The Role of Ras Signaling in Associative Learning and Memory in *Drosophila*

by

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ABSTRACT

Ras is an evolutionally conserved signaling molecule that has been implicated in a variety of cellular events, such as cell proliferation, differentiation, and survival. Recent studies also suggest roles of Ras in neuronal plasticity. I investigated the role of Ras in associative learning and memory in *Drosophila*. Flies carrying hypomorphic *ras* mutations were impaired in memory, with normal learning performance. The severity of the memory impairment correlated with molecular lesions in the *ras* gene. Studies on synaptic morphology at larval neuromuscular junctions revealed an increased number of presynaptic varicosities in the *ras* mutants. Flies carrying a dominant-negative or -active form of a *ras* transgene showed a learning impairment, when expression of the transgene occurred in the mushroom bodies (MBs), the center for the associative learning and memory. Acute induction of dominant-active *ras* expression also resulted in a learning impairment. Simple sensorimotor functions and overall MB morphology were normal in all the flies that showed learning or memory impairments. These results collectively suggest a direct role of Ras signaling in associative learning and memory in *Drosophila*. Here, I also present the results of characterization of a strong eye phenotype resulting from inhibition of Ras signaling. When a dominant-negative form of mammalian *ras* was expressed in the eye imaginal disc, flies developed eyes that are severely reduced in size. The phenotype was modified in a way sensitive to the level of Ras signaling. It was based on massive cell death resulting from inhibition of EGFR/Ras-dependent signaling pathways, including the MAPK and PI3-K pathways. Additional components such as Amnesiac and Rap appeared to modulate the signaling machinery associated with the phenotype. Oncogenic *ras* has been implicated in many types of tumors. This Ras-associated small eye phenotype may provide a new powerful tool to help develop therapeutic strategies for human cancers, as well as further understand the complexity of Ras signaling in basic biological events.

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Acknowledgments

When I came to the United States 10 years ago, I did never dream of graduating from MIT with a Ph.D. in brain and cognitive sciences. I was a physical therapist with a B.A. in English literature. My bachelor's thesis was about D.H. Lawrence. I finished my Master with a paper on biomechanics of human motor control. Now, I am presenting a Ph.D. thesis on genetic analysis of learning and memory in fruit flies. I believe that everything I have learned will serve as a valuable asset for me to pursue next levels of challenges in my life.

I would like to thank many people who helped me to go through the last several years here at MIT. First, I would like to thank my thesis advisor, Chip Quinn. As his graduate student, I was privileged to enjoy his great intelligence and unique personality. Without him, I could not have finished my research as it is presented here. Second, I would like to thank Ann Graybiel, Susumu Tonegawa, and Troy Littleton for their intellectual support and encouragement as my thesis committee members. Especially, I would like to give my special thanks to Ann, who was my first-year advisor in the graduate program. Without her, I could neither have joined this extraordinary academic community nor initiated the thesis research as smoothly as I could.

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CHAPTER 1

Introduction

1-1. Biology of Ras

ras encodes a small G-protein that is evolutionary conserved from yeasts to humans. The gene product contains guanine nucleotide binding, effector recognition, and membrane attachment domains. The amino acid sequences of Ras homologs are highly similar between organisms, particularly in the amino (N) -terminal region that includes the nucleotide binding and effector domains. Humans and rodents carry three functional *ras* genes, designated *H-ras*, *K-ras*, and *N-ras*. These isoforms all encode 21 kilo-dalton (kD) proteins, which are highly homologous each other. The fruit fly, *Drosophila melanogaster*, has only one *ras* homolog, known as *ras1*, also encoding a 21-kD protein. The N-terminal region of the Ras1 protein is 95% identical to that of human H-, K-, or N-Ras (Barbacid, 1987; Lowy & Willumsen, 1993).

The biochemical properties of Ras closely resemble those of G-proteins mediating transmembrane signal transduction. Ras cycles between the GDP-bound inactive conformation and the GTP-bound active conformation. It binds to the inner face of the plasma membrane and functions downstream of transmembrane receptors to transduce extracellular signals to intracellular effectors (Barbacid, 1987; Lowy & Willumsen, 1993). Recent studies suggest that the interaction of Ras with the plasma membrane is highly dynamic. Ras proteins are synthesized as cytosolic precursors and undergo a series of post-translational processing that transfer them to the plasma membrane for activation.

In some cases, however, Ras also generates a signal from intracellular membranes (Hancock, 2003; Hingorani & Tuveson, 2003; Silvius, 2002).

The function of Ras is regulated directly by two classes of proteins. One is the guanine nucleotide exchange factor (GEF) and the other is the GTPase activating protein (GAP). The GEF class includes Sos, Ras-GRF, and Ras-GRP. They mediate the conformational change from the inactive Ras to the active Ras by facilitating the exchange of Ras-bound GDP with GTP. The GAP class includes Gap, NF1 and p120 Ras-GAP. They accelerate the hydrolysis of Ras-bound GTP by the intrinsic Ras GTPase activity, leading to inactivation of Ras. Ras can activate a number of downstream effectors, such as Raf, Ral-GDS, and Phosphoinositide 3-kinase (PI3-K). Although the Raf pathway that leads to the activation of the mitogen-activated protein kinase (MAPK) appears to be the major pathway that mediates Ras signaling, other pathways are also believed to assume significant roles. Each of these effector pathways appears to be activated in different cellular contexts (i.e., cell type, developmental stage, intensity of Ras activation, etc.). This differential activation or more possibly, combinatorial activations of these pathways may provide a mechanism of how activation of Ras can result in different biological consequences as illustrated below (Malumbres & Barbacid, 2003; Shields et al., 2000).

Ras in cell proliferation, differentiation, and survival

Cell proliferation. The biological importance of Ras was first appreciated in studies on human cancers. In the early 1980s, researchers found that NIH-3T3 mouse fibroblasts were transformed with DNA isolated from human tumor cell lines, and that a specific element of DNA was responsible for the transformation (Shih et al., 1979; Shih et al., 1981; Perucho et al., 1981). The molecular cloning of transforming genes from human bladder tumor cells directly demonstrated the existence of such a genetic element in human tumors (Goldfarb et al., 1982; Pulciani et al., 1982; Shih & Weinberg, 1982). The

identities of these oncogenes were pursued using probes for well-characterized retroviral oncogenes to hybridize DNA from NIH-3T3 cells that had been transformed with DNA from various human tumors. Cells transformed with bladder or lung carcinoma DNA contained extra sequences that hybridized to probes specific for the v-*h-ras* or the v-*k-ras* oncogenes, respectively, showing that the human oncogenes were related to the cellular homologs of the retroviral oncogenes (Der et al., 1982; Parada et al., 1983; Santos et al., 1982). Subsequent studies identified that the oncogenic property of human Ras could be conferred by a single point mutation (Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982; reviewed by Malumbres & Barbacid, 2003).

Molecular cloning of the *ras* genes facilitated biochemical characterizations of Ras proteins. It was found that Ras proteins were GTPases, and the GTPase activity was greatly reduced in the transforming alleles (Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984). With the observed structural homologies between Ras and the α-subunit of G proteins (Hurley et al., 1984), these findings led to a hypothesis that Ras mediates transmembrane signal transduction that leads to cell proliferation. In fact, epidermal growth factor (EGF), an extracellular mitogenic signal, stimulated binding of GTP to Ras proteins (Kamata & Feramisco, 1984). Microinjection of a monoclonal anti-Ras antibody blocked serum-induced DNA synthesis (Mulcahy et al., 1985). Furthermore, Ras activity was required for transformation induced by oncogenes that were derived from transmembrane growth factor receptor tyrosine kinases, but not by cytoplasmic kinases (Smith et al., 1986; reviewed in Malumbres & Barbacid, 2003).

The mechanism linking growth factor receptor and Ras activations was revealed by the identifications of GEFs and adaptor proteins. The first Ras-GEF, Cdc25, was identified by genetic studies in yeast (Broek et al., 1987; Robinson et al., 1987). Mammalian Ras-GEFs, such as Sos1 and Sos2, were isolated by functional complementation of Cdc25 or by sequence homology (Bowtell et al., 1992; Martegani et al., 1992; Wei et al., 1992). Sem-5 in *Caenorhabditis elegans* (*C. elegans*) and its

mammalian homolog Grb2/ASH were identified through various screens as proteins containing a single src-homology-2 (SH2) domain and two SH3 domains, which had been known to be involved in protein-protein interactions (Clark et al., 1992; Lowenstein et al., 1992; Matuoka et al., 1992). They were readily characterized as adaptor proteins that bind receptor tyrosine kinases through the SH2, and Sos through the SH3 domains, revealing the signaling pathway, growth factor receptor-Grb2-Sos-Ras (reviewed in McCormick, 1993; Malumbres & Barbacid, 2003).

The identification of Raf as a downstream effector of Ras was a next major breakthrough in the field of Ras research. It was shown that Raf, which had also been known as the cellular homolog of a retroviral oncogene, specifically interacted with GTP-bound active Ras (Moodie et al., 1993; Warne et al., 1993; Zhang et al., 1993). A number of studies found that Raf activates MAPK via MAPK kinase, which then activates both cytoplasmic and nuclear targets. These studies, taken together, clearly provided a molecular explanation for how external mitogenic signals are transduced into the cell, as well as a possible explanation for tumorigenesis (reviewed by Malumbres & Barbacid, 2003).

Cell differentiation. Ras has also been shown to mediate cellular differentiation in several experimental systems. The PC12 cell line, derived from a pheochromocytoma tumor of the rat adrenal medulla, is a useful model system for studying signal transduction leading to biological events, such as cell proliferation, differentiation, and survival (Vaudry et al., 2002). When treated with nerve growth factor (NGF), PC12 cells halt dividing and acquire sympathetic neuron-like characteristics, developing electrically excitable neurites and functional synapses (Dichter et al., 1977; Greene & Tischler, 1976; Schubert et al., 1977). Surprisingly, introducing *ras* oncogenes into PC12 cells mimicked the NGF-induced neuronal differentiation, that is, halted-cell division and neurite outgrowth (Bar-Sagi & Feramisco, 1985; Guerrero et al., 1986; Noda et al., 1985).

Furthermore, introduction of an anti-Ras antibody into PC12 cells inhibited NGF-induced neuronal differentiation, indicating that Ras is required for this event (Hagag et al., 1986). Subsequent studies revealed that the NGF signal is mediated by the receptor tyrosine kinase TrkA and then Grb2 and Sos. They also revealed that the activation of Ras leads to the activation of a subfamily member of MAPK, ERK (reviewed in more detail by Patapoutian & Reichardt, 2001; Vaudry et al., 2002).

Induction of vulva differentiation in *C. elegans* also requires Ras. In vulva formation in *C. elegans*, three of six vulva precursor cells (VPCs) are induced by a signal from an anchor cell, and then the induced VPCs undergo three rounds of mitosis to give rise to the mature vulva. Loss-of-function mutations of *ras* (*let-60*) cause a vulvaless phenotype, and can suppress extra-vulva (multivulva) phenotypes. To the contrary, gain-of-function mutations of *ras* cause a multivulva phenotype, and can suppress vulvaless phenotypes (Beitel et al., 1990; Eisenmann & Kim, 1997; Ferguson & Horvitz, 1985; Han et al., 1990; Han & Sternberg, 1990; Han & Sternberg, 1991; Jongeward et al., 1995). A *C. elegans* homolog of the EGF receptor (*let-23*) is also required for vulval induction. Gain-of-function mutations of *ras* suppress the vulvaless phenotype of the EGF receptor mutants (Ferguson et al., 1987; Han et al., 1990). Activated EGF receptor functions are suppressed by dominant-negative mutations of *ras* (Katz et al., 1996). These findings indicate that Ras mediates EGF receptor signaling to mediate vulval induction in *C. elegans*. To date, a number of other genes, including *grb2* (*sem-5*), *raf* (*lin-45*), *MAPK kinase* (*mek-2*), and *MAPK* (*mpk-1*), have been identified based on genetic interactions with the *ras* pathway that mediates vulval induction (reviewed in Moghal & Sternberg, 2003; Sternberg & Han, 1998).

Finally, studies on the cell fate specification of *Drosophila* R7 photoreceptor neurons provide another good example of a critical role of Ras in cellular differentiation. The *Drosophila* compound eye is composed of about 750 subunits called ommatidia. Each ommatidium contains eight photoreceptor neurons (R1-R8) and a complement of

non-neural support cells, which are arranged in a fixed pattern. The photoreceptor cells are individually distinct, each of which is defined by its position in the ommatidium, pattern of gene expression, and spectral property. The R7 neuron, the last photoreceptor to differentiate, is situated adjacent to R1, R6 and R8, and is the only photoreceptor cell sensitive to UV light (reviewed in Freeman, 1997; Voas & Rebay, 2004; Zipursky & Rubin, 1994). Mutant flies having a mutation in the gene *sevenless* (*sev*) were isolated in a screen for abnormal UV phototaxis (Campos-Ortega et al., 1979; Harris et al., 1976). Their R7 precursor cells are transformed into non-neuronal cone cells rather than R7 photoreceptor neurons (Tomlinson & Ready 1986; Tomlinson & Ready 1987). Genetic mosaic analysis revealed that the function of the *sev* gene is cell-autonomous, acting only in the R7 cell (Campos-Ortega et al., 1979; Tomlinson & Ready 1987). Consistent with this, molecular studies revealed that the gene encodes a receptor tyrosine kinase (Hafen et al., 1987), which binds to the ligand Boss that is expressed in the adjacent R8 cell (Reinke & Zipursky, 1988). Subsequent genetic screens for enhancers of the *sev* phenotype identified the *Drosophila* homolog of *ras*, *ras1* (Simon et al., 1991). Expression of a constitutively-active *ras1* transgene in the eye imaginal disc resulted in the transformation of cone cell precursors into R7, as well as suppressing the *sev* phenotype in sev^- mutants (the cell fates of the other photoreceptor neurons were not affected)(Fortini et al., 1992). These results mimicked the consequence of expression of a constitutively-active form of a *sev* transgene in sev^- mutants: rescue of R7 development and cone cell transformation into R7 (Basler et al., 1991). Now, we have a more detailed understanding of the Sev-Ras signaling pathway with its additional components, such as the adaptor protein Drk, GEF Sos, and the Raf/MAPK effector pathway (reviewed in Freeman, 1997; Voas & Rebay, 2004; Zipursky & Rubin, 1994).

Cell survival. Viability of cells is actively regulated by cell survival and cell death-inducing signals. For example, in *C. elegans*, two proteases, Ced-3 and Ced-4, play a

critical role in executing an intrinsic cell-death program or apoptosis, while the mammalian Bcl-2-like protein, Ced-9, protects the cell from dying by downregulating the proteases (Ellis & Horvitz, 1986; Ellis et al., 1991; Hengartner, 1996; Hengartner & Horvitz, 1994). Involvement of Ras in such regulatory processes has been demonstrated in a number of studies for the past several years, establishing a new field in Ras research. Accumulating evidence indicates that Ras can promote both pro-apoptotic and anti-apoptotic processes. The end-result appears to be the sum of those processes that depend on many variables including cell type, intensity of Ras activation, and the state of the cell when Ras is activated.

In many cell types, the Ras effector PI3-K provides a survival signal through the protein kinase Akt (also known as PKB). In general, activation of the PI3-K/Akt pathway promotes cell survival and inhibition of that pathway enhances apoptosis (reviewed in Cox & Der, 2003; Downward, 1998; Marte & Downward, 1997). The anti-apoptotic function of the PI3-K/Akt pathway has been thought to be mediated by phospholylation of a pro-apoptotic member of the Bcl-2 family, Bad. The phosphorylation of Bad by Akt leads to binding of Bad to 14-3-3 protein, forming an inactive complex and preventing Bad from inactivating anti-apoptotic proteins Bcl-2 and Bcl-X_L that inactivate cell death-effector proteases (Datta et al., 1997; del Peso et al., 1997; Zha et al., 1996; reviewed in Cox & Der, 2003; Downward, 1998). The PI3-K/Akt pathway is also thought to mediate the activation of NF-κB downstream of Ras to promote cell survival (Romashkova & Makarov, 1999). NF-κB is a transcription factor whose activation can lead to expression of anti-apoptotic proteins such as inhibitor of apoptosis proteins (IAP)(reviewed in Cox & Der, 2003; Downward, 1998).

Another downstream effector pathway of Ras, the Raf/ERK pathway, can be either anti- or pro-apoptotic, depending on cellular contexts. When fibroblasts are induced to undergo apoptosis by c-Myc expression during serum starvation, Ras activates a death-inducing pathway through Raf/ERK signaling, as well as a survival pathway

through PI3-K/Akt (Kauffmann-Zeh et al., 1997). In brown adipocytes, oncogenic Ras induces apoptosis during serum withdrawal, and dominant-negative Raf blocks the Ras-induced apoptosis (Navarro et al., 1999). In contrast to these observations, in hematopoietic cells, activated Ras/Raf/ERK signaling prevents apoptosis induced by IL3 withdrawal (Kinoshita et al., 1997; Terada et al., 2000). In Ras-transformed cells that show high resistance to p53-mediated apoptosis, oncogenic Ras suppresses p53 with the Raf/ERK cascade (Ries et al., 2000). Raf and PI3-K signaling may converge at several points downstream of Ras to protect cells from apoptosis. For example, the protein kinase Rsk (a target of ERK) and Akt can phosphorylate Bad at Ser112 and at Ser136, respectively, in order to inhibit the pro-apoptotic function of Bad (Bonni et al., 1999; Blume-Jensen et al., 1998; Fang et al., 1999; Tan et al., 1999). Rsk and Akt also can phosphorylate CREB at Ser133 to upregulate transcription of pro-survival genes (Bonni et al., 1999; Du & Montminy, 1998). Whether Ras activates Raf or PI3-K signaling to promote survival may depend on the nature of apoptosis induction. In sympathetic neurons, apoptosis induced by NGF withdrawal is suppressed by enhanced PI3-K signaling (Mazzoni et al., 1999; Xue et al., 2000). On the other hand, apoptosis induced by cytosine arabinoside (a DNA-damaging agent) is suppressed by enhanced Raf signaling (Mazzoni et al., 1999; Xue et al., 2000; reviewed in Cox & Der, 2003).

The RASSF1/Nore1/Mst1 pathway is a recently identified pathway that mediates Ras promotion of apoptosis. RASSF1 is a putative tumor suppressor protein and can dimerize with the closely related protein Nore1. They can exist with Mst1 in a protein complex. Under serum stimulation, Ras binds to the complex to stimulate apoptosis (Khokhlatchev et al., 2002; reviewed in Cox & Der, 2003; Feig & Buchsbaum, 2002).

As illustrated above, much effort has been made to delineate the nature of Ras-regulation of apoptosis. But, many questions remain to be answered. For example, although differential or combinatorial activations of distinct effector pathways appear to be at least part of the mechanism underlying the dual effects of Ras activation on

apoptosis (i.e., prevention vs. promotion), the principle underlying the distribution of Ras signaling is poorly understood. Also, the role of Ras in regulating survival or apoptosis has hardly been understood *in vivo*. Further studies are still needed to answer these questions.

Ras in neuronal and behavioral plasticity

Recently, studies on roles of Ras in regulating neuronal and behavioral plasticity have established another field in Ras research. In vertebrates and invertebrates, Ras is highly expressed in adult brains (Manabe et al., 2000; Segal & Shilo, 1986; Sudol, 1988). It can be activated by Ca^{2+} after membrane depolarization in neurons, and several signaling molecules that link Ca^{2+} and Ras have been identified (Chen et al., 1998; Ebinu et al., 1998; Farnsworth et al., 1995; Kim et al., 1998; reviewed in Grewal et al., 1999). These findings strongly suggest that Ras plays an important role in Ca^{2+}-dependent neuronal events, such as synaptic transmission and synaptic modification in mature neurons. In fact, there is accumulating evidence that suggests Ras may mediate both pre- and post-synaptic plasticity. For example, in rat hippocampal neurons, Ras acts downstream of NMDA receptor and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) signaling to drive synaptic delivery of AMPA receptor subunits for long-term potentiation (LTP) of synaptic transmission (Zhu et al., 2002). In *Drosophila* larval neuromuscular junctions, the level of the Ras-MAPK pathway is critical to regulate the number of varicosities at the axon termini (Koh et al., 2002). This regulation of presynaptic structure by this pathway is mediated by Fasciclin II (FasII) at pre-synaptic varicosities, suggesting that the Ras-MAPK pathway controls FasII-mediated cell adhesion that has been implicated in synaptic growth (Koh et al., 2002).

Ras has also been implicated in learning and memory behavior, which has been assumed based on neuronal plasticity. Mice heterozygous for a null mutation of *K-ras* show a contextual learning deficit, when coupled with pharmacological inhibition of a

downstream effector (Ohno et al., 2001). Mice lacking a Ca^{2+}-dependent Ras-specific GEF (*Ras-GRF1*) are impaired in various forms of memories (Brambilla et al., 1997; Giese et al., 2001). *NF1* is a member of the GAP family that specifically accelerate inactivation of GTP-bound Ras. Mutations in this gene cause NF1 disease in humans, and about half of NF1 patients exhibit learning impairments (Costa & Silva, 2003; Dasgupta & Gutmann, 2003). Animal models of NF1 disease also show significant impairments in learning. Mice deficient in *NF1* are impaired in spatial learning (Costa et al., 2002; Costa et al., 2001). Flies deficient in *NF1* are impaired in associative olfactory learning (Guo et al., 2000). Although loss of NF1 expression is associated with neurofibromas in certain types of cells, the learning defects appear to be independent of NF1-associated tumor formations (Costa & Silva, 2003; Dasgupta & Gutmann, 2003). SynGAP, another member of the Ras-GAP family, is a molecule that links the NMDA receptor and Ras (Chen et al., 1998; Kim et al., 1998). Mice deficient in *SynGAP* show a spatial learning deficit (Komiyama et al., 2002). Because NF1 and SynGAP are GAPs that inactivate Ras, it is suggested that the learning impairments associated with the deficiency in these genes result from hyperactivity of Ras (Costa & Silva, 2003; Costa et al., 2002; Costa et al., 2001; Dasgupta & Gutmann, 2003).

Another line of evidence that implicates Ras in learning and memory comes from studies on downstream effectors of Ras. A subfamily of MAPK, ERK, is an intensively-studied downstream effector of Ras. In the Ras/ERK pathway, ERK is activated by Raf through MEK (MAPK/ERK kinase). In *Aplysia*, ERK signaling is required for long-term facilitation of synaptic transmission between sensory and motor neurons (Martin et al., 1997). In rat, ERK is required for induction of both NMDA receptor-dependent and -independent LTP in hippocampal and amygdala neurons (reviewed in Adams & Sweatt, 2002). A recent study shows that the NMDA receptor is coupled to the ERK pathway through Ras-GRF (Krapivinsky et al., 2003). The evidence that ERK signaling plays an important role in learning and memory has also been accumulating. An MEK inhibitor

blocks hippocampus- and amygdala-dependent learning and memory in rodents (reviewed in Adams & Sweatt, 2002; Atkins et al., 1998; Blum et al., 1999; Schafe et al., 1999; Schafe et al., 2000, Selcher et al., 1999). Mice lacking *ERK1* show enhanced synaptic plasticity in the striatum and striatum-dependent learning and memory (Mazzucchelli et al., 2002). Conditional expression of a dominant-negative form of *MEK1* in mouse forebrain results in selective deficits in the translation-dependent phase of hippocampal LTP and hippocampus-dependent long-term memory (Kelleher et al., 2004). In flies, *leonardo* mutants in the *14-3-3 zeta* gene that can activate the ERK pathway are impaired in olfactory learning and memory (Philip et al., 2001; Skoulakis & Davis, 1996). PI3-K is another Ras downstream effector that has been implicated in learning and memory. Pharmacological inhibitions of PI3-K impair long-term fear memory, as well as hippocampal and amygdala LTP (Lin et al., 2001; Sanna et al., 2002). Furthermore, cocaine-induced behavioral sensitization is attenuated by inhibition of PI3-K in rats (Izzo, 2002).

