Thirty years of olfactory learning and memory research in Drosophila melanogaster

Sean E. McGuire a,*, Mitch Deshazer b, Ronald L. Davis a,c

a Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, USA
b Department of Neuroscience, Baylor College of Medicine, Houston, Texas 77030, USA
c Department of Psychiatry and Behavioral Sciences, Baylor College of Medicine, Houston, Texas 77030, USA

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Abstract

The last 30 years have witnessed tremendous progress in elucidating the basic mechanisms underlying a simple form of olfactory learning and memory in Drosophila. The application of the mutagenic approach to the study of olfactory learning and memory in Drosophila has yielded insights into the participation of a large number of genes in both the development of critical brain regions as well as in the physiology underlying the acquisition, storage, and retrieval of memory. Newer sophisticated molecular-genetic tools have further allowed for the specification and functional dissection of the neuronal circuitry involved in these processes at a systems level. With these advances in our understanding of the genes, neurons, and circuits involved in learning and memory, the field of Drosophila memory research is nearing a state of integration of the bottom up and top down approaches to understanding this form of behavioral plasticity.

Keywords: Drosophila melanogaster; Olfactory learning and memory; Behavior

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Abbreviations: STM, short term memory; MTM, middle term memory; LTM, long term memory; ARM, anesthesia resistant memory; ASM, anesthesia sensitive memory; CS, conditioned stimulus; US, unconditioned stimulus; EMS, ethyl methane sulfonate; mbD, mushroom body deranged; mbm, mushroom body miniature; rut, rutabaga; PKA, protein kinase A; leo, leonardo; Vol, Volado; fascII, fasciclin II; DOR, Drosophila odorant receptors; ORNs, olfactory receptor neurons; GABA, gamma amino butyric acid; PN, projection neurons; ACT, antennal–cerebral tract; MARCM, mosaic analysis with a repressible cell marker; iACT, internal antennal–cerebral tract; MB, mushroom body; GAL4, galactose regulated gene 4; DPM, dorsal paired medial neuron; PACAP, pituitary adenylyl cyclase activating peptide; ChAT, choline acetyltransferase; Ach, acetylcholine; spH, synapto-pHluorin; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescent resonance energy transfer; KC, Kenyon cell; LFP, local field potential; OPNs, omnipause neurons; LH, lateral horn; dnc, dendra; eAMP, cyclic adenosine monophosphate; ANP, antero-notopleural; mEPSC, miniature excitatory post-synaptic current; NMJ, neuromuscular junction; PTP, post-tetanic potentiation; BMP, bone morphogenic protein; CaMKII, calmodulin sensitive kinase II; gbb, glass-bottom boat; CREB, cAMP response element binding protein; ann, anneries; Shh, temperature-sensitive Shhner; UAS, upstream activating sequence; AMI, age-related memory impairment; NFI, neurofibromatosis I; PKC, protein kinase C; aPKM, atypical protein kinase M; rsh, radish; lat, latheo; lio, linotte; Adf1, Nalyot; PL-A2, phospholipase A2; N, Notch; stau, staufen; puun, pumilio; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; eIF-5C, elongation factor 5C; CPEB, cytoplasmic polyadenylation element binding protein; DSCR1, Down’s Syndrome critical region 1; DS, Down’s Syndrome; nla, nebulata; cer, cranner; Scs-fp, synthetase flavoprotein subunit; syn, synapsins; S6KII, S6 Kinase II; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; OCT, 3-octanol; MCH, 4-methocyclohexanol; BA, benzaldehyde; ala, alpha lobes absent; TβH, tyramine β hydroxylase

* Corresponding author. Tel.: +1 713 256 3681.
E-mail address: seanmcguire@sbcglobal.net (S.E. McGuire).

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The idea that certain behaviors can be selected for or against, either naturally or artificially, dates back conceptually to the time of Darwin and in practice even further back to the early domestication of animals. The subsequent observations by Mendel that certain traits are discrete and can be transmitted in a hereditary fashion led to the notion that genes may underlie...
behavioral differences between individuals. This notion spurred early attempts at behavior-genetic analysis in order to quantify the impact of genes on behavior (for review see Hirsch, 1967). Largely through selective breeding experiments, the behavior-geneticists attempted to quantify the number of genetic loci influencing a given behavior. They determined that specific behavioral traits could be selected for, and were likely to result from many interacting loci. Beyond this general conclusion, little information was ever gleaned regarding the identity of these loci.

The modern era of the genetic dissection of behavior began when Seymour Benzer proposed that genes involved in complex behaviors in Drosophila might be identified by chemical mutagenesis to produce phenotypic mutants bearing single gene defects (reviewed in Benzer, 1971, 1973). The advantage of a mutagenic approach over the selective breeding method of the behavior-geneticists was several-fold. First, the ability to produce single mutations allowed examination and understanding of the effects of a single gene on behavior. Second, the mutagenic approach permitted the identification of major-effect genes that might be fixed in a population and thus elude selective breeding experiments, which depend on allelic variation. Finally, the chemical approach can frequently produce mutant alleles of greater strength (e.g. null) than those found in the naturally occurring allelic population, allowing easier identification of loci involved in behavior (for a review and comparison of the two approaches see Tully, 1996). Early success with the forward-genetic approach was achieved in identifying mutants defective for phototaxis (Benzer, 1967) and circadian rhythms (Konopka and Benzer, 1971).

2. Olfactory associative learning in Drosophila

2.1. Operant conditioning

The application of the forward-genetic approach to the study of associative learning and memory in Drosophila began some 30 years ago with the development of a reliable assay to measure olfactory learning and memory by Quinn and colleagues (Quinn et al., 1974). In this assay (Fig. 1A), a group of approximately 40 flies were placed into a small start tube. At the beginning of the conditioning period, the flies were aroused and allowed to run toward a light source at the end of a second connected tube. The second tube contained an electrifiable grid painted with an odor (odor A). Flies that ran into the second tube received electric shock while being exposed to odor A for 30 s. After a short rest the flies were returned to the start tube and allowed to run into a different tube containing a shock grid painted with odor B for 30 s without electric shock. A second group was trained reciprocally to associate the shock with odor B and the absence of shock with odor A. The flies were then tested by being presented with the opportunity to run into a tube painted with either odor A or odor B. A performance index was calculated by subtracting the fraction of flies that avoided the unpaired odor from the fraction that avoided the shock-paired odor. Importantly, prior to conditioning, flies are first tested to demonstrate that they possess both the sensory acuity and motor capacity to perform in the assay, to rule out non-associative effects.

Although the learning observed in the operant assay of Quinn et al. was reliable, the assay tended to produce index scores in the range of 0.3 (Quinn et al., 1974). These generally low scores were attributed to the fact that only a portion of the flies that were tested actually ran into the odor tubes and received the intended reinforcement, and that the effects of training needed to be strong enough to overcome Drosophila's strong phototactic drive during testing (Tully and Quinn, 1985).

2.2. Classical conditioning

To address the limitations of the operant assay and to expand behavioral analyses in Drosophila, Tully and Quinn (1985) developed a classical conditioning paradigm. In this assay (Fig. 1B), approximately 100 flies were placed in a tube whose internal surface was comprised of an electrifiable copper grid. The flies were subsequently exposed to odor A for one minute in the presence of 12 pulses of electric shock (CS+) followed by...
conditioning assay (for reviews, see Aceves-Pina et al., 1983; The mutagenesis was followed by screening of approximately one lethal mutation in 30% of X chromosomes. The chemical mutagen ethyl methane sulfonate (EMS) to produce mutations on the X chromosome with a frequency of 0.7 to 0.9. Employed the chemical mutagen ethyl methane sulfonate and later in the laboratory of Quinn. In flies, these screens were carried out initially in the laboratory of Benzer learning were carried out initially in the laboratory of Benzer and later in the laboratory of Quinn. In flies, these screens employed the chemical mutagen ethyl methane sulfonate (EMS) to produce mutations on the X chromosome with a frequency of one lethal mutation in 30% of X chromosomes. The mutagenesis was followed by screening of approximately 4000 strains for defects in associative learning in the operant conditioning assay (for reviews, see Aceves-Pina et al., 1983; Waddell and Quinn, 2001a,b). The first single gene mutant defective in associative learning, dunce, was identified in Benzer’s lab (Dudai et al., 1976). Subsequently, a second allele of dunce was identified (Byers et al., 1981). Four additional mutants were identified at Princeton, including rutabaga, radish, cabbage, and turnip (Aceves-Pina et al., 1983) while a sixth mutant, amnesiac, was identified in an assay for later memory defects (Quinn et al., 1979). Flies of these six strains showed normal olfactory acuity and shock reactivity, which is defined as avoidance of noxious odors or isolated shock stimuli, respectively, equivalent to wild-type.

3. Screens for mutants

3.1. Chemical mutagenesis

The mutagenic approach to dissecting behavior proposed by Benzer was an extension of the tremendously successful genetic screens employed in bacteria, phage, and yeast. The first systematic screens for mutants defective in associative learning were carried out initially in the laboratory of Benzer and later in the laboratory of Quinn. In flies, these screens employed the chemical mutagen ethyl methane sulfonate (EMS) to produce mutations on the X chromosome with a frequency of one lethal mutation in 30% of X chromosomes. The mutagenesis was followed by screening of approximately 4000 strains for defects in associative learning in the operant conditioning assay (for reviews, see Aceves-Pina et al., 1983; Waddell and Quinn, 2001a,b). The first single gene mutant defective in associative learning, dunce, was identified in Benzer’s lab (Dudai et al., 1976). Subsequently, a second allele of dunce was identified (Byers et al., 1981). Four additional mutants were identified at Princeton, including rutabaga, radish, cabbage, and turnip (Aceves-Pina et al., 1983) while a sixth mutant, amnesiac, was identified in an assay for later memory defects (Quinn et al., 1979). Flies of these six strains showed normal olfactory acuity and shock reactivity, which is defined as avoidance of noxious odors or isolated shock stimuli, respectively, equivalent to wild-type.

