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BREAKTHROUGHS AND VIEWS

Genes for Sexual Behavior

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The mating behavior of *Drosophila melanogaster* is a stereotyped sequence of fixed action patterns, composed of orientation, tapping, singing, licking, attempted copulation and copulation. Mutations that block a unique aspect of mating behavior were isolated and analyzed at the cellular and molecular levels. The wild-type counterparts of the mutated genes were shown to rescue the phenotypes by their ubiquitous or targeted expression in some of the mutants. This strategy of artificial control of fly behavior opens up an avenue for studies to identify the neural center for individual behavioral actions.

The relative influence of genetic and environmental factors on the development of behavioral and neuronal sex differences has long been a controversial issue. Recent revolutions in reproductive technology and genetic engineering have made the gene knockout technique a conventional approach for exploring the genetic basis of behavior in mice (1). In humans, the establishment of many DNA polymorphic markers has greatly improved the reliability of linkage studies, leading to isolation of some genes linked to heritable disorders with behavioral abnormalities (2). Success in identifying the genes responsible for impaired behavior in mice and humans has shed light on the potential of behavioral genetics.

However, identification of the genes does not necessarily mean that the functions of the genes or the gene products are resolved at organism, cellular and molecular levels. For example, we still do not know exactly what the biochemical roles of presenilin (3), huntingtin (2), or α-synuclein (4) are, which have been determined in human genetics to cause Alzheimer, Huntington, or Parkinson diseases when mutated, respectively.

Another aspect of behavioral genetics utilizes model organisms such as *C. elegans, Drosophila melanogaster* and zebra fish, with which extensive analysis of functions of individual genes is possible by combining the molecular, biochemical and genetic approaches. These model organisms are amenable to forward genetic strategy, which starts with isolation of mutants with specific defects in selected behavioral elements. This allows us not only to identify novel genes for behavior but to also precisely define their functions in the organism. Behavioral genetics in such model organisms complements complex behavior studies in mammals.

For thorough understanding of the genetic basis of sexual behavior, we have taken advantages of forward genetics using *Drosophila*. In this article, we review the current situation in the study of sexual behavior mutants of *Drosophila* with special reference to the molecular biology of the responsible genes.

DROSOPHILA SEXUAL BEHAVIOR IS A STEREOTYPED SEQUENCE OF FIXED ACTION PATTERNS

The mating behavior of *Drosophila melanogaster* males is composed of several discrete steps, i.e., orientation toward a female, tracking the female, tapping the female abdomen with the forelegs, unilateral wing vibration to generate courtship songs, licking of the female genitalia, gripping the female wings to attempt mounting, mounting followed by copulation, and disengagement (5, 6). These elementary acts take place in the sequence described here, although tapping and licking may be skipped in a few exceptional cases.

The females may accept or reject courting males depending on their physiological status or "mood" (5). Female sexual receptivity develops in first two days after

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eclosion (7). When courted by a male, sexually immature females display rejection behavior, such as decamping, fending, kicking and flicking, whereas recently fertilized females refuse copulation typically by extruding their ovipositor (8). In response to the male's attempt, receptive females raise their wings allowing the male to grip them and open the vaginal plate for copulation (5, 6).

Mutations affecting specific aspects of the mating behavior have been isolated by us and others (9, 10). In the following we survey phenotypic, genetic and molecular characteristics of these mutants.

MUTATIONS THAT IMPAIR INITIATION OF MATING BEHAVIOR

The most extreme example of this class of mutations is *satori*, which was isolated in our screen. The *satori* males do not exhibit courtship behavior toward female flies (11, 12). Genetic analysis of *satori* revealed that it is a mutation in the *fruitless* (*fru*) locus (thus referred to as fru^{sat} hereafter), which is represented by multiple alleles and a range of abnormalities in male courtship behavior (5). Interestingly, males of all *fru* alleles other than fru^{sat} court females to varying degrees, although most of them do not copulate. A unique exception is the males of fru^{2}, some of which copulate with females producing offspring. An anomaly common to all *fru* alleles is enhanced male-to-male courtship. Even the fru^{sat} males engage in homosexual courtship although they do not display sexual reactions toward females.

