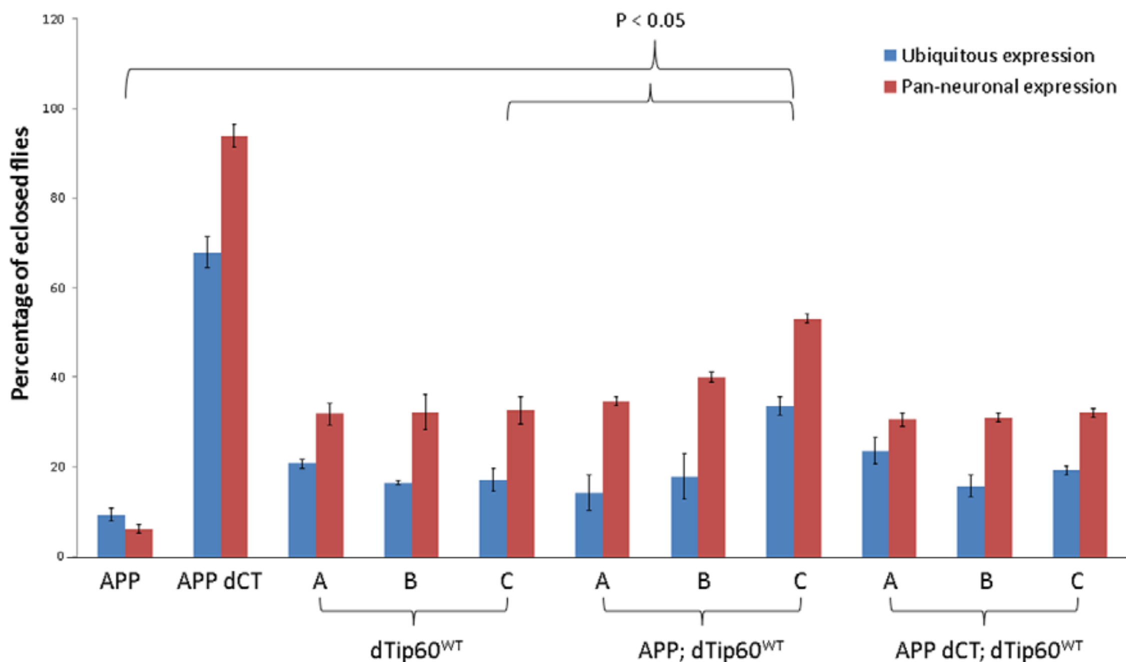
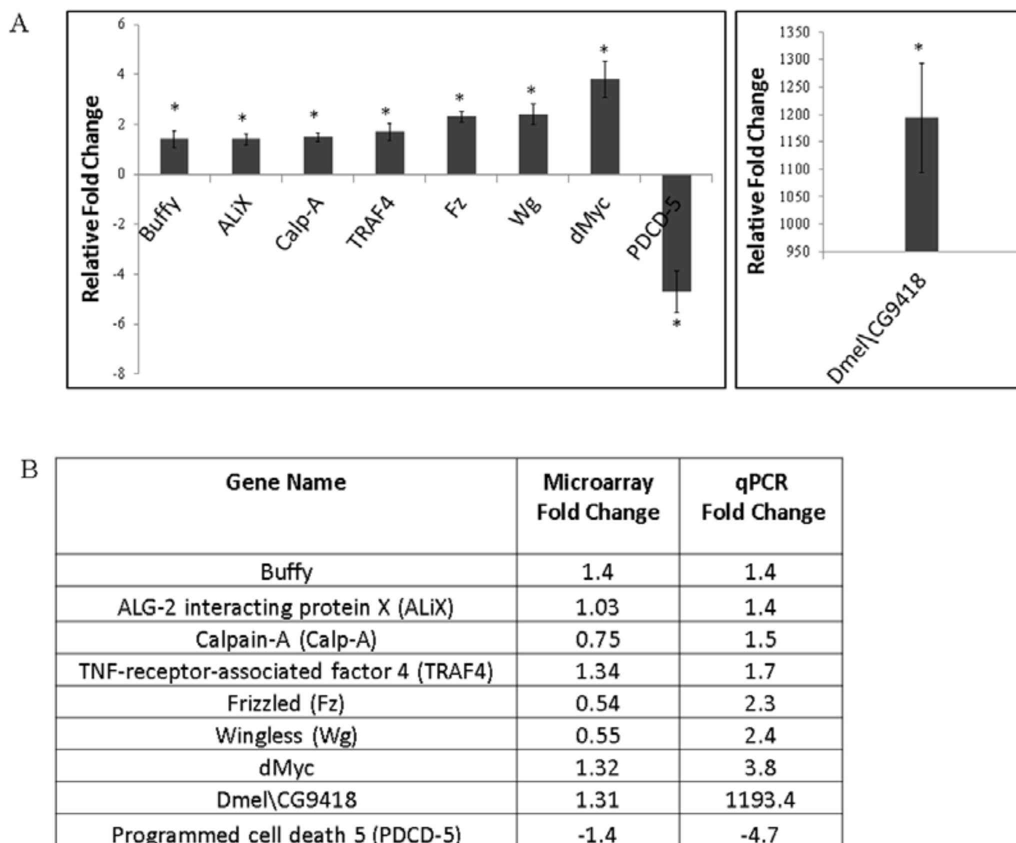


Figure 3: Viability analysis indicates genetic interaction between Tip60 and APP in *Drosophila*. The indicated transgene was expressed ubiquitously in the fly using 337-Gal4 driver or pan-neuronally using 179y-Gal4 driver. The number of F1 progeny that eclosed were counted daily. The percentage of eclosed flies was calculated relative to the wild type control (w^{1118}). All crosses were carried out in triplicate at 25° C. Overexpression of APP drastically reduced viability to < 10% while no effect was observed due to expression of truncated version of APP lacking its C-terminal domain. Overexpression of varying levels of wild type dTip60 ($dTip60^{WT}$) also reduced viability in a dose independent manner. However, co-expression of $dTip60^{WT}$ with APP partially rescued the lethal effects induced by APP expression in a dose dependent manner with the maximum effect observed with high levels of $dTip60^{WT}$. In the presence of APP lacking the C-terminus, overexpression of $dTip60^{WT}$ had similar effects seen in flies that overexpressed $dTip60^{WT}$ alone.



Gene Name	Microarray Fold Change	qPCR Fold Change
Buffy	1.4	1.4
ALG-2 interacting protein X (ALiX)	1.03	1.4
Calpain-A (Calp-A)	0.75	1.5
TNF-receptor-associated factor 4 (TRAF4)	1.34	1.7
Frizzled (Fz)	0.54	2.3
Wingless (Wg)	0.55	2.4
dMyc	1.32	3.8
Dmel\CG9418	1.31	1193.4
Programmed cell death 5 (PDCD-5)	-1.4	-4.7

Figure 4. Quantitative RT-PCR validation of selected apoptosis related genes identified by microarray analysis. **(A)** Histogram showing relative fold change in expression level of apoptosis related target genes in flies expressing dTip60^{E431Q} A. Staged second instar larvae were used for cDNA preparation. RT-PCR reactions were carried out in triplicate and the fold change was calculated using the 2-ΔΔCT method using RP49 as control. **(B)** List of selected apoptosis related target genes identified by microarray analysis and validated in the dTip60^{E431Q} A line using quantitative RT-PCR.

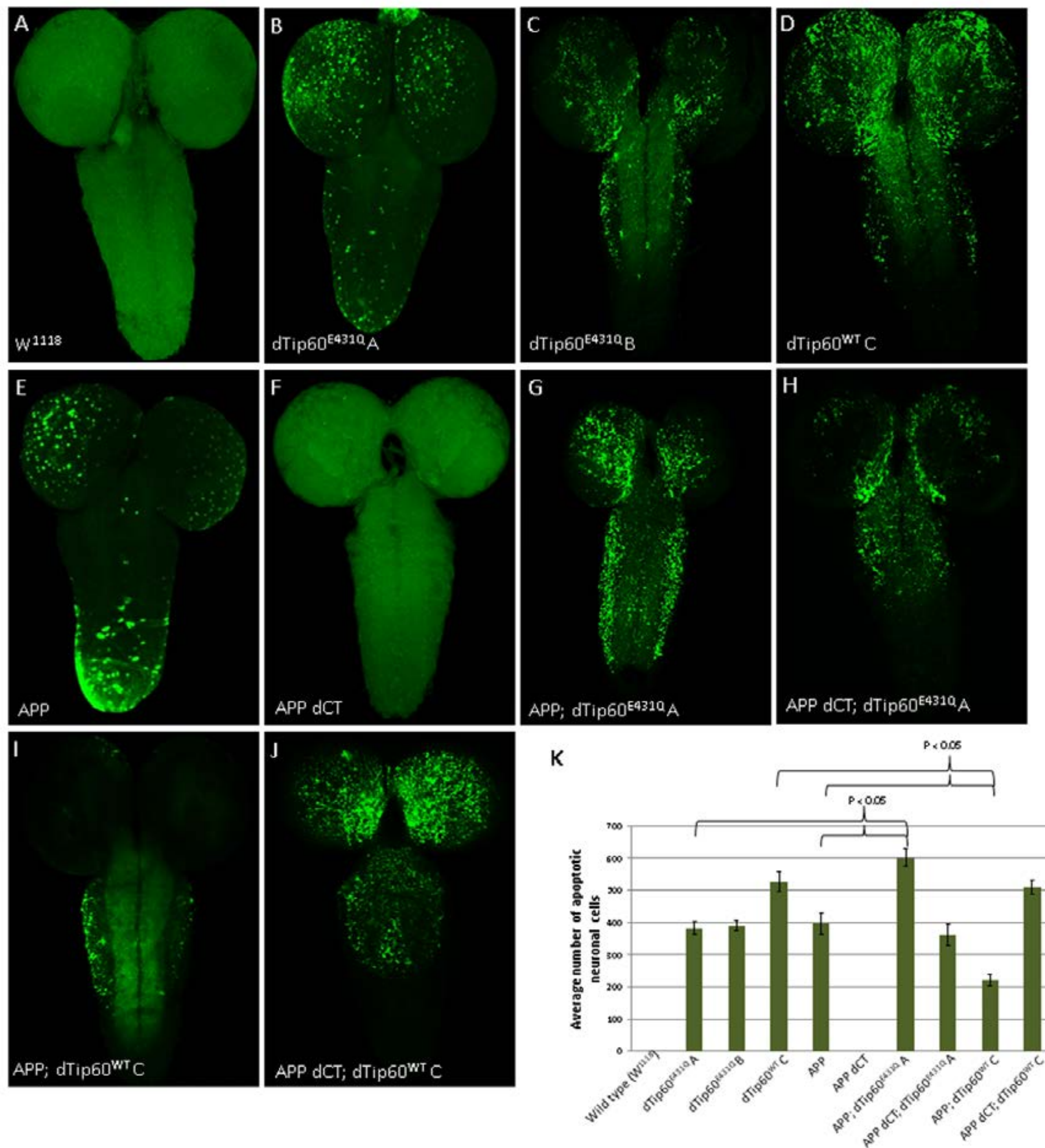


Figure 5. dTip60 mediates APP induced apoptotic neuronal cell death in the *Drosophila* central nervous system. Representative confocal images of neuronal apoptosis visualized by TUNEL staining of brains from staged third instar larvae expressing indicated transgenes driven by pan-neuronal driver 179-GAL4 . The w^{1118} larvae used as genetic background control showed no apoptosis (**A**). Pan neuronal

Figure 5 (Continued)

expression of dTip60^{E431Q} induces apoptosis in a dose independent manner as evident from comparable levels of apoptosis seen in fly lines expressing low **(B)** or high **(C)** levels of dTip60^{E431Q}. Overexpression of wild type dTip60 increased neuronal cell death due to apoptosis (**D**). The C-terminal domain of APP induces apoptosis as evident from TUNEL positive apoptotic cells in flies overexpressing APP **(E)** while no apoptosis was observed in flies expressing a truncated version of APP lacking the C-terminal domain (**F**). Co-expression of APP with low levels of dTip60^{E431Q} (dTip60^{E431Q} A) enhances the severity of apoptosis phenotype in a synergistic manner **(G)** that is dependent on the APP C-terminal domain. Co-expression of APP lacking C-terminus with dTip60^{E431Q} A exhibited apoptosis that was comparable to that seen when dTip60^{E431Q} A was expressed alone **(H)**. Overexpression of wild type dTip60 in the APP overexpressing background partially rescued the apoptosis phenotype **(I)** but in the presence of APP lacking C-terminus exerted similar severity seen in flies overexpressing wild type dTip60 alone. Images shown represent projections of 1 um confocal slices. Apoptotic cells in the different genotypes were quantified by counting the number of TUNEL positive cells in the entire fly brain (**K**).

Chapter 3 Figures

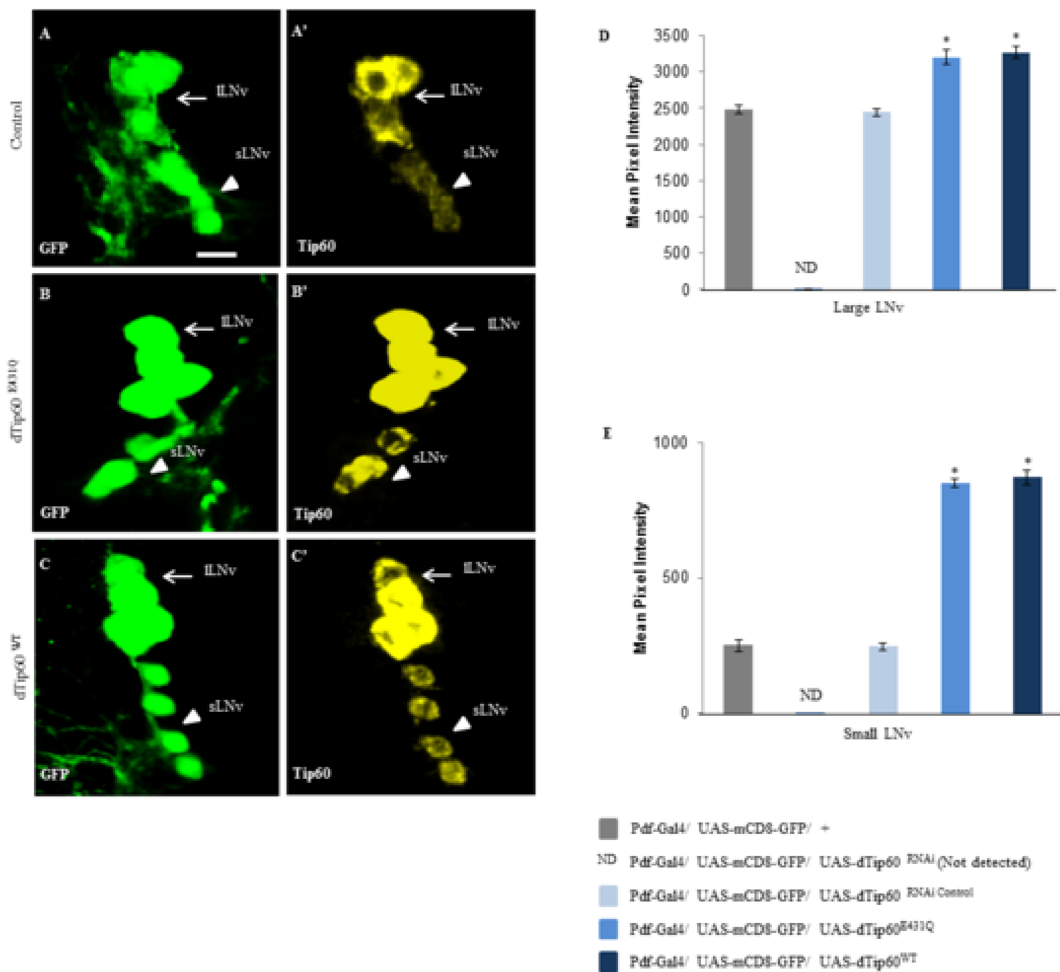


Figure 1. Tip60 immunolocalization in the *Drosophila* ventrolateral neurons (LNvs). Representative confocal image of LNvs in adult brain from Pdf-Gal4/ UAS-mCD8-GFP/+ (control) flies stained with anti-Tip60 antibody showing strong Tip60 immunoreactivity in lLNv (arrow) and relatively weaker expression in sLNv (arrowhead). GFP is shown in green (**A'**) and Tip60 in yellow (**A'**). Scale bar, 10uM. GFP and Tip60 staining of LNvs in adult brains of Pdf-Gal4/ UAS-mCD8-GFP/UAS-dTip60^{E431Q} flies (**B** and **B'**) and Pdf-Gal4/ UAS-mCD8-GFP/UAS-dTip60^{WT} flies (**C** and **C'**). Quantification of Tip60 levels in LNvs (**D** and **E**, * $P < 0.05$ compared to Control) expressing dTip60^{RNAi}, dTip60$^{RNAi\ Control}$, overexpressing HAT mutant dTip60^{E431Q} or wild type dTip60^{WT}.

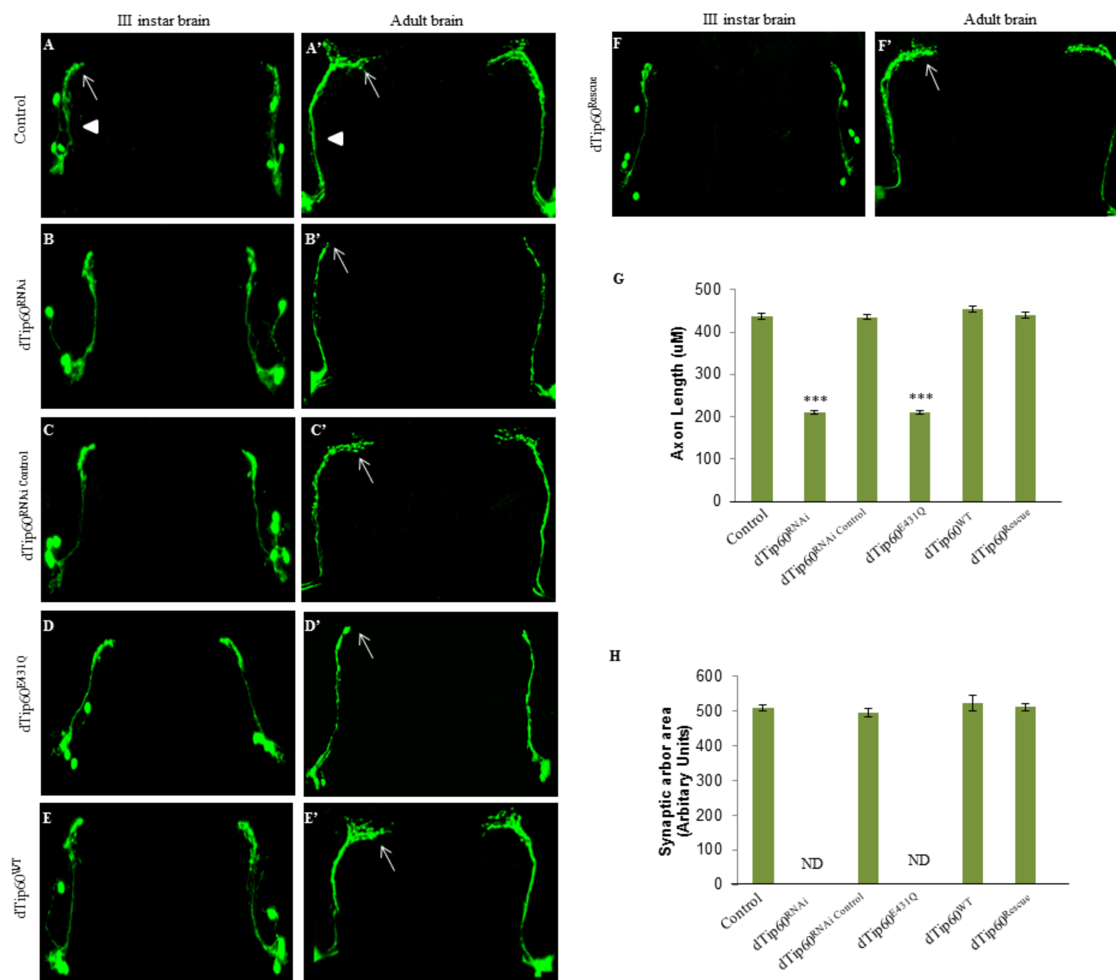


Figure 2. Loss of dTip60 HAT activity decreases axonal arborization in the adult sLNv. Representative confocal images of sLNv axon morphology in the Control (Pdf-Gal4/UAS-mCD8-GFP/+) third instar larval brain (**A**) and in the adult brain (**A'**), showing dorsally projecting axons (arrowhead) and terminal synaptic arbors (arrow). LNv specific induction of Tip60 RNAi response (dTip60^{RNAi})or expression of the HAT defective dominant negative dTip60 (dTip60^{E431Q}) has no effect on sLNv morphology in third instar larva (**B** and **D**) but leads to collapse of synaptic arbor in the adult brain (**B'** and **D'**). Expression of corresponding Tip60 RNAi control construct (dTip60$^{RNAi\ Control}$) or overexpression of wild type dTip60 (dTip60^{WT}) in the LNvs has no significant effect on sLNv axonal growth in the third instar larva (**C** and **E**) or the adult (**C'**and **E'**).