3.2. P-element mediated mutagenesis

Although EMS mutagenesis has the advantage of generating mutations at a high rate and the potential to mutate any gene throughout the genome, it suffers from the difficulty in identifying the single base change generated in the background of the entire genome, making the identification of the mutated gene extremely challenging. For this reason, a different approach to generating mutants was performed in the lab of Tully, initially at Brandeis and later at Cold Spring Harbor (for review, see Tully et al., 1990). This approach employed P-element transposition to disrupt gene function. The advantage of this approach is that it provides a molecular tag to identify where the mutation has occurred. Initially, the general location of the P-element was determined by hybridization of polytene chromosome squashes with sequences from the P-element. However, the development of additional techniques including plasmid rescue, inverse PCR, and the completion of the Drosophila genome sequencing project, has made the precise localization of any P-element insertion facile. The limitation, however, is that P-element mutagenesis is less efficient than chemical mutagenesis. The P-element mediated mutagenesis screen has thus far identified three poorly performing mutants: latheo, linotte, and nalyot (Boynton and Tully, 1992; DeZazzo et al., 2000; Dura et al., 1993). More recently, Dubnau et al. (2003a,b) used a combination of microarray and P-element screening to investigate the genetic basis of LTM. The authors report 42 candidate genes which show expression changes after spaced training on the arrays, as well as 60 lines of flies derived from the P-element screen that perform poorly in tests of LTM. Four specific genes thus far have been identified that demonstrate gene expression changes after spaced training and have defective LTM: staufen, pumilio, oskar, and elf-5c (discussed in Section 4.6.3).

3.3. Brain structure mutants

An altogether different approach to identifying mutants defective in conditioned behavior has been to screen histologically for chemically induced mutants with structural defects in the brain, with the goal of perturbing a brain structure required for learning and memory (Heisenberg and Boehl, 1979; Heisenberg et al., 1985). This approach has the advantage of being much faster than behavioral screens to identify candidate mutants, as well as allowing a linkage to be made between a behavioral defect with a particular brain structure. Two mutants with defects in the MBs, mushroom body deranged (mbd) and mushroom bodies miniature (mbm), were identified from this screen and subsequently shown to be impaired in olfactory learning (Heisenberg et al., 1985), implicating the MBs as important centers in Drosophila for olfactory conditioning. However, the identification of the genes responsible for the defects in brain structure has proven difficult.

3.4. Enhancer-detection

Finally, in an attempt to combine the relative ease of histological screening with the advantages of P-element mutagenesis, the Davis lab at Baylor College of Medicine performed an enhancer detector screen for genes preferentially expressed in the MBs. Several lines of evidence had implicated the involvement of the MBs in olfactory conditioning (see Section 5.3.1). Early work in the honeybee had demonstrated that local cooling of the MBs after olfactory conditioning produced amnesia (Erber and Menzel, 1980). Additionally, the structural mutants mbd and mbm identified by Heisenberg and colleagues demonstrated defects in olfactory conditioning (Heisenberg et al., 1985). Moreover, the MBs receive olfactory input from the antennal lobes, placing them in the pathway of information flow in during olfactory conditioning. Finally, it was shown that dunce was preferentially expressed in the MBs (discussed in Section 4.1.1; Nighorn et al., 1991).
Enhancer detection is based upon the random insertion of a P-element carrying a reporter gene near an endogenous enhancer that normally drives the expression of a nearby gene in a particular tissue. Proximity of the P-element to this enhancer causes reporter gene expression to be driven in the same fashion as the endogenous gene (O’Kane and Gehring, 1987; Bellen et al., 1989; Wilson et al., 1989). Frequently, the enhancer detector P-element causes a disruption of the endogenous gene resulting in the simultaneous production of a mutant carrying a tagged marker that facilitates its cloning (Han et al., 1992). The enhancer detector lines thus derived were considered to be candidate learning and memory mutants and were screened in the classical olfactory conditioning assay of Tully and Quinn. To date, this approach has identified additional alleles of rut, DCO (the catalytic subunit of PKA), leonardo (a 14-3-3 protein), Volado (an α-integrin subunit), and fasciclin II, as being preferentially expressed in the MBs, as well as involved in olfactory conditioning (see Fig. 4 below; Han et al., 1992, Skoulakis et al., 1993; Skoulakis and Davis, 1996; Grotewiel et al., 1998; Cheng et al., 2001). More recently crammer (an inhibitor of cathepsins) has been identified in a MB enhancer detector screen, although currently its role in memory appears to map outside of the MB (Comas et al., 2004).

4. Genes involved in learning and memory

4.1. cAMP pathway

4.1.1. dunce (dnc)

The first learning and memory mutant to be isolated was dunce (Dudai et al., 1976). Dunce was initially identified in the Benzer lab as a poor learner that demonstrated normal olfactory avoidance behavior and shock reactivity. Initial genetic mapping of dnc placed it on the distal arm of the X chromosome between the markers yellow and chocolate (Dudai et al., 1976). Independent studies of cAMP phosphodiesterase activity (Davis and Kiger, 1978; Kiger and Golanty, 1977) and female sterility (Mohler, 1977) had identified mutations that mapped to the region of 3C12-3D4 of the X chromosome. One allele of dnc also caused defects in female fertility and subsequent mapping with deficiency chromosomes demonstrated that deletions of the 3C12-3D4 recovered the dnc learning phenotype. Biochemical tests then demonstrated that the dnc and dnc2 mutants were deficient in one form of cAMP phosphodiesterase activity, suggesting for the first time that the dnc mutations produced a specific defect in cAMP phosphodiesterase activity (Byers et al., 1981; Davis and Kiger, 1981). Unambiguous evidence that the dnc locus did indeed encode a cAMP phosphodiesterase came with the identification of cDNA clones that demonstrated significant homology to known phosphodiesterases (Chen et al., 1986) and the generation of functional cAMP phosphodiesterase activity with expression of the dnc cDNAs in yeast. Additional confirmation that the dnc gene was responsible for the learning and memory phenotype was shown by the fact that inducible transgenes carrying either the Drosophila dnc+ cDNA or a rat homologue rescued the learning and memory defect (Dauwalder and Davis, 1995).

Perhaps most importantly, studies on the expression pattern of dnc provided the first link between learning and memory mutants and the MBs (Nighorn et al., 1991). By performing immunohistochemistry on Drosophila brains with an anti-Dnc antibody, Nighorn et al., demonstrated preferential expression of the Dnc protein in the MBs. This was confirmed by RNA in situ hybridization experiments demonstrating that dnc RNA is elevated in the MBs of wild type flies, but not in flies carrying a deficiency in the region of the dnc locus (Nighorn et al., 1991).

4.1.2. Rutabaga (rut)

The next mutant to be molecularly characterized was rutabaga. The original rut mutation was genetically mapped to 12F5-7. Because the dnc mutations had been shown to perturb the cAMP signaling pathway, subsequent mutants were tested to see whether they demonstrated defects in cAMP metabolism. These studies demonstrated that rut flies were deficient in the activity of a Ca2+/calmodulin-sensitive adenylyl cyclase (Livingstone et al., 1984). The cloning of adenylyl cyclases from Drosophila based on homology to mammalian cyclases revealed that one of the cyclases mapped to the X chromosome at the region from 12F5-13A1. Subsequent cloning of the rut locus demonstrated that it encodes a type I adenylyl cyclase responsive to both Ca2+/calmodulin and Gs proteins. Sequencing of the locus from rut2 mutant flies revealed a single point mutation resulting in the substitution of an arginine for a conserved glycine at amino acid 1026 that abolished the catalytic activity of the cyclase (Levin et al., 1992).

In addition to the original rut' allele, seven different alleles of rut have been identified by the mushroom body enhancer detector screen of Han et al. (Han et al., 1992). Since the enhancer detector element presumably reports the expression pattern of the disrupted gene, Han et al. examined the expression pattern of rut by RNA in situ hybridization and immunohistochemistry and demonstrated that, similar to dnc, rut is preferentially expressed in the MBs. Recent proof that the type I adenylyl cyclase encoded by rut is responsible for the rut memory phenotype came from rescue experiments demonstrating that the expression of a rut cDNA specifically in the MBs of rut mutant flies was sufficient to rescue the associated memory defect (Zars et al., 2000). More recently it has been shown that induction of rut expression in the adult mushroom bodies is sufficient to rescue the memory defect, while expression only during development is not (McGuire et al., 2003; Mao et al., 2004). The function of rutabaga as a molecular coincidence detector of the convergence of the conditioned stimulus and the unconditioned stimulus in the MB’s has been proposed (Dudai et al., 1988).

