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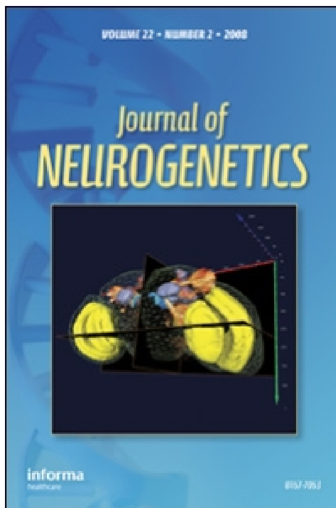

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The *white* Gene of *Drosophila melanogaster* Encodes a Protein with a Role in Courtship Behavior

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Abstract: The *white* gene of *Drosophila melanogaster* has been extensively studied, yet it is still not understood how its ectopic overexpression induces male-male courtship. To investigate the cellular basis of this behavior, we examined the sexual behavior of several classes of mutants. We find that male-male courtship is seen not only in flies overexpressing the *white* gene, but also in mutants expected to have mislocalized White protein. This finding confirms that mislocalizing White transporter in the cells in which it is normally expressed will produce male-male courtship behaviors; the courtship behavior is not an indirect consequence of aberrant physiological changes elsewhere in the body. Male-male courtship is also seen in some mutants with altered monoamine metabolism and deficits in learning and memory, but can be distinguished from that produced by White mislocalization by its reduced intensity and locomotor activity. Double mutants overexpressing *white* and with mutations in genes for serotonergic neurons suggest that male-male courtship produced by mislocalizing White may not be mediated exclusively by serotonergic neurons. We also find decreased olfactory learning in *white* mutants and in individuals with mutations in the genes for White's binding partners, *brown* and *scarlet*. Finally, in cultured *Drosophila* and mammalian cells, the White transporter is found in the endosomal compartment. The additional genes identified here as being involved in male-male courtship increase the repertoire of mutations available to study sexual behavior in *Drosophila*.

Keywords: Drosophila melanogaster, white, endosome, courtship, learning and memory

INTRODUCTION

Since its initial discovery (Morgan, 1910), the *white* gene has become one of the most intensively studied genes at the genetic, cytological, and molecular levels because of the dramatic eye-color phenotype of mutants (reviewed by Phillips & Forrest, 1980; Hazelrigg, 1987; Green, 1996; Ewart & Howells, 1998). Yet, despite the intense investigation of the gene, both in its own right and for its extensive use as a genetic marker, it was not until 1995 that an additional role in neural function was suggested. Zhang and Odenwald (1995) reported that transgenic male flies in which the *white* gene was ectopically overexpressed from a heat-shock promoter (*hs-mini-white*$^{+}$) begin to court each other, forming long courtship chains. Acquisition of homosexual courtship behavior was not a phenotype expected in flies overexpressing a gene hitherto thought to be involved only in the pigmentation of eyes and internal organs.

The predicted structure of the *white* gene product yielded the first clue as to the biological basis of the behavioral phenotype; the *white* gene encodes a member of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transmembrane transporter proteins (Mount, 1987; Pepling & Mount, 1990). These proteins use energy generated by ATP hydrolysis to transport specific substrate molecules through the lipid bilayer (Schmitz et al., 2001). Each transporter is characterized by two ATP-binding folds and two hydrophobic membrane-spanning domains, each consisting of five to six membrane-spanning alpha helices (Mount, 1987; Hyde et al., 1990; Schmitz et al., 2001). These elements can all be present in one protein or split between two polypeptides that dimerize, as is the case for the *Drosophila* White protein (Mount, 1987; Schmitz et al., 2001). Genetic analysis of the pigmentation phenotypes of *white*, *brown*, and *scarlet* mutants led to the proposition that the White protein dimerizes, separately, with the product of the *brown* gene to transport

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the guanidine triphosphate (GTP)-based precursors of pteridine pigments and the product of the *scarlet* gene to transport the tryptophan-based precursors of the ommochrome pigment (Nolte, 1952). Subsequent molecular analysis confirmed that *brown* and *scarlet* indeed encode half-ABC transporter pumps (Dreesen et al., 1988; Tearle et al., 1989). Tryptophan and GTP are not only pigment precursors, but they are also precursors of small monoamine neurotransmitters or cofactors in neurotransmitter biosynthesis (Joh, 1997). Tryptophan is the direct precursor of 5-hydroxytryptamine (5-HT) or serotonin, and GTP is a precursor of 4 hydroxybiopterin, an essential cofactor required for tyrosine hydrolase to produce serotonin and the catecholamine dopamine (Goodwill et al., 1998). While the pigment phenotypes of these mutants predict that the *white*-encoded ABC transporter transports GTP and tryptophan-derived compounds, the specificity of the White transporter is largely determined by its binding partner (Ewart & Howells, 1998). White appears to have a role in the transport of cyclic guanosine monophosphate (cGMP) (Evans et al., 2008), and depending on its binding partner, the White protein could potentially transport many other small metabolites, including the amino acids that are the precursors of other neurotransmitters.

The second clue as to how overexpression of the *white* gene might give rise to altered courtship behavior came from the finding that mutants for another classical eye pigment gene, *garnet*, also show male-male courtship behaviors (Lloyd et al., 1999). These behaviors were indistinguishable from those seen when the *white* gene was ectopically overexpressed and were dependent on the presence of the White protein (Lloyd et al., 1999). The *garnet* gene encodes one subunit of the *Drosophila* adaptor protein (AP)-3 complex (Simpson et al., 1997; Ooi et al., 1997; Lloyd et al., 1999) involved in shunting proteins from the general cellular export pathway at the trans-Golgi network to the endosomal compartment (Odorizzi et al., 1998). The presence of a diagnostic dileucine residue (Odorizzi et al., 1998; Schmitz et al., 2001) in the N-terminal region of the White protein suggests that it is targeted for transport to the endosomal compartment by the AP-3 complex. The pigment granules of the pigment cells are derived, or receive their cargo, from endosomes (Shoup, 1966; Schraermeyer & Dohms, 1993), as do synaptic vesicles in neurons (Regnier-Vigouroux & Huttner, 1993). In *garnet* mutants, the normal AP-3 shunt mechanism is compromised, so that the White protein that would normally be transported to the Golgi body would likely be transported to the plasma membrane by the default export pathway instead.

The original explanation for the male-male courtship seen in the *hs-mini-white*$^+$ strain studied by Zhang and Odenwald (1995) was that it arose from the induced ectopic expression of the White transporter that indirectly altered the availability of serotonin, or its precursors, in neurons. Based on our previous findings of male-male courtship in *garnet* mutants, we would instead propose that mistargeting White protein to different regions, but of the same cells that normally express this protein, can produce a surplus of White protein at the plasma membrane and a shortage in endosomal derivatives, such as the pigment granules and potentially synaptic vesicles. This rerouting of the White protein would cause a decrease in the amount of White transmembrane transporter in pigment granules that produces the reduced pigmentation diagnostic of *garnet* and the other granule group mutants (Lloyd et al., 1998, 2002). However, it is the mislocalized, surplus, protein that causes the change from obligate male-female courtship to male-male courtship; flies completely lacking the White protein do not show this behavior (Lloyd et al., 2002). The same mislocalization of the White transporter could arise from ubiquitous overexpression of the *hs-mini-white*$^+$ transgene from the heat-shock promoter, as reported by Zhang and Odenwald (1995). In this case, an excess of White protein could overwhelm the usual sorting pathway to the endosomes, and the surplus White protein would, again, be transported to the plasma membrane. The *white* gene is strongly expressed in the eyes and other pigmented organs (Fjose et al., 1984; Chintapalli et al., 2007), but low levels of *white* mRNA have also been detected in nonpigmented organs, such as the fat body and in the heads of flies lacking pigmented organs, suggesting that it is also expressed in neural tissue (Campbell & Nash, 2001, Chintapalli et al., 2007). The male-male courtship of *garnet* mutants shows that mislocalization of the White protein only in the cells normally expressing it is sufficient to induce male-male courtship.

Mislocalization of a metabolite transporter provides an explanation for the behavioral and other phenotypes of these mutant flies. However, this mislocalization model has been inferred solely from genetic data. To demonstrate a neural function for the *white* eye-color gene and explain, at the cellular level, how its overexpression induces male-male courtship in *Drosophila,* we, therefore, sought to confirm three main predictions of this mislocalization model:

1. White protein localizes to endosomes where it could, directly or indirectly, impact neurotransmitter systems.
2. Mutants with altered expression and localization of the *white* gene also show alterations in nonsexual behaviors, as expected for a gene active in the central nervous system.
3. Males mutant for other genes responsible for the localization and function of the White protein show male-male courtship behavior.

The results of these studies confirm that mislocalization of the White protein causes the male-male courtship phenotype, that its absence also has behavioral consequences, and, finally, the monitoring of courtship and locomotor behavior of flies

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with mutations in genes involved in learning, memory, and monoamine neurotransmitter metabolism suggest that the basis of male-male courtship behavior is complex, and that White encodes a relatively nonspecific transporter.

