Associative learning and memory in Drosophila: beyond olfactory conditioning

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Abstract

The associative learning abilities of the fruit fly, Drosophila melanogaster, have been demonstrated in both classical and operant conditioning paradigms. Efforts to identify the neural pathways and cellular mechanisms of learning have focused largely on olfactory classical conditioning. Results derived from various genetic and molecular manipulations provide considerable evidence that this form of associative learning depends critically on neural activity and cAMP signaling in brain neuropil structures called mushroom bodies. Three other behavioral learning paradigms in Drosophila serve as the main subject of this review. These are (1) visual and motor learning of flies tethered in a flight simulator, (2) a form of spatial learning that is independent of visual and olfactory cues, and (3) experience-dependent changes in male courtship behavior. The present evidence suggests that at least some of these modes of learning are independent of mushroom bodies. Applying targeted genetic manipulations to these behavioral paradigms should allow for a more comprehensive understanding of neural mechanisms responsible for diverse forms of associative learning and memory.

Keywords: Classical conditioning; Mushroom body; Optomotor learning; Spatial learning; Courtship

1. Introduction

The fruit fly, Drosophila melanogaster, can modify multiple aspects of its behavior as a result of experience. This has been convincingly demonstrated in the contexts of at least a dozen different associative and non-associative learning paradigms (Davis, 1996; Wolff et al., 1998). In non-associative learning, an animal learns about the properties of a stimulus presented alone, and either decreases (habituation) or increases (sensitization) its responses to that stimulus after repeated exposure (Rescorla, 1988). Classical and operant conditioning are two categories of associative learning that are distinguished by what an animal learns. In classical conditioning an animal learns that one stimulus predicts the occurrence of another. For example, learning to associate an odor (a conditioned stimulus) with electric shock (an unconditioned stimulus) is a well-known form of classical conditioning in Drosophila (Tully and Quinn, 1985). In operant conditioning, an animal learns a relationship between its own action and a reinforcing stimulus, so that it changes the frequency of that action to control the reinforcer. Flies can learn to modify their motor behaviors to avoid aversive heat in two different operant conditioning paradigms (Wolff and Heisenberg, 1991; Wustmann et al., 1996). Both classical and operant learning contribute to a flight simulator training paradigm, when flies are conditioned to turn away
from inherently neutral visual stimuli paired with aversive reinforcement (Brembs and Hessenberg, 2000). Male flies can even learn to modify the intensity of their courtship behavior as a result of experience courting inappropriate targets, through a classical associative learning mechanism (Tompkins et al., 1983). The relative simplicity of these learning paradigms in comparison to both implicit and explicit forms of memory in mammals, and the availability of unique genetic tools in Drosophila, make it an especially attractive system for dissecting neural mechanisms of learning and memory. In fact, progress in identifying neural substrates of learning in Drosophila has been truly impressive only for the olfactory classical conditioning paradigm, in which flies learn to avoid odors that are paired with electric shocks. As a result of 20 years of intensive investigations involving diverse experimental approaches and levels of analysis in several different labs, a detailed model has emerged of the essential neural substrates and processes underlying this form of olfactory classical conditioning (Davis, 1996; Dubnau and Tully, 2001; Roman and Davis, 2001; Waddell and Quinn, 2001; Dubnau et al., 2003). We will outline its major features here in order to provide a frame of reference for a more detailed discussion of other forms of associative learning in Drosophila, which comprise the major focus of this review. Our goal is to summarize and interpret the evidence regarding forms of associative learning that are currently strong candidates for further genetic and neurobiological investigations. Specifically, we will consider (1) visual and motor learning of flies tethered in a flight simulator, (2) a simple form of spatial learning, and (3) experience-dependent aspects of male courtship behavior. While the current state of evidence regarding their underlying neural mechanisms is relatively limited, behavioral studies have established these as reliable learning paradigms, making them prime targets for a new wave of serious investigations of the neural substrates of learning and memory.

2. Olfactory conditioning

Groups of flies are trained in the olfactory conditioning paradigm by exposing them alternately to two volatile odors (the conditioned stimuli, CS) and pairing one of these (the CS+) with a series of electric shocks (the unconditioned stimuli, US). Memory is assessed later in a T-maze, where most wild-type flies will avoid the shock-paired odor for several hours after a single training session (Tully and Quinn, 1985; Tully, 1987). Multiple training sessions produce longer lasting effects, for example, a form of long-term memory that persists up to 7 days and depends on protein synthesis (Tully et al., 1994). Behavioral, genetic, and pharmacological manipulations can be used to distinguish short-term (minutes), middle-term (hours), and long-term (days) phases of memory, as well as an anesthesia-resistant form of memory that persists for many hours after flies have been cold-shocked (Tully et al., 1994; Greenspan, 1995; Dubnau et al., 2003).

