Kismet/CHD7 regulates axon morphology, memory and locomotion in a *Drosophila* model of CHARGE syndrome

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CHARGE syndrome (CS, OMIM #214800) is a rare, autosomal dominant disorder, two-thirds of which are caused by haplo-insufficiency in the *Chd7* gene. Here, we show that the *Drosophila* homolog of *Chd7*, *kismet*, is required for proper axonal pruning, guidance and extension in the developing fly's central nervous system. In addition to defects in neuroanatomy, flies with reduced *kismet* expression show defects in memory and motor function, phenotypes consistent with symptoms observed in CS patients. We suggest that the analysis of this disease model can complement and expand upon the existing studies for this disease, allowing a better understanding of the role of *kismet* in neural developmental, and *Chd7* in CS pathogenesis.

INTRODUCTION

CHARGE Syndrome (CS, OMIM #214800) is a rare disorder, two-thirds of which are caused by haplo-insufficiency in the *Chromodomain Helicase DNA Binding Protein 7* (*Chd7*) gene. Heterozygous loss-of-function mutations in the *Chd7* gene were first described as associated with CS in 2004 (1), and since have been estimated to be causative for nearly two-thirds of all CS diagnoses (2), underscoring the importance of analysis of *Chd7* function in this disease. CS is characterized by a variety of clinical symptoms and is prevalent in approximately one in every 10 000 live births (3). Although the clinical symptoms of CS vary from case to case, the clinical definition of CS initially relied on six basic symptoms: coloboma, heart malformation, choanal atresia, retardation of growth and/or development, genital anomalies and ear anomalies, making CS a common cause of congenital anomalies (2). Though these clinical symptoms alone can often suggest a diagnosis of CS, the literature stresses the importance of coupling this clinical diagnosis with a molecular diagnosis, through mutational analysis of *Chd7* (2–5). *Chd7* encodes an evolutionarily conserved protein thought to play a role in the regulation of gene expression through chromatin remodeling (1). Recent studies of Chd7 DNA-binding sites on chromatin have shown that Chd7 binding is correlated to areas of mono- and dimethylated lysine 4 of histone H3 (6), consistent with Chd7 serving a role in mediating transcription through chromatin remodeling.

Attempts to understand the complex etiology of many human diseases have been improved through the study of model organisms. The fruit fly, *Drosophila melanogaster*, has been tremendously important and influential in furthering our understanding of the molecular and cellular mechanisms of gene function in a variety of human diseases, including neurodegenerative diseases and forms of hereditary mental retardation. We therefore sought to utilize the fly in an attempt to understand the basic functions of the *Drosophila* homolog of mammalian *Chd7* in behavior and neural development. Literature suggests that the *Drosophila* homolog of *Chd7* is the *kismet* (*kis*) gene (7–14). Searches of the NCBI Homologene database and information hyperlinked over proteins databases also list *kis* as the fly homolog of human *Chd7*. Finally, an NCBI Protein BLAST of the human CHD7 protein (NP_060250.2) lists the Kismet protein as the protein with the highest degree of similarity and identity within *Drosophila* (E value 0.0).

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CHD7 belongs to a subfamily of proteins in mammals that includes CHD6, CHD7, CHD8 and CHD9 (13), collectively referred to as subgroup III (12). Literature has suggested that Kismet protein function in flies is carried out by the proteins within this subfamily III group in mammals (12,13), consistent with the protein sequence homology shared by Kismet and this group of proteins. Taken together, this suggests that the Kismet protein is most closely related to the Bilaterian ancestral protein that fly Kismet and human CHD6, CHD7, CHD8 and CHD9 evolved from ∼670 million years ago (15). Recently, Batsukh *et al.* (12) have shown that CHD7 interacts with family member CHD8 both directly and indirectly to form a potential CHD7/CHD8 complex. Based on the literature showing that in other diseases (Hereditary Spastic Paraplegia and Cornelia de Lange syndrome), interacting partners are involved in the underlying cause of the disease, these authors suggest that a CHD7/CHD8 containing complex may be involved in the pathogenesis of CS (12). A major advantage of *Drosophila* as a model system for the study of human disease is the ability to study the functions of genes whose mammalian homologs exist in multi-gene families, in order to gain a deeper understanding of the evolutionarily conserved functions and relationships between these genes. Given that 1) CHD7 is the closest human homolog based on sequence identity to fly Kismet; 2) CHD7 and CHD8 family members are both homologous to the Kismet protein; and 3) CHD7 and CHD8 interact to potentially contribute to CHARGE pathogenesis, determining the functions of *kismet* in neural development and behavior may significantly contribute to our understanding of the function of both CHD7 and CHD8 in CS pathogenesis.

The *Drosophila kismet* gene encodes for two protein products (Kis-L and Kis-S) which share a common C terminal stretch of ∼2100 amino acids (16). The common C terminal segment contains a BRK domain of unknown function, whereas the N-terminal domain of Kis-L also contains an ATPase domain similar to those found in other chromatin remodeling enzymes and two chromodomains which can recognize Histone H3 methylation (16). Importantly, the BRK domain, as well as the ATPase and chromodomains found in the Kismet protein are also conserved in human *Chd7* (8), suggesting that the *Drosophila* protein is a good candidate to study in order to better understand human Chd7 protein function.

kis was initially identified in a genetic screen as a suppressor of a dominant homeotic phenotype and as a member of the *trithorax* group of transcriptional activators (17). Subsequent analysis of *kis* mutations revealed an essential role for *kis* in embryonic segmentation and adult and larval cell fate specification (17,18). *kis* mutations have been identified in genetic screens as modifiers of the Ras and Notch signal transduction pathways (19,20), as modifiers of the proneural gene *atonal* in retinal development (11) and as a regulator for vein specification in developing wings (21). Kis protein is proposed to facilitate an early step in transcriptional elongation through alterations in chromatin structure (16).

Here, we utilize *Drosophila* to better understand the role of *kis* in fly behavior and neural development. We report the first evidence that adult flies with decreased Kis protein exhibit a number of robust and genetically tractable phenotypes that are similar to symptoms observed in CS patients, including defects in gross motor coordination and defective learning and memory. Histological analysis of *kis* mutant neurons show defects in cell and axonal migration in multiple neuronal populations in the fly central nervous system, suggesting a possible anatomical mechanism for the behavioral defects we observe in adult flies. We suggest that analysis of this disease model can complement and expand upon the studies done in cell culture and vertebrate model organisms for this disease, allowing a better understanding of the role of *kis* in neural developmental and *Chd7* in CS pathogenesis.