4.1.3. PKA

A third component of the cAMP signaling pathway has also been implicated in Drosophila olfactory learning and memory. The cAMP-dependent protein kinase (PKA) is a major mediator of signaling through the cAMP pathway (Taylor et al., 1990). Following the demonstration that dnc encoded a cAMP phosphodiesterase (Chen et al., 1986) and that rut mutants were deficient in a Ca2+/calmodulin dependent
adenylyl cyclase activity (Livingstone et al., 1984) Drain et al. (1991) tested whether an inducible inhibitor of PKA activity could disrupt olfactory learning and memory. Using a heat-shock transgene, they demonstrated that induction of a peptide inhibitor of PKA acutely before the conditioning procedure could disrupt memory, whereas the induction of a mutated form of the inhibitor did not. This provided the first evidence for an acute physiological role for PKA activity in learning and memory.

Subsequent work by Skoulakis and colleagues focused on mutants in the catalytic domain of PKA (Skoulakis et al., 1993). One mutant, DCO581, was identified in the same enhancer detector screen as rut. Genetic combinations of this allele with another point mutant allele of DCO decreased PKA activity to 20% of normal activity, and flies carrying these alleles demonstrated a significant impairment of memory performance (Skoulakis et al., 1993). Of particular interest was the demonstration that the catalytic subunit of PKA, like dnc and rut, was preferentially expressed in the mushroom bodies (Skoulakis et al., 1993), placing three important components of the cAMP signaling pathway in the biochemical cascade underlying olfactory learning in the mushroom bodies.

PKA, as the primary downstream effector of cAMP modulation, has many targets that may act in concert to bring about the cellular and circuit-level changes that learning and memory require. At the minimum, PKA has been shown in Drosophila to modulate the Ca2+-dependent K+ channel (Zhou et al., 2002), as well as enhance spontaneous transmitter release via increases in Ca2+ influx (Yoshihara et al., 2000). Yao and Wu (2001) have shown that PKA mutations disrupt frequency coding in cultured neurons and cause variable firing patterns in response to identical current stimuli. Another intriguing function of PKA has recently come to light. In an investigation of postsynaptic PKA action, Davis et al. (1998) have shown a retrograde regulation of presynaptic release in a NMJ preparation. Decreases or increases in PKA signaling via expression of PKA mutants in the post-synaptic muscle increases and decreases, respectively, quantal size in the pre-synaptic neuron. Baines (2004) has subsequently demonstrated at central synapses that PKA signaling can regulate synaptic strength both positively and negatively, and also that such a phenotype can be mimicked by post-synaptic rut expression. This effect was phenocopied by expressing a constitutively active TGF-β receptor (thickvein) presynaptically, as well as overexpressing glass bottom boat (gbb), a TGF-β ligand, in the post-synaptic cell. Recent studies have shown that PKA activity can be regulated by TGF-β signaling via the Smad family of proteins (Zhang et al., 2004), raising the possibility that the retrograde signal, initiated by PKA activity in the post-synaptic cell, is mediated in the pre-synaptic cell by altered PKA activity, creating a model in which PKA activity in the two cells is regulated in a coordinated fashion. This series of findings raises the intriguing possibility that cAMP pathway mediated activity may have both anterograde and retrograde roles in regulating synaptic strength and controlling the neural plasticity underlying learning and memory in Drosophila.

4.1.4. CREB

One of the major phosphorylation targets of PKA is the transcription factor CREB. Phosphorylation of CREB by PKA leads to transcriptional activation by the phosphorylated CREB protein. This activated peptide has been implicated in a host of important interactions (see Lonze and Ginty, 2002). In Drosophila, CREB was first cloned by Yin and colleagues, who found that CREB encodes several isoforms (Yin et al., 1995a,b). One putative isoform, dCREB2-α, is predicted to encode a CREB activator protein. A second isoform, dCREB2-β, functions as a dominant negative CREB that blocks PKA-dependent activation of CREB-mediated transcription. Experiments in Drosophila using a heat shock inducible transgene bearing the dominant negative dCREB2-β gene have been shown to specifically block the formation of the protein synthesis-dependent component of long-term memory (Yin et al., 1994). Yin and colleagues have also reported that the induced expression of the dCREB2-α enhances the formation of long-term memory (Yin et al., 1995a,b). However, the sequence of the CREB activator transgene has recently been demonstrated to contain an early frame-shift mutation that results in a premature N-terminal stop codon (Perazzona et al., 2004). Attempts to reproduce the enhanced long-term memory effects with over-expression of the mutant dCREB2-α or a corrected version of the dCREB2-α have been unsuccessful, leaving the role of CREB activator protein in the formation of LTM unclear at the present time (Perazzona et al., 2004).

4.1.5. amnesiac (amn)

An additional link to the cAMP pathway has been identified with the amnesiac mutant. The original mutant was identified by Quinn et al. (1979) in a screen for mutants that are normal for learning, but defective in short-term memory. The amn locus was genetically mapped to the end of the X chromosome at 19A1, but further characterization of the original mutation remained elusive (Quinn et al., 1979). The locus was finally cloned by means of a P-element screen for genetic suppressors of the dnc female sterility phenotype. This screen yielded a P-element insert at 19A1 that failed to complement the amn1 memory phenotype. Analysis of the locus demonstrated that it encodes three putative neuropeptides, one of which has homology to the pituitary adenyl cyclase activating peptide (PACAP) (Feany and Quinn, 1995). This immediately suggested a potential mechanistic link between amn and dnc via modulation of cAMP levels by neuropeptide signaling through a G protein-coupled receptor that signals through the rut adenyl cyclase (Kandel and Abel, 1995).

In an elegant study, Waddell and colleagues demonstrated that the amnesiac-encoded polypeptide is expressed in two neurons of the Drosophila brain, which they termed the dorsal paired medial (DPM) neurons. They demonstrated that transgenic expression of amn in the DPM neurons was sufficient to rescue the amn memory phenotype. Additionally, they demonstrated that disrupting fast neurotransmission from these neurons using a temperature-sensitive Shibire transgene (Shi25) that encodes a dynamin GTP-ase required for synaptic vesicle recycling (Kitamoto, 2001), during memory.
consolidation phenocopied the amn memory phenotype (Waddell et al., 2000), demonstrating an acute role for the DPM neurons in memory processing. Together, these data have added an important new piece to the circuitry of learning and memory in the Drosophila brain.

DeZazzo and colleagues have previously reported that developmental expression of amn rescues the amn memory phenotype (DeZazzo et al., 1999). This conclusion was based on the ability to rescue the amn memory defect in amn-28A mutants, which contain a GAL4 driver inserted at the amn locus, with a UAS-amn transgene, combined with the inability to see rescue when using a heat-shock promoter to drive amn only in the adult. Notably, the authors were not able to turn off expression of the amn28 GAL4 in adults, leaving open the possibility that amn is also required during adulthood. The temporal dynamics of amn expression, as well as the identity of the peptide(s) encoded at the amn locus that are responsible for the phenotype, remain to be elucidated.

4.1.6. NF1

Mutations in the neurofibromatosis 1 gene produce a dominant disorder in humans characterized by nervous system tumors and learning defects. The gene product encoded at the NF-1 locus has been shown to be a component of the presenilin proteins. This potentially connects dysfunctional Notch signaling to the memory loss observed in Alzheimer’s disease. Intriguingly, two recent reports have tied Notch into long-term memory in Drosophila. Temperature sensitive mutants for Notch showed no defects in short term memory immediately after training, but demonstrated impaired long term memory after spaced training (Presente et al., 2004; Ge et al., 2004). A dominant negative Notch molecule was demonstrated to impair 24 h memory after spaced training, but not after massed training, suggesting a specific lesion of the LTM form of consolidated memory and not ARM. Additionally, overexpression of a wild type Notch was shown to enhance 24 h memory after a single training cycle, and this effect was blocked by protein synthesis inhibitors, demonstrating an involvement of Notch in protein synthesis dependent LTM (Ge et al., 2004). Finally, an RNAi construct directed against Notch impaired long term memory when expressed specifically in the mushroom bodies, demonstrating that Notch function in these structures is required for LTM. Thus Notch represents an exciting new member of the learning and memory gene family. However, the mechanisms regulating the activation of Notch, as well as its downstream effects in LTM, remain unknown.

4.2. Cell adhesion molecules and membrane receptors

4.2.1. Volado (Vol)

New evidence for the role of cell adhesion molecules in short-term memory has come to light with the identification of Volado. This gene encodes an α-integrin subunit that disrupts STM when mutated (Grotewiel et al., 1998). Vol was isolated on the basis of preferential expression in the mushroom bodies. Rescue experiments have demonstrated that acute expression of Vol just prior to associative conditioning is sufficient to rescue the memory phenotype, however it has not been shown where this expression is required in order to achieve the rescue effect. Vol has been shown to regulate synaptic transmission and plasticity at the larval neuromuscular junction (Rohrbough et al., 2000).

4.2.2. fasciclin II (fasII)

A second cell adhesion molecule, encoded by the fasciclin II gene, was identified in the mushroom body enhancer trap screen. Behavioral screening demonstrated that mutations in fasII, like those in Vol, disrupt short-term memory. While likely to have developmental roles in the brain, induction of a heat shock fasII transgene prior to, but not after, training was sufficient to correct the memory phenotype, suggesting a role for fasII in memory formation (Cheng et al., 2001). fasII is a homologue of Aplysia ApCAM, which has been shown to be involved in Aplysia plasticity (reviewed in Martin and Kandel, 1996). Dynamic regulation of fasII levels has been shown to regulate pre-synaptic plasticity (Schuster et al., 1996). The FasII protein is expressed strongly in the α/β lobes and weakly in the γ lobe of the mushroom body (discussed in Section 8.3.3). However, it remains to be determined whether the function of fasII in short-term memory localizes to the MB’s.