So what neural mechanisms mediate olfactory associative learning and memory? Insects detect olfactory stimuli through olfactory receptor neurons in the antennae and maxillary palps that project to glomeruli in the antennal lobes (Stocker, 1994; Hildebrand and Shepherd, 1997; Jeffries et al., 2002). Specific odorants induce patterns of oscillating activity in ensembles of neurons that project from the antennal lobes to the dorsal and lateral protocerebrum (MacLeod and Laurent, 1996; Stopfer et al., 1997). The dorsal projections synapse on the dendrites of the Kenyon cells in the calyces of the paired mushroom bodies (MBs). The MBs are distinctive central brain neuropil structures defined by the dendritic and axonal projections of the Kenyon cells, whose approximately 2500 cell bodies are compactly clustered in the dorsal posterior cortex of each brain hemisphere (Davis, 1993; Strausfeld et al., 1995). Their dendrites arborize in the calyces, neuropil structures just ventral to the cell bodies, where they are contacted by synaptic terminals of projection neurons carrying chemosensory information from the antennal lobes (Jeffries et al., 2002). Their axons project anteriorly and ventrally via fiber tracts called peduncles, and branch into distinctive medial and dorsal lobes (Ito et al., 1997; Crittenden et al., 1998; Lee et al., 1999). The neural targets of the MB lobes project to “higher” centers in the medial and lateral protocerebrum (Ito et al., 1998). As the lateral protocerebrum also receives direct input from antennal lobe projection neurons (Stocker et al., 1990), the MBs provide an indirect pathway for processing chemosensory inputs that converges with this direct pathway in the lateral protocerebrum (Heimbeck et al., 2001).
While the direct projections from the antennal lobe to the lateral protocerebrum are essential for detecting volatile odorants (Heimbeck et al., 2001), compelling evidence indicates that the MBs are the critical sites for the formation and expression of associative olfactory memories (Dubnau and Tully, 2001; Roman and Davis, 2001; Waddell and Quinn, 2001). MBs can be ablated pharmacologically (by feeding larvae hydroxyurea) or severely disrupted genetically (e.g. mutations like mushroom body miniature), and olfactory conditioning is completely eliminated by such disruptions (Heisenberg et al., 1985; deBelle and Heisenberg, 1994). MB neurons express most of the genes that are known to be critical for olfactory learning and memory (Davis, 1993; Dubnau and Tully, 2001; Waddell and Quinn, 2001). Many of these encode enzymes that function in cAMP-protein kinase A signaling, including dunces (cAMP phosphodiesterase), rutabaga (type I 
Ca
++-calmodulin-dependent adenyl cyclase), and the genes encoding both regulatory and catalytic subunits of protein kinase A (Davis, 1996; Roman and Davis, 2001). Genetic manipulations specifically targeted to MB neurons with the GAL4–UAS system (Brand and Perrimon, 1993) have been used to demonstrate that cAMP signaling in these neurons is both necessary and sufficient for normal olfactory learning. Necessity was demonstrated by MB-targeted expression of a mutant constitutively active Gα, which eliminated learning without impairing the basic sensory and motor functions required for this behavior (Connolly et al., 1996). Genetic evidence for sufficiency of a molecular pathway in a specific structure can be obtained by attempting to rescue the behavioral defects of a mutant with spatially or temporally restricted expression of the wild-type gene. This strategy was used to demonstrate that wild-type rutabaga (rut+) expression in a subset of MB neurons was sufficient to rescue the olfactory learning defect of rut mutants (Zars et al., 2000a).

Olfactory learning also depends on activity in a pair of large modulatory neurons, the dorsal paired medial (DPM) cells, that densely innervate the lobes of the MBs and express amnesiac, another gene that is required for olfactory memory (Waddell et al., 2000). These DPM cells may influence the output from the synaptic terminals of the MB neurons (Waddell and Quinn, 2001; Dubnau et al., 2003). Such output is necessary for expression of olfactory memory, but not for learning or storage (Dubnau et al., 2001; Mcguire et al., 2001). This conclusion is based on results obtained with another powerful neurogenetic tool, shibirets (shi+), a temperature-sensitive mutation that allows for temporal control of neurotransmitter release (Kitamoto, 2001). When the GAL4–UAS system was used to express shi+ in MB neurons, results indicated that the output from synaptic terminals of MB neurons is required for normal performance during testing, but not during training or in the interval between training and testing (Dubnau et al., 2001; Mcguire et al., 2001).

The evidence is consistent with a model that attributes behavioral significance to specific neural structures. In particular, there exists substantial support for the hypothesis that the essential molecular changes that mediate olfactory associative learning occur within MB neurons (Roman and Davis, 2001; Waddell and Quinn, 2001; Dubnau et al., 2003). When flies are trained by pairing an odor with electric shock, inputs from the antennal lobes (encoding odors) and the DPM neurons (possibly activated by shock) converge on MB neurons and trigger molecular events involving cAMP and PKA signaling which produce changes in the activity or excitability of these cells. Subsequently, when flies are exposed to the shock-paired odor, the responses of the MB neurons are modified, as are the outputs of their synaptic terminals in the lobes and the responses of their neural targets. Many of these targets project to the lateral protocerebrum, where they converge on direct targets of antennal lobe projection neurons, and there may participate in expressing a fly’s memory of olfactory conditioning. The relevant evidence has been summarized and integrated in several recent reviews (Dubnau and Tully, 2001; Roman and Davis, 2001; Waddell and Quinn, 2001).