RESULTS

Adult flies with decreased kismet expression display numerous phenotypes

Null mutations in *kis* are embryonic lethal, presumably due to the fact that *kis* gene function is required to maintain states of homeotic gene transcription in early embryogenesis (18). We hypothesized that if we were to partially knock down *kis* gene function in specific tissues, we could allow flies to overcome the requirement for *kis* in the embryo, while sufficiently hindering *kis* gene function in later stages of development, to generate viable adults with prominent phenotypes. To accomplish this, we utilized the Gal4/UAS system (22). We expressed two different publicly available *kis RNAi* strains from the Vienna *Drosophila* RNAi Center (*kis RNAi.a*, and *kis RNAi.b*). To examine the extent to which we are able to knock down Kis protein expression with these constructs *in vivo*, we first expressed them in the posterior compartment of developing wing imaginal discs by using the *engrailed-Gal4* driver (Supplementary Material, Fig. S1). *engrailed* is expressed only in the posterior compartment of tissues (23,24), leaving the anterior compartment as a critical internal control for this assay and allowing us to estimate the amount of knock down these constructs have *in vivo*. The Kis protein is normally expressed ubiquitously in the developing wing imaginal disc, with roughly equivalent expression levels in the posterior and anterior compartment (Supplementary Material, Fig. S1A and B). We found that the two different *kis RNAi* constructs knockdown Kis protein expression differentially. *kis RNAi.a* shows a 60% knockdown (Supplementary Material, Fig. S1C and D), whereas *kis RNAi.b* shows a very strong 90% knockdown (Supplementary Material, Fig. S1E and F). These results are consistent with recently published data that also show significant knockdown of Kis protein using these constructs (10).

In order to generate viable adult flies with decreased Kis expression, we first expressed both of these *kis RNAi* constructs ubiquitously with the *daughterless-Gal4* driver (*da-Gal4*). Expression of *kis RNAi.b* with *da-Gal4* at 25°C leads to late pupal lethality, suggesting that Kis is required for some critical function during pupal development. However, expression of *kis RNAi.a* with *da-Gal4* at 25°C produces viable adults with obvious morphological defects (Fig. 1). These flies are unable to fly, and exhibit a prominent postural defect where they hold their wings apart and below their bodies with 100% penetrance (Fig. 1C and E). This
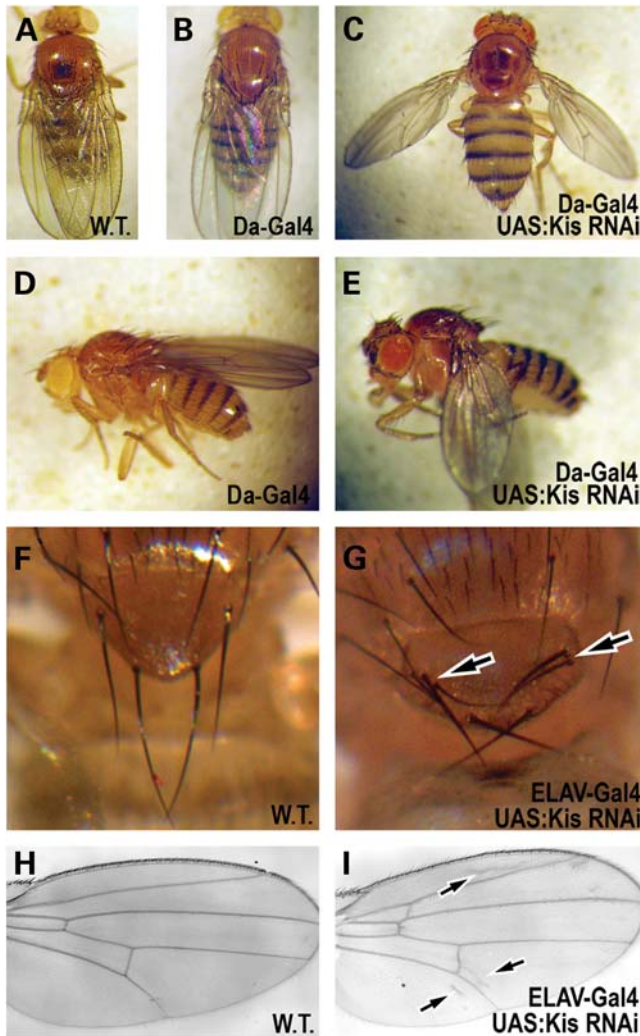
Figure 1. Adult phenotypes in Kismet knockdown flies. (**A**–**E**) Adult female flies. (A–C) Dorsal view. (D–E) Lateral view. (A) Wild-type (Canton S) fly. Note the position of the adult wings. (B and D) A fly expressing only the *Daughterless-Gal4* reagent (*Da-Gal4/ +*) holds its wings normally. (C and E) A fly where Kis protein is ubiquitously knocked down (*Da-Gal4/UAS:kis RNAi.a*) holds its wings abnormally apart from its body. (**F** and **G**) Dorsal view of bristles on scutellum in (F) wild-type flies and (G) flies where Kis protein is knocked down in nervous tissue (*Elav-Gal4/UAS:kis RNAi.a*). Arrow notes duplication of bristles in Kis knockdown flies. (**H** and **I**) Adult wings from (H) wild-type and (I) Kis knockdown flies (*Elav-Gal4/UAS:kis RNAi.a*). Arrows denote presence of extra vein tissue near wing vein L2 (top arrow), the posterior crossvein (middle arrow) and wing vein L5 (bottom arrow). In cases not shown, *Da-Gal4/+* and *ELAV/+* flies show wild type phenotypes.

postural defect is reminiscent of defects associated in muscle cells, and is also observed in mutants for other chromatin remodeling factors in flies (25,26). Further, this phenotype is consistent with hypotonia-related posture problems observed in CHARGE patients (27).

To initially assess *kis* function in the developing nervous system, we expressed both of the RNAi constructs using *ELAV-Gal4*, a pan-neural driver. Expression of *kis RNAi.b* at 25°C leads to late pupal lethality, suggesting that the cause of the lethality observed at this stage with the *da-Gal4*

driver may be due to loss of Kis protein within the nervous system. Expression of *kis RNAi.a* with *ELAV-Gal4* produces viable flies with various morphological defects (Fig. 1). These flies exhibit duplicated bristles on the adult thorax [Fig. 1G, 24% penetrance ($n = 50$) compared with 0% in sibling controls ($n = 50$)]. This is a defect in the development of peripheral nervous system structures and has been previously observed in other regulators of chromatin structure in *Drosophila* (28,29). These flies also display extra vein differentiation in adult wings (Fig. 1I). These defects were associated mostly near wing veins L2 [45% ($n = 50$) compared with 0% in sibling controls ($n = 70$)] and L5 [24% ($n = 50$) compared with 0% in sibling controls ($n = 70$)]. This is also consistent with previously reported loss-of-function *kis* effects in wing vein differentiation (21).

Kismet function is required in muscles for early climbing behavior

Patients with CHARGE display delayed or impaired motor coordination and adaptive motor skills, musculoskeletal anomalies and hypotonia (3,27). To specifically test motor control of our CS flies, we utilized a simple, yet powerful behavioral assay based on climbing ability (30). Because we are interested in looking for early defects that may be associated with developmental problems due to loss of *kis* function, we restricted our analysis of climbing ability to the first 10 days after eclosion (early climbing behavior, days 1–10). Flies with ubiquitous expression of *UAS:kis RNAi.a* by *Da-Gal4* exhibit a significant decrease in climbing behavior within 10 days after eclosion (Fig. 2A) compared with controls. To more precisely determine specific tissue(s) in which loss of *kis* function affects this behavior, we expressed the *UAS:kis RNAi* constructs with a number of different tissue specific Gal4 drivers (Fig. 2, Table 1). Although the expression of *kis RNAi.b* leads to late pupal lethality with the pan-neural *ELAV-Gal4* (see above), the expression of *kis RNAi.a* with this driver has no significant effect on early climbing behavior (Table 1). Further, specific knockdown of Kis protein within motor neurons, dopaminergic neurons, glutamatergic neurons, cholinergic neurons, mushroom body neurons and glial cells all have no significant effect on early climbing behavior (Table 1). However, knockdown of Kis within muscles shows a strong effect (Fig. 2B, Table 1), reducing climbing frequency by nearly half compared with control flies. These data suggest that *kis* function is critically required in *Drosophila* muscles for coordinated motor function.