4.2.3. Notch (N)

Notch receptors have been shown to have a diverse array of functions in both invertebrates and vertebrates ranging from cell type specification via lateral inhibition to regulation of neurite outgrowth. Notch is activated by cleavage of the cytoplasmic domain by gamma-secretase activity, which is a component of the presenilin proteins. This potentially connects dysfunctional Notch signaling to the memory loss observed in Alzheimer’s disease. Intriguingly, two recent reports have tied Notch into long-term memory in Drosophila. Temperature sensitive mutants for Notch showed no defects in short term memory immediately after training, but demonstrated impaired long term memory after spaced training (Presente et al., 2004; Ge et al., 2004). A dominant negative Notch molecule was demonstrated to impair 24 h memory after spaced training, but not after massed training, suggesting a specific lesion of the LTM form of consolidated memory and not ARM. Additionally, overexpression of a wild type Notch was shown to enhance 24 h memory after a single training cycle, and this effect was blocked by protein synthesis inhibitors, demonstrating an involvement of Notch in protein synthesis dependent LTM (Ge et al., 2004). Finally, an RNAi construct directed against Notch impaired long term memory when expressed specifically in the mushroom bodies, demonstrating that Notch function in these structures is required for LTM. Thus Notch represents an exciting new member of the learning and memory gene family. However, the mechanisms regulating the activation of Notch, as well as its downstream effects in LTM, remain unknown.

4.3. Additional mutants

4.3.1. Atypical PKM (aPKM)

Atypical PKM has recently been shown to influence olfactory memory in Drosophila. Studies of LTP in the rat had previously shown that a persistently active isoform of atypical PKC, known as atypical PKM (aPKM), was increased and maintained throughout the maintenance phase of LTP (Sacktor et al., 1993). Drier et al. (2002) recently examined the role of aPKM in memory formation in Drosophila. They found
that induction of either a mouse or *Drosophila* aPKM transgene shortly after training produced an enhancement of 24 h and 4 day memory following massed training to levels equivalent with those produced by spaced training, but they saw no additional improvement of LTM after spaced training. They showed that this effect was not blocked in *radish* (rsh, Section 4.6.1) mutants, demonstrating that aPKM does not function upstream of rsh. Interestingly, the investigators demonstrated that induction of a dominant negative aPKM, or the use of the PKM inhibitor chelerythrine, inhibited 24 h memory after massed training, suggesting an inhibition of ARM (the only memory form present at 24 h after massed training) in the absence of aPKM, leading the authors to favor a model in which the aPKM functions in the same pathway downstream of rsh. Whether aPKM activity results in the production of protein synthesis dependent LTM after massed training was not investigated. Although the normal physiological role of *Drosophila* aPKM in consolidated memory is difficult to assess without genetic mutants, the data strongly demonstrate that aPKM is sufficient to enhance memory, and thus likely to be involved in memory stability under physiological circumstances.

### 4.3.2. *leonardo* (*leo*)

*The leonardo* gene was isolated from the MB enhancer trap screen in the Davis lab on the basis of its expression in the MBs. Cloning of the *leo* gene demonstrated that it encodes an isoform of the 14-3-3 protein family. Mutant alleles of the *leo* gene are associated with defects in olfactory learning, implicating other pathways such as the MAPK and protein kinase C pathways in *Drosophila* olfactory learning and memory (Skoulakis and Davis, 1996). *Leo* mutants have decreased synaptic transmission at the larval neuromuscular junction, as well as defects in synaptic transmission, fidelity, and post-tetanic potentiation, reminiscent of the electrophysiological defects in the cAMP pathway (Broadie et al., 1997). These may be due to its documented interaction with the Slowpoke Ca$^{2+}$ activated potassium channel (Zhou et al., 1999, 2003). Recent experiments have shown that induction of a heat shock-*leo* transgene in adults is sufficient to rescue the learning and memory defect (Philip et al., 2001).

### 4.3.3. *Nalyot*

*Nalyot* encodes a Myb-related *Adf1* transcription factor. *Nalyot* mutants demonstrate mild deficits in early memory but severe deficits in long-term memory. Studies at the neuromuscular junction have demonstrated that *Adf1* plays a role in synaptic growth as well. The lethal phenotype of *Nalyot* null mutants generated by P-element excision was rescued by a heat shock-*Adf1* transgene. Similarly, the memory phenotype in *Nalyot* mutants was rescued by low levels of leaky expression from a heat shock-*Adf1* transgene in adult flies. Interestingly, unlike the case with *CREB, overexpression of Adf1* in the adult can be detrimental to memory (DeZazzo et al., 2000).

### 4.3.4. *radish* (*rsh*)

*radish* represents another member of the original “vegetable” mutants generated in the Quinn lab. Folkers et al. showed that *radish* mutants were selectively deficient in anesthesia resistant memory (ARM) following application of a cold-shock, whereas *amnesiac* mutants were shown to be deficient in the anesthesia-sensitive component (ASM) of consolidated memory (Folkers et al., 1993, for discussion of memory phases, see section 7.3.4). They additionally showed by deficiency mapping that the mutation mapped to a 180 kb region at 11 D-E. Recently, Chiang et al. (2004) have identified a P-element insertion in Phospholipase-A2 (c133) in this region that fails to complement the original *rsh* mutation. The gene product is expressed in a previously uncharacterized set of neurons, a subset of which send projections to the mushroom bodies. The authors showed that they could rescue the c133 phenotype by driving a UAS-PL-A2 transgene; however, whether the PL-A2 can rescue the *rsh* mutant remains to be shown. Additionally, the nature of the *rsh* lesion in the PL-A2 gene remains to be identified.

Folkers, Quinn and colleagues have recently identified a different locus as containing the *rsh* gene (personal communication). They sequenced all open reading frames in *rsh* flies within the interval previously shown to contain the *rsh* mutation and found one change, an amber mutation in the novel gene CG15720. They subsequently showed that heat-shock induced expression of CG15720 in *rsh* mutant flies is sufficient to correct the *rsh* memory phenotype. A polyclonal antibody raised to the peptide inferred from the CG15720 sequence downstream of the amber mutation was shown to label the lobes of the mushroom bodies in wild type flies, and this immunostaining was absent in *rsh* flies. It has yet to be shown that the effect of induced expression of the CG15720 transgene does not generally improve learning and memory performance in wild type flies, which would lend support to the identification of CG15720 as the *rsh* gene.

Currently, it is unclear which locus is responsible for the *rsh* phenotype. The PL-A2 c133 mutants appear to demonstrate a phenotype that mimics the *rsh* memory phenotype. However in the absence of controls for sensory acuity and motor function, it is difficult at present to ascertain whether the deficits seen in these flies are associative or non-associative in nature. The rescue of the c133 phenotype by expression of a PL-A2 transgene demonstrates that the c133 phenotype is linked to this gene. However, the inability at present to identify a lesion in or around the PL-A2 gene in *rsh* flies leaves open the question of the relationship between the *rsh* mutation and this locus. The identification of the amber mutation in *rsh* flies and the subsequent rescue of the memory defect by expression of the CG15720 in these flies suggests that this locus is responsible for the memory phenotype in *rsh* flies, provided that it is demonstrated that CG15720 does not have a general effect on memory performance in wild type flies. At present, the true identity of the *rsh* gene remains unresolved. It is possible at this point that both the PL-A2 and CG15720 gene products are involved in the phenotype in a way that we do not currently understand.

### 4.3.5. stau/pumilio Pathway (*stau/pum*)

Recently, several new genes whose products function in the localization and translation of mRNA were isolated based on a parallel screening process. These genes were identified in either
of the MBs, but rather only seen when nebula expression was reduced. Quanti-
tative PCR demonstrated that expression of scs-fp was normal upstream of succinyl-coenzyme A synthetase flavoprotein subunit (Scs-fp) and 53 bp downstream of CG10460. Qual-
titative PCR demonstrated that expression of scs-fp was normal in this line while expression of the CG10460 was reduced. Sequence comparison revealed that the CG10460 gene predicts a protein product similar to the amino-terminal regions of cathepsins, which normally are synthesized as proenzymes with an inhibitory N-terminal region. This observation led the authors to hypothesize that the CG10460 gene, named crammer (cer), might function as a trans-inhibitor of cathepsins. Indeed, in vitro experiments demonstrated that cer could inhibit cathepsins L and B competitively at nanomolar concentrations. The authors subsequently showed that they could rescue the cer<sup>P</sup> phenotype with a single copy of a genomic construct containing the cer<sup>P</sup> gene. They additionally showed that overexpression of cer<sup>P</sup> in a wild-type background could impair LTM in the presence of two copies of the cer<sup>P</sup> transgene. They demonstrated that this effect was not due to overexpression in the MBs, but rather only seen when cer was overexpressed in glial cells under the control of the repo-GAL4 driver, suggesting a role for glial cells in LTM. At present, it is not clear whether this is due to an effect on the development of glial cells, or due to an acute perturbation of the physiology underlying LTM, although the authors show that global cer mRNA levels are decreased 3 h after spaced training. Also, where cer expression is required for the rescue of the loss of function allele remains unknown. It is not clear what role cer expression in the MB has for learning and memory. These experiments raise the exciting possibility that additional cellular and molecular players exist in the LTM pathway, but at present many questions remain to elucidate more clearly the possible role of cathepsins in this process.