1-2. Olfactory learning and memory in *Drosophila*

Genetic dissection of *Drosophila* learning and memory was initiated in the mid-1970s, when a behavioral assay for classical conditioning was developed for flies (Quinn et al., 1974). Classical conditioning is a type of learning, in which an animal makes an association between two previously-unrelated stimuli. In Ivan Pavlov's original experiment, dogs were conditioned so that sound of a bell was associated with provision of food. Before the experiment, dogs salivated with food, but not with the bell sound. However, after repeated trials of this simultaneous stimulus presentation, the dogs became salivating even with the bell sound alone. This phenomenon indicates that the dogs predicted the presentation of food from the bell sound by making an association between the two stimuli. Now, a stimulus that plays a role as the bell sound is called a conditioned stimulus (CS), and a stimulus such as food is called an unconditioned stimulus (US). In flies, animals were exposed alternately to two different odors, one of which was presented with electric shock. In this paradigm, the odors were CS and electric shock was US. Without electric shock, the two odors were equally mild repellents to flies. The assumption was that after training, flies would respond to the shock-associated odor as if they respond to shock (i.e., avoiding being exposed to the stimulus). After several minutes of the training sessions, flies were exposed again to each odor, but this time with no electric shock at all. In this testing session, a number of flies avoided the odor that had been paired with electric shock, but only a few avoided the other odor that had not been paired with shock, indicating that similar to Pavlov's dogs, the flies could make a CS-US association. The established olfactory memory lasted for a few hours with gradual decay.

The development of this olfactory learning and memory assay in *Drosophila* prompted researchers to identify genes linked to learning, which was far more difficult to do in higher organisms. Flies were mutated with chemicals and their progeny were tested

for the olfactory learning and memory. The first genetic screen yielded five informative mutants, named *dunce* (Dudai et al., 1976), *rutabaga* (Livingstone et al., 1984), *amnesiac* (Quinn et al., 1979), *radish* (Folkers et al., 1993) and *turnip* (Choi et al., 1991). Detailed behavioral analysis revealed that *dunce* and *rutabaga* are impaired in learning (i.e., making an association) and the other three mutants are impaired in memory. Their simple olfactory, somatosensory, and motor performances required to complete the task were normal, indicating that their learning or memory impairments resulted from their inability in making an association or maintaining an established association over time, respectively.

Mutants were defective in the cAMP pathway

Molecular studies on the learning and memory mutants revealed the biochemical identities of the *dunce*, *rutabaga*, and *amnesiac* genes. The identification of the *radish* and *turnip* genes awaits further investigations.

The *dunce* gene encodes a phosphodiesterase that degrades cyclic adenosine 3,5' monophosphate (cAMP) (Byers et al., 1981; Chen et al., 1986). The *rutabaga* gene encodes a Ca^{2+}/calmodulin-dependent adenylyl cyclase that synthesizes cAMP (Livingstone et al., 1984; Levin et al., 1992). The *dunce* and *rutabega* mutations impaired the functions of the corresponding genes. Biochemical analysis revealed that cAMP synthesis is upregulated in *dunce* and downregulated in *rutabega* mutants. These findings indicate that the regulation of cAMP level is critical for associative learning in *Drosophila*. This was astonishing, particularly considering the critical involvement of the cAMP pathway in neuronal and behavioral plasticity in sea slug *Aplysia*. (Kandel, 2001).

The gene identification of the memory mutant *amnesiac* further shed light on the cAMP pathway as a mediator for learning and memory. The *amnesiac* gene encodes a pre-pro-neuropeptide protein that has homology to the mammalian pituitary adenylyl cyclase-activating peptide (PACAP)(Feany & Quinn, 1995; Moore et al., 1998; Vaudry et

al., 2000). Given that neuropeptides stimulate intracellular signaling cascades for longer than conventional transmitters (e.g., glutamate), this finding led to the hypothesis that the Amnesiac peptide activates Rutabega adenylyl cyclase so that activation of the cAMP pathway is prolonged to maintain acquired memory (Waddell & Quinn, 2001a). Indeed, mutations in *amnesiac* suppress female sterility resulting from severe alleles of *dunce* (Feany & Quinn, 1995). Furthermore, feeding *amnesiac* flies with the adenylyl cyclase agonist, forskolin, reverts their hypersensitivity to ethanol (another *amnesiac* phenotype) to the wild-type level (Moore et al., 1998). These findings provide evidence that the Amnesiac peptide activates the cAMP pathway.

More evidence on the cAMP pathway: PKA and CREB

Protein kinase A (PKA) and cAMP-responsive-element-binding (CREB) proteins are downstream effectors of cAMP. cAMP activates PKA, and then PKA activates CREB. These two effectors have been implicated in various forms of learning and memory in a variety of organisms (Kandel, 2001). In flies, acute heat-shock induction of a dominant-negative form of a *PKA* transgene impairs olfactory learning (Drain et al., 1991). Similar learning impairments have been reported subsequently for flies that carry mutations in the *PKA* genes (Goodwin et al., 1997; Skoulakis et al., 1993). The duration of PKA activation is believed to correlate with the duration for which memory lasts (Li et al., 1996). In honeybees, it has been demonstrated that repetitive, spaced training results in persistent PKA activation that leads to more permanent memory (Muller, 2000). CREB is a transcription factor that can be phosphorylated by PKA. A number of studies have implicated CREB in activity-dependent transcription required for long-term memory (LTM) formation (Kandel, 2001). In flies, acute induction of a dominant-negative form of a CREB transgene impairs protein-synthesis-dependent LTM (Yin et al., 1994), while acute induction of a constitutively-active CREB transgene enhances LTM (Yin et al.,

1995). These findings provide converging evidence that the cAMP pathway is critical for olfactory learning and memory in *Drosophila*.

Other genes involved in olfactory learning and memory

Other genetic screens and transgenic studies have identified additional genes for olfactory learning and memory. Some of them are postulated to elaborate the cAMP pathway and the others are not.

***latheo*, *linotte*, and *nalyot*.** These mutants were identified in a screen using mutagenesis induced by *P*-elements, transposable elements in *Drosophila* (Cooley et al., 1988 for *P*-element mutagenesis). Although this method is less efficient than chemically-induced mutagenesis (Waddell & Quinn, 2001b), the mutated genes are more clonable with this method, because the genes that have undergone mutations are tagged by the known nucleotide sequence of the transposons. Chemically-induced mutagenesis usually causes point mutations. The *latheo* gene encodes a component of the origin recognition complex for DNA replication (Boynton & Tully, 1992; Pinto et al., 1999). The *linotte* and *nalyot* genes each turned out to be an allele of a known gene: *linotte*, the *derailed* receptor tyrosine kinase (Dura et al., 1993; Dura et al., 1995) and *nalyot*, the *Adf1* transcription factor (DeZazzo et al., 2000). All the mutants show olfactory learning or memory impairments, but their precise roles in mediating learning and memory remain to be determined (reviewed in Waddell & Quinn, 2001b).

***leonardo*, *volado*, and *fasII*.** These mutants were identified through a *P*-element-based enhancer-trap screen for genes that are preferentially expressed in the mushroom bodies (MBs), fly brain structures that had been implicated in olfactory learning and memory (The role of the MBs in learning and memory is discussed later). The *leonardo* gene, which encodes the zeta isoform of 14-3-3 protein, is expressed preferentially in the MBs, and *leonardo* mutant flies are defective in olfactory learning and memory (Philip et al.,

2001; Skoulakis et al., 1996). Members of the 14-3-3 protein family can regulate protein kinase C (PKC) activity (Aitken et al., 1995; Xiao et al., 1995). They also can activate rate-limiting enzymes in catecholamine and serotonin biosynthesis (Ichimura et al., 1995). A number of reports have demonstrated that they can activate Raf, a target of Ras (Fantl et al., 1994; Freed et al., 1994; Irie et al., 1994; Li et al., 1995). In the *Drosophila* larval neuromuscular junction (NMJ), the Leonardo protein is enriched in the presynaptic terminal (Broadie et al., 1997) and interacts with the voltage-sensitive Ca^{2+}-dependent K^+ channel Slowpoke via the Slowpoke-binding protein Slob (Zhou et al., 1999). Mutant flies defective in *leonardo* have reduced synaptic transmission at the larval NMJ (Broadie et al., 1997). These findings in the *Drosophila* NMJ collectively suggest that Leonardo may mediate Ca^{2+}-dependent presynaptic vesicle exocytosis required for transmitter release (reviewed in Waddell & Quinn, 2001b).

The other two genes, *volado* and *fasII*, both encode cell-surface receptors that mediate cell adhesion. They are expressed preferentially in the MBs. The *volado* gene encodes two splice variants of α-integrin (Grotewiel et al., 1998). *volado* mutant flies are significantly impaired in olfactory learning (Grotewiel et al., 1998). The learning impairment in *volado* mutants can be rescued by heat-shock induction of a *volado* cDNA transgene right before training, indicating that Volado is acutely required for learning. *Drosophila* integrins, including Volado, also regulate the morphology and function of the NMJ (Beumer et al., 1999; Rohrbough et al., 2000). The *fasII* gene encodes three isoforms of a cell adhesion receptor similar to the vertebrate NCAM isoforms (Cheng et al., 2001). All three isoforms share the same extracellular domains and two of them have a transmembrane domain with divergent intracellular tails (the remaining one is missing the transmembrane domain and is attached to the cellular membrane via a GPI linkage)(Grenningloh et al., 1991; Lin & Goodman, 1994; reviewed in Cheng et al., 2001). In *Drosophila*, FasII has been implicated in many aspects of neuronal development, such as neuronal migration (Grenningloh et al., 1990, 1991; Holmes &

Heilig, 1999; Wright et al., 1999), axon bundling (Lin et al., 1994; Lin & Goodman, 1994), and synapse stabilization and growth (Schuster et al., 1996). Adult *fasII* mutant flies show an olfactory learning impairment. This defect can be rescued by acute heat-shock induction of the cDNA for the transmembrane form of FasII (Cheng et al., 2001). These findings indicate that FasII is required for both normal neuronal development and adult olfactory learning. How receptor proteins such as Volado and FasII that mediate cell adhesion are involved in acute olfactory learning remains unknown. However, these proteins have also been implicated in cell signaling, and it has been speculated that they may mediate learning by regulating intracellular signal transduction cascades (Cheng et al., 2001; Grotewiel et al., 1998).

NF1. The *Drosophila* homolog of the *NF1* gene was isolated through a combination of various DNA library screens using a human *NF1* probe (The et al., 1997). Individuals with mutations in *NF1* are predisposed to tumors in the nervous system, and typically show characteristic pigmentary abnormalities in childhood. About 50% of them have learning disabilities, and about 10% develop skeletal abnormalities (Dasgupta & Gutmann, 2003). The most common tumors in NF1 patients are the neurofibroma associated with a peripheral nerve, which is composed of Schwann cells, fibroblasts, and mast cells, and the optic pathway glioma, composed of neoplastic astrocytes (Dasgupta & Gutmann, 2003). The human *NF1* gene was identified based on positional cloning and found to encode a Ras-GAP (Ballester et al., 1990; Xu et al., 1990a; Xu et al., 1990b). Loss of NF1 protein expression in *NF1*-associated tumors or *NF1*-deficient mouse cells is associated with elevated Ras activity and increased cell proliferation (reviewed in Dasgupta & Gutmann, 2003). However, in *Drosophila*, studies show that the NF1 protein can also function as an activator of the cAMP pathway. *NF1* mutant flies were generated by mobilizing a *P*-element which had been inserted nearby the *NF1* locus. Flies carrying null mutations in *NF1* show a variety of phenotypes such as reductions in body size (The

et al., 1997), a cellular response to the neuropeptide PACAP38 (Guo et al., 1997), and olfactory learning (Guo et al., 2000). Interestingly, these flies have lower G protein-stimulated adenylyl cyclase activity, and increasing cAMP signaling can normalize their phenotypes, as well as heat shock-induced expression of wild-type *NF1* (Guo et al., 1997; Guo et al., 2000; The et al., 1997).

pumilio, oskar, elF-5C, staufen, moesin, orb, and elF-2G. These genes were recently identified by a combination of screens using DNA microarrays and *P*-element mutagenesis (Dubnau et al., 2003a). The DNA microarrays were used to detect genes that are transcribed during olfactory long-term memory formation in normal flies. The *P*-element mutagenesis was used for a genome-wide behavioral screen to identify mutant flies defective in olfactory long-term memory. All of the genes are involved in subcellular localization of mRNA translation.

pumilio, *oskar*, and *elF-5C* were identified from both screens. *pumilio* is a transcript-specific translational repressor (Dubnau et al., 2003a; Macdonald, 1992; Nakahata et al., 2001; Sonoda & Wharton, 1999). Expression of the *pumilio* gene increases during long-term memory formation, and the memory mutants *milord-1* and *milord-2* carry mutations in the *pumilio* gene (Dubnau et al., 2003a). *oskar* is a gene involved in Staufen-dependent mRNA translocation (Micklem et al., 2000), and the memory mutant *norka* carries a mutation in the *oskar* gene. elF-5C is a translation initiation factor, and the memory mutant *krasavietz* carries a mutation in the *elF-5C* gene (Dubnau et al., 2003a).

All the other genes were identified by DNA microarrays. *staufen* is a gene involved in mRNA translocation in oocytes and in neurons (Ferrandon et al., 1994; St Johnston et al., 1995; Li et al., 1997; reviewed in Roegiers & Jan, 2000), and temperature-sensitive mutants of *staufen* show a long-term memory deficit (Dubnau et al., 2003a). Moesin is an actin binding protein required for proper localization of Staufen

(Polesello et al., 2002). Orb is the *Drosophila* homolog of CPEB, a protein with a conserved role in localized translation in both oocytes and neurons (Chang et al., 1999; Chang et al., 2001; Christerson & McKearin, 1994; Huang et al., 2002; Lantz et al., 1992; Lantz et al., 1994). eIF-2G is a translation initiation factor that mediates CPEB-dependent translation (Stebbins-Boaz et al., 1999).

These findings collectively suggest that translocation of newly-transcribed mRNAs and local regulation of their translation appear to be involved in olfactory long-term memory formation in *Drosophila* (Dubnau et al., 2003a).

Neuroanatomy of olfactory learning and memory

Mapping between properties of behavior and areas of the brain has been a central interest of brain scientists for hundreds of years. It is a requisite for building models that describe mechanisms underlying behavior. By combining sophisticated genetic tools available in *Drosophila*, recent studies in fruit flies are now linking genes, neurons, and behavior to generate a more comprehensive understanding of olfactory learning and memory.

Mushroom bodies (MBs). One of the most-well studied regions of the insect brain is the MBs. In the insect central brain, the MBs and another structure, the central complex, are clearly visible. They are separated by glial sheaths from many discrete neuropil regions surrounding them. (Heisenberg, 2003). In flies, the MBs are two synapses away from sensory receptors. Signals from chemical receptors on the antennae and maxillary palps travel dorsocaudally to the antennal lobes and then to the MB calyces. Each MB calyx contains dendrites of about 2,500 Kenyon cells, the intrinsic MB neurons. Axons of the Kenyon cells extend rostroventrally, forming the stalk-like peduncle through the central brain and splitting frontally into vertical (α and α') and medial (β, β', and γ) lobes. The output from the MBs is less obvious. The lobes and the rostral parts of the peduncles are

innervated by extrinsic neurons that probably provide input to, as well as receive the output from, the MBs (reviewed in Heisenberg, 2003; Waddell & Quinn, 2001b).

It is now the converging notion that the MBs play a critical role in olfactory learning and memory. Mutant flies defective in MB anatomy show impaired olfactory learning with normal simple sensorimotor performance (deBelle & Heisenberg, 1994; Heisenberg et al., 1985). Genes involved in the cAMP pathway, such as *dunce*, *rutabega*, and *PKA* (*DCO*), are expressed preferentially in the MBs (Han et al., 1992; Nighorn et al., 1991; Skoulakis et al., 1993). *leonardo*, *volado*, and *fasII* were identified for their expression in the MBs (Cheng et al., 2001; Grotewiel et al., 1998; Skoulakis et al., 1996). In fact, altering the level of cAMP signaling in the MBs (but not in the central complex) using the GAL4-UAS system (Brand & Perrimon, 1993) results in alterations in olfactory learning performance. Expression of a constitutively-active form of adenylyl cyclase-stimulatory G protein (Gs) in the MB Kenyon cells abolishes olfactory learning without apparent developmental or simple sensorimotor abnormalities (Connolly et al., 1996). Expression of a wild-type form of a *rutabega* cDNA transgene in subpopulations of the Kenyon cells is sufficient to restore olfactory learning in a *rutabega* mutant background (Zars et al., 2000).

The more precise role of the MBs in olfactory learning and memory has been pursued with refined techniques. Anatomical studies suggest that the MBs can act as a center in which multimodal inputs are integrated to generate a directed behavior (Crittenden et al., 1998; Ito et al., 1998). In olfactory learning, the MBs have been hypothesized to provide the domain in which odor-shock associations take place (reviewed in Dubnau et al., 2003b; Dubnau & Tully, 2001; Heisenberg, 2003). Indeed, the identification of *Drosophila* odor receptor genes in sensory neurons in the antennae has confirmed the olfactory pathway leading to the MBs (Vosshall et al., 2000), although the pathway carrying the shock stimulus remains to be identified (Dubnau et al., 2003b; Dubnau & Tully, 2001; Heisenberg, 2003).

Recent behavioral studies demonstrate that the odor-shock association in olfactory learning can be established without neurotransmission from the MBs to their output neurons (Dubnau, et al., 2001; McGuire et al., 2001). In those studies, a temperature-dependent dominant-negative *shibire* transgene (*shibire^{ts1}*) (Kitamoto, 2001) was expressed in MB neurons using the GAL4-UAS system, in order to block the output from the MBs in a conditional manner. The *shibire* gene encodes a dynamin GTPase that is required for synaptic vesicle recycling (Chen et al., 1991; van der Bliek & Meyerowitz, 1991) and maintenance of the readily releasable pool of synaptic vesicles (Kawasaki et al., 2000). The temperature-sensitive allele *shibire^{ts1}* has a mutation in the GTPase domain and the Shibire^{ts1} protein reversibly blocks synaptic transmission at an elevated temperature (Grant et al., 1998; Koenig & Ikeda, 1989). The switch between the normal and dominant-negative states takes only a few minutes after temperature shifts. Behavioral analyses using various heat-shock protocols revealed that the neurotransmission from the MBs is required only for the expression of a learned behavior (Dubnau, et al., 2001; McGuire et al., 2001). Association between odor and electroshock, as well as retention of once-established associative memory, did not require the output transmission from the MBs. These findings strongly suggest that coincident detection of odor and electroshock in associative olfactory learning occur anatomically upstream of synaptic output from MB neurons (Dubnau et al., 2003b; Dubnau & Tully, 2001). Sites for input to the MBs (e.g., the dendrites of the Kenyon cells) and/or antennal lobe neurons appear to be strong candidates for where the association takes place (Dubnau et al., 2003b; Dubnau & Tully, 2001).

How the odor-shock association takes place at the molecular level is entirely an open question. However, the converging notion is that Rutabega adenylyl cyclase is likely to play a role as a coincident detector (Dubnau et al., 2003b; Heisenberg, 2003). In a cellular model of classical conditioning, CS and US converge at the same neuron. The coincident stimulation of CS and US increases the synaptic efficacy for the CS pathway,

so that after the pairing, CS alone can activate the postsynaptic neuron as if US does (e.g., Johnston & Wu, 1995). adenylyl cyclase can be activated either by Ca^{2+} or G-protein coupled receptor, but the level of activation is much enhanced when the two stimuli are given simultaneously. In *Aplysia*'s gill withdrawal reflex, this nature of adenylyl cyclase activation has been used to explain classical conditioning (i.e., US is represented by Ca^{2+} and CS by G-protein coupled receptor)(Kandel, 2001). In olfactory learning in *Drosophila*, expression of Rutabega adenylyl cyclase in MB neurons has been shown critical (Zars et al., 2000). Although in mammalian neurons, strengthening of a CS pathway in classical conditioning has been rather hypothesized mediated by NMDA receptor-dependent LTP (e.g., Johnston & Wu, 1995), there has been no direct evidence that the *Drosophila* homologs of the NMDA receptor are involved in synaptic or behavioral plasticity.

Dorsal paired medial (DPM) neurons. These neurons were identified in an effort to collect *P*-element based *GAL4* enhancer trap lines that express GAL4 protein preferentially in the adult brain (Waddell et al., 2000). When these *GAL4* driver lines were crossed to *UAS*-lines that carry reporter genes under the *UAS* sequence, two lines showed predominant expression of GAL4 in a pair of large neurons now called the DPM neurons that are situated medially to the MBs. Intriguingly, the axon of each DPM neuron was found to project broadly with extensive ramifications to the axonal lobes (but not the dendritic calyx) of the ipsilateral MB (Waddell et al., 2000).

The role of the DPM neurons in olfactory learning and memory has been tested using the temperature-sensitive dominant-negative dynamin *shibire^{ts1}* (see above for *shibire^{ts1}*). By crossing a *UAS-shibire^{ts1}* line to one of the DPM-specific *GAL4* drivers, *shibire^{ts1}* was expressed in the DPM neurons, in order to block synaptic transmission from the DPM neurons in a temperature-dependent manner. At a permissive temperature, the flies were normal both in olfactory learning and memory. However, when the

temperature was shifted to a restrictive range, they showed a severe memory deficit with normal immediate learning, reminiscent of the memory phenotype of *amnesiac* mutants (Waddell et al., 2000; reviewed in Davis, 2001; Dubnau et al., 2003b; Dubnau & Tully, 2001).

Indeed, striking convergence was seen between the DPM neurons and *amnesiac* when the tissue localization of the Amnesiac neuropeptide was investigated. Immunohistochemistry revealed that Amnesiac is expressed quite specifically in the DPM neurons in the adult brain (Waddell et al., 2000). Furthermore, transgenic expression of a wild-type form of *amnesiac* in the DPM neurons rescued the memory deficit of *amnesiac* mutants (Waddell et al., 2000).

Because the axons of the DPM neurons project onto the axonal lobes of the MBs, these findings prompt a scenario that the Amnesiac neuropeptide is released from the DPM neurons to modulate neurotransmission from the MBs that is required for expression of olfactory memory (see above for the role of synaptic output of the MBs). Indeed, modulation of MB neurophysiology by Amnesiac has been suggested experimentally (Davis, 2001; Rosay et al., 2001). Furthermore, combined with our knowledge on the biochemical property of Amnesiac (i.e., a PACAP-like neuropeptide), these findings suggest a more integrative model in which Amnesiac released from the DPM neurons activates the adenylyl cyclase-cAMP pathway in MB axons to facilitate the role of the MBs in olfactory memory. Substantiation of such a model requires further studies. But such presynaptic neuromodulation through cAMP signaling has been well demonstrated in *Aplysia* (Dubnau & Tully, 2001; Kandel, 2001).

1-3. The aims of the research

As reviewed in the first section above (1.1. The biology of Ras), Ras plays an important role as a signaling molecule from yeasts to humans. It transduces extracellular signals to intracellular effectors. Although it was originally identified as a proto-oncogene, studies have shown that Ras also assumes critical roles in regulating a variety of cellular events, such as cell proliferation, differentiation, and apoptosis required for normal organismal development. Recent studies have also suggested important roles of Ras in neuronal and behavioral plasticity.

To obtain a further insight into the neuronal function of Ras, I investigated the role of Ras signaling in associative learning and memory in *Drosophila*. As illustrated above (see Section 1-2), *Drosophila* is a well-established model organism for genetic studies of biology, including studies of the biological basis of behavioral traits. Associative learning and memory is a type of behavior that can be studied in this organism. An integrative model that synthesizes from molecules to behavior is emerging with identification of genes and anatomical loci underlying this type of learning and memory. In the following two chapters, I will provide evidence that Ras plays a critical role in olfactory learning and memory in *Drosophila*.

In Chapter 2, I will demonstrate that reduced Ras signaling results in memory deficits, while learning tested immediately after associative training is intact. The severity of the memory impairment was correlated with molecular lesions in the *ras* gene. The memory impairment was rescued by pan-neural expression of wild-type *ras* in the *ras* mutant background, indicating that the memory impairment is due to inadequate Ras signaling. Examination of synaptic morphology at neuromuscular junctions revealed an increased number of presynaptic varicosities with normal target recognition by motor neurons in the *ras* mutant.