In addition to identifying a requirement for *kis* function in muscles, we also observe a strong decrease in early climbing ability by simultaneously reducing *kis* function in the ventral nerve cord, Kenyon neurons (neurons involved in learning and memory) and pars intercerebralis (the major neuroendocrine gland in flies) (Fig. 2C, Table 1). We also observe a small but significant decrease in early climbing ability when we knock down Kis in the giant fiber circuit with the *OK307-Gal4* reagent (Fig. 2D, Table 1). The giant fiber system in *Drosophila* is a pair of symmetrical inter-neurons that mediate the flight escape response by relaying sensory visual information to the thoracic flight muscles and leg extensor muscles of the thorax (31–33). We also observe that
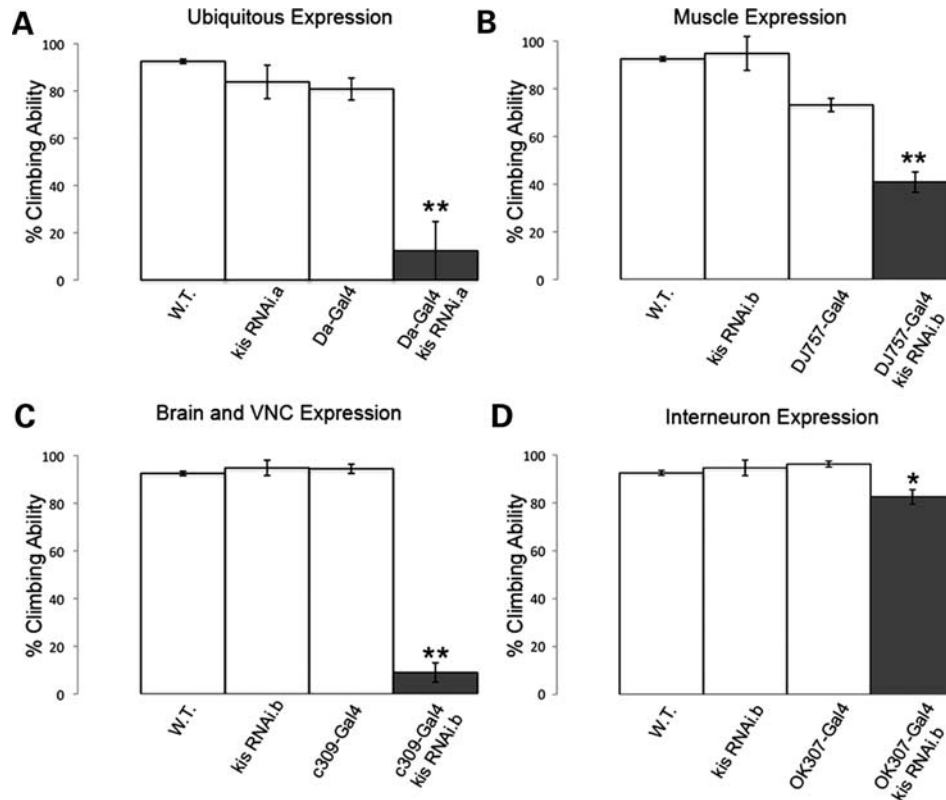
Figure 2. Kismet knockdown in muscles causes defective climbing behavior. (**A–D**) shows compiled climbing ability of wild-type, control and Kis knockdown flies at days 2–10. (A) Ubiquitous expression of *UAS:Kis RNAi.a* (*Da-Gal4/UAS:Kis RNAi.a*) shows severely reduced climbing ability compared with wild-type, *Da-Gal4/+* and *UAS:Kis RNAi.a/+* outcrossed control flies. (B) Kis knockdown restricted to muscles (*DJ757-Gal4/UAS:Kis RNAi.b*) also shows a strong reduction in climbing ability compared with wild-type, *DJ757-Gal4/+* and *UAS:Kis RNAi.b/+* outcrossed controls. (C) Kis knockdown in mushroom body neurons and the ventral nerve cord (*c309-Gal4/UAS:Kis RNAi.b*) shows a strong reduction in climbing ability compared to wild-type, *c309-Gal4/+* and *UAS:Kis RNAi.b/+* outcrossed controls. (D) Kis knockdown in the giant fiber inter-neurons (*OK307-Gal4/UAS:Kis RNAi.b*) shows a small but significant effect compared with wild-type, *OK307-Gal4/+* and *UAS:Kis RNAi.b/+* outcrossed controls. Error bars represent $\pm$ SEM. In all cases, double asterisk indicates $P < 0.001$ and asterisk indicates $P < 0.05$ compared with controls.

roughly half of these flies exhibit abnormal postural defects where they hold their wings out to the side of their bodies (53%, $n = 62$). Taken together, these data show that Kis function is also required outside of muscles in these inter-neuronal populations within the brain and ventral nerve cord to control climbing behavior and wing posture. However, because we restricted our analysis of *kis* function to the first 10 days post-eclosion, we cannot rule out the possibility that *kis* is required within the tissues we tested at later times during the fly's life cycle.

Kismet protein is broadly expressed in the developing fly brain

To begin to explore Kis protein function in the fly brain, we first analyzed Kis protein expression in the developing central nervous system. Because we had identified *kis* as a regulator of *atonal* and *daughterless* proneural gene expression in the retina (11), we compared Kis protein expression in the brain with the expression pattern of both these proneural proteins. We find that Kis protein is strongly expressed in many areas of the larval brain, including the cortex, the brain lobes and the ventral nerve cord (Fig. 3), and strongly

co-localized with both Atonal and Daughterless protein expression in this tissue. Our analysis of Ato protein expression is consistent with what has been previously reported in this tissue (34). Ato is expressed in the inner proliferation center of the optic lobe (lower arrow in Fig. 3A), as well as in a group of 20–30 cells in the dorso-lateral region of the central brain (upper arrow in Fig. 3A). This group of 20–30 cells is known as the dorsal cluster neurons (DCNs), which send out bundles of axons ventrally and then contralaterally toward the opposite optic lobe (34). We find that the Da protein is strongly co-localized to both the Ato expressing neurons in the optic lobe as well as in the central brain (Fig. 3A–F). Da protein is also significantly expressed in neurons outside of the Ato-specific neurons, within regions of higher expression in both the cortex and central brain (Fig. 3B). Similarly, Kis protein expression is also co-localized to sites of Ato expression (Fig. 3A). Like Da protein expression, Kis is also significantly expressed outside of the Ato-specific expression domains, but unlike Da, Kis expression is more uniform in the brain (Fig. 3C). Kis protein is also expressed within the neurons of the Kenyon cells (Fig. 3G–I), which are required for learning and memory in the fly.