4.3.8. synapsins (syn)

Synapsins are synaptic vesicle associated phosphoproteins that have a variety of functions in vertebrates, including neurite elongation, synaptic formation, and regulation of synaptic release (for review, see Ferreira and Rapoport, 2002). In vertebrates there are 3 isoforms which, when individually
deleted, show normal development but specific alterations in synaptic structure and plasticity. In an effort to avoid compensation effects, Godenschwege et al. (2004) deleted the single synapsin isoform found in Drosophila and discovered that while these flies showed no structural or synaptic transmission alterations, various forms of behavior were altered. Specifically, syn mutants showed an increase in ethanol tolerance, a phenotype seen in many cAMP pathway mutants, as well as a deficit in 3-min memory after classical conditioning, suggesting that plasticity may be regulated at the level of neurotransmitter release.

4.3.9. S6KII

S6KII is a member of the Ribosomal S6 Kinase (RSK) family of proteins and has been implicated in a variety of important cell functions, including cell survival, protein synthesis, and cell cycle progression (Frodin and Gammeltoft, 1999). Importantly, they have also been implicated in synaptic and behavioral plasticity via participation in the well-characterized ERK/MAPK pathway (Impy et al., 1999). Putz et al. (2004) isolated S6KII in a screen designed to identify genes that disrupt operant conditioning. This screen involved conditioning in an operant "heat box" apparatus, in which the fly is allowed to roam freely inside a small box that is heated to 40 °C when the fly crosses to the "punishment" half (Wustmann et al., 1996). The results of behavioral assays were complicated, but in general, flies missing S6KII are without any operant learning phenotype, but those missing the 5' half of the gene have a dominant impairment in operant conditioning. Thus, it is likely that the alleles that are deficient in the 5' part of the gene are impaired due to a gain-of-function. However, both the gain-of-function and the loss-of-function alleles recessively impair performance after olfactory classical conditioning.

4.3.10. latheo (lat)

latheo was the first mutant identified from the P-element insertional mutagenesis behavioral screen carried out by Tully and colleagues (Boynton and Tully, 1992). The recent cloning of lat has demonstrated that it encodes a component of the origin recognition complex (ORC), suggesting a role in DNA replication (Pinto et al., 1999). This result might account for the developmental defects observed in the brains of lat mutants, which show a reduction in mushroom body volume. Unexpectedly, the lat protein was also found at the synapse of motor neurons, and lat mutants were shown to have defects in synaptic transmission and plasticity at the neuromuscular junction (Rohrbough et al., 1999). It is not clear at present why a member of the origin recognition complex would be present at the synapse. Additionally, the mechanism by which this protein exerts its effects at the synapse remains to be discovered.

4.3.11. linotte (lio)

linotte was also identified from the P-element behavioral screen (Dura et al., 1993). Competing claims have been made that lio encodes either an allele of the receptor tyrosine kinase derailed (Moreau-Fauvarque et al., 1998; Simon et al., 1998) or a novel gene (Bolwig et al., 1995). Developmental defects in brain structure, including the mushroom bodies, have been observed in lio mutants (Moreau-Fauvarque et al., 1998; Simon et al., 1998), and these defects have been rescued with the receptor tyrosine kinase (Moréau-Fauvarque et al., 1998). The learning phenotype was initially reported to be rescued with a cDNA of the novel gene (Bolwig et al., 1995) although this result has not been reproducible (Bolwig et al., 2002). Therefore, current evidence suggests that lio encodes a receptor tyrosine kinase required for normal brain development.

5. Neuronal circuitry of olfactory learning and memory

Crucial to an understanding of how and where olfactory learning and memory occurs in the brain is an understanding of the mechanisms by which odors are detected and discriminated. Recent work has produced great strides in elucidating the molecules and circuits underlying olfactory information processing in the Drosophila brain (Fig. 2).

5.1. Olfactory receptor neurons

In Drosophila, odors are detected by a family of G-protein coupled receptors known as Drosophila odorant receptors (DOR). To date, 61 DOR genes have been described (Clyne et al., 1999; Vosshall et al., 1999, 2000; Larsson et al., 2004)
They are expressed on specialized dendrites of approximately 1200 olfactory receptor neurons (ORNs) found inside sensilla covering the third antennal segment and maxillary palps (Stockler, 1994). Most ORN’s express a single unique odorant receptor, along with a common related protein encoded by OR83b (Larsson et al., 2004), allowing each ORN to be distinctly identified by the particular receptor that it expresses. More recently it has been established that one class of ORN’s co-expresses two receptor genes, potentially expanding the coding capacity of these ORN’s (Goldman et al., 2005). The ORNs then project their axons to the antennal lobe, which consists of 43 distinct glomeruli. The observation that ORNs arrive at specific antennal lobe glomeruli prior to the expression of the DOR genes supports the idea that the development of the adult olfactory system does not require sensory input from ORNs (Clyne et al., 1999).

5.2. Projection neurons of the antennal lobe

In the antennal lobe, incoming ORN axons synapse in glomeruli that contain the dendritic fields of antennal lobe projection neurons (PNs), as well as local interneurons (LI). Approximately 180 PNs gather information in the antennal lobe and project to higher centers of the brain through one of three antennal-cerebral tracts (ACT). PN axons that pass through the internal ACT (iACT) form synapses in the MB calyx and the lateral horn (LH). PN axons that pass through the middle and lateral ACTs bypass the MB and travel directly to the LH.

5.3. Interneurons of the antennal lobe

Stocker et al. (1990) have reported that antennal lobe interneurons ramify throughout the antennal lobe, with connections both within and between glomeruli. Local interneurons are largely GABAergic (Jackson et al., 1990). They lack a distinct axon, and appear to receive excitatory input from PNs and provide reciprocal inhibitory connections (Sun et al., 1997; Ng et al., 2002; Wilson et al., 2004). These connections suggest that the antennal lobe may possess computational abilities in addition to serving as a conduit to higher brain centers.

5.4. Mushroom body intrinsic neurons

The MBs of Drosophila are bilaterally symmetric structures consisting of approximately 2500 intrinsic neurons, also known as Kenyon cells, per brain hemisphere (Fig. 2). The cell bodies of these neurons are located in the dorsal posterior aspect of the brain. Just anterior and ventral to the cell bodies, the MB neurons give rise to a dendritic field known as the calyx which receives input from the PNs traveling from the antennal lobe via the iACT. The axons of the neurons project to the anterior portion of the brain via a dense structure known as the peduncle, where they turn and give rise to the lobes of the MB (Crittenden et al., 1998; Lee et al., 1999).

Several studies have demonstrated that the MB lobes can be subdivided in structure on the basis of the expression of different genes or reporter constructs that reflect gene activity in the different lobes. In a screen for enhancer detector GAL4 lines, Yang and colleagues identified a number of GAL4 lines that selectively marked different subsets of the MBs (Yang et al., 1995). An immunohistochemical study of the expression pattern of different genes expressed in the MBs has similarly shown that specific proteins localize to specific subsets of neurons and to specific regions within neurons, allowing definition of the α/β, α’/β’ and γ lobes, as well as suggesting that functional compartmentalization may exist both between and within neurons of the MB (Crittenden et al., 1998). More recently, Strausfeld and colleagues have identified an additional medial lobe that is situated just posterior to the γ lobe, noted for being intensely taurine immunopositive and aspartate immunonegative, in contrast to the other medially-projecting β, β’, and γ lobes. They have termed this structure the β0’ lobe. They also identify an aspartate immunonegative core to the α/β lobes that is strongly positive to anti-glutamate antibodies. They label these distinct glutamatergic core neurons as αc/βc (Strausfeld et al., 2003).

Cell lineage experiments have demonstrated that the MB is derived from four neuroblasts, each of which contributes autonomously to all parts of the overall structure (Ito et al., 1997). Lee et al. (1999) have extended these findings by clonal analysis and demonstrated that each neuroblast sequentially produces three types of neurons. The earliest born neurons, from larval hatching through the mid-third instar larval stage, project to the γ lobe. Neurons born between the mid-third instar
larval stage and puparium formation project into the α′β′ lobes. Finally, neurons born after puparium formation project into the α/β lobes (Lee et al., 1999). The developmental progression of the newly defined β′′ and αε/βε lobes has not yet been determined.

The calyx of the MB has been shown to contain three major types of processes. The first type includes the dendrites of the intrinsic neurons of the MB. The second type includes the extrinsic PNs from the antennal lobe that form large synaptic boutons on the intrinsic MB neurons and are immunoreactive against choline acetyltransferase and vesicular acetylcholine transporter. The PNs form divergent synapses upon many MB cell dendrites. The third type includes GABA immunoreactive neurons that synapse upon the MB dendrites and occasionally upon the PN boutons (Yasuyama et al., 2002).

5.5. Lateral horn neurons

Virtually nothing is known regarding the identity or function of the LH neurons that receive input from the antennal lobe. GABA immunoreactive neurons have been observed projecting from the LH to the MB calyces in the locust suggesting that there is a functional inhibitory input to the MBs in these insects that may play a role in the local field potentials of the MBs (Perez-Orive et al., 2002). However, in the absence of the MBs, flies still respond to odors, suggesting that innate responses to odors may be mediated by the LH (DeBelle and Heisenberg, 1994). The mechanism by which the LH connects to motor regions of the brain is unknown.