In Chapter 3, I will show that enhanced Ras signaling can impair immediate learning in a tissue-specific manner. Flies expressing a dominant-active form of *ras* in a subset of the intrinsic MB neurons exhibited a learning impairment, when tested right after associative training. Flies expressing the same dominant-active *ras* construct in neurons in the central complex showed normal learning, indicating that the effect of the transgene expression on the behavior is tissue-specific. Simple sensorimotor functions and overall MB morphology were normal in the learning-impaired transgenic flies. Acute induction of dominant-active *ras* expression before training also resulted in a learning impairment, suggesting that expression of the activated *ras* may disrupt acute processes required for initial association. Although the Ras/Raf/MAPK pathway has recently been implicated in neuronal or behavioral plasticity, expression of a dominant-active form of *raf* in the same set of the MB neurons did not result in a learning impairment. Because activation of other known downstream pathways (i.e., the Ral-GDS and PI3-K pathways) did not result in a learning impairment, the effect of enhanced Ras signaling in the MBs on learning may be mediated in a cooperative way by the known downstream effectors or as yet unknown effectors of Ras.

During the course of behavioral studies, I also unexpectedly found a remarkable non-behavioral phenotype. When a mammalian form of dominant-negative *ras* was expressed in all the post-mitotic neurons, the flies developed eyes that are severely reduced in size. In Chapter 4, I will describe the results of characterization of this small eye phenotype. In brief, the phenotype was assumed to result from inhibition of Ras signaling by the expression of dominant-negative *ras*. The reduced eye size was modified in a way sensitive to the level of Ras signaling. The phenotype was based on massive cell death in the developing eye, as well as blocking of EGFR/Ras-dependent signaling pathways, including the MAPK and PI3-K pathways. Additional components such as Amnesiac and Rap appeared to modulate the signaling machinery implicated in the small eye phenotype.

This small eye phenotype provides an opportunity to identify novel components that are involved in the Ras pathway. With the ease of scoring the phenotype and the demonstrated sensitivity to the level of Ras signaling, it provides a very efficient and powerful system for genetic screens. Genes that are identified associated with the Ras pathway through this system could be readily tested for olfactory learning and memory. Such systematic approach using multiple assays should facilitate to uncover how Ras mediates olfactory learning and memory in *Drosophila*. Furthermore, *ras* has been highly implicated in tumorigenesis in humans. The Ras-associated small eye phenotype may also provide a powerful tool to help develop therapeutic strategies for human cancers, as well as further understand the complexity of Ras signaling in basic biological events.

1-4. References

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CHAPTER 2

Ras is Required for Olfactory Learning and Memory in *Drosophila*

2-1. Introduction

ras genes are evolutionary conserved eukaryotic genes, identified in a number of organisms, such as yeasts, worms, flies, mice, and humans (Barbacid, 1987; Lowy & Willumsen, 1993). The biochemical property of Ras closely resembles that of G-proteins, cycling between the GDP-bound inactive and GTP-bound active conformations (Barbacid, 1987; Lowy & Willumsen, 1993). It binds itself to the inner face of the plasma membrane and functions downstream of transmembrane receptors to transduce extracellular signals to intracellular effectors. This role of Ras has been well appreciated in the signaling pathway that begins with growth-factor activation of receptor tyrosine kinases (Malumbres & Barbacid, 2003). In this pathway, activation of Ras leads to activation of the Raf/MEK/MAPK pathway. The biological significance of this pathway has been well demonstrated in a variety of basic cellular processes, such as cell proliferation, differentiation, and survival (Cox & Der, 2003; Downward, 1998; Malumbres & Barbacid, 2003; Sternberg & Han, 1998; Vaudry et al., 2002; Voas & Rebay, 2004).

Recently, studies on roles of Ras in regulating neuronal functions have been establishing a new field in Ras research (Grewal et al., 1999; Orban et al., 1999). In matured animals, Ras is expressed highly in the central nervous system (CNS) (Manabe, et al., 2000; Segal & Shilo, 1986; Sudol, 1988). Identification of increasing numbers of

Ca^{2+}-dependent Ras signaling pathways strongly suggests an important role of Ras in activity-dependent neuronal events, such as synaptic plasticity (Chen et al., 1998; Downward, 1998; Ebinu et al., 1998; Farnsworth et al., 1995; Finkbeiner & Greenberg, 1996; Kawasaki et al., 1998; Kim et al., 1998). In fact, it has been shown that Ras acts downstream of NMDA receptor and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) signaling to drive synaptic delivery of AMPA receptor subunits for long-term potentiation (LTP) in rat hippocampal neurons (Zhu et al., 2002). Behavioral studies suggest that Ras may play an important role in mediating learning and memory. For example, mice lacking the Ca^{2+}-dependent Ras-GEF, *Ras-GRF*, are impaired in amygdala- or hippocampus-dependent memories (Brambilla et al., 1997; Giese et al., 2001). Genetic or pharmacological inhibition of the MAPK pathway affects amygdala-, hippocampus- or striatum-dependent memories in rodents (Atkins et al., 1998; Mazzucchelli et al., 2002; Ohno et al., 2001; Schafe et al., 2000).

In *Drosophila*, Ras1, the homolog of human H-, K, or N-Ras was originally identified in a genetic screen for genes involved in receptor tyrosine kinase-dependent R7 photoreceptor cell differentiation in the compound eye (Fortini et al., 1992; Simon et al., 1991). Subsequent studies have continuously identified additional roles of Ras1 in regulating various cellular events. In the developing eye and wing, Ras1 has also been implicated in cellular proliferation and apoptosis (Bergmann et al., 1998; Karim & Rubin, 1998; Kurada & White, 1998; Prober & Edgar, 2000). In the larval neuromuscular junction (NMJ), Ras1 is required for a postsynaptic cellular response to an adenylyl cyclase (AC)-activating neuropeptide (Zhong, 1995). Altering the level of Ras1 signaling in motor neurons results in alterations in the number of presynaptic varicosities in the axon termini (Koh et al., 2002). These findings in the neuromuscular synapse suggest that Ras1 may also be involved in pre- and post-synaptic processes in the CNS for behavioral plasticity. Indeed, the AC/cAMP pathway and structural modification of synapses have both been highly implicated in learning and memory in general (Kandel, 2001). In flies,

involvement of the AC/cAMP pathway in olfactory learning has been well demonstrated (Dubnau et al., 2003; Heisenberg, 2003; Waddell & Quinn, 2001). Several studies have shown that genes that can associate with the Ras pathway are also involved in olfactory learning. Mutant flies for the *14-3-3 zeta* gene (*leonardo*) that can activate the MAPK pathway (Philip et al., 2001; Skoulakis & Davis, 1996) or for the *NF1* gene that can inactivate Ras itself (Guo et al., 2000) are impaired in olfactory learning.

These findings from rodents and flies collectively suggest a role of Ras in learning and memory. However, all the evidence is rather circumstantial. Here, I directly tested whether Ras is required for olfactory learning and memory, using a variety of genetic tools available in *Drosophila.*

2-2. Methods

Fly stocks

The following *Drosophila* strains were used for this study: $ras1^{ix12a}$ (Schnorr & Berg, 1996), $ras1^{5703}$ (Karpen & Spradling, 1992), $ras1^{\Delta C40B}$ (Hou et al, 1995), *UAS-*$ras1^{N17}$ (Lee et al., 1996), *UAS-*$ras1^{WT}$ (Karim & Rubin, 1998), *UAS-lacZ* (Brand & Perrimon, 1993), *247* (Zars et al., 2000), *c747* (Connolly et al., 1996), *c232* (O'Dell et al., 1995), *c155* (Lin & Goodman, 1994), and *c316* (Waddell et al., 2000). The *Canton-S* strain was used as a wild-type control. All the flies were raised on standard cornmeal food at 25 °C on a 12 hr : 12 hr light : dark cycle.

Behavioral assays

Olfactory learning and memory were assayed essentially as described previously (Quinn et al., 1974). This assay is based on the classical conditioning paradigm, in which animals make an association between conditioned (CS) and unconditioned (US) stimuli. In brief, in the paradigm I used, flies are trained so that they become to discriminate an odor that is paired with electroshock from another odor not paired with any shock. In my

experiments, about 30 adult flies (3-7 days old) were exposed to two odors for 30 seconds alternately interspersed with a 30-second rest interval. The cycle was repeated 3 times. One odor was presented with 90-volt electroshock, and another odor without electroshock. The odors used were 3-octanol (OCT) and 4-methylcyclohexanol (MCH). OCT was diluted 1:200 and MCH 1: 100 in ethyl ether. Flies were tested 30 seconds after training for learning, or 1 or 2 hours after training for memory. Flies' learning or memory performance was quantified by dividing the difference between the number of flies that avoided the odor that was presented with electroshock and the number of flies that avoided the other odor that was presented without electroshock by the total number of flies tested. To control an odor bias, two independent sessions were carried out. An odor presented with shock (or without shock) was switched for the other odor between the sessions. The resulting two scores were averaged to generate a single performance index. Flies were also tested for sensorimotor functions required to complete the learning or memory tasks (Quinn et al., 1974; Waddell et al., 2000). To test odor acuities, an untrained group of flies (n = ~100) were given 60 seconds to choose between fresh air and odor used for the learning or memory assay. An avoidance index was calculated as the difference between the number of flies that chose fresh air and the number of flies that chose odor, divided by the total number of flies. Electroshock reactivity was tested and quantified similarly. Untrained flies were given 60 seconds to choose between a tube containing an electrified grid and a tube containing a nonelectrified grid. Running performance was assayed essentially as described previously (Benzer, 1967). In the presence of light, 5 runs were made. Each run lasted for 30 seconds. About 50 flies were tested in a single experiment. Flies were given a score according to the number of times that they run toward the light (flies that run 5 times were given a score of 5, 4 times a score of 4, and so on). To calculate the running index (RI), the running scores given to individual flies were summed and the total value was divided by the value of the total number of flies tested multiplied by 5. Thus, the RI ranged from 0 to 1, 0 representing

none of the flies run at all and 1 representing all the flies run 5 times. All experiments were conducted at 25 ˚C.

Immunohistochemistry

Mushroom bodies (MBs). Adult brains were dissected from flies aged 3-7 days old under phosphate-buffered saline (PBS). They were transferred to 2% paraformaldehyde for 30 minutes and then washed in PBS + 0.3 % Triton X-100 (Sigma) several times for at least one hour total time. Primary antibodies were diluted in PBS + 0.3 % Triton X-100 + 3 % Normal Goat Serum and incubated overnight at 4 °C. Primary antibodies were either rabbit anti-β-galactosidase (Cappel) 1: 2000 or mouse anti-Fasciclin II (FasII) 1:20 (Schuster et al., 1996). On the following day, brains were washed several times (minimum 2 hours) in PBS + 0.3 % Triton X-100. Secondary antibodies were diluted in PBS + 0.3% Triton X-100 and incubated overnight at 4 °C. Secondary antibodies used were goat anti-rabbit IgG-conjugated with Alexa Fluor 488 or goat anti-mouse IgG-conjugated with Alexa Fluor 488 (Molecular Probes Inc.). Finally, brains were washed in PBS + 3 % Triton X-100 for 2-4 hours before mounting in VectaShield (Vector Labs Inc.).

NMJs. Wandering third instar larvae were dissected in *Drosophila* saline (NaCl, 70 mM; KCl, 5 mM; $MgCl_2$, 4 mM; $NaHCO_3$, 10 mM; Trehalose, 5 mM; sucrose, 115 mM; HEPES-NaOH, 5 mM, pH 7.2, modified from HL3) and fixed in 4% formaldehyde in HL3 (Stewart et al., 1994) for 45 minutes. Goat anti-HRP IgG conjugated to fluorescein (Cappel) was used to label neuronal cell membrane. Preparations were incubated for one hour in blocking solution (1% BSA in PBS + 0.5% Triton-X), and then in antibody solution (anti-HRP diluted 1:1000 in the blocking solution) for two hours at room temperature with gentle agitation. Immunoreactive proteins were visualized on a Zeiss Pascal Confocal using fluorescent secondary antibodies (Molecular Probes Inc.).

2-3. Results

Flies carrying hypomorphic mutations in *ras1* are impaired in olfactory memory

To examine whether Ras signaling is essential to olfactory learning and memory in *Drosophila*, I first tested flies carrying *P*-element-based mutations in the endogenous *ras1* locus (Figure 2-1A). The $ras1^{5703}$ strain was generated in a *P*-element mutagenesis screen (Karpen & Spradling, 1992). It contains a *P*-element at 85D10, located 28 bp upstream of a putative *ras1* transcription start site, disrupting expression of the *ras1* gene (Schnorr & Berg, 1996). Homozygous $ras1^{5703}$ females lay eggs with ventralized eggshells, which can hatch normal larvae. $ras1^{ix12a}$ and $ras1^{\Delta C40B}$ were generated by mobilizing the *P*-element in the $ras1^{5703}$ strain (Schnorr & Berg, 1996). $ras1^{ix12a}$ consists of a large internal deletion of the *P*-element, retaining 2 kb of the original 15-kb transposon sequence. Consistent with the molecular lesions, *ix12a* has been characterized as a weaker allele than *5703* in the eggshell phenotype (Schnorr & Berg, 1996). $ras1^{\Delta C40B}$ contains a deletion of the *ras1* open reading frame, and is homozygous lethal (Hou et al., 1995; Schnorr & Berg, 1996).

I tested these alleles in various combinations for olfactory learning and memory. The allelic combinations tested were *ΔC40B/+*, *ix12a/ix12a*, *ix12a/5703*, and *ix12a/ΔC40B*. Homozygous *5703* or *5703/ΔC40B* flies were virtually lethal. The results are shown in Figure 2-1B. When flies were tested right after training sessions, all the *ras* mutants showed normal performance in comparison to the wild-type control, indicating that the *ras* mutants have an ability to make an association between odor and electroshock. However, when they were tested at later times for the memory retention, the mutants tended to perform poorly, so that their memory loss correlates with the molecular lesions that disrupt expression of *ras1*. At 2 hours after training, the memory deficit of *ix12a/ΔC40B* flies was statistically significant, comparing to wild-type or *ΔC40B/+* flies ($p < .01$, Figure 2-1B). The simple sensorimotor functions required to

perform the learning or memory task were indistinguishable between the wild-type and *ras* mutants (Figure 2-2). Histological analysis revealed that the overall morphology of the MBs (the center for olfactory learning and memory) was also indistinguishable between the wild-type and mutants (Figure 2-3). These results collectively suggest that Ras is essential to cellular processes required for the retention of once-established olfactory memory in *Drosophila*.

Neural tissues that require Ras for olfactory learning and memory

The data I have presented suggested a requirement of Ras in normal olfactory memory in *Drosophila*. To gain a further insight into the role of Ras, I attempted to rescue the memory phenotype of the *ras1* mutant $ras^{ix12a}/ras1^{\Delta C40B}$ in a tissue-specific manner. I used the GAL4-UAS system (Brand & Perrimon, 1993) to drive expression of a wild-type form of a *ras1* transgene ($ras1^{WT}$) in the *ix12a/ΔC40B* background (Figure 2-4A). The *GAL4* drivers used were *c155*, *c747*, *c316*, and *c232*. The line *c155* contains a *GAL4* *P*-element insertion in the *elav* gene that is expressed virtually in all the post-mitotic neurons in *Drosophila* (Lin & Goodman, 1994). The line *c747* has been characterized to express GAL4 predominantly in MB neurons (Connolly et al., 1996). *c316* and *c232* have been used as drivers for specific expression for the dorsal-paired medial (DPM) neurons and the central complex (CC), respectively (O'Dell et al., 1995; Waddell et al., 2000). The MBs are brain structures that have been shown to play a central role in olfactory learning and memory (de Belle & Heisenberg, 1994; Dubnau et al., 2001; McGuire et al., 2001), and the DPM neurons are a pair of large neurons that innervate MB axons and are required specifically for retention of olfactory memory (Waddell et al., 2000). The CC is another major fly brain structure and has been implicated in locomotor control as well as olfactory learning (Martin et al., 1999).

The results are shown in Figure 2-4B. Only pan-neural expression of $ras1^{WT}$ rescued the memory deficit of *ix12a/ΔC40B*. None of the MB-, DPM-, or CC-specific

expression rescued the phenotype. This result may reflect a broad expression pattern of the *ras1* transcript in the *Drosophila* adult brain (Segal & Shilo, 1986). That is, Ras may be required for multiple brain structures constituting the neuronal circuitry for olfactory memory.

In an effort to obtain a further insight into what brain tissues require Ras, I also conducted another set of experiments, expressing a dominant-negative form of *ras1* (*ras1*^{N17}, Feig & Cooper, 1988) under the GAL4-UAS control. Although expression of wild-type *ras1* in a subset of brain cells was not sufficient to restore the memory performance of *ras* mutants, disruption of Ras signaling, on the other hand, could result in a memory deficit, even if it occurred only in part of the memory circuitry. Using the same set of *GAL4* drivers used for the rescue experiments, I expressed *ras1*^{N17} in MB, DPM or CC neurons. Expression of *ras1*^{N17} in MB neurons by the *c747* driver resulted in a significant learning (immediate memory) deficit (Figure 2-5). The sensorimotor functions (Figure 2-6) and overall MB morphology (Figure 2-7) of the learning-impaired flies were indistinguishable from those of the other transgenic flies that showed normal learning. Unfortunately, the *UAS-ras1*^{N17} strain itself showed a 1 h-memory deficit without *GAL4* drivers (data not shown), the tissue-specific effects of inhibition of Ras signaling on the retention of memory remained to be determined. Nevertheless, the experiments using the dominant-negative *ras1*^{N17} revealed a requirement of Ras signaling in the MBs for olfactory memory formation in *Drosophila*.

The effects of reducing Ras dosage on synaptic morphology

Activity-dependent modifications of synaptic morphology have been believed to provide a basis for long-lasting memory stored in the nervous system (Kandel, 2001). The *Drosophila* neuromuscular junction (NMJ) provides an excellent model system for analysis of gene functions in regulation of presynaptic morphology. Flies deficient in olfactory learning and memory, such as *dunce* and *fasII*, have been shown to have altered

presynaptic morphology at the larval NMJ (Schuster et al., 1996). To obtain an insight into involvement of Ras signaling in regulation of presynaptic morphology, I performed a histological analysis for the larval NMJ of the $ras1^{ix12a}/ras1^{\Delta C40B}$ mutant. The NMJ of the *ras1* mutant had an expanded branch pattern and increased number of varicosities at the motor neuron axon terminal, comparing to the wild-type (Figure 2-8).

2-4. Discussion

I tested if Ras signaling is required for an associative form of olfactory learning and memory in *Drosophila*. I demonstrated that flies carrying hypomorphic mutations in the *ras1* gene are impaired in the ability to maintain memory that has been established previously. Their learning (i.e., the ability to make a CS-US association) tested immediately after training was entirely normal (Figure 2-1). Consistent with this, the simple sensorimotor functions required to perform the learning task and overall morphology of the MBs (the center for multimodal associations) were also normal in the *ras1* mutant (Figures 2-2 and 2-3). The severity of the memory impairment was correlated with the degree of molecular lesions in the *ras1* gene. Furthermore, the memory impairment was rescued by pan-neural expression of a wild-type *ras1* transgene (Figure 2-4). These results indicate that the memory impairment of the *ras1* mutant results from an inadequate level of Ras signaling.

Ras has been suggested to play an important role in learning and memory behavior in mammals. Mice heterozygous for a null mutation of *K-ras* show a contextual learning deficit, when coupled with pharmacological inhibition of MEK, a downstream effector of Ras (Ohno et al., 2001). Mice lacking a Ca^{2+}-dependent Ras-specific GEF (*Ras-GRF1*) are impaired in various forms of memories (Brambilla et al., 1997; Giese et al., 2001). NF1 and SynGAP are members of the GAP family that specifically accelerate inactivation of GTP-bound Ras. Mutations in the *NF1* gene cause NF1 disease in humans, and about half of NF1 patients exhibit learning impairments (Costa & Silva,

2003; Dasgupta & Gutmann, 2003). Mice lacking *NF1* or *SynGAP* show spatial learning deficits (Costa et al., 2002; Costa et al., 2001; Komiyama et al., 2002). The ERK type of MAPK is an intensively-studied downstream effector of Ras. Mice lacking *ERK1* show enhanced striatum-dependent learning and memory (Mazzucchelli et al., 2002). Conditional expression of a dominant-negative form of *MEK1* (an activator of ERK) in mouse forebrain results in a selective deficit in hippocampus-dependent long-term memory (Kelleher et al., 2004). My study demonstrates that Ras is required for associative olfactory memory in *Drosophila*.

My study also revealed that inhibition of Ras signaling in the MBs can impair associative learning in *Drosophila* (Figure 2-5). The result that it affected learning rather than memory (unlike the hypomorphic *ras1* mutations) may be due to stronger suppression of Ras signaling by the dominant-negative $Ras1^{N17}$ when expressed with the *c747 GAL4* driver.

The MB forms an insect brain structure that in *Drosophila* consists of about 2,500 intrinsic neurons (Davis, 1993). Residing in each hemisphere, it receives olfactory input and presumably somatosensory input that transmits electroshock (Davis, 1993). Chemical or genetic ablation of the MBs in *Drosophila* results in an olfactory learning impairment (de Belle & Heisenberg, 1994; Heisenberg et al., 1985). Recent studies suggest that intrinsic MB neurons may provide where the odor-shock association takes place in olfactory learning (Dubnau et al., 2001; McGuire et al., 2001). A number of *Drosophila* genes that are implicated in olfactory learning, such as *dunce* (cAMP phosphodiesterase), *rutabega* (adenylyl cyclase, AC), *DCO* (PKA-C1), *leonardo* (14-3-3 protein), *volado* (α-integrin), and *fasII*, are expressed preferentially in MB neurons (Cheng et al., 2001; Grotewiel et al., 1998; Han et al., 1992; Nighorn et al., 1991; Skoulakis et al., 1993; Skoulakis & Davis, 1996). Deregulation of the AC/cAMP pathway in MB neurons leads to a learning impairment (Connolly et al., 1996). Expression of the Rutabaga AC in MB neurons is sufficient to rescue the olfactory learning deficit of the *rutabaga* mutant (Zars

et al., 2000). My finding that expression of a dominant-negative form of *ras1* in MB neurons impairs olfactory learning updates the list of *Drosophila* genes for learning, so as to include *ras1*.

Recent studies suggest that Ras controls both pre- and post-synaptic plasticity. Ras can be activated by Ca^{2+} following membrane depolarization in neurons (Farnsworth et al., 1995). Several signaling molecules that link Ca^{2+} and Ras have been identified (Chen et al., 1998; Ebinu et al., 1998; Farnsworth et al., 1995; Kim et al., 1998). In the mammalian hippocampus, Ras can regulate NMDA receptor-dependent synaptic potentiation (Manabe et al., 2000; Zhu et al., 2002). In the *Drosophila* larval NMJ, Ras is required for a postsynaptic cellular response to an AC-activating neuropeptide (Zhong, 1995). Another study demonstrates that the activity level of the Ras/MAPK pathway is critical to regulate the number of varicosities at the NMJ axon termini (Koh et al., 2002). The regulation of the presynaptic structure by this pathway is shown to be mediated by FasII localized at the presynaptic termini, suggesting that the Ras/MAPK pathway controls FasII-mediated cell adhesion that has been implicated in synaptic growth (Koh et al., 2002). The increased number of presynaptic varicosities in the hypomorphic *ras1* mutants in my study mimicked the result in flies expressing dominant-negative *ras1* in motor neurons (Koh et al., 2002). Whether similar processes are involved in the *Drosophila* brain is entirely an open question. However, these findings may provide a foundation to pursue how Ras functions in central neurons (e.g., MB neurons) to mediate olfactory learning and memory in *Drosophila*.