MATERIALS AND METHODS

Mutant Strains and Chromosomes

All the mutations used in this study are described in Flybase (Grumbling et al., 2006) and were obtained from the Bloomington *Drosophila* Stock Center, (Indiana University, Bloomington, Indiana, USA), with the exception of strain $PHSBJB_3$, hereafter referred to as *hs-mini-white*$^+$. The $PHSBJB_3$ strain, generously provided by W. Odenwald, is an X-chromosome insertion of the $PHSBJB_1$ transgene, which contains *pollux (plx)* cDNA in an antisense orientation to the CaSperR *mini-white* gene (Zhang & Odenwald, 1995). The CaSperR *mini-white* gene construct contains the *white* gene from which most of the large first intron has been removed, leaving an intact coding region coupled to the hsp-70 promotor and 300 and 630 bp of the 5′ and 3′ regulatory regions, respectively (Pirrotta, 1988). When possible, mutant strains were tested as single mutations, but in some cases, the mutant stocks contained other (known) mutations. The full genotypes of these strains are given in the relevant table legends. The strains tested for courtship were generally not backcrossed to homogenize the genetic background.

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Crosses

All crosses were performed at 22°C, unless otherwise stated. Culture medium was standard cornmeal/molasses medium supplemented with 0.04% tegosept as a mold inhibitor.

Sexual Behavior Test

Sexual behavior tests for male-male courtship were performed with young adult (5 + 2 days old) males. *O.R* wild-type and the *hs-mini-white*$^+$ males were used as negative and positive controls, respectively. After brief anesthetization by ether vapor, five groups were set up of either 50 males in glass 100-mm petri dishes or 25 males in 60-mm plastic petri dishes. In all cases, the five sexual behavior trials were performed on different days and, in some cases, by different observers to control for environmental or observer biases. Results were generally consistent between trials and observers. The Petri dish lids were secured with Parafilm (Fisher Scientific Canada, Ottawa, Ontario, Canada), and once the flies had recovered from anesthesia, the Petri plates were placed on a damp paper towel in a plastic container. The container was sealed and placed at 37°C for 60 minutes to induce the expression of genes under heat-shock control and to increase humidity. After the heat shock, the container with flies was placed at room temperature, 22° + 5° C, for 4 + 2 hours to allow recovery. The Petri plates were removed from the tray, and each group of flies was continuously visually monitored for courtship activity for 10 minutes. For consistency, all strains were heat shocked; in our hands, the frequency, but not target, of sexual behavior displays is increased by the high humidity resulting from the heat-shock procedure. The courtship index was calculated as the number of courtship chains, multiplied by the number of individuals in the chain excluding the leader (i.e., a chain of 4 individuals was counted as three courtship attempts), within the 10-minute period, averaged over five trials. A courtship chain was defined as any instance in which a fly pursued a potential suitor and displayed one of more of the following courtship behaviors: wing vibrated or held at 90 degrees to the body, licking of the genitalia of the preceding fly, and attempted or successful mounting, as described by Hall (1994). Because the chains were transitory, individuals might participate in several chains over the 10-minute observation period. After the sexual behavior tests, the flies were discarded. The frequencies of male-male courtship between each mutant and wild type were analyzed by using the Student's *t*-test. As the probability of a type-1 error (e.g., incorrectly detecting statistical significance) increases with the number of *t*-tests performed, the results were also analyzed with a one-factor analysis of variance (ANOVA), using the number of courtship attempts for each trial as the dependent variable and the mutant strain as the independent variable. Differences from the *O.R* control were tested by using the post-hoc Dunnett's *t*-test. The data presented in Tables 2, 3 and 5, and 6 were analyzed separately.

Sexual Preference Test

Three-day-old males, *O.R* wild-type, *hs-mini-white*$^{+}$, g^{1}, g^{2}, g^{50e}, or g^{53d}, were placed on ice to cool the flies and individual males rapidly transferred to separate wells of six-well Nunc microtiter plates (Fisher Scientific Canada, Ottawa, Ontario, Canada) kept on an ice pack. The *hs-mini-white*$^{+}$ males were heat shocked, as described above, and allowed to recover for 1 hour before testing. To each well, two 3-day-old virgin females and two 3-day-old virgin males were added, again following immobilization by exposure of approximately 1 hour to 4°C. The microtiter plate was removed from the ice pack and allowed to come to room temperature. As the plate warmed, the flies rapidly became active. After the onset of activity, the males were visually monitored for courtship attempts, as described above, for 10 minutes. The total number of courtship attempts and the sex of the fly courted were noted. A total of 10 trials were carried out for each strain.

Locomotor Ability Tests

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To test geotaxis, 100 young adult (5-±2-day old) male flies were isolated with light ether anesthesia and divided into groups of 10. Each group was placed into a clean, dry 60-cm-long glass tube with a 15-mm internal diameter, which had previously been calibrated into 5-cm segments. The ends of the tube were sealed with corks so that the remaining available length was 50 cm. The tubes were vertically mounted on ring stands and 1 hour was allowed for the flies to recover, acclimate, and freely disperse within the tube. After 1 hour had passed, the vertical distribution of the flies in the tube was noted for each 5-cm segment. The tube was then pounded vertically on a foam pad to knock all the flies to the bottom of the tube, and the time required for the fastest fly to reach the top of the tube was noted. This was repeated for a total of five trials for each of the 10 tubes. The time taken for the fastest fly to reach the top of the tube was averaged for each of the five repeats with the same set of flies. This value was then averaged across the 10 sets of flies. Horizontal locomotor ability was tested by placing 50 flies in a 60-mm plastic petri dish marked across the middle with a line. The number of times a fly crossed the center line within a 5-minute period was recorded. A minimum of five sets of 25 flies was tested for each strain. The results were analyzed by a one-way ANOVA, followed by a post-hoc Dunnett's *t*-test to assess the statistical significance of the results. For those trials in which the flies were heat shocked, heat shock was carried out in a humid chamber at 37°C for 1 hour, followed by a 3-hour recovery period.

Olfactory Learning Test

The olfactory learning test was based on the procedure reported by Tempel et al. (1983). Wild-type and experimental males and females, 5+2 days old, were separated and placed in dry containers without food for 17 hours. To train, groups of 10 surviving males or females were placed in the stem of a plastic Y-tube connector (Nalgene; Fisher Scientific Canada, Ottawa, Ontario, Canada) with a 1-cm internal diameter and arms 3.7 cm long and the end sealed with a wad of Kimwipe®, (Nalgene; Fisher Scientific Canada, Ottawa, Ontario, Canada) which occluded 1 cm of the arm. Immediately before the flies were introduced, 200 μL of an odorant, plus 10% sucrose, was added to a wad of Kimwipe in one arm of the Y-tube and 200 μL of the other odorant was added to the other arm. Both arms were then sealed with Parafilm. The odorants were 0.5% 3-octanol and 1% 4-methylcyclohexanol. In half the trials, the sucrose was added to the octanol and in the other half to the hexanol. The tubes with odorants and flies were stored upright in a fume hood for 24 hours. The following day, the flies were removed from the training tubes by cooling the Y-tubes on ice and shaking the flies out the stem opening. Flies were rapidly transferred to testing tubes: Y-tubes prepared as above but lacking sucrose, the stem sealed with a wad of Kimwipe, and the tube placed upright at room temperature. The proportion of flies choosing each odorant arm within 2 minutes was recorded. ''Learning'' was calculated as the proportion choosing the odorant previously associated with sucrose. A minimum of 10 trials was performed for each sex and genotype.