Table 1. Gal4 lines tested for climbing behavior

Name	BL#	Organ/tissue	Climbing affected
ELAV-Gal4	458	Pan-neuronal	No
Ple-Gal4	8845	Dopaminergic neurons	No
Cha-Gal4	6798/6793	Cholinergic neurons	No
OK371-Gal4	26160	Glutamatergic neurons	No
OK107-Gal4	854	entire MB	No
1471-Gal4	9465	Gamma lobes of MB	No
D42-Gal4	8816	Motor neurons	No
Pdf-Gal4	6900	Ventrolateral neurons	No
Repo-Gal4	7415	Glial cells	No
C309-Gal4	6906	MB, thoracic ganglion, eye	Yes
OK307-Gal4	6488	Giant fiber interneuron	Yes
A51-Gal4	8764	Muscles and motor neurons	Yes
DJ757-Gal4	8184	Muscles	Yes

The effect of different Gal4 lines tested for climbing behavior with *UAS:kis RNAi* constructs. BL, Bloomington Stock number; MB, mushroom body. Climbing is affected when compared with wild-type flies, as well as Gal4 and UAS controls outcrossed to w^-.

Kismet function is required for immediate recall memory

Based on the expression pattern of Kis in the developing brain, and the fact that patients with CS often suffer from intellectual disability, we tested the learning and memory ability of adult flies with reduced *kismet* function. To test for deficits in learning and memory in these flies, we performed the conditioned courtship suppression assay (35). This assay is an associative conditioning procedure that is ethologically based and capable of measuring both learning and memory in individual flies (36). The conditioning aspect of this assay is based on the observation that male courtship behavior is modified by exposure to a previously mated female that is unreceptive to courting (35,37). Thus, after 1 h of unsuccessful courting of a mated female, wild-type males suppress their courtship behavior, even toward subsequent receptive virgin females, for 1–3 h (35,38–40).

In order to knock down Kis protein in this assay, we expressed each of the *UAS:kis RNAi* constructs continuously during development with the *OK107-Gal4* driver. This driver is expressed in discrete populations of neurons during development in the fly brain, including the Kenyon cells (41), neurons of the Ventral Ganglion (42), and early in the optic lobes, central brain regions and protocerebrum (43). 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 first 10 min of this training and compared with the last 10 min of the training period. Wild-type (Canton S) control flies show a significant drop in courtship behavior in the last 10 min of training when compared with the first 10 min (Fig. 4A), indicative of an appropriate learning response. Similarly, outcrossed control flies (Fig. 4A and B) and Kis knockdown flies (Fig. 4C) also show a significant decrease in courtship behavior in the last 10 min of the training period compared with the first 10. Importantly, this indicates that our Kis knockdown flies are capable of successful perception and interpretation of the sensory stimuli required in this

assay and that these flies are able to alter their behavior appropriately (learn) in response to this training.

There have been five phases of memory defined in *Drosophila*, immediate recall (0–2 min post-training), short-term memory (out to 1 h post-training), medium-term memory (out to 6 h), anesthesia-resistant memory (out to 2 days) and long-term memory (out to 9 days) (44,45). In order to test the earliest phase of memory first, we assayed Kis knockdown flies for their immediate recall memory by transferring trained male flies to clean mating chambers with a receptive virgin female within 2 min of training and assaying their courtship behavior for 10 min. Trained wild-type males show a clear decrease in courtship activity when compared with parallel sham-trained wild-type flies (Fig. 4D), indicating a change in behavior consistent with normal immediate recall memory of training. Similarly, outcrossed *Gal4* and *UAS* control flies also show a significant decrease in their courtship activity compared with genotype equivalent outcrossed sham-trained flies (Fig. 4D and E). However, both Kis knockdown flies show no significant decrease in their courtship activity within 2 min of training (Fig. 4F). Because these flies are capable of successful perception, and experience-dependent alteration of their behavior in the learning component of this assay, their inability to suppress their courtship frequency in the second component of this assay indicates that these flies are defective in their immediate recall memory of this learning.

Kismet mutants display abnormal axonal pruning and migration during development of Kenyon cells

Because the *kis* gene encodes for a transcription factor, these results suggest that *kis* may be regulating the gene expression of some critical factor(s) important for immediate recall memory formation during development. This could either be through the control of neurogenesis of appropriate neurons in this memory circuit, control of circuit connectivity once these neurons have already differentiated and/or control of the development and/or morphology of these neuronal target tissues. To begin to address these questions, we first analyzed the effect of loss of function in *kis* on neuronal development and morphology of the Kenyon neurons, as these neurons are associated with learning and memory in multiple experimental paradigms in *Drosophila* (46–50). We utilized the MARCM technique (51) to create clones of neurons that are homozygous mutant for kis^{LM27}, a protein null mutant (11). The population of Kenyon cells consists of three distinct groups of neurons, each with its own characteristic developmental morphology (41). During larval development, the γ subset of Kenyon neurons are differentiated first, and these neurons undergo highly stereotyped dendritic and axonal pruning during pupal development (41,52). Pruning of these neurons occurs by localized degeneration (53) and is evident by 18 h after puparium formation in wild-type neurons (Fig. 5A, arrow shows remnants of γ axonal projects into the α lobes). Although γ neurons mutant for *kis* undergo proper dendritic pruning, they display defects in their axonal pruning (arrow in Fig. 5B) and continue to display unpruned γ axons projecting into α lobes 40% of the time (Table 2).
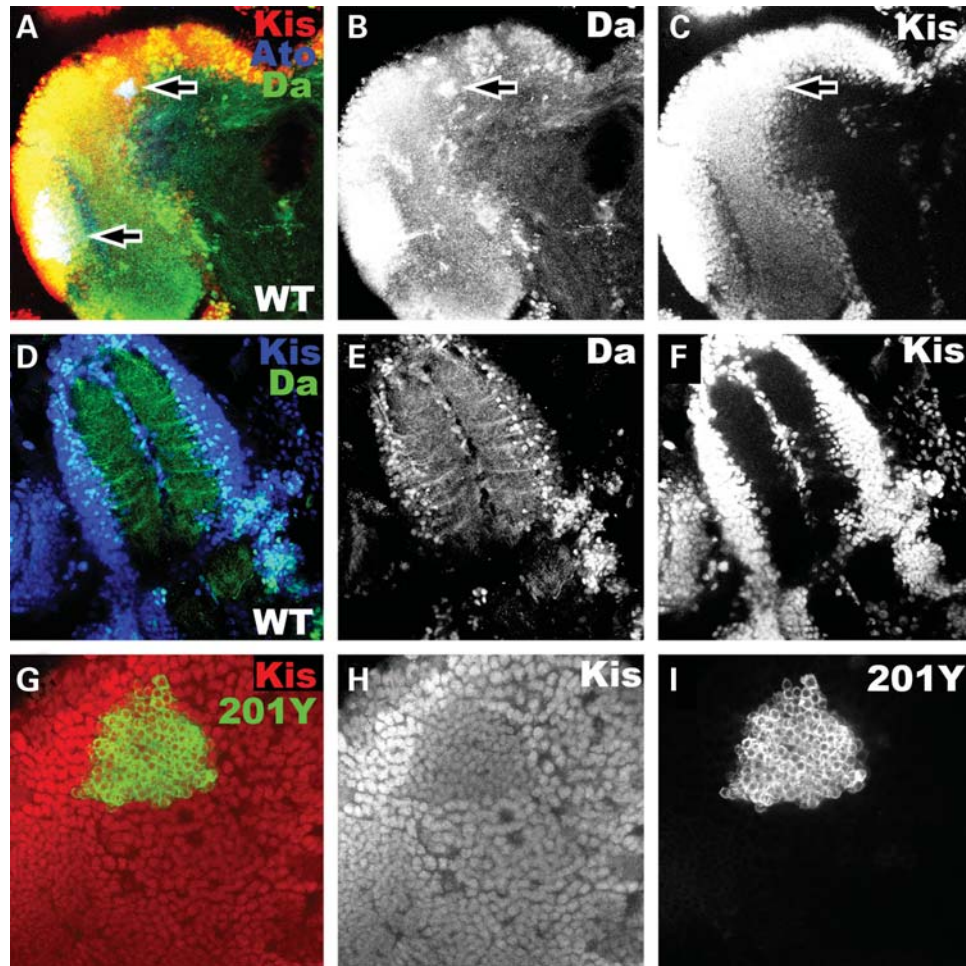
Figure 3. Kismet is widely expressed in the developing larval central nervous system. (**A**−**C** and **G**−**I**) Developing brain lobe of wild-type late third instar central nervous system. (**D**−**F**) Developing ventral nerve cord of wild-type late third instar larval central nervous system. (A−F) are whole brain reconstructions from individual confocal image slices. (A) Kis expression (red), Atonal expression (blue) and Daughterless expression (green) in wild-type brain lobe. Upper arrow denotes the Atonal-expressing DCNs. Lower arrow denotes the developing optic lobe. (B) Daughterless expression (white) from (A). Arrow denotes Daughterless expression in DCNs. (C) Kis expression (white) from (A). Arrow denotes Kis expression in DCNs. (D) Kis expression (blue) and Daughterless expression (green) in developing ventral nerve cord. (E) Daughterless expression (white) from (D). (F) Kis expression (white) from (D). (G) GFP expression (green) in the membrane as driven from the *201Y-Gal4* driver (*201Y-Gal4/UAS:CD8-GFP*) to denote the location of the γ-Kenyon neurons. Kis expression (red). Note strong co-localization. (H) Kis expression (white) from (G). (I) GFP expression (white) from (G).