3. Visual learning in the flight simulator

Visual learning in Drosophila has been most successfully studied with an experimental set-up known as a “flight simulator” in which a single fly is suspended in midair by a wire tether glued to its dorsal thorax (Heisenberg and Wolf, 1988; Wolf and Heisenberg, 1991). As the fly attempts to turn right or...
left in flight, it applies torque on the tether; this “yaw torque” is measured using a torque meter (Heisenberg and Wolf, 1988). It provides a feedback signal to the flight simulator, which functions to rotate the surrounding visual environment in the opposite direction of the torque the fly applies, so that the tethered fly detects rotations of its visual field that simulate appropriate movements. The cylinder surrounding the tethered fly usually contains four “T” shapes, two upright and two inverted, toward which the fly can orient itself with the application of appropriate yaw torque (Wolf and Heisenberg, 1991). Visual learning at the flight simulator occurs when turns towards upright T-shapes, for example, are negatively reinforced with heat, thereby conditioning flies to escape heat by turning towards the inverted T-shapes (Wolf and Heisenberg, 1991). Memory is measured as a significant tendency to turn away from the heat-reinforced visual pattern after the reinforcement has been discontinued (Wolf and Heisenberg, 1991; Brembs and Heisenberg, 2000). Performance hits an asymptotic level after four 2-min training sessions (Brembs and Heisenberg, 2000).

Like olfactory learning, retention of visual conditioning in the flight simulator appears to improve with repeated training sessions and to be consolidated in different memory stages. Xia et al. (1997) tested a combination of spaced and massed training sequences to identify an optimal protocol for recall longevity. Memory from their new procedure lasted up to 48 h following training. Exposing flies to cold shock within 20 min of training disrupted memory, but later cold treatments did not. Additionally, flies fed cyclohexamide to inhibit protein synthesis were not able to retain memory longer than 3 h following training. These results indicate that visual learning in the flight simulator can induce at least three stages of memory, which are analogous to those reported for olfactory conditioning (Tully et al., 1994): (1) anesthesia-sensitive short-term memory lasting up to 20 min, (2) anesthesia-resistant memory from 20 min to 3 h after training, and (3) protein synthesis-dependent memory, developing after 3 h (Xia et al., 1997).

3.1. Learning in the flight simulator is a combination of operant and classical conditioning

Because visual learning in the flight simulator involves using particular motor behaviors to control the heat reinforcement, it might be primarily an operant conditioning paradigm. On the other hand, it is possible that visual cues in the flight simulator might function as conditioned stimuli in a classical conditioning mode, such that a fly learns to associate T’s in a particular orientation with the aversive unconditioned stimulus. The relative contributions of classical and operant conditioning to this learning paradigm have been assessed in a series of experiments, with the results indicating that both modes of associative learning contribute to a fly’s learned preference to orient towards a particular visual cue after heat reinforcement (Brembs and Heisenberg, 1991; Brembs and Heisenberg, 2000).

Three related issues have been addressed in these studies: (a) whether flies can be operantly conditioned to turn right or left in the absence of visual stimuli, (b) whether they can be classically conditioned to associate visual cues with heat when tethered to a torque meter, and (c) the extent to which classical and operant mechanisms contribute to learning and memory in the standard flight simulator training paradigm. Purely operant (motor) learning was demonstrated with flies in a uniformly illuminated flight simulator without any visual cues; they quickly learned how to avoid the heat when either “right-hand” or “left-hand” turns were negatively reinforced, and continued to avoid turns in that direction after reinforcement (Wolf and Heisenberg, 1991; Wolf et al., 1998).

To assess purely classical conditioning, flies were tethered to a torque meter that did not provide feedback to control the surrounding environment. They were trained by a predetermined schedule of visual cues and heat reinforcement, independently of their motor behaviors. Then, to test memory after training, flies were allowed to express their learned preference for a particular visual stimulus by connecting the feedback from the torque meter to control the rotation of the environment. When training consisted of a straightforward 3-s alternation between upright and inverted Ts, one of which was paired with heat, flies learned to avoid the visual cue that was paired with heat (Wolf et al., 1998). The memory scores obtained through this protocol were equivalent to those obtained after the standard visual conditioning protocol in the flight simulator (when the position of visual cues is controlled by the fly’s motor behavior during training). Thus, flies tethered to a torque meter can learn to associate visual patterns (the CS) with
heat (the US) using a purely classical conditioning paradigm.