Figure 2 (Continued)

Expression of equivalent levels of wild type dTip60 in the dTip60^{E431Q} background does not affect the sLNv axons in the third instar larva (**F**) but rescues the HAT mutant induced retraction of sLNv axons in the adult brains (**F'**). Quantification of sLNv axon length (**G**, *** P<0.001 compared to Control) and synaptic arbor area (**H**) was done using NIH ImageJ software, ND – not determined. Error bars represent the 95% confidence interval.

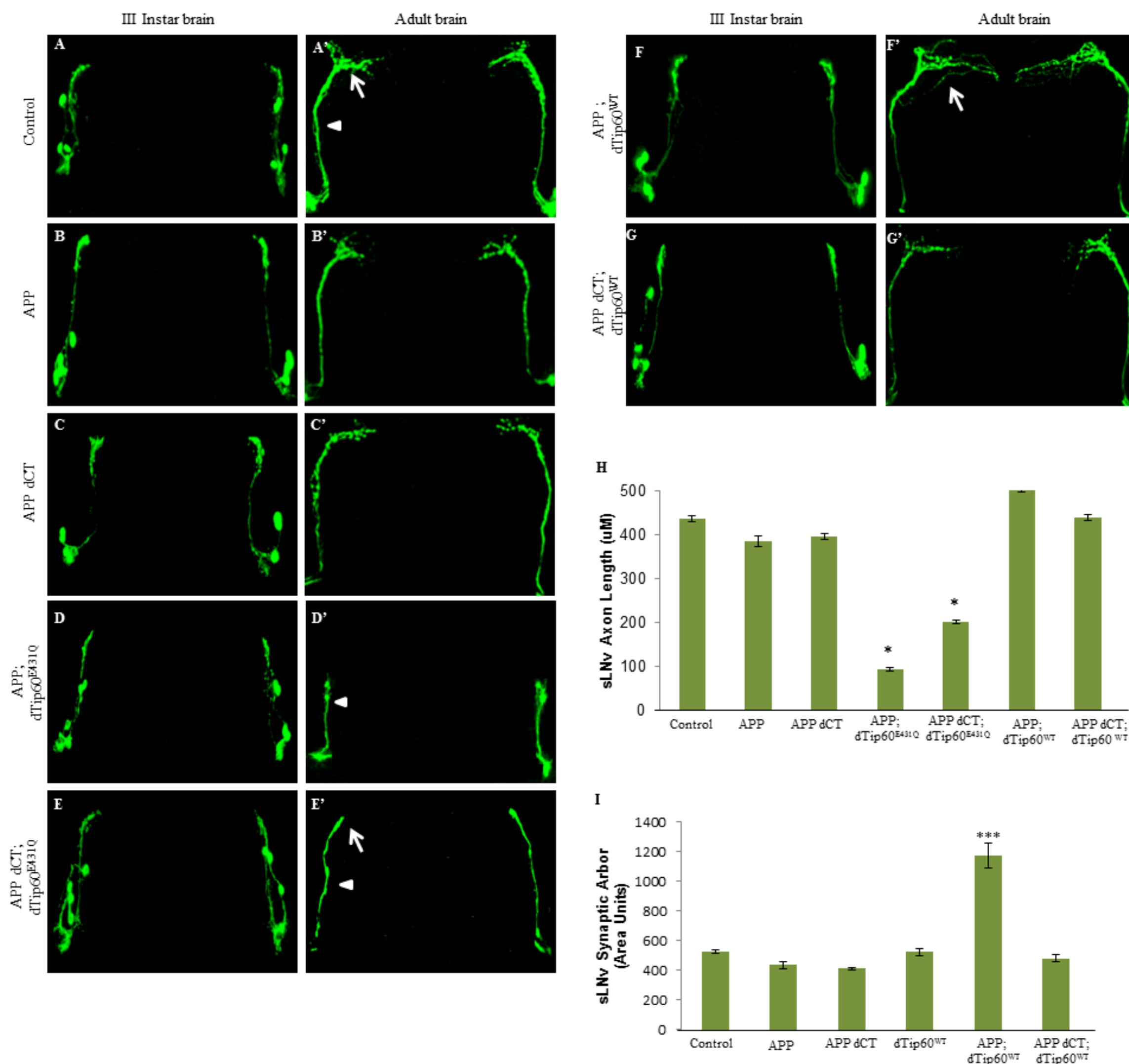


Figure 3. dTip60 modulates APP mediated effects on sLNv axonal growth post developmentally in the *Drosophila* CNS. Representative images of sLNv axons in adult *Drosophila* brains expressing UAS-mCD8-GFP reporter gene at 25°C in conjunction with each of the different GAL4 responsive transgenes under the control of the PDF-Gal4 driver. sLNv axonal arborization pattern in Control (Pdf-Gal4/ UAS-mCD8-GFP/ +) **(A)** third instar larval brain and **(A')** adult, respectively. LNv specific expression of neuronal isoform of **(B,B')** APP or **(C,C')** APP lacking the C-terminus (APP dCT) has no observable effect on sLNv axon structure in both third instar larvae and adult. **(D)** Co-

Figure 3 (Continued)

expression of full length APP with HAT activity deficient mutant dTip60 (dTip60^{E431Q}) has no effect in third instar larvae stage but **(D')** causes severe retraction of the sLNv synaptic arbor in the adult brain resulting in much shorter axons. **(E)** Co-expression of APP dCT with dTip60^{E431Q} causes no effects in third instar larvae **(E')** but causes shortening of the sLNv similar to shortening observed for dTip60^{E431Q} alone. Overexpression of APP with wild type dTip60 (dTip60 ^{WT}) causes **(F)** no effect in third instar larvae but **(F')** in adult brain causes the sLNv axons to extend further and arborize over a larger area, an effect that was dependent on the Tip60 interacting APP C-terminus as seen from the lack of any significant effect due to expression of APP dCT with dTip60^{WT} **(G, G')**. Histogram showing quantification of the sLNv axon length shows significant reduction of sLNv axons in flies co-expressing dTip60^{E431Q} with APP or APP dCT (* $P<0.05$ compared to Control) (**H**) and quantification of two dimensional area of the terminal axonal arbor using NIH Image J shows robust increase in flies co-expressing APP and dTip60^{WT} (**I**, ***$P<0.001$ compared to Control). Error bars represent 95% confidence interval.

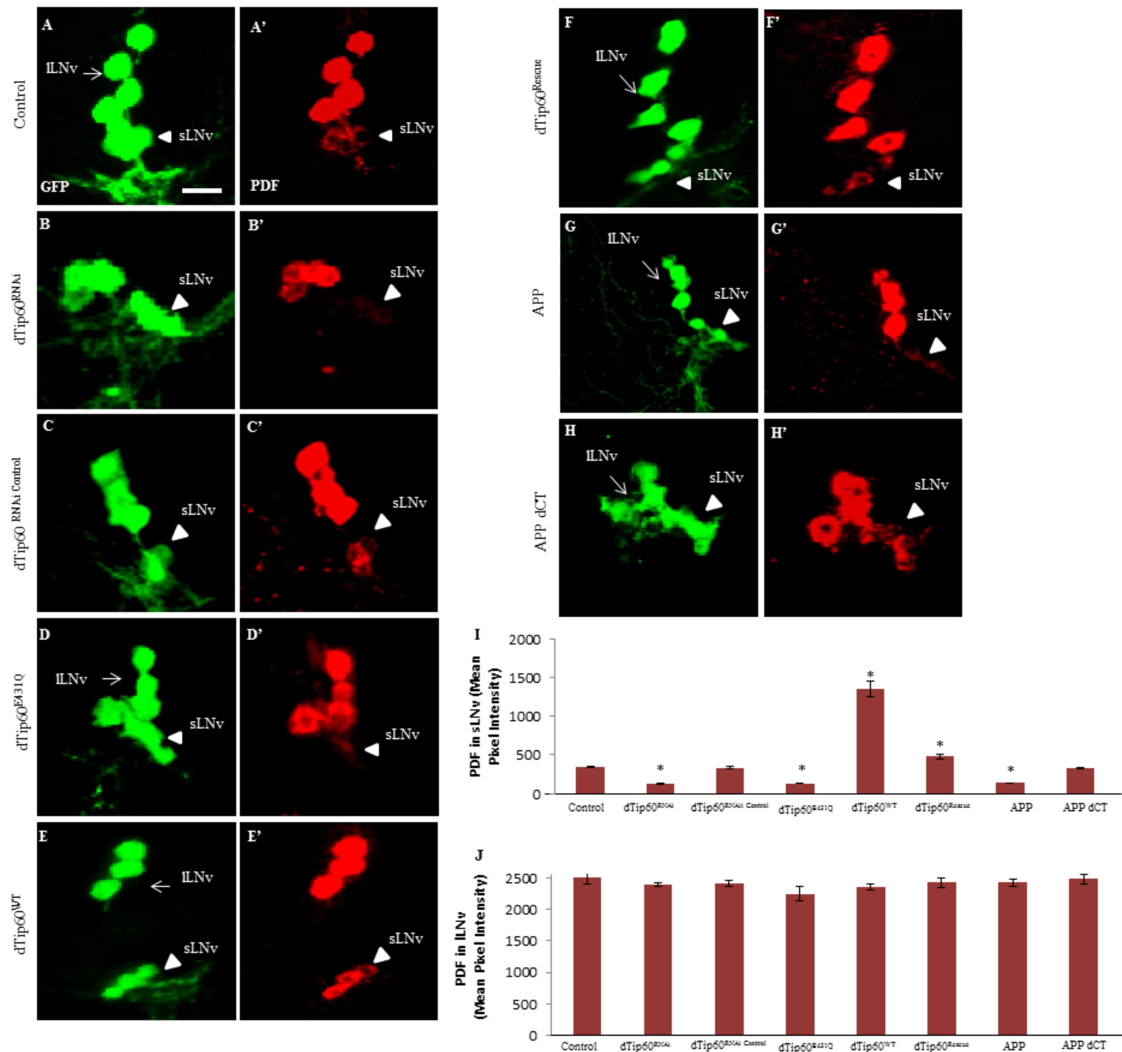


Figure 4. dTip60 or APP expression in the LNv subsets selectively affects PDF immunoreactivity (IR) in the sLNv and not the lLNv. Representative images of anti-GFP or anti-PDF staining in lLNv (arrow) and sLNv (arrow head) soma in adult flies expressing each of the different transgenes (indicated next to each panel) under the control of the Pdf-Gal4 driver. Panels **(A-H)** show anti-GFP staining used as marker to localize the lLNv and sLNv in the adult brains. Scale bar = 20 microns. **(A')** shows anti-PDF staining in lLNv (arrow) and sLNv (arrow head) soma in control flies (Pdf-Gal4/ UAS-mCD8-GFP/ +). **(B')** LNv specific induction of $dTip60^{RNAi}$ results in partial loss of PDF IR in sLNv while **(C')** expression of the corresponding $dTip60^{RNAi\ Control}$ has no

Figure 4 (Continued)

effect on PDF. **(D')** Expression of HAT activity defective dTip60^{E431Q} results in partial loss of PDF IR in sLNv while **(E')** overexpression of wild type dTip60 (dTip60^{WT}) significantly increases PDF IR in the sLNv. **(F')** Expressing equivalent amounts of wild type Tip60 with mutant dTip60^{E431Q} rescues dTip60^{E431Q} induced loss of PDF IR in sLNv in the dTip60^{Rescue} flies. **(G')** Expression of APP also resulted in partial reduction in sLNv PDF IR, an effect dependent on its C-terminus as seen from **(H')** lack of any observable effect on PDF IR due to expression of APP lacking the C-terminus (APP dCT). PDF expression in lLNv was largely unaffected in each of the different genotypes. Quantification of PDF-IR in sLNv **(I)** and in the lLNv **(J)**. Values represent average of four sLNvs and four lLNv PDF-IR from 15 brains for each genotype. A student t-test revealed significant decrease in sLNv PDF-IR in flies expressing dTip60^{E431Q} or APP compared to control (* $P<0.05$). Error bars represent 95% confidence interval.

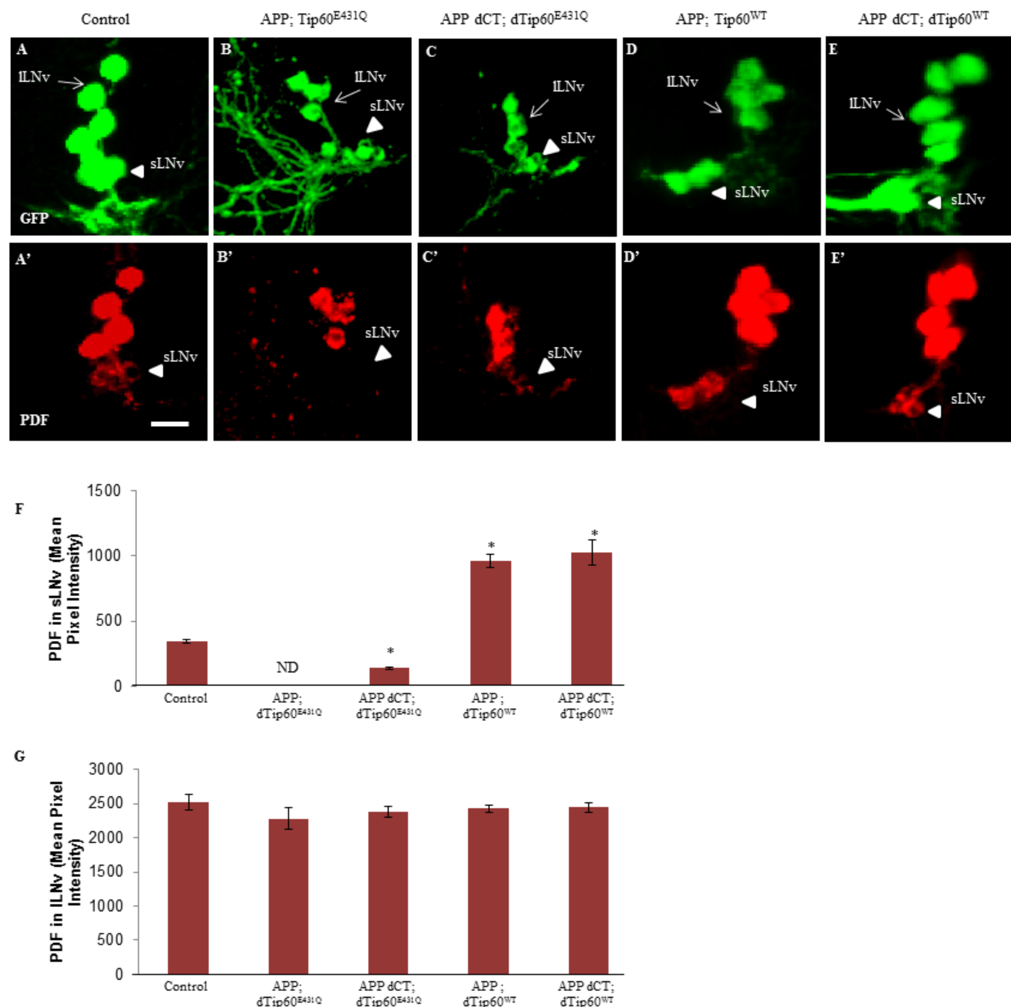


Figure 5. **dTip60 and APP functionally interact to regulate PDF expression in the sLNv.** Representative images of anti-GFP or anti-PDF staining of lLNv (arrow) and sLNv (arrowhead) in adult flies expressing the different transgenes under the control of the Pdf-Gal4 driver as indicated above each column. Panels **(A-D)** show anti-GFP staining used as marker to localize the lLNv and sLNv in the adult brains. Scale bar = 20 microns. **(A')** shows anti-PDF staining in lLNv (arrow) and sLNv (arrow head) soma in control flies (Pdf-Gal4/ UAS-mCD8-GFP/ +). **(B')** Co-expression of APP with HAT activity defective dTip60^{E431Q} resulted in selective loss of PDF immunoreactivity (IR) in the sLNv although PDF-IR in the lLNv was not affected. **(C')** dTip60^{E431Q} in the presence of APP dCT resulted in only a partial reduction of sLNv PDF IR similar to that observed when only dTip60^{E431Q} was expressed in the LNvs. **(D')** Expressing full length

Figure 5 (Continued)

APP or **(E')** APP lacking the C-terminus with wild type dTip60^{WT} both increased sLNv PDF IR but had no effect on PDF IR in the lLNv. Quantification of PDF IR in the sLNv **(F)** and the lLNv **(G)** revealed significant difference in PDF expression in the different genotypes compared to control (*P<0.05). Error bars represent 95% confidence interval. Scale bar = 20 microns.

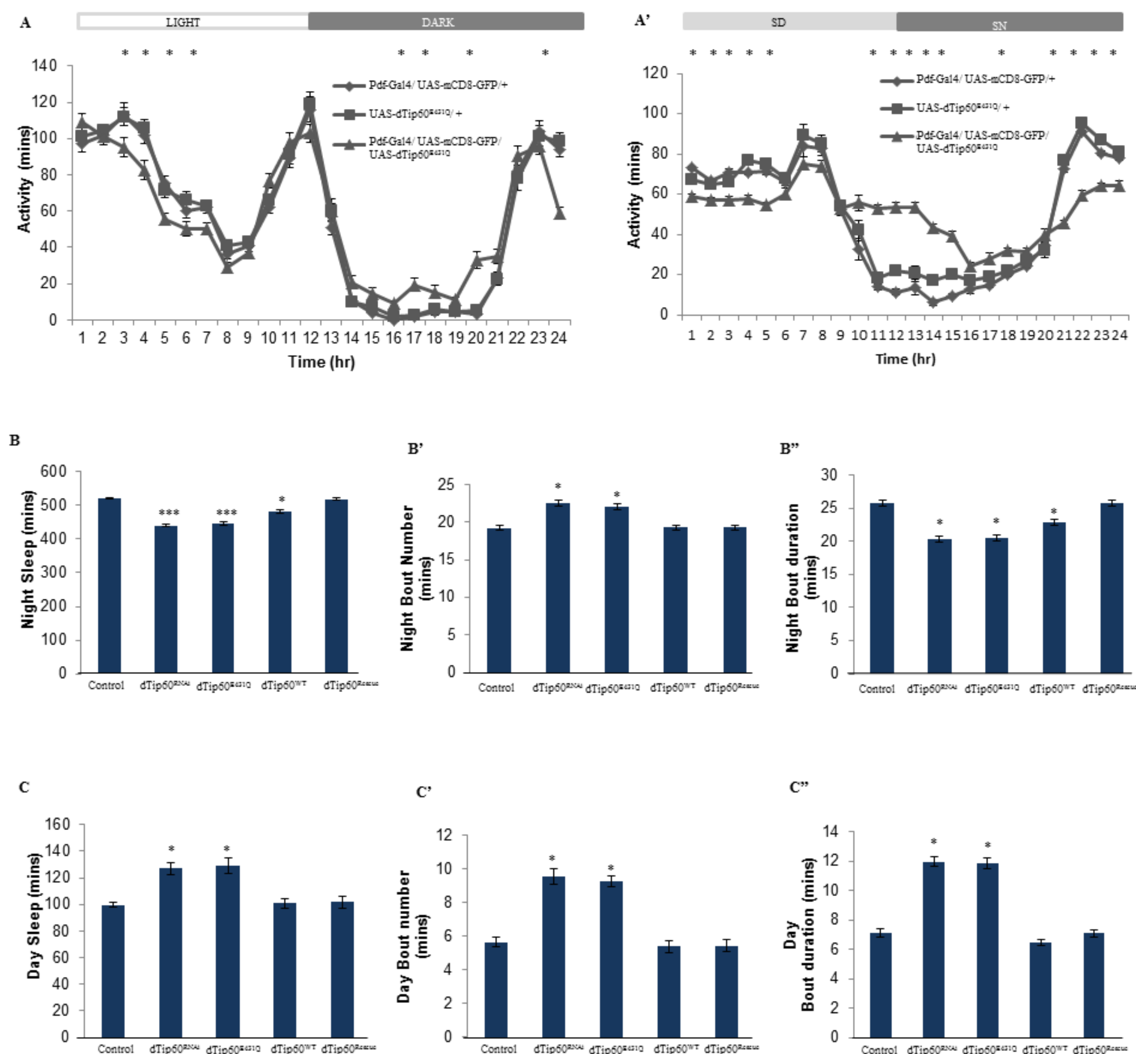


Figure 6. LNv specific modulation of dTip60 HAT activity has no effect on generation of biphasic locomotor rhythm but leads to sleep defects in *Drosophila*. Locomotor activity records of control (Pdf-Gal4/ UAS-mCD8-GFP/ + and UAS-dTip60^{E431Q}/ +) and HAT mutant dTip60^{E431Q} (Pdf-Gal4/ UAS-mCD8-GFP/ UAS-dTip60^{E431Q}) flies show persistence of morning and evening anticipatory behavior in both **(A)** Light:Dark (LD) and **(A')** Dark:Dark (DD) conditions. Under LD conditions, dTip60^{E431Q} flies are less active during the day but also exhibit increased activity during the night **(A)**. Similar activity pattern persists during the subjective day (SD) and

Figure 6 (Continued)

subjective night (SN) in DD **(A')**. **(B)** Digital video analysis of sleep in LD in flies expressing dTip60^{RNAi} or HAT mutant dTip60^{E431Q} and flies overexpressing dTip60^{WT} revealed marked decrease in sleep during the night compared to control flies (Pdf-Gal4/ UAS-mCD8-GFP/ +) (n=28) while dTip60^{Rescue} flies did not have any effect on night sleep. Sleep assessment was based on behavioral immobility lasting 5 mins or longer. Night time sleep in dTip60^{RNAi} and dTip60^{E431Q} flies was characterized by increased bout number **(B')** and decrease in bout duration **(B'')** indicating fragmentation of sleep during the night. dTip60^{WT} flies also exhibited reduced consolidation of night sleep as seen from the decrease in night bout duration **(B'')**. Flies expressing dTip60^{RNAi} or dTip60^{E431Q} displayed increase in daytime sleep although dTip60^{WT} overexpressing flies and dTip60^{Rescue} flies did not have any observable effect on daytime sleep **(C)**. Daytime sleep in dTip60^{RNAi} and dTip60^{E431Q} flies was characterized by an increase in both bout number **(C')** and bout duration **(C'')**. ***P<0.001; *P<0.05 compared to control as determined by student t-test. Error bars represent 95% confidence interval.