The final adult structure of the mushroom body axonal projections consist of five distinct projections to the α, α′, β, β′ and γ lobes (Fig. 5C). To determine whether structures other than γ lobes are affected by mutations in *kis*, we created MARCM clones mutant for *kis*[LM27] using the *OK107-Gal4* driver, which is expressed in the α, β and γ neurons. While Kenyon cell dendrite morphology was normal in these *kis* mutant neurons, we did observe axonal migration defects in these cells where axons cross the midline of the brain to migrate into the opposite brain lobe (arrow in Fig. 5D, 55%, Table 2).

Kismet mutants display abnormal axon extension and migration in DCNs and photoreceptor axons

The Kis protein is widely expressed in the fly brain (Fig. 3). Given that mutations in *kis* affect axonal pruning and axonal migration in developing Kenyon neurons, we analyzed the effect of *kis* loss of function in other neuron populations in the developing and adult brain to determine whether the Kis protein may play a broader role in regulating neuronal morphology. Based on the co-localization of Kis and Atonal proteins in the developing larval brain and that *kis* function is known to regulate *atonal* transcription in the developing retina (11), we created MARCM clones mutant for *kis* function in the DCNs, a subset of Atonal-expressing neurons in the larval and adult brain (34). These neurons form a highly stereotypical pattern of connections in the fly brain that innervate the optic lobes (34,54). The regular array of these neuronal connections has been extremely useful to determine how genes function to regulate neurite extension, outgrowth, neuronal morphology and arborization patterns (54,55). During larval development, DCN soma forms a distinct cluster of roughly 20−30 cells in the dorso-lateral region of the central brain (34). These neurons then extend a bundle of neurites posteriorly, and then form a bundle of commissural
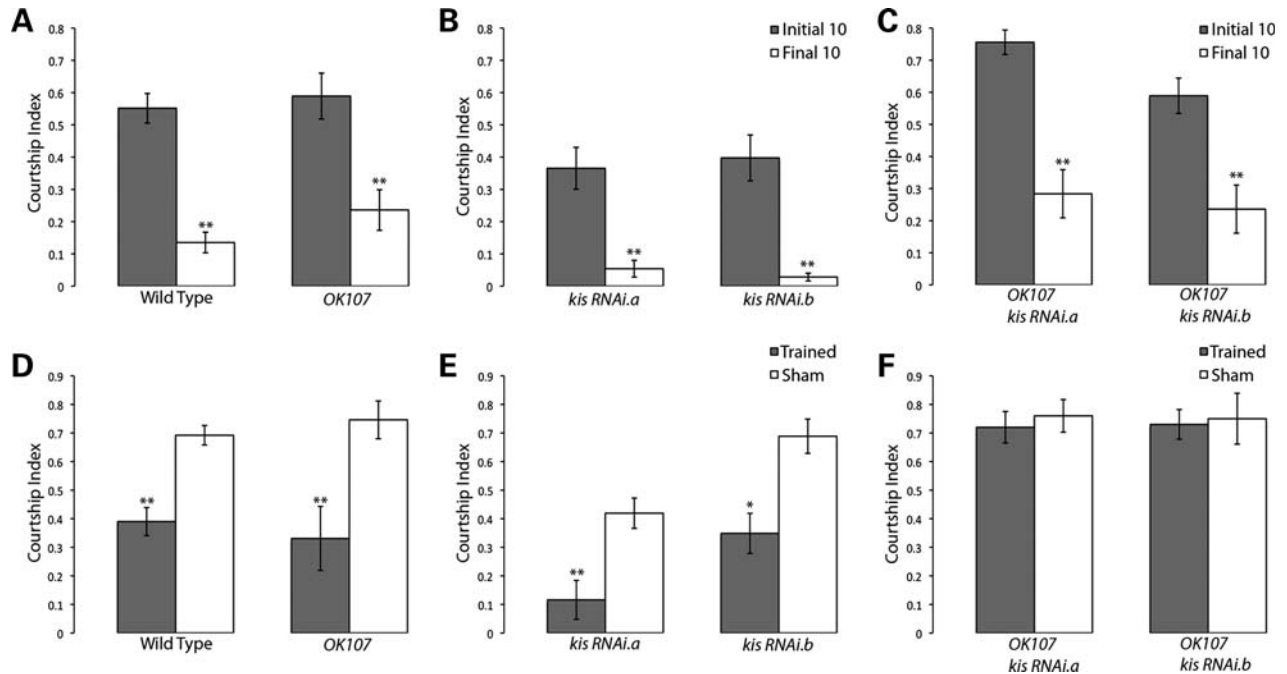
Figure 4. Kismet knockdown flies can learn, but are deficient in their immediate recall memory. (**A–C**) denote learning during the first 10 (gray columns) and last 10 minutes (white columns) of the training phase during the courtship suppression assay. Genotypes are indicated. Note normal response of Kis knockdown mutants. (**D–F**) Panels denote immediate recall memory (0–2 min post-training) of trained flies (gray columns) when compared with sham-trained matching genotypes (white columns). Kis knockdown showed no significant difference between trained and sham-trained flies, indicative of no immediate recall memory of training. Error bars represent ± SEM. In all cases, double asterisk indicates $P < 0.001$ and asterisk indicates $P < 0.05$ compared with controls.

axons that migrate contralaterally to innervate targets of the opposite brain lobe (Fig. 6A). Developing larval DCNs mutant for kis^{LM27} exhibit several morphological abnormalities indicative of abnormal axon development (Table 2, Supplementary Material, Table S1). These neurons display abnormal commissural axon projections (arrow in Fig. 6B, Table 2), consistent with the defects in axonal migration observed in *kis* mutant Kenyon neurons. *kis* mutant cells also display abnormal positioning within the brain, appearing laterally in more than half of the clones observed (left arrow in Fig. 6B, Table 2). Interestingly, these neurons appear to send their initial neurite bundles to the proper location for initial branching however (Fig. 6B). Additionally, we occasionally observe groups of DCN soma that are not part of the larger soma cluster (arrowhead in Fig. 6B, Table 2). We observe similar morphological defects in larval DCNs where *kis* is knocked down with both *UAS:kis RNAi* constructs (Supplementary Material, Fig. S1), providing further validation of the specificity of these defects for *kis* function in these cells.