Construction of Ds-Red *and* EGFP-*Tagged* white *and* garnet *Vectors*

All molecular techniques were performed by using standard procedures (Sambrook et al., 1989). Enzymes and buffers were from MBI Fermentas (Fermentas International Inc, Burlington Ontario, Canada). The *DsRed*- and *EGFP*-tagged

white gene plasmids, p*DsRed1-N1-w*$^{+}$ and p*EGFP-N1-w*$^{+}$, were generated by isolating the *mini-white* gene as a 3396-bp *Hind*III-*Apa*I fragment from the 7776 bp pP(CaSper) vector (Genbank accession number, X81644). This fragment was then ligated into the pBluescript II KS$\pm$(pBs) vector (Stratagene; Agilent Technologies, La Jolla, Callifornia, USA) multiple cloning site between the *Hind*III and *Apa*I restriction sites. DNA sequencing, using the T7 and T3 primers, confirmed the presence and orientation of the *white* gene. To generate the pBs-*w*$^{+}$*-DsRed* and pBs-*w*$^{+}$*-EGFP* plasmids, the *DsRed1* and *EGFP* genes were amplified via polymerase chain reaction (PCR) from the p*DsRed1-N1* and p*EGFP-N1* vectors (Clontech; Mountain View California, USA), using primers 5′ccgcgggcccgggatccaccggtcgcc3′ and 5′ggcgggccctgatctagagtcgcggcc3′, which introduced an *Apa*I site at the 3′ end of the end of the gene. The 738-bp PCR product was isolated, digested with *Apa*I, and ligated into the *Apa*I site in the pBs–*w*$^{+}$ plasmid polylinker 3′ to the *white* gene. This resulted in the substitution of the last three amino acids of the White protein with either DsRed or EGFP proteins. The presence of in-frame *DsRed* was initially detected by restriction digestion with *Apa*I and confirmed by DNA sequencing. To generate the pRmHa-3-*white–DsRed* vector for S2 cell transformation, a 4150-bp *Eco*RI–*Kpn*I fragment from the pBs–*w*$^{+}$*–DsRed* plasmid construct was isolated and ligated into the pRmHa-3 (Bunch et al., 1988) multiple cloning site between the *Eco*RI and *Kpn*I restriction enzyme sites. To generate the *white*$^{+}$*-DsRed* (p*DsRed1-N1-w*$^{+}$) and *white*$^{+}$*-EGFP* (p*EGFP-N1-w*$^{+}$*)* vectors for COS-1 cell transformation, the 3396-bp *Hind*III-*Apa*I fragment from the 7776-bp pP(CaSper) vector (described above) was ligated directly into the p*DsRed1-N1* and p*EGFP-N1* (Clontech) vectors, substituting the last three amino acids of the White protein with either the DsRed or EGFP proteins. To construct the *garnet–DsRed* vector for COS-1 cell transformation, the p*EGFP-N1-g*^{8} plasmid was created by isolating the *garnet* cDNA as a 2265-bp *Eco*RI-*Sal*I fragment from the 6005-bp pUC19-*garnet* vector (Genbank accession number, U31351; Lloyd et al., 1999). This fragment was then ligated into the p*DsRed1-N1* vector (Clontech) multiple cloning site between the *Eco*RI and *Sal*I restriction sites.

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Intracellular Localization of White

Transformation of COS-1 Cells

Cos-1 African green monkey kidney cells were maintained following standard procedures in a water-jacketed incubator with 5% carbon dioxide in 25-mL vented culture flasks (Falcon (BD Falcon; Fisher Scientific Canada, Ottawa, Ontario, Canada) to allow gas exchange. Cells were raised on Dulbecco's modified Eagle's medium (DMEM; GIBCO; Invitrogen Corporation, Carlsbad, California, USA), supplemented with 10% fetal bovine serum (FBS; GIBCO) and a 2% penicillin/streptomycin antibiotic mixture (GIBCO). Cells were subcultured approximately every 4–5 days. For transfection, 700 ng of plasmid DNA was added to 4 μL of Superfect™ transfection reagent (Qiagen; Qiagen Canada, Mississauga, Ontario, Canada) and vigorously mixed before being added to a Cos-1 subculture in a sterile six-well culture plate (Falcon), with each well containing a sterile cover slip. Cells were then cultured for 48–72 hours before fixation, DAPI staining, and inspection, using a Zeiss LSM 410 confocal microscope. (Carl Zeiss Inc, Carl Zeiss Canada Ltd., Toronto, Canada)

Transformation of S2 Cells

Drosophila S2 (Schneider's 2) cells were maintained following the standard procedures (Cherbas & Cherbas, 2000) at 27C in 25-mL vented culture flasks (Falcon), and grown in Shield and Sang's (M3) medium (Sigma; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), supplemented with 10% FBS (GIBCO) and a 2% penicillin/streptomycin antibiotic mixture (GIBCO). S2 cells were subcultured approximately every 3 days, when the cells reached a 70% confluence. Transfection was carried out by vortexing 1 mL of M3 medium containing no sera or antibiotics, 150 ng of plasmid DNA, and Cellfectin™ transfection reagent (Invitrogen; Invitrogen Corporation, Carlsbad, California, USA) for 30 seconds in a 10-mL sterile tube (Falcon). After 30 minutes, concentrated S2 cells (harvested at 50–60% confluence) were added and the tube incubated horizontally at 27°C for 4 hours. After incubation, the cells were pelleted and gently resuspended in 3 mL of M3 containing 10% FBS and 2% penicillin/streptomycin and aliquoted into three wells of a sterile six-well culture plate (Falcon), with each well containing a sterile cover slip. The culture plate was then wrapped in Parafilm and placed at 27°C overnight. The following day, the plates were retrieved, and sterilized $CuSO_4$–$5H_20$ was added to each well to a final concentration of 0.5–0.7 mM. Plates were rewrapped in Parafilm and returned to the 27°C incubator for 72 hours before fixation, DAPI staining, and inspection, using a Zeiss LSM 410 confocal microscope. Fixation, staining with DAPI, and detection of GFP or DsRed fluorescence was done according to the standard procedures.

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RESULTS

The initial observation of energetic male-male courtship behavior in *hs-mini-white Drosophila* (Zhang & Odenwald, 1995) was surprising and provided the first suggestion that the *white* gene might play a role in the nervous system, in addition to its more obvious function in pigmentation. To further assess a neural role for the White protein, we examined the ability of *white*, *brown*, and *scarlet* males to learn using a simple T-tube olfactory learning paradigm. Flies were given the opportunity to associate sugar with the presence of an odorant and then subsequently tested for a preference for that odorant in the absence of food. Approximately 80% of wild-type (*O.R*) males learned to associate the odorant with the presence of food and to show a subsequent preference for that odorant. In contrast, *white*, *brown*, and *scarlet* mutant males showed a preference for the conditioned odorant that did not differ significantly from random choice (Table 1). Although these data do not exclude the possibility that these mutants are defective in their ability to detect either the odorant or sucrose, the results do suggest that *white*, *brown*, and *scarlet* males are defective in learning or memory, a prediction consistent with the reduced performance of *white* mutants in spatial learning (Diegelmann et al., 2006).

The deficit in learning behavior shown above was found in flies lacking expression of the *white* gene, whereas male-male courtship behavior is seen in flies that ectopically overexpress the *white* gene. To investigate the process whereby overexpressing the *white* gene triggers male-male courtship, we wished first to confirm if this switch in courtship behavior reflects perturbation of cells in which White is normally expressed, or if it is an indirect effect of general physiological disruption following the induction of *white* expression in all cells of the body.

First, we confirmed that the heat shock–induced overexpression of the *hs-mini-white* gene throughout the body induces vigorous male-male courtship. Male *hs-mini-white*$^+$ males heat shocked even once as adults show frequent bouts of male-male courtship occurred (Table 2, *hs-mini-white*$^+$, row 2). If the expression of the *hs-mini-white*$^+$ gene was induced throughout development by daily heat shocks, even higher levels of male-male courtship occured (Table 2, *hs-mini-white*$^+$, row 3). Flies in which the *hs-mini-white*$^+$ gene was induced only when adults had pale orange eyes (data not shown), presumably due to only low-level background transcription from the heat-shock promotor, whereas flies heat shocked throughout development had fully pigmented eyes (data not shown). In this latter group, sufficient White protein is transported to pigment granules to allow for normal pigmentation, so levels of White protein in other endosomal organelles may also be normal.

Individuals mutant for the *garnet (g)* gene also share this male-male courtship phenotype (Table 2; Lloyd et al., 2002). The *garnet* gene is a member of a group of unusual eye-color genes, called the granule group, that are involved in the transport of proteins to endosomes (Lloyd et al., 1998). Mutations in these genes decrease the level of both the ommochrome and pteridine pigments, which precludes a role in pigment biosynthesis, as the two pathways do not share enzymes. Further, many of these granule-group genes are also highly pleiotropic and essential, which eye pigmentation is not, and molecular analysis has shown that a number are involved in intracellular trafficking (Shestopal et al., 1997; Ooi et al., 1997; Simpson et al., 1997; Sevrioukov et al., 1999; Lloyd et al., 1999; Narayanan et al., 2000; Kretzschmar et al., 2000; Sriram et al., 2003; Falcon-Perez et al., 2007; Syrzycka et al., 2007). The pigmentation phenotype of the granule-group mutants resembles hypomorphic mutations of the *white* gene, indicating that the White protein is a likely cargo protein transported by the products of these genes (Lloyd et al., 2002). Given that the White protein should be mislocalized in these granule-group mutants, we tested the mutants for male-male courtship behavior. Table 2 shows that male mutants of many members of the granule group of genes did indeed show male-male courtship behavior. In addition to *garnet* (*g*), less vigorous male-male courtship behavior was shown by males mutant for two other of the four components of the AP-3 adaptor complex: *orange (or)* and *ruby (rb)*. The lower frequency of male-male courtship exhibited by these mutants was statistically significant, when compared with the *O.R* wild type by a *t*-test, although not if the significance level was reduced to correct for multiple comparisons, whereas *carmine (cm)* males did not show significant levels of male-male courtship. Additional alleles in a homogenized genetic background would have to be tested

Table 1. Olfactory in *white, brown* and *scarlet* mutant males

Strain	Learning[a] (%)
O.R	79 ± 2.5
w^{1118}	56 ± 4.4
bw^1	58 ± 3.1
st^1	51 ± 2.1

[a]Learning is defined as the percentage of flies choosing an odor previously associated with food given a choice of two odors, as described in Materials and Methods.

