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Epigenetic crosstalk: Pharmacological inhibition of HDACs can rescue defective synaptic morphology and neurotransmission phenotypes associated with loss of the chromatin reader Kismet

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ABSTRACT

We are beginning to appreciate the complex mechanisms by which epigenetic proteins control chromatin dynamics to tightly regulate normal development. However, the interaction between these proteins, particularly in the context of neuronal function, remains poorly understood. Here, we demonstrate that the activity of histone deacetylases (HDACs) opposes that of a chromatin remodeling enzyme at the *Drosophila* neuromuscular junction (NMJ). Pharmacological inhibition of HDAC function reverses loss of function phenotypes associated with Kismet, a chromodomain helicase DNA-binding (CHD) protein. Inhibition of HDACs suppresses motor deficits, overgrowth of the NMJ, and defective neurotransmission associated with loss of Kismet. We hypothesize that Kismet and HDACs may converge on a similar set of target genes in the nervous system. Our results provide further understanding into the complex interactions between epigenetic protein function *in vivo*.

1. Introduction

Regulation of chromatin dynamics is a vital aspect of normal developmental processes as dysregulation of chromatin dynamics is associated with a number of pathological conditions (Haberland et al., 2009; Kazantsev and Thompson, 2008; Machado-Vieira et al., 2011). In the field of neurobiology, epigenetic mechanisms represent a new frontier of understanding not only for simple cellular processes, but also complex multimodal cognitive-behavioral circuits (Day and Sweatt, 2012; Marcus et al., 1994; Ronan et al., 2013). Several studies have concluded that chromatin regulators make up a vast and complicated interconnected network (Maze et al., 2013; Venkatesh and Workman, 2015; Wenderski and Maze, 2016; Allis and Jenuwein, 2016). For this reason, it is critical to better understand the interactions between individual members of this network.

Proteins implicated in regulating chromatin dynamics can be divided into two major classes: chromatin remodelers and chromatin modifiers (Henikoff et al., 2011; Jenuwein and Allis, 2001). Chromatin remodelers alter chromatin structure by moving, ejecting, or inserting nucleosomes, which are made up of DNA wrapped around histone octamers (Clapier and Cairns, 2009). The chromodomain helicase DNA-binding (CHD) family is one of five major families of chromatin remodelers (Ho and Crabtree, 2010; Bartholomew, 2014). The two defining characteristics of this family are: 1) ATPase activity and 2) dual chromodomains. The latter recognizes histone N-terminal tail methylations, which confers the title of epigenetic 'readers' to this class of proteins (Flanagan et al., 2005). *Drosophila melanogaster* have three distinct CHD proteins, compared to nine identified in mammals (Clapier and Cairns, 2009; Brehm et al., 2004; Srinivasan et al., 2005; Bouazoune and Brehm, 2006). One of these *Drosophila* proteins, Kismet (Kis), is important for proper nervous system development and function (Ghosh et al., 2014; Melicharek et al., 2008; Melicharek et al., 2010). Decreasing Kis in motor neurons produces motor function deficits, aberrant synaptic morphology, and defects in postsynaptic glutamate receptor clustering and localization (Ghosh et al., 2014). Additionally, reducing Kis levels in the central nervous system (CNS) leads to axonal pruning and migration abnormalities and defects in higher-order behaviors like immediate recall memory (Melicharek et al., 2010). These studies indicate a vital role for Kis in mature CNS neurons.

The second class of proteins necessary for maintaining chromatin

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dynamics is chromatin modifiers, which are considered 'writers' and 'erasers' and are responsible for modifications of histone N-terminal tails. These post-translational modifications can have direct or indirect effects on chromatin structure. One of the most widely studied modifications is lysine acetylation, which mitigates the positive charge on the lysine residue (Haberland et al., 2009; Shahbazian and Grunstein, 2007; Tapias and Wang, 2017). This modification decreases association of the positively charged histone tail with the negatively charged DNA backbone making removal or movement of the nucleosome more likely. Lysine acetylation is usually enriched at actively transcribed genes as it allows for unobstructed access of promotor or enhancer DNA sequences to transcription factors and enhancers (Haberland et al., 2009; Tapias and Wang, 2017; Chuang et al., 2009; Peserico and Simone, 2011). Histone acetyltransferases (HATs) are the 'writers' that catalyze the addition of the acetyl groups and histone deacetylases (HDACs) are the counteracting 'erasers' (Tapias and Wang, 2017; Peserico and Simone, 2011). HDACs, in particular, have been widely studied as potential therapeutic targets in the nervous system (Haberland et al., 2009; Kazantsev and Thompson, 2008; Machado-Vieira et al., 2011). Class II HDACs were shown to be essential for learning and memory formation and synaptic plasticity in mice (Kim et al., 2012; Sando et al., 2012). Additionally, pharmacological inhibition of HDACs has been shown to be effective in mouse models of genetic disorders including Rubinstein-Taybi syndrome, Rett syndrome, and Friedreich's ataxia (Herman et al., 2006; Vecsey et al., 2007; Kavalali et al., 2011). Furthermore, general HDAC inhibitors (HDACi) like Valproate were FDA approved for the treatment of complex psychiatric disorders such as major depressive disorder, bipolar disorder, and epilepsy (Vasudev et al., 2012; Vigo and Baldessarini, 2009). Despite the importance of these enzymes for nervous system function, little is known about their interactions with other classes of chromatin associated proteins such as chromatin remodelers.