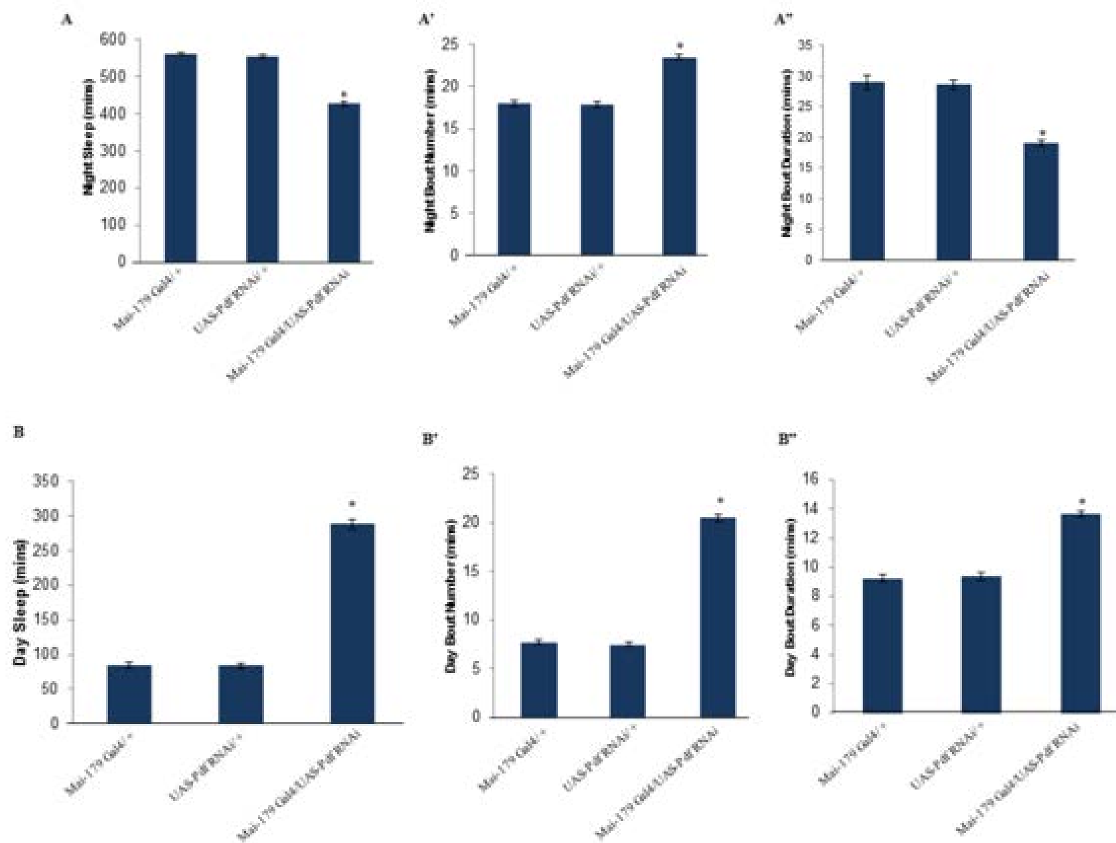


Figure 7. RNAi knock down of Pdf in the sLNv recapitulates dTip60^{E431Q} mediated sleep deficits. **(A)** Mai-179-Gal4 driven knock down of Pdf in the sLNv (Mai-179 Gal4/ UAS-Pdf RNAi) results in marked decrease in sleep during the night compared to the controls (Mai-179 Gal4/ + and UAS-Pdf RNAi/ +). **(A')** Night sleep was highly fragmented as inferred from the increase in bout number and **(A'')** decrease in bout duration. The night time sleep deficit was accompanied by **(B)** increase in sleep during the day that was characterized by **(B')** increase in bout number and **(B'')** bout duration (n=28). *P<0.05 compared to control flies (Mai179-Gal4/+ and UAS-Pdf RNAi/ +). Error bars represent 95% confidence interval.

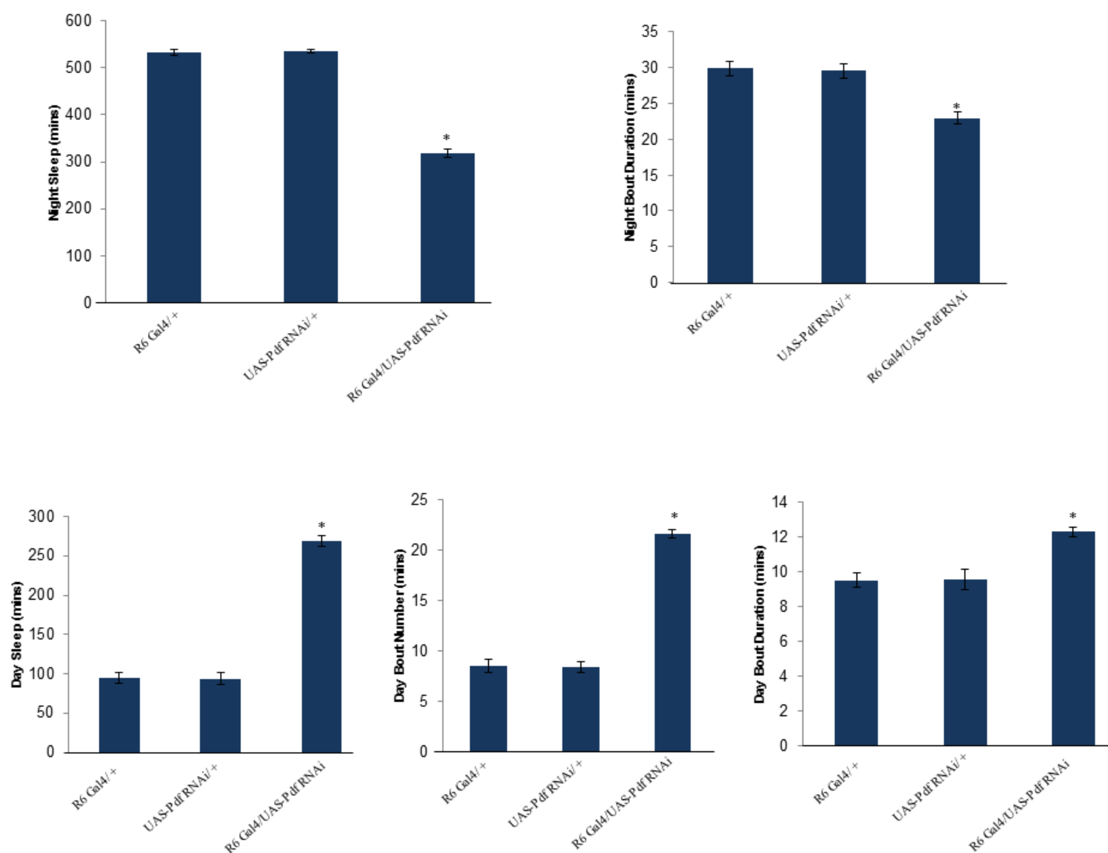


Figure 8. R6-Gal4 driven RNAi knock down of Pdf in the sLNv also results in sleep defects similar to dTip60^{E431Q}. (A) Pdf knock down flies exhibit significant reduction in sleep during the night with **(A')** marked decrease in bout duration, indicative of defects in sleep maintenance. This was accompanied by **(B)** increase in daytime sleepiness with **(B')** significant increase in both bout number and (B'') bout duration. Sample size n=28. *P<0.05 compared to control (R6-Gal4/+). Error bars represent 95% confidence interval.

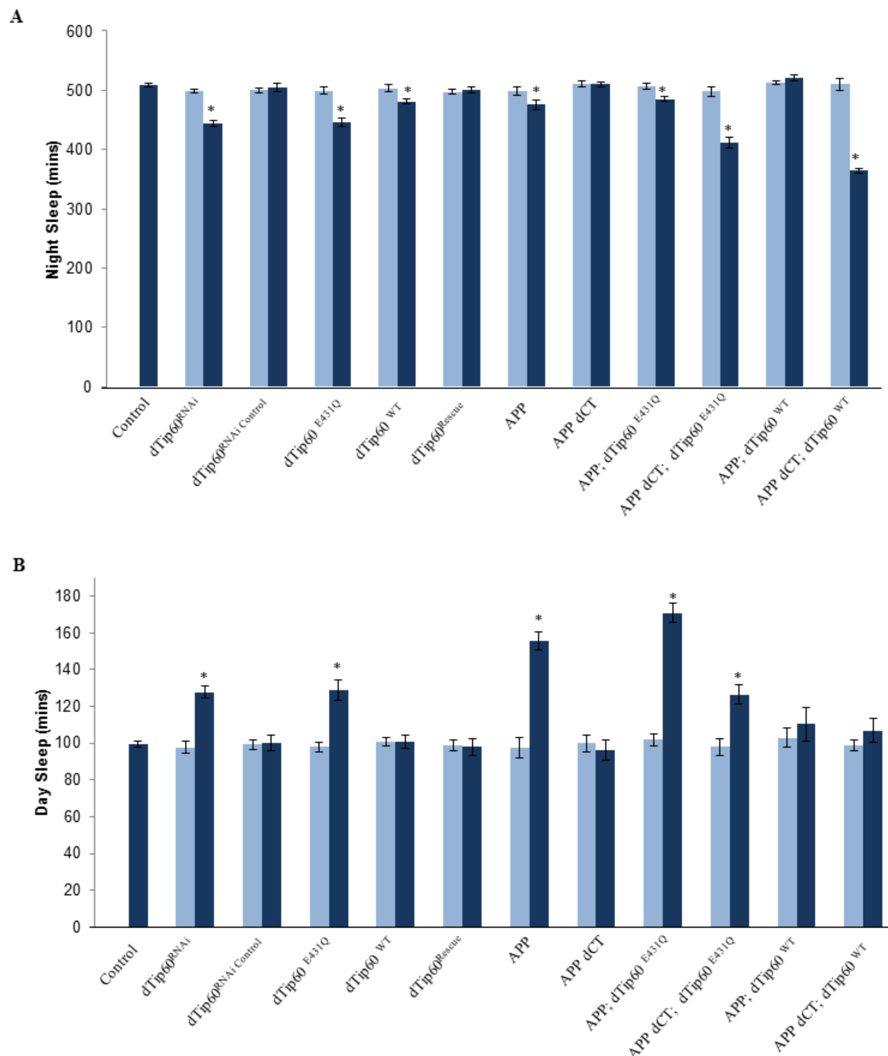


Figure 9. dTip60 and APP functionally interact to mediate day and night time sleep. (A) dTip60 and APP functionally interact to mediate night time sleep deficits. Adult flies were entrained to cycles of 12 hr LD, and their sleep was monitored for 3 days in 12 hr LD. Shown is a histogram depicting the average sleep during the dark period in control (Pdf-Gal4/ UAS-mCD8-GFP/ +) and flies expressing each of the different transgenes under the control of Pdf-Gal4 driver (dark blue bars). Shown in light blue bars is the average sleep during the night in the respective UAS controls for each transgenic line (UAS-transgene/ +). Asterisks indicate the values that are statistically different from those of control flies (Pdf-Gal4/ UAS-mCD8-GFP/ + and the respective UAS control), *$P<0.05$. All data bars represent mean ± SEM. **(B)** Night time sleep defects in

Figure 9 (Continued)

dTip60^{RNAi}, dTip60^{E431Q} and APP flies are accompanied by an increase in daytime sleep. Digital video analysis of sleep for 3 days in adult flies entrained to cycles of 12 hr LD. Shown is a histogram depicting the average sleep during the light period in flies expressing each of the different transgenes under the control of Pdf-Gal4 driver (dark blue bars) and the respective UAS control (light blue bar). Asterisk indicates statistically significant values compared to control flies (Pdf-Gal4/ UAS-mCD8-GFP/ + and the respective UAS controls), *$P<0.05$. All data bars represent mean ± SEM.

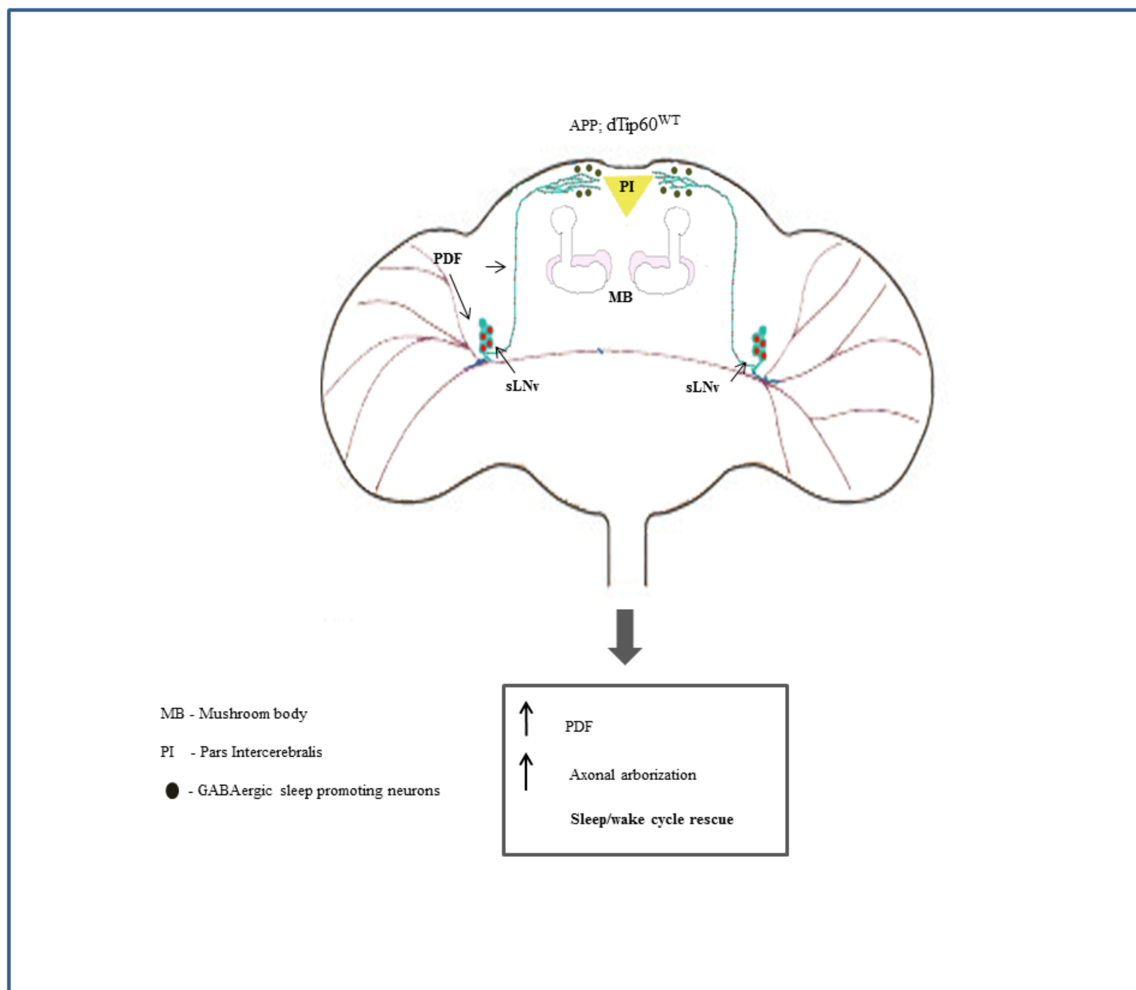


Figure 10. Schematic representation of proposed events for sleep/wake cycle rescue by Tip60 under APP overexpressing conditions. Significant exacerbation of axonal arborization in APP;dTip60^{WT} flies may serve as sites for neural inputs that counteract PDF mediated sleep disruption through activation of compensatory sleep promoting mechanisms. Since sLNv axon terminals in the protocerebrum express post-synaptic $GABA_B$ receptor, neural input from sleep promoting GABAergic cells in the vicinity may be such a compensatory mechanism.

Chapter 4 Figures

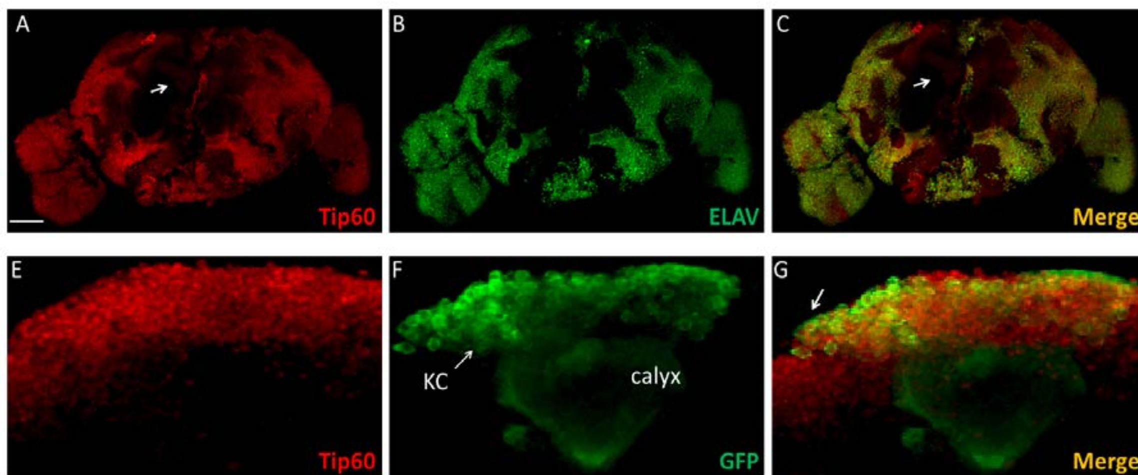


Figure 1: Tip60 expression in the adult *Drosophila* brain. Frontal view of a wild type (Canton S) adult Drosophila brain stained with an antibody to Tip60 (red) and counterstained with anti-ELAV antibody (green). Tip60 is widely expressed in the adult fly brain (**A**) including the mushroom body lobes (**A**, arrow), with an expression pattern similar to the pan-neuronal ELAV protein (**B** and **C**). (**A-C**) are whole brain reconstruction of individual confocal image slices, scale bar 100uM. A single confocal plane through the mushroom body at the level of the calyx (approximately center of the Z stack) in flies that express mCD8-GFP under the control of OK107-GAL4 driver shows Tip60 expression in Kenyon cell (KC) nuclei with a halo of GFP expression in the cell membrane and calyx (dendritic processes) (**E-G**).