Besides the change in male sexual orientation, the *fru* mutations cause malformation of a male-specific muscle, the Muscle of Lawrence (MOL), in the male adult abdomen (13). The failure in copulation by the males of some *fru* alleles cannot be ascribed to the MOL defect because MOL is dispensable for any aspect of mating behavior in *melanogaster* males (14). Severity of the phenotype varies depending on the allele, from the complete absence in fru^{sat} to a moderate reduction in size of the MOL in fru^{1} (11, 13). The MOL formation is under the control of the sex determination cascade as is the case of other sexually dimorphic characters (15). In *transformer* (*tra*) mutant females, the MOL develops, indicating that the MOL formation is repressed by the female-determining factor Tra. Unlike other sexual characters, however, mutations in the Tra-target *doublesex* (*dsx*) gene have no effect on MOL development: the *dsx* males possess normal-looking MOLs (15). Taken together, *fru* appears to be a novel element of the sex determination cascade contributing to a previously unknown branch downstream of *tra* but not of *dsx* (12, 16).

Sex-mosaic experiments have demonstrated that the MOL is formed when the motorneurons innervating it are male, regardless of the sex of the MOL (17). Thus the site of *fru* action may be in the neurons rather than in the muscle. These considerations lead to the hypothesis that *fru* is a gene that determines the sex of a class of neurons. This hypothesis would also explain homosexual or bisexual courtship in male flies possessing the mutated *fru* gene, as a consequence of inadequate sex determination of certain central neurons that are involved in sexual orientation (18).

Molecular cloning of the *fru* gene provided evidence supporting this hypothesis (11, 19). The *fru* gene produces several transcripts including sex-specific forms. In the 5′ non-coding region of a female-specific transcript we identified a triplicate 13 nucleotide repeat very similar to the Tra-binding consensus sequence previously found in the *dsx* primary transcript. The existence of a Tra-binding site was a prerequisite for *fru* to be a candidate for a Tra target. The primary structure of the Fru protein deduced from the cDNA sequence suggests that it is a transcription factor with an N-terminal BTB domain and two C-terminal Zn-finger motifs. This implies that Fru occupies a position in the sex determination cascade similar to Dsx, which has been known to directly regulate the "realizer" genes, for male or female development, as a transcription factor.

Expression of the sex-specific mRNA is confined to the nervous system, including the antennal lobe, of which sexual transformation from male to female by targeted expression of tra^{+} has been shown to induce bisexual courtship in male flies (20). These results are consistent with the hypothesis that Fru is a transcription factor in a novel branch of the sex-determination cascade downstream of Tra.

The other mutation that alters male sexual orientation is *dissatisfaction* (*dsf*): The *dsf* males court vigorously other males as well as females (21). These males rarely copulate with females since they are unable to bend their abdomen sufficiently as a consequence of poor extension of motor nerve endings on the ventral longitudinal muscles of the A5 segment (21). Similarly abnormal motor terminals develop on the A5 muscles in *dsf* females when they are masculinized by the tra^{-} mutation. This fact indicates that *dsf* is placed downstream of *tra* in the sex-determination cascade. Since other muscles including MOLs are innervated normally in *dsf* male flies, *dsf* is not regulated by *fru*. *dsf* is not under the *dsx* control because motor inervation of the A5 muscle was normal in *dsf* females that had been masculinized by the dsx^{D} mutation, which constitutively produces the male-type Dsx protein regardless of chromosomal sex. These observations are in accord with the hypothesis that the *dsf* gene constitutes the third branch of the sex-determination cascade downstream of Tra (21).

There are several mutant males of which rarely court females (or males). These include *he's not interested* (*hni*), *tapered* (*ta*), *pale* (10, 22, 23), cuckold (cuc; 51), and *courtless* (S. Orgad and D. Segal, personal communication). However, courtship activity in males is not completely blocked in any of these mutants. In the case

of *ta*, for instance, males do not court for about a week after eclosion, exhibiting a low level of courtship thereafter without copulation (22).

Two of these genes have been characterized at the molecular level. The *courtless* gene encodes a ubiquitin-conjugating enzyme (S. Orgad and D. Segal, personal communication). The *pale* gene encodes tyrosine hydroxylase that catalyses the synthesis of dopamine, an important monoamine neurotransmitter (24). It is interesting to investigate whether transcription of these genes is regulated in a sexually dimorphic manner by the sex determination genes, such as *fru* and *dsf*.