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2-6. Figures

Figure 2-1

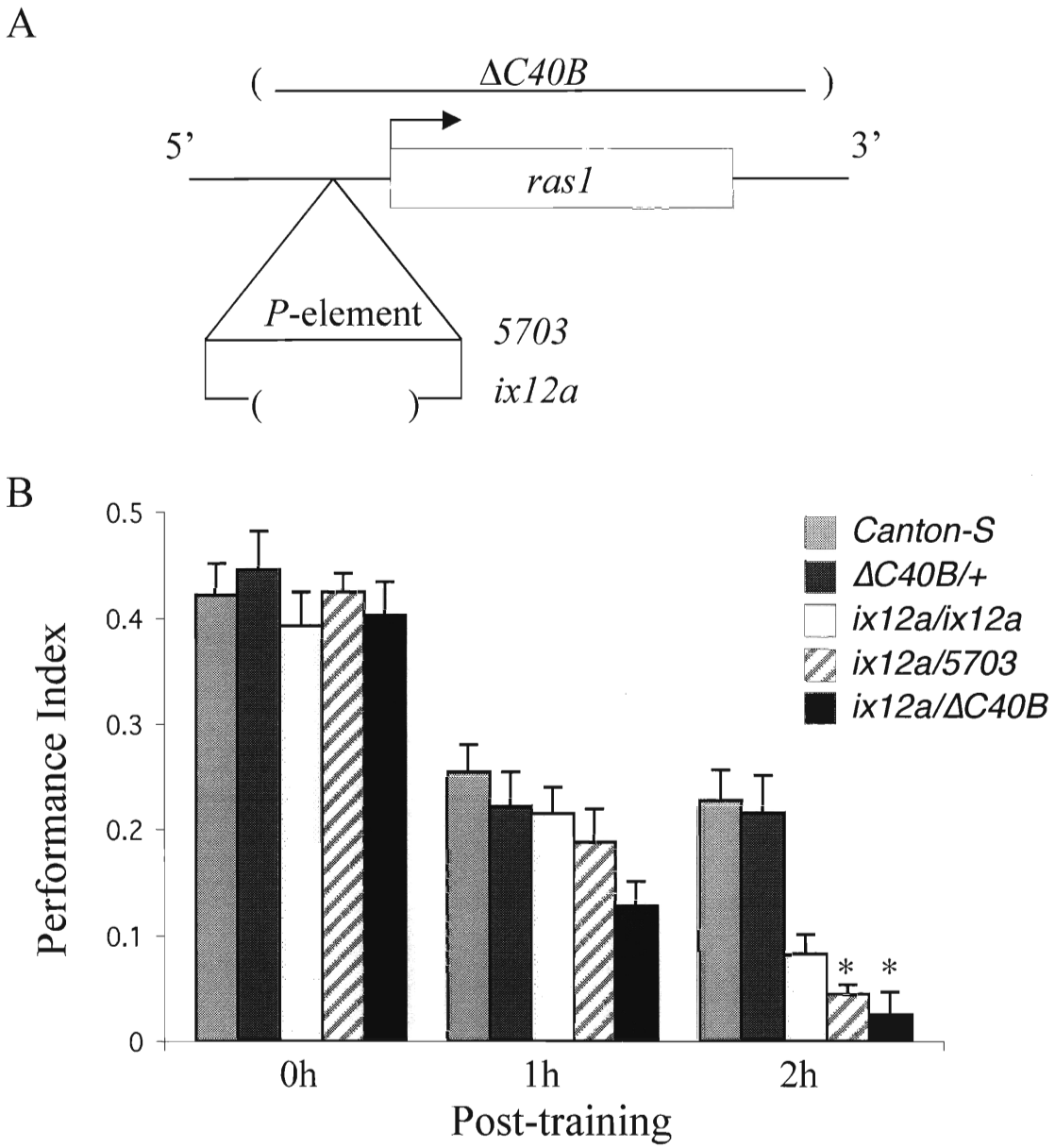
A
ΔC40B
5'
3'
ras1
P-element
5703
ix12a
B
Performance Index
0.5
0.4
0.3
0.2
0.1
0
Canton-S
ΔC40B/+
ix12a/ix12a
ix12a/5703
ix12a/ΔC40B
*
*
0h
1h
2h
Post-training

Figure 2-1. Hypomorphic *ras1* mutant flies are impaired in olfactory memory. (A) A schematic representation of the mutant alleles used for the experiments. The allele $ras1^{5703}$ contains a *P*-element 28 bp upstream of the *ras1* transcription start site that disrupts expression of *ras1*. $ras1^{ix12a}$ and $ras1^{\Delta C40B}$ were generated by imprecise excisions of the *P*-element. $ras1^{ix12a}$ is a milder allele, consisting of an internal deletion, leaving 2 kb of the original 15 kb transposon. $ras1^{\Delta C40B}$ is a severer allele, consisting of a deletion of *ras1*. By combining these alleles, an allelic series was made to investigate the relationship between levels of *ras1* expression and learning and memory performance. (B) All the *ras1* mutants showed normal learning performance (0 h post-training), but their memory tended to decay faster than the *Canton-S* control, except heterozygous Δ*C40B*. The memory loss of the mutants appeared to be correlated with the degree of their molecular lesions in the *ras1* gene. * $p \leq .05$ by Schuffe's post-hoc comparisons with *Canton-S* or Δ*C40B*/+ following a one-way ANOVA for 2 h post-training. Each of the data bars shows a mean ± SEM from 6 independent experiments.

Figure 2-2

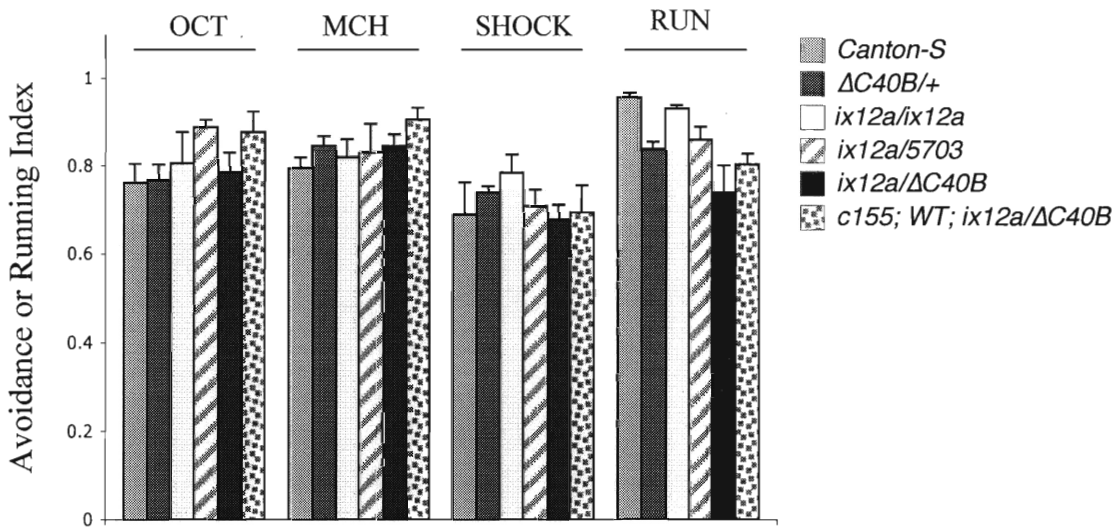

Figure 2-2. The *ras1* mutants are normal in simple sensorimotor functions. Reactivity to the stimuli used for the learning and memory experiments was quantified as Avoidance Index, and running performance as Running Index (see Methods). The labels on the upper right of the figure represent *ras1* alleles. *c155; WT; ix12a/ΔC40B* represent *c155; UAS-ras1*^{WT} in the *ras1*^{ix12a}/*ras1*$^{\Delta C40B}$ background. See Figure 4 for their rescued memory performance. Each of the data bars shows a mean ± SEM from 6 independent experiments.

Figure 2-3

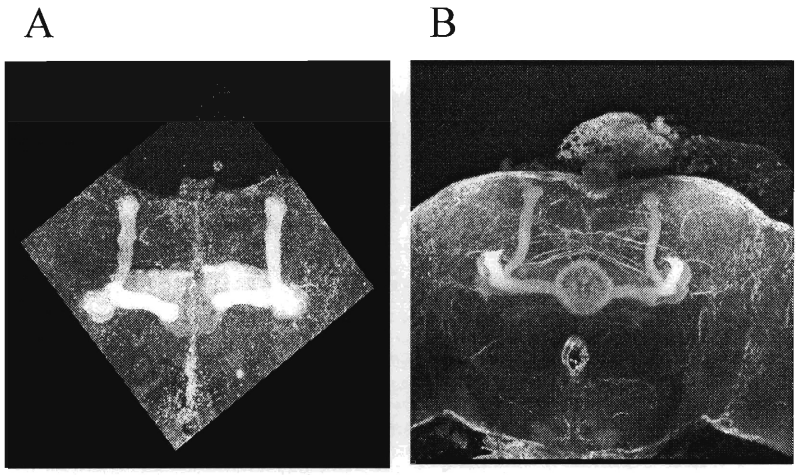

Figure 2-3. The overall MB morphology of the *ras1*^{ix12a}/*ras1*$^{\Delta C40B}$ mutant is normal. (A) Wild-type (*Canton-S*). (B) *ras1*^{ix12a}/*ras1*$^{\Delta C40B}$. The MBs were visualized with an anti-FasII antibody. The image of *Canton-S* was scanned with high contrast. Examination of single sections showed essentially the same pattern between the wild-type and mutant.

Figure 2-4

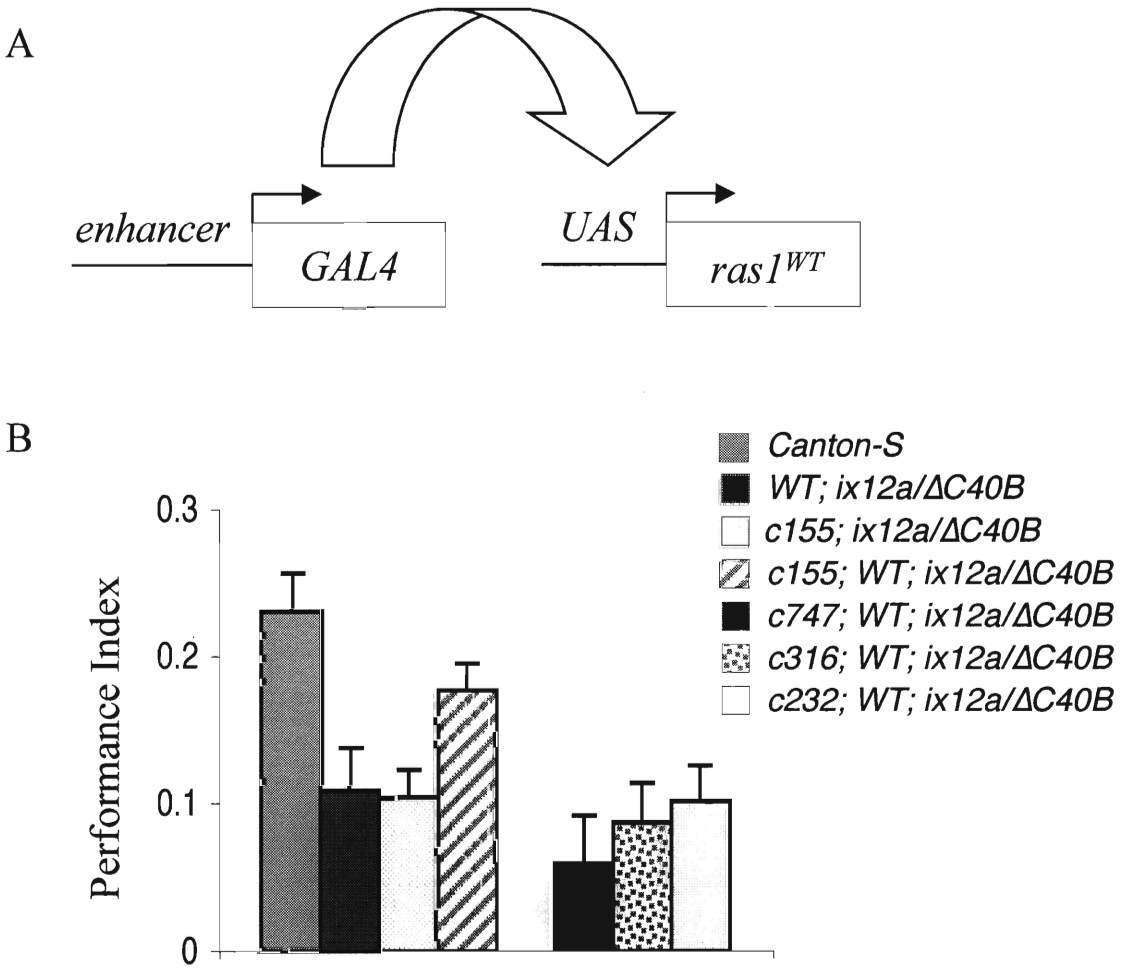
A
enhancer
GAL4
UAS
ras1WT
B
Performance Index
0.3
0.2
0.1
0
Canton-S
WT; ix12a/ΔC40B
c155; ix12a/ΔC40B
c155; WT; ix12a/ΔC40B
c747; WT; ix12a/ΔC40B
c316; WT; ix12a/ΔC40B
c232; WT; ix12a/ΔC40B

Figure 2-4. The memory deficit of $ras1^{ix12a}/ras1^{\Delta C40B}$ is rescued by expression of wild-type *ras1* ($ras1^{WT}$). (A) The GAL4-UAS system was used for tissue-specific expression of $ras1^{WT}$. In this system, the yeast gene *GAL4*, foreign to *Drosophila*, can be expressed specifically in various tissues, dependent on the neighboring enhancer. The expressed GAL4 protein activates the *UAS* promotor that drives expression of the gene placed in its downstream. (B) Pan-neural expression of $ras1^{WT}$ by the *c155 GAL4* driver resurrected the 1 h memory of *ix12a/ΔC40B*. Statistical significance of the memory deficit of *ix12a/ΔC40B* ($p = .005$) disappeared with a combination of *c155* and *UAS-*$ras1^{WT}$ ($p = .751$, compared with *Canton-S* by Scheffe's post-hoc tests). Expression of $ras1^{WT}$ in more restricted brain regions (the right-most 3 groups) did not result in rescue. The data are shown in mean ± SEM. Eight independent experiments were done for the left 4 groups in the figure and 6 experiments for the rest (see Figure 2 for the sensorimotor performance of *c155; WT; ix12a/ΔC40B*).

Figure 2-5

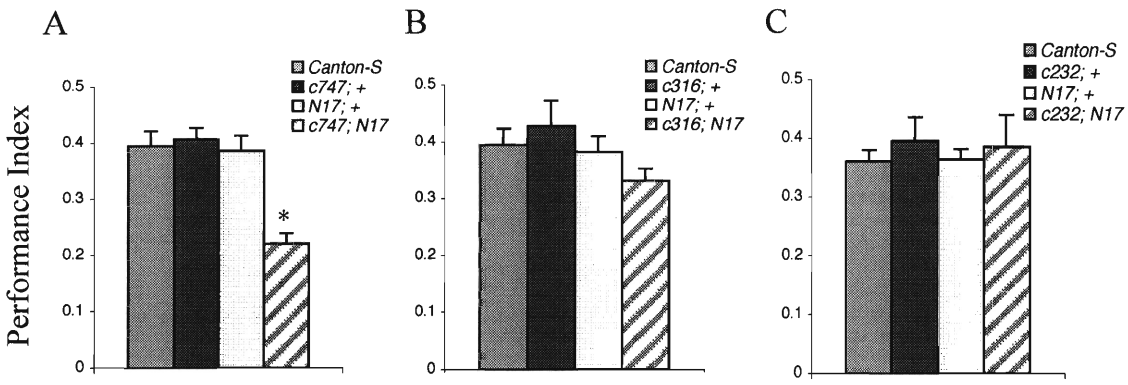

Figure 2-5. Inhibition of Ras signaling in the MBs impairs olfactory learning. (A-C) Using the GAL4-UAS system, a dominant-negative form of *ras1* ($ras1^{N17}$) was expressed in MB (A), DPM (B) or CC (C) neurons. Independent ANOVAs were performed for each set of the experiments (i.e., A, B, and C) * $p < .001$, compared with *Canton-S* by Scheffe's post-hoc analysis. Each of the data bars represents a mean ± SEM from 6 independent experiments.

Figure 2-6

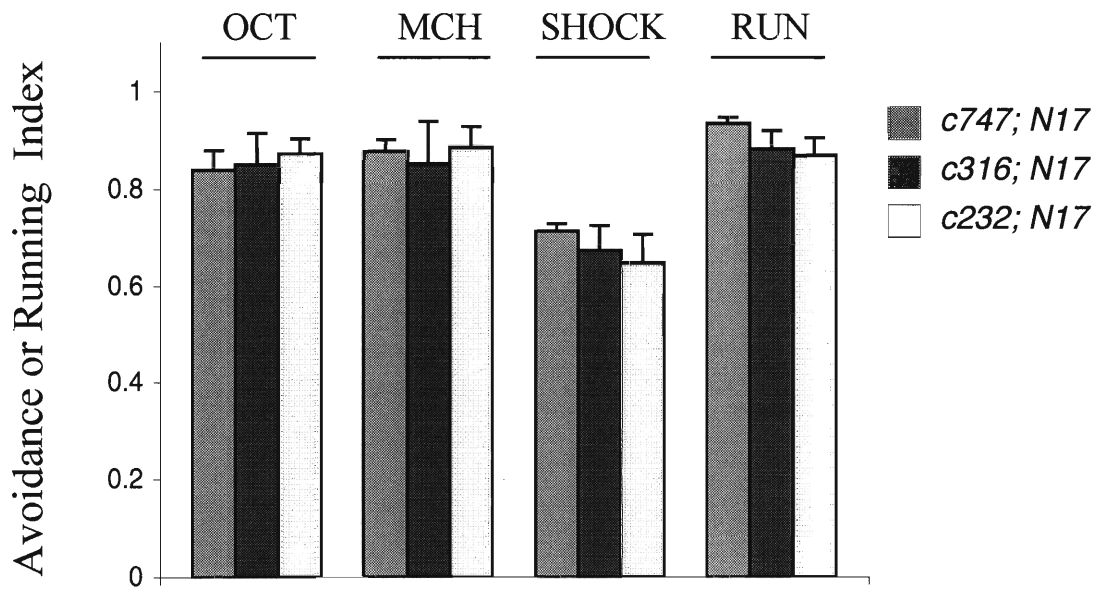

Figure 2-6. Inhibition of Ras signaling in the MBs does not affect simple sensorimotor functions. No significant differences between the genotypes were detected for each stimulus or running. Each of the data bars represents a mean ± SEM from 6 independent experiments.

Figure 2-7

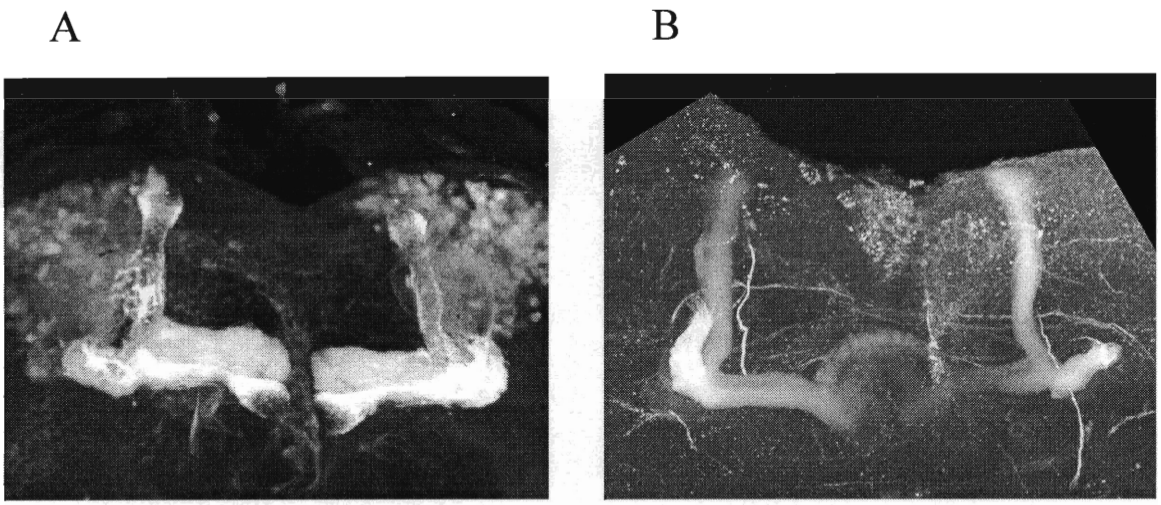

Figure 2-7. Overall MB morphology is normal in the learning-impaired *ras1*^{N17} transgenic flies. (A) Control. *UAS-lacZ* was expressed by the *GAL4* driver *247*. The MBs are visualized with an anti-LacZ antibody. (B) The MBs of *c747; UAS-ras1*^{N17}, visualized with an anti-FasII antibody.

Figure 2-8

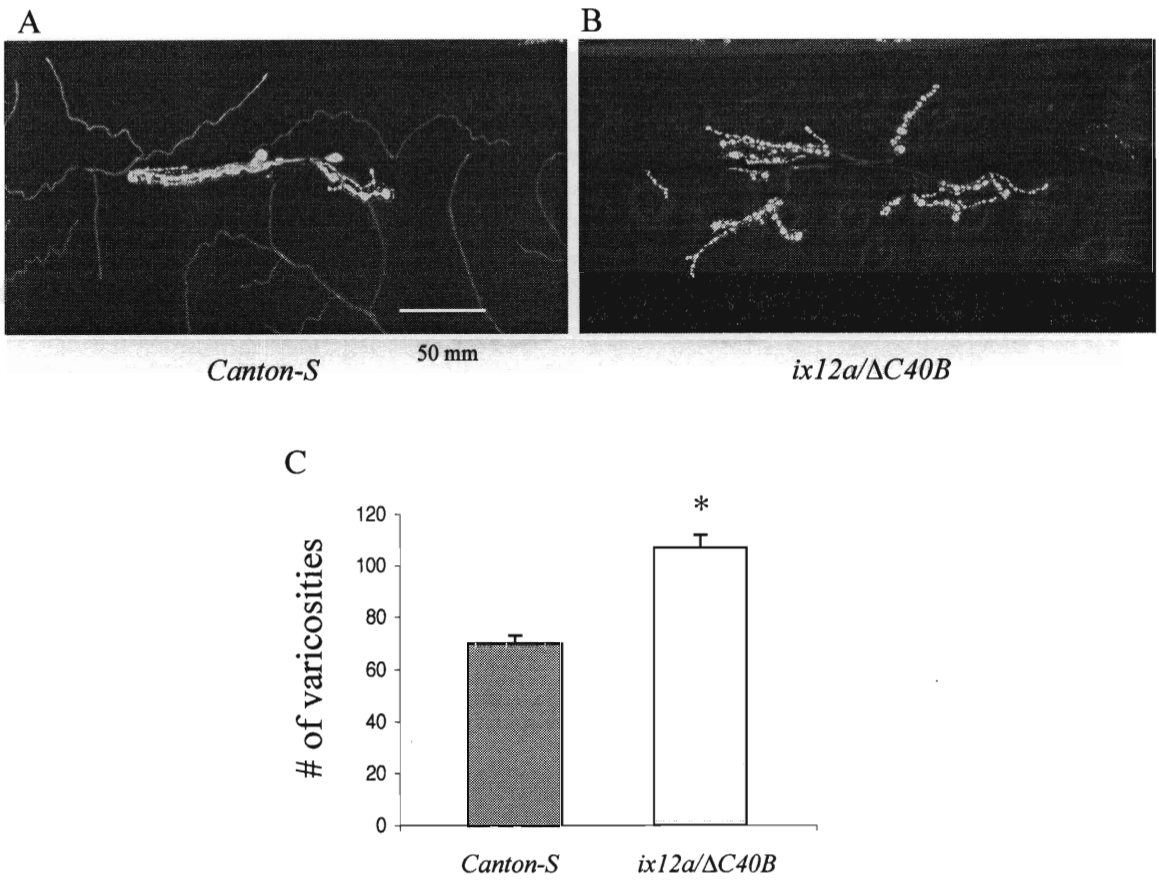

Figure 2-8. An increased number of presynaptic varicosities at the neuromuscular junction (NMJ) of $ras1^{ix12a}/ras1^{\Delta C40B}$. (A and B) Shown is the morphology of motor neuron axon termini innervating the abdominal muscles 6 and 7 at the segment A3 of third instar larvae. The neurons are visualized with an anti-HRP antibody. (C) *ix12a/ΔC40B* had the greater number of varicosities than *Canton-S* ($p < .001$, t-test). The data (shown in mean ± SEM) were collected from 16 animals for *Canton-S* and 11 for *ix12a/ΔC40B*.