Table 2. Male-Male Courtship in Granule-Group Mutants

Strain[a]	Courtship index[b]
O.R	0.5 ± 0.2
hs-mini-white^{+} (*adult heat shock*)	94.0 ± 10.8*
hs-mini-white^{+} (*repeated heat shocks*)	234.5 ± 26.1*
g^{1}	6.9 ± 1.7
g^{2}	24.5 ± 4.1*
g^{2}(CS)[c]	30.8 ± 2.7*
g^{50e}	9.0 ± 3.5
g^{53d}	18.4 ± 3.8*
rb^{1}	3.0 ± 1.0
cm^{1}	0.6 ± 0.4
or^{1}	0.4 ± 0.3
or^{495}	6.0 ± 2.0
dor^{1}	0.4 ± 0.3
car^{1}	2.6 ± 0.4
p^{1}	1.6 ± 0.3
p^{p}	14.6 ± 9.9*
cd^{1}	1.4 ± 1.1
pd^{1}	0.8 ± 0.2
pn^{1}	5.2 ± 0.9
rs^{1}	2.2 ± 0.6
ca^{1}	3.2 ± 0.6
cin^{1}	1.6 ± 0.8
pr^{1}	0.8 ± 0.7
v^{1}	1.4 ± 0.4
cn^{1}	10.8 ± 2.7
cin^{1}	1.6 ± 0.8
mal^{1}	26 ± 8.1*
$Pu^{2}/+$	167.2 ± 13.8*
Pu^{r1}	28.0 ± 16.4*

[a]Individuals with known mutations in only the indicated gene were tested in all cases, except for or^{1}, ca^{1}, and Pu^{2}, which were tested as a^{1} px^{1} or^{1}, ca^{1} awd^{1}, and $wg^{Sp\text{-}1}$ Bl^{1} L^{rm} Bc^{1} Pu^{2}/CyO strains, respectively.

[b]Courtship index was calculated as explained in Materials and Methods, by using five groups of 50 males monitored for 10 minutes, plus or minus the standard error of the mean. *Indicates a significant difference from the wild-type (*O.R*) courtship index calculated by using an analysis of variance, followed by a post-hoc Dunnett's *t*-test, as described in Materials and Methods.

[c]Strain in a Canton-S genetic background.

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to determine if this result were due to the weakness of the cm^{1} allele or the accumulation of compensatory modifiers. While only deemed significant when assessed using generous statistical tests, these low levels of male-male courtship behaviors may still be biologically meaningful. In *garnet* mutants, the lowered fertility associated with male-male courtship leads to the accumulation of genetic modifiers reducing male-male courtship (Table 2 and data not shown). Comparison between the frequency of male-male courtship in males with a homogenized genetic background versus those without suggest that this may be true for other genes as well (Tables 2 and 3). Additionally, the number of courtship bouts correlates with allele strength for *garnet* (Lloyd et al., 2002; Table 2), *brown*, and *scarlet* (Table 3). The number of mutants tested precluded exhaustive testing of multiple alleles and homogenization of background for each, but as these factors both act to reduce the incidences of male-male courtship attempts, even low frequencies of these behaviors may be biologically meaningful.

In addition to components of the AP-3 complex, *pink (p)* mutant males also showed significant levels of male-male courtship. Males mutant for *carnation (car), prune (pn), rose (rs)*, and *claret (ca)* showed low-frequency incidents of male-male courtship, statistically significant when compared to the *O.R* wild type by a simple *t*-test but not if more conservative statistical tests were applied. The single, hypomorphic alleles of *deep orange (dor), cardinal (cd)*, *cinnamon (cin)*, and *purpleoid (pd)* did not exhibit male-male courtship; however, additional alleles would have to be tested to determine if this was an allele-specific effect. In addition to the granule-group genes, males mutant for the purine metabolism genes, *Punch* (*Pu*) and *maroon-like* (*mal*), showed significant levels of male-male courtship. Males mutant for the pigment biosynthesis genes, *cinnabar (cn)* and *vermillion (v)*, also showed low-frequency male-male courtship

Table 3. Male-Male Courtship in *brown*, *scarlet*, and *white* Mutants

Strain[a]	Courtship index[b]
O.R	0.5 ± 0.2
C.S	7.4 ± 2.1
hs-mini-white$^+$	74.8 ± 26*
bw^1	45.0 ± 18.1*
bw^1*(CS)*[c]	32.0 ± 6.6*
bw^8	80.0 ± 27.6*
bw^{45a}	16.0 ± 5.0
bw^{2b}	6.0 ± 1.2
bw^{2b}*(CS)*[c]	43.4 ± 6.1*
st^1	21.2 ± 7.5
st^1*(CS)*[c]	68.8 ± 11.2*
w^{1118}	0.6 ± 0.4
w^1	4.2 ± 1.8
w^1*(CS)*[c]	11.3 ± 4.6
w^a	2.6 ± 1.8
w^{Bwx}	5.0 ± 2.2
w^e	2.0 ± 1.0
w^{sat}	1.2 ± 0.6

[a]Individuals with known mutations in only the indicated gene were tested in all cases.

[b]Courtship index was calculated, as explained in Materials and Methods, by using five groups of 50 males monitored for 10 minutes, plus or minus the standard error of the mean. *Indicates a significant difference from the wild-type (*O.R*) courtship index calculated by using an analysis of variance, followed by a post-hoc Dunnett's *t*-test, as described in Materials and Methods.

[c]Strain in a Canton-S genetic background.

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attempts (Table 2), although, again, these values are statistically significant only if not corrected for multiple comparisons. The number of genes surveyed precluded testing alleles backcrossed into a uniform genetic background. However, a few key genes with a cantonized wild-type genetic background were tested and showed similar or greater levels of male-male courtship (Tables 2 and 3). Collectively, the male-male courtship seen in these pigmentation mutants implicates the White ABC transporter as a key player in courtship behavior and shows that, rather than its absence or ectopic expression, it is the mislocalization of the White protein in cells in which it is normally expressed that induces male-male courtship behavior.

To confirm that male-male courtship results from the relocation of White in the cell, rather than its absence, we examined hypomorphic *white* mutants for male-male courtship activity. The pigmentation phenotype of hypomorphic *white* mutants is indistinguishable from that of the granule group mutants and reflects the reduced amount of White protein available to be placed in pigment granules. Presumably, other endosomal derivatives, such as synaptic vesicles, also have reduced amounts of White protein. Neither *white* null (w^{1118} and w^1) nor hypomorphic alleles (w^a, w^{Bwx}, w^e, and w^{sat}) showed significant incidences of male-male courtship (Table 3). This shows that the absence of the White protein at its normal location does not of itself cause male-male courtship; rather, its mislocalization to other sites, probably the plasma membrane, is required instead.

The White protein is thought to complex with the Brown and Scarlet proteins and, possibly, others to form a transmembrane pump for small metabolites (Ewart & Howells, 1998). In the absence of these binding partners, the localization and function of the White protein should also be disrupted. We, therefore, examined courtship behavior in *brown* and *scarlet* mutants. Table 3 shows that *brown* and *scarlet* mutant males showed male-male courtship behavior similar to the *hs-mini-white*$^+$ males. The frequency of the observed courtship, again, depended on the allele and genetic background and tended to be lower than that seen in the *hs-mini-white*$^+$ males; however, in all cases, it differed significantly from wild-type male-male courtship levels. The lower frequency of male-male courtship bouts, particularly for the weaker alleles, might explain why this activity was not detected by Zhang and Odenwald (1995).

Zhang and Odenwald (1995) proposed that the male-male courtship observed in males overexpressing the *white* gene resulted from an altered serotonin metabolism that results when the mislocalized White transmembrane pump alters the amount of tryptophan, which is the precursor of serotonin. To investigate the relationship between the male-male courtship seen in *white* overexpressing males and serotonin metabolism, we first made double mutant strains, mutant for both *hs-mini-white*$^+$ and either *fruitless (fru)*, *eagle (eg)*, or *huckebein (hkb)*. The *fruitless* gene product directs innervation of the abdominal ganglia by serotonergic neurons (Lee & Hall, 2001), whereas *eg* and *hkb* specify the formation of serotonergic

Table 4. Male-Male Courtship in *hs-mini-white*$^{+}$ and *eagle (eg)*, *huckebein (hbn)*, and *fruitless (fru)* Single and Double Mutants

Strain	Courtship index[a]
O.R	0.5 ± 0.2
hs-mini-w^{+}; *H/TM3*	27.4 ± 8.9*
eg^{2}/*eg*^{2}	0.9 ± 0.4
hs-mini-w^{+}; *eg*^{2}/*eg*^{2}	45.4 ± 3.6*
hkb^{1}/*hkb*^{1}	6.2 ± 1.2*
hs-mini-w^{+}; *hkb*^{1}/*hkb*^{1}	21.4 ± 8.0*
fru^{3}/*fru*^{3}	8.0 ± 5.2*
hs-mini-w^{+}; *fru*^{3}/*fru*^{3}	48.6 ± 11.5*

[a]Courtship index was calculated, as explained in Materials and Methods, by using five groups of 50 males monitored for 10 minutes, plus or minus the standard error of the mean. *Indicates a significant difference from the wild-type (*O.R*) courtship index calculated by using the Student's *t*-test, as described in Materials and Methods.