There are mutations that reduce "motivation" for mating in virgin female flies. The *dsf* mutation affects not only male courtship but also sexual receptivity in females (21). The *dsf* females resist the males' attempts to copulate in a way similar to that seen in tra^{-} females that were incompletely rescued by artificial expression of tra^{+} as driven by the *hsp70* promoter. The *dsf* females suffer from a second deficit in the peripheral nervous system: the motor innervation of the uterine muscle is diminished significantly, making the mutant females unable to oviposit. Although this second phenotype is evidently independent from the receptivity phenotype, it leads to the inference that the reduced willingness to copulate in *dsf* females originates from aberrant synapse formation in the CNS circuit controlling the female mating behavior.

We isolated two mutants, *spinster* (*spin*) and *chaste* (*cht*), for enhanced mate refusal in virgin females (7, 10). The *spin* females avoid courting males typically by decamping, fending, flicking, kicking and curling without any sign of extrusion. Thus their rejection behavior resembles that of immature virgin females rather than that of fertilized females (7, 8). The *spin* females remain rejective of courting males throughout their life, suggesting that sexual receptivity is never turned on in the *spin* females (7). The *spin* gene product is a novel protein with multiple putative transmembrane domains expressed in some glial cells and a few neurons in the central nervous system (Y. Nakano, unpublished data). An experiment with a temperature-sensitive *spin* allele demonstrated its requirement in the pupal stage, suggesting the possible role for $spin^{+}$ in development of the adult nervous system. Genetic epistasis between *dsf* and *spin* need to be analyzed because of the marked phenotypic similarity between the two mutants.

MUTANTS WITH ABERRANT COURTSHIP SONGS

The most overt courtship retual in the male is generation of courtship songs. Mutations known to completely block male singing are the fru^{3} and fru^{4} alleles of *fru* (25). Although males with other *fru* alleles sing at least toward males, fru^{3} and fru^{4} males do not regardless of the sex of the presented target. Thus some neurons that are subjected to *fru*-dependent sex-determination are involved in courtship song generation. Indeed, *in situ* hybridization analysis using *fru*-specific probes revealed *fru*-expressing neurons in the thoracic ganglion in which the courtship songs are produced (19). It should be noted, however, that not all the neurons contributing to the song circuit are *fru*-dependent, because the sine song, a component of the courtship songs, is eliminated by mutations in the *dsx* gene (26), a key player in a *fru*-independent branch of the sex-determination cascade.

Several mutations are known to alter the pattern of singing by males. Unlike the wild-type songs in which the pulse song is composed of an array of single-peaked pulses, pulses in the songs produced by the mutants *cacophony* (*cac*), *dissonance* (*diss*), and *croaker* (*cro*) are often multi-peaked (27-30). These mutants exhibit a reduced level of mating success, which may be a consequence of longer delays in initiating courtship by males. The song defects contribute only partially to the low mating success. In *cro*, motor nerve conduction, chemical transmission at the glutamatergic neuromuscular junction, and generation of Ca-dependent action potentials in the skeletal muscle were all normal when examined electrophysiologically with the aid of microelectrodes. These results imply that the *cro* mutant is not associated with generalized neural disfunctioning. Rather, it appears to have a localized effect, presumably in the CNS. In contrast, *cac* and *diss* mutants suffer from visual defects that are detectable during behavioral assays as well as on electroretinogram (ERG) recordings. The fact that both motor (reflected in the song) and sensory (vision) functions are affected in these two mutants suggest that *cac* and *diss* wild-type products play roles in many neurons.

Genetic and molecular analysis revealed that *cac* is a mutation in the gene encoding the α 1-subunit of a voltage-dependent Ca channel (31, 32). *diss* was determined to be a mutation in the *no-on-transient A* (*nonA*) locus, which produces a protein with two RNA-recognition motifs, RRM1 and RRM2 (33, 34). Each RNA-recognition motif is composed of two conserved features called RNP1 and RNP2. Adjacent to the C-terminal side of RRM1 there is a charged domain named DBHS (*Drosophila* Behavior/Human Splicing).