5.6. Extrinsic neurons

A number of different MB extrinsic neurons are likely to be involved in olfactory learning and memory. One recently described set of extrinsic neurons shown to be involved in olfactory memory is the dorsal paired medial (DPM) neurons described by Waddell et al. (2000). Each brain hemisphere contains a single DPM neuron that decorates all of the lobes of the MB. These neurons are unusual in that they have no obvious dendritic field, and ramify only to the MBs (Keene et al., 2004). These neurons express PACAP-like peptides, encoded by the amnesiac gene, and exhibit ChAT gene expression, suggesting dual transmitter release (Keene et al., 2004). A role for the PACAP-like peptides olfactory memory has been established by the deficits observed in amnesiac mutants (Quinn et al., 1979). Furthermore, fast neurotransmission has been shown to be required from these neurons, presumably involving acetylcholine as the transmitter (Waddell et al., 2000; Keene et al., 2004).

There are at least two other classes of neuromodulatory neurons that are thought to synapse onto the MB neurons and affect their physiology. Nassel and Elekes (1992) first described dopaminergic neurons whose axonal terminals ramify broadly throughout the MB lobes, offering the possibility that dopamine may be released upon the axons and axon terminals of MB neurons to modulate their function. In addition, Hammer and Menzel (1991) have described octopaminergic neurons in the honeybee that innervate the antennal lobes, the MB calyces, and the LH. Schweizer and colleagues have recently demonstrated a behavioral role for both dopaminergic and octopaminergic neurons in olfactory memory in the fly (Schweizer et al., 2004).

Ito and colleagues have identified by enhancer detection and golgi staining a number of extrinsic neurons that primarily appear to connect the lobes of the MBs to the superior and inferior medial protocerebrum and the superior lateral protocerebrum. A single line was found to identify a neuron projecting from the α-lobe to the LH (Ito et al., 1998). Intriguingly, a GABA-ergic neuron has also been identified that appears to connect the α-lobe to the calyx (Yasuyama et al., 2002). To date, though, the function of these neurons remains unknown.

6. Coding of olfactory information

6.1. Spatial coding

6.1.1. Olfactory receptor neurons

As previously mentioned, most ORN’s express one unique odorant receptor, in addition to a common protein, immediately suggesting how odors in the environment are detected and subsequently channeled via specific neurons to higher centers of the brain, based upon the subset of neurons activated in response to a particular odor (Vosshall et al., 1999; Clyne et al., 1999). Quite strikingly, ORNs expressing a given receptor project to one or two invariant glomeruli in the antennal lobe, demonstrating that the brain maintains a spatial map of receptor activation that can potentially be decoded by the pattern of glomerular activity in the antennal lobe. (Gao et al., 2000; Vosshall et al., 2000).

6.1.2. Antennal lobe

The question of whether the spatial segregation of odorant channels established by the ORNs is preserved in the PNs emanating from the glomeruli of the antennal lobe was recently investigated by Jefferis and colleagues. Using an elegant technique known as MARCM (Lee and Luo, 1999) to generate labeled clones of neurons, they demonstrated that PNs are prespecified by both their neuroblast lineage and their birth order within that lineage to project their dendrites to one or two invariant glomeruli and thus form a synapse with a specific class of ORNs (Jefferis et al., 2001). Thus, the organization of PNs in the antennal lobe is designed to maintain the spatial mapping of odorant receptor activation from the periphery.

Functional studies have confirmed the presence of a spatial map of activation within the antennal lobe as well. Early studies using 2-deoxyglucose mapping in the antennal lobe indicated that different odors elicit specific patterns of glomerular activity (Rodrigues and Buchner, 1984). Subsequent studies using calcium sensitive dyes in the honeybee also demonstrated glomerular specific responses to single odors as well as combinations of odors (Galizia et al., 1999).

Newer generations of optical reporters have been developed that are genetically encoded. A recent study by Ng et al. (2002)
utilized the pH sensitive mutant of GFP fused to vesicular synaptobrevin, known as synapto-pHluorin (spH), to visualize synaptic transmission from olfactory receptor neurons, PNs, and inhibitory interneurons in the antennal lobe. In response to complex odors, they observed synaptic signaling from ORNs that was glomerular specific. Each odor produced a different pattern of synaptic responses that was reproducible and conserved from fly to fly. Surprisingly, the authors found that when spH was driven by the PN-specific GAL4 driver GH146, they observed signal in the antennal lobe, where the PNs send dendritic processes. This is due to the fact that the PN processes in the glomeruli form both receptive and transmissive junctions with the processes from other neurons. The authors utilized the signal from the PN antennal lobe processes to compare responses of ORNs and PNs to the same odorants. They found that the pattern of ORN responses in the antennal lobe was transmitted to the PNs almost completely intact, suggesting that at the level of odorant responses, the spatial map of neuronal activity is transferred to the PNs (Ng et al., 2002). The group also measured the activity patterns of local inhibitory GABA-ergic neurons (LN) and found that these responses were much broader across the antennal lobe than were the ORN and PN responses (Ng et al., 2002). A similar study using the synapto-pHluorin has looked at pure odorants at different concentrations and demonstrated odor-specific and concentration-specific responses in the antennal lobe (Yu et al., 2004). Finally, using the Cameleon reporter, a chimera containing the enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), fused to a calmodulin sequence, that in the presence of calcium allows fluorescent resonance energy transfer (FRET) from ECFP to EYFP, Fiala and colleagues demonstrated reproducible patterns of calcium signals in the antennal lobe glomeruli in response to specific odorants (Fiala et al., 2002).

6.1.3. Mushroom bodies

The question of whether a spatial representation of odors exists in the MBs has also recently been addressed. Wang et al. (2002) used the calcium sensitive dye Calcium Green-1 to detect calcium responses in the cell bodies of the MB neurons in response to different odors. The group found that unique patterns of calcium signal could be detected for different odors and different concentrations, indicating that at least some odors produce a spatial fingerprint of activation. Although these signals appear to be reproducible within the same individual, they appear to vary considerably between individuals (Wang et al., 2002). Fiala et al. (2002) used the genetically encoded Cameleon reporter to investigate calcium signaling in the synaptic boutons of PNs in the calyx of the MB. They found that specific odors produce spatially organized patterns of calcium signaling in the calyx that were reproducible within individuals. Whether these patterns directly correlate with the pattern of firing in the MB neurons, or whether there is sparsening of this representation as observed in the locust MB’s (Perez-Orive et al., 2002) remains unknown. Additionally, the issue of consistency across flies was not addressed (Fiala et al., 2002).

To address the issue of stereotypical spatial patterns across flies, Wang et al. (2004) engineered a transgenic fly expressing a GFP-based Ca^{2+} sensor, which would allow single cell resolution of Ca^{2+} by two-photon microscopy. They found that at the level of the MB soma layer, odor-specific responses were spatially restricted. Furthermore, by using selected odors (cineole and MCH) that activated very few MB neurons, they determined that the spatial stereotypy extends to the single cell level and is conserved across multiple individual flies. Taken together, these studies suggest that there is a spatial representation of the glomerular activity pattern in the MB, although the details of each odor’s representation remain to be solved.

Tanaka et al. (2004) have investigated the internal organization of the MB calyx by isolating GAL4 strains that label subsets of antennal lobe PNs and defining the target area of these neurons in the MB calyx. By comparing the dendritic arborization of specific MB neurons in relation to these PN target zones, they demonstrated that individual MB neurons cover multiple target zones. In tracing these MB cells to their eventual projection sites in the MB lobes, they found that each lobe probably has access to much of the output of the entire antennal lobe. This global sampling may be important for the integrative properties of the MBs.

6.1.4. Lateral horn

Wong et al. (2002) and Marin et al. (2002) have recently examined how the glomerular spatial map is represented in the lateral horn (LH). By labeling clones of PNs and following their projections in the brain, both groups identified an invariant stereotypical pattern of projections in the LH that was specified by the glomerulus from which the PN was derived. Interestingly, sensory deprivation resulting from removal of the antennae and maxillary palp from newly eclosed adults does not disrupt this patterning, suggesting that the maintenance of the projection pattern is not activity dependent (Wong et al., 2002). Tanaka et al. (2004) have confirmed and extended these findings by demonstrating that the LH intrinsic neurons have input zones that are spatially restricted to specific sets of antennal lobe glomeruli, unlike the MBs.

6.2. Temporal coding of olfactory information

6.2.1. Olfactory receptors

In addition to the spatial coding implicit in the organization of ORNs, temporal coding of olfactory information appears to take place in the olfactory receptor neurons (ORNs). Odorant stimulation of these neurons produces an array of responses, which can include excitation, inhibition, and responses that vary in temporal dynamics and intensity. Electrophysiological analysis of ectopically expressed odorant receptor molecules in a receptor-null mutant ORN has demonstrated that the receptor type is responsible not only for the basal firing rates and odorant spectrum response, but also the coding response variations of the ORN (Hallem et al., 2004).