To what extent, then, does classical associative learning about a predictive relationship between visual cues and heat contribute to memory in the standard training protocol for visual learning in the flight simulator? This question was initially addressed using a variation on the basic paradigm that eliminated any potential operant contributions to learning, but was otherwise identical to the basic conditioning paradigm (Wolf and Heisenberg, 1991). Flies tethered to a torque meter that did not control the surrounding environment were simply exposed to the same sequence of visual cues and heat reinforcements that had been recorded in a previous experiment with a fly in the normal flight simulator training mode (Wolf and Heisenberg, 1991; Brembs and Heisenberg, 2000). Flies that were unable to control the stimuli by feedback from the torque meter during training showed no significant learning, indicating that the ability to control the visual environment (i.e. their operant behavior) played a significant role in the basic learning paradigm (Wolf and Heisenberg, 1991). However, flies in an analogous play-back experiment with additional repetitions of training demonstrated significant learning (Brembs and Heisenberg, 2000). Flies that were unable to control the stimuli by feedback from the torque meter during training showed no significant learning, indicating that the ability to control the visual environment (i.e. their operant behavior) played a significant role in the basic learning paradigm (Wolf and Heisenberg, 1991). However, flies in an analogous play-back experiment with additional repetitions of training demonstrated significant learning (Brembs and Heisenberg, 2000). Brembs and Heisenberg (2000) suggest therefore that the basic visual learning paradigm in the flight simulator has both classical and operant components. Through analysis of torque spikes, they concluded that operant behavior facilitates the formation of classical associations. In other words, learning about relationships between sensory stimuli is enhanced when the fly actually controls its exposure to these stimuli by its own motor behaviors (Heisenberg et al., 2001).

3.2. Variations on short-term visual learning in the flight simulator

The impressive flexibility of the flight simulator training apparatus allows for the use of complex conditioned stimuli and reinforcers that act through different sensory modalities. Variations to the original procedure have demonstrated successful learning in the flight simulator when the basic T-shapes were replaced by other visual cues, such as a vertical stripe (Wolf and Heisenberg, 1991), continuous patterns (Dill et al., 1995), or colors (Wolf and Heisenberg, 1997; Brembs and Heisenberg, 2001). Different reinforcers also can be effective, including both appetitive and aversive olfactory stimuli (Wolf and Heisenberg, 1991; Guo and Gotz, 1997), and aversive visual field oscillations (Wolf et al., 1998). Various stimulus-reinforcer combinations have been reported to result in learning scores similar to those achieved in the basic flight simulator paradigm, demonstrating a robust ability to associate visual stimuli towards which a fly orients itself with visual, olfactory, or thermal aversive stimuli. In addition, complex conditioned stimuli have been used to demonstrate the breadth and limitations of Drosophila’s associative learning abilities, e.g. to transfer the learned associations to new conditioned stimuli or contexts (Liu et al., 1999; Brembs and Heisenberg, 2001), as well as impressive visual discrimination and choice behaviors (Tang and Guo, 2001).

3.3. Does visual learning in the flight simulator require mushroom bodies?

Wild-type flies without MBs (hydroxyurea-treated) or mushroom body miniature (mbm1) mutants performed normally when tested in most variations of visual learning in the flight simulator (Wolf et al., 1998). When flight direction guided by T-shapes was reinforced with either heat or visual oscillations, hydroxyurea-treated flies and mbm1 mutants demonstrated normal learning scores. Even when flies were trained with a purely classical paradigm (in which the presentation of visual cues and heat during training was controlled independently of the fly’s motor behavior), learning ability was still normal in hydroxyurea-treated flies. Thus, even though visual input is known to affect the growth of MBs (Barth and Heisenberg, 1997), memory in these visual associative learning paradigms does not require MB function. Hydroxyurea-treated flies also performed normally in the purely motor learning task, in that they learned to associate right or left turns with heat in the absence of visual cues (Wolf et al., 1998).

A requirement for MBs was revealed, however, when the visual memory task was made more complicated. Drosophila are capable of limited context generalization in the flight simulator training paradigm, that is, they display the normal learned preference to avoid orienting towards a heat-reinforced pattern...
even when contextual features of the visual environment change between training and testing (Liu et al., 1999). Thus, flies have normal memory scores when they are trained in monochromatic blue or green light and tested in white light (or vice versa), or when dark flashes are applied during training but not during testing (Liu et al., 1999). It is this ability to generalize the memory of training to a different visual context during testing that requires MB function: three different methods of disrupting MBs yielded flies with no memory when the visual context changed, even though they performed well in a constant visual environment (Liu et al., 1999).

Even more demanding than the ability to generalize a learned preference to a new visual context is the ability to choose between conflicting cues in compound stimuli. MBs are also essential for a fly’s ability to make such choices in the flight simulator (Tang and Guo, 2001). Flies were trained with compound stimuli that differed both in pattern and color. They were tested with patterns of the opposite colors, and the relative salience of the two cues was varied systematically. Wild-type flies made discrete choices in favor of the more salient stimulus. However, mbm flies were unable to make discreet choices to favor the more salient stimulus (Tang and Guo, 2001).

Thus, while MBs are not required for simple forms of visual associative learning in the flight simulator, the evidence suggests that they are essential for more demanding tasks that require flies to assess the relative importance of different features of a complex visual environment.

4. Spatial learning in the heat-box

While the spatial learning capabilities of rodents are the focal point of most neuroscientists’ efforts to understand learning and memory, the spatial learning abilities of fruit flies have received little attention until recently. The ability of Drosophila to learn spatial preferences that are independent of olfactory and visual cues can be demonstrated in an apparatus called a heat-box (Wustmann et al., 1996; Putz and Heisenberg, 2002). The heat-box training paradigm emerges as a sufficiently robust behavioral learning assay to offer excellent prospects for a detailed analysis of the neural substrates of spatial learning. Initial efforts in this direction indicated that flies lacking MBs (after hydroxyurea treatment) still exhibited significant spatial preferences when tested immediately following training (Wolf et al., 1998; Putz and Heisenberg, 2002).