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CHAPTER 3

Enhanced Ras Signaling Impairs Associative Learning in *Drosophila*

3-1. Introduction

Ras is a small GTPse whose structure and basic cellular function is highly conserved from yeasts to humans. It serves as a molecular switch in the cell, by cycling between the GDP-bound inactive and GTP-bound active conformations. Located in the inner face of the plasma membrane, it functions to transduce extracellular signals to intracellular effectors (Lowy & Willumsen, 1993). Disruption of normal Ras function leads to a variety of aberrant phenotypes, apparently dependent on a context in which Ras is functioning in the cell (e.g., cell type, developmental stage, intensity of Ras activation) (Bergmann et al., 1998; Fortini et al., 1992; Halfar et al., 2001; Karim et al., 1998). Importantly, in humans, deregulation of Ras or related signaling pathways has been implicated in cognitive disorders as well as in cancers (Shields et al., 2000; Weeber et al., 2002).

Roles of Ras in post-mitotic neurons have been studied intensively. The observed high level of Ras expression in adult brains (Manabe et al., 2000; Segal & Shilo, 1986; Sudol, 1988) and recent discoveries of Ca^{2+}-dependent Ras signaling pathways (Chen et al., 1998; Ebinu et al., 1998; Farnsworth et al., 1995) have strongly suggested an important role of Ras in activity-dependent neuronal events, such as synaptic transmission and synaptic modification. Most recently, it has been suggested that Ras acts downstream of NMDA receptor and Ca^{2+}/calmodulin-dependent protein kinase II

signaling to drive synaptic delivery of AMPA receptor subunits for long-term potentiation (LTP) in rat hippocampal neurons (Zhu et al., 2002). In *Drosophila*, it has been suggested that Ras controls fine presynaptic morphology in axon termini at neuromuscular junctions (Koh et al., 2002). These two functions of Ras at pre- and post-synaptic sites are apparently mediated by the mitogen-activated protein kinase (MAPK), the most well-studied downstream effector of Ras.

A number of molecular-genetic or pharmacological studies suggest that Ras plays an important role in learning and memory behavior. Mice lacking a Ca^{2+}-dependent positive regulator of Ras, *Ras-GRF*, are impaired in amygdala- or hippocampus-dependent memories (Brambilla et al., 1997; Giese et al., 2001). Genetic or pharmacological inhibition of the MAPK pathway affects amygdala-, hippocampus- or striatum-dependent memories (Atkins et al., 1998; Mazzucchelli et al., 2002; Schafe et al., 2000). In flies, *leonardo* mutants in the *14-3-3 zeta* gene that can activate the MAPK pathway are impaired in associative olfactory learning (Philip et al., 2001).

Another line of evidence that suggests a role of Ras in learning and memory comes from studies on negative regulators of Ras. The *NF1* gene is a member of the Ras GTPase-activating protein (GAP) family that specifically accelerate inactivation of GTP-bound Ras. Mutations in this gene cause NF1 disease in humans, and about half of NF1 patients exhibit learning impairments (Costa & Silva, 2003; Dasgupta & Gutmann, 2003). Animal models of NF1 disease also show significant impairments in learning. Mice deficient in NF1 function are impaired in spatial learning (Costa et al., 2002; Costa et al., 2001). Flies deficient in *NF1* are impaired in associative olfactory learning (Guo et al., 2000). Although loss of NF1 expression is associated with neurofibromas in certain types of cells, the learning defects appear to be independent of NF1-associated tumor formations (Costa & Silva, 2003; Dasgupta & Gutmann, 2003). SynGAP, another member of the GAP family, is a molecule that links the NMDA receptor and Ras (Chen et al., 1998; Kim et al., 1998). Mice deficient in *SynGAP* show a spatial learning deficit

(Komiyama et al., 2002). Because NF1 and SynGAP are GAPs that inactivate Ras, it is suggested that the learning impairments associated with the deficiency in these genes result from hyperactivity of Ras (Costa & Silva, 2003; Costa et al., 2002; Costa et al., 2001; Dasgupta & Gutmann, 2003). However, there has been no direct evidence that enhanced Ras signaling can impair learning.

Here we report that, in *Drosophila*, enhanced Ras signaling can lead to a significant impairment in an associative form of olfactory learning without developmental complications. Our findings may be a behavioral manifestation of the roles of Ras in synaptic plasticity that have been hypothesized previously. Also, our findings may provide insights into the pathology of Ras-related cognitive impairments in humans.

3-2. Methods

Flies

All the transgenic insertions used in this study have been described previously. *UAS-ras1^{WT}*, *UAS-ras1^{V12}*, *UAS-ras1^{V12S35}*, UAS-*ras1^{V12G37}*, and *UAS-ras1^{V12C40}* were generated by Karim et al. (Karim et al., 1998). *UAS-raf^{gof}* (Brand & Perrimon, 1994) and *UAS-lacZ* (Brand & Perrimon, 1993) were generated by Brand et al. *HS-ras1^{V12}* was a gift from Norbert Perrimon (Miller & Cagan, 1998). The *GAL4* line *247* is described by Zars et al (Zars et al., 2000), and *c747* (Connolly et al., 1996) and *c232* (O'Dell et al., 1995) are *GAL4* enhancer-trap lines. All the transgenic flies were isogenized by outcrossing at least 5 generations to a *white* stock with the *Canton-S* genetic background. *GAL4-UAS* flies were constructed when necessary by crossing a *UAS* stock to a *GAL4* one (The only exception was *247; UAS-lacZ*. For brain staining, we constructed *247; UAS-lacZ* double homozygotes and maintained them as a stock). All the flies were raised on standard cornmeal food at 25 ˚C on a 12 hr: 12 hr light: dark cycle.

Behavioral assays

Olfactory learning and simple sensorimotor functions were assayed essentially as described previously (Quinn et al., 1974; Waddell et al., 2000). In this study, we used adult flies, mixed males and females, 3-7 days old. Flies were tested for learning immediately after training. 3-octanol (ICN Biomedicals Inc.) was diluted to 1:200 and 4-methylcyclohexanol (ICN Biomedicals Inc.) 1:100 both in ether. All experiments were conducted at 25 ˚C. In experiments involving heat shock, flies were placed in a chamber at 37 ˚C for 1 h, given a 3 h-recovery period at 25 ˚C, and then tested for learning. Running performance was also assayed essentially as described previously (Benzer, 1967). Briefly, in the presence of light, 5 runs were made. Each run lasted for 30 s. About 50 flies were tested in a single experiment. Flies were given a score according to the number of times that they run toward the light (flies that run 5 times were given a score of 5, 4 times a score of 4, and so on). To calculate the running index (RI), the running scores given to individual flies were summed and the total value was divided by the value of the total number of flies tested multiplied by 5. Thus, the RI ranged from 0 to 1, 0 representing none of the flies run at all and 1 representing all the flies run 5 times.

Immunohistochemistry

Adult brains were dissected from flies aged 3-7 days old under phosphate-buffered saline (PBS). They were transferred to 2% paraformaldehyde for 30 min and then washed in PBS + 0.3 % Triton X-100 (Sigma) several times for at least one hour total time. Primary antibodies were diluted in PBS + 0.3 % Triton X-100 + 3 % Normal Goat Serum and incubated overnight at 4 ˚C. Primary antibodies were either rabbit anti-β-galactosidase (Cappel) 1: 2000 or mouse anti-Fasciclin II 1:20 (Schuster et al., 1996). On the following day, brains were washed several times (minimum 2 hours) in PBS + 0.3 % Triton X-100. Secondary antibodies were diluted in PBS + 0.3% Triton X-100 and incubated overnight at 4 ˚C. Secondary antibodies used were goat anti-rabbit IgG-conjugated with Alexa

Fluor 488 or goat anti-mouse IgG-conjugated with Alexa Fluor 488 (Molecular Probes Inc.). Finally, brains were washed in PBS + 3 % Triton X-100 for 2-4 hours before mounting in VectaShield (Vector Labs Inc.).

3-3. Results

ras1^{V12} expression in mushroom body neurons impairs olfactory learning

Olfactory learning can be assayed efficiently in *Drosophila*. If flies are exposed to an odor and simultaneously given electric shock, the majority of the flies afterward avoid the shock-associated odor (Quinn et al., 1974). This type of olfactory learning requires the MBs, apparent brain loci for the odor-shock association in *Drosophila* (Dubnau et al., 2003; Heisenberg, 2003).

Drosophila ras1 is a homolog of mammalian *H-ras*, *K-ras*, and *N-ras*. To examine the effect of enhanced Ras signaling on learning, we expressed a constitutively-active form of *ras1*, *ras1^{V12}* (Seeburg et al., 1984), in the MBs using the yeast GAL4-UAS system (Brand & Perrimon, 1993), and tested the flies in the olfactory learning paradigm. A similar approach has been used previously in flies for the G-protein α subunit that activates adenylyl cyclase (Connolly et al., 1996). We used two different *GAL4* driver lines, *247* and *c747*, which have been shown to express the GAL4 protein predominantly in Kenyon cells in the MBs (Connolly et al., 1996; Zars et al., 2000). When transgenic flies homozygous with a *UAS-ras1^{V12}* construct were crossed to flies homozygous with a *247 GAL4* construct, their progeny showed a severe learning impairment (Figure 3-1A). Furthermore, when *UAS-ras1^{V12}* were crossed to *c747*, their progeny showed a similar learning impairment (Figure 3-1B). Because the learning of singly-transgenic control flies (i.e., *UAS-ras1^{V12}; +*, *247; +* or *c747; +*) were indistinguishable from that of *Canton-S* (wild-type), the learning impairments of *247; UAS-ras1^{V12}* or *c747; UAS-ras1^{V12}* were considered due to the expression of *ras1^{V12}* driven by the MB *GAL4* drivers. We also tested the effect of expression of a wild-type

form of *ras1* (*ras1^{WT}*) in the MBs using the same *GAL4* drivers. Such simple overexpression of *ras1^{WT}* did not result in a significant learning impairment (Figure 3-1D and E), which indicates that the effect of *ras1^{V12}* expression on learning is not due to abundance of Ras proteins by transgene expression, but is a dominant effect by *ras1^{V12}*.

We then expressed *ras1^{V12}* in another brain structure, called central complex (CC), in order to examine whether the effect of *ras1^{V12}* expression on learning is specific to the MBs. The CC has been implicated in locomotor control as well as olfactory learning (Martin et al., 1999). To drive expression of *ras1^{V12}* in the CC, we crossed *UAS-ras1^{V12}* to the *GAL4* line *c232* (O'Dell et al., 1995) that has been shown to express the GAL4 protein predominantly in CC neurons. The learning performance of the resulting *c232; UAS-ras1^{V12}* was indistinguishable from that of *Canton-S* (Figure 3-1C). The learning of similarly generated *c232; UAS-ras1^{WT}* was also indistinguishable from that of *Canton-S* (Figure 3-1F). These results indicate that the effect of *ras1^{V12}* expression on learning occurs in the MBs.

Comparisons of simple sensorimotor functions between transgenic and *Canton-S* lines indicated that odor acuity, electroshock reactivity, and running performance were normal in the flies that showed a learning impairment (i.e., *247; UAS-ras1^{V12}* and *c747; UAS-ras1^{V12}*) (Figure 3-2). Furthermore, histological comparisons of the MBs between the learning-impaired and the control indicated no abnormalities in overall MB morphology (Figure 3-3). These results collectively indicate that enhanced Ras signaling in the MBs by *ras1^{V12}* expression impairs olfactory learning without affecting non-associative forms of simple sensorimotor functions and gross MB morphology.

Acute induction of *ras1^{V12}* expression impairs olfactory learning

Although the GAL4-UAS approach we used is an elegant way to achieve tissue-specific expression of transgenes, the temporal profiles of transgene expression depend on the enhancers that drive *GAL4* expression, and resulting phenotypes could be due to the

chronic expression of the transgenes during development (Brand & Perrimon, 1993). Although we did not observe any abnormality in gross MB morphology in the *GAL4-UAS* flies with a learning impairment, it is still possible that their neuronal development and function is affected by chronic expression of $ras1^{V12}$, and that this indirectly leads to the learning phenotype. To check whether acutely-enhanced Ras signaling can affect olfactory learning, we tested flies carrying $ras1^{V12}$ whose expression is driven by a heat-shock promotor (*HS-*$ras1^{V12}$). The flies were heat-shocked for 1 h at 37 ˚C and tested at 25 ˚C for learning 3 h later. Without heat shock, the learning performance of *HS-*$ras1^{V12}$ was not distinguishable from *Canton-S*. After heat shock, however, *HS-*$ras1^{V12}$ showed a significant learning impairment, suggesting that $ras1^{V12}$ expression can acutely impair learning (Figure 3-4A). Because their simple sensorimotor functions were not significantly affected by the heat-shock treatment (Figure 3-4B), the learning impairment of *HS-*$ras1^{V12}$ appears to result from an impairment in establishing association between odor and electric shock.

Effector signaling pathways that mediate the effects of $Ras1^{V12}$ on learning

Ras can activate multiple effectors, such as Raf, Ral-GDS, and Phosphoinositide 3-kinase (PI3-K). These effectors can then transduce the Ras signal to MAPK, Phospholipase D and Akt-1, respectively (Shields et al., 2000). Three different mutations have been identified in the effector-loop domain of Ras (i.e., T35S, E37G, and Y40C). These Ras^{S35}, Ras^{G37}, and Ras^{C40} mutants selectively activate Raf, Ral-GDS, and PI3-K, respectively (Joneson et al., 1996; Rodriguez-Viciana et al., 1997; White et al., 1995). By combining these effector-loop mutations with the V12 mutation (i.e., by constructing Ras^{V12S35}, Ras^{V12G37} and Ras^{V12C40}), the downstream effector signaling pathways that mediate Ras^{V12} signaling have been identified in various mammalian and *Drosophila* systems (Figure 3-5A)(Bergmann et al., 1998; Halfar et al., 2001; Joneson et al., 1996; Karim & Rubin, 1998; Koh et al., 2002; Rodriguez-Viciana et al., 1997; White et al.,

1995). In order to investigate what effector signaling pathways relay Ras1^{V12} signaling that impairs olfactory learning, we tested transgenic flies carrying a *UAS-ras1^{V12S35}*, *UAS-ras1^{V12G37} or UAS-ras1^{V12C40}* construct with *GAL4* driver *247*. Only *247; UAS-ras1^{V12S35}* showed a significant learning impairment. However, their impairment was much milder than that of *247; UAS-ras1^{V12}* (Figure 3-5B). The simple sensorimotor functions of *247; UAS-ras1^{V12S35}* were indistinguishable from those of *247; UAS-ras1^{V12}* or *Canton-S* (data not shown). Also, we did not detect any abnormality in gross MB morphology in *247; UAS-ras1^{V12S35}* (data not shown). Because Ras1^{V12S35} transduces its signal only to Raf, we then tested transgenic flies carrying a constitutively-active form of *raf* (*raf^{gof}*) in a *UAS* construct. Surprisingly, *247; UAS-raf^{gof}* showed no learning impairment (Figure 3-5B). Thus, overall, none of the downstream effector signaling pathways that we examined exactly mimicked the learning phenotype of *247; UAS-ras1^{V12}*, and none could be claimed as a strong candidate pathway that mediates Ras1^{V12} signaling in the MBs that impairs olfactory learning (see Discussion).

3-4. Discussion

Recent studies have shown that disruption of molecular signaling that regulates Ras activation can impair learning and memory. For example, mice lacking a positive regulator of Ras, *Ras-GRF*, are impaired in long-term memories (Brambilla et al., 1997; Giese et al., 2001). A loss-of-function mutation of a negative regulator of Ras, *NF1*, can lead to learning impairments in flies, mice, and humans (Costa & Silva, 2003; Costa et al., 2002; Costa et al., 2001; Dasgupta & Gutmann, 2003; Guo et al., 2000). These findings suggest that regulation of Ras is critical for learning and memory. Here, we directly examined the effects of deregulating Ras signaling on learning by expressing constitutively-active *ras1* (*ras1^{V12}*) in the *Drosophila* brain. We found that *ras1^{V12}* expression in the MBs severely impaired an associative form of olfactory learning without affecting gross MB morphology. We also found that acute induction of *ras1^{V12}*

expression before behavioral training led to a moderate, but significant, learning impairment in the same olfactory learning paradigm.

In *Drosophila*, the MBs are critical brain structures for olfactory learning that requires association of an odor cue with footshock. Recent studies plausibly suggest that MB neurons may be the site where the odor-shock association takes place (Dubnau et al., 2003; Heisenberg, 2003). Given this hypothesis, our behavioral results may reflect that enhanced Ras signaling disrupts molecular processes that establish the odor-shock association in MB neurons.

Ras can activate a number of effector signaling pathways, each of which apparently mediates independent cellular events (Shields et al., 2000). Among others, the MAPK pathway and more recently the PI3-K pathway have been strongly implicated in both synaptic and behavioral plasticity (Atkins et al., 1998; Izzo et al., 2002; Lin et al., 2001; Mazzucchelli et al., 2002; Sanna et al., 2002; Schafe et al., 2000). In this study, we wished to determine a signaling pathway that mediates enhanced Ras signaling that impairs olfactory learning. To this end, we used three different transgenic strains (*UAS-ras1^{V12S35}*, *UAS-ras1^{V12G37}*, and *UAS-ras1^{V12C40}*). Ras^{V12S35}, Ras^{V12G37}, and Ras^{V12C40} interact selectively with Raf (MAPK kinase kinase), Ral-GDS, and PI3-K, respectively (see Figure 3-5A) (Joneson et al., 1996; Rodriguez-Viciana et al., 1997; White et al., 1995). By crossing the *UAS* strains to *GAL4* driver *247*, we generated flies that express the *UAS* transgenes in the MBs. We found that only *247; UAS-ras1^{V12S35}* showed a significant learning impairment, suggesting that the Raf-MAPK pathway mediates Ras1^{V12} signaling to impair learning. However, the learning impairment of these flies was much less severe than that of *247; UAS-ras1^{V12}* (i.e., ~35% versus ~80% loss, comparing to their controls). Furthermore, when we tested *247; UAS-raf^{gof}* (flies expressing a constitutively-active form of *raf* in the MBs), we found that their learning is normal. The differences in learning performance observed between these genotypes might merely reflect differences in the level of *UAS* transgene expression between them.

But, we think this is unlikely because of the following reasons: 1) the level of transgene expression is reported similar between the *UAS* insertions we used; 2) when their expression is driven by other *GAL4* drivers, all the *UAS* insertions can modify phenotypes in a tissue-dependent manner; and 3) *UAS-ras1*^{V12S35} and *UAS-raf*^{gof} can produce phenotypes as aberrant as *UAS-ras1*^{V12} (Bergmann et al., 1998; Halfar et al., 2001; Karim et al., 1998; Koh et al., 2002). Alternatively, we speculate that Ras1^{V12S35} can activate not only the Raf-MAPK signaling pathway, but also other pathways, possibly including a yet unknown pathway, that may mediate Ras1^{V12} signaling in the MBs to impair learning. Besides the pathways examined in this study, Ras can activate adenylyl cyclase in yeasts (Lowy & Willumsen, 1993) and Rin1 in mice (Dhaka et al., 2003). In flies, the adenylyl cyclase pathway is strongly implicated in olfactory learning (Dubnau et al., 2003; Heisenberg, 2003) and is postulated to be activated by the Ras-GAP NF1 (Guo et al., 2000). Mice lacking *Rin1* show enhanced amygdala LTP and amygdala-dependent aversive memory formation (Dhaka et al., 2003). Further investigations in the *Drosophila* MB system might reveal the interactions of Ras1 with these effectors or a novel Ras effector signaling pathway involved in learning.

Although the effect of deregulating Ras signaling on learning was robust in our study, we do not really know how Ras mediates learning in *Drosophila*. Recent studies suggest that Ras controls both pre- and post-synaptic plasticity. In mammals, it is hypothesized that Ras regulates NMDA receptor-dependent synaptic plasticity through downregulating NMDA receptor phosphorylation (Manabe, et al., 2000) or upregulating AMPA receptor trafficking (Zhu et al., 2002). In these hypotheses, enhanced Ras signaling could lead to an impairment in activity-induced synaptic potentiation by either downregulating Ca^{2+} influx or enhancing a basal level of synaptic transmission occuluding activity-induced potentiation. It would be tempting to assume these mechanisms also in *Drosophila* olfactory learning, because the NMDA receptor is a coincidence detector of pre- and post-synaptic activation that may underlie classical

conditioning (e.g., Refs. Dubnau et al., 2003 and Johnston & Wu, 1995 for review). However, the *Drosophila* homologs for the *NMDA receptor* (i.e., *Nmdar1* and *Nmdar2*) have not yet been functionally implicated in learning. A recent study in *Drosophila* larval neuromuscular junctions suggest that Ras is a critical regulator of presynaptic morphology (Koh et al., 2002); Chronic expression of $ras1^{WT}$ or $ras1^{V12}$ transgenes in the presynaptic neurons led to a ~60% or ~100% increase in the number of synaptic boutons, respectively. With this observation, the learning impairment of our *247; UAS-*$ras1^{V12}$ *or c747; UAS-*$ras1^{V12}$ might be in part caused by, if any, such morphological alterations in MB axon termini. Our finding that learning impairments are much severer in *UAS-*$ras1^{V12}$ than in *HS-*$ras1^{V12}$ might reflect the additive effects of functional and structural abnormalities in *UAS-*$ras1^{V12}$.

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3-6. Figures

Figure 3-1

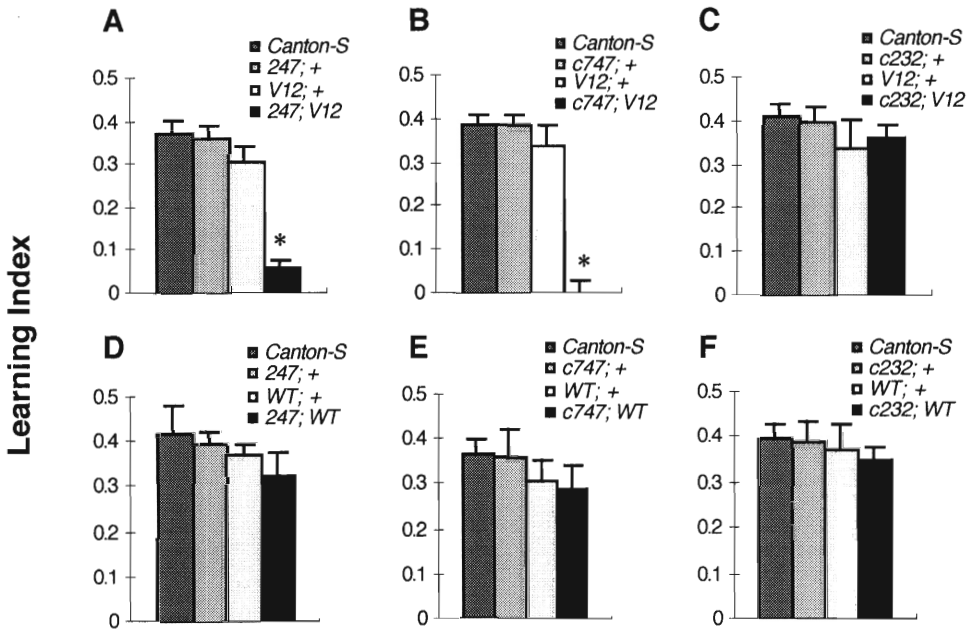

Figure 3-1. *ras1*^{V12} expression in MB neurons impairs olfactory learning. (A) To express a constitutively-active form of *ras1* (*ras1*^{V12}) in MB neurons, we crossed *UAS-ras1*^{V12} to the *GAL4* line *247* that expresses GAL4 protein predominantly in the MBs. The resultant progeny showed a severe learning impairment, comparing to the other genotypes (*$p < .001$). Singly-transgenic control flies *247; +* or *UAS-ras1*^{V12}*; +* were constructed by crossing homozygous *247* or *UAS-ras1*^{V12} to *Canton-S*, respectively. In the graph labels, *UAS-ras1*^{V12} is abbreviated as *V12*. (B) We found a similar learning impairment (*$p < .001$) using another *GAL4* line *c747* that expresses GAL4 predominantly in the MBs. The control line *c747; +* was constructed by crossing homozygous *c747* to *Canton-S*. (C) *ras1*^{V12} expression in CC neurons does not affect learning ($p = .61$). Expression of *UAS-ras1*^{V12} was driven by the *GAL4* line *c232* that expresses GAL4 protein predominantly in the CC. *c232; +* was constructed by crossing homozygous *c232* to *Canton-S*. (D–F) Expression of a wild-type form of *ras1* (*ras1*^{WT}) in the MBs (D and E) or CC (F) does not affect learning. (D) $p = .59$; (E) $p = .59$; (F) $p = .87$. *UAS-ras1*^{WT}*; +* was constructed by crossing homozygous *UAS-ras1*^{WT} to *Canton-S*. In the graph labels, *UAS-ras1*^{WT} is abbreviated as *WT*. One-way ANOVA was used to detect statistically significant differences in learning between genotypes. For A and B, Scheffe's post-hoc comparisons were made. Each of the data bars represents a mean ± SEM from 6 independent experiments.