We sought to determine the interaction between one chromatin remodeler, the epigenetic 'reader' Kis, and a class of chromatin modifiers, HDACs, in *Drosophila*. Our lab previously showed that ubiquitous Kis knockdown produces morphological, postural, and motor-function defects (Ghosh et al., 2014; Melicharek et al., 2010). Using these phenotypes, we performed a small-scale screen with a group of drugs that target epigenetic proteins. From this screen, we identified a number of HDAC inhibitors capable of suppressing the phenotypes associated with Kis knockdown. We then selected the top general HDAC inhibitor (suberoylanilide hydroxamic acid, SAHA) and top specific inhibitor (suberohydroxamic acid, SBHA) from the HDAC inhibitors we tested to evaluate further. Using the well-defined *Drosophila* neuromuscular junction (NMJ) as a model of glutamatergic synaptic function, we show both SAHA and SBHA alleviate defects in synaptic morphology, motor behavior, and neurotransmission associated with decreased Kis levels. These results provide further understanding into the complex interactions between epigenetic reader and eraser functions *in vivo* and lead us to hypothesize that Kis and HDACs may converge on a similar set of target genes in the nervous system.

2. Materials and methods

2.1. Drosophila stocks and genetics

All *Drosophila* stocks and crosses were maintained at 25 °C in a 12:12 light:dark cycle with 60% humidity. To drive the expression of transgenes in *Drosophila*, the Gal4/UAS bipartite system was used as previously described (Brand and Perrimon, 1993). All Gal4 stocks (*Da-Gal4*, *elav-Gal4*), w^{1118}, and *Canton S* (wild-type) were obtained from Bloomington *Drosophila* Stock Center. *UAS:kis.RNAi.a* (VDRC #10762) was obtained from the Vienna *Drosophila* Resource Center and is previously described in (Melicharek et al., 2010). Control outcrosses were generated from either the *Da-Gal4* or *elav-Gal4* drivers or *UAS:kis.RNAi.a* and w^{1118} (BL#3605). Each transgene was heterozygous in all experiments.

2.2. Drug treatment of Drosophila

Fly media were prepared from dried instant food (Nutri-Fly Instant from Genesee Scientific) with water containing 1.6% of 10% w/v tegosept (methyl p-hydroxybenzoate in 95% ethanol) and 0.1% of DMSO vehicle or 10 μM drug treatment. In order to maximize exposure to drug treatments, *Drosophila* were raised on drug containing food for their entire lifespan. Adult flies were set on drug food and allowed to lay for 3 days. These adults were then moved to vials containing newly prepared drug food, and allowed to lay for an additional 3 days. This cycle was repeated for 2 weeks per cross. F1 progeny were exposed to drug from egg laying until metamorphosis. Adults that hatched from pupal cases were kept in food vials containing the respective drug for their lifespan.

2.3. Adult climbing assessment

A modified version of the climbing assay procedure described by Le Bourg and Lints was utilized (Le Bourg and Lints, 1992). Flies were collected and maintained on the same treatment food every 24 h and assayed every other day beginning the day following collection (considered day 2) for 14 days. Groups of 20 or fewer flies were transferred from their treatment food vial to an empty one. Each vial was tapped flat on the bench top and flies were given 18 s to climb 5 cm during the assay. The number of flies that climbed to or above the 5 cm line was recorded and an end total of 50 flies per condition were assayed. Food vials were changed every other climbing period to ensure longevity of flies.

2.4. Larval body wall muscle contractions

Wandering third instar larvae were briefly rinsed with PBS and allowed to acclimate for one minute on a 4% agar surface. An individual larva was then transferred to the center of a fresh 100-mm Petri dish containing 4% agar. The larval contraction assay was performed on a clean and flat agar surface, under a constant white light to avoid any chemotactic, geotatic and phototactic cues as previously described (D'Rozario et al., 2016). A contraction was considered as one full body wall contraction, either forward or backward. The number of contractions was manually counted using a Leica Mz 125 stereomicroscope for 30 s for each larva. A minimum of 60 larvae were analyzed per experimental condition.

2.5. Neuromuscular junction morphology

Wandering third instar larvae were pinned down in Sylgard lined Petri dishes for fillet dissections and then fixed in 4% paraformaldehyde for 25 min as described (Mhatre et al., 2014). Larvae were washed with PBS containing 0.1% Triton X-100 (PBT) and subsequently incubated in PBT containing TRITC-conjugated Phalloidin at 1:300 (Sigma-Aldrich) to stain muscles and fluorescein-conjugated HRP at 1:100 (Jackson ImmunoResearch Labs) to stain neuronal membranes for 1 h at 25 °C. After two 5-min washes with PBT, dissected larvae were mounted in Vectashield (Vector Labs, H-1000). Imaging was performed using an Olympus FluoView FV1000 laser scanning confocal microscope. The number of axonal branches, the type of branching and the number of synaptic boutons, were quantified using ImageJ software as previously described (D'Rozario et al., 2016; Mhatre et al., 2014). The 6/7 NMJ of hemisegments A3 were used for all studies.

2.6. Quantitative reverse transcriptase - PCR

Wandering third instar larvae were washed with PBS and immediately transferred to RNA Later (Abion) and stored in − 20 °C. Isolation of total RNA was done using phenol:chloroform extraction followed by alcohol precipitation for purification. RNA was stored in