Thus, spatial learning in Drosophila appears to be independent of MBs. Nonetheless, it shares with olfactory associative learning a requirement for type IC a2+/calmodulin-dependent adenylyl cyclase, as rutabaga mutants were significantly impaired in tests of spatial memory (Zars et al., 2000b). In order to identify neural structures in which rut expression is sufficient to rescue the learning defect of rut mutants, expression of rut cDNA was targeted to different brain regions of rut mutants with the GAL4–UAS system (Brand and Perrimon, 1993). Results implicated the antennal lobes, the median bundle, and the ventral ganglion as structures where type I Ca2+/calmodulin-dependent adenylyl cyclase activity is sufficient for spatial learning (Zars et al., 2000b). By testing the effects of other mutations in this paradigm, and targeting the expression of mutant and wild-type transgenes to these and other structures, it should be possible to assess the involvement of other signaling molecules and other brain systems in acquiring and expressing spatial preferences.
5. Experience-dependent changes in male courtship behavior

Male reproductive success depends on appropriate responses to the aphrodisiac and anti-aphrodisiac cues of potential courtship targets. Studies of behavioral changes produced by courtship experience indicate that male responsiveness to these stimuli can be modified by both associative and non- associative learning processes.

A male fly performs several characteristic behaviors in the presence of a potential courtship target (Sturtevant, 1915; Bastock and Manning, 1955; Tompkins, 1984; Hall, 1994; Greenspan and Ferveur, 2000). He initially orients towards and follows an active female, and taps her cuticle with his foretarsi. The tapping behavior may be a mechanism to sample the hydrocarbons of the target fly’s cuticle (Hall, 1994; Ferveur, 1997) with his tarsal taste bristles (Stocker, 1994). Female cuticular hydrocarbons include potent courtship-stimulating pheromones, while males express higher levels of courtship-inhibiting hydrocarbons (Antony and Jallon, 1982; Antony et al., 1985). Thus, by tapping, a courting male provides chemosensory feedback to the neural systems controlling his courtship behavior. If the feedback is positive, he proceeds to vibrate one wing to generate a courtship song. Receptive females respond to the song of a conspecific male by decreasing their locomotion (Tompkins et al., 1982). A less mobile female presents opportunities for the male to approach from the rear, lick her abdominal cuticle and genitalia with his mouthparts, and attempt copulation (Bastock and Manning, 1955; Hall, 1994). Licking also allows the male to sample the target’s pheromones, through olfactory sensilla on the maxillary palps and gustatory sensilla on the proboscis (Stocker and Gendre, 1989; Stocker, 1994). These behavioral interactions are repeated, with some variation, until successful copulation. Courtship behavior can be quantified by scoring each of these behaviors separately, or more typically, by summing the time engaged in all courtship behaviors and expressing it as a percentage of a defined observation interval (either 5 or 10 min) to compute a courtship index (CI).

The neural systems that generate the motor programs for these courtship behaviors are likely to be widely distributed (Hall, 1979; Kido and Ito, 2002), and may receive competing inputs from excitatory and inhibitory regulatory systems. Aphrodisiac cues of virgin females activate the excitatory systems, and anti-aphrodisiac cues of mature males activate the inhibitory systems. Recent evidence that potent courtship-inhibiting systems normally suppress male–male courtship derives from the dramatic induction of homosexual courtship after conditional inactivation of synaptic transmission from peripheral cholinergic neurons (Kitamoto, 2002).

Socially naive males will court immature males (Cook and Cook, 1975; Jallon and Hotta, 1979) and previously mated females (Bastock and Manning, 1955; Connolly and Cook, 1973), but they gradually learn to decrease their courtship of these inappropriate targets. Experience with immature males produces a form of habituation known as experience-dependent courtship modification (Gailey et al., 1982; Tompkins, 1989), while experience with mated females results in a form of associative learning called conditioned courtship suppression or courtship conditioning (Siegel and Hall, 1979; Tompkins et al., 1983). While these forms of learning and memory bear obvious relevance to male reproductive success, the “natural” behavioral and sensory interactions that constitute the training experiences present unique challenges to mechanistic analyses. Both experience-dependent courtship modification and courtship conditioning involve changes in male responses to stimuli presented by another behaving fly, rather than stimuli defined by a rational experimental design. Thus, the first challenge in deciphering the mechanisms of these intriguing forms of behavioral plasticity has been to identify the relevant stimuli and sensory systems that transduce them.

5.1. Habituation to immature male pheromones

Very young males (within 3–4 h of eclosion) stimulate older males to perform all of their normal courtship behaviors (Cook and Cook, 1975; Jallon and Hotta, 1979; Tompkins et al., 1980; McRobert and Tompkins, 1983). Yet 30 min of experience with an immature male is sufficient to specifically eliminate this misdirected courtship, and this suppression lasts for 4 h; the effect is specific in that a male’s courtship of virgin females is not altered by this experience.
Even 30 min of rest in a chamber that had previously been occupied by immature males was sufficient to suppress subsequent courtship of immature males (Gailey et al., 1982), suggesting that this form of experience-dependent courtship modification is the result of habituation to courtship-stimulating pheromones that are specifically synthesized by immature males (Tomkins, 1989). This hypothesis was confirmed (Vaisas et al., 1993) after two such compounds (11-tritriacontene and 13-tritriacontene) were identified among the cuticular hydrocarbons of immature males (Schaner et al., 1989). Simply exposing a mature male to either or both of these hydrocarbons, even if he did not perform any courtship behaviors or interact with another fly, was sufficient to significantly reduce his courtship of immature males (Vaisas et al., 1993).