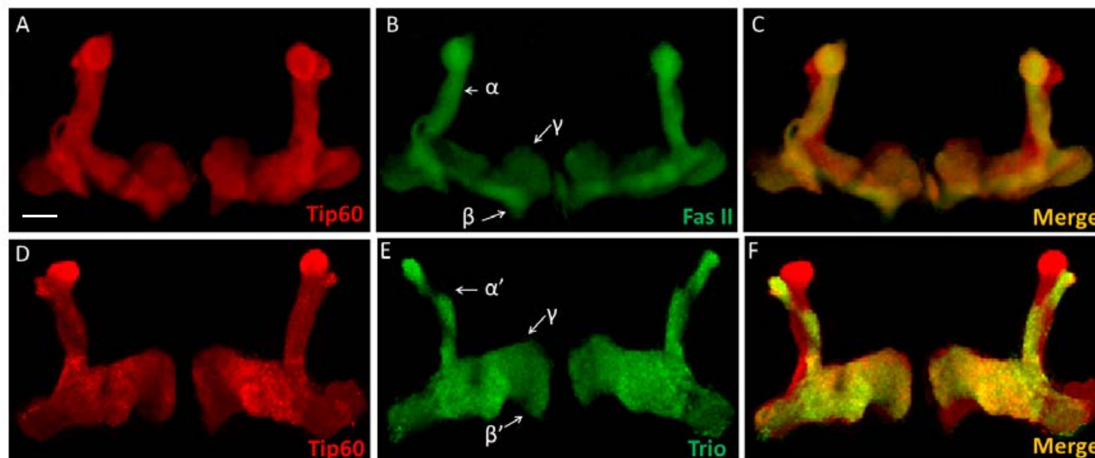


Figure 2. Tip60 is expressed in the mushroom body lobes. Adult mushroom body lobes in wild type (Canton S) *Drosophila* brain stained with Tip60 antibody (**A** and **D**) and co-stained with antibodies to either Fasciclin II (Fas II) (**B**) or Trio (**E**) antibodies, scale bar 10 uM. Fas II is a cell adhesion molecule that is expressed strongly in the mushroom body α/β lobes and weakly in the γ-lobe. Trio is a Dbl family protein that activiates Rho family GTPases and is expressed strongly in the α'/β' lobes and weakly in the γ-lobe. Tip60 is expressed in all the lobes of the mushroom body and co-localizes with Fas II and Trio in the α/β (**C**) and α'/β' (**F**) lobes, respectively. Tip60 also co-localizes with Fas II and Trio in the γ-lobes (**C** and **F**).

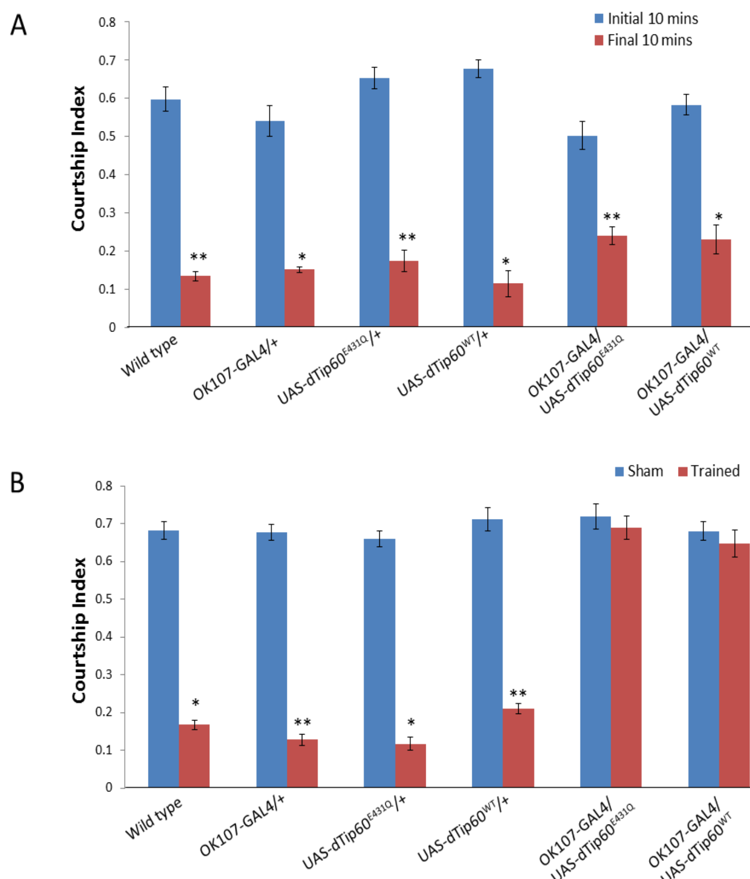


Figure 3: Misregulation of Tip60 in *Drosophila* MB does not affect learning but leads to defects in immediate learning memory. Panel (**A**) denotes learning during the initial 10 mins (blue columns) and final 10 minutes (red columns) of the training phase during the courtship suppression assay. Genotypes are indicated. Flies expressing either the mutant Tip60 defective in its HAT activity (dTip60^{E431Q}) or additional copies of wild type Tip60 (dTip60^{WT}) exhibit marked decrease in courtship index during final 10 mins compared to the initial 10 mins, indicative of normal learning response. This is comparable to response observed in wild type (Canton S) flies as well as the corresponding GAL4 and UAS background controls. Panel (**B**) denotes immediate recall memory (0-2 mins post training) of trained males compared to untrained (sham) males of the same genotype. dTip60^{E431Q} and dTip60^{WT} flies show no significant difference between trained and sham males, indicative of no immediate recall of training. Error bars represent 95% confidence interval. In panel (**A**), single asterisk indicates $P < 0.05$ and double asterisk indicates $P < 0.001$ compared with initial 10 mins. In panel (**B**), single

Figure 3 (Continued)

asterisk indicates P < 0.05 and double asterisk indicates P < 0.001 compared with sham males, n=20 for trained and untrained males in each genotype.

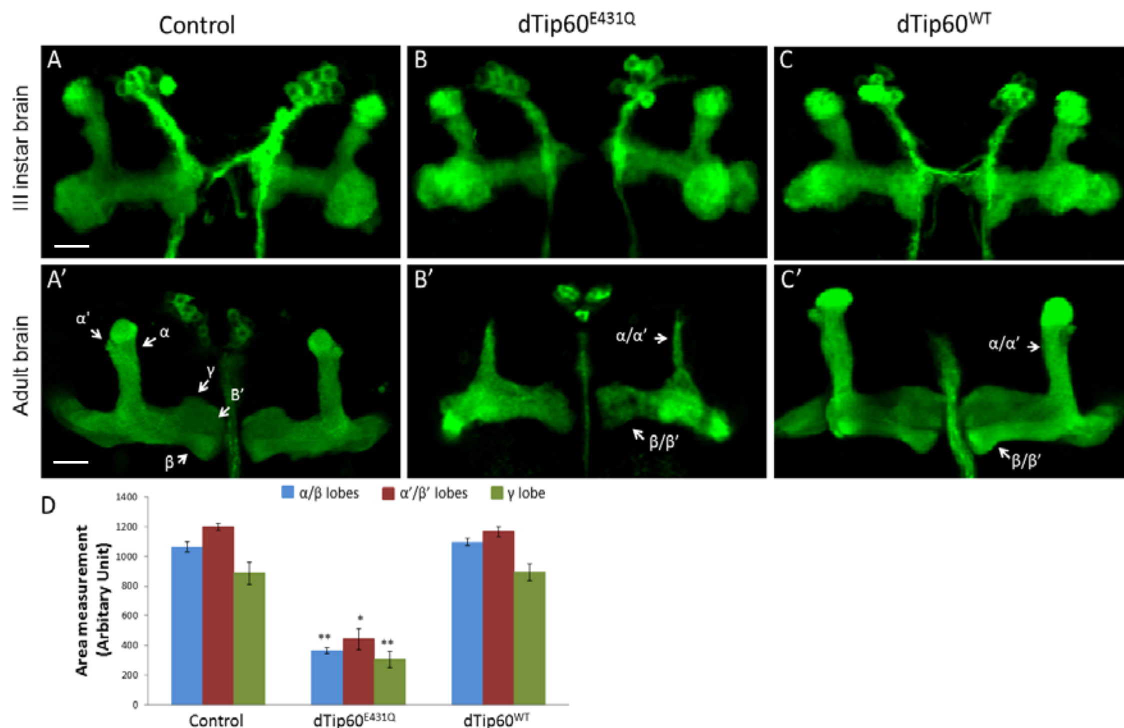


Figure 4: Tip60 is required for normal structures of the adult mushroom body. Larval and adult mushroom body visualized with mCD8-GFP driven by pan-MB driver, OK107-GAL4. Third instar larval brain in control flies (**A**). Scale bar 10 uM. Flies expressing mutant Tip60 defective in its HAT activity (dTip60^{E431Q}) (**B**) or additional copies of wild type Tip60 (dTip60^{WT}) (**C**) show no effect on mushroom body structure in the third instar larva. GFP labeling shows similar widths of and α/β lobes in (**A'**) adult control brains (OK107-GAL4; UAS-GFP), whereas adult flies expressing mutant dTip60^{E431Q} display severe reduction in length and width of both α and β lobes (arrow) (**B'**) while overexpressing dTip60^{WT} did not have any effect on the MB in the adult flies (**C'**), scale bar 10 uM. (**D**) Quantification of area in the different genotypes in adult flies, quantification of α/β and γ lobe lobes was done using Fas II labeling. Trio labeling was used for α'/β' lobes quantification(n=20). Error bars represent 95% confidence interval. Single asterisk indicates $P < 0.05$ and double asterisk indicates $P < 0.001$ compared to respective MB lobes in the control.

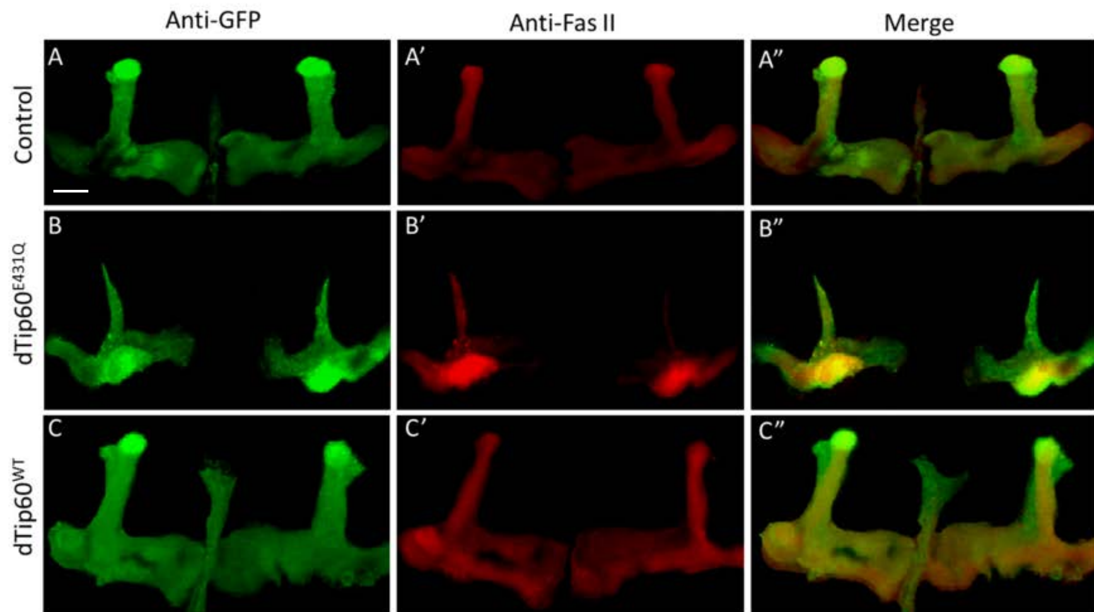


Figure 5. Fasciclin II labeling in the mushroom body. OK107-GAL4 driver carrying GFP construct was used to drive expression of dTip60^{E431Q} or dTip60^{WT} and effect on mushroom body structure was visualized using GFP and Fas II staining. Compared to control brains (UAS-mCD8-GFP; OK107-GAL4), dTip60^{E431Q} flies exhibit marked decrease in α/β and γ lobes. dTip60^{WT} flies did not exhibit any significant effect on the MB lobes. Scale bar 10 uM. Fas II labeling was used for quantifying area measurements in the different genotypes.

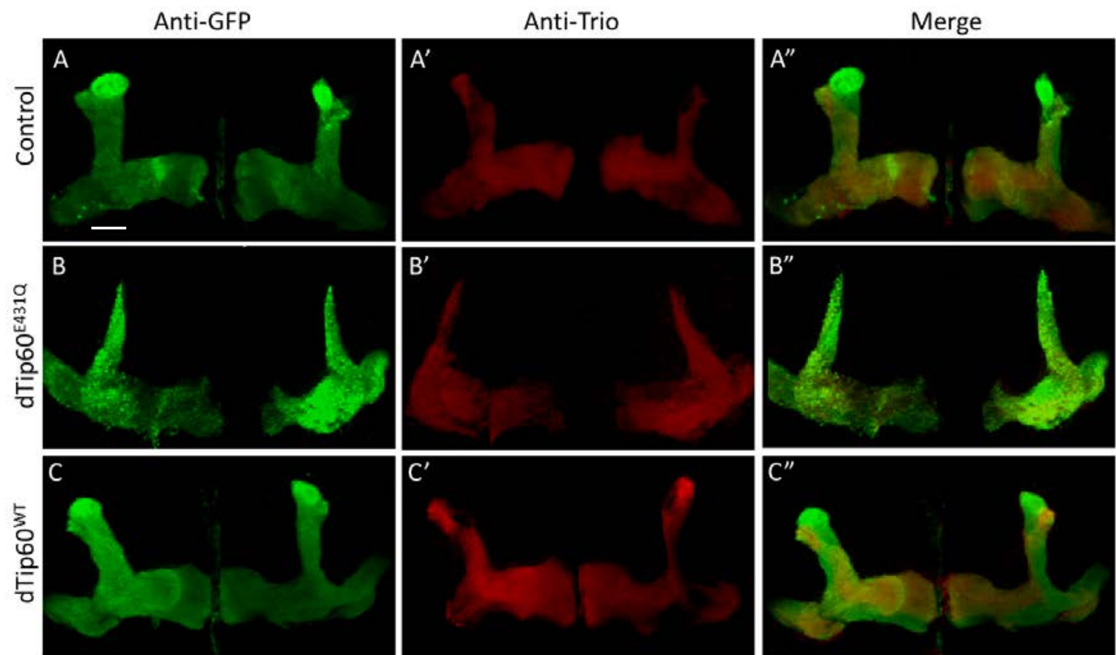


Figure 6. Trio labeling in the mushroom body. OK107-GAL4 driver carrying GFP construct was used to drive expression of dTip60^{E431Q} or dTip60^{WT} and effect on mushroom body structure was visualized using GFP and Trio staining. Compared to control brains (UAS-mCD8-GFP; OK107-GAL4), dTip60^{E431Q} flies exhibit marked decrease in α'/β' while dTip60^{WT} flies did not exhibit any marked effect on these lobes. Scale bar 10 uM. Trio labeling was used for quantifying area measurements in the different genotypes.

Work in Progress Figures

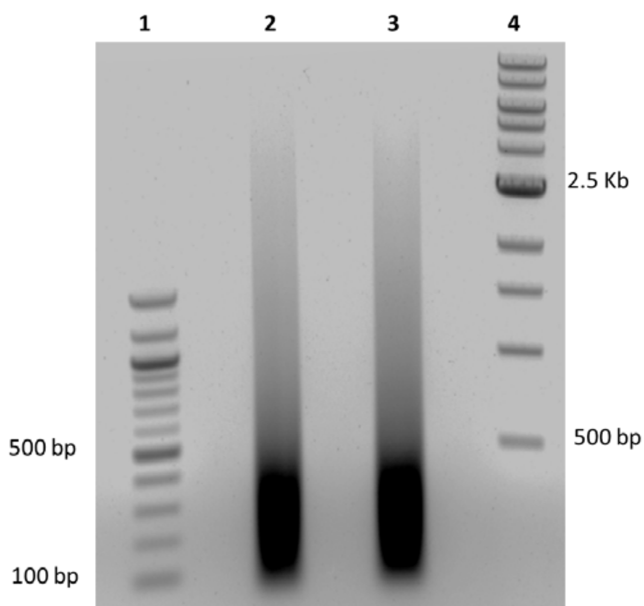


Figure 1: Gel analysis of chromatin prepared by sonication. *Drosophila* S2 cells were fixed for 10 mins with 1% formaldehyde and chromatin was prepared using the ChIP-IT Express kit (Active Motif). Chromatin was sheared by sonication on ice. The sheared chromatin was subjected to cross-link reverasl, treated with Proteinase K and Rnase A. DNA was then precipitated by phenol-chloroform extracted. Samples were electrophoresed through 1% agarose gel to check for optimal shearing that yields fragments that range in size between 150 -1500 bp. Lanes 1 and 4 contain 100bp and 1 Kb ladders. Lanes 2 and 3 contain 10 ul of sheared chromatin prepared from replicate *Drosophila* S2 cells.

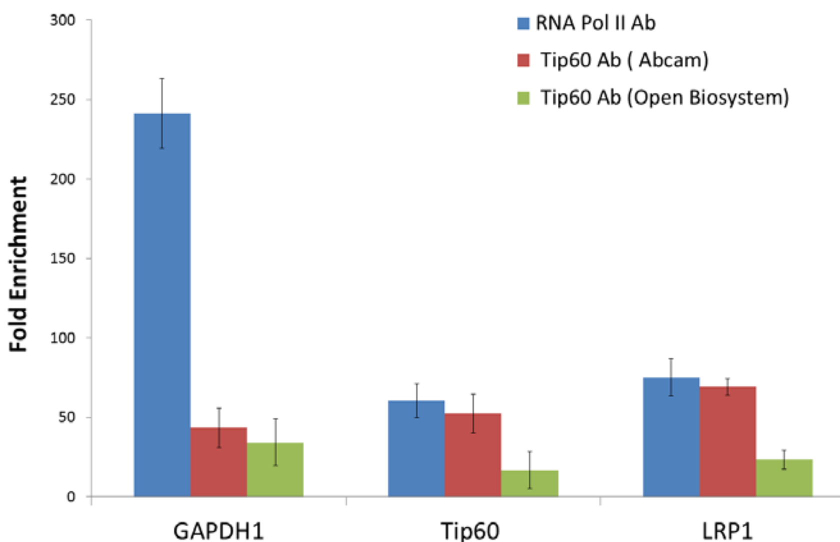


Figure 2: ChIP enrichment of target genes by RNA Polymerase II and Tip60 antibodies. *Drosophila* S2 cells were fixed for 10 mins using 1% formaldehyde and chromatin was prepared by sonication. ChIP was performed using ChIP-IT Express kit (Active Motif) using RNA polymerase II (RNA Pol II) and two different Tip60 antibodies that target the C-terminus of Tip60. A mock reaction containing no antibody was used as control. Real-time PCR was performed on DNA purified from each of the ChIP reactions using primer pairs specific for *Drosophila* GAPDH, Tip60 and low density LRP1 (lipoprotein receptor related protein 1) genes. Fold enrichment of the respective genes was calculated relative to the mock control. These results demonstrate that ChIP performed using the RNA pol II and Tip60 antibodies enriched for the target genes.

APPENDIX A: MODULATING EPIGENETIC HAT ACTIVITY: A PROMISING THERAPEUTIC OPTION FOR NEUROLOGICAL DISEASE?

The epigenome (epi- derived from Greek for 'over' or 'above') with its rich cache of highly regulated structural modifications to the DNA, histone residues and histone variants, defines the three-dimensional structure of chromatin, the genetic material within the eukaryotic cell nucleus, and serves as the molecular bridge between transcriptional gene control and our environment [3]. Only a few years ago, such epigenetic gene control mechanisms were primarily viewed in the context of cell division and fate specification as they were thought to function primarily in maintaining "cell memory" as the cell steers through elaborate pathways during early development and differentiation, and seemed to bear little relevance to adult brain function, as the mature brain is primarily composed of post-mitotic and already highly differentiated neuronal cells committed to specialized functions that collectively determine neuronal responses to external stimuli. However, recent explorations of the brain epigenome are providing unprecedented insights into the importance of specific epigenetic modification patterns in controlling gene expression not only in early brain development, but in adult brain function as well, calling into place a 'reprogramming process' that allows for plasticity at many levels of the neural circuitry in response to environmental cues [4]. One issue to consider with reference to the mature brain and cognitive disorders is how the course of normal maturation as well as aging affects the brain epigenome. Indeed, an increasing body of evidence indicates that substantial reorganization of the brain epigenome occurs during aging and such age related epigenetic drift could further exacerbate an individual's vulnerability to neurodegenerative diseases. However, unlike age related accumulation of somatic mutations and structural changes to the DNA that are likely irreversible, most if not all of the epigenetic modification marks studied to date are in fact reversible, making targeting of the neural epigenome a promising strategy for neuroprotection and/or neuroregeneration both early in development as well as during the aging process [3].