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neurons (Dittrich et al., 1997). If the male-male courtship seen in *white* overexpressing males results from defects in serotonin metabolism in these serotonergic neurons, *eg, hkb*, and *fru* mutants should all show identical male-male courtship behaviors. Further, *eg, hkb*, and *fru* mutations would be predicted to be epistatic to the *hs-mini-white*$^{+}$ phenotype, because altered serotonin metabolism should be irrelevant if the serotonergic neurons do not form. Table 4 shows that *fru* and *hkb* males did indeed show male-male courtship behaviors. The frequency of male-male courtship activity was, however, much lower than that of *hs-mini-white*$^{+}$ males. Double mutants between these genes and *hs-mini-white*$^{+}$ showed courtship frequencies that were equivalent to the *hs-mini-white*$^{+}$ strain alone. Although interpretation of this result is complicated by the fact that the *eg, hkb*, and *fru* alleles used here are necessarily viable, weak alleles that compromise, but would not be expected to eliminate, the formation of serotonergic neurons, our findings do show that these mutations are not epistatic to *hs-mini-white*$^{+}$.

To clarify how overexpression of the *hs-mini-white* gene affects courtship behavior, if not exclusively through action in serotonergic neurons, a number of mutants listed in Flybase (Grumbling et al., 2006) as having altered monoamine neurotransmitter and neuromodulator metabolism were examined for male-male courtship behavior. The broad nature of this survey precluded testing more than one allele of each of these genes, so that low frequencies of male-male courtship in weak alleles might be missed. In addition, the genetic background was not controlled, so genetic modifiers, which reduce male-male courtship, could have obscured a male-male courtship phenotype. As a result, the absence of male-male courtship in these mutants is not definitive. Positive results, however, are meaningful. Table 5 shows that mutations *dunce (dnc), ether a go-go (eag), Frequenin 1 (Frq1), rutabaga (rut),* and *Tyramine receptor (TyrR)* all showed significant frequencies of male-male courtship activity. Interestingly, the frequency of male-male courtship in these mutants, while significantly higher than in the wild type, does not approach that seen in *hs-mini-white*$^{+}$ flies. This more moderate level of male-male courtship was repeatable over several trials and with different investigators.

The locomotor activity of a subset of these strains was investigated to determine if these flies also resembled *hs-mini-white*$^{+}$ flies in nonsexual behaviors. Table 6 shows that, once heat shocked, *hs-mini-white*$^{+}$ males climbed vertically and moved horizontally as well as, or better than, wild-type flies. Heat shock seems to be necessary to induce high levels of activity (Table 6, compare rows 2 and 3, *hs-mini-white*$^{+}$ before and after heat shock). Similarly, granule-group mutants and other eye-color mutants, all of which showed high frequencies of male-male courtship, also performed locomotor tasks as well as, or better than, wild-type flies (Table 6), despite the formation of courtship chains, which impeded efficient completion of the trial (data not shown). In contrast, the non-eye color mutants showing male-male courtship showed significantly less locomotor activity, whereas those that demonstrated a lack of male-male courtship had normal locomotor ability (Table 6). Although the lower frequencies of male-male courtship and reduced locomotor activity could simply result from the flies being unhealthy, the overall fecundity, longevity, and vigor of these strains seemed not to be diminished, compared with the strains that demonstrate no male-male courtship and normal locomotor activity. Thus, males mislocalizing the White protein showed wild-type or higher frequencies of locomotor activity. In contrast, males with altered monoamine metabolism that exhibited male-male courtship showed reduced frequencies of locomotor activity, whereas those that did not exhibit male-male courtship showed wild-type locomotor levels.

As well as having altered monoamine metabolism, the *dunce (dnc), ether a go-go (eag)*, and *rutabaga (rut)* genes are all involved in learning. Male *Drosophila* normally engage in male-male courtship but quickly learn to repress this behavior, a process impaired in *dnc*, *eag*, and *rut* mutants (Mehren et al., 2004). This suggests that

Table 5. Male-Male Courtship in Amine Neurotransmitter Mutants

Strain[a]	Courtship index[b]
O.R	0.7 ± 0.04
hs-mini-white^{+}	28 + 10*
CG8291^{KG07083}	3.1 ± 1.3
Dat^{lo}	0.2 ± 0.2
Ddc^{DE1}	0.7 ± 0.3
dnc^{1}	12.4 ± 3.2*
e^{1}	0.4 + 0.5
eag^{1}	12.3 ± 4.0*
Frq1^{1}	6.5 ± 1.1*
Hn^{r}	0.5 ± 0.5
Hn^{r3}	0.5 ± 0.7
iav^{1}	2.0 ± 1.2
norpA^{7}	3.4 ± 1.1
numb^{1}/+	0.7 ± 0.4
per^{JC43}	2.3 ± 1.0
ple^{4}/+	0.0 ± 0.0
qs^{2}	0.0 ± 0.0
rut^{1}	11.3 ± 2.6*
Sh^{5}	5.6 ± 2.5
stnA^{1}	5.6 ± 2.5
svr^{1}	1.2 ± 0.7
t^{1}	0.0 ± 0.0
TyrR^{neo30}/+	21.5 ± 4.0*
tyr1^{1}	0.9 ± 0.4

[a]Individuals with known mutations in only the indicated gene were tested when possible. The full genotypes for the other strains are: CG8291: *y*^{1} *w*^{67c23}*; P{y*$^{+mDint2}$ *w*$^{BR.E.BR}$ *=SUPor-P}CG8291*^{KG07083}, Dat: *bw*^{1} *Dat*^{lo}, Frq: *T(1;Y)V7, y*^{1} *w*^{1} *f*^{1} *Frq1*^{1}*: y*$^{+}$ *B*^{S}, Hn^{r}: *jv*^{1} *Hn*^{r} *h*^{1}, Hn^{r3}: *Hn*^{r3} *sr*^{1}, per: *T(1;4)JC43, l(1)3Ed*^{3} *per*^{JC43}, ple: *ple*^{4} *st*^{1} *e*^{1}*/TM3, Sb*^{1}, stnA: *stnA*^{1}*/Dp(1;Y)y*$^{+}$*mal*^{106}, TyrR: *mwh*^{1} *P{hsneo}TyrR*^{neo30} *P{hsneo}CG11367*^{neo30} *red*^{1} *e*^{1}*/TM3, ry*^{RK} *Sb*^{1} *Ser*^{1}*;* tyr1: *b*^{1} *tyr1*^{1}

[b]Courtship index was calculated, as explained in Materials and Methods, by using five groups of 25 males monitored for 10 minutes, plus or minus the standard error of the mean. *Indicates a significant difference from the wild-type (*O.R*) courtship index calculated by using an analysis of variance, followed by a post-hoc Dunnett's *t*-test, as described in Materials and Methods.

male-male courtship behavior could stem from a failure to suppress normal male-male courtship. In that case, one would expect that courtship would be directed toward both males and females, rather than being switched from obligate male-female courtship to obligate male-male courtship. Thus, we examined the courtship behavior of *hs-mini-white*$^{+}$ flies in more detail. Although Zhang and Odenwald (1995) reported that *hs-mini-white*$^{+}$ males show male-male courtship in the presence of females, many subsequent studies have studied courtship behaviors only in groups of isolated males. We find that when given a choice between male or female potential mates, male *hs-mini-white*$^{+}$ *Drosophila* chose female mates in 82% of trials ($n = 34$) versus 100% for the *O.R* wild type ($n = 44$). Likewise, *garnet* males showed frequent male-male courtship bouts (Table 2) but, if given a choice, greatly preferred female partners (80% for *g*^{1}, 87% for *g*^{2}, 88% for *g*^{53d}, and 94% for *g*^{50e}; $n = 15$–34). Sexual behavior was not seen to be altered in *hs-mini-white*$^{+}$ females, although in *g*^{2} and *g*^{53d} females, rare (12 and 3%, respectively) female-female approaches, with behaviors resembling male courtship, were seen. The time taken for *garnet* males to initiate courtship with wild-type (*O.R*) females did not generally differ from wild-type males, although the time between the initiation of courtship and copulation with either or both wild-type or *garnet* females was significantly longer for *g*^{1}, *g*^{2}, and *g*^{50e} males (data not shown).