The aphrodisiac hydrocarbons produced by immature males are 31-carbon alkenes (Schaner et al., 1989). Like the 27- and 29-carbon dienes produced specifically by females (Antony and Jallon, 1982; Antony et al., 1985), these are likely to be detected by gustatory sensilla in male foretarsi and/or proboscis (Hall, 1994; Ferveur, 1997). Such chemoreceptors provide an important source of sensory feedback to the neural systems that modulate courtship behavior. While habituation to immature male hydrocarbons could theoretically be mediated entirely at the level of peripheral chemoreceptors, some evidence suggests that the relevant changes involve central brain structures. Dopaminergic modulatory neurons may be involved, as dopamine-depleted males do not habituate to immature males (Neckameyer, 1998). A requirement for cAMP signaling pathways is suggested by the severely impaired habituation phenotypes of dance mutants (Gailey et al., 1982). Two other olfactory memory mutants, rutabaga and amnesiac, also were moderately impaired in their habituation to immature males (Gailey et al., 1982). The expression of these genes in MBs and their associated DPM cells (Davis, 1993; Waddell et al., 2000) suggests a possible role of MBs in the habituation to immature male hydrocarbons. Evidence that MB-ablated males did not habituate to immature males (Neckameyer, 1998) further suggests that this non-associative form of learning may involve a MB-dependent inhibitory modulation of the courtship sensorimotor system.

5.2. Experience with mated females elicits conditioned courtship suppression

Males that have habituated to immature male pheromones still court females avidly (Gailey et al., 1982). By contrast, after 30 min of experience with a mated female, males avoid courting other mated females for more than 8 h (Gailey et al., 1982; Kamyshev et al., 1999), and suppress their courtship towards virgin females for about 2 h (Siegel and Hall, 1979; Kamyshev et al., 1999). Yet courtship of immature males is not altered by this experience (Gailey et al., 1984), indicating that it is not simply a generalized suppression of all courtship behavior. Rather, behavioral evidence suggests that it is a form of classical conditioning, in which males learn to associate some female aphrodisiac cue(s) (a conditioned stimulus) with an anti-aphrodisiac cue (an unconditioned stimulus) of the mated female (Tomkins et al., 1983; Ackerman and Siegel, 1986).

Previously mated females differ from virgins in their behavioral responses to male courtship, which includes frequent ovipositor extrusions (Bastock and Manning, 1955; Connolly and Cook, 1973; Siegel and Hall, 1979; Bubis et al., 1998). But a male does not have to see the mated female’s extrusion behaviors in order to be conditioned, as training either normal or visually impaired males in either normal light or darkness (or red light) results in similarly strong suppression of their subsequent courtship of virgins (Tomkins et al., 1983; Joiner and Griffith, 1997). Indeed, the conditioning can be mediated entirely by chemosensory cues: a 30-min exposure to an organic extract of mated females in the presence of another fly (even an immobilized male!) elicited active courtship of the target fly and a significant suppression of subsequent courtship towards the test virgins; either stimulus alone or sequential 30-min presentations had no effect (Tomkins et al., 1983). Both the mated-female extract and a courtship target had to be present simultaneously in order to elicit a suppression of subsequent courtship towards the test virgins; either stimulus alone or sequential 30-min presentations had no effect (Tomkins et al., 1983), strongly suggesting an associative learning process. In similar controlled experiments, males were successfully conditioned by extracts of virgin females paired with specific aversive odorants (quinine or cis-vaccenyl acetate), but only if they were actively courting during training (Ackerman and Siegel,
1986; Zawistowski, 1988). These studies clearly demonstrated that an aversive olfactory stimulus can function as an unconditioned stimulus to produce a conditioned suppression of courtship towards virgins.

The consistent requirement for active courtship during training in these behavioral studies (Tompkins et al., 1983; Ackerman and Siegel, 1986; Zawistowski, 1988) suggests that males may learn to associate an aversive chemosensory cue with the act of courtship, which would be a form of operant learning. But another key observation argues against operant conditioning; notably, males conditioned by either mated females or quinine-plus-virgin females do not suppress all courtship behavior. They still court actively in the presence of immature males (Gailey et al., 1984; Ackerman and Siegel, 1986). Indeed, recent efforts to identify a conditioned stimulus have provided positive evidence for a classical mode of conditioning. In studies involving genetic manipulations of cuticular hydrocarbons (Ferveur and Sureau, 1996; Savarit et al., 1999), we found that males conditioned by normal mated females did not suppress their courtship of hydrocarbon-depleted virgin females; however, they did exhibit apparently normal conditioned courtship suppression in tests with male targets expressing a characteristically female pheromonal profile (K.K.S., L.L. and J.-F. Ferveur, unpublished data). These results indicate that the conditioned stimulus is among the blend of cuticular hydrocarbons typically expressed by Canton-S females. Because these pheromones are likely to be detected by gustatory receptors on the male foretarsi or proboscis, it makes perfect sense that he must be courting actively (i.e. tapping and licking) in order to be trained.