Cognitive decline, particularly in memory capacity, is a normal part of aging and has been associated with aberrant changes in gene expression in the brain's hippocampus and frontal lobe [323]. Of the epigenetic modifications identified so far in the nervous system, histone acetylation, mediated by the counteractive effects of histone acetyltransferases (HATs) and histone deacetylases (HDACs) have been unequivocally associated with the transcriptional control of genes that facilitate learning and memory [31,324]. An emerging hypothesis is that age related accumulation of aberrant epigenetic marks in chromatin in the adult brain cause gene misregulation that drives cognitive decline and memory impairment. Over the past decade, several studies have also reported reduced histone acetylation in animal models of neurodegeneration that exhibit cognitive decline, including models for Alzheimer's disease (AD) [259]. Accordingly, pharmacological treatments using non-selective HDAC inhibitors like valproic acid, trichostatin A and Sodium Butyrate have been demonstrated to have promising effects in reversing such cognitive deficits in some of these models likely by increasing "global" acetylation levels and potentially HDAC inhibitor dependent genetic programs [151]. Similarly, restoring acetylation status through HDAC inhibition has been shown to ameliorate disease progression in models of Parkinson's and Huntington's disease [115,119,120,325]. These studies in turn have ignited enormous interest in the therapeutic potential of HDAC inhibitors for various neurodegenerative conditions. However, there is also widespread speculation about the target specificity of HDAC inhibitors as HDACs function as classes of proteins with individual members being able to compensate for each other's functions [152]. Thus, the current use of pan-HDAC inhibitors that act by increasing global acetylation levels can also disrupt cellular acetylation homeostasis with subsequent negative consequences. Moreover, targeting a particular class of HDACs or individual members is currently an arduous task as the causative agents of memory impairing histone acetylation changes and hence, the best targets for pharmacological strategies, remain unknown [259]. Additionally, class-specific modulation of HDAC activity may lead to very different and potentially opposing clinical implications. For

example, activation and/or overexpression of class I HDACs 2 and 3 is associated with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and neural cell toxicity [126,158], while inhibition of another member of this class, HDAC 1 has been found to lead to neurodegeneration [107,159]. Another issue to consider in terms of HDAC based therapeutic efficacy is that although HDAC inhibitors are generally considered to promote neuronal growth and differentiation, they also exhibit toxicity in various cell types of the central nervous system. For instance, there is evidence that they could have potentially detrimental effects on the orderly maturation of astrocytes and oligodendrocytes [161-163]. Moreover, like their counterparts, the HATs – class I, II and III of HDACs also regulate lysine acetylation of non-histone proteins that exert neuroprotective effects [165,326] adding a further layer of complexity to the interpretation of therapeutic potentials of currently available broad spectrum or even class specific HDAC inhibitors for neurodegenerative diseases. Thus, the specificity and side-effect profiles of inhibitors of HDACs require additional investigation to fully gauge their neuroprotective abilities. Further exploration of isoform-selective HDAC inhibitors that are also region-specific may provide a therapeutic advantage in targeting specific cell and tissue functions under pathological conditions.

It has become increasingly clear that chromatin acetylation status can be impaired during the lifetime of neurons through loss of function of specific HATs with negative consequences on neuronal function [152]. Once the acetylation balance is disturbed by the loss of HAT dose, the HAT: HDAC ratio tilts in favor of HDAC in terms of availability and enzymatic functionality, a fact highlighted by amelioration of several neurodegenerative conditions by various HDAC inhibitors [75]. In fact, a clue to explain the net deacetylation observed during neurodegeneration came with the finding that dying neurons exhibit progressive loss of HAT activity and/or expression, particularly that of the HAT CREB binding protein (CBP) and to a lesser extent the HAT p300. Notably, overexpression of CBP under apoptotic conditions delays neuronal cell

death, an event that was dependent on the HAT function of CBP [77,166]. CBP overexpression has also been shown to protect neurons from polyglutamine induced toxicity in Huntington's disease [111,327,328]. We have also reported a similar effect for Tip60, a multifunctional HAT that forms a transcriptionally active complex with the AD associated amyloid precursor protein (APP) intracellular domain (AICD). Neuronal loss of the histone acetylase activity of Tip60 under APP induced neurodegenerative conditions enhances apoptotic neuronal cell death in a *Drosophila* AD model, an effect predominantly mediated through transcriptional dysregulation of pro-apoptotic and essential genes. Remarkably, overexpression of the HAT competent Tip60 leads to a marked decrease in APP induced apoptosis highlighting a neuroprotective role for Tip60 HAT function in AD associated pathogenesis [78]. Specific HATs are also emerging as regulators that gate access to genes regulating specific neuronal processes that are essential for maintaining neuronal health and for mediating higher order brain functions. Such processes are also affected in neurodegenerative conditions with detrimental consequences. For instance, CBP has been shown to mediate specific forms of hippocampal long term potentiation, a form of synaptic plasticity thought to underlie memory storage [167]. In contrast, the HAT p300 has been shown to constrain synaptic plasticity in the prefrontal cortex and reduced function of this HAT is required for formation of fear extinction memory [168]. Importantly, overexpression of p300 but not HDAC inhibition has been shown to promote axonal regeneration in mature retinal ganglion cells following optic nerve injury, an effect mediated by p300 induced hyperacetylation of histone H3 and p53 that consequently leads to increased expression of selected pro-axonal outgrowth genes [169]. Overexpression of Tip60 under APP induced neurodegenerative conditions also induces intrinsic axonal arborization of the *Drosophila* small ventrolateral neurons, a well characterized model system for studying axonal growth [329]. The acetyltransferase Elp3 known to acetylate microtubules has been shown to be involved in the regulation of synaptic bouton expansion during neurogenesis [171] and recent studies suggest that regulation of microtubule acetylation by the ELP3 might be commonly affected in neurological diseases making it a

potential target for acetylation modulator based therapies (reviewed in [172]). Tip60 has also been recently shown to play a causative role in synaptic plasticity partly through acetylation of microtubules [173]. Together, these studies raise the possibility that modulation of expression levels and/or activity of specific HATs such as Tip60 could be an alternative therapeutic option for neurological conditions. Targeting HATs can also be beneficial because unlike HDACs, HATs have non-redundant functions under physiological conditions and thus the presence of specific modulators can have more direct effects. In a study by [174], it was reported that the total protein amount and activity of various HDACs is not altered by mutant huntington protein expression in primary cortical neurons. Thus, the neurodegeneration associated tilt in HAT: HDAC does not appear to include augmentation of HDAC protein level. Therefore, activation of specific HATs may restore acetylation balance in addition to activating specific gene expression programs that consequently have neuroprotective effects. In fact, a number of recent studies conclude that HDAC inhibitor induced hyperacetylation alone may not be sufficient to produce beneficial effects. In a study by [330], it was reported that HDAC inhibition mediated enhancement of synaptic plasticity and hippocampus dependent memory formation requires the presence of at least one wild type allele of *cbp* highlighting the requirement of HATs like CBP for site specific acetylation and the recruitment of the basal transcriptional machinery. However, increasing neuronal dosage of specific HATs to reinstate acetylation homeostasis calls for the same concern as does the utilization of HDAC inhibitors. Non-specific enhancement of HAT levels and/or activity may lead to further complications by skewing the acetylation balance in the neighboring cell population towards hyperacetylation. Therefore, in order to reap the full potential of specific HAT activators, it is also essential to quantify HAT-HDAC dose in specific cell populations that are vulnerable to different degenerative etiology [75].

Canonically, HATs have been associated with active and HDACs with inactive genes. However, recent genome wide mapping of HATs and HDACs binding on chromatin indicates

that the majority of HDACs in the human genome are associated with active genes, and only a minor fraction are detected in silent genes. A major function of HDACs at active genes appears to be the removal of acetyl groups added by high levels of HATs during the process of transcriptional initiation and elongation and reset the chromatin structure required for the next round of transcription. Notably, HATs also transiently and frequently bind inactive genes to acetylate histones and HDACs remove the acetyl groups to keep the genes inactive [331]. Excessive acetylation in transcribed regions can destabilize chromatin and increase cryptic initiation of transcription. Given the dynamic cycling between acetylation and deacetylation by transient HAT/HDAC binding, it is likely that excessive acetylation induced by HDAC inhibitor treatment not only destabilizes the chromatin architecture at active genes but also at genes that are repressed under basal conditions. Thus, it is important to realize that modulation of HAT/HDAC levels and/or activity may alter the expression of many genes. While the induction of expression of some genes may constitute a "cassette" of neuroprotective agents, it is likely that there is also induction or de-repression of genes that have detrimental effects. In fact, in the case of the HAT Tip60, overexpression of wild type Tip60 but not the HAT defective mutant increases survival in a Drosophila AD model, an effect that was mediated via enhanced repression of pro-apoptotic genes and induction of pro-survival factors like Bcl-2. This indicates that Tip60's HAT activity exerts a neuroprotective effect by tipping the cell fate control balance in favor of cell survival [78]. Similar mechanisms may underlie the neuroprotective effects observed with other HATs like CBP and p300.

A major challenge with utilization of modifiers of cellular acetylation levels is the identification of bona fide targets of HATs and HDACs and the integration of histone and transcription factor acetylation into a broader context of neuronal, and importantly, cellular homeostasis [175]. Although still in its infancy, the neuroprotective effects displayed by HATs like CBP, p300 and Tip60 and specificity of these effects for particular neuronal processes is much more promising

that currently available non-selective HDAC inhibitors. However, determining the genes or "cassettes" of genes that are regulated by such HATs and characterizing the survival or degenerative effects such genes have would subsequently facilitate the development of novel drugs and specific therapeutic strategies with lower adverse side effects than those currently available.

APPENDIX B: A HAT FOR SLEEP? – EPIGENETIC REGULATION OF SLEEP BY TIP60 IN *DROSOPHILA*

ABSTRACT

Sleep disturbances are a major and early hallmark of age- related neurodegenerative diseases such as Alzheimer's disease (AD), and are thought to drive neuronal cell death and cognitive decline. Unfortunately, how AD is mechanistically linked with interference of the body's natural sleep rhythms remains unclear. Our recent findings provide insight into this question by demonstrating that sleep disruption associated with AD is driven by epigenetic changes mediated by the histone acetyltransferase (HAT) Tip60. In this study, we show that Tip60 functionally interacts with the AD associated amyloid precursor protein (APP) to regulate axonal growth of *Drosophila* small ventrolateral neuronal (sLNv) pacemaker cells, and their production of neuropeptide pigment dispersing factor (PDF) that stabilizes appropriate sleep-wake patterns in the fly. Loss of Tip60 HAT activity under APP neurodegenerative conditions causes decreased PDF production, retraction of the sLNv synaptic arbor required for PDF release and disruption of sleep-wake cycles in these flies. Remarkably, excess Tip60 in conjunction with APP fully rescues these sleep-wake disturbances by inducing overelaboration of the sLNv synaptic terminals and increasing PDF levels, supporting a neuroprotective role for Tip60 in these processes. Our studies highlight the importance of epigenetic based mechanisms underlying sleep disturbances in neurodegenerative diseases like AD.

Neurons, while being subjected to a variety of stimuli, are also able to convert such cues into higher order functions such as controlling behavior, storing memories and decision making. These unique properties are based on the highly flexible nature of neurons, a characteristic that is regulated by networks of extrinsic and intrinsic molecular pathways that together orchestrate precise gene expression profiles required for neuronal plasticity. Epigenetic control, which largely involves events of chromatin remodeling, appears to be one way in which transcriptional regulation of gene expression can be controlled in neurons [4]. Of the epigenetic modifications identified so far in the nervous system, histone acetylation mediated by the antagonistic activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes [74], has been unequivocally shown to play a crucial role in regulating neuronal gene expression profiles critical for neuronal functions [94,332]. HATs generally promote chromatin decondensation by catalyzing the transfer of an acetyl group from acetyl-CoA to the ε-amino group of specific lysine residues within the N-terminal tails of nucleosomal histones. This modification weakens histone–DNA as well as neighboring nucleosomal contacts to promote chromatin disruption that, in turn, facilitates factor binding and transcriptional activation. HATs also exhibit distinct substrate preference for specific histone, lysine, and gene targets and thereby generate different acetylation patterns within the genome [333,334]. Such HAT generated acetylation patterns together with other DNA and histone modifications is thought to serve as a molecular bar code to recruit chromatin remodeling complexes and downstream regulatory factors that drive gene expression profiles required for particular cellular events, a paradigm referred to as the ‘histone-code hypothesis’[26,335,336]. As such, loss of function of specific HATs with

vital neuronal functions has been reported to impair neuronal acetylation status and contribute to degenerative effects in various cellular and animal models of neurodegenerative diseases [103,152,337].

The HAT Tip60 (Tat interactive protein, 60 KDa) is a member of the MYST family of proteins that are related by a ~300 aminoacid domain containing atypical zinc finger and HAT domains [338]. The HAT activity of Tip60 exerts pleiotropic cellular effects that include a variety of chromatin mediated processes such as transcription regulation, cell cycle check-point control, DNA damage repair and apoptosis to name a few (reviewed in [177]). In 2007, we first isolated the *Drosophila* homologue of Tip60 and further demonstrated an essential role for Tip60 during multicellular development [222]. Subsequent work from our laboratory has demonstrated that Tip60 is robustly produced in the developing embryonic nervous system as well as in specific regions of the adult fly brain. Moreover, our studies further revealed that Tip60's HAT activity is critical for nervous system development and function, an effect primarily mediated *via* transcriptional regulation of genes enriched for a variety of specific neuronal functions[69]. Accordingly, we found that Tip60's HAT activity controls synaptic plasticity [173] and regulates apoptosis to prevent unwanted cell death in the developing *Drosophila* central nervous system (CNS) [78]. Consistent with our findings, Tip60 has been implicated in neurodegenerative diseases such as spinocerebellar ataxia (SCA1) [70] and the age-related neurodegenerative Alzheimer's disease (AD) [144]. Tip60's role in the latter stems from observations that Tip60 forms a transcriptionally active complex with a cytosolic fragment derived from proteolytic processing of the AD-associated amyloid precursor protein (APP), termed the APP intracellular domain (AICD)[144,149].

The Tip60/AICD complex has been shown to increase histone acetylation [213] and coactivate gene promoters which are linked to apoptosis and neurotoxicity associated with AD [180]. Moreover, misregulation of certain putative target genes of the Tip60/AICD complex has been linked to AD related pathology [147,214]. More recently, our laboratory has demonstrated that Tip60 and APP functionally interact to mediate lethality and apoptotic mediated neurodegeneration in the central nervous system (CNS) of an AD fly model, *in vivo* [78]. Together, these studies support the concept that neuropathology associated with AD is due, at least in part, to epigenetic dysregulation, Tip60 being a likely candidate mediating such effects. However, little is known about how aberrant alterations of the neural epigenome by Tip60 in particular, affect specific neural circuits under AD linked neurodegenerative conditions.

Sleep abnormalities are a major and early feature of neurodegenerative diseases like AD that are also characterized by cognitive decline. While the causes of such sleep disturbances are unknown, they are thought to further exacerbate the effects of a fundamental process leading to neurodegeneration [255]. Sleep dependent mechanisms of neural plasticity are believed to contribute to memory consolidation and thus are likely critical for learning and memory [339,340]. As such, analysis of sleep disturbances may offer important insights into the pathological mechanisms underlying such neurodegenerative diseases. *Drosophila* has become a well-accepted behavioral model for sleep research as it shares many features with mammalian sleep [341,342] and is thus well suited to examine the fundamental functions of sleep, and the mechanisms that regulate it [343,344]. In *Drosophila*, the small- and large- ventrolateral neurons (LNv) (henceforth referred to as sLNv and lLNv, respectively) are part of the well-characterized

fly circadian circuitry [215] as well as the "core" sleep circuitry in the fly [216,217]. Both the circadian and sleep regulatory effects of the LNvs are mediated via the neuropeptide pigment dispersing factor (PDF) that serves as the main functional output from the LNvs to coordinate neural circuits that operate downstream of the LNvs [218,219]. A limited number of other fly brain regions have been proposed to contribute to sleep. These include the mushroom body and pars intercerebralis in the central brain and importantly, are both regions thought to receive rhythmic signal from the sLNv axon terminals [345]. These features bear resemblance to the regulatory effects that the mammalian pacemaker, the suprachiasmatic nucleus (SCN) has on controlling sleep-wake cycles as well as coordinating this with other brain areas to enhance behavioral adaptation [346]. All of these features make the *Drosophila* LNv sleep circuit a powerful model to study the mechanisms underlying sleep regulation.

In the study by [170], we set out to test the hypothesis that APP and Tip60 are both required to mediate selective neuronal processes such as sLNv morphology and function that when misregulated, are linked to AD pathology. We found that that both sLNv and lLNv cell types endogenously express Tip60 and disruption of the epigenetic HAT function of Tip60 ($Tip60^{mut}$) in the LNvs causes sleep disturbances consisting of fragmented night sleep and daytime sleepiness, reminiscent of those observed in AD. Furthermore, our analysis revealed that the sLNvs are particularly susceptible to loss of Tip60's HAT activity and exhibit diminished expression of PDF as well as retraction of the sLNv axon terminals that are required for pre-synaptic release of PDF in the dorsal protocerebrum. These neuroanatomical defects likely contributed to the sleep disturbances by disrupting PDF-mediated interaction of the sLNvs with downstream

circuits. Intriguingly, disruption of Tip60 HAT activity under APP induced neurodegenerative conditions (APP; $Tip60^{mut}$) was found to exacerbate retraction of the sLNv axonal terminals and further caused complete loss of PDF, although the sleep disturbances were same as in flies exhibiting only loss of Tip60 HAT activity in their sLNvs. Importantly, the anatomical defects we observed were dependent on the presence of the C-terminus of APP that is required for generation of the Tip60 interacting AICD fragment. While the pathogenesis of sleep disturbances associated with AD is unclear, neurodegeneration in brain regions that are involved in sleep regulation are thought to be linked to sleep abnormalities. In this regard, the degenerative effects we observe specifically in the sLNvs suggest that Tip60 mediated epigenetic dysregulation can render selective neuronal populations more vulnerable to APP induced neurodegeneration with detrimental consequences on associated behavioral outputs.

In light of these observations, we hypothesized that overexpression of HAT competent Tip60 under APP overexpressing conditions would override APP mediated neurodegenerative effects and alleviate the observed sleep disturbances. LNv directed overexpression of Tip60 ($Tip60^{OE}$) enhanced PDF expression in the sLNv with no marked effect on the sLNv axon growth. These flies also exhibit impaired ability to maintain sleep at night, an effect we speculate could be mediated through untimely activation of downstream arousal promoting neural circuits by the excess PDF. Overexpression of wild type Tip60 in the LNvs in conjunction with APP containing its C-terminus (APP; $Tip60^{OE}$) also increased sLNv PDF expression. Additionally, these flies also exhibited extensive arborization of the sLNv axon terminals in the dorsal protocerebrum. However, despite these anatomical changes, co-expression of Tip60

along with APP that contained its C-terminus restored the normal sleep-wake cycle, consistent with our hypothesis.

So how can Tip60 overexpression in conjunction with APP rescue the night time sleep disruption and day time sleepiness we observe in AD model flies or when Tip60 itself is misregulated? A clue to this question may come from our observation that significant exacerbation of axonal arborization and normal sleep-wake patterns were only observed as a result of co-expression of wild type Tip60 and APP, and not when Tip60 was overexpressed alone. Based on these findings, we propose a model by which such APP/Tip60 induced axonal overelaboration could play a role in restoring the sleep-wake cycles by increasing growth of the sLNv axon terminals into the dorsal protocerebrum. These additional synaptic terminals might provide additional neural input sites for sleep promoting signals. Consistent with this model, the sLNv axon terminals have been reported to express postsynaptic $GABA_B$ receptors and GABAergic sleep promoting neurons have also been observed in the vicinity of the sLNv axon terminals in the adult CNS [252], suggesting that the sLNvs might receive slow inhibitory GABAergic input from such neurons in the vicinity through the dorsal terminals. Furthermore, recent electron microscopy studies also indicate the presence of sparsely distributed input synapses at the sLNv axon terminals [251] that could also play a role in transducing sleep promoting neural signals.

Accumulating evidence indicates that axonal dysfunction and degeneration in AD may persist long before the disease related neuropathologies are detectable, and it is believed that these early axonal dystrophies in the affected neurons may significantly contribute to disease symptoms [277]. In this regard, our observation that loss of Tip60

HAT activity causes retraction and loss thereof of sLNv axonal synaptic terminals and/or PDF signaling suggests that disruption of neuronal connectivity within this particular axonal circuit may be an early event in the AD process, accounting for the sleep abnormalities that occur in AD patients long before pathophysiological manifestation of the disease sets in. Together, our data demonstrating the modulatory effects that Tip60 HAT activity or lack thereof have on the sLNvs under APP induced neurodegenerative conditions provides novel mechanistic insights into epigenetic regulation of neural circuits that underlie the sleep abnormalities that AD patients experience early in the disorder. Future investigation into the downstream mechanism by which Tip60 regulates axonal growth as well as its apparent neuroprotective role in maintaining normal sleep-wake cycles under APP induced neurondegenerative conditions should serve as the groundwork when exploring the utility of specific HAT activators as early intervention therapeutic strategies to prevent or delay the progression of age-linked neurodegenerative disorders.