Finally, as a first step in correlating the behavior of flies with altered White protein levels and intracellular locations, we determined the intracellular location of the wild-type White protein in cultured *Drosophila* and mammalian cells. To do this, we generated DsRed- and EGFP-tagged versions of the White protein. The generation of these fusion proteins results in the substitution of the last three C-terminus amino acids of the White protein, to which no function has been ascribed (Ewart et al., 1994). When expressed in *Drosophila* S2 embryonic cells, *garnet-EGFP* and *white-DsRed* fluorescence was seen in a virtually identical cytoplasmic perinuclear distribution (Figure 1A). When expressed in the larger mammalian Cos-1 cells, the fluorescence of the tagged White protein was clearly punctate and perinuclear consistent with an endosomal location (Figure 1B). This distribution largely overlapped that

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Table 6. Locomotor Activity

Strain	Courtship index[a]	Geotaxis	Horizontal locomotion
O.R	–	30.1 ± 1.3	140 ± 22.8
hs-mini-white$^+$ (*not heat shocked*)	–	20.7 ± 1.3	101.4 ± 12.6
hs-mini-white (*heat shocked*)	+	27.0 ± 2.2	229.6 ± 11.5
g^1	–	30.0 ± 2.0	ND
g^2	+	31.2 ± 4.3	ND
g^{50e}	–	33.1 ± 1.3	ND
g^{53d}	+	34.7 ± 4.7	ND
or^{495}	–	ND	195.0 ± 38.1
bw^1	+	26.3 ± 0.5	296.2 ± 74.4*
st^1	+	43.2 ± 4.7*	ND
w^{1118}	–	30.5 ± 1.0	ND
cn^1	–	33.4 ± 1.7	ND
pr^1	–	28.3 ± 1.2	ND
v^1	–	25.9 ± 0.4*	ND
cin^1	–	ND	94.0 ± 36.0
mal^1	+	ND	319.2 ± 28.9*
Pu^2	+	ND	424.6 ± 27.9*
CG8291	–	ND	155.3 ± 33.5
Ddc^1	–	ND	116.7 ± 3.0
eg^1	–	ND	136.5 ± 9.6
hkb^1	+	ND	45.8 ± 17*
fru^1	+	ND	47.5 ± 34.6*
Frq^1	+	ND	45.4 ± 7.0*
Hn^r	–	ND	222.2 ± 16.8
$numb^1$	–	ND	109.3 ± 5.0
$TyrR^1$	+	ND	68 ± 13.5*

*Indicates a significant difference from the wild-type (*O.R*) values calculated by using an analysis of variance, followed by a post-hoc Dunnett's *t*-test, as described in Materials and Methods.

ND = not done.

[a]"+" indicates male-male courtship activity significantly higher than the *O.R* wild-type;

"-" indicates courtship activity not significantly different from wild-type.

of a DsRed-tagged Garnet protein cotransfected into the Cos-1 cells; approximately 50% of the Garnet-containing bodies also contain White protein and no White protein was seen in bodies lacking Garnet (Figure 1B). As the *garnet* gene encodes the delta subunit of the AP-3 adaptor complex that shuttles cargo from the trans-Golgi network to endosomes (Simpson et al., 1997; Ooi et al., 1997; Lloyd et al., 1999), this suggests that the Garnet transports White to a subset of endosomes. Further, the fact that the Garnet and White proteins appeared to colocalize to endosome-like structures in mammalian Cos-1 cells shows a remarkable conservation of protein structure and function in the AP-3 intracellular sorting mechanism. The transformed Cos-1 cells possess a *Drosophila garnet*/delta AP-3 subunit, but in order for the *Drosophila* subunit to function, it must form a working complex with the other three AP-3 subunits, which in this case, are mammalian. The overlap between the Garnet and White fluorochromes implies the *Drosophila* DsRed-tagged Garnet protein is recognized by the other members of the mammalian AP-3 complex, and that this hybrid AP-3 complex is able to function correctly and recognize the tagged *Drosophila* White protein.

In summary, we have shown that flies mutant for *white*, as well as *brown* and *scarlet*, show decreased ability to learn. Male-male courtship results from the mislocalization of the White protein, rather than its absence, and mislocalization of the White protein in only the cells in which it is normally expressed is sufficient to produce high frequencies of male-male courtship. Male-male courtship is also seen in a subset of mutants in genes involved in learning. These flies show a lower frequency of male-male courtship and locomotor activity than males in which the White protein is mislocalized. Finally, when expressed in cultured cells, the White protein is found in the endosomal compartment, which would be consistent with White being targeted to sites where it might exert an effect on the pumping of neurotransmitters.

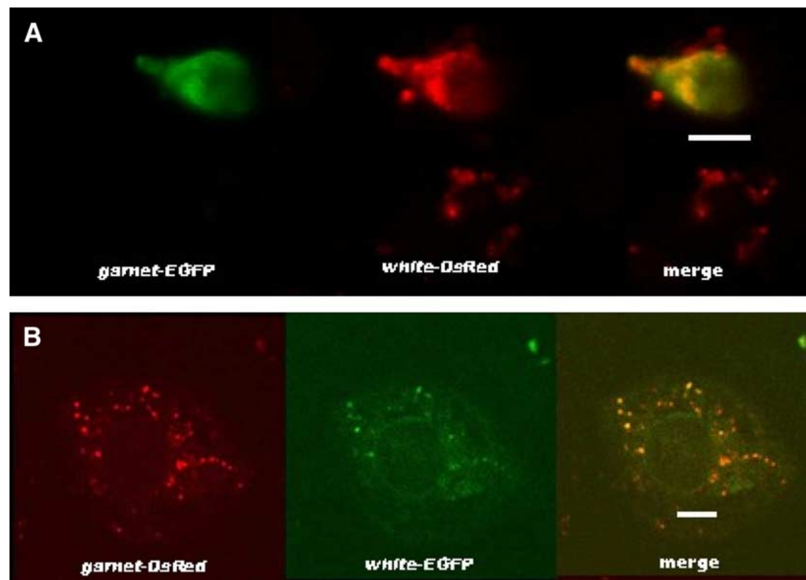

Figure 1. Intracellular location of the White protein. A. Co-transfected Garnet-EGFP (left-most panel) and White-DsRed (middle panel) are found in a cytoplasmic perinuclear distribution in Drosophila S2 cells. Scale bar is 10μm. B. Co-transfected Garnet-DsRed (left-most panel) and White-EGFP (middle panel) co-localize to a punctate perinuclear pattern consistent with endosomes in COS cells. Scale bar is 10μm.

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DISCUSSION

The *white* gene has been the focus of hundreds of studies for nearly a century. Yet, only in 1995 was a role in the nervous system, with resulting effects on courtship behavior, intimated. Although we find that *white*, as well as *brown* and *scarlet* mutants, appear to have a deficit in olfactory learning, null and hypomorphic *white* mutations do not induce any obvious change in male or female sexual behavior. To explain at the cellular level how overexpression of the *white* eye-color gene in *Drosophila* induces male-male courtship, we examined the behavior of four classes of mutants: those mutant for the *white* gene, those mutant for genes encoding the White binding partners, those mutant for genes responsible for correct intracellular localization of White, and those mutant for genes with roles in learning and monoamine neurotransmitter metabolism. We also examined the intracellular location of the *white*-encoded ABC-type transmembrane transporter.

The Location of White

When the male-male courtship behavior of the *hs-mini-white*$^+$ gene was first noted, it was proposed to result from heat shock–induced ectopic expression of the *white* gene in cells in which it was not normally expressed (Zhang and Odenwald, 1995). Thus, while interesting, the male-male courtship behavior did not of itself require *white* to have a neurological role normally. Indeed, male courtship has been shown to depend not only on neural identity and activity, but also on proteins secreted into the hemolymph (Lazareva et al., 2007). However, the *white* gene is transcribed in the heads of flies lacking eyes and ocelli (Campbell & Nash, 2001), so it seems likely that *white* is transcribed in neural tissue. We have shown previously (Lloyd et al., 2002) that male-male courtship is seen in *garnet* mutants in which the intracellular localization of White is disrupted only in the cells normally producing White. The male-male courtship of *garnet* mutants is strictly dependent on the presence of the White protein; *garnet* and *white* double mutants show no male-male courtship (Lloyd et al., 2002). Here, we extend these results to show that male-male courtship is seen in many ''granule-group'' mutants, mutants that have defects in the intracellular trafficking of various proteins, including White. The male-male courtship, sexual preference, and unimpaired general locomotion seen in these mutants mimic the behaviors seen in *hs-white*$^+$ males. The seemingly identical male-male courtship behaviors in males overexpressing and mislocalizing the White protein shows that male-male courtship can result from the relocation of the White transmembrane protein within the cells normally producing it, rather than exclusively being an indirect effect of metabolic perturbation of other cells in the body. This evidence, along with our finding of learning defects in *white*, *brown*, and *scarlet* mutants, the reduced performance of *white* mutants in spatial learning reported by Diegelmann et al. (2006), and finding that *white* and *brown*

mutants are resistant to the effects of a volatile general anesthetic (Campbell & Nash, 2001), together strongly support the assertion that *white* normally acts in the nervous system.