In adults, the *atonal* expressing neurons form highly complex dendritic and axonal fields (Fig. 6C and E) (34,55). Adult DCNs project neurite bundles that branch ipsilaterally to form the dendritic field (Fig. 6C), and also project a large bundle of commissural axons contralaterally to innervate the lobula and medulla in a stereotypical fan-like pattern (Fig. 6E) (34,54,55). Adult DCNs homozygous mutant for *kis* do not display abnormal dendrite formation (Fig. 6D). However, *kis* mutant DCNs show a severe reduction in the number of axons extending into the lobula, and subsequently extending from the lobula into the medulla (arrow Fig. 6F,

Table 2). Wild-type MARCM clones exhibit 13.5 ± 1.51 neurites extending from the lobula into the medulla, whereas *kis* mutant MARCM clones exhibit 5.5 ± 2.59 neurites extending from the lobula to the medulla, a significantly lower number ($P = 0.00018$). These results are similar to those observed in Kenyon cells, in that the dendrites develop normally, whereas the axons do not.

To further validate the axonal defects we observe in *kis* mutant neurons, we analyzed the axonal projections of photoreceptor cells into the optic lobe of developing larval brains, a standard technique for analyzing photoreceptor axonal guidance (56,57). We dissected third instar retina–brain complexes from developing larvae and stained these tissues for the MAP1B-like protein Futsch (58). Wild-type photoreceptor cells extend their axons from retinal cells away from retinal tissue toward the optic stalk where the axons then fasciculate into a bundle. These axons then migrate to innervate the lamina and medulla of the larval brain in a highly stereotypical fashion (Fig. 7A). We then created clones of kis^{LM27} homozygous mutant cells in the developing retina and analyzed migration of photoreceptor axons into the brain. In these retinas, photoreceptor axons exhibit a severe defect in axonal migration into the optic lobe (Fig. 7B), with photoreceptor axons extending beyond their normal targets and radiating out into the surrounding brain areas. These axons also show abnormal defasciculation of axonal bundles in the optic stalk (arrow in Fig. 7B). Interestingly, the photoreceptor cells migrate normally into their proper positions in the ommatidial clusters in *kis* mutant retinal tissue (Fig. 7C, non-green tissue) and initially send their axons to properly bundle in
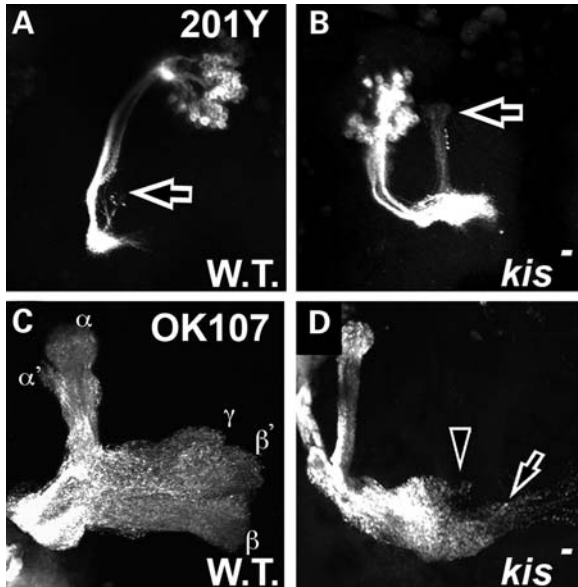
Figure 5. Kismet is required for proper axon pruning and axon migration in developing mushroom bodies. All panels show GFP in Kenyon neurons by MARCM analysis. (**A** and **B**) Pupal brains 18–20 h after puparium formation (apf). (**C** and **D**) Adult brains 48 h after eclosion. (A) Wild-type MARCM clones (FRT 40A) in pupal brains driven by *201Y-Gal4* show remnants of proper axonal pruning of γ neurons that were previously populating the α lobes (arrow). (B) *kis^{LM27}* homozygous mutant MARCM clones display unpruned γ axons that continue to populate the α lobes (arrow). (C) Wild-type MARCM clones (FRT 40A) in adult brains driven by *OK107-Gal4* show normal pattern of α, α′, β, β′ and γ axons innervating the mushroom body lobes, as labeled. (D) *kis^{LM27}* homozygous mutant MARCM clones in these adult brains often display abnormal axon migration beyond the midline (demarcated by the arrowhead).

Table 2. Neuronal defects observed in *kismet* MARCM mutants

Neuron	Gal4-line	n	Defects observed	%	P-value
γ neurons, MB	201Y				
control (pupal)		8	Unpruned axons	0%	—
kis mutant (pupal)		10	Unpruned axons	40%	<0.05
α, β, γ neurons, MB	OK107				
Control (adult)		9	Lobe structure	0%	—
			Cell migration	0%	—
			Axon migration	11%	—
kis mutant (adult)		11	Lobe structure	55%	<0.05
			Cell migration	36%	<0.05
			Axon migration	55%	<0.05
DCNs	Atonal				
Control (larval)		9	Cell position	0%	—
			Axon migration	0%	—
			Cells out of cluster	0%	—
Control (adult)		6	Axon extension (13.5 axons in medulla)	0%	—
kis mutant (larval)		19	Cell position	53%	<0.001
			Axon migration	32%	<0.05
			Cells out of cluster	32%	<0.05
kis mutant (adult)		6	Axon extension (5.5 axons in medulla)	100%	<0.001

Quantification of defects observed in different *kismet* mutant neurons. MB, mushroom body. Unpruned axons refers to the number of clones with unpruned γ axons extending into the α lobe at 18–20 h APF. Lobe structure indicates missing or altered lobe morphology in α, β or γ lobes. Cell position indicates the number of soma that appear lateral to their appropriate locations by the developmental time indicated. Axon migration indicates either axons crossing the midline for adult Kenyon cells or commissural axons failing to cross the midline or failing to migrate to their proper contralateral targets in larval DCNs. Axon extension indicates the presence of axons failing to extend from lobula into the medulla in adult brains. *P*-values are indicated from a two-tailed Student's *t*-test comparing *kis* mutant with control.

the optic stalk. Further, these clones in the adult eye do not show any abnormal external development of mutant ommatidia (Fig. 7E and F).

Greater than 80% of *CHD7*-positive CHARGE patients display coloboma of the eye, retina or both tissues (3,59). Though external adult eye morphology appears normal when discrete patches of eye tissue are mutant for *kis*, we tested whether loss of function in *kis* throughout the whole eye would show abnormal external morphology. We expressed *UAS:kis RNAi.b* in all cells posterior to the morphogenetic furrow using the *GMR:Gal4* driver at 25°C. At this temperature, *GMR:Gal4* eyes alone display normal external eye morphology (Fig. 7G), as do *UAS:kis RNAi.b* flies (data not shown). However, when the Kis protein is significantly reduced throughout development of the eye, adult morphology is disrupted (Fig. 7H). These eyes have a slightly rough and significantly glassy appearance (Fig. 7H), although eye size is not affected. Taken together, our results suggest that proper *kis* function is required both for normal eye development, as well as normal photoreceptor axon guidance in the developing fly eye.