LIST OF REFERENCES

1. Berger SL (2007) The complex language of chromatin regulation during transcription. Nature 447: 407-412.
2. Riccio A (2010) Dynamic epigenetic regulation in neurons: enzymes, stimuli and signaling pathways. Nat Neurosci 13: 1330-1337.
3. Jakovcevski M, Akbarian S (2012) Epigenetic mechanisms in neurological disease. Nat Med 18: 1194-1204.
4. Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P (2008) Decoding the epigenetic language of neuronal plasticity. Neuron 60: 961-974.
5. Graff J, Kim D, Dobbin MM, Tsai LH (2011) Epigenetic regulation of gene expression in physiological and pathological brain processes. Physiol Rev 91: 603-649.
6. Peterson CL, Laniel MA (2004) Histones and histone modifications. Curr Biol 14: R546-551.
7. Cheung P, Allis CD, Sassone-Corsi P (2000) Signaling to chromatin through histone modifications. Cell 103: 263-271.
8. Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403: 41-45.
9. Wood MA, Hawk JD, Abel T (2006) Combinatorial chromatin modifications and memory storage: a code for memory? Learn Mem 13: 241-244.
10. Bernstein BE, Meissner A, Lander ES (2007) The mammalian epigenome. Cell 128: 669-681.
11. Ruthenburg AJ, Li H, Patel DJ, Allis CD (2007) Multivalent engagement of chromatin modifications by linked binding modules. Nat Rev Mol Cell Biol 8: 983-994.
12. Trojer P, Reinberg D (2006) Histone lysine demethylases and their impact on epigenetics. Cell 125: 213-217.
13. Lessard J, Wu JI, Ranish JA, Wan M, Winslow MM, et al. (2007) An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron 55: 201-215.
14. Putignano E, Lonetti G, Cancedda L, Ratto G, Costa M, et al. (2007) Developmental downregulation of histone posttranslational modifications regulates visual cortical plasticity. Neuron 53: 747-759.
15. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, et al. (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. Nat Genet 40: 897-903.
16. Andersson R, Enroth S, Rada-Iglesias A, Wadelius C, Komorowski J (2009) Nucleosomes are well positioned in exons and carry characteristic histone modifications. Genome Res 19: 1732-1741.
17. Kolasinska-Zwierz P, Down T, Latorre I, Liu T, Liu XS, et al. (2009) Differential chromatin marking of introns and expressed exons by H3K36me3. Nat Genet 41: 376-381.
18. Spies N, Nielsen CB, Padgett RA, Burge CB (2009) Biased chromatin signatures around polyadenylation sites and exons. Mol Cell 36: 245-254.
19. Tilgner H, Nikolaou C, Althammer S, Sammeth M, Beato M, et al. (2009) Nucleosome positioning as a determinant of exon recognition. Nat Struct Mol Biol 16: 996-1001.
20. Sun ZW, Allis CD (2002) Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature 418: 104-108.
21. Latham JA, Dent SY (2007) Cross-regulation of histone modifications. Nat Struct Mol Biol 14: 1017-1024.
22. Margueron R, Trojer P, Reinberg D (2005) The key to development: interpreting the histone code? Curr Opin Genet Dev 15: 163-176.

23. Fuks F (2005) DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev 15: 490-495.
24. Lo WS, Trievel RC, Rojas JR, Duggan L, Hsu JY, et al. (2000) Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. Mol Cell 5: 917-926.
25. Zocchi L, Sassone-Corsi P (2010) Joining the dots: from chromatin remodeling to neuronal plasticity. Curr Opin Neurobiol 20: 432-440.
26. Fischle W, Wang Y, Allis CD (2003) Histone and chromatin cross-talk. Curr Opin Cell Biol 15: 172-183.
27. Wysocka J, Allis CD, Coonrod S (2006) Histone arginine methylation and its dynamic regulation. Front Biosci 11: 344-355.
28. Linggi BE, Brandt SJ, Sun ZW, Hiebert SW (2005) Translating the histone code into leukemia. J Cell Biochem 96: 938-950.
29. Fischle W, Wang Y, Allis CD (2003) Binary switches and modification cassettes in histone biology and beyond. Nature 425: 475-479.
30. Chwang WB, O'Riordan KJ, Levenson JM, Sweatt JD (2006) ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. Learn Mem 13: 322-328.
31. Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, et al. (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279: 40545-40559.
32. Miller CA, Sweatt JD (2007) Covalent modification of DNA regulates memory formation. Neuron 53: 857-869.
33. Miller CA, Campbell SL, Sweatt JD (2008) DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. Neurobiol Learn Mem 89: 599-603.
34. Schreiber SL, Bernstein BE (2002) Signaling network model of chromatin. Cell 111: 771-778.
35. Meaney MJ, Ferguson-Smith AC (2010) Epigenetic regulation of the neural transcriptome: the meaning of the marks. Nat Neurosci 13: 1313-1318.
36. Nelson ED, Kavalali ET, Monteggia LM (2008) Activity-dependent suppression of miniature neurotransmission through the regulation of DNA methylation. J Neurosci 28: 395-406.
37. Guan Z, Giustetto M, Lomvardas S, Kim JH, Miniaci MC, et al. (2002) Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. Cell 111: 483-493.
38. Crosio C, Heitz E, Allis CD, 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: 4905-4914.
39. Tsankova NM, Kumar A, Nestler EJ (2004) Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. J Neurosci 24: 5603-5610.
40. Levenson JM, Sweatt JD (2005) Epigenetic mechanisms in memory formation. Nat Rev Neurosci 6: 108-118.
41. Erraji-Benchekroun L, Underwood MD, Arango V, Galfalvy H, Pavlidis P, et al. (2005) Molecular aging in human prefrontal cortex is selective and continuous throughout adult life. Biol Psychiatry 57: 549-558.
42. Tang B, Chang WL, Lanigan CM, Dean B, Sutcliffe JG, et al. (2009) Normal human aging and early-stage schizophrenia share common molecular profiles. Aging Cell 8: 339-342.
43. Gonzalo S (2010) Epigenetic alterations in aging. J Appl Physiol 109: 586-597.
44. Han S, Brunet A (2012) Histone methylation makes its mark on longevity. Trends Cell Biol 22: 42-49.

45. Winnefeld M, Lyko F (2012) The aging epigenome: DNA methylation from the cradle to the grave. Genome Biol 13: 165.
46. Wang CM, Tsai SN, Yew TW, Kwan YW, Ngai SM (2010) Identification of histone methylation multiplicities patterns in the brain of senescence-accelerated prone mouse 8. Biogerontology 11: 87-102.
47. Lu T, Pan Y, Kao SY, Li C, Kohane I, et al. (2004) Gene regulation and DNA damage in the ageing human brain. Nature 429: 883-891.
48. Copray S, Huynh JL, Sher F, Casaccia-Bonnefil P, Boddeke E (2009) Epigenetic mechanisms facilitating oligodendrocyte development, maturation, and aging. Glia 57: 1579-1587.
49. Fischer A, Sananbenesi F, Mungenast A, Tsai LH (2010) Targeting the correct HDAC(s) to treat cognitive disorders. Trends Pharmacol Sci 31: 605-617.
50. Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, et al. (2000) Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci 20: 3993-4001.
51. Hall J, Thomas KL, Everitt BJ (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci 3: 533-535.
52. Steward O, Worley PF (2001) Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation. Neuron 30: 227-240.
53. Linnarsson S, Bjorklund A, Ernfors P (1997) Learning deficit in BDNF mutant mice. Eur J Neurosci 9: 2581-2587.
54. Dickey CA, Loring JF, Montgomery J, Gordon MN, Eastman PS, et al. (2003) Selectively reduced expression of synaptic plasticity-related genes in amyloid precursor protein + presenilin-1 transgenic mice. J Neurosci 23: 5219-5226.
55. Palop JJ, Chin J, Bien-Ly N, Massaro C, Yeung BZ, et al. (2005) Vulnerability of dentate granule cells to disruption of arc expression in human amyloid precursor protein transgenic mice. J Neurosci 25: 9686-9693.
56. Rosi S, Ramirez-Amaya V, Vazdarjanova A, Worley PF, Barnes CA, et al. (2005) Neuroinflammation alters the hippocampal pattern of behaviorally induced Arc expression. J Neurosci 25: 723-731.
57. Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, et al. (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. J Neurosci 23: 3807-3819.
58. Small SA, Chawla MK, Buonocore M, Rapp PR, Barnes CA (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: 7181-7186.
59. Rowe WB, Blalock EM, Chen KC, Kadish I, Wang D, et al. (2007) Hippocampal expression analyses reveal selective association of immediate-early, neuroenergetic, and myelinogenic pathways with cognitive impairment in aged rats. J Neurosci 27: 3098-3110.
60. Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J Neurosci 28: 10576-10586.
61. Penner MR, Roth TL, Barnes CA, Sweatt JD (2010) An epigenetic hypothesis of aging-related cognitive dysfunction. Front Aging Neurosci 2: 9.
62. Mai A, Valente S, Meade S, Carafa V, Tardugno M, et al. (2009) Study of 1,4-dihydropyridine structural scaffold: discovery of novel sirtuin activators and inhibitors. J Med Chem 52: 5496-5504.
63. Migliore L, Coppede F (2009) Environmental-induced oxidative stress in neurodegenerative disorders and aging. Mutat Res 674: 73-84.

64. Desplats P, Spencer B, Coffee E, Patel P, Michael S, et al. (2011) Alpha-synuclein sequesters Dnmt1 from the nucleus: a novel mechanism for epigenetic alterations in Lewy body diseases. J Biol Chem 286: 9031-9037.
65. Tong L, Thornton PL, Balazs R, Cotman CW (2001) Beta -amyloid-(1-42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival Is not compromised. J Biol Chem 276: 17301-17306.
66. Vitolo OV, Sant'Angelo A, Costanzo V, Battaglia F, Arancio O, et al. (2002) Amyloid beta -peptide inhibition of the PKA/CREB pathway and long-term potentiation: reversibility by drugs that enhance cAMP signaling. Proc Natl Acad Sci U S A 99: 13217-13221.
67. Caccamo A, Maldonado MA, Bokov AF, Majumder S, Oddo S (2010) CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 107: 22687-22692.
68. McCampbell A, Taylor JP, Taye AA, Robitschek J, Li M, et al. (2000) CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet 9: 2197-2202.
69. Lorbeck M, Pirooznia K, Sarthi J, Zhu X, Elefant F (2011) Microarray analysis uncovers a role for Tip60 in nervous system function and general metabolism. PLoS One 6: e18412.
70. Gehrking KM, Andresen JM, Duvick L, Lough J, Zoghbi HY, et al. (2011) Partial loss of Tip60 slows mid-stage neurodegeneration in a spinocerebellar ataxia type 1 (SCA1) mouse model. Hum Mol Genet 20: 2204-2212.
71. Ryu H, Lee J, Hagerty SW, Soh BY, McAlpin SE, et al. (2006) ESET/SETDB1 gene expression and histone H3 (K9) trimethylation in Huntington's disease. Proc Natl Acad Sci U S A 103: 19176-19181.
72. Hu Y, Chopra V, Chopra R, Locascio JJ, Liao Z, et al. (2011) Transcriptional modulator H2A histone family, member Y (H2AFY) marks Huntington disease activity in man and mouse. Proc Natl Acad Sci U S A 108: 17141-17146.
73. Graff J, Mansuy IM (2009) Epigenetic dysregulation in cognitive disorders. Eur J Neurosci 30: 1-8.
74. Legube G, Trouche D (2003) Regulating histone acetyltransferases and deacetylases. EMBO Rep 4: 944-947.
75. Saha RN, Pahan K (2006) HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis. Cell Death Differ 13: 539-550.
76. Wang Y, Wang X, Liu L (2009) HDAC inhibitor trichostatin A-inhibited survival of dopaminergic neuronal cells. Neurosci Lett 467: 212-216.
77. Rouaux C, Jokic N, Mbebi C, Boutillier S, Loeffler JP, et al. (2003) Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. EMBO J 22: 6537-6549.
78. Pirooznia SK, Sarthi J, Johnson AA, Toth MS, Chiu K, et al. (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: e41776.
79. Kontopoulos E, Parvin JD, Feany MB (2006) Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. Hum Mol Genet 15: 3012-3023.
80. Li F, Macfarlan T, Pittman RN, Chakravarti D (2002) Ataxin-3 is a histone-binding protein with two independent transcriptional corepressor activities. J Biol Chem 277: 45004-45012.
81. Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A, et al. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature 413: 739-743.
82. Mbebi C, See V, Mercken L, Pradier L, Muller U, et al. (2002) Amyloid precursor protein family-induced neuronal death is mediated by impairment of the neuroprotective

calcium/calmodulin protein kinase IV-dependent signaling pathway. J Biol Chem 277: 20979-20990.
83. Song C, Kanthasamy A, Anantharam V, Sun F, Kanthasamy AG (2010) Environmental neurotoxic pesticide increases histone acetylation to promote apoptosis in dopaminergic neuronal cells: relevance to epigenetic mechanisms of neurodegeneration. Mol Pharmacol 77: 621-632.
84. Brady S, Morfini G (2010) A perspective on neuronal cell death signaling and neurodegeneration. Mol Neurobiol 42: 25-31.
85. Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ (2007) Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. Neurology 68: 1501-1508.
86. Arendt T (2009) Synaptic degeneration in Alzheimer's disease. Acta Neuropathol 118: 167-179.
87. Shankar GM, Walsh DM (2009) Alzheimer's disease: synaptic dysfunction and Abeta. Mol Neurodegener 4: 48.
88. Sananbenesi F, Fischer A (2009) The epigenetic bottleneck of neurodegenerative and psychiatric diseases. Biol Chem 390: 1145-1153.
89. Xu K, Dai XL, Huang HC, Jiang ZF (2011) Targeting HDACs: a promising therapy for Alzheimer's disease. Oxid Med Cell Longev 2011: 143269.
90. Francis YI, Fa M, Ashraf H, Zhang H, Staniszewski A, et al. (2009) Dysregulation of histone acetylation in the APP/PS1 mouse model of Alzheimer's disease. J Alzheimers Dis 18: 131-139.
91. Van Raamsdonk JM, Pearson J, Slow EJ, Hossain SM, Leavitt BR, et al. (2005) Cognitive dysfunction precedes neuropathology and motor abnormalities in the YAC128 mouse model of Huntington's disease. J Neurosci 25: 4169-4180.
92. Giralt A, Puigdellivol M, Carreton O, Paoletti P, Valero J, et al. (2012) Long-term memory deficits in Huntington's disease are associated with reduced CBP histone acetylase activity. Hum Mol Genet 21: 1203-1216.
93. Koshibu K, Graff J, Beullens M, Heitz FD, Berchtold D, et al. (2009) Protein phosphatase 1 regulates the histone code for long-term memory. J Neurosci 29: 13079-13089.
94. Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, et al. (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328: 753-756.
95. Barrett RM, Malvaez M, Kramar E, Matheos DP, Arrizon A, et al. (2011) Hippocampal focal knockout of CBP affects specific histone modifications, long-term potentiation, and long-term memory. Neuropsychopharmacology 36: 1545-1556.
96. Alarcon JM, Malleret G, Touzani K, Vronskaya S, Ishii S, et al. (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: 947-959.
97. Korzus E, Rosenfeld MG, Mayford M (2004) CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron 42: 961-972.
98. Oliveira AM, Wood MA, McDonough CB, Abel T (2007) Transgenic mice expressing an inhibitory truncated form of p300 exhibit long-term memory deficits. Learn Mem 14: 564-572.
99. Maurice T, Duclot F, Meunier J, Naert G, Givalois L, et al. (2008) Altered memory capacities and response to stress in p300/CBP-associated factor (PCAF) histone acetylase knockout mice. Neuropsychopharmacology 33: 1584-1602.
100. Bousiges O, Vasconcelos AP, Neidl R, Cosquer B, Herbeaux K, et al. (2010) Spatial memory consolidation is associated with induction of several lysine-acetyltransferase (histone acetyltransferase) expression levels and H2B/H4 acetylation-dependent transcriptional events in the rat hippocampus. Neuropsychopharmacology 35: 2521-2537.

101. Schiltz RL, Mizzen CA, Vassilev A, Cook RG, Allis CD, et al. (1999) Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. J Biol Chem 274: 1189-1192.
102. Owen DJ, Ornaghi P, Yang JC, Lowe N, Evans PR, et al. (2000) The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. EMBO J 19: 6141-6149.
103. Stilling RM, Fischer A (2011) The role of histone acetylation in age-associated memory impairment and Alzheimer's disease. Neurobiol Learn Mem 96: 19-26.
104. Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, et al. (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci 983: 84-100.
105. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, et al. (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459: 55-60.
106. McQuown SC, Barrett RM, Matheos DP, Post RJ, Rogge GA, et al. (2011) HDAC3 is a critical negative regulator of long-term memory formation. J Neurosci 31: 764-774.
107. Kim D, Frank CL, Dobbin MM, Tsunemoto RK, Tu W, et al. (2008) Deregulation of HDAC1 by p25/Cdk5 in neurotoxicity. Neuron 60: 803-817.
108. Benn CL, Butler R, Mariner L, Nixon J, Moffitt H, et al. (2009) Genetic knock-down of HDAC7 does not ameliorate disease pathogenesis in the R6/2 mouse model of Huntington's disease. PLoS One 4: e5747.
109. Dietz KC, Casaccia P (2010) HDAC inhibitors and neurodegeneration: at the edge between protection and damage. Pharmacol Res 62: 11-17.
110. McCampbell A, Fischbeck KH (2001) Polyglutamine and CBP: fatal attraction? Nat Med 7: 528-530.
111. Jiang H, Nucifora FC, Jr., Ross CA, DeFranco DB (2003) Cell death triggered by polyglutamine-expanded huntingtin in a neuronal cell line is associated with degradation of CREB-binding protein. Hum Mol Genet 12: 1-12.
112. Cong SY, Pepers BA, Evert BO, Rubinsztein DC, Roos RA, et al. (2005) Mutant huntingtin represses CBP, but not p300, by binding and protein degradation. Mol Cell Neurosci 30: 12-23.
113. Pallos J, Bodai L, Lukacsovich T, Purcell JM, Steffan JS, et al. (2008) Inhibition of specific HDACs and sirtuins suppresses pathogenesis in a Drosophila model of Huntington's disease. Hum Mol Genet 17: 3767-3775.
114. Mielcarek M, Benn CL, Franklin SA, Smith DL, Woodman B, et al. (2011) SAHA decreases HDAC 2 and 4 levels in vivo and improves molecular phenotypes in the R6/2 mouse model of Huntington's disease. PLoS One 6: e27746.
115. Hockly E, Richon VM, Woodman B, Smith DL, Zhou X, et al. (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: 2041-2046.
116. Ferrante RJ, Kubilus JK, Lee J, Ryu H, Beesen A, et al. (2003) Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. J Neurosci 23: 9418-9427.
117. Thomas EA, Coppola G, Desplats PA, Tang B, Soragni E, et al. (2008) The HDAC inhibitor 4b ameliorates the disease phenotype and transcriptional abnormalities in Huntington's disease transgenic mice. Proc Natl Acad Sci U S A 105: 15564-15569.
118. Moore DJ, West AB, Dawson VL, Dawson TM (2005) Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci 28: 57-87.
119. Monti B, Gatta V, Piretti F, Raffaelli SS, Virgili M, et al. (2010) Valproic acid is neuroprotective in the rotenone rat model of Parkinson's disease: involvement of alpha-synuclein. Neurotox Res 17: 130-141.