6.2.2. Antennal lobe

While it has become evident that the Drosophila brain, in similar fashion to the vertebrate brain, maintains odor-topic maps in the brain, there is also evidence that odor information is...
encoded in insect brains by the temporal response patterns of PNs emerging from the antennal lobe (Laurent and Naraghi, 1994). In vivo response recordings of PNs to specific odors in the locust antennal lobe have revealed complex temporal patterns of firing that evolve over time. These patterns consist of periods of inhibited firing and periods of enhanced firing. Additionally, a given PN representing the activity of a specific ORN will respond to different odorsants with a completely different temporal pattern of firing (Perez-Orive et al., 2002). The temporal pattern of PN firing is believed to be generated based on the integration of excitatory input from ORNs and inhibitory input from local neurons. The firing patterns of the PNs occur in synchrony with oscillations in the local field potential (LFP). The disruption of inhibitory signaling in the antennal lobe by picrotoxin results in a loss of synchronization of the LFP and the firing pattern of PNs (Laurent et al., 1998).

Wilson et al. (2004) used an electrophysiological approach to address the tuning properties of the antennal lobe in Drosophila. Recordings from PNs revealed a broad range of responses to individual odors, with two general categories: "specialists", which responded to few or none of the 33 odors tested, and "generalists", which responded to many. Of note, they found that PNs could be strongly excited by odors that were poor stimuli for their presynaptic ORN, and that the shape of the response histograms were often different between a particular ORN and its PN. This nonlinear transformation was ascribed to the GABAergic reciprocal interneurons. Overall, the PNs were more broadly tuned than the individual ORNs that projected to them. This broader tuning contrasts with the imaging studies noted above, a finding the authors attribute to the increased sensitivity of their approach.

6.2.3. Mushroom body and lateral horn neurons

In contrast to the robust and broad responses of PNs to odorants, Perez-Orive et al. (2002) report that MB neuron responses in the locust are sparse due in part to inhibitory input from GABAergic neurons from the LH, suggesting that the LH may have a gating function in olfactory processing. It is possible that the antennal lobe acts as the central director, activating the MB responses as well as inhibiting the tonic repression from the LH. These results have suggested a mechanism whereby the combinatorial input of excitatory drive from PNs and inhibitory input from the LH lead to a sparsening of odor representation in the MB neuron firing pattern.

The responses of MB neurons are also phase-locked to the local field potential (LFP) in the locust. In the presence of picrotoxin administered near the MB calyx, the MB showed a broader response pattern to the same odors, and this pattern was no longer phase-locked to the LFP (Perez-Orive et al., 2002). Functionally, it has been observed that desynchronization of PN neuron firing by the administration of picrotoxin to the antennal lobe of honeybees does not affect the ability of the bee to discriminate between dissimilar odors, but does impair the discrimination of similar odors, suggesting that the oscillations may be involved in fine discrimination of odorants (Stopfer et al., 1997).

7. Functional roles in olfactory learning and memory

7.1. Antennal Lobe

Recent studies have indicated that the antennal lobes are not simple transit stations for the passage of olfactory information. Yu et al. (2004) investigated the responses of projection neurons in the antennal lobes to classical conditioning using fluorescent synaptic transmission reporter, synapto-pHluorin (Ng et al., 2002). They found that odor-specific subsets of the antennal lobe glomeruli were activated in response to either octanol or methylcyclohexanol, yet when each odor was associated with forward conditioning, an additional glomerulus was activated (D and VA1, respectively). The duration of these memory traces was approximately 7 min and backward conditioning controls were unable to elicit activation of D or VA1. This work builds on prior studies in the honeybee that have suggested that memory traces may be formed in the antennal lobe (Faber and Menzel, 2001), and argues for a novel mechanism of memory trace formation via synaptic recruitment.

7.2. DPM neurons

Waddell et al. (2000) demonstrated that the activity of DPM neurons is not required for initial learning but is required during the interval between training and testing for normal levels of memory to be observed beginning approximately thirty minutes after conditioning. In an extension of that work, Keene et al. (2004) demonstrated that DPM activity is dispensable for learning or recall, but vital only during the interim period, when storage or consolidation is thought to occur. They further showed that this requirement is odor specific. Replacing either octanol or methylcyclohexanol with benzaldehyde (BA) changed the DPM output requirement from the consolidation period to the training interval. The authors attribute this difference at least partially to the different mechanism by which BA is sensed, via both classical olfactory receptors on the antenna and maxillary palps as well as by chemosensory bristles defined by the pox-neuro (poxn) mutant. This non-canonical input pathway appears to change how this scent is processed, raising questions about additional circuitry for olfaction.

7.3. Mushroom bodies

7.3.1. Early Studies

A large collection of evidence has now been gathered that removes any doubt that the MBs play an important role in olfactory memory. Speculations on the importance of the MBs date back as early as the 1850’s with Dujardin. Early studies using localized cooling in the honeybee demonstrated that cooling of the MBs disrupts memory after conditioning in an olfactory reward paradigm (Erber and Menzel, 1980). The subsequent demonstration that mutants with defects in MB structure were deficient in olfactory learning (Heisenberg et al., 1985) supported this link between olfactory memory and the MBs. The observation that the dnc-encoded cAMP phosphodiesterase
was preferentially expressed in the MBs provided the first link between the learning and memory mutants identified through behavioral screening and the MBs (Nighorn et al., 1991). Hydroxyurea ablation of the mushroom bodies during development was also shown to abolish olfactory learning (DeBelle and Heisenberg, 1994). More recently, targeted expression of a constitutively active Gαs subunit to the MBs was shown to impair olfactory learning (Connolly et al., 1996). Additionally, it has been shown that the expression of a rut transgene specifically in the MBs of rut mutants is sufficient to rescue the memory defect in these mutants (Zars et al., 2000). Finally, the identification of the DPM neurons as sufficient for ann rescue and critical for memory with their specific projection to the MBs provides another layer of evidence for the critical role of the MB neurons. Taken together, these data provide strong evidence that the MBs are critical neurons in the circuitry underlying olfactory learning and memory (Fig. 4).

7.3.2. Mushroom bodies and memory acquisition, storage, and retrieval

The question of when MB signaling is required for memory acquisition, storage, and retrieval was addressed recently by Dubnau and colleagues, and McGuire and colleagues. Both groups used Shi²⁵ to transiently disrupt synaptic transmission from the MBs during the course of conditioning. Both groups found that disruption of synaptic transmission from the MBs specifically impaired memory retrieval, but not memory acquisition or storage, suggesting that retrieval of short-term memories requires signaling through the MBs and that acquisition and storage of STM occurs upstream of the output of the MB neurons (Dubnau et al., 2001; McGuire et al., 2001). McGuire and colleagues also found that disruption of signaling in the αβ lobes could completely block memory retrieval at 3 h, suggesting a role for these neurons in the retrieval of consolidated memory. Schwaerzel and colleagues found a similar result using the more broadly expressed GAL4 drivers, and also confirmed that 3 h consolidated memory could be completely blocked (Schwaerzel et al., 2002).

Schwaerzel and colleagues went on to show that extinction of a memory trace could occur even when MB synaptic transmission was blocked, suggesting that extinction also occurred upstream of the MB output. Extinction was blocked when the UAS-Shi²⁵ was driven in PNs from the antennal lobe which synapse on both the MB calyces and the lateral horn.

Fig. 4. Molecular and cellular model of olfactory memory formation in the Mushroom Bodies. Olfactory information (the conditioned stimulus) is transmitted from the antennal lobes to the mushroom body calyx via antennal lobe projection neurons along the antennal-cerebral tract. Coincident reinforcement from the unconditioned stimulus (electric shock or sucrose) is projected to the MB’s via dopaminergic (negative reinforcement) or octopaminergic (positive reinforcement) neurons. The coincidence of these events is detected by the synergistic activation of the rutabaga-encoded adenyl cyclase by simultaneous G-protein signaling, mediated through NF1, and Ca²⁺ influx, leading to elevated levels of cAMP. Appropriate modulation of cAMP levels by the dunce-encoded phosphodiesterase leads to the activation of PKA, which in turn phosphorylates a number of substrates involved in STM. Modulation of protein phosphorylation by the calcipressin family member nebula is important for STM. The cell adhesion proteins FasII and the α integrin-encoding volado are also involved in STM, as is the leonardo-encoded 14-3-3 protein. Consolidation of STM into middle-term memory (MTM or ASM) requires persistant signaling from the annesia expressing DPM neurons. The formation of anaesthesia-resistant memory (ARM) involving the radish pathway has yet to be localized, but may occur in the MB’s. Protein synthesis-dependent LTM involves modulation of the activity of the CREB repressor protein and possibly the CREB activator protein. Additional molecules involved in mRNA translocation (stau, oskar), protein synthesis (pum, eIF-5C), and cell adhesion/cell signaling (notch) are also required for normal LTM. Additionally, the glial expressed cysteine protease inhibitor crammer is the first non-neuronal gene shown to be required for normal LTM. STM and ARM are intact in the presence of either the medially-projecting β/β' collaterals or the vertically projecting α/α' collaterals. LTM induced by spaced training requires the vertical α/α' lobes and is intact in the absence of the β/β' lobes. Figure adapted from Davis (2004).
(LN), suggesting that synaptic transmission to one or both of these neuropils is required for extinction to occur (Schwaerzel et al., 2002). The simplest explanation favored by the authors is that extinction is a process of memory trace suppression occurring in the same neurons in which memory formation takes place.