Figure 3-2

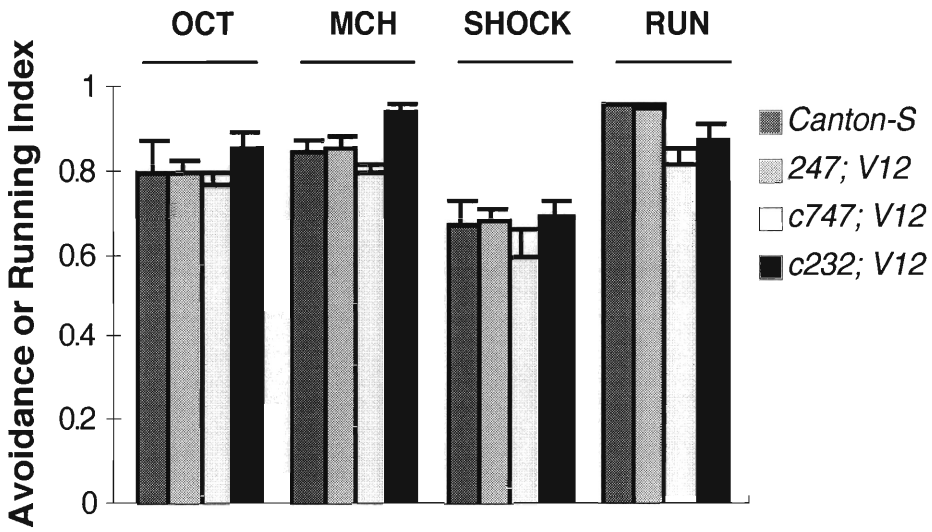

Figure 3-2. Simple sensorimotor functions of *UAS-ras1*^{V12}. OCT = 3-octanol; MCH = 4-methylcyclohexanol; SHOCK = electroshock; RUN = running. Although there were statistically significant differences in RUN between *Canton-S* and *c747; UAS-ras1*^{V12} (p = .02), this difference did not appear to explain the "zero" learning in *c747; UAS-ras1*^{V12} (There was no difference in RUN between *c747; UAS-ras1*^{V12} and *c232; UAS-ras1*^{V12}, but the learning of *c232; UAS-ras1*^{V12} was entirely normal). Scheffe's post-hoc comparisons were made following overall ANOVAs for each stimulus and RUN. Each of the data bars represents a mean ± SEM from 6 independent experiments.

Figure 3-3

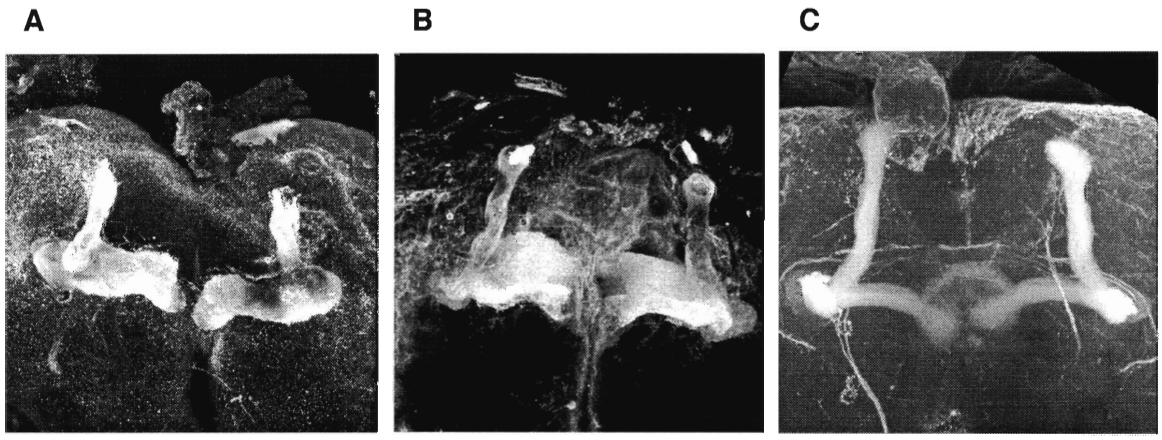

Figure 3-3. Gross morphology of the MBs is normal in the *ras1*^{V12} transgenic flies that exhibit a learning impairment. (A) *247; UAS-lacZ.* (B) *247; UAS-lacZ; UAS-ras1*^{V12}. (C) *c747; UAS-ras1*^{V12}. All the pictures show a frontal view of projections of optical sections of the fly brain. The MBs are visualized with an anti-LacZ antibody (A and B) or anti-Fasciclin II antibody (C).

Figure 3-4

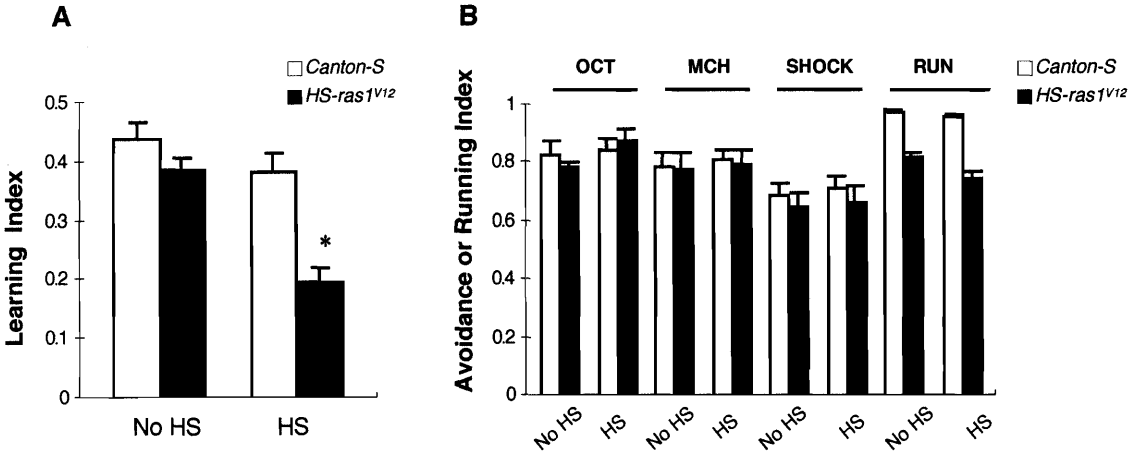

Figure 3-4. Heat-shock (HS) induction of *ras1*^{V12} expression before behavioral training leads to a learning impairment. (A) In experiments involving HS (the right-hand bars), both *Canton-S* and *HS-ras1*^{V12} were placed in a chamber at 37 °C for 1 h, 3 h before training. Such HS led to a learning impairment in *HS-ras1*^{V12} (*$p < .001$). The left-hand data bars represent the results without HS ($p = .15$). Bonferroni's planned comparisons ($\alpha = .025$) were made for each of the HS conditions following a two-way ANOVA ($p < .001$ for genotype; $p < .001$ for HS; $p = .02$ for interaction). (B) Simple sensorimotor functions of *HS-ras1*^{V12}. Two-way ANOVA (genotype x HS) was used to detect the main effects and interaction for each stimulus and RUN. For RUN, there was a main effect in genotype ($p < .001$), but no main effect in HS ($p = .10$) or interaction ($p = .28$). Thus, the learning impairment of *HS-ras1*^{V12} after HS is not likely due to their running performance. Each of the data bars shows a mean ± SEM from 6 independent experiments.

Figure 3-5

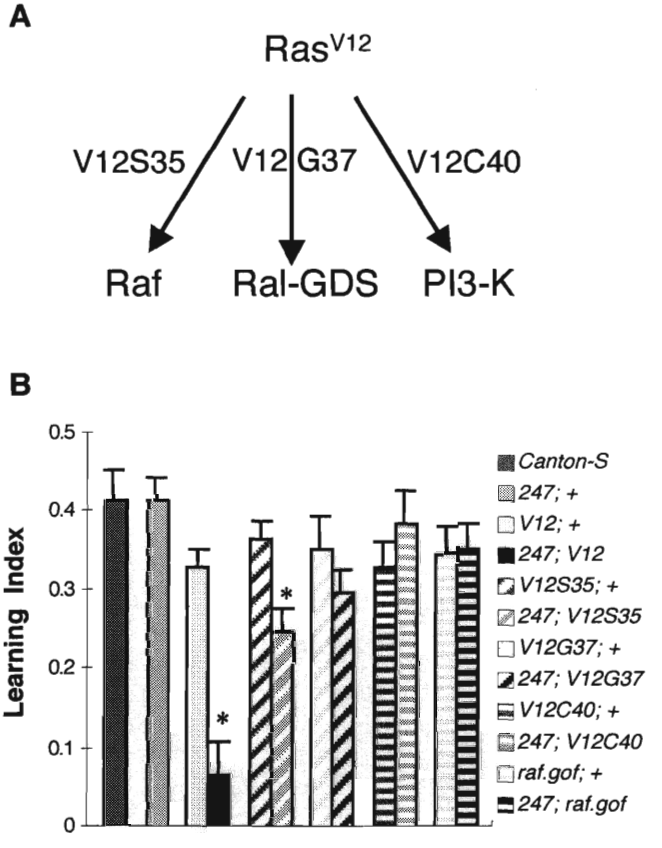

Figure 3-5. None of the *ras1*^{V12} variants that affect known downstream effector signaling pathways mimicked the learning impairment of *ras1*^{V12}. (A) Multiple signaling pathways downstream of Ras. Effector-loop mutants Ras^{V12S35}, Ras^{V12G37}, and Ras^{V12C40} can transduce the Ras^{V12} signal selectively to Raf, Ral-GDS, and PI3-K, respectively (see text). (B) The results of olfactory learning. Flies carrying *UAS-ras1*^{V12}, *UAS-ras1*^{V12S35}, *UAS-ras1*^{V12G37}, *UAS-ras1*^{V12C40}, or *UAS-raf*^{gof} were crossed to the *GAL4* line *247* or *Canton-S*, and their progeny were tested for learning. In the graph labels, *UAS-ras1*^{V12}, *UAS-ras1*^{V12S35}, *UAS-ras1*^{V12G37}, *UAS-ras1*^{V12C40}, and *UAS-raf*^{gof} are abbreviated as *V12*, *V12S35*, *V12G37*, *V12C40*, and *raf.gof*, respectively. An ANOVA detected a significant difference between the genotypes ($p < .001$). Bonferroni's planned comparisons ($\alpha = .01$) following the overall ANOVA identified significant differences between *UAS-ras1*^{V12}; + and *247; UAS-ras1*^{V12} (*$p < .001$), and between *UAS-ras1*^{V12S35}; + and *247; UAS-ras1*^{V12S35} (*$p = .01$). It should be noted that the learning deficit of *247; UAS-ras1*^{V12S35} is much less than that of *247; UAS-ras1*^{V12}. Each of the data bars shows a mean ± SEM from 6 independent experiments.

Acknowledgments

I would like to thank Douglas Armstrong for brain histology. I would also like to thank Andrea Bergmann (*UAS-ras1^{WT}*, *UAS-ras1^{V12}*), Jerry Rubin (*UAS-ras1^{V12S35}*, *UAS-ras1^{V12G37}*, *UAS-ras1^{V12C40}*), Scott Waddell (*247*, *c747, c232*), Norbert Perrimon (*HS-ras1^{V12}*), and the Bloomington Stock Center (*UAS-raf^{gof}*) for their generous donations of the fly stocks.

CHAPTER 4

Ablation of the Compound Eye by Inhibition of Ras Signaling in *Drosophila*

4-1. Introduction

Devising useful genetic screens is essential to identifying genes involved in a biological process of interest. In conventional screens, genes are mutagenized and mutated animals are examined for defined biological events. Aberrant phenotypes caused by the gene mutations can then be used to identify additional genes based on their ability to modify the phenotypes. Relatively simple organisms, such as yeast, nematode, and fly, have been demonstrated particularly useful for such screens. In these organisms, genome-wide, large scale screens based on random mutagenesis can be conducted relatively easily with their short life cycles and abundant genetic tools available in them. Identification of genes based on such screens can lead to novel hypotheses for molecular mechanisms underlying complex biological processes, as exemplified by screens for cell cycle (Hartwell et al., 1974), apoptosis (Ellis & Horvitz, 1986), and embryonic development (Nusslein-Volhard & Wieschaus, 1980).

In *Drosophila*, mutations in the endogenous *ras1* gene or ectopic expression of *ras1* transgenes can cause a variety of aberrant phenotypes. In a sensitized *sevenless* (*sev*) mutant background, loss-of-function mutations of the *ras1* gene can generate the *sev* phenotype, the absence of R7 cells in the adult compound eye (Simon et al., 1991). Expression of a constitutively-active form of the *ras1* transgene ($ras1^{V12}$) under the control of a *sev* enhancer results in a rough eye phenotype with supernumerary R7 cells

through transformation of cone cell precursors into R7s (Fortini et al., 1992). Expression of the same *ras1*^{V12} allele during larval wing imaginal disc development is sufficient to drive ectopic cell proliferation and hyperplastic tissue growth followed by widespread cell death in the wing disc, which results in ablation of adult wing structures (Karim & Rubin, 1998). Clonal analysis shows that in the larval wing, cells lacking *ras1* are smaller, have reduced growth rates, and undergo apoptosis (Prober & Edgar, 2000). Finally, altering the level of Ras signaling with *ras1* mutations or transgenic expression of various alleles of *ras1* in subsets of the neural tissues can result in learning and memory deficits in adult (Sakamoto, this thesis), as well as abnormal numbers of presynaptic varicosities in the larval neuromuscular junction (Koh et al., 2002).

Screens for genes that interact with the Ras signaling pathway have been carried out extensively in the *Drosophila* eye, based on the Ras-associated R7 phenotypes (Dickson et al., 1996; Huang & Rubin, 2000; Karim et al., 1996; Neufeld et al., 1998; Rebay et al., 2000; Simon et al., 1991; Therrien et al., 1998; Therrien et al., 2000). The *Drosophila* eye confers several advantages for genetic screens. It is dispensable for viability or fertility, allowing establishment of lines even with severe eye abnormalities. Also, it is an easy tissue to screen, while undergoing complex cellular processes to develop. The *Drosophila* eye is composed of a regular array of about 800 units, called ommatidia. Each ommatidium contains eight photoreceptor neurons (designated as R1 to R8), four lens-secreting cone cells, and three different types of pigment cells (designated as primary, secondary, and tertiary cells). The adult eye develops from the larval eye imaginal disc, a monolayer of undifferentiated cells, through a series of cell-cell interactions that involves a variety of inter- and intra-cellular signaling pathways (reviewed in Voas & Rebay, 2004). The specification of the presumptive R7 cell initiated by signaling from the R8 cell provides an example. Normal differentiation of the R7 cell requires activation of the Sev receptor tyrosine kinase expressed on the R7 cell by its ligand Boss expressed on the adjacent R8. The activation of Sev leads to activation of the

Ras/MAPK pathway to activate downstream effectors required for R7 differentiation (reviewed in Zipursky & Rubin, 1994). Genetic screens based on the Ras-dependent R7 development have identified many essential components, such as *sos*, *gap1*, *drk*, *Dsor1*, *ksr*, *PTP-ER*, *cnk*, *yan*, and *sina*, which are all associated with the Sev/Ras/MAPK signaling in the R7 cell (Dickson et al., 1996; Huang & Rubin, 2000; Karim et al., 1996; Neufeld et al., 1998; Rebay et al., 2000; Simon et al., 1991; Therrien et al., 1998; Therrien et al., 2000).

More recently, another set of genetic screens has implicated Ras in programmed cell death during eye development in *Drosophila*. Apoptosis in *Drosophila* is mediated by at least three genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim* (Chen et al., 1996; Grether et al., 1995; White et al., 1994). These genes are clustered in a small region of the genome, and deletion of the region blocks virtually all apoptosis in the embryo (White et al., 1994). Ectopic expression of any one of these genes in the developing eye can result in small eye phenotypes due to excessive apoptosis (Chen et al., 1996; Grether et al., 1995; Hay et al., 1995; White et al., 1996). Genetic screens for modifiers of the *hid*-inducing or *rpr*-inducing eye phenotypes identified *ras1* as a suppressor of the phenotypes, together with other components of the Ras signaling pathway, such as *Epidermal growth factor receptor* (*Egfr*) and *MAPK* (*rl*) (Bergmann et al., 1998; Kurada & White, 1998). Further analyses revealed that the anti-apoptotic effect of the Ras pathway is mediated by downregulation of Hid through MAPK (Bergmann et al., 1998; Kurada & White, 1998; see also Sawamoto, et al., 1998). These findings suggest that the EGFR/Ras/MAPK pathway mediates signals promoting cell survival during *Drosophila* eye development. However, the demonstrated anti-apoptotic effect of the pathway is largely based on rather artificially-induced apoptosis (i.e., apoptosis induced by ectopic or overexpression of cell death inducers). Furthermore, the role of EGFR or Ras1 in promoting cell survival may be mediated also by signaling pathways other than the MAPK pathway that leads to downregulation of Hid.

Here, I show that blocking Ras signaling itself is sufficient to cause a small eye phenotype that mimics the one induced by ectopic expression of *hid*, *rpr*, or *grim*. The eye ablation resulting from the inhibition of Ras can be suppressed by blocking apoptosis-inducing machinery, suggesting that the phenotype is based on excessive cell death. The phenotype is also suppressed by enhancing any of the MAPK, Ral-GDS, or PI3-K pathways, indicating that the inhibition of Ras blocks multiple Ras downstream effector pathways to generate the small eye. Interestingly, mutations or transgenic expression of the *amnesiac* or *rap1* genes, which have not been linked to Ras signaling *in vivo*, significantly modified the Ras-associated small eye phenotype. Mutational activation of *ras* has been highly implicated in human cancers, in which viability of affected cells is increased abnormally, as well as the rate of proliferation (Hoffman & Liebermann, 1994). Also, deregulation of apoptosis has been implicated in the pathogenesis of a variety of other human diseases (Thompson, 1995). The small eye phenotype resulting from direct inhibition of endogenous Ras signaling provides a new genetic tool to help understand precise mechanisms underlying Ras-regulated biological events, as well as develop therapeutic strategies for human diseases that are associated with deregulated apoptosis.

4-2. Methods

Fly stocks

The following *Drosophila* strains were used in this study: *c155* (Lin & Goodman, 1994), *GMR-GAL4* (Freeman, 1996), *UAS-ras*^{N17}, *UAS-ras1*^{N17} (Lee et al., 1996), *UAS-ras1*^{WT}, *UAS-ras1*^{V12}, *UAS-ras1*^{V12S35}, *UAS-ras1*^{V12G37}, *UAS-ras1*^{V12C40} (Karim & Rubin, 1998), *ras1*$^{\Delta C40B}$ (Hou et al., 1995), *sev-ras1*^{V12} (Fortini et al., 1992), *Df(3L)H99* (White et al., 1994), *GMR-DIAP1*, *GMR-DIAP2* (Hay et al., 1995), *GMR-p35* (Hay et al., 1994), *GMR-hid* (Grether et al., 1995), *rl*^{sem} (Brunner et al., 1994), *argos*^{W11} (Freeman, 1992), *sprouty*^{226} (Kramer et al., 1995), *Egfr*^{F2} (Nusslein-Volhard et al., 1984), *pnt*$^{\Delta 88}$ (Scholz et

al., 1993), *UAS-pntP2* (Scholz et al., 1997), *UAS-yan^{ACT}* (Rebay & Rubin, 1995), *yan^{1}* (Rogge et al., 1995), *amn^{x8}* (Moore et al., 1998), *amn^{EP346}* (Kraut et al., 2001), *rap1^{CD3}* (Asha et al., 1999), *UAS-rap1 ^{V12}* (Hariharan, I.K., unpublished), *Df(2R)E3363* (Rubin, G.M., personal communication to FlyBase available from http://flybase.bio.indiana.edu/. bin/fbpcq.html?FBrf0077918). *Canton-S* was used as a wild-type control. Flies were raised at 25C° in standard cornmeal food.

Construction of the *c155, UAS-ras^{N17}* and the *UAS-ras^{N17}; GMR-GAL4* lines

I generated these fly stocks for experiments to identify modifiers of their small eye phenotypes. The *c155, UAS-ras^{N17}* line was obtained by meiotic recombination. *c155* adult males were crossed to *UAS-ras^{N17}* virgins en masse, and the F1 progeny were sibcrossed (i.e., *UAS-ras^{N17}/Y x c155/UAS-ras^{N17}*). F2 recombinant males that had small eyes (i.e., *c155, UAS-ras^{N17}/Y*)(n = 3) were selected and then crossed to female flies carrying the *FM7a* balancer chromosome, establishing the *c155, UAS-ras^{N17}/FM7a* stock. The estimated map distance between *c155* and *UAS-ras^{N17}* insertions was 29 cM. The *UAS-ras^{N17}; GMR-GAL4* line (*UAS-ras^{N17}* on the X chromosome and *GMR-GAL4* on the 2nd chromosome) was obtained through a series of crosses. *GMR-GAL4* males were crossed to *UAS-ras^{N17}* virgins en masse, and the F1 males were crossed to *FM7a* females. F2 virgins that had small bar eyes (i.e., *UAS-ras^{N17}/FM7a; GMR-GAL4/+*) were crossed to male flies carrying the *CyO* balancer chromosome. F3 males with small eyes and curly wings (i.e., *UAS-ras^{N17}/Y; GMR-GAL4/CyO*) were backcrossed to *UAS-ras^{N17}/FM7a; GMR-GAL4/+* virgins. Finally, F4 progeny that had small eyes and curly wings were selected and then sibcrossed to establish the *UAS-ras^{N17}/FM7a; GMR-GAL4/CyO* stock.

Evaluation of eye size

Adult flies (2~3 days old) were examined for their overall eye size under a microscope. Pictures were taken using a digital camera (OLYMPUS, BX51WI) attached to a 10 X zoom lens and processed by the application software Magnafire (Karl Storz Imaging).

Viability assay

The viability of F1 progeny from a cross was quantified as follows: Five adult males from one strain were incubated with five virgin adult females from another strain in a food vial for five days. After evacuating the parental flies, the progeny were raised to adulthood in the same vial. F1 progeny that survived to adulthood were evacuated from the vial and the number of them was counted under CO_2 anesthesia daily for five consecutive days, starting on the day the first progeny eclosed. The total number of flies collected from the period of five days was taken as a measure of viability for the F1 progeny from the cross. The data were shown as the mean ± standard error of the mean (SEM) from five independent experiments (or crosses).