Both the vision defect and courtship song abnormality in the *diss* mutants are rescued by ubiquitous expression of the wild-type NonA protein from a transgene ($nonA^{+}$) driven by the *hsp70* promoter (35). Interestingly, heat-shock induction of $nonA^{+}$ prior to adult eclosion can rescue the *diss* mutant phenotypes just as induction after eclosion does (35). Such apparent lack of time specificity in the rescuing effect could be due to perdurance of the NonA protein expressed during development. Alternatively, the NonA protein possibly acts as a switch for a molecular cascade for the induction of behavior which remains active once triggered during development or adulthood. The *diss*

mutation (nonA^{diss}) has a point mutation in the DBHS domain (R to C at amino acid 548, R548C). Expression of a mutant *nonA* transgene with a conservative replacement at a.a. 548 (R to K) in the absence of the intrinsic *nonA*$^{+}$ gene alleviated the courtship song phenotype, although it did not improve the visual defect (33). Substitutions of single residues in the RRM1 or RRM2 domain demonstrated that both RRM1 and RRM2 are necessary for normal visual physiology whereas RRM2 is dispensable for singing behavior (34). Thus the domains in the NonA protein required for generation of normal courtship song is distinct from, although overlaps with, those for the visual function.

The *nonA* gene is expressed ubiquitously in neuronal nuclei throughout the nervous system, but causes specific song abnormalities when mutated. This specificity would reflect a unique function of the NonA protein in the neurons of the song circuit where its target RNA may be different from those in other neurons involved in other functions, such as vision. There is no evidence that expression of the nonA gene controlled by the sex-determination cascade, although we cannot exclude the possibility that NonA physically interacts with Sx1, Tra, or Tra-2 which is also an RNA-binding protein functioning as a splicing regulator. In fact, a mammalian splice factor Psf is a close relative of NonA with extensive homology throughout the protein. HeLa-cell-derived p54^{nrb}, a non-POU-containing octamer-binding protein (NonO), and *Drosophila* NAhomo are highly homologous to NonA and Psf, defining a NonA family RNA-binding proteins (33, 34).

MUTATIONS CAUSING COPULATION DEFECTS

Males of some mutants are unable to copulate despite courting females vigorously. *celibate* (*cel*) (9) and *platonic* (*plt*) (10) represent this class of mutations. Another class of mutations has problems maintaining or terminating copulation. The duration of copulation is within a range of 10-20 min in wild-type flies. Longer or shorter copulatory events are rarely seen. When the males with the mutations *coitus interuptus* (*coi*) (9), *fickle* (*fic*) (10), or *okina* (*ok*) (10) are involved, copulation tends to terminate prematurely. For example, half of the copulatory events observed with *fic* males had durations indistinguishable from those of wild-type flies, but the rest had extremely variable durations ranging from approx. 1 min to 50 min, although the longer-than-normal copulatory events are rare. In about 8% of pairs mated with *fic* males, copulation was repeated twice or more within 1 hr-observation period. This repetitive copulation may be a consequence of premature uncoupling, because even wild-type flies copulate again soon after separation if the preceding copulation has been interrupted artificially about 5 min after the start of copulation (36). In wild-type flies, re-copulation with females is prevented by a male accessory gland product, the sex-peptide, which is transferred to the female during copulation and then acts on the nervous system to shut down sexual receptivity in the female (37-39). The transmission of the sex peptide from male to female commences after several minutes delay after the copulation starts. It is possible that the premature termination of copulation interfered with the transfer of the sex peptide from male to female, thereby inducing the repetitive copulation in *fic*.

The last class of mutations to be discussed is those which interfere with disengagement of copulating pairs. There have been two mutations reported, *stuck* (*sk*) (9) and *lingerer* (*lig*) (10), of this class. In both mutations, the male and female pairs engaged in mating behavior which appears normal, before and during copulation. At the end of copulation, the males cannot easily withdraw their genitalia. The male often dismounts the female to which it is joined by genital connection (Table 1). Although the most males with the *sk* and *lig* mutations finally succeed in withdrawing their genitalia, a few males die without genital uncoupling. Hall et al. (9) considered that this failure of uncoupling in *sk* mutants is of neural origins. First, no overt morphological defects are found in the genitalia and its associates of the *lig* mutant. Furthermore, the male genital clasper appears to hold the female genitalia too strongly in the *sk* mutant, because application of strong shaking to copulating pairs is much less effective in dissociating the male from the female in *sk* than in wild-type flies. This fact implies that tonic neuronal control of male genital muscles is altered by the *sk* mutation.

Similarly the *lig* mutation most likely affects neuronal control of the male genitalia. The *lig* males in copulation are less reactive to kicking by the partner female, an action which usually urges male's dismounting (H. Kuniyoshi, unpublished data). Thus, these mutants are considered to be useful tools for probing the neural machinery for sensorimotor coordination in copulatory behavior. Molecular cloning of the *lig* gene is currently underway in our laboratory.