We find that when a tagged version of the White protein is expressed in cultured cells, it appears to be localized to endosomes. A primarily endosomal location of the native White protein is supported by genetic analysis (Lloyd et al., 2002) and consistent with immunocytochemical evidence localizing White to endosomal derivatives in pigment cells and similar vesicular structures in Malpighian tubules (Mackenzie et al., 2000; Evans et al., 2008). Further, *white* mutants display distinctive, abnormal, large pigment granules reminiscent of the giant lysosomes found in Chediak-Higashi Syndrome individuals (Nolte, 1961; Shoup, 1966; Stark & Sapp, 1988), and at least one of the human homologs of *white*, *ABCG1*/*ABC8*, is also found in a punctate perinuclear distribution consistent with an vesicular location (Lorkowski et al., 2001), as is another human homolog, *ABCC4* (Jedlitschky et al., 2004). Interestingly, the labeling pattern we see for White is very similar to that shown in cultured cells for one of the splice variants of the *Drosophila* vesicular monoamine transporter gene (VMATA); the second splice variant, VMATB, by contrast, localizes to the plasma membrane (Greer et al., 2005). VMATA is qualified to act as a vesicular transporter for 5-HT *in vivo*, as well as two other amines, dopamine and octopamine (Greer et al., 2005), and the similarity in its pattern of expression to that for White suggests that the latter may also be associated with synaptic vesicles or with other endosomal compartments. An endosomal location for the White protein could be expected to alter the morphology, number, or neurotransmitter loading of synaptic vesicles and would thus simplify models proposed to explain the effect of *white* on behavior. But, this role would need to be demonstrated. Moreover, immunocytochemistry of the native White protein, using an antibody against part of the extracellular loop, between putative transmembrane helices 5 and 6 (Mackenzie et al., 2000), fails to label the synaptic boutons of the neuromuscular junction (J.A. Borycz, unpublished). Thus, if White is indeed a synaptic vesicle protein, it is not an abundant one, a finding consistent with its low level of transcription (Campbell & Nash, 2001). Previous biochemical analysis of the uptake of small metabolites in *white* mutants led to the suggestion that White protein functions in the plasma membrane (Sullivan et al., 1974; Sullivan & Sullivan 1975; Sullivan et al., 1979; Sullivan et al., 1980); however, transport of metabolites across an epithelium can be mediated, indirectly, by intracellular transporters (Mackenzie et al., 2000; Evans et al., 2008). A plasma-membrane location, however, is compatible with the location of other members of the ABC family (Castanys-Muñoz et al., 2007) and the immunoexpression of native White protein in the glial cells that surround terminals of photoreceptors (Borycz et al., 2008), whereas the terminals themselves, which use a fourth biogenic amine, histamine (Hardie, 1987; Sarthy, 1991), fail to express either isoform of VMAT (Borcyz & Meinertzhagen, unpublished). These conflicting localization data could be partly reconciled if the *white* gene product is only transiently associated with the plasma membrane, as is the case for the yeast ABC transporter, STE6 (Krsmanovic et al., 2005) Or if White's cellular location differs for different cells or different neurons.

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The Behavioral Outcomes of Altered white *Expression*

While the absence or reduction of White protein does have behavioral consequences, it is only its relocation that induces male-male courtship. The different behavioral responses to the absence versus mislocalization of the White protein could arise from multiple causes. There are many transmembrane pumps capable of transporting tryptophan (Sullivan et al., 1980; Greer et al., 2005) and VMAT has been identified as the major vesicular monoamine transporter (Greer et al., 2005). If neurotransmitter loading relies on a number of proteins, the absence of White from the synaptic vesicle may have much less effect than its inappropriate relocation to the plasma membrane. Another alternative is that different behavioral effects of the inappropriate placement of White stem from the availability of binding partners in each compartment, since the specificity of the transporter is conferred not by White, but by its binding partner (Ewart & Howells, 1998; Schmitz et al., 2001). Insofar as the White protein can combine with a number of partners to transport a variety of substrates, a change in the intracellular localization of White could perturb the distribution of a broad spectrum of neurotransmitters and other small metabolites. Indeed, Hoyer at al. (2008) show that *white*-null males are defective in octopamine-associated aggressive behavior. A final possibility is that the male-male courtship behavior is a direct consequence of reduced learning.

Courtship behavior in *Drosophila* is complex and largely invariant, but it can be modified by learned behaviors (Mehren et al., 2004). Normally, courtship between mature males is suppressed by a combination of rejection from other males, antiaphrodisiac pheromones, and learning to refrain from male-male courtship after courting immature males, a process termed experience-dependent courtship modulation or courtship conditioning (Spieth, 1974; Scott & Richmond, 1988; Gailey et al., 1982; Hirsch & Tompkins, 1994; Neckameyer, 1998). This male-male courtship may function to transmit the nuances of the courtship song to the young males, but it is without apparent benefit to the older male

(McRobert & Tompkins, 1988). As a result, wild-type males rapidly learn to repress this behavior, and the obligate male-female courtship seen in wild-type flies is, in part, a learned behavior.

Our finding, consistent with that of Nilsson et al. (2000), that flies in which White is mislocalized do not switch their sexual preference, but simply reduce the specificity of targeting their courtship attempts, suggests a learning deficit. Further, *vermillion*, *cinnabar*, and *raised* mutants show both male-male courtship (Table 2, McRobert et al., 2003) and a decreased ability to learn (Savvateeva et al., 2000; McRobert et al., 2003), although their neurological functions remain undefined. The neurological functions of the proteins encoded by *Frequinin 1, Tyramine receptor*, *rutabaga*, *ether a go-go*, and *dunce*, which we find also show male-male courtship, are well defined. *Frequinin 1* encodes a synaptic calcium-binding protein essential for signaling and neuromodulation (Cremona & De Camilli, 2001). Unfortunately, effects on learning or memory have not been reported. *Tyramine receptor* encodes a receptor that signals in response to octopamine and tyramine and, possibly, other amines (Brody & Cravchik, 2000). Octopamine has a role in learning and memory, as well as aggression (Dudai et al., 1987; Schwaerzel et al., 2003; Hoyer at al., 2008). *rutabaga (rut)*, *ether a go-go* (*eag*), and *dunce* (*dnc*) encode a protein with adenylate cyclase activity, a plasma-membrane voltage-gated potassium channel and 3′5′-cyclic nucleotide phosphodiesterase, respectively (Mehren et al., 2004; Grumbling et al., 2006). All of these mutants have defects in learning and/or memory (Mehren et al., 2004), and *rutabaga* and *dunce* have been shown to have defects in specific aspects of learned courtship behavior (Mehren et al., 2004).

Thus, the findings that the loss of White protein causes a decreased ability to learn, and that courtship alterations are found in other mutants with decreased capacity for learning, strengthens the link between learning and courtship. This link could be either indirect or direct. Both learning and courtship behavior may be independently influenced by a neurotransmitter, levels of which are influenced by the White ABC transporter, or the male-male courtship seen in flies mislocalizing the White transporter is a consequence of failure to remember previous nonproductive courtship interactions with other males. We find that when the White protein is overproduced from a heat-shock promoter throughout development, wild-type amounts of eye pigment are deposited. This suggests that normal amounts of the White transporter are available in endosomal and related organelles, presumably including synaptic vesicles, so that learning should be normal. Yet, the frequency of male-male courtship in these flies is higher than in those in which the *white* gene is only transiently overexpressed (Table 2). This suggests that male-male courtship may not be a direct consequence of impaired learning but, instead, both depend on the proper levels of neurotransmitters and neuromodulators. As such, the identification of numerous additional genes that induce male-male courtship behavior suggests that indiscriminate sexual behavior may be the default state, and further analysis of these genes may help clarify the link between neural function and courtship behavior. An additional implication of these results is that effects due to the *white*-null background of many transgene strains, with the *mini-white* gene of the transformation vector providing only partial rescue, or strains marked with visible eye color markers, such as *brown*, *scarlet*, or members of the granule group, should be monitored in behavioral assays.