While behavioral studies have shown that mated-female extracts contain an aversive unconditioned stimulus (Tompkins et al., 1983), this stimulus has not been specifically identified. The cuticular hydrocarbons of mated females are very similar to those of virgins (Tompkins and Hall, 1981; Tompkins and Hall, 1984). The aversive cue that functions as an unconditioned stimulus apparently is not constitutively expressed by mated females, but is released upon exposure to males (Tompkins et al., 1983; Gailey et al., 1984). Mated females may produce more 7-tricosene, a male-predominant anti-aphrodisiac hydrocarbon, in response to male courtship (Scott, 1986; Scott and Jackson, 1990), but the ability of this substance to function as an aversive reinforcer in courtship conditioning remains to be tested.

5.3. Neural substrates of conditioned courtship suppression

The impaired courtship conditioning phenotypes of the original learning mutants, amnesiac, rutabaga, and dune (Siegel and Hall, 1979; Gailey et al., 1984), suggested early on that this learning paradigm involves some of the same molecular players as olfactory conditioning. Thus, the courtship of amnesiac mutants is significantly suppressed in tests immediately after training, but memory decays rapidly (Siegel and Hall, 1979), very much like the rapid memory loss these mutants display after olfactory conditioning (Tully, 1987). Both dune and rutabaga mutants were abnormal in tests with virgin females immediately after courtship conditioning (Gailey et al., 1984). These phenotypes suggest a role for cAMP signaling in conditioned courtship suppression, although the precise neural systems in which it functions have not been identified.

The role of MBs has been addressed in studies involving chemical ablations by hydroxyurea treatment (McBride et al., 1999) or GAL4-targeted expression of a specific CaM kinase inhibitor (Joiner and Griffith, 1999, 2000). The results suggest that different components of memory of courtship conditioning are differentially sensitive to MB disruption. MB-disrupted males had apparently normal memory of courtship conditioning when tested immediately after training in normal light (McBride et al., 1999; Joiner and Griffith, 2000). Tests at later times, however, revealed a requirement for the MBs in 30- and 60-min memory of courtship conditioning (McBride et al., 1999). Thus, some structures critical for learning in this paradigm remained functional after MB disruptions, while consolidation or retrieval of later memory was severely impaired. The early, MB-independent component of memory might reflect transient changes in antennal lobe representations of chemosensory aphrodisiacs; alternatively, it may reflect short-lasting, experience-dependent changes in the ability of non-olfactory female
cues to activate courtship excitatory systems. In fact, the evidence supports both possibilities. Evidence that antennal lobe structures contribute to memory of courtship conditioning was gleaned from careful histological analysis of the effects of HU treatment in behaviorally tested males. This analysis indicated that partial damage to antennal lobes was correlated with memory defects in MB-ablated males (McBride et al., 1999). Behavioral effects of partial antennal lobe damage were evident only in MB-ablated males tested 30 min after training and not at 60 min, suggesting that antennal lobe functions may contribute directly to 30-min memory in the absence of MBs, but dissipate after 60 min (McBride et al., 1999).

Although normal memory was observed in MB-disrupted males tested immediately after training in normal light (McBride et al., 1999; Joiner and Griffith, 2000), no memory was detected in males expressing the CaM kinase inhibitor in MBs when experiments were performed in red light, which effectively deprives flies of visual input (Joiner and Griffith, 1999). This important result indicates that all non-visual memory of courtship conditioning requires CaM kinase function in MBs, and strongly suggests that the MB-independent suppression of courtship observed in the other studies (McBride et al., 1999; Joiner and Griffith, 2000) depends largely on visual input during either training or testing. Thus, although visual cues from the mated female are not necessary to elicit conditioned courtship suppression in normal males (Tompkins et al., 1983; Joiner and Griffith, 1997), they may be sufficient to produce a significant suppression of courtship immediately after training. This visually mediated component of courtship suppression might be unmasked in males without MBs, where longer lasting chemosensory associations are eliminated.

Taken together, the results suggest that immediately after conditioning in normal light, male courtship is suppressed by two temporally overlapping and anatomically distinct forms of memory. One is the result of the reduced effectiveness of visual cues that stimulate courtship; this is independent of MBs and decays rapidly after training. In addition, associative memories involving aphrodisiac and anti-aphrodisiac chemosensory cues require CaM kinase function in MBs; these processes induce a conditioned suppression of courtship that lasts for several hours. While this model provides a reasonable framework for interpreting the available evidence, it will require far more experimental testing to approach the current level of support for the role of MBs in olfactory associative learning as outlined above (Roman and Davis, 2001; Waddell and Quinn, 2001).