DISCUSSION

In summary, the data presented here describe a requirement for *kis* gene function in adult *Drosophila* early climbing behavior, memory, eye development and neural development. Our results suggest that the reduced early climbing ability observed in *kis* mutants can at least partially be attributed to a requirement for *kis* gene function in muscle cells. This is particularly of interest when we consider that knockdown of *kis* function in motor neurons, as well as pan-neural knockdown of *kis* function has no effect on the early climbing behavior we analyzed. Thus, *kis* may either be regulating the expression of critical post-synaptic target gene(s) in muscle cells required to facilitate synaptic transmission and muscle coordination and/or may be required for the morphology and/or development of the muscle cells themselves. Further, the postural defect we observe in adult flies with reduced Kismet protein is also reminiscent of defects associated with muscle cells, and both phenotypes are consistent with hypotonia, impaired motor coordination and muscle-related posture problems observed in CHARGE patients (27). Based on these similarities, our results may suggest that there is a similar requirement for *Chd7* function in muscles of vertebrates and may help to elucidate a possible mechanism for these symptoms often observed in CHARGE patients.

Our data suggest that decreased *kis* function does not alter the fly's ability to learn, although it does have an effect on immediate recall memory. Our data have also shown that *kis*
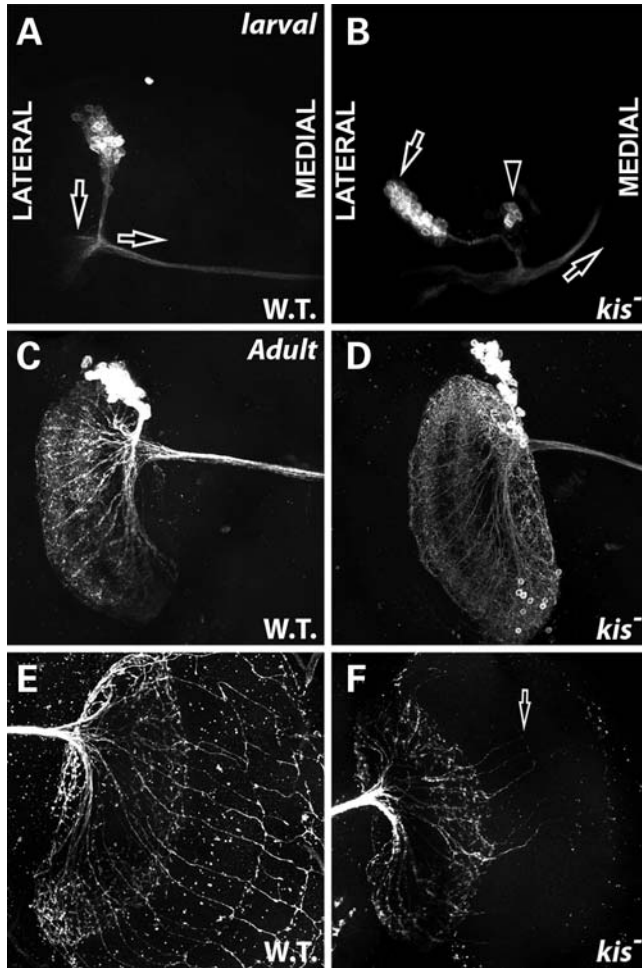
Figure 6. Kismet is required for proper DC position and axon migration in developing DCNs. All panels show GFP in DCNs by MARCM analysis. (**A** and **B**) Late third instar larval brains. Both images show the left hemisphere. Anterior is up. Medial is right. Lateral is left. (**C**–**F**) Adult brains 48 h after eclosion. (A) Wild-type MARCM clones (FRT 40A) in larval DCNs. Note position of the soma cluster axon bundles (parallel to vertical arrow) compared with commissural axon bundles (below horizontal arrow). (B) kis^{LM27} homozygous mutant MARCM clones display abnormal positioning of soma cluster compared with commissural axon bundles (left arrow), soma that have developed outside of the normal cluster (arrowhead) and disrupted commissural axon migration (right arrow). (C) Wild-type MARCM clones (FRT 40A) in adult DCNs displaying normal dendritic morphology on ipsilateral brain hemisphere. (D) kis^{LM27} homozygous mutant MARCM clones in adult brains. Overall morphology of dendritic field appears normal. (E) Wild-type MARCM clones (FRT 40A) in adult DCNs displaying normal axonal morphology on contralateral brain hemisphere. (F) kis^{LM27} homozygous mutant MARCM clones in adult brains. Arrow displays abnormal and reduced number of axonal extensions from lobulla into the medulla.

Figure 7. Kismet is required for proper photoreceptor axon migration and eye development. (**A**–**D**) Late third instar larval retinas with attached brains. (**E**–**H**) Adult eyes, anterior right. (A) Wild-type MARCM clone with attached larval brain stained for the Futsch protein (white) display normal axonal bundles in the optic stalk that innervate the developing optic lobes. (B) kis^{LM27} homozygous mutant clones in the developing retina stained for Futsch (white) display abnormal axonal migration into the developing brain. Arrow indicates defasciculation of axon bundles in the optic stalk. (C) Cross-section of kis^{LM27} homozygous mutant clones in the developing retina posterior to the morphogenetic furrow. Heterozygous (control) tissue is denoted by the presence of GFP (green). Homozygous clones are denoted by lack of GFP. Retinas are stained with Futsch (magenta). Note normal positioning of ommatidia clusters near the apical (top) portion of the retina. (D) Futsch stain (white) from (C). (E) Normal eye expressing only *eyeless:Flip*. (F) Adult eye showing kis^{LM27} homozygous mutant clones (in white). (G) *GMR-Gal4* adult eye raised at 25°C displays normal morphology. (H) *GMR-Gal4* / *UAS:kis RNAi.b* adult eye raised at 25°C displays abnormal morphology and glassy appearance.