Additional evidence supporting the idea that memory formation takes place in the MB’s has come from studies on the rut gene product. McGuire et al. (2003) and Mao et al. (2004) demonstrated that the expression of rut only in the MBs and only during adulthood is sufficient to rescue the rut learning impairment. They utilized new transgenic techniques for spatial and temporal regulation of gene expression (McGuire et al., 2003; Mao et al., 2004; see McGuire et al., 2004, for a general review) to control the expression of rut in the MBs of the adult fly, but not during development. Taken together, these results strongly suggest that the acute events mediated by the cAMP pathway underlying learning and short-term memory occur within the MBs (McGuire et al., 2003; Mao et al., 2004).

7.3.3. Mushroom body substructure

Zars and colleagues showed using the UAS/GAL4 approach that rut expression in the MBs is sufficient to rescue the learning impairment by rut mutation (Zars et al., 2000). They further showed that expression of rut in the γ lobes using the H24 GAL4 driver was sufficient to rescue the rut memory defect. In contrast, they found that expression in a subset of the α/β lobes with the GAL4 drivers 17D and 189Y was not sufficient to rescue the memory defect. Based on these results, the authors proposed that the γ lobes were the principle sites for short-term olfactory memory.

The first hint that the α/β lobes might be important for short-term memory came from studies of fasII, which shows very strong staining of the α/β lobes of the MBs and very weak expression in the γ lobes. The first functional demonstration of a specific role for the α/β lobes in short-term memory came when McGuire et al. (2001) showed that the expression of Shi\(^{\alpha}\) specifically in the α/β lobes of the MBs with the GAL4 driver c739 impaired retrieval of immediate memory and completely blocked retrieval of 3 h memory (McGuire et al., 2001). Isabel, Preat and colleagues found that expression of Shi\(^{\alpha}\) with the c739 driver also reduced memory retrieval 24 h after training (Isabel et al., 2004). Moreover, Chang et al. (2003) showed by using the c739 GAL4 line that they could both rescue the nla memory defect and impair immediate memory by over-expressing nla in α/β neurons in a mutant or wildtype background respectively, demonstrating the importance of α/β neurons for nla mediated short-term memory.

In a separate set of experiments, McGuire et al. (2003) used both the α/β specific c739 GAL4 line and the γ lobe-preferential H24 GAL4 line for rescue experiments of the rut memory impairment. Using either GAL4 driver alone, the authors failed to see significant rescue of the rut memory defect, in contrast to the complete rescue observed with the 247 GAL4 driver, which drives expression in a subset of both the α/β and γ lobes. However, when the c739 and H24 GAL4 drivers were combined in the same fly, providing expression in both the α/β and γ lobes, the authors observed complete rescue of the immediate memory defect, demonstrating that both lobe sets are required for the rut mediated memory pathway (McGuire et al., 2003, Supplemental data). It is possible that the rut-mediated short-term memory pathways require both the α/β and γ neurons, while nla is required only the α/β lobes. It should be noted however that Chang and colleagues did not report any attempts to rescue the nla deficiency with a γ-lobe specific driver.

Pascual and Preat (2001) have subsequently suggested, based on experiments with the alpha-lobes absent mutant (ala), that long-term memory requires the MB’s and specifically the α and α’ vertical lobes. Isabel et al. (2004) have shown that blocking neurotransmission in the MBs with Shi\(^{\alpha}\) produces a defect in long-term memory after spaced training. They found that expression of Shi\(^{\alpha}\) with the α/β specific c739 GAL4 driver produced a moderate defect in long-term memory performance, demonstrating a role for these neurons in long-term memory as well as short-term memory, as previously demonstrated. Using the GAL4 driver 247, which drives expression in a majority of MB neurons, they found an approximately 50% reduction in LTM. This raises the possibility that some of the memory observed after spaced training localizes to outside of the MB’s.

7.3.4. Memory phases

One model of memory processing in Drosophila (Fig. 3A) has postulated that following either single trial or massed training, three distinct memory phases are induced. These include short-term memory (STM), middle-term memory (MTM), and anesthesia-resistant memory (ARM). STM is observed shortly after training and is disrupted in the dnc (dnce) and rutabaga (rut) mutants. MTM follows STM and is impaired in the ann mutant, also linked to the cAMP pathway. ARM initially coexists with MTM but persists longer, lasting out to and beyond 24 h, and is disrupted by the rsh mutation. This model envisions a linear pathway beginning with STM, followed by MTM (ASM and ARM), and finally ending with ARM (or protein synthesis-independent LTM). Protein synthesis-dependent LTM is specifically induced by a spaced training protocol and has been shown to coexist with ARM 24 h after training (Quinn and Dudai, 1976; Tully et al., 1994). A more recent model has been proposed by Isabel et al. (2004, Fig. 2B). They note that ARM is normal in rut and ann mutants, leading to the suggestion that rsh identifies a pathway independent of the cAMP encoded STM-MTM pathway. The second change they propose involves consolidated memory. Previous work has shown that consolidated memory consists of two components. The first is anesthesia resistant memory (ARM), which is produced by single-trial, massed, and spaced training and is specifically disrupted by the rsh mutation. The second type of consolidated memory is produced by spaced training, and is inhibited by protein synthesis inhibitors and the dCREB repressor protein. Following spaced training, the use of protein synthesis inhibitors results in a 50% reduction in observed consolidated memory. Similarly, loss of radish protein results in a 50% reduction of memory. The combination of protein synthesis inhibitors or dCREB-2b
expression with the radish mutation results in a complete loss of consolidated memory, arguing that two independent forms of consolidated memory coexist (Tully et al., 1994). The challenge to this model offered by Isabel et al. (2004) was based on surprising results with ala mutants. As previously shown (Pascual and Preat, 2001), ala mutants lacking a/a' lobes fail to demonstrate LTM after spaced training. However, these same mutants show normal ARM five hours after single trial training or massed training. Surprisingly, when these mutants are subjected to spaced training, memory at 5 h after training is also abolished. They reason that since much of the memory observed at 5 h is ARM, then spaced training, or LTM, must erase ARM, in contrast to the earlier suggestion that they coexist. These results suggest that spaced training results in a/a' lobe-dependent memory, while single trial training and massed training produce memory that is normal in the absence of the a/a' lobes (Fig. 3B). While intriguing, these results are based on very small numbers of the ala mutants missing only the vertical lobes and clearly require independent verification with mutants that demonstrate a higher penetration of loss of the a/a' lobes, or other methods that selectively block output from the vertical lobes.

7.4. Unconditioned stimulus (US) pathways

Schwaerzel et al. (2004) have recently added much needed information at the systems level of how information representing the unconditioned stimulus is transmitted to the MBs, both for aversive (shock) conditioning and appetitive (sucrose) conditioning, using the same odor conditioned stimulus. First, the group demonstrated that both aversive and appetitive conditioning require rut expression in the same subset of MB neurons for learning and short-term memory to occur. They then demonstrated that blocking synaptic transmission from these same sets of MB neurons blocked retrieval, but not acquisition or storage, of both aversive and appetitive memories. This demonstrated that both aversive and appetitive learning (and short-term memory) were occurring within the same subset of approximately 700 MB neurons. Next, they sought to elucidate the biogenic amine pathways that differentiated the opposite responses to the same odors under appetitive or aversive reward conditioning paradigms. For this, they first employed flies carrying a mutation in tyramine-β-hydroxylase (TβH), a gene encoding an enzyme required for octopamine synthesis. They then tested these flies in an assay adapted from earlier work on reward learning in Drosophila that employed sucrose as a reward (Temple et al., 1983). They found that this mutation blocked appetitive conditioning, but not aversive conditioning, and could be rescued by heat shock of a TβH transgene or by feeding flies octopamine just prior to, but not after, training. This result bears some analogy to findings in the honeybee, where octopaminergic signaling has been shown to convey the US signal in appetitive sucrose conditioning (Hammer and Manzel, 1998). Strikingly, Schwaerzel and colleagues then demonstrated that blocking dopaminergic signaling using a tyrosine hydroxylase-GAL4 driver/UAS-Shi combination, which is expressed in dopaminergic neurons, results in a defect in acquisition, but not retrieval, of aversive olfactory memory while leaving appetitive learning intact. These studies have added a new piece to the puzzle of olfactory learning by demonstrating a differential role for these two biogenic amines in mediating the acquisition of either an aversively or appetitively conditioned memory. It remains to be determined which specific subsets of dopaminergic and octopaminergic neurons carry this information, and whether they ramify on the same sets of MB cells, or different subsets within the approximately 700 neurons studied in these experiments.

8. Summary

A large body of information has emerged over the past thirty years that has given insight into the genes, enzymes, biochemical pathways, neurons, and systems-level circuitry that underlie olfactory learning and memory in Drosophila. A model is presented in Fig. 4 that attempts to incorporate much of this information. It is now beyond dispute that the mushroom bodies have a central role in olfactory learning and memory in Drosophila. Additionally, a much finer view of the circuitry underlying the perception of olfactory information from the environment and how it is communicated in the brain has emerged. We are beginning to glean some functional insights into these pathways as well, as new technologies emerge that allow the experimenter to probe the activity and function of individual neurons, as well as control the expression of genes in both time and space. Unfortunately, we still do not have a clear grasp yet of how specific odors are represented in the MB’s or how a given odor can be positively or negatively reinforced in the MB. Furthermore, we do not yet understand how odors are processed and transformed before they reach the MB’s. Nor do we know the identity of downstream effector neurons that elicit the behaviors we study, and almost certainly additional extrinsic neurons that factor into to the memory process will come to light. A great headway has been made, however, in light of what we still do not know, the next 30 years promise to be as exciting as the last.

References