120. Outeiro TF, Kontopoulos E, Altmann SM, Kufareva I, Strathearn KE, et al. (2007) Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. Science 317: 516-519.
121. Chou SM (1997) Neuropathology of amyotrophic lateral sclerosis: new perspectives on an old disease. J Formos Med Assoc 96: 488-498.
122. Ikemoto A, Hirano A, Akiguchi I (2000) Neuropathology of amyotrophic lateral sclerosis with extra-motor system degeneration: characteristics and differences in the molecular pathology between ALS with dementia and Guamanian ALS. Amyotroph Lateral Scler Other Motor Neuron Disord 1: 97-104.
123. Orrell RW, King AW, Hilton DA, Campbell MJ, Lane RJ, et al. (1995) Familial amyotrophic lateral sclerosis with a point mutation of SOD-1: intrafamilial heterogeneity of disease duration associated with neurofibrillary tangles. J Neurol Neurosurg Psychiatry 59: 266-270.
124. Rouaux C, Panteleeva I, Rene F, Gonzalez de Aguilar JL, Echaniz-Laguna A, et al. (2007) Sodium valproate exerts neuroprotective effects in vivo through CREB-binding protein-dependent mechanisms but does not improve survival in an amyotrophic lateral sclerosis mouse model. J Neurosci 27: 5535-5545.
125. Ryu H, Smith K, Camelo SI, Carreras I, Lee J, et al. (2005) Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice. J Neurochem 93: 1087-1098.
126. Janssen C, Schmalbach S, Boeselt S, Sarlette A, Dengler R, et al. (2010) Differential histone deacetylase mRNA expression patterns in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 69: 573-581.
127. Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297: 353-356.
128. Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 8: 101-112.
129. Gotz J, Ittner A, Ittner LM (2012) Tau-targeted treatment strategies in Alzheimer's disease. Br J Pharmacol 165: 1246-1259.
130. Ittner LM, Gotz J (2011) Amyloid-beta and tau--a toxic pas de deux in Alzheimer's disease. Nat Rev Neurosci 12: 65-72.
131. Oddo S, Vasilevko V, Caccamo A, Kitazawa M, Cribbs DH, et al. (2006) Reduction of soluble Abeta and tau, but not soluble Abeta alone, ameliorates cognitive decline in transgenic mice with plaques and tangles. J Biol Chem 281: 39413-39423.
132. Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, et al. (2010) Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. Cell 142: 387-397.
133. Trinchese F, Fa M, Liu S, Zhang H, Hidalgo A, et al. (2008) Inhibition of calpains improves memory and synaptic transmission in a mouse model of Alzheimer disease. J Clin Invest 118: 2796-2807.
134. Mastroeni D, Grover A, Delvaux E, Whiteside C, Coleman PD, et al. (2011) Epigenetic mechanisms in Alzheimer's disease. Neurobiol Aging 32: 1161-1180.
135. Ricobaraza A, Cuadrado-Tejedor M, Perez-Mediavilla A, Frechilla D, Del Rio J, et al. (2009) Phenylbutyrate ameliorates cognitive deficit and reduces tau pathology in an Alzheimer's disease mouse model. Neuropsychopharmacology 34: 1721-1732.
136. Ricobaraza A, Cuadrado-Tejedor M, Garcia-Osta A (2011) Long-term phenylbutyrate administration prevents memory deficits in Tg2576 mice by decreasing Abeta. Front Biosci (Elite Ed) 3: 1375-1384.
137. Kilgore M, Miller CA, Fass DM, Hennig KM, Haggarty SJ, et al. (2010) Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease. Neuropsychopharmacology 35: 870-880.

138. Donmez G, Wang D, Cohen DE, Guarente L (2010) SIRT1 suppresses beta-amyloid production by activating the alpha-secretase gene ADAM10. Cell 142: 320-332.
139. Muller M, Cardenas C, Mei L, Cheung KH, Foskett JK (2011) Constitutive cAMP response element binding protein (CREB) activation by Alzheimer's disease presenilin-driven inositol trisphosphate receptor (InsP3R) Ca2+ signaling. Proc Natl Acad Sci U S A 108: 13293-13298.
140. Marambaud P, Wen PH, Dutt A, Shioi J, Takashima A, et al. (2003) A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. Cell 114: 635-645.
141. Saura CA, Choi SY, Beglopoulos V, Malkani S, Zhang D, et al. (2004) Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. Neuron 42: 23-36.
142. Fischer A, Sananbenesi F, Wang X, Dobbin M, Tsai LH (2007) Recovery of learning and memory is associated with chromatin remodelling. Nature 447: 178-182.
143. Muller T, Meyer HE, Egensperger R, Marcus K (2008) The amyloid precursor protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics-relevance for Alzheimer's disease. Prog Neurobiol 85: 393-406.
144. Cao X, Sudhof TC (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 293: 115-120.
145. von Rotz RC, Kohli BM, Bosset J, Meier M, Suzuki T, et al. (2004) The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. J Cell Sci 117: 4435-4448.
146. Ryan KA, Pimplikar SW (2005) Activation of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. J Cell Biol 171: 327-335.
147. Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, et al. (2002) Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. Cell 110: 55-67.
148. Muller T, Concannon CG, Ward MW, Walsh CM, Tirniceriu AL, et al. (2007) Modulation of gene expression and cytoskeletal dynamics by the amyloid precursor protein intracellular domain (AICD). Mol Biol Cell 18: 201-210.
149. Slomnicki LP, Lesniak W (2008) A putative role of the Amyloid Precursor Protein Intracellular Domain (AICD) in transcription. Acta Neurobiol Exp (Wars) 68: 219-228.
150. Giliberto L, Zhou D, Weldon R, Tamagno E, De Luca P, et al. (2008) Evidence that the Amyloid beta Precursor Protein-intracellular domain lowers the stress threshold of neurons and has a "regulated" transcriptional role. Mol Neurodegener 3: 12.
151. Kazantsev AG, Thompson LM (2008) Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. Nat Rev Drug Discov 7: 854-868.
152. Selvi BR, Cassel JC, Kundu TK, Boutillier AL (2010) Tuning acetylation levels with HAT activators: therapeutic strategy in neurodegenerative diseases. Biochim Biophys Acta 1799: 840-853.
153. Broide RS, Redwine JM, Aftahi N, Young W, Bloom FE, et al. (2007) Distribution of histone deacetylases 1-11 in the rat brain. J Mol Neurosci 31: 47-58.
154. Fischle W, Dequiedt F, Hendzel MJ, Guenther MG, Lazar MA, et al. (2002) Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell 9: 45-57.
155. Lagger G, O'Carroll D, Rembold M, Khier H, Tischler J, et al. (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 21: 2672-2681.
156. Montgomery RL, Hsieh J, Barbosa AC, Richardson JA, Olson EN (2009) Histone deacetylases 1 and 2 control the progression of neural precursors to neurons during brain development. Proc Natl Acad Sci U S A 106: 7876-7881.

157. Akhtar MW, Raingo J, Nelson ED, Montgomery RL, Olson EN, et al. (2009) Histone deacetylases 1 and 2 form a developmental switch that controls excitatory synapse maturation and function. J Neurosci 29: 8288-8297.
158. Bardai FH, D'Mello SR (2011) Selective toxicity by HDAC3 in neurons: regulation by Akt and GSK3beta. J Neurosci 31: 1746-1751.
159. Bates EA, Victor M, Jones AK, Shi Y, Hart AC (2006) Differential contributions of Caenorhabditis elegans histone deacetylases to huntingtin polyglutamine toxicity. J Neurosci 26: 2830-2838.
160. Chawla S, Vanhoutte P, Arnold FJ, Huang CL, Bading H (2003) Neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5. J Neurochem 85: 151-159.
161. Hsieh J, Nakashima K, Kuwabara T, Mejia E, Gage FH (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. Proc Natl Acad Sci U S A 101: 16659-16664.
162. Liu J, Casaccia P (2010) Epigenetic regulation of oligodendrocyte identity. Trends Neurosci 33: 193-201.
163. Pedre X, Mastronardi F, Bruck W, Lopez-Rodas G, Kuhlmann T, et al. (2011) Changed histone acetylation patterns in normal-appearing white matter and early multiple sclerosis lesions. J Neurosci 31: 3435-3445.
164. Pfister JA, Ma C, Morrison BE, D'Mello SR (2008) Opposing effects of sirtuins on neuronal survival: SIRT1-mediated neuroprotection is independent of its deacetylase activity. PLoS One 3: e4090.
165. Dokmanovic M, Marks PA (2005) Prospects: histone deacetylase inhibitors. J Cell Biochem 96: 293-304.
166. Anne-Laurence B, Caroline R, Irina P, Jean-Philippe L (2007) Chromatin acetylation status in the manifestation of neurodegenerative diseases: HDAC inhibitors as therapeutic tools. Subcell Biochem 41: 263-293.
167. Wood MA, Kaplan MP, Park A, Blanchard EJ, Oliveira AM, et al. (2005) Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage. Learn Mem 12: 111-119.
168. Marek R, Coelho CM, Sullivan RK, Baker-Andresen D, Li X, et al. (2011) Paradoxical enhancement of fear extinction memory and synaptic plasticity by inhibition of the histone acetyltransferase p300. J Neurosci 31: 7486-7491.
169. Gaub P, Joshi Y, Wuttke A, Naumann U, Schnichels S, et al. (2011) The histone acetyltransferase p300 promotes intrinsic axonal regeneration. Brain 134: 2134-2148.
170. Pirooznia SK, Chiu K, Chan MT, Zimmerman JE, Elefant F (2012) Epigenetic regulation of axonal growth of Drosophila pacemaker cells by histone acetyltransferase tip60 controls sleep. Genetics 192: 1327-1345.
171. Singh N, Lorbeck MT, Zervos A, Zimmerman J, Elefant F (2010) The histone acetyltransferase Elp3 plays in active role in the control of synaptic bouton expansion and sleep in Drosophila. J Neurochem 115: 493-504.
172. Nguyen L, Humbert S, Saudou F, Chariot A (2010) Elongator - an emerging role in neurological disorders. Trends Mol Med 16: 1-6.
173. Sarthi J, Elefant F (2011) dTip60 HAT activity controls synaptic bouton expansion at the Drosophila neuromuscular junction. PLoS One 6: e26202.
174. Hoshino M, Tagawa K, Okuda T, Murata M, Oyanagi K, et al. (2003) Histone deacetylase activity is retained in primary neurons expressing mutant huntingtin protein. J Neurochem 87: 257-267.
175. Langley B, Gensert JM, Beal MF, Ratan RR (2005) Remodeling chromatin and stress resistance in the central nervous system: histone deacetylase inhibitors as novel and

broadly effective neuroprotective agents. Curr Drug Targets CNS Neurol Disord 4: 41-50.

176. 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. Virology 216: 357-366.
177. Sapountzi V, Logan IR, Robson CN (2006) Cellular functions of TIP60. Int J Biochem Cell Biol 38: 1496-1509.
178. 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: 827-839.
179. Alves da Costa C, Sunyach C, Pardossi-Piquard R, Sevalle J, Vincent B, et al. (2006) Presenilin-dependent gamma-secretase-mediated control of p53-associated cell death in Alzheimer's disease. J Neurosci 26: 6377-6385.
180. Kinoshita A, Whelan CM, Berezovska O, Hyman BT (2002) The gamma secretase-generated carboxyl-terminal domain of the amyloid precursor protein induces apoptosis via Tip60 in H4 cells. J Biol Chem 277: 28530-28536.
181. Wolozin B, Iwasaki K, Vito P, Ganjei JK, Lacana E, et al. (1996) Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. Science 274: 1710-1713.
182. Checler F (1999) Presenilins: multifunctional proteins involved in Alzheimer's disease pathology. IUBMB Life 48: 33-39.
183. Guo Y, Livne-Bar I, Zhou L, Boulianne GL (1999) Drosophila presenilin is required for neuronal differentiation and affects notch subcellular localization and signaling. J Neurosci 19: 8435-8442.
184. Alves da Costa C, Paitel E, Mattson MP, Amson R, Telerman A, et al. (2002) Wild-type and mutated presenilins 2 trigger p53-dependent apoptosis and down-regulate presenilin 1 expression in HEK293 human cells and in murine neurons. Proc Natl Acad Sci U S A 99: 4043-4048.
185. Bookout AL, Mangelsdorf DJ (2003) Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl Recept Signal 1: e012.
186. Irizarry RA, Ooi SL, Wu Z, Boeke JD (2003) Use of mixture models in a microarray-based screening procedure for detecting differentially represented yeast mutants. Stat Appl Genet Mol Biol 2: Article1.
187. Gunawardena S, Goldstein LS (2001) Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in Drosophila. Neuron 32: 389-401.
188. Merdes G, Soba P, Loewer A, Bilic MV, Beyreuther K, et al. (2004) Interference of human and Drosophila APP and APP-like proteins with PNS development in Drosophila. EMBO J 23: 4082-4095.
189. Blum D, Hemming FJ, Galas MC, Torch S, Cuvelier L, et al. (2004) Increased Alix (apoptosis-linked gene-2 interacting protein X) immunoreactivity in the degenerating striatum of rats chronically treated by 3-nitropropionic acid. Neurosci Lett 368: 309-313.
190. Hemming FJ, Fraboulet S, Blot B, Sadoul R (2004) Early increase of apoptosis-linked gene-2 interacting protein X in areas of kainate-induced neurodegeneration. Neuroscience 123: 887-895.
191. Montero L, Muller N, Gallant P (2008) Induction of apoptosis by Drosophila Myc. Genesis 46: 104-111.
192. Xu L, Chen Y, Song Q, Xu D, Wang Y, et al. (2009) PDCD5 interacts with Tip60 and functions as a cooperator in acetyltransferase activity and DNA damage-induced apoptosis. Neoplasia 11: 345-354.
193. Jellinger KA, Stadelmann C (2001) Problems of cell death in neurodegeneration and Alzheimer's Disease. J Alzheimers Dis 3: 31-40.

194. Sykes SM, Stanek TJ, Frank A, Murphy ME, McMahon SB (2009) Acetylation of the DNA binding domain regulates transcription-independent apoptosis by p53. J Biol Chem 284: 20197-20205.
195. Sax JK, El-Deiry WS (2003) Identification and characterization of the cytoplasmic protein TRAF4 as a p53-regulated proapoptotic gene. J Biol Chem 278: 36435-36444.
196. Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, et al. (2000) Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 102: 463-473.
197. 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: 165-173.
198. Ai W, Zheng H, Yang X, Liu Y, Wang TC (2007) Tip60 functions as a potential corepressor of KLF4 in regulation of HDC promoter activity. Nucleic Acids Res 35: 6137-6149.
199. Fazzio TG, Huff JT, Panning B (2008) An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell 134: 162-174.
200. Salminen A, Tapiola T, Korhonen P, Suuronen T (1998) Neuronal apoptosis induced by histone deacetylase inhibitors. Brain Res Mol Brain Res 61: 203-206.
201. Boutillier AL, Trinh E, Loeffler JP (2003) Selective E2F-dependent gene transcription is controlled by histone deacetylase activity during neuronal apoptosis. J Neurochem 84: 814-828.
202. Rouaux C, Loeffler JP, Boutillier AL (2004) Targeting CREB-binding protein (CBP) loss of function as a therapeutic strategy in neurological disorders. Biochem Pharmacol 68: 1157-1164.
203. Kim HS, Kim EM, Lee JP, Park CH, Kim S, et al. (2003) C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. FASEB J 17: 1951-1953.
204. Bhanot P, Fish M, Jemison JA, Nusse R, Nathans J, et al. (1999) Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development. Development 126: 4175-4186.
205. Inestrosa NC, Varela-Nallar L, Grabowski CP, Colombres M (2007) Synaptotoxicity in Alzheimer's disease: the Wnt signaling pathway as a molecular target. IUBMB Life 59: 316-321.
206. Boonen RA, van Tijn P, Zivkovic D (2009) Wnt signaling in Alzheimer's disease: up or down, that is the question. Ageing Res Rev 8: 71-82.
207. Inestrosa NC, Toledo EM (2008) The role of Wnt signaling in neuronal dysfunction in Alzheimer's Disease. Mol Neurodegener 3: 9.
208. Grewal SS, Li L, Orian A, Eisenman RN, Edgar BA (2005) Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. Nat Cell Biol 7: 295-302.
209. Galindo KA, Lu WJ, Park JH, Abrams JM (2009) The Bax/Bak ortholog in Drosophila, Debcl, exerts limited control over programmed cell death. Development 136: 275-283.
210. Xiao H, Chung J, Kao HY, Yang YC (2003) Tip60 is a co-repressor for STAT3. J Biol Chem 278: 11197-11204.
211. Li B, Samanta A, Song X, Iacono KT, Bembas K, et al. (2007) FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci U S A 104: 4571-4576.
212. Squatrito M, Gorrini C, Amati B (2006) Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends Cell Biol 16: 433-442.
213. Kim HS, Kim EM, Kim NJ, Chang KA, Choi Y, et al. (2004) Inhibition of histone deacetylation enhances the neurotoxicity induced by the C-terminal fragments of amyloid precursor protein. J Neurosci Res 75: 117-124.

214. Hernandez F, Nido JD, Avila J, Villanueva N (2009) GSK3 inhibitors and disease. Mini Rev Med Chem 9: 1024-1029.
215. Nitabach MN, Taghert PH (2008) Organization of the Drosophila circadian control circuit. Curr Biol 18: R84-93.
216. Parisky KM, Agosto J, Pulver SR, Shang Y, Kuklin E, et al. (2008) PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron 60: 672-682.
217. Sheeba V, Fogle KJ, Kaneko M, Rashid S, Chou YT, et al. (2008) Large ventral lateral neurons modulate arousal and sleep in Drosophila. Curr Biol 18: 1537-1545.
218. Lin Y, Stormo GD, Taghert PH (2004) The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the Drosophila circadian system. J Neurosci 24: 7951-7957.
219. Lear BC, Zhang L, Allada R (2009) The neuropeptide PDF acts directly on evening pacemaker neurons to regulate multiple features of circadian behavior. PLoS Biol 7: e1000154.
220. Crocker A, Sehgal A (2010) Genetic analysis of sleep. Genes Dev 24: 1220-1235.
221. Leyssen M, Ayaz D, Hebert SS, Reeve S, De Strooper B, et al. (2005) Amyloid precursor protein promotes post-developmental neurite arborization in the Drosophila brain. EMBO J 24: 2944-2955.
222. 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: 1229-1240.
223. Peng IF, Berke BA, Zhu Y, Lee WH, Chen W, et al. (2007) Temperature-dependent developmental plasticity of Drosophila neurons: cell-autonomous roles of membrane excitability, Ca2+ influx, and cAMP signaling. J Neurosci 27: 12611-12622.
224. Zimmerman JE, Raizen DM, Maycock MH, Maislin G, Pack AI (2008) A video method to study Drosophila sleep. Sleep 31: 1587-1598.
225. Sheeba V (2008) The Drosophila melanogaster circadian pacemaker circuit. J Genet 87: 485-493.
226. Fernandez MP, Berni J, Ceriani MF (2008) Circadian remodeling of neuronal circuits involved in rhythmic behavior. PLoS Biol 6: e69.
227. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell 99: 791-802.
228. Chung BY, Kilman VL, Keath JR, Pitman JL, Allada R (2009) The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. Curr Biol 19: 386-390.
229. Pfeiffenberger C, Lear BC, Keegan KP, Allada R (2010) Processing sleep data created with the Drosophila Activity Monitoring (DAM) System. Cold Spring Harb Protoc 2010: pdb prot5520.
230. Gonzales E, Yin J (2010) Drosophila Models of Huntington's Disease exhibit sleep abnormalities. PLoS Curr 2.
231. Dubruille R, Emery P (2008) A plastic clock: how circadian rhythms respond to environmental cues in Drosophila. Mol Neurobiol 38: 129-145.
232. Shafer OT, Taghert PH (2009) RNA-interference knockdown of Drosophila pigment dispersing factor in neuronal subsets: the anatomical basis of a neuropeptide's circadian functions. PLoS One 4: e8298.
233. Helfrich-Forster C (1997) Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of Drosophila melanogaster. J Comp Neurol 380: 335-354.
234. Saxena S, Caroni P (2007) Mechanisms of axon degeneration: from development to disease. Prog Neurobiol 83: 174-191.