Beyond the MBs, results of Joiner and Griffith (1999, 2000) provide additional clues as to neural structures required for expressing the chemosensory component of memory of courtship conditioning. When the CaM kinase inhibitor was expressed selectively in the central complex, the results were similar to those obtained with the MB-specific GAL4 drivers: memory was normal in males trained and tested in white light (Joiner and Griffith, 2000), but was impaired in the absence of visual input (Joiner and Griffith, 1999). The implications are similar: normal CaM kinase activity in central complex structures is required for the non-visual component of memory. Parallel results were obtained with a GAL4 line with a unique expression pattern, restricted to cells in the pars intercerebralis, fibers in the median bundle and some commissural fibers that terminate in the lateral protocerebrum, where targets of MB neurons also terminate (Ito et al., 1998). These structures are likely to be involved in expressing the effects of the chemosensory associations formed during courtship conditioning.

5.4. Suppressed courtship of mated females

When effects of training with a mated female are assayed in tests with virgin females, male courtship returns to normal levels after about 3 h (Siegel and Hall, 1979). By contrast, courtship towards another mated female remains suppressed for at least 8 h after training (Gailey et al., 1982; Kamyshev et al., 1999). These different time courses may reflect distinct experience-dependent changes in the male nervous system, which decay in different neural structures with different time constants. Kamyshev and coworkers (1999) presented a model in which these different time courses are taken to represent two different forms of associative memory, involving associations between aphrodisiac cues and either the internal representation of anti-aphrodisiac mated female cues, or higher centers that mediate aversive responses to these anti-aphrodisiacs. While strong evidence supports the
associative nature of the conditioned suppression of courtship towards virgin females (see above), the proposed associative nature of the longer lasting suppression of courtship towards mated females is currently based largely on theoretical arguments (Kamyshev et al., 1999). The evidence remains consistent with a non-associative increase in a male’s responsiveness to the anti-aphrodisiacs of mated females, a form of sensitization, which persists for 8 h or more. Further experiments involving independent manipulations of putative conditioned and unconditioned stimuli will be required to evaluate these alternatives.

The effects of certain genetic manipulations suggest that a male’s responses to mated females during training are modified by a different mechanism from the associative processes that produce a suppression of subsequent courtship towards virgins. Males with globally expressed mutations or transgenes that disrupted either CaM kinase or protein kinase C (PKC) activity did not decrease their courtship towards mated females during training (Joiner and Griffith, 1997; Kane et al., 1997). Yet these same genotypes exhibited normal conditioned courtship suppression in subsequent tests with virgin females (Joiner and Griffith, 1997; Kane et al., 1997). These results suggest that a male’s aversive responses to mated–female anti-aphrodisiacs may increase during training by processes that require normal PKC or CaM kinase activity, while the concurrent associative learning processes that result in suppressed courtship towards virgins can proceed even when these kinases are globally inhibited.

Overall, behavioral evidence indicates that the male nervous system sustains multiple experience-dependent changes as a result of courtship with inappropriate targets. These develop and decay with different time constants, and are likely to involve different neural substrates. Rapid habituation to chemosensory aphrodisiacs of immature males probably involves dopamine, cAMP signaling, and MBs. Experience with a mated female produces a suppressed responsiveness to courtship-stimulating visual cues, which, like other forms of visual learning (Wolf et al., 1998), does not require MB functions. Also resulting from experience with mated females, associative connections develop through antennal lobe and MB-dependent processes, between the representations of chemosensory aphrodisiacs and either the anti-aphrodisiac representations or higher courtship-inhibiting centers (Kamyshev et al., 1999). As a result, aphrodisiac cues can contribute to the activation of the inhibitory systems in subsequent tests with virgins. Mechanisms producing a longer lasting suppression of courtship towards mated females remain entirely unexplored, even though this effect may actually provide some functional benefit to experienced males. Ultimately, the structures and molecules that mediate experience-dependent aspects of courtship behavior must interact with neural systems that generate the motor programs for courtship behavior. While recent results of targeted genetic manipulations indicate that these systems are widely distributed (Kido and Ito, 2002), they also justify an expectation that essential features of the fly’s courtship neural systems will be elucidated by this approach.

6. Conclusions and future prospects

Many forms of associative learning are well documented in Drosophila. In olfactory classical conditioning, flies learn to associate odors with shock. In a flight simulator, they can learn to associate either visual cues or their own turning behavior with aversive reinforcers, indicating that both classical and operant learning are possible. A simple form of spatial learning in a heat-box provides another clear example of purely operant learning, in the absence of visual and olfactory cues. Finally, the courtship conditioning paradigm demonstrates a remarkable ability to form associative memories involving naturally occurring appetitive and aversive stimuli presented by another fly.

Just as different forms of memory in vertebrates involve distinct (and sometimes overlapping) neural systems, so too each of these forms of behavioral plasticity in Drosophila is likely to be mediated by different neural substrates. A mechanism for visual associative learning must exist that is distinct from the processes in MB neurons that establish memory of olfactory conditioning, since basic forms of visual associative memory are independent of MBs. The neural systems that mediate operant learning, or experience-dependent modulation of courtship behavior, are certain to be complex, but current prospects for deciphering them are excellent. Consider the results of the integrated experimental assault that has been brought to bear on olfactory memory in the past.
References


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