4-3. Results

This study was initiated unexpectedly. As part of the behavioral studies for Ras, I attempted to express a dominant-negative form of mammalian *ras*, ras^{N17} (Lee et al., 1996) broadly in the central nervous system in *Drosophila* using the GAL4-UAS system (Brand & Perrimon, 1993). To this end, I crossed flies carrying a *UAS-*ras^{N17} insertion to ones carrying the *GAL4* insertion *c155*. Both insertions are X-linked and the *GAL4* line *c155* have the *P*-element insertion in the *elav* gene that is expressed virtually in all the post-mitotic neurons in *Drosophila* (Lin & Goodman, 1994). Immunostaining with an anti-Elav antibody visualizes the photoreceptor neurons in the compound eye (Bier et al., 1988; Rebay & Rubin, 1995; Robinow & White, 1991). The F1 female progeny from the cross (i.e., *c155/UAS-*ras^{N17}) had eyes that are severely reduced in size and largely missing ommatidial morphology (Figure 4-1F). To explore the effects of other alleles of

ras, I also crossed various *UAS-ras* strains to *c155*. However, none of the F1 progeny from such crosses showed the small eye phenotype similar to *c155/UAS-ras^{N17}*. The only abnormality observed in them was rough eye (Figures 4-1B-E). Despite the severe eye phenotype, the viability of *c155/UAS-ras^{N17}* was not distinguishable from that of other strains that had normal eye size (Figure 4-1G).

The eye ablation phenotype of *c155/UAS-ras^{N17}* results from inhibition of Ras signaling

Ras^{N17} is an allele of Ras, which contains a substitution of serine by asparagine at codon 17, and has been used extensively as a dominant-negative form of Ras (Feig & Cooper, 1988; reviewed in Feig, 1999). The mechanism assumed for inhibition of Ras signaling is sequestration of endogenous Ras from Ras-specific guanine nucleotide exchange factors (GEFs). That is, Ras^{N17}, which itself cannot activate downstream effectors of Ras, binds more tightly to Ras-GEFs than wild-type Ras, preventing formation of GTP-bound active Ras (reviewed in Feig, 1999). To determine whether the small eye phenotype of *c155/UAS-ras^{N17}* results from inhibition of Ras signaling, I altered the level of Ras signaling in the *c155/UAS-ras^{N17}* background and examined a change in overall eye size. To do this, I constructed the *c155, UAS-ras^{N17}* stock by meiotic recombination and crossed it to various *ras* strains. The proposed hypothesis was that if Ras^{N17} blocked endogenous Ras1 as postulated, doubling a *ras^{N17}* dosage or removing a copy of the endogenous *ras1* gene would result in enhancement of the small eye phenotype of *c155, UAS-ras^{N17}*. On the contrary, increasing a *ras1* dosage or expressing a constitutively-active (GTP-bound) form of *ras1 (ras1^{V12})* would suppress the eye phenotype by competing with Ras^{N17} for GEFs or bypassing GEFs for activation of the Ras pathway, respectively. As expected, when I doubled a *UAS-ras^{N17}* dosage or reduced an endogenous *ras1* dosage to 50%, the small eye phenotype of *c155, UAS-ras^{N17}* was severely enhanced (Figures 4-2B and C). On the contrary, overexpression of a wild-type

(using *UAS-ras1*^{WT}) or a constitutively-active form of *ras1* (using *sev-ras1*^{V12}, *ras1*^{V12} placed downstream of eye-specific *sev* enhancers) greatly suppressed the *c155, UAS-ras*^{N17} phenotype (Figures 4-2D-F). These results are consistent with the proposed model for the dominant-negative function of Ras^{N17}, and I assumed that the eye ablation of *c155, UAS-ras*^{N17} results from inhibition of Ras signaling by Ras^{N17}.

Suppression of the eye ablation by blockade of cell death-inducing machinery

The small eye phenotype that I observed in *c155/UAS-ras*^{N17} is reminiscent of the one caused by eye-specific ectopic expression of *Drosophila* apoptosis-inducing genes, such as *hid* (Grether et al., 1995). Indeed, Ras1 has been shown to assume anti-apoptotic function in *Drosophila* eye by inhibiting Hid (Bergmann et al., 1998; Kurada et al., 1998; Sawamoto et al., 1998). To examine whether the Ras^{N17}-inducing phenotype is based on apoptosis, I crossed *c155, UAS-ras*^{N17} to the chromosomal deletion line *Df(3L)H99 (H99),* whose deletion encompasses the genomic region 75C1, 2 that contains the loci of the three cell-killing genes, *hid*, *rpr*, and *grim* (Chen et al., 1996; Grether et al., 1995; White et al., 1994; White et al., 1996). If the Ras^{N17}-inducing phenotype resulted from deregulated activity of the apoptotic genes, reducing dosage for those genes by 50% should result in suppression of the phenotype. In fact, the progeny heterozygous for *H99* in the *c155, UAS-ras*^{N17} background showed enlarged eyes comparing to *c155, UAS-ras*^{N17} (Figure 4-3B). I further tested the effect of enhancing anti-apoptotic machinery by expressing the baculovirus gene *p35* in *c155, UAS-ras*^{N17}. In both vertebrate and invertebrate cells, the P35 protein has been shown to block apoptosis apparently by inhibiting the Ced-3/ICE (interleukin-1 converting enzyme) family of proteases (Hay et al., 1994; Rabizadeh et al., 1993; Sugimoto et al., 1994). Expression of *p35* under the control of the eye-specific *GMR* promotor (*GMR-p35*)(Hay et al., 1994) substantially suppressed the small eye phenotype of *c155, UAS-ras*^{N17} (Figure 4-3C). Expression of the *Drosophila* homologs of the *p35* gene (*DIAP1* and *DIAP2*) in the eyes also suppressed

the *c155, UAS-ras^{N17}* phenotype (Figures 4-3D and E). These results indicate that the small eye phenotype resulting from inhibition of Ras1 is based on excessive apoptosis executed by proteases. Finally, I also confirmed the effect of ectopic expression of *hid* in the eyes of *c155, UAS-ras^{N17}*. Expression of a single copy of *GMR-hid* severely enhanced the *c155, UAS-ras^{N17}* phenotype, to the extent that their eyes mimicked the ones of flies homozygous for *GMR-hid* (Figures 4-3F-H). These results are consistent with the role of Ras1 as an anti-apoptotic protein that inhibits Hid.

The small eye phenotype is modified by mutations that affect EGFR signaling

In *Drosophila*, Ras1 can be activated by the receptor tyrosine kinase EGFR, and EGFR/Ras signaling has been implicated in various aspects of *Drosophila* eye development, including promotion of cell survival (Baker & Rubin, 1989; Baker &Yu, 2001; Freeman, 1996; Bergmann et al., 1998; Kurada & White, 1998; Sawamoto et al., 1998; reviewed in Voas & Rebay, 2004). To obtain insights into whether the small eye phenotype of *c155, UAS-ras^{N17}* results from inhibition of EGFR/Ras signaling, I crossed *c155, UAS-ras^{N17}* to flies that carry a mutation in a gene that regulates EGFR/Ras signaling (Figure 4-4A). If there were some residual Ras signaling in *c155, UAS-ras^{N17}*, enhancement of EGFR activity could suppress the small eye phenotype. Conversely, suppression of EGFR activity could result in enhancement of the phenotype. Activity of EGFR is regulated by multiple ligands. Argos (Freeman et al., 1992; Okano et al., 1992) and Sprouty (Hacohen et al., 1998) are known to be negative regulators. When I crossed *c155, UAS-ras^{N17}* to flies carrying a loss-of-function mutation in the *argos* gene, the small eye phenotype of *c155, UAS-ras^{N17}* was substantially suppressed (Figures 4-4B and C). A similar level of suppression was seen with a loss-of-function mutation in the *sprouty* gene (Figure 4-4E). Conversely, a loss-of-function mutation in *Egfr* itself enhanced the phenotype (Figure 4-4D). These results are consistent with the assumption

that the small eye phenotype of *c155, UAS-ras^{N17}* results from the blockade of EGFR/Ras signaling.

Activation of Ras effectors suppresses the small eye

Ras can activate multiple effectors, such as Raf, Ral-GDS, and PI3-K. These effectors can then transduce the Ras signal to MAPK, Phospholipase D and Akt-1, respectively (e.g., reviewed in Shields et al., 2000). Three different mutations have been identified in the effector-loop domain of Ras (i.e., T35S, E37G, and Y40C). These Ras^{S35}, Ras^{G37}, and Ras^{C40} mutants selectively activate Raf, Ral-GDS, and PI3-K, respectively (Joneson et al., 1996; Rodriguez-Viciana et al., 1997; White et al., 1995). By combining these effector-loop mutations with the G12V mutation that makes Ras constitutively-active (Seeburg et al., 1984), the downstream effector signaling pathways that mediate Ras signaling have been identified in various mammalian and *Drosophila* cells (Figure 4-5A)(Bergmann et al., 1998; Joneson et al., 1996; Karim & Rubin, 1998; Koh et al., 2002; Rodriguez-Viciana et al., 1997; White et al., 1995). To examine what effector pathway(s) are essential to prevent the Ras-associated small eye, I crossed *c155, UAS-ras^{N17}* to flies carrying a Ras effector-loop mutant transgene downstream of the *UAS* promotor. The underlying assumption was that if the small eye resulted from inhibition of a specific pathway, selective activation of that pathway should suppress the phenotype. Similar analysis has been made for *hid*-induced small eyes, and the Raf/MAPK pathway has been identified as the predominant pathway that mediates the anti-apoptotic signal of Ras1 (Bergmann et al., 1998). My result was that activation of any single downstream pathways was sufficient to make a substantial suppression in overall eye size (Figures 4-5B-E)(Because the progeny from a cross with *UAS-ras1^{V12S35}* did not survive to adulthood, flies carrying an activated-form of *MAPK*, *rl^{sem}* were used instead to test the Raf/MAPK pathway). Thus, unlike in the *hid*-induced small eye, all of the Ras

downstream pathways tested appear to function in a rather redundant way to prevent the phenotype in *c155, UAS-ras^{N17}*.

MAPK-regulated transcription factors are modifiers of the small eye

Regulation of gene expression is one of the functions of MAPK. Two transcription factors, Pointed (Pnt) and Yan, are targets of MAPK in *Drosophila* (Klambt, 1993; O'Neill et al., 1994; Rebay & Rubin, 1995). In the compound eye, Pnt mediates Ras/MAPK signaling to promote R7 photoreceptor differentiation (O'Neill et al., 1994). On the other hand, Yan is negatively regulated by MAPK and inhibits R7 differentiation (O'Neill et al., 1994; Rebay & Rubin, 1995)(Figure 4-6A). They have also been implicated in regulation of apoptosis. It has been argued that Pnt mediates Ras/MAPK signaling to inhibit transcription of *hid*, thereby inhibiting *hid*-induced apoptosis, while activation of Yan with low levels of Ras/MAPK signaling promotes *hid* transcription (Kurada & White, 1998). To examine whether the small eye phenotype of *c155, UAS-ras^{N17}* is sensitive to activity levels of these transcription factors, I crossed *c155, UAS-ras^{N17}* to flies carrying a loss-of-function mutation in *pnt* (*pnt$^{\Delta 88}$*) or *yan* (*yan^{1}*). *pnt$^{\Delta 88}$* enhanced the small eye of *c155, UAS-ras^{N17}*, while *yan^{1}* suppressed it (Figures 4-6B, C and E). I also examined the effects of enhancing Pnt or Yan signaling by expressing a wild-type form of a *pnt* or activated form of a *yan* (*yan^{ACT}*) transgene in the eyes of *c155, UAS-ras^{N17}*, respectively. Substantial enhancement was seen with expression of *yan^{ACT}* (Figure 4-6D). For Pnt, the tested constructs (*UAS-pntP1* and *UAS-pntP2*) were both dominant-lethal when combined with *c155, UAS-ras^{N17}* (see Figure 4-9J for further experiments with expression of *pnt*. *UAS-pntP2* survived to adulthood in another line having a small eye phenotype resulting from inhibition of Ras1 and suppressed the phenotype). These results indicate that Pnt prevents the small eye, while Yan promotes it, and suggest that inhibition of the Ras/MAPK pathway in *c155, UAS-ras^{N17}* impairs

normal regulation of the transcription machinery, leading to biased activation of Yan and hence the small eye phenotype.

Alterations in levels of Amnesiac or Rap signaling modify the small eye phenotype of *c155, UAS-ras^{N17}*

So far, I have shown the results of characterization of the small eye phenotype of *c155, UAS-ras^{N17}*, largely based on the knowledge that Ras1 relays EGFR signaling to MAPK to promote cell differentiation or cell survival in the developing *Drosophila* eye. My results have suggested that a blockade of the EGFR/Ras/MAPK pathway is certainly a cause of the small eye in *c155, UAS-ras^{N17}*. My results have also suggested that a blockade of other Ras-dependent pathways, such as the Ral-GDS or PI3-K pathways, may as well contribute to the development of the small eye. In an effort to identify additional components that modulate the Ras pathways associated with the small eye, I performed a preliminary F1 screen using fly strains that were maintained in our lab. The genes in which mutations or whose transgenic expression modified the eye size of *c155, UAS-ras^{N17}* were *amnesiac* (*amn*) and *rap1*. The *amn* gene encodes a preproneuropeptide with some sequence similarity to pituitary-adenylyl-cyclase-activating peptide (PACAP)(Feany & Quinn, 1995; Moore et al., 1998). In *Drosophila*, it has been known to function in olfactory memory formation (Quinn et al., 1979; Waddell et al., 2000), control of ethanol sensitivity (Moore et al., 1998), and of female fertility (Feany & Quinn, 1995). In the *c155, UAS-ras^{N17}* background, a deletion allele of *amn* (*amn^{X8}*) dominantly enhanced the small eye phenotype (Figures 4-7A and B). Conversely, overexpression of wild-type *amn* using *amn^{EP346}* (an *amn* allele with an insertion of the *UAS* sequence in the 5' regulatory region) resulted in suppression of the small eye (Figure 4-7C). The *rap1* gene encodes a member of the Ras superfamily of small GTPases (Hariharan et al., 1991). In mammalian cell lines, Rap proteins were originally characterized as antagonists of oncogenic Ras (Cook et al., 1993; Kitayama et al., 1989).

However, recent studies suggest that Rap can also function independent of Ras (reviewed in Bos et al., 2001; Caron 2003). The role of Rap proteins *in vivo* is poorly understood. In *Drosophila*, Rap1 has been implicated in regulation of morphogenesis in various tissues including the developing eye (Asha et al., 1999; Boettner et al., 2003; Knox & Brown, 2002), but the underlying mechanisms are largely unknown. In my experiments, a constitutively-active form of *rap1*, when co-expressed with ras^{N17}, strongly suppressed the *c155, UAS-*ras^{N17} phenotype, although a loss-of-function mutation in the *rap1* gene tested did not appear to affect the phenotype (Figures 4-7D and E). Finally, from the screen, I also identified the deletion line *Df(2R)E3363* (*E3363*) as a dominant modifier of the *c155, UAS-*ras^{N17} phenotype. *E3363* removes a 47A-47F region on the second chromosome and was originally identified as a modifier of rough eyes in *sev-*$ras1^{V12}$ (Rubin, G.M., personal communication to FlyBase available from http://flybase.bio.indiana.edu/.bin/fbpcq.html?FBrf0077918). Inclusion of a copy of the chromosome bearing the deletion in *c155, UAS-*ras^{N17} strongly suppressed the small eye phenotype (Figures 4-7F and 4-8B). The eyes of the *amn*, *rap1* or *E3363* mutants themselves were apparently normal, so were those of flies expressing amn^{EP346} or $rap1^{V12}$ under the control of *c155* (data not shown).

Expression of *UAS-*ras^{N17} with *GMR-GAL4* also results in small eyes

The *GMR-GAL4* strain expresses GAL4 protein under the control of the *glass multimeric reporter* (*GMR*) promotor. The expression occurs in virtually all the cells in the eye imaginal disc posterior to the morphogenetic follow (Freeman, 1996). Expression of a dominant-negative form of *Egfr* (Freeman, 1996) with this driver results in a small eye phenotype. Expression of a dominant-active form of *yan* (Rebay & Rubin, 1995) or a wild-type form of cell death inducers (Chen et al., 1996; Grether et al., 1995; White et al., 1996) under the direct control of the *GMR* promotor (e.g., *GMR-*yan^{ACT}) also results in small eyes. Based on this knowledge, I crossed *UAS-*ras^{N17} to *GMR-GAL4*. I found that

their F1 progeny show small eyes, although the size defect is milder than that in *c155, UAS-ras^{N17}* (Figure 4-9B). As in the case with the *c155* driver, only the expression of *ras^{N17}* resulted in small eyes, among other *ras* alleles tested. The only eye abnormality observed was rough eyes, when *ras1^{V12G37}*, *ras1^{V12C40}*, or *ras1^{N17}* was expressed (data not shown). Expression of *ras1^{WT}* did not appear to affect eye development. Expression of *ras1^{V12}* or *ras1^{V12S35}* killed the flies during development. The viability of *GMR-GAL4; UAS-ras^{N17}* was not distinguishable from that of the other *GMR-GAL4; UAS-ras* flies that had normal eye size (Figure 4-9S). The small eye phenotype of *GMR-GAL4; UAS-ras^{N17}* was modified by additional genetic changes in a similar (qualitatively identical) fashion to that of *c155, UAS-ras^{N17}* (Figures 4-9C-Q). Finally, I also tested for the eye size when *UAS-ras^{N17}* is expressed with *sev-GAL4*, another well-used driver for studies of *Drosophila* eye development (Freeman, 1996; Sun & Artavanis-Tsakonas, 1997). *sev-GAL4* flies expresses GAL4 protein under the control of the *sev* promotor in a subset of cells posterior to the morphogenetic follow in the eye disc. Expression of *ras1^{N17}* or *ras1^{V12}* under the direct control of the *sev* promotor (i.e., *sev-ras1^{N17}* or *sev-ras1^{V12}*) results in rough eyes (Fortini et al., 1992; Karim et al., 1996). Expression of *ras^{N17}* using the *sev-GAL4* driver did not result in a significant reduction in eye size, but only caused rough eyes (Figure 4-9R).

4-4. Discussion

I found that the progeny from a cross between flies carrying the *GAL4* driver *c155* and flies carrying a dominant-negative form of mammalian *ras* under the *UAS* sequence had a remarkable small eye phenotype (Figure 4-1). The progeny were fully viable to adult and fertile so that I could establish the *c155, UAS-ras^{N17}* stock. Their eye size was modified in such a way that further reduction of Ras signaling enhances, and supplement of it suppresses the phenotype (Figure 4-2). This correlation between the severity of the phenotype (eye size) and the level of Ras signaling indicated that the small eye in *c155,*

UAS-ras^{N17} resulted from inhibition of Ras signaling. The small eye was also greatly suppressed by blocking apoptosis-inducing machinery, indicating that massive cell death is involved in the development of the phenotype (Figure 4-3). The small eye appeared to be the result of blocking EGFR/Ras-dependent signaling pathways, including the MAPK, Ral-GDS, and PI3-K pathways (Figures 4-4 to 4-6). Additional components such as Amnesiac and Rap1 appeared to modulate the signaling machinery implicated in the small eye phenotype (Figure 4-7). Figure 4-10 summarizes the findings of this study.

Cellular events underlying the small eye

Coordinated cellular events involving cell proliferation, differentiation, and survival establish the development of the *Drosophila* compound eye. The inhibition of Ras signaling in *c155, UAS-ras*^{N17} is supposed to impair some part of the processes to generate the small eye. The *c155 P*-element is inserted in the *elav* gene that is expressed in all of the post-mitotic neurons in *Drosophila* (Bier et al., 1988; Lin & Goodman, 1994). The line *c155* has been used extensively as a pan-neural *GAL4* driver in the GAL4-UAS system. In the eye, the Elav protein is used to visualize all the photoreceptor neurons (Bier et al., 1988; Rebay & Rubin, 1995). Therefore, expression of the dominant-negative *ras* transgene by *c155* is assumed to selectively affect cellular processes through inhibition of Ras signaling in the post-mitotic photoreceptor neurons in the eye. Based on this assumption, the expression of *ras*^{N17} is not likely to impair proliferation of the photoreceptors, because the cells are already post-mitotic. Also, photoreceptor differentiation should not be impaired if *ras*^{N17} is expressed in already differentiated neurons. However, there is a possibility that *elav* expression starts at the onset of neurogenesis, and photoreceptor differentiation is blocked by *ras*^{N17} expression. EGFR signaling has been demonstrated to be required for differentiation of the photoreceptor neurons except the R8 neuron (Freeman, 1996).

Reduced viability of the differentiated photoreceptor neurons is certainly a candidate for the primary cause of the small eye phenotype of *c155, UAS-ras^{N17}*. Expression of anti-apoptosis genes (i.e., baculovirus *p35* or its *Drosophila* homologs) in the developing eye or removal of endogenous pro-apoptotic genes (i.e., *hid*, *rpr* and *grim*) substantially suppressed the small eye phenotype of *c155, UAS-ras^{N17}* (Figure 4-3). Furthermore, EGFR/Ras/MAPK signaling has been postulated to prevent apoptosis by inhibiting Hid in various tissues including eye cells in *Drosophila* (Bergmann et al., 1998; Bergmann et al., 2002; Kurada & White, 1998). Several explanations are however possible for massive cell death leading to the small eyes of *c155, UAS-ras^{N17}*. First, the inhibition of Ras signaling in differentiated photoreceptor neurons might induce apoptosis specifically in those *ras^{N17}*-expressing photoreceptor neurons after ommatidial assembly, leaving the development of other non-neural cells intact. Second, the inhibition of Ras signaling in differentiated photoreceptor neurons might induce apoptosis in those photoreceptors during ommatidial assembly, and thus also prevent cell-cell signaling events initiated by photoreceptor neurons required for proliferation (i.e., the second mitotic wave), differentiation, and survival of surrounding non-neural cells. Third, the inhibition of Ras signaling might be initiated before photoreceptor cells are fully differentiated into neurons, and the inhibition of photoreceptor differentiation might block ommatidial development that follows. Fourth, the inhibition of Ras signaling might affect both differentiation and survival of developing photoreceptor neurons, so the small eye may be the result of summation of both effects. These possibilities should be explored by further histological analysis.

Toward further understanding of Ras signaling pathways *in vivo*

Accumulating evidence from a number of studies has revealed the complexity of the Ras signaling pathway. An increasing number of regulators or effectors of Ras have been identified in a variety of experimental systems (Malumbres & Barbacid, 2003). In the

Drosophila eye, Ras1 was originally identified as a mediator of Sev receptor tyrosine kinase specifying R7 photoreceptor cell fate (Simon et al., 1991). Subsequent studies identified the MAPK pathway as a major downstream pathway mediating the Sev/Ras signaling (Brunner et al., 1994; Rebay & Rubin, 1995). In regulation of apoptosis, Ras1 relays anti-apoptotic EGFR signaling to the MAPK pathway, in order to inhibit pro-apoptotic Hid activity (Bergmann et al., 1998; Kurada & White, 1998). These findings indicate importance of the Ras/MAPK pathway downstream of receptor tyrosine kinases in *Drosophila* eye development. However, the exact signaling mechanisms underlying the roles of Ras1 are not fully understood, and existence of additional, as yet unidentified, molecular components that mediate (or modulate) Ras signaling has been suggested (Kurada & White, 1999; Voas & Rebay, 2004).