function is required for the proper development of the Kenyon cells, as *kis* mutants show defective axonal pruning and axonal migration in these neuronal populations. The Kenyon neurons are associated with learning and memory in multiple experimental paradigms in *Drosophila*, including the conditioned courtship suppression behavior (46–50,60). Thus, it may be that the defects we observe in Kenyon cell development are responsible for the impaired learning behavior we observe in adult flies with decreased *kis* function. *kis*-mediated transcriptional regulation of genes involved in the pruning and/or
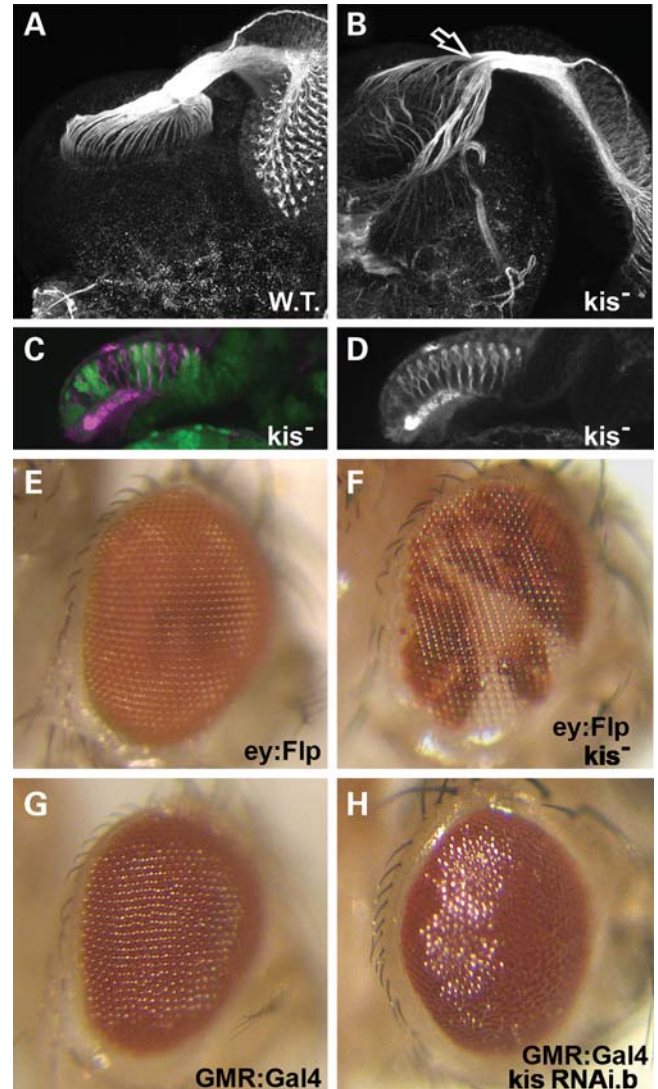
migration of axons in this cell population would therefore make attractive targets for further investigation into this memory defect.

In analyzing the morphology of neuronal populations mutant for *kis*, we consistently observe defects in axon morphology and positioning. These defects may be due to abnormal axonal

pruning observed in some *kis* mutant neurons or may be due to defective axonal migration or defects in axonal extension and/or retraction. Interestingly, in each of the neuronal populations studied, dendritic development was normal. When taken together, these data suggest that *kis* may function to regulate the expression of target gene(s) normally required for axon morphology and connectivity, as opposed to dendritic morphology, at least in the neuronal populations and developmental times studied here. The specific target genes that Kismet regulates most likely differ between different neuronal populations. However, the defects observed in *kis* mutant DCNs suggest that *kis* functions in DCN positioning as well as axonal morphology. Abnormal positioning of larval DC soma is also observed in larval brains mutant for *ato* gene function (34). As *kis* regulates *ato* transcription in the larval retina (11), this neuron migration defect may be due to *kis* mediated regulation of *ato* transcription in these cells as well.

Taken together, we suggest that the analyses presented here can complement and expand upon the studies done in cell culture and vertebrate model organisms toward a better understanding of the role of *kis* in neural developmental and *Chd7* in CS pathogenesis.

MATERIALS AND METHODS

Drosophila stocks and genetics

All flies were maintained at 25°C in a 12:12 light:dark cycle at 60% humidity. All crosses were carried out at 25°C. Normal food consisted of a standard cornmeal, yeast, molasses recipe. BL# refers to Bloomington Stock Center stock number (http://flystocks.bio.indiana.edu/bloomhome.htm). VDRC# refers to the Vienna Drosophila Resource Center stock number (http://stockcenter.vdrc.at/control/main). Wild-type flies used were Canton S. Stocks used are described: kis^{LM27} (11), *UAS:kis RNAi.a* (VDRC #10762) and *UAS:kis RNAi.b* (VDRC #46685).

All Gal4 stocks were obtained from the *Drosophila* Bloomington Stock Center. The tissues affected and Bloomington Stock number are listed in Table 1.

Clones in developing retinas to analyze axonal guidance were generated using *ey:FLP* as previously described (56). MARCM mosaic analysis was performed as previously described (51,61). Genotypes for MARCM analysis were:

(1) y^-,w^-,*hs:Flp, UAS:CD8-GFP; Gal80,Frt40A/Cyo; +/+; OK107-Gal4*
(2) y^-,w^-,*hs:Flp, UAS:CD8-GFP; Gal80,Frt40A, 201Y-Gal4*
(3) y^-,w^-,*hs:Flp, UAS:CD8-GFP; Gal80,Frt40A/Cyo; Ato-Gal4(14a)/TM6B*
(4) w^-; kis^{LM27},*Frt40A/Cyo*
(5) w^-; *Frt40A*

Immunohistochemistry and antibodies

Kis antibody was a kind gift from J. Tamkun and is described in Srinivasan *et al*. (16). Daughterless antibody was a kind gift from C. Cronmiller and is described in Cronmiller *et al*. (62). Futsch antibody was obtained from the Iowa Developmental Hybridoma Bank (22C10). Secondary antibodies for immunohistochemistry used were goat anti-mouse TRITC (# 115-116-072, 1:150), goat anti-rabbit TRITC (# 111-116-144, 1:250), goat anti-rabbit Cy5 (#111-176-144, 1:1000) and goat anti-mouse Cy5 (# 115-176-072, 1:500). All secondary antibodies were from Jackson ImmunoResearch.

Adult and larval brains were dissected, fixed and prepared essentially as described (63). Adult and larval brains were dissected directly in fix. Brains were mounted in vectashield (Vector Laboratories, H-1000). All fluorescent imaging was done using an Olympus FluoView FV1000 laser scanning confocal microscope.

Behavioral testing and training

For climbing assays, a modified version of Le Bourg and Lints (30) was used. Flies were collected between 0 and 8 h after eclosion and assayed every 2 days. Groups of 10 or fewer flies were transferred to a clean, empty vial and given 18 s to climb 5 cm. The number of flies that successfully reach the 5 cm line were recorded.

For courtship behavioral training, virgin male flies of the appropriate genotype were collected between 0 and 6 h after eclosion and transferred to individual food vials. All flies were maintained at 25°C in a 12:12 light:dark cycle at 60% humidity. All behavioral tests were performed in a separate room maintained at 25°C and 60% humidity and illuminated under a constant 130 V white light Kodak Adjustable Safelight Lamp mounted above the courtship chambers. All behavior was digitally recorded using a Sony DCR-SR47 Handycam with Carl Zeiss optics. Subsequent digital video analysis of time spent performing courtship behavior was quantified using iMovie software (Apple). The total time that a male performed courtship activity was measured and scored. The courtship index was calculated as the total time observed performing courting behavior divided by the total time assayed, as described (35).

Virgin female wild-type (Canton S) flies were collected and kept in normal food vials in groups of 10. Male flies were aged for 5 days before behavioral training and testing. All tests were performed during the relative light phase. 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 female Canton S targets used were 4 days old. Male flies were assigned to random groups the day of training, and assays were set up and scored blind. 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 the flies were recorded for 10 min.

Statistical analysis

All statistical analyses were performed on SPSS version 17. To determine the significance between multiple different

genotypes, a one-way ANOVA analysis was performed with Tukey *post hoc* analysis. Genotype is the independent variable. To determine the significance between different measures of the same genotype, a two-tailed paired Student's *t*-test was performed. An unpaired Student's *t*-test was performed between two groups of different genotypes. Significance was determined at the 95% confidence interval.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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