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The Role of the White Transporter in Serotonergic Synaptic Action

The evidence for a behavioral role for the White protein is clear, and from its biochemical function as a transporter and its intracellular localization to endosomes, a role for White in moderating the loading of synaptic vesicles with neurotransmitter seems plausible. However, the biochemical details of White's action are not yet clear. Numerous studies have linked serotonin to sexual behavior. Feeding male rats or cats *p*-chlorophenylalanine, an inhibitor of serotonin biosynthesis, and feeding rabbits a diet lacking tryptophan, the amino-acid precursor of serotonin, both induce male homosexual mounting behavior (Tagliamonte et al., 1969; Ferguson et al., 1970; Fratta et al., 1977; Gessa & Tagliamonte, 1974) superficially similar to the male-male courtship observed here in *Drosophila*. In *Drosophila*, serotonin has also been implicated in sexual behavior. Mosaic flies, in which some cells in the brain have been converted from male to female, either by chromosome loss (Hall, 1979) or by selective expression of sex-determination genes (Ferveur et al., 1995), display male-male courtship. Among the key regions of the brain identified in this way was the mushroom body, a region of the brain associated with courtship behavior and containing serotonergic neurons (Hall, 1979; Ferveur et al., 1995). Genetic evidence suggests that the White-Scarlet heterodimer pumps tryptophan or tryptophan-derived 3-hydroxykynurenine (Ewart & Howells, 1998), and in serotonergic neurons, tryptophan is converted into serotonin. This evidence led Zhang and Odenwald (1995) to postulate that overexpression of the *white* gene alters tryptophan availability to serotonergic neurons But despite some similarities between the neurobiology of male sexual behavior in mammals and *Drosophila,* the involvement of serotonin in male-male courtship is still ambiguous.

The courtship behavior seen in some of the mutants tested in this work does not necessarily support a role for serotonin as the key neurotransmitter involved in male-male courtship in *Drosophila*. The Scarlet protein is believed to

dimerize with White to form a tryptophan transporter (Mackenzie et al., 2000). Thus, while a dearth of White-Scarlet dimer could lead to synaptic vesicles deficient in tryptophan and serotonin, male-male courtship occurs only in the absence of Scarlet but not White. This discrepancy could be due to altered localization of White in the absence of its binding partner. Superficially, the male-male courtship, albeit weak, seen in *cinnabar* and *vermillion* males, is also consistent with the involvement of serotonin in male-male courtship; both mutants interrupt the tryptophan pathway. Scenarios linking the male-male courtship behavior seen in *brown* and *Punch* mutants to a diminution of serotonin levels become more contrived, however. *Punch* encodes GTP cyclohydrolase 1 (Grumbling et al., 2006), and the Brown-White heterodimer is believed to transport GTP (Ewart & Howells, 1998). GTP is not a precursor of serotonin, although it is a precursor of tetrahydrobiopterin, a cofactor required for the synthesis of the serotonin as well as dopamine and nitric oxide (Kwon et al., 1989; Kumer & Vrana, 1996; Campbell & Nash, 2001). If, however, mutations in the *brown* gene were to cause male-male courtship behavior by decreasing the intravesiclar concentrations of a cofactor for serotonin biosynthesis, serotonin biosynthesis would have to occur in the synaptic vesicle and not in the cytoplasm, as currently thought (Joh, 1997). Other explanations, which could rationalize how a decrease in the Brown and Scarlet proteins could lower intravesicular serotonin levels, include the possibility that dimerization of Brown and White is necessary for the correct sorting of the White protein or that a decrease in transcription of the *brown* and *scarlet* genes induce transcriptional upregulation of the *white* gene, causing mislocalization of the excess White protein. Although the *brown*, *scarlet*, and *white* genes appear to be concordantly regulated (Rabinow et al., 1991), neither the amount nor the location of the White protein has been reported in *brown* and *scarlet* mutants. Finally, although only one allele was tested here, significant levels of male-male courtship were not observed in *Ddc* mutant males that have greatly decreased amounts of serotonin (Livingstone & Tempel, 1983).

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Conclusions on the role of serotonin in mutants previously found to show male-male courtship are also conflicting. In *Drosophila*, only a limited number of mutations have previously been shown to cause a switch from male-female to male-male courtship behavior. In addition to the male-female mosaics mentioned above (Hall, 1979; Ferveur et al., 1995), *doublesex* (*dsx*) mutants also show male-male courtship (Villella & Hall, 1996). As *doublesex* is involved in somatic sex determination, this effect is probably also mediated by alterations of sexually dimorphic neurons. Similarly, the *fruitless* gene is involved in somatic sex determination, and mutants exhibit distinctive and, for some alleles, exclusive male-male courtship. These mutants show reduced serotonin levels in a subset of abdominal serotonergic neurons; however, the serotonergic neurons of the mushroom body do not express a male-specific form of the Fruitless protein (Lee & Hall, 2001). Further, Kitamoto (2002) was able to induce male-male courtship behavior by selective disruption of cholinergic peripheral neurons but not neurons in the mushroom body. The *dissatisfaction*, *prospero*, *quick-to-court*, and *raised* mutants also show male-male courtship (Finley et al., 1997; Balakireva et al., 1998; Grosjean et al., 2001; Gaines et al., 2000; McRobert et al., 2003); however, the role of these genes, if any, in serotonin metabolism has yet to be elucidated. The *dissatisfaction*, *prospero*, and *quick-to-court* genes are all expressed in neurons (Finley et al., 1997; Balakireva et al., 1998; Gaines et al., 2000), and *prospero* and *dissatisfaction* appear to encode transcription factor or related proteins (Finley et al., 1998; Demidenko et al., 2001), but it is not known if these gene products are involved in the specification of serotonergic neuron fate. Thus, the relationship between the White transporter protein, serotonin metabolism, and male-male courtship behavior remains suggestive but unclear.

To attempt to clarify the relationship between serotonergic neurons and male-male courtship behavior resulting from White mislocalization, we made double mutants with the *hs-mini-white*$^+$ and three mutations involved in the formation of serotonergic neurons, *huckebein, eagle*, and *fruitless*. Our finding that males mutant for *huckebein* or *fruitless* show male-male courtship superficially supports a role for serotonin in male-male courtship in *Drosophila*. However, the male-male courtship behavior shown by the *huckebein* and *fruitless* mutant flies can be distinguished from that shown by *white* mislocalization mutants in both the lower frequency of male-male courtship and the reduced locomotor activity. Further, double mutants combining mutant alleles of *fruitless*, *eagle* and *huckebein*, and the *hs-mini-white*$^+$ gene show that the effects of *fruitless*, *eagle*, and *huckebein* are not epistatic, as might be expected if the White transporter protein acted primarily in serotonergic neurons. Although the fact that the alleles used are viable hypomorphs, and so may have only a modest impact on serotonergic neurons, is an important caveat, if mislocalization of the White transporter acts on serotonin metabolism, its effects should be nullified or greatly reduced in mutants with fewer serotonergic neurons. Our data did not show this. However, these results differ from those reported by Nilsson et al. (2000), however, who found that double mutants between a *mini-white*$^+$ transgene and the *fru*^{sat} allele showed reduced male-female and male-male courtship characteristic of the *fru*^{sat} allele alone. Given that the *fruitless* gene is complex with many sex-specific alternately spliced products (Lee & Hall, 2001), it is possible that this discrepancy reflects the *fru* allele tested. The *fru*^{sat} allele used by Nilsson et al. (2000) and *fru*^{3} allele used in this work are produced by transposon inserts into different portions of the gene, which are included in different sets of mRNAs (Lee & Hall, 2001). Thus, our data, and conflicting data in the literature, on the role of serotonin in *Drosophila* courtship behavior, suggest that it is possible that male-male

courtship may result from alterations in more than one neuronal pathway or by altering more than one type of neurotransmitter. Because the White protein appears to be a fairly nonspecific transporter, it may affect the transport of different neurotransmitters. Direct examination of the contents of monoamine neurotransmitters in *white* mutant brains is the subject of a parallel study that has revealed a reduced amine content in the heads of *white* mutants, as well as a redistribution of the amines in head homogenates, from being concentrated in a synaptosome-rich fraction of the wild type to a supernatant fraction in the mutant (Borycz et al., 2008). These results are also consistent with the abolition of male aggression, an octopamine-associated behavior, recently reported in *white*-null mutants (Hoyer et al., 2008).

Human Homologs

There are at least eight human homologs of the *Drosophila white* gene (Schmitz et al., 2001). Polymorphisms in one of these, ABCG1, have been associated with mood and panic disorders in males (Nakamura et al., 1999). Although a direct demonstration of altered *white* mRNA or protein levels has not been reported, the association of the human homolog of *white* with panic disorders is reminiscent of the anesthetic resistance reported by Campbell and Nash (2001) and the modest hyperactivity found in the locomotor tests here. Further investigation into the role of *white/ABCG1* in humans might also reveal a subtle neural function. More recent work has implicated *ABCG1* in other conditions, such as cholesterol and phospholipid metabolism problems and as a contributor to the etiology of Tangier disease (Schmitz et al., 2001). Thus, while the differences between human and *Drosophila* brains and behaviors are incontestable, the *white* gene, the oldest and most prosaic mutation in *Drosophila*, is highly conserved in both structure and function, and as a metabolite transporter with wide specificity, may have multiple biological roles, including a hitherto largely unacknowledged neural role in *Drosophila*.

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