In my study, the small eye phenotype of *c155, UAS-ras^{N17}* was strongly suppressed by activation of MAPK-independent as well as -dependent pathways (Figure 4-4). In mammalian cells, Ras^{V12G37} and Ras^{V12C40} predominantly activate the Ral-GDS and PI3-K pathways, respectively (Joneson et al., 1996; Rodriguez-Viciana et al., 1997; White et al., 1995), although recent studies suggest that in *Drosophila*, Ras^{V12G37} rather activates PI3-K, while the target of Ras^{V12C40} remains to be determined (Halfar et al., 2001; Prober & Edgar, 2002). In either case, activation of the MAPK pathway is substantially blocked in those two effector-loop mutants (Prober & Edgar, 2002; White et al., 1995). My findings are different from those in the *hid*-induced small eye phenotype, in which only the activation of the MAPK pathway resulted in strong suppression (Bergmann et al., 1998). My findings suggest that Ras downstream pathways, other than the MAPK pathway, play an important role in *Drosophila* eye development, specifically in differentiation or survival of photoreceptor neurons (remember that the effector-loop mutants were co-expressed with *ras^{N17}* in photoreceptor cells).

A screen for modifiers of the small eye phenotype of *c155, UAS-ras^{N17}* also identified genes, such as *amn* and *rap1* (Figure 4-7). Amn is a preproneuropeptide

homologous to mammalian PACAP, and has been believed to function in neuronal plasticity (Feany & Quinn, 1995; Moore et al., 1998; Quinn et al., 1979; Waddell et al., 2000). In *Drosophila* neuromuscular junctions, bath application of PACAP-like neuropeptides can activate the Ras pathway as well as the cAMP pathway in the postsynaptic muscle cell (Zhong et al., 1995). However, to my knowledge, Amn has never been implicated in the Ras-dependent photoreceptor development in the fly eye. Rap is a member of the Ras superfamily of small GTPases that was originally characterized as an antagonist of oncogenic Ras (Cook et al., 1993; Kitayama et al., 1989). Recent studies suggest that Rap can also function independent of Ras (Bos et al., 2001; Caron 2003). In *Drosophila*, Rap1 has been implicated in regulation of morphogenesis, rather than cell proliferation or cell survival (Asha et al., 1999; Boettner et al., 2003; Knox & Brown, 2002). In the developing eye, it has been suggested that Ras1 and Rap1 function in distinct pathways (Asha et al., 1999). My findings, nevertheless, suggest that Rap1 may modulate Ras1 signaling to regulate cell differentiation or cell survival. Co-expression of activated *rap1* with ras^{N17} in the same photoreceptor cells strongly suppressed the ras^{N17}-inducing small eye phenotype. In PC12 cells, Rap activates the MAPK pathway to promote neuronal differentiation downstream of cAMP or nerve growth factor (Vossler et al., 1997; York et al., 1998). Further investigations using the eye of the *c155, UAS-*ras^{N17} fly might help to understand the nature of the interaction between Ras and Rap in regulating cellular events *in vivo*.

Implications of the small eye phenotype for screens for genes and drugs

As illustrated above, the Ras pathway associated with the Ras^{N17}-induced small eye phenotype appears to be composed of many genes, including ones functioning independent of the well-characterized EGFR/Ras/MAPK pathway. I believe that further genetic screens using the small eye phenotype should identify additional components that help to fully understand the nature of the complexity of Ras signaling.

As demonstrated in this study, the severity of the phenotype (i.e., eye size) is well correlated with the level of Ras signaling, making the phenotype a sensitive measure for Ras levels *in vivo*. With this fact, the Ras^{N17}-induced small eye may provide a sensitive tool to identify Ras-associated genes that cannot be easily identified using other Ras phenotypes. The rough eye seen in flies carrying a dominant-active form of *ras1* under the *sev* promoter (i.e., *sev-ras1*^{V12}) has been used most extensively for screens for genes downstream of Ras1 (Fortini et al., 1992; Karim et al., 1996). However, the same rough eye phenotype results in flies carrying dominant-negative *ras1* (i.e., *sev-ras1*^{N17})(Karim et al., 1996). Therefore, it is not always possible to estimate the amount of suppression or enhancement of Ras signaling by modifiers, based on the roughness of their eyes. If suppression of Ras signaling is overshot in *sev-ras1*^{V12}, for example, flies can still exhibit rough eyes. Furthermore, evaluating "roughness" under a dissection microscope appears to be a task of distinguishing subtleties, comparing to evaluating eye size. In fact, I found that the deletion *E3363*, which only weakly enhanced the *sev-ras1*^{V12} rough eye, strongly suppressed the small eye phenotype of *c155, UAS-ras*^{N17} (Figure 4-8).

Another advantage of using the small eye phenotype for identification of Ras-signaling genes is that the phenotype is based on the GAL4-UAS system. In *c155, UAS-ras*^{N17}, expression of *ras*^{N17} is driven by the *c155* GAL4 protein. Therefore, while still suitable for testing mutants generated by conventional methods of mutagenesis (e.g., chemical, X-ray, or non-GAL4-UAS-based *P*-element mutagenesis), *c155, UAS-ras*^{N17} enables us to test the effects of expression of any additional *GAL4* or *UAS* transgenes on its eye size by simply crossing them to the strains to be tested. When *UAS* transgenes are used, they must be co-expressed with *ras*^{N17} in the same set of cells by the *c155* driver (in the eye, photoreceptor neurons). This feature makes *c155, UAS-ras*^{N17} a suitable strain for genome-wide GAL4-UAS-based gain-of-function screens, such as the EP screen, in which endogenous genes are expressed through GAL4-UAS control (Rorth et al., 1998). The increased repertoire of screens available for *c155, UAS-ras*^{N17} heightens its potential

to help identify modifiers for Ras signaling, in comparison to previously-used fly strains, such as *sev* or *GMR* promotor-controlled transgenic animals (Bergmann et al., 1998; Fortini et al., 1992; Freeman, 1996; Karim et al., 1996; Kurada et al., 1998; Rebay & Rubin, 1995; Sawamoto et al., 1998).

Expression of ras^{N17} by the *GMR-GAL4* driver (i.e., *GMR-GAL4*; *UAS-*ras^{N17}) also resulted in a small eye phenotype, although the reduction of eye size was not as severe as in *c155*, *UAS-*ras^{N17} (Figure 4-9*B*). In contrast to the selective GAL4 expression of the *c155* driver in the photoreceptor neurons, expression of the *GMR* driver occurs in all of the cells in the eye disc posterior to the morphogenetic follow (Freeman, 1996). The less severe phenotype of the *GMR*-driven ras^{N17} strain may be due to a lower level of GAL4 expression by *GMR*. Cellular processes affected may be similar in the *c155* and *GMR* flies, because they showed a similar pattern of genetic interactions when crossed with other fly strains. When combined with *c155, UAS-*ras^{N17}, the *GMR*-driven ras^{N17} strain should assume a complementary role in screens for modifiers. Indeed, in this study, *UAS-pntP2* flies were lethal when combined with *c155, UAS-*ras^{N17}. But when combined with *GMR-GAL4*; *UAS-*ras^{N17}, they revealed to suppress the Ras^{N17}-induced small eye phenotype in adult (Figure 4-9J).

The findings in this study carry strong biomedical implications. Mutationally-activated Ras signaling has been believed to trigger tumorigenesis in certain types of cells. (Malumbres & Barbacid, 2003; Shields et al., 2000). About 20% of human tumors are known to be associated with mutations that activate *ras* (Bos, 1989; Downward, 2003). In those tumors, the mutated Ras protein contributes substantially to the deregulation of cellular processes, such as uncontrolled cell proliferation, cell survival, and blood vessel formation (Downward, 2003; Shields et al., 2000). With these observations, current therapeutic approaches to cancers have been exclusively oriented to regulation of Ras signaling. Many therapeutic agents designed to suppress Ras pathways are being developed (Downward, 2003). My study here demonstrates that the small eye

phenotype resulting from inhibition of Ras signaling can be modified with alterations in the level of Ras signaling. Together with the relatively short generation time of *Drosophila* (10~12 days) and ease of scoring of the phenotype (required is a quick scan under a dissection microscope), the small eye-bearing fruit flies may serve as a powerful tool to assay the effectiveness of those Ras-targeting therapeutic agents *in vivo*. Furthermore, with the fact that the small eye phenotype is based on massive cell death, they may also be useful to make drug screens for other cell death-associated human diseases, such as viral infections, autoimmune, and neurodegenerative diseases (Thompson, 1995).

4-5. References

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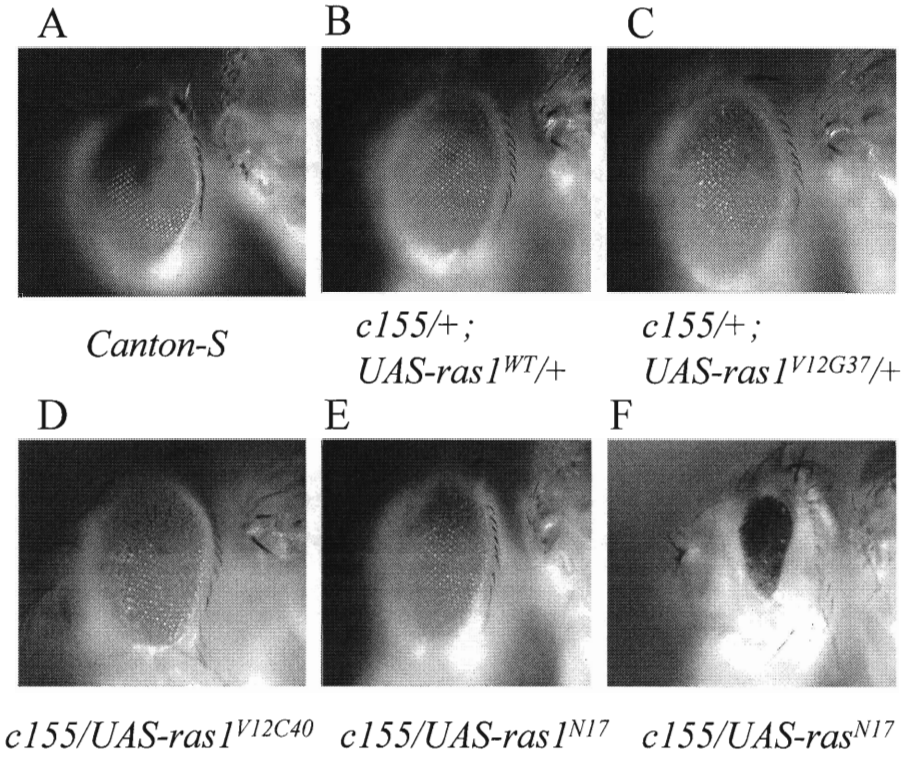
4-6. Figures

Figure 4-1


A
B
C
Canton-S
c155/+;
UAS-ras1WT/+
c155/+;
UAS-ras1V12G37/+
D
E
F
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c155/UAS-ras1N17
c155/UAS-rasN17

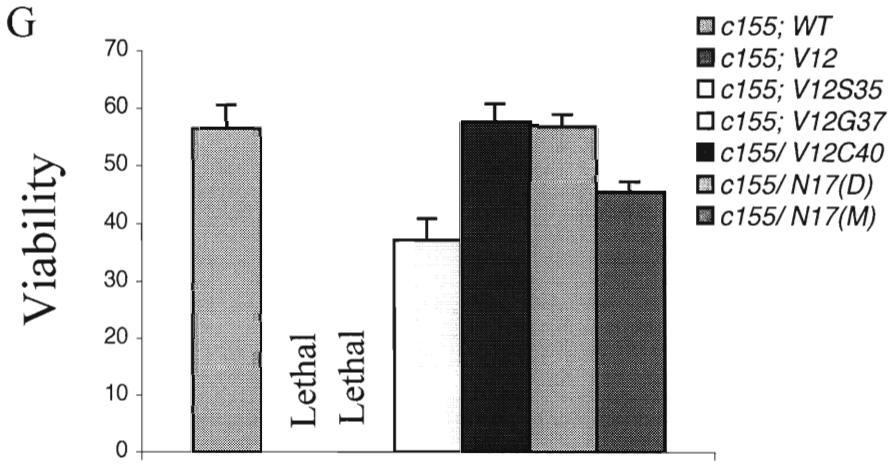

G
Viability
70
60
50
40
30
20
10
0
Lethal
Lethal
c155; WT
c155; V12
c155; V12S35
c155; V12G37
c155/ V12C40
c155/ N17(D)
c155/ N17(M)

Figure 4-1. Expression of mammalian *ras*^{N17} by the *c155 GAL4* driver results in a small eye phenotype. (A) *Canton-S* (wild-type). (B-E) Various *Drosophila ras1* alleles were expressed by *c155*. (B) Expression of a wild-type form of *ras1* did not affect eye development. Expression of activated forms of *ras1* with effector-loop mutations (C and D) or of a dominant-negative form of *ras1* (E) resulted in rough eyes with apparently normal eye size. (F) Dramatic reduction of eye size by expression of a mammalian form of dominant-negative *ras*. Normal ommatidial structures are largely missing. (G) The viability of *c155/UAS-ras*^{N17} was similar to controls. Flies expressing dominant-active *ras1* (*ras1*^{V12}) or another active *ras1* with an effector-loop mutation (*ras1*^{V12S35}) through *c155* did not survive to adulthood. In the labels in the graph, the words "*UAS*" and "*ras*" are omitted before the allele identifiers. "*N17(D)*" and "*N17(M)*" represent *Drosophila ras1*^{N17} and mammalian *ras*^{N17}, respectively.

Figure 4-2

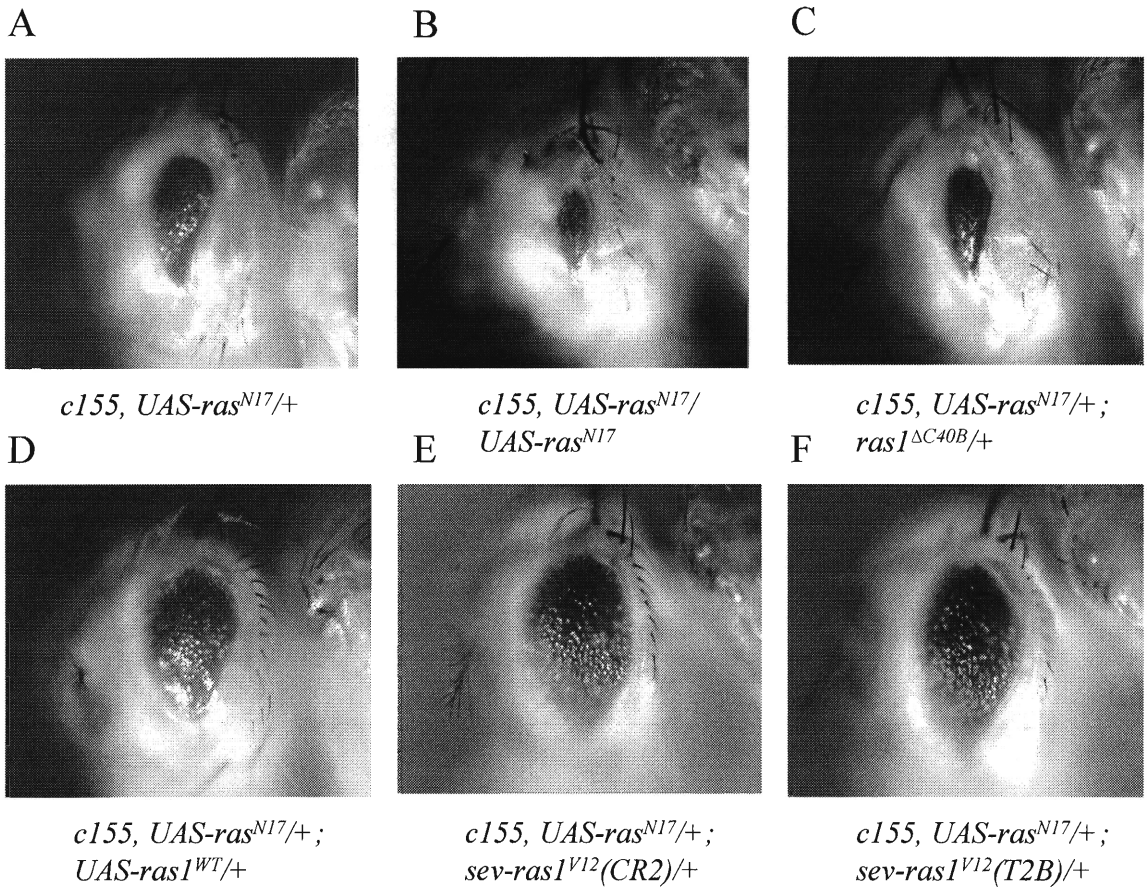


Figure 4-2. A correlation between eye size and the level of Ras signaling in the *c155, UAS-ras^{N17}* background. (A) The small eye of *c155, UAS-ras^{N17}*. The phenotype was enhanced by expression of one more copy of *ras^{N17}* (B) or removal of a copy of the endogenous *ras1* gene (C). The phenotype was suppressed by expression of wild-type *ras1* (D) or activated *ras1* (E and F). Two independent *sev-ras1^{V12}* lines (CR2 and T2B) were tested.

Figure 4-3

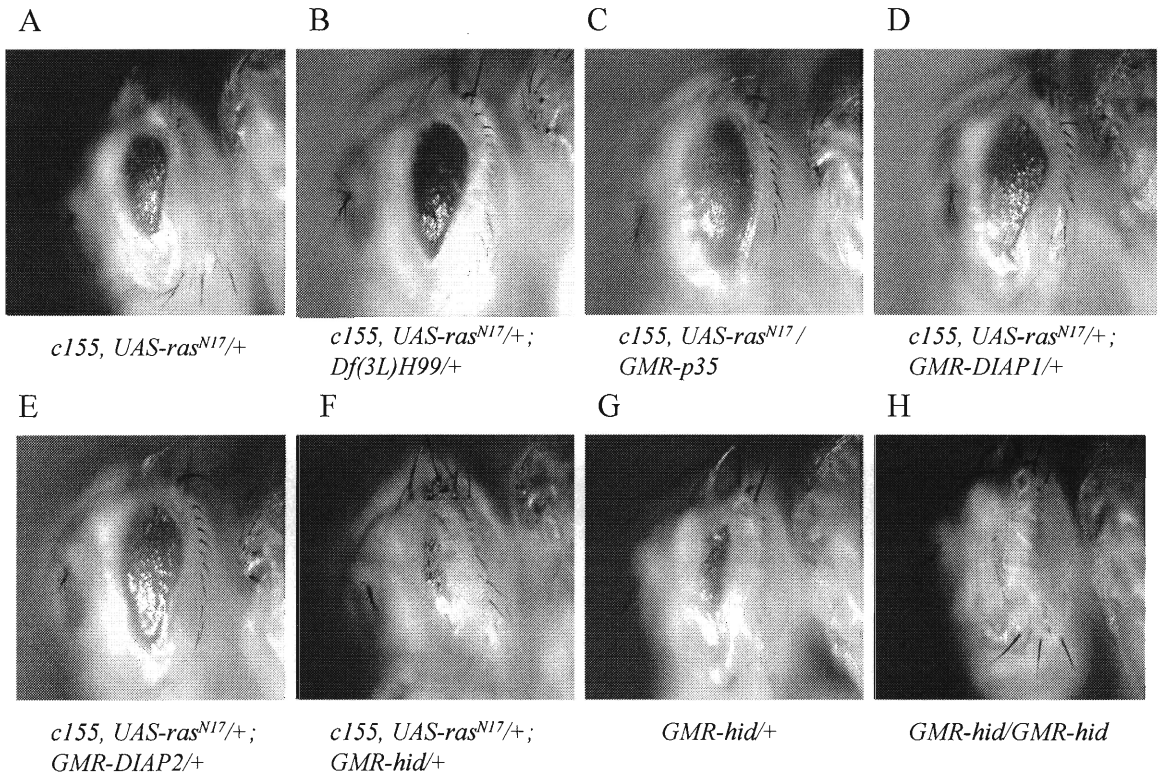


Figure 4-3. Massive cell death is involved in the small eye phenotype of *c155, UAS-ras^{N17}*. (B) Removal of a copy of the chromosomal region containing the pro-apoptotic genes, *hid*, *rpr*, and *grim*, resulted in suppression of the *c155, UAS-ras^{N17}* phenotype (compare with A). (C-E) Ectopic expression of anti-apoptotic genes, such as baculovirus *p35*, *DIAP1*, and *DIAP2*, also resulted in suppression. (F) Overexpression of the *hid* gene resulted in enhancement. (G and H) Heterozygous or homozygous *GMR-hid*.

Figure 4-4

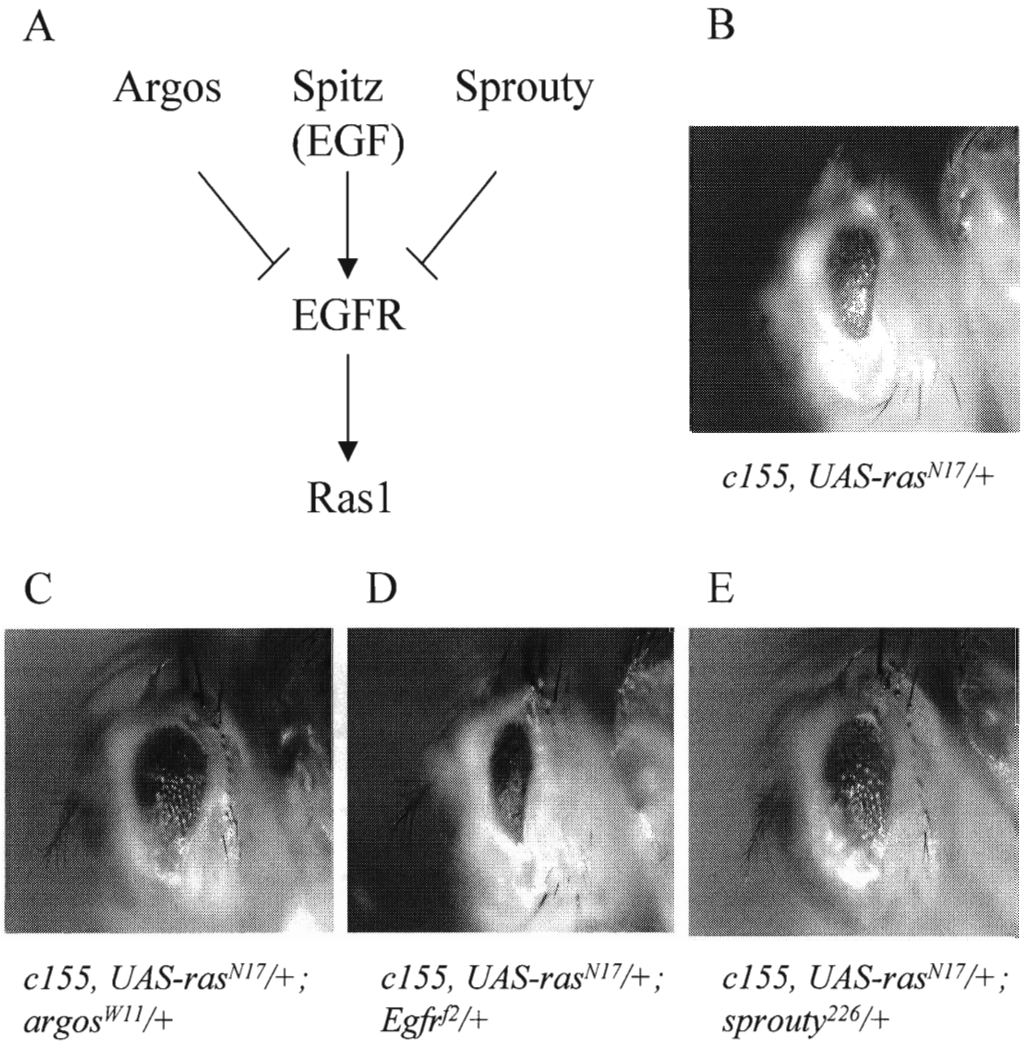


Figure 4-4. The small eye phenotype of *c155, UAS-ras^{N17}* is modified with mutations in components of the EGFR signaling pathway. (A) EGFR activity is regulated by positive (Spitz) and negative (Argos and Sprouty) ligands. The small eye phenotype of *c155, UAS-ras^{N17}* (B) was suppressed by loss-of-function mutations in the *argos* (C) or *sprouty* (E) genes, and enhanced by a loss-of-function mutation in the *Egfr* gene (D).

Figure 4-5