235. Yan T, Feng Y, Zhai Q (2010) Axon degeneration: Mechanisms and implications of a distinct program from cell death. Neurochem Int 56: 529-534.
236. Mandelkow EM, Stamer K, Vogel R, Thies E, Mandelkow E (2003) Clogging of axons by tau, inhibition of axonal traffic and starvation of synapses. Neurobiol Aging 24: 1079-1085.
237. Yan Y, Barlev NA, Haley RH, Berger SL, Marmorstein R (2000) Crystal structure of yeast Esa1 suggests a unified mechanism for catalysis and substrate binding by histone acetyltransferases. Mol Cell 6: 1195-1205.
238. Chokroverty S (2009) Sleep and neurodegenerative diseases. Semin Neurol 29: 446-467.
239. Thannickal TC, Nienhuis R, Siegel JM (2009) Localized loss of hypocretin (orexin) cells in narcolepsy without cataplexy. Sleep 32: 993-998.
240. Ohno K, Sakurai T (2008) Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. Front Neuroendocrinol 29: 70-87.
241. Fronczek R, van Geest S, Frolich M, Overeem S, Roelandse FW, et al. (2011) Hypocretin (orexin) loss in Alzheimer's disease. Neurobiol Aging.
242. Park JH, Helfrich-Forster C, Lee G, Liu L, Rosbash M, et al. (2000) Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc Natl Acad Sci U S A 97: 3608-3613.
243. Sheeba V, Fogle KJ, Holmes TC (2010) Persistence of morning anticipation behavior and high amplitude morning startle response following functional loss of small ventral lateral neurons in Drosophila. PLoS One 5: e11628.
244. Henny P, Jones BE (2006) Innervation of orexin/hypocretin neurons by GABAergic, glutamatergic or cholinergic basal forebrain terminals evidenced by immunostaining for presynaptic vesicular transporter and postsynaptic scaffolding proteins. J Comp Neurol 499: 645-661.
245. Sakurai T (2007) The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. Nat Rev Neurosci 8: 171-181.
246. Schwartz JR, Roth T (2008) Neurophysiology of sleep and wakefulness: basic science and clinical implications. Curr Neuropharmacol 6: 367-378.
247. Franken P, Dijk DJ (2009) Circadian clock genes and sleep homeostasis. Eur J Neurosci 29: 1820-1829.
248. Carter ME, Adamantidis A, Ohtsu H, Deisseroth K, de Lecea L (2009) Sleep homeostasis modulates hypocretin-mediated sleep-to-wake transitions. J Neurosci 29: 10939-10949.
249. Prober DA, Rihel J, Onah AA, Sung RJ, Schier AF (2006) Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. J Neurosci 26: 13400-13410.
250. Isaac RE, Johnson EC, Audsley N, Shirras AD (2007) Metabolic inactivation of the circadian transmitter, pigment dispersing factor (PDF), by neprilysin-like peptidases in Drosophila. J Exp Biol 210: 4465-4470.
251. Yasuyama K, Meinertzhagen IA (2010) Synaptic connections of PDF-immunoreactive lateral neurons projecting to the dorsal protocerebrum of Drosophila melanogaster. J Comp Neurol 518: 292-304.
252. Hamasaka Y, Wegener C, Nassel DR (2005) GABA modulates Drosophila circadian clock neurons via GABAB receptors and decreases in calcium. J Neurobiol 65: 225-240.
253. McCarthy EV, Wu Y, Decarvalho T, Brandt C, Cao G, et al. (2011) Synchronized bilateral synaptic inputs to Drosophila melanogaster neuropeptidergic rest/arousal neurons. J Neurosci 31: 8181-8193.
254. Shang Y, Griffith LC, Rosbash M (2008) Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci U S A 105: 19587-19594.
255. Kang JE, Lim MM, Bateman RJ, Lee JJ, Smyth LP, et al. (2009) Amyloid-beta dynamics are regulated by orexin and the sleep-wake cycle. Science 326: 1005-1007.

256. Barrett RM, Wood MA (2008) Beyond transcription factors: the role of chromatin modifying enzymes in regulating transcription required for memory. Learn Mem 15: 460-467.
257. Graff J, Franklin TB, Mansuy IM (2010) [Epigenetics and memory]. Biol Aujourdhui 204: 131-137.
258. Peixoto L, Abel T (2013) The role of histone acetylation in memory formation and cognitive impairments. Neuropsychopharmacology 38: 62-76.
259. Graff J, Rei D, Guan JS, Wang WY, Seo J, et al. (2012) An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature 483: 222-226.
260. Cao X, Sudhof TC (2004) Dissection of amyloid-beta precursor protein-dependent transcriptional transactivation. J Biol Chem 279: 24601-24611.
261. Fitzsimons HL, Scott MJ (2011) Genetic modulation of Rpd3 expression impairs long-term courtship memory in Drosophila. PLoS One 6: e29171.
262. Heisenberg M (2003) Mushroom body memoir: from maps to models. Nat Rev Neurosci 4: 266-275.
263. Mehren JE, Ejima A, Griffith LC (2004) Unconventional sex: fresh approaches to courtship learning. Curr Opin Neurobiol 14: 745-750.
264. Melicharek DJ, Ramirez LC, Singh S, Thompson R, Marenda DR (2010) Kismet/CHD7 regulates axon morphology, memory and locomotion in a Drosophila model of CHARGE syndrome. Hum Mol Genet 19: 4253-4264.
265. Lee T, Lee A, Luo L (1999) Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development 126: 4065-4076.
266. Fushima K, Tsujimura H (2007) Precise control of fasciclin II expression is required for adult mushroom body development in Drosophila. Dev Growth Differ 49: 215-227.
267. Crittenden JR, Skoulakis EM, Han KA, Kalderon D, Davis RL (1998) Tripartite mushroom body architecture revealed by antigenic markers. Learn Mem 5: 38-51.
268. Awasaki T, Saito M, Sone M, Suzuki E, Sakai R, et al. (2000) The Drosophila trio plays an essential role in patterning of axons by regulating their directional extension. Neuron 26: 119-131.
269. Bates KE, Sung CS, Robinow S (2010) The unfulfilled gene is required for the development of mushroom body neuropil in Drosophila. Neural Dev 5: 4.
270. Siegel RW, Hall JC (1979) Conditioned responses in courtship behavior of normal and mutant Drosophila. Proc Natl Acad Sci U S A 76: 3430-3434.
271. Broughton SJ, Tully T, Greenspan RJ (2003) Conditioning deficits of CaM-kinase transgenic Drosophila melanogaster in a new excitatory courtship assay. J Neurogenet 17: 91-102.
272. Siwicki KK, Riccio P, Ladewski L, Marcillac F, Dartevelle L, et al. (2005) The role of cuticular pheromones in courtship conditioning of Drosophila males. Learn Mem 12: 636-645.
273. Kamyshev NG, Iliadi KG, Bragina Iu V, Savvateeva-Popova EV, Tokmacheva EV, et al. (1999) [Isolation of memory-deficient Drosophila mutants in conditioned courtship suppression paradigm]. Ross Fiziol Zh Im I M Sechenova 85: 84-92.
274. Aso Y, Grubel K, Busch S, Friedrich AB, Siwanowicz I, et al. (2009) The mushroom body of adult Drosophila characterized by GAL4 drivers. J Neurogenet 23: 156-172.
275. Greenspan RJ (1995) Flies, genes, learning, and memory. Neuron 15: 747-750.
276. McBride SM, Choi CH, Wang Y, Liebelt D, Braunstein E, et al. (2005) Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. Neuron 45: 753-764.
277. Luo L, O'Leary DD (2005) Axon retraction and degeneration in development and disease. Annu Rev Neurosci 28: 127-156.

278. Graff J, Mansuy IM (2008) Epigenetic codes in cognition and behaviour. Behav Brain Res 192: 70-87.
279. Sweatt JD (2009) Experience-dependent epigenetic modifications in the central nervous system. Biol Psychiatry 65: 191-197.
280. Scott EK, Lee T, Luo L (2001) enok encodes a Drosophila putative histone acetyltransferase required for mushroom body neuroblast proliferation. Curr Biol 11: 99-104.
281. Watts RJ, Hoopfer ED, Luo L (2003) Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. Neuron 38: 871-885.
282. Watts RJ, Schuldiner O, Perrino J, Larsen C, Luo L (2004) Glia engulf degenerating axons during developmental axon pruning. Curr Biol 14: 678-684.
283. Baas PW, Luo L (2001) Signaling at the growth cone: the scientific progeny of Cajal meet in Madrid. Neuron 32: 981-984.
284. Dent EW, Gertler FB (2003) Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron 40: 209-227.
285. Conde C, Caceres A (2009) Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci 10: 319-332.
286. Creppe C, Malinouskaya L, Volvert ML, Gillard M, Close P, et al. (2009) Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin. Cell 136: 551-564.
287. Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol A 157: 263-277.
288. Davis RL (1993) Mushroom bodies and Drosophila learning. Neuron 11: 1-14.
289. Dubnau J, Tully T (1998) Gene discovery in Drosophila: new insights for learning and memory. Annu Rev Neurosci 21: 407-444.
290. McBride SM, Giuliani G, Choi C, Krause P, Correale D, et al. (1999) Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron 24: 967-977.
291. Rybak J, Menzel R (1993) Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. J Comp Neurol 334: 444-465.
292. Hu A, Zhang W, Wang Z (2010) Functional feedback from mushroom bodies to antennal lobes in the Drosophila olfactory pathway. Proc Natl Acad Sci U S A 107: 10262-10267.
293. Guan Z, Buhl LK, Quinn WG, Littleton JT (2011) Altered gene regulation and synaptic morphology in Drosophila learning and memory mutants. Learn Mem 18: 191-206.
294. Technau G, Heisenberg M (1982) Neural reorganization during metamorphosis of the corpora pedunculata in Drosophila melanogaster. Nature 295: 405-407.
295. Technau GM (1984) Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. J Neurogenet 1: 113-126.
296. Heisenberg M, Heusipp M, Wanke C (1995) Structural plasticity in the Drosophila brain. J Neurosci 15: 1951-1960.
297. Barth M, Heisenberg M (1997) Vision affects mushroom bodies and central complex in Drosophila melanogaster. Learn Mem 4: 219-229.
298. Valouev A, Johnson DS, Sundquist A, Medina C, Anton E, et al. (2008) Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. Nat Methods 5: 829-834.
299. MacQuarrie KL, Fong AP, Morse RH, Tapscott SJ (2011) Genome-wide transcription factor binding: beyond direct target regulation. Trends Genet 27: 141-148.
300. Furey TS (2012) ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions. Nat Rev Genet 13: 840-852.
301. Park PJ (2009) ChIP-seq: advantages and challenges of a maturing technology. Nat Rev Genet 10: 669-680.

302. Erlich Y, Mitra PP, delaBastide M, McCombie WR, Hannon GJ (2008) Alta-Cyclic: a self-optimizing base caller for next-generation sequencing. Nat Methods 5: 679-682.
303. Cock PJ, Fields CJ, Goto N, Heuer ML, Rice PM (2010) The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res 38: 1767-1771.
304. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9: 357-359.
305. Ji H, Jiang H, Ma W, Wong WH (2011) Using CisGenome to analyze ChIP-chip and ChIP-seq data. Curr Protoc Bioinformatics Chapter 2: Unit2 13.
306. Ji H, Jiang H, Ma W, Johnson DS, Myers RM, et al. (2008) An integrated software system for analyzing ChIP-chip and ChIP-seq data. Nat Biotechnol 26: 1293-1300.
307. Thomas-Chollier M, Darbo E, Herrmann C, Defrance M, Thieffry D, et al. (2012) A complete workflow for the analysis of full-size ChIP-seq (and similar) data sets using peak-motifs. Nat Protoc 7: 1551-1568.
308. Yin H, Sweeney S, Raha D, Snyder M, Lin H (2011) A high-resolution whole-genome map of key chromatin modifications in the adult Drosophila melanogaster. PLoS Genet 7: e1002380.
309. Huang da W, Sherman BT, Tan Q, Kir J, Liu D, et al. (2007) DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res 35: W169-175.
310. Haggarty SJ, Tsai LH (2011) Probing the role of HDACs and mechanisms of chromatin-mediated neuroplasticity. Neurobiol Learn Mem 96: 41-52.
311. Day JJ, Sweatt JD (2011) Epigenetic mechanisms in cognition. Neuron 70: 813-829.
312. Day JJ, Sweatt JD (2012) Epigenetic treatments for cognitive impairments. Neuropsychopharmacology 37: 247-260.
313. Ribeiro S (2012) Sleep and plasticity. Pflugers Arch 463: 111-120.
314. Tononi G, Cirelli C (2003) Sleep and synaptic homeostasis: a hypothesis. Brain Res Bull 62: 143-150.
315. Born J, Feld GB (2012) Sleep to upscale, sleep to downscale: balancing homeostasis and plasticity. Neuron 75: 933-935.
316. Vyazovskiy VV, Cirelli C, Pfister-Genskow M, Faraguna U, Tononi G (2008) Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. Nat Neurosci 11: 200-208.
317. Liu ZW, Faraguna U, Cirelli C, Tononi G, Gao XB (2010) Direct evidence for wake-related increases and sleep-related decreases in synaptic strength in rodent cortex. J Neurosci 30: 8671-8675.
318. Ganguly-Fitzgerald I, Donlea J, Shaw PJ (2006) Waking experience affects sleep need in Drosophila. Science 313: 1775-1781.
319. Donlea JM, Ramanan N, Shaw PJ (2009) Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science 324: 105-108.
320. Fraser P, Bickmore W (2007) Nuclear organization of the genome and the potential for gene regulation. Nature 447: 413-417.
321. Manuelidis L (1984) Different central nervous system cell types display distinct and nonrandom arrangements of satellite DNA sequences. Proc Natl Acad Sci U S A 81: 3123-3127.
322. McGuire SE, Mao Z, Davis RL (2004) Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci STKE 2004: pl6.
323. Sweatt JD (2010) Neuroscience. Epigenetics and cognitive aging. Science 328: 701-702.
324. Peixoto L, Abel T (2012) The Role of Histone Acetylation in Memory Formation and Cognitive Impairments. Neuropsychopharmacology.

325. Gardian G, Browne SE, Choi DK, Klivenyi P, Gregorio J, et al. (2005) Neuroprotective effects of phenylbutyrate in the N171-82Q transgenic mouse model of Huntington's disease. J Biol Chem 280: 556-563.
326. Zhao W, Kruse JP, Tang Y, Jung SY, Qin J, et al. (2008) Negative regulation of the deacetylase SIRT1 by DBC1. Nature 451: 587-590.
327. Jiang YM, Yamamoto M, Kobayashi Y, Yoshihara T, Liang Y, et al. (2005) Gene expression profile of spinal motor neurons in sporadic amyotrophic lateral sclerosis. Ann Neurol 57: 236-251.
328. Jiang H, Poirier MA, Liang Y, Pei Z, Weiskittel CE, et al. (2006) Depletion of CBP is directly linked with cellular toxicity caused by mutant huntingtin. Neurobiol Dis 23: 543-551.
329. Pirooznia SK, Chiu K, Chan MT, Zimmerman JE, Elefant F (2012) Epigenetic Regulation of Axonal Growth of Drosophila Pacemaker Cells by Histone Acetyltransferase Tip60 Controls Sleep. Genetics.
330. Vecsey CG, Hawk JD, Lattal KM, Stein JM, Fabian SA, et al. (2007) Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. J Neurosci 27: 6128-6140.
331. Wang Z, Zang C, Cui K, Schones DE, Barski A, et al. (2009) Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell 138: 1019-1031.
332. Feng J, Fouse S, Fan G (2007) Epigenetic regulation of neural gene expression and neuronal function. Pediatr Res 61: 58R-63R.
333. Sterner DE, Berger SL (2000) Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 64: 435-459.
334. Roth SY, Denu JM, Allis CD (2001) Histone acetyltransferases. Annu Rev Biochem 70: 81-120.
335. Nowak SJ, Corces VG (2000) Phosphorylation of histone H3 correlates with transcriptionally active loci. Genes Dev 14: 3003-3013.
336. Rice JC, Allis CD (2001) Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol 13: 263-273.
337. Konsoula Z, Barile FA (2012) Epigenetic histone acetylation and deacetylation mechanisms in experimental models of neurodegenerative disorders. J Pharmacol Toxicol Methods 66: 215-220.
338. Miyagi S, Mishima Y, Iwama A (2011) [Structure and function of MYST family histone acetyltransferases]. Rinsho Ketsueki 52: 490-496.
339. Walker MP, Stickgold R (2006) Sleep, memory, and plasticity. Annu Rev Psychol 57: 139-166.
340. Stickgold R, Walker MP (2007) Sleep-dependent memory consolidation and reconsolidation. Sleep Med 8: 331-343.
341. Hendricks JC, Sehgal A (2004) Why a fly? Using Drosophila to understand the genetics of circadian rhythms and sleep. Sleep 27: 334-342.
342. Shaw P (2003) Awakening to the behavioral analysis of sleep in Drosophila. J Biol Rhythms 18: 4-11.
343. Ho KS, Sehgal A (2005) Drosophila melanogaster: an insect model for fundamental studies of sleep. Methods Enzymol 393: 772-793.
344. Cirelli C (2009) The genetic and molecular regulation of sleep: from fruit flies to humans. Nat Rev Neurosci 10: 549-560.
345. Helfrich-Forster C, Wulf J, de Belle JS (2002) Mushroom body influence on locomotor activity and circadian rhythms in Drosophila melanogaster. J Neurogenet 16: 73-109.
346. Moore RY (2007) Suprachiasmatic nucleus in sleep-wake regulation. Sleep Med 8 Suppl 3: 27-33.

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2004	M.Sc., Genomics, Madurai Kamaraj University, India (Dissertation: *Subcloning and Characterization of ppm (post plating mutagenesis) mutant gene in Escherichia Coli: evidence to support mistranslation due to ppm*)
2002	B.Sc., Biotechnology, Lady Doak College, India

PUBLICATIONS

1. Lorbeck, M., **Pirooznia, K.**, Sarthi, J., Zhu, X. and Elefant, F. (2011) Microarray Analysis Uncovers a Role for Tip60 in Nervous System Function and General Metabolism. PLoS One 6: e18412

2. **Pirooznia, S. K.**, Sarthi, J., Johnson, A., Lorbeck, M., Chiu, K., Koduri, S. and 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

3. **Pirooznia, S. K.**, Chiu, K., Zimmerman, J. E. and Elefant, F (2012) The histone acetyltransferase Tip60 epigenetically regulates axonal growth of *Drosophila* pacemaker cells to control sleep. Genetics 192(4):1327-45

4. **Pirooznia, S. K**., and Elefant, F. Modulating Epigenetic HAT Activity: A Promising Therapeutic Option for Neurological Disease? J Mol Cloning Genet Recomb 2012, 1:1

5. **Pirooznia, S. K.,** and Elefant, F. HATs and aging associated neurodegenerative diseases: translating basic biology to therapeutic possibilities. Review. Under preparation

6. **Pirooznia, S. K.,** Koduri, S., Chiu, K., Menon, T., and Elefant, F. Tip60 mediated neuronal gene expression changes underlie memory formation in *Drosophila*. Manuscript, under preparation

ABSTRACTS

Pirooznia, S. K., Chiu, K, Sarthi, J, Zervos, A, Reube, W, Koduri, S and Elefant, F. An Epigenetic Role for Tip60 in APP mediated axonal outgrowth of clock neurons that control sleep. To be presented at the 10th EMBL Conference – Transcription and Chromatin, Advanced Training Center, Heidleburg, Germany. (August 25-28, 2012)

Chiu, K., Koduri, S., **Pirooznia, S. K**., and Elefant, F. An Epigenetic Role for Tip60 in Learning and Memory in *Drosophila*. Annual Research Day, Drexel University College of arts and sciences, (2012)

Pirooznia, S. K., Chiu, K and Elefant, F. Tip60 HAT activity modulates APP mediated effects on *Drosophila* small ventrolateral neurons and affects sleep-wake cycle. Presented at Annual Research Day, Drexel University College of arts and sciences, (2012)

Sarthi, J., **Pirooznia, S. K**., Zervos, A and Elefant, F. An Epigenetic Role for Tip60 in the control of Synapstic Plasticity: Implications for Learning and Memory. BIT's 2nd Annual World Congress of Neuro Talk, Dalian, China (2011)

Pirooznia, S. K., and Elefant, F. Role of dTip60 in APP mediated axonal outgrowth. Presented at the Keystone symposium. Histone Code: Fact or Fiction ?, Midway, Utah, (2011).

Pirooznia, S. K., and Elefant, F. Role of Tip60 in APP mediated axonal arborization in *Drosophila melanogaster*. Presented at Annual Research Day, Drexel University College of arts and sciences, (2011). *Received best poster award*

Pirooznia, S. K., and Elefant, F. Role of Tip60 in APP mediated axonal arborization in *Drosophila melanogaster*. Presented at 51st Annual *Drosophila* Research Conference, Washington DC, (2010).

Chandrasekar, S.K., and Shearer, G. Isolation and characterization of the Ku70, Ku80 and ligD gene orthologs of the pathogenic fungus *Histoplasma capsulatum*. Presented at the Fall Meeting of the Texas/South Central Branches of The American Society for Microbiology (November 2008)

Chandrasekar, S. K., Ellender, R. D., and Wang, S. Overcoming the Inhibitor Problem in PCR Detection of *Salmonella* in Recreational Waters: A Volume Based Approach. Presented at the 108th American Society for Microbiology General Meeting (May 2008)

Chandrasekar, S. K., Ellender, R. D., and Wang, S. Detection of *Salmonella* spp. in Recreational Waters Using a Real-Time PCR Assay. Presented at the 107th American Society for Microbiology General Meeting (2007)

Chandrasekar, S. K., Ellender, R. D., and Wang, S. Detection of *Salmonella* spp. in Coastal Waters. Presented at the 106th American Society for Microbiology General Meeting (2006)

INVITED ORAL PRESENTATIONS

- An epigenetic role for dTip60 in APP mediated axonal outgrowth that controls circadian rhythm. Department of Biology graduate seminar series, Drexel University (2010)
- Tip60 HAT activity plays an active role in axonal outgrowth during Drosophila development. Penn Epigenetics Program, University of Pennsylvania, PA (2010)
- An epigenetic role for Tip60 in APP mediated axonal outgrowth of clock neurons that control sleep. Department of Biology graduate seminar series, Drexel University (2012)
- An epigenetic role for Tip60 in sleep and memory formation in *Drosophila*. Mood Disorder group, Johns Hopkins University, MD (2012)
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RESEARCH EXPERIENCE

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