CONCLUDING REMARKS

Molecular cloning of the genes responsible for aberrant mating behavior in mutants led to the discovery of novel proteins required for the development and functioning of the postulated "neural center" for the respective behavioral elements. There are two immediate issues to be addressed in future experiments that represent the central questions in "molecular ethology" (the term introduced by C. Kyriacou (45)): What is the neural network that constitutes the neural center for each behavioral action?; How do individual proteins determine the structure and properties of the neural center? The first question deals with the anatomy of the neural center or the cellular correlations with behavior. The second question is concerned with the signaling

TABLE 1

A List of Genes for Sexual Behavior

Phenotype	Mutant	Gene product	Reference
Altered Sexual Orientation	*fruitless (fru)*	BTB-ZnFinger	11,19
	dissatisfaction (dsf)	?	21
Reduced Courtship	*he's not interested (hni)*	?	referred in 10
	cuckold (cuc)	?	51
	courtless (col)	ubiquitin-conjugating enzyme	S. Orgad and D. Segal, personal communication
	pale	tyrosine hydroxilase	24
	tapered (ta)	?	23
Reduced Female Receptivity	*spinster (spin)*	transmembrane protein	Y. Nakano, unpublished data
	dissatisfaction (dsf)	?	21
	chaste (cht)	?	10
Aberrent Courtship Song	*fruitless (fru)*	BTB-ZnFinger	11,19
	double sex (dsx)	transcription factor with DM domain	26
	cacophony (cac)	voltage-dependent Ca channel alpha 1 subunit	31,32
	dissonance (diss)	(=nonA)RNA binding motif (RRM1, RRM2), DBHS	33-35
	croaker	?	30
Copulation Defect	*celibate (cel)*	?	9
	platonic (plt)	?	10
	coitus interuptus (coi)	?	9
	fickle (fic)	cytoplasmic tyrosine kinase	10
	okina (ok)	?	10
	stuck (sk)	?	9
	lingerer (lig)	?	10

mechanism underlying the formation or functioning of the neural center, i.e. the molecular basis for behavior.

Identification of the proteins encoded by the mutated genes provides clues for studies to address both these questions. The anatomical localization of the neural center for a particular behavioral action can be inferred from localized protein distribution in the nervous system. If the developmental stage when the protein is required for expression of normal behavior is precisely defined, the candidate brain "focus" for the behavior can be further narrowed. This is accomplished by investigating whether the behavioral defects can be rescued by ubiquitous expression of the cloned wild-type genes in the mutants at a given developmental stage, with the aid of a conditional promoter such as *hsp70*.

Definite anatomical determination of the neural center for a behavioral act could be performed using cell-by-cell expression of wild-type cDNA in the corresponding mutant brain to determine the neurons that rescue the behavioral phenotype of the mutants when they are driven to express the cDNA. Cell-by-cell expression of a given cDNA is now possible, by means of a targeted expression system utilizing yeast GAL4 and UAS sequences (46, 47). With this strategy, Ferveur et al. (20) succeeded in specifying the glomeruli in the antennal lobe which are critically involved in the determination of sexual orientation in male flies. Thus, a combination of expression analysis, temporally controlled cDNA expression for rescue and cell-specific cDNA expression for rescue will allow us to determine the neuronal networks controlling different aspects of mating behavior.

The second question relates to the biochemical mechanism whereby the protein concerned instructs the neuron expressing it to acquire the properties required in the neural center for the given behavior. The first step toward this analysis would be to determine the biochemical activity of the relevant protein in the cell. Since the majority of the proteins identified as those that induce aberrant mating behavior when mutated are novel, it is necessary to search for partner proteins with known functions that interact directly with these, to infer their biological activities. One approach for this search uses yeast two hybrid screens, or purification of the binding proteins by affinity columns (48).

An alternative approach to screen for interacting molecules is based on modifier genetics (48); by isolating second site mutations that strengthen (enhancers) or weaken (suppressors) the original mutant phenotypes, the genes encoding upstream, downstream or partner elements for the respective proteins can be identified. Such modifier screens have been the conventional approach for elucidating the molecular cascades of developmental genes in *Drosophila* (49, 50). We believe that modifier genetics is similarly useful for the study of the molecular signaling that controls behavior in *Drosophila*. The first attempt to isolate modifiers of mating behavior mutants has begun in our laboratory.

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