Epigenetic control of learning and memory in *Drosophila* by Tip60 HAT action

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ABSTRACT

Disruption of epigenetic gene control mechanisms in the brain causes significant cognitive impairment that is a debilitating hallmark of most neurodegenerative disorders including Alzheimer’s disease (AD). Histone acetylation is one of the best characterized of these epigenetic mechanisms that is critical for regulating learning and memory associated gene expression profiles, yet the specific histone acetyltransferases (HATs) that mediate these effects have yet to be fully characterized. Here, we investigate an epigenetic role for the HAT Tip60 in learning and memory formation using the Drosophila CNS mushroom body (MB) as a well-characterized cognition model. We show that Tip60 is endogenously expressed in the Kenyon cells, the intrinsic neurons of the MB and in the MB axonal lobes. Targeted loss of Tip60 HAT activity in the MB causes thinner and shorter axonal lobes while increasing Tip60 HAT levels cause no morphological defects. Functional consequences of both loss and gain of Tip60 HAT levels in the MB are evidenced by defects in immediate recall memory. Our ChIP-Seq analysis reveals that Tip60 target genes are enriched for functions in cognitive processes and accordingly, key genes representing these pathways are misregulated in the Tip60 HAT mutant fly brain. Remarkably, we find that both learning and immediate recall memory deficits that occur under AD associated amyloid precursor protein (APP) induced neurodegenerative conditions can be effectively rescued by increasing Tip60 HAT levels specifically in the MB. Together, our findings uncover an epigenetic transcriptional regulatory role for Tip60 in cognitive function and highlight the potential of HAT activators as a therapeutic option for neurodegenerative disorders.
INTRODUCTION

The ability of living organisms to respond to a constantly changing environment and fine-tune their complex behaviors accordingly is crucial for their adaptation and survival throughout development (AL-SAIGH et al.; LEVENSON AND SWEATT 2005; BORRELLI et al. 2008; WEST AND GREENBERG 2011). One of the most important of such experience-driven behavioral changes is learning and memory formation, as it directly impacts cognitive ability (LEVENSON AND SWEATT 2005; CARULLI et al. 2011; NELSON AND L.M.MONTEGIA 2011; WEST AND GREENBERG 2011). In the brain, external stimuli are converted into intracellular signals that program coordinated expression of specific gene sets that promote sustained neuroplasticity and cognitive adaptation (SWEATT 2009; RICCIO 2010; CARULLI et al. 2011). Disruption of these response programs result in significant cognitive impairment disorders (WEST AND GREENBERG 2011; EBERT AND GREENBERG 2013; PIROOZNA AND ELEFANT 2013a). Epigenetic post-translational modifications (PTMs) of histone proteins that control nuclear chromatin packaging and gene expression profiles are emerging as a fundamental mechanism by which neurons adapt their transcriptional response to environmental cues(FENG et al. 2007; SWEATT 2009; BOURSIGES et al. 2010; MEANEY AND FERGUSON-SMITH 2010; RICCIO 2010; NELSON AND L.M.MONTEGIA 2011; PIROOZNA AND ELEFANT 2012). One of the best characterized forms of epigenetic chromatin modification in the learning and memory field is histone acetylation (PEIXOTO AND ABEL 2013), which is regulated by the antagonistic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (LEGUBE AND TROUCHE 2003). Blocking histone acetylation has been reported to impair both long lasting synaptic plasticity as well as behavioral performance (KORZUS et al. 2004). Notably, inhibition of histone deacetylase activity reverses such deficits and improves memory formation (KORZUS et al. 2004; LEVENSON et al. 2004), thus highlighting the importance of histone acetylation for memory formation.

Disruption of epigenetic gene control mechanisms in the brain causes significant cognitive impairment that is a debilitating hallmark of most neurodegenerative disorders including Alzheimer’s disease (AD). Sporadic cases of reduced histone acetylation levels are found in the brains of animal models for multiple types of neurodegenerative diseases, that include AD. These changes have been shown to cause an epigenetic blockade of transcription that results in cognitive impairment. (GRAFF et al. 2012a). Pharmacological treatments aimed at increasing histone acetylation levels by inhibiting histone deacetylase
action in these models have shown promising effects in reversing cognitive deficits (KAZANTSEV AND THOMPSON 2008). 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) (CAO AND SUDHOF 2001; CAO AND SUDHOF 2004).

Tip60 (Tat interactive protein, 60KDa) is a multifunctional HAT that has been shown by others and us to epigenetically regulate genes essential for neurogenesis (LORBECK et al. 2011; PIROOZNIA et al. 2012b). 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 for AD by activating pro-survival factors while concomitantly repressing activators of cell death (PIROOZNIA et al. 2012b). Overexpression of Tip60 also promotes axonal growth of the Drosophila circadian neurons, the small ventrolateral neurons (sLNvs) and rescues axonal outgrowth and transport as well as associated behavioral phenotypes such as sleep and locomotion under APP induced neurodegenerative conditions (PIROOZNIA et al. 2012a). 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 focused on molecular dissection of components of memory formation due to the availability of reproducible memory assays and genetic tools that enable restricting gene expression manipulation to specific subregions of the brain (SIWICKI 2003; FIALA 2007; KAHSAI 2011). The Drosophila MB is known to regulate a range of behavioral and physiological functions that range from olfactory learning, courtship conditioning to decision making under uncertain conditions (HEISENBERG 2003; MARGULIES C. 2005; AKALAI 2006; FARRIS S. 2013; GUVEN-AZKAN T. 2014). 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 (Dubna J. 2001; Mehren et al. 2004; Keene 2007; Busto 2010).

In this study, we focus on the Drosophila mushroom body (MB) as a well-characterized cognition model to investigate an epigenetic role for Tip60 HAT action in learning and memory formation. We find that Tip60 is robustly produced in the MB of the adult fly brain. Targeted loss of Tip60 HAT activity in the MB causes abnormal development of the MB axonal lobes, while increasing Tip60 HAT levels causes no morphological defects. Further, we show that misregulation of Tip60 HAT activity in the MB leads to immediate recall memory deficits in adult flies. Our ChIP-Seq analysis reveals that Tip60 target genes are enriched for functions in cognitive processes and accordingly, key genes representing these pathways are misregulated in the Tip60 HAT mutant fly brain. Importantly, we show that both learning and memory defects in Drosophila that occur under AD associated APP induced neurodegenerative conditions can be effectively rescued by increasing Tip60 HAT levels in the Drosophila MB. Together, our studies uncover an epigenetic transcriptional regulatory role for Tip60 HAT action in cognitive function and highlight the therapeutic potential of utilizing specific HAT activators for treatment of cognitive deficits in neurodegenerative disorders.

METHODS

Fly Stocks and Maintenance

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 wild-type controls. OK107-GAL4 and UAS-GFP stocks were obtained from the Bloomington Drosophila stock center (Indiana University). The generation and characterization of UAS-dTip60E431Q and UAS-dTip60WT flies are described in (Lorbeck et al. 2011). Generation and characterization of the double transgenic UAS-Tip60;APP fly lines are described in (Pirooznia et al. 2012b). Double transgenic lines carrying the UAS-mCD8-GFP and either UAS-dTip60E431Q or UAS-dTip60WT constructs were generated according to standard procedures. All of the UAS-dTip60 fly lines described here are contained within a w^{1118} genetic background. Additionally, for all experiments, transgene expression levels for each of the UAS fly lines were assessed as described (Lorbeck et al. 2011; Pirooznia et al. 2012a; Pirooznia et al. 2012b; Johnson et al. 2012).
(2013) to ensure that the different transgenic lines used for phenotype comparisons show equivalent dTip60\textsubscript{E431Q}, dTip60\textsubscript{WT}, APP or APP-CT expression levels.

**Courtship Suppression Assay:**

Assays were performed as described in (McBride et al. 2005; Melicharek et al. 2010). 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 hrs 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 1μM serial Z-sections. Consecutive subsets of the Z-stacks were utilized for the final projection. Images were adjusted for brightness and contrast using the ImageJ program to more clearly define MBs. Projections from unprocessed images are found in Figure S1. Area of the mushroom body lobes in the different genotypes was measured using NIH ImageJ software.

**Real-time PCR analysis.**

Total RNA was isolated from adult fly heads using the RNeasy Plus Mini Kit (QIAGEN). cDNA was prepared using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions with 1 μg total RNA and 0.2μg/ml random hexamer primers (Roche Applied Science). PCRs were performed in a 20 μl reaction volume containing cDNA, 1μPower SYBR Green PCR Master Mix (Applied Biosystems), and 10 μM both forward and reverse primers (primer pairs available upon request). PCR was performed using an ABI 7500 Real-Time PCR system (Applied Biosystems) following the manufacturer’s instructions. Fold change in mRNA expression was determined by the ΔΔCt method (Livak and Schmittgen, 2001).
**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)

**ChIP-Seq**

Chromatin from S2 cells was prepared as follows. 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 then 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 5μL 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 yielding sheared chromatin fragments ranging from 200-500bp.

Chromatin precipitation assays were performed using ChIP-IT Express Kit (Active Motif), following the manufacturer’s instructions. Briefly, 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 (Thermo Fisher Scientific, Huntsville, AL). A mock reaction containing all reagents except the antibody was also set up as a control. The chromatin was immunoprecipitated using the ChIP IT Express kit (Active Motif) exactly following the manufacturer’s specifications. The eluted material from the
immunoprecipitation was then purified using QIAquick PCR purification kit (Qiagen) and was directly used for real-time PCR.

DNA library preparation and sequencing of ChIP DNA samples was carried out by the Jefferson Kimmel Cancer Center (KCC), Cancer Genomics Shared Resource (CGSR). Sequencing of ChIP DNA samples was performed using the Illumina HiSeq2000 platform.

**Bioinformatic Analysis**

**Peak-calling**

MACS v1.4 peak-calling algorithm was used to find over-represented sequence regions (peaks) representing likely in vivo binding sites for Tip60 or RNA Pol II, and the final set of significant peaks was produced in interval (BED) format. Prior to peak calling, BAM files of biological replicates for each antibody or input were merged together. Peak calling with MACS was done using the online Galaxy platform with the following parameters: mfold=5, tag size=35nt, p-value cutoff=1e-05, genome size=130e+06, FutureFDR=true.

**Gene annotation of ChIP-Seq peaks**

ChIP-Seq peaks in the form of genomic intervals (BED file) were intersected with genomic coordinates of genes in the *D. melanogaster* genome annotation release R5.56 (ftp://ftp.flybase.net/releases/FB2014_02/) using the Galaxy platform (https://usegalaxy.org/) and gene identifiers were confirmed and converted where necessary for further analysis using the UCSC table browser (http://genome.ucsc.edu/).

**Functional annotation and cluster enrichment**

Functional annotation and enrichment cluster analysis of Tip60-associated gene list was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, and Gene Annotation Co-occurrence Discovery (GeneCodis) v3 (http://genecodis.cnb.csic.es/). P-values for significant enrichment were obtained with a hypergeometric test and corrected for multiple comparisons by FDR. Pathway enrichment was determined using the pathway enrichment module of the FlyMine integrated genomics database platform.
Tissue-specific expression profiles

Tissue-specific expression data for the 321 Tip60-associated genes were obtained from FlyAtlas (Chintapalli et al., 2007) using the FlyMine integrated genomics database (Lyne et al., 2007).

TF motif enrichment

TF motif enrichment analysis was performed on Tip60-associated neuronal genes using the MEME-ChIP suite (Machanick & Bailey, 2011), RSAT (Thomas-Chollier et al., 2011; 2012) and Motif Enrichment Tool. TF motif databases utilized for comparison with discovered motifs included JASPAR Core Insects, FlyFactorSurvey, DMMPMM, and IDMMPMM drosophila databases. As each tool employs a different algorithm for determining significance of motif enrichment and database match, where database matches are identified by more than one tool, significance values reported in this work represent the most conservative significance value found.

RESULTS

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

Given the importance of brain specific histone acetylation profiles in cognitive function, we wished to examine whether the HAT Tip60 was produced in the adult fly brain. Tip60 production in the adult fly brain was characterized by immunohistochemistry on whole mount Canton S adult brains using an anti-Drosophila Tip60 antibody. We found that Tip60 was robustly and widely produced throughout the adult brain in a pattern similar to the pan-neuronal ELAV protein including the mushroom body (MB) lobes (Figure 1, A-C). To examine Tip60 concentration in the MB, immunohistochemistry for Tip60 was performed on brains expressing mCD8-GFP under control of the MB specific driver OK107-GAL4 that marks MB structure. In the MB neurons, called Kenyon cells, mCD8-GFP 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 (Lee et al. 1999). 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 (Fushima and Tsujimura 2007) and is expressed strongly in the α/β lobes (Figure 2B) (Crittenden et al. 1998). 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) (Awasaki et al. 2000). Both markers are produced weakly in the γ lobe as well (Figure 2B and 2E)(Bates et al. 2010). Tip60 production pattern in the α/ β and α’/β’ lobes followed the pattern of Fas-II and Trio, respectively (Figure 2C and 2F).

**Appropriate Tip60 HAT levels are required for immediate recall memory.**

Our finding that Tip60 is endogenously and robustly produced in the adult MB prompted us to ask whether Tip60 epigenetically regulates memory formation. To address this question, we used the conditioned courtship suppression assay (Siegel and Hall 1979). This assay is an associative conditioning procedure that measures both learning and memory in individual flies (Broughton et al. 2003). 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 (Siegel and Hall 1979; Siwicki et al. 2005). 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 (Siegel and Hall 1979)

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 (dTip60E431Q), or wild-type dTip60 (dTip60WT) (Siegel and Hall 1979; Lorbeck et al. 2011). 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 (Aso et al. 2009). 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-dTip60E431Q/+ and UAS-dTip60WT/+ ) and in the wild type Canton S flies (Figure 3A). Male flies expressing either the Tip60 HAT mutant (dTip60E431Q ) or additional copies of wild type Tip60 (dTip60WT ) 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) (Greenspan 1995; McBride et al. 1999; McBride et al. 2005). In order to test for the earliest phase of memory first, we assayed male flies expressing either the Tip60 HAT mutant (dTip60E431Q ) or wildtype Tip60 (dTip60WT ) 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-dTip60E431Q/+ and UAS-dTip60WT/+ ) 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 (dTip60E431Q ) or additional copies of the HAT competent wild type Tip60 (dTip60WT ) (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 HAT activity 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 (Luo and O’Leary 2005). Accordingly, degeneration of neural circuits essential for learning and memory may lead to impaired behavioral plasticity. We have previously reported that Tip60 HAT function promotes axonal growth of the Drosophila small ventrolateral neurons (sLNv), a well characterized model system for axonal growth (Pirooznia et al. 2012a). 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-dTip60E431Q) or wild type Tip60 (UAS-mCD8-GFP; UAS-dTip60WT). Expression of the respective transgenes was directed by the MB specific 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’). While the stereotyped morphology of the MB lobes was detected in third instar brain of flies expressing either the HAT mutant dTip60E431Q or the wild type dTip60WT (Figure 4B and 4C), severe axonal defects were observed in the adult flies. GFP staining of adult brains from the dTip60E431Q 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 dTip60E431Q 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. While the defects we observed were present in all of the dTip60E431Q MBs we inspected, in some MBs the defects were more severe than in others. Therefore, to more accurately quantify the changes as well as to examine which particular lobe(s) were specifically affected in the dTip60E431Q 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 dTip60E431Q flies compared to the control flies (OK107-GAL4; UAS-GFP)(Figure 4D). On the contrary, adult brains from the dTip60WT 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, production 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 outgrowth.

**Tip60 associated target genes are enriched for functions in cognition linked neuronal processes.**

Our prior findings demonstrated that gene control is a key mechanism by which Tip60 exerts its action in promoting function in a variety of neuronal processes. To determine whether Tip60 associates with target genes enriched for cognition-linked processes, we conducted genome-wide analysis of Tip60 occupancy using ChIP-Seq analysis. We also carried out ChIP-Seq analysis for the transcriptionally active elongation version of RNA polymerase II occupancy to assess which Tip60 associated gene targets were transcriptionally active. To identify early developmental targets, we used the Drosophila S2 embryonic cell line that robustly produces endogenous Tip60 (Zhu et al. 2007) and is comprised of mixed cell types for an unbiased enrichment analysis.

To identify Tip60 associated target genes, gene annotation of Tip60 and RNA Pol II ChIP-Seq peaks was performed using the D. melanogaster annotation database R5.5 and UCSC table browser, producing a list of 321 gene targets for Tip60 and 2210 for RNA Pol II.
Functional annotation clustering analysis was performed on the list of 321 Tip60-associated genes using both DAVID and GeneCodis3 (TABAS-MADRIDE et al. 2012). Analysis of these functional clusters (Figure 7A) revealed that they were enriched for a variety of neuronal processes all linked to cognition. Next, tissue-specific expression data for the 321 Tip60-associated genes was obtained through FlyAtlas. Figure 7B shows the percentage of Tip60-associated genes that are up-regulated and down-regulated in the various fly tissue types. Importantly, these data show that in neuronal tissues (brain, head, larval CNS and thoracicoabdominal ganglion), approximately 60-65% of Tip60 target genes are robustly expressed, while in other tissue types, the majority of Tip60 target genes show low level expression. In order to produce a comprehensive list of Tip60 associated 'neuronal genes', we combined the list of 46 neuronal genes identified through GO functional annotation with additional Tip60-target genes that are upregulated in neuronal tissues relative to whole fly, as determined based on tissue-specific expression data from FlyAtlas. This produced a comprehensive list of 178 unique neuronal genes targeted by Tip60. Importantly, 132 out of 178 neuronal genes targeted by Tip60 showed co-localization of RNA Pol II, suggesting that the majority of Tip60-target neuronal genes are actively transcribed (Figure 7C).

Further analysis using the DAVID and Gene Codis 3 classification system revealed that a substantial number of these genes were functionally linked to specific cognition linked pathways (Table 1), many of which were activity-dependent signaling pathways. Full lists of total Tip60 gene targets and neuronal Tip60 gene targets are shown in Tables S1 and S2, respectively. To identify the enrichment of known transcription factor DNA-binding motifs within Tip60-target-gene intervals, a motif enrichment analysis was performed using the MEME-ChIP suite as well as RSAT and Motif Enrichment Tool. DNA recognition sequences and statistical significance for top hit TFs involved in various neuronal functions are shown in Figure S2.

Based on our ChIP-Seq analysis, we hypothesized that the memory disruption and mushroom body morphological defects we observed upon Tip60 HAT misregulation was caused by inappropriate expression of genes required for these processes. To test this hypothesis, we performed quantitative real-time PCR analysis of ten genes identified in our ChIP-Seq analysis that were well characterized representatives of a particular cognition linked pathway, this time using adult fly heads expressing dTip60E431Q under the control of the elav-GAL4 driver (Figure 8). Each of the ten genes we tested were significantly downregulated in response to Tip60 HAT. These genes included those required for axonal
extension, guidance and target recognition such as ben, brat, CadN2 and pyd, consistent with the MB axonal outgrowth defects we observed in Tip60\textsuperscript{E431Q} mutant fly brains (Figure 4). Moreover, genes required for memory such as rut, ptp10d, fas2 as well as a core dendrite morphogenesis gene chinmo and neurotransmitter release gene aux were also negatively affected, consistent with the memory defects we observed in the Tip60\textsuperscript{E431Q} flies (Figure 3). Taken together, our findings support a transcriptional regulatory role for Tip60 HAT activity in the activation of genes essential for cognitive processes.

**Increasing Tip60 levels in the mushroom body of the fly CNS rescues learning and memory defects under APP induced neurodegenerative conditions.**

We previously reported that Tip60 plays a neuroprotective role in AD neurodegenerative progression by demonstrating that increased levels of Tip60 HAT activity suppress AD deficits that include apoptotic induced neurodegeneration, axonal outgrowth and transport defects and associated sleep and locomotion behavioral phenotypes in a *Drosophila* model overexpressing APP (PIROOZMIA et al. 2012a; PIROOZMIA et al. 2012b; JOHNSON et al. 2013; PIROOZMIA AND ELEFANT 2013b). Given the importance of histone acetylation in cognitive ability (PIROOZMIA AND ELEFANT 2012; PEIXOTO 2013; PIROOZMIA AND ELEFANT 2013a; A. 2014) and our finding that appropriate levels of Tip60 HAT activity are required for short term memory (Figure 3A,B), we hypothesized that excess Tip60 might also rescue AD linked cognitive deficits. To test this hypothesis, we again used the conditioned courtship suppression assay to assess learning and memory, this time using a unique AD fly model generated in our laboratory that co-expresses equivalent levels of dTip60\textsuperscript{WT} or dTip60\textsuperscript{E431Q} with either APP or APP dCT (APP lacking the C terminus that forms the transcriptional regulatory Tip60/AICD complex) (SIEGEL AND HALL 1979). We found that flies exhibited both learning and short-term memory defects when APP expression was targeted specifically to the fly MB using GAL4 driver OK-107 GAL4 and that these cognitive deficits were dependent upon the presence of the Tip60 interacting C-terminus of APP. Remarkably, our studies revealed that excess levels of Tip60 in the fly MB rescued such APP induced learning and memory defects (Figure 9 A,B) and that this rescue was dependent upon both a functioning Tip60 HAT domain and the Tip60 interacting C terminus of APP (Figure 9A,B). (GUNAWARDENA AND GOLDSTEIN 2001; MERDES et al. 2004). Taken together, our results support a neuroprotective role for Tip60 HAT activity in APP induced cognitive deficits associated with AD.
DISCUSSION

Our previous work using a Drosophila model system demonstrated that Tip60 HAT function is critical for a variety of cognition linked processes that include synaptic plasticity, axonal outgrowth and transport and neuronal cell apoptotic control in the central nervous system (CNS) (SARTHI AND ELEFANT 2011; PIROOZNIA et al. 2012a; PIROOZNIA et al. 2012b; JOHNSON et al. 2013; PIROOZNIA AND ELEFANT 2013a). Our present study extends these findings to demonstrate that Tip60 HAT activity plays an integral functional role in memory formation in Drosophila. Here, we show that loss of dTip60 HAT activity or increased levels of dTip60 HAT activity in all the lobes of the mushroom body (MB), the learning and memory center of the Drosophila CNS, disrupt immediate recall memory while there is no effect on learning. In the dTip60\textsuperscript{E431Q} HAT mutant flies, memory defects are also accompanied by axonal growth defects that are evident in dorsal $\alpha/\alpha'$ and medial $\beta/\beta'$ and $\gamma$ lobes of the adult MB in these flies with no marked effect on the larval MB structure. The $\alpha$, $\beta$, and $\gamma$ neurons composing the mushroom body undergo considerable structural reorganization during embryonic, larval and pupal development. The $\gamma$ neurons are the earliest born and develop during first instar larval stages while development of $\alpha'/\beta'$ axons and $\alpha/\beta$ axons takes place during the third instar larval and pupal stages, respectively (SCOTT et al. 2001). Although the $\alpha/\beta$ lobes appear much later in development than the $\alpha'/\beta'$ lobes, the dramatic effects we observe on both these lobes in the dTip60\textsuperscript{E431Q} adult flies indicate that Tip60 HAT activity may be crucial for development of $\alpha/\beta$ lobes as well as for maintaining branch stability in the larval born $\alpha'/\beta'$ lobes as development proceeds. During metamorphosis, the $\gamma$ neurons undergo a stereotypical process of axon elimination wherein the dorsal and medial segments of its axon are pruned back (WATTS et al. 2003; WATTS et al. 2004). The $\gamma$ axons subsequently re-extend medially during pupal remodeling. Tip60 HAT activity likely mediates regeneration of $\gamma$ 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\textsuperscript{E431Q} mutant. Consistent with these findings, we recently reported a similar effect in the axons of Drosophila small ventrolateral neurons (sLNv), a well characterized model system for studying axonal growth, wherein Tip60 HAT loss inhibits sLNv axon outgrowth in the adult flies while no axonal defects were observed in the third instar larva (PIROOZNIA et al. 2012a). Together, these results support a role for Tip60 HAT activity in mediating MB axonal outgrowth required for proper adult MB axonal formation.
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 (GRAFF AND MANSUY 2008; SWEATT 2009). As such, epigenetic post-translational modifications (PTMs) of histone proteins, most notably histone acetylation, that control nuclear chromatin packaging and gene expression profiles are emerging as a fundamental mechanism by which neurons adapt their transcriptional response to environmental cues (FENG et al. 2007; SWEATT 2009; BOURSIGES et al. 2010; MEANEY AND FERGUSSON-SMITH 2010; RICCIO 2010; NELSON AND L.M.MONTEGGIA 2011; PIROOZIA AND ELEFANT 2012). The implicit hypothesis is that environmental signals drive changes in histone acetylation modifications that are inherently dynamic in nature. Such changes allow for activity-dependent transcriptional 'plasticity' that mediates sustained variation in neural function (SWEATT 2009; RICCIO 2010; GRAFF et al. 2012b; BOURSIGES et al. 2013). One of the most important of such experience-driven behavioral changes is learning and memory formation, as it directly impacts cognitive ability (LEVENSON AND SWEATT 2005; CARULLI et al. 2011; NELSON AND L.M.MONTEGGIA 2011; WEST AND GREENBERG 2011). Importantly, the MB is a highly plastic brain region in the Drosophila CNS that is well known for its role in multimodal sensory integration and associative learning. Thus, it serves as a powerful model to study not only developmental synaptic reorganization but also experience-dependent cognition linked plasticity (TECHNAU 1984; HEISENBERG et al. 1995; BARTH AND HEISENBERG 1997). Here we show that robust production of Tip60 is localized in the MB Kenyon cell nuclei. Moreover, our ChIP-Seq analysis reveals that Tip60 direct target genes are enriched for functions in cognitive processes, and that key genes representing these pathways are misregulated in the adult Tip60 HAT mutant fly brain. We do acknowledge that because the Tip60 target genes we show here were identified in S2 cells, they cannot be assumed to be the same in vivo. However, our findings are consistent with our previous microarray study performed on Tip60 HAT mutant second instar larvae in vivo that showed misregulation of genes enriched for neuronal function (LORBECK et al. 2011). Intriguingly, we found that many of the pathways enriched for Tip60 associated genes are activity dependent (Table 1). Taken together, our studies uncover an epigenetic transcriptional regulatory role for Tip60 HAT action in cognitive function and suggest that Tip60 function in gene control may rely on experience driven modes of action.

Several other brain regions in addition to the MB have been identified to be important for courtship learning. Basic courtship involves communication between the projection
neurons from the antennal glomeruli with higher centers in the lateral protocerebrum and mushroom bodies (Mehren et al. 2004). 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 (Rybak and Menzel 1993; Hu et al. 2010). Furthermore, such functional feedbacks from the Kenyon cells are thought to be mediated by the β and γ lobes (Hu et al. 2010), which are also severely affected in the dTip60E431Q 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 (Guan et al. 2011). Thus, the axonal growth defects we observe in the dTip60E431Q 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 (dTIP60WT) did not have an observable effect on the MB structure per se, the dTip60WT expressing flies exhibit defects in immediate recall memory similar to the dTip60E431Q flies. Importantly, under normal conditions, maintaining the balance between HAT and HDAC levels and activity is critical for establishing proper histone modification patterns that serve to regulate both stable and rapidly changing gene expression profiles crucial for both neuronal homeostasis and appropriate neurophysiological response outputs such as long term potentiation, learning, and memory, respectively (Morris 2003). Thus, we speculate that increasing Tip60 mediated acetylation in the MB can lead to complex changes in the chromatin landscape causing misregulation of genes that are induced following patterned synaptic stimulation, such as behavioral experiences, and play a critical role in translating the activity in neural circuits into accessible memories in the brain (Pirooznia and F. 2012) (Pirooznia and Elefant 2012; Pirooznia and Elefant 2013a).

Outgrowth and stabilization of axons during development of the nervous system and reorganization of axonal connections in the adult in response to environmental cues critical for cognition are based on the dynamic rearrangement of the cytoskeleton (Baas and Luo 2001; Dent and Gertler 2003). Axon growth and elongation depends, among other factors, on microtubules polymerization (Conde and Caceres 2009) and acetylation of α-tubulin has been reported to stabilize microtubules and promote polymerization (Creppe et al. 2009). Accordingly, reducing levels of HDAC6 has been reported to restore learning and memory as well as α-tubulin acetylation in a mouse model for AD (Govindarajan 2013). Consistent with these findings, we previously reported that Tip60 partially acetylates microtubules in the larval
neuromuscular junction (NMJ), an effect that was dependent on its HAT function (Sarthi and Elefant 2011), although we did not observe this effect in motor neuron axons of the Drosophila CNS (Johnson et al. 2013). Here, our analysis reveals that Tip60 is localized not only within the nucleus of the Kenyon cells in the MB but is also present within all the MB axonal lobes, indicative of both nuclear and cytoplasmic localization for Tip60. Such cytoplasmic and nuclear Tip60 localization is recapitulated in the Drosophila NMJ, where we previously reported Tip60 to be localized both pre and post synaptically (Sarthi and Elefant 2011). Therefore, we cannot rule out the possibility that Tip60 may also mediate 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. Of note, neural activity has been shown to modulate chromatin acetylation in hippocampal neurons in part, by controlling the shuttling of certain HDACs in and out of the nucleus that in turn, influences their activity in gene control (Chawla et al. 2003; Riccio 2010). Thus, Tip60 may also utilize a similar cytoplasmic/nuclear shuttling mechanism, an area that we are currently exploring.

Tip60 has been implicated in AD via its interaction with the APP intracellular domain (AICD), a fragment generated by the processing of APP by γ-secretase that is subsequently released into the cytoplasm (Muller et al. 2008). AICD forms a transcriptional competent protein complex with the HAT Tip60 (Cao and Sudhof 2001) and is recruited to the promoters of specific neuronal target genes where it acts to acetylate select histone proteins to epigenetically regulate gene transcription (Cao and Sudhof 2001; von Rotz et al. 2004; Ryan and PimpiKarak 2005). Importantly, misexpression of certain Tip60/AICD target genes such as neprilysin, EGFR, LRP and KAI-1 have been associated with AD pathophysiology (Baek et al. 2002; Muller et al. 2007; Slomnicki and Lesnick 2008). Based on these findings, it has been proposed that the inappropriate AICD/Tip60 complex formation and/or its recruitment may contribute or lead to AD pathology via epigenetic transcriptional misregulation of target genes required for neuronal functions (Pirooznia and Elefant 2012; Johnson et al. 2013; Pirooznia and Elefant 2013a). Here, we report that while misregulation of Tip60 HAT activity alone causes short term memory deficits in the fly, excess production of Tip60 in conjunction with APP rescue both APP-induced learning and immediate recall memory deficits in a fly model overexpressing APP. Importantly, reversal of these cognitive deficits is dependent upon the C terminus of APP that forms the transcriptional regulatory Tip60/AICD complex as well as the functional HAT activity of Tip60. Accordingly, we show by ChIP-Seq that Tip60 associated genes are enriched for cognition function and that key Tip60 genes representing learning and memory linked pathways are misregulated in the Tip60
HAT mutant fly brain, in vivo. Consistent with these findings, we previously reported that increasing in vivo Tip60 HAT levels in the nervous system of flies under APP induced neurodegenerative conditions rescues Tip60 mediated cognition linked processes impaired in AD that include apoptotic neurodegeneration (PIROOZNIA et al. 2012b), axonal outgrowth (PIROOZNIA and ELEFANT 2013b) and transport (JOHNSON et al. 2013) and restores associated disrupted complex functional abilities that include sleep cycles (PIROOZNIA et al. 2012a; PIROOZNIA and ELEFANT 2013b) and locomotor function (JOHNSON et al. 2013). Gene expression analysis revealed that these flies exhibit repression of cassettes of pro-apoptotic genes and induction of pro-survival genes (PIROOZNIA and ELEFANT 2012; PIROOZNIA et al. 2012b; PIROOZNIA and ELEFANT 2013a). Together, our results point to an all encompassing neuroprotective role for Tip60 in early AD progression and support a model by which Tip60 epigenetic reprograms select gene sets that redirect neuronal cell fate from cell death towards cell survival and function (PIROOZNIA and ELEFANT 2013a). Tip60 might exert such gene reprogramming either by itself or by complexing with other peptides such as AICD for its recruitment to select genes via promoter bound TFs such as those we identified in our ChIP-Seq analysis. Our findings that Tip60 exerts neuroprotective effects under neurodegenerative conditions via epigenetic gene reprogramming are not unprecedented. Indeed, gene transfer of CBP was shown to ameliorate learning and memory deficits in a mouse AD model by increasing brain derived neurotrophic factor (BDNF) (CACCAMO et al. 2010) and p300 but not HDAC inhibitors were found to enhance axonal regeneration by inducing axonal outgrowth genes (GAUB et al. 2011). In light of these findings, it is important consider that modulation of specific HAT levels and/or activity may alter the expression of many genes or “cassettes” of specific genes that act together to produce a neuroprotective effect, as indicated by our ChIP-Seq analysis for Tip60 (PIROOZNIA and ELEFANT 2012; PIROOZNIA and ELEFANT 2013a). Therefore, it will be critical to determine the identity of specific cognition linked gene target sets regulated by Tip60 to further dissect their neuroprotective nature for more effective design of HAT based therapeutic strategies.

FUNDING

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ACKNOWLEDGMENTS

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FIGURE LEGENDS

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. Representative confocal images of 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. 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 activates 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 recall 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 (dTIP60E431Q) or additional copies of wild type Tip60 (dTIP60WT) 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. dTIP60E431Q and dTIP60WT 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 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.**
Representative confocal images of larval and adult mushroom body visualized with mCD8-GFP driven by pan-MB driver, OK107-GAL4. Images were displayed as projections of 1uM serial Z-sections and were adjusted for brightness and contrast using the ImageJ program to more clearly define the MBs. Unprocessed images are shown in Figure S1. Third instar larval brain in control flies (A). 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'). (D) Quantification of area in the different genotypes in adult flies. MBs stained with GFP to delineate MB structure were counterstained with either Fas II staining (Figure 5) for quantification of area of α/β and γ-lobes or Trio staining (Figure 6) for quantification of area of α'/β' lobes where ($n=20$). 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.** Representative confocal images of OK107-GAL4 driver carrying GFP construct was used to drive expression of dTip60$^{E431Q}$ or dTip60$^{WT}$ and effect on mushroom body structure visualized using GFP and Fas II staining. Images were displayed as projections of 1uM serial Z-sections and were adjusted for brightness and contrast using the ImageJ program to more clearly define MBs. Unprocessed images are shown in Figure S1. Compared to control brains (UAS-mCD8-GFP; OK107-GAL4), dTip60$^{E431Q}$ flies exhibit marked decrease in α/β and γ lobes while dTip60$^{WT}$ flies did not exhibit any effects on α/β and γ lobes. Fas II labeling was used for quantifying area measurements in the different genotypes.

**Figure 6. Trio labeling in the mushroom body.** Representative confocal images of OK107-GAL4 driver carrying GFP construct was used to drive expression of dTip60$^{E431Q}$ or dTip60$^{WT}$ and effect on mushroom body structure visualized using GFP and Trio staining. Images were displayed as projections of 1uM serial Z-sections and were adjusted for brightness and
contrast using the ImageJ program to more clearly define MBs. Unprocessed images are shown in Figure S1. Compared to control brains (UAS-mCD8-GFP; OK107-GAL4), dTip60\textsuperscript{E431Q} flies exhibit marked decrease in $\alpha'$/$\beta'$ while dTip60\textsuperscript{WT} flies exhibit no significant effects on $\alpha'$/$\beta'$ lobes. Trio labeling was used for quantifying area measurements in the different genotypes.

**Figure 7. Tip60 associates with genes enriched for neuronal functions.** (A) Functional annotation clustering of Tip60 associated genes using GeneCodis. Only clusters containing more than two genes and having significant enrichment (indicate p value here) are shown. (B) Tissue-specific expression profiles for Tip60 associated genes. Expression profiles were obtained through FlyAtlas. Histogram analysis depicts that the majority of Tip60-associated genes are robustly expressed specifically in tissues with neuronal function. (C) Proportions of different gene categories in Tip60-target genes. 132 neuronal genes targeted by Tip60 showed co-localization of active elongation version of RNA Pol II, suggesting that these Tip60-target neuronal genes are actively transcribed.

**Figure 8. Tip60 HAT loss in the CNS causes misregulation of neuronal genes.** (A) Real-time PCR was performed on cDNA isolated from adult fly heads expressing dTip60\textsuperscript{E431Q} under the elav C155 pan-neuronal GAL4 driver. Histogram represents relative fold change in expression level of neuronal target genes. Real-time PCRs were performed in triplicate, and the fold change was calculated using the $\Delta\Delta$CT method. Statistical significance was calculated using an unpaired Student’s $t$ test: * $p < 0.05$, ** $p < 0.01$. (B) List of selected learning and memory related genes and their functions that are identified in ChIP-seq analysis and validated in adult head tissue using qRT-PCR.

**Figure 9. Increased level of Tip60 HAT activity rescues learning and memory deficits under APP induced neurodegenerative conditions.** 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 hAPP or co-expressing equivalent levels of hAPP with HAT mutant dTip60\textsuperscript{E431Q} exhibit no marked decrease in courtship during final 10 mins compared to initial 10 mins, indicative of learning defects. Flies co-expressing hAPP with dTip60\textsuperscript{WT} exhibit marked decrease in courtship index during final 10 mins compared to the initial 10 mins, indicative of normal learning response. Flies expressing either hAPP dCT or co-expressing and hAPPdCT; dTip60\textsuperscript{E431Q} exhibit marked decrease in courtship index during final 10 mins compared to initial 10 mins, indicating that learning effects are dependant upon the C-terminal of hAPP. Panel (B) Immediate recall
memory (0-2 mins post training) of trained males compared to untrained (sham) males of the same genotype. Both hAPP and hAPP;dTip60\textsuperscript{E431Q} flies show no significant difference between trained and sham males, indicative of no immediate recall memory, while hAPP; dTip60\textsuperscript{WT} show significant difference, indicative of memory rescue. hAPP dCT with dTip60\textsuperscript{E431Q} and dTip60\textsuperscript{WT} flies show no significant difference between trained and sham males, indicative of no immediate recall of training. Error bars represent 95% confidence interval. Single 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.
Table 1. Activity-dependant cognition linked pathways enriched for Tip60 associated genes.

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Figure 2
**Figure 3**

A

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B

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* **: p < 0.05
** **: p < 0.01
Figure 4
Figure 5

Control

Anti-GFP

Anti-Fas II

Merge

A

A'

A"

B

B'

B"

dTip60<sup>E431Q</sup>

dTip60<sup>WT</sup>

AnS‐GFP

AnS‐Fas II

II

Merge

A

A'

A"

B

B'

B"

C

C'

C"

Figure 5
Figure 6
Figure 7
Figure 8

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<td>-5</td>
<td>synaptic vesicle uncoating</td>
<td>protein serine/threonine kinase activity</td>
</tr>
<tr>
<td>chinmo</td>
<td>chronologically inappropriate morphogenesis</td>
<td>-2.27</td>
<td>dendrite morphogenesis and mushroom body developmental defect</td>
<td>metal ion binding</td>
</tr>
<tr>
<td>pyd</td>
<td>polychaetoid</td>
<td>-2.63</td>
<td>adherens junction strength</td>
<td>cell adhesion molecule binding</td>
</tr>
</tbody>
</table>
**Figure 9**

**A** LEARNING PHASE

- Initial 10 min
- Last 10 min

**B** MEMORY PHASE

- Sham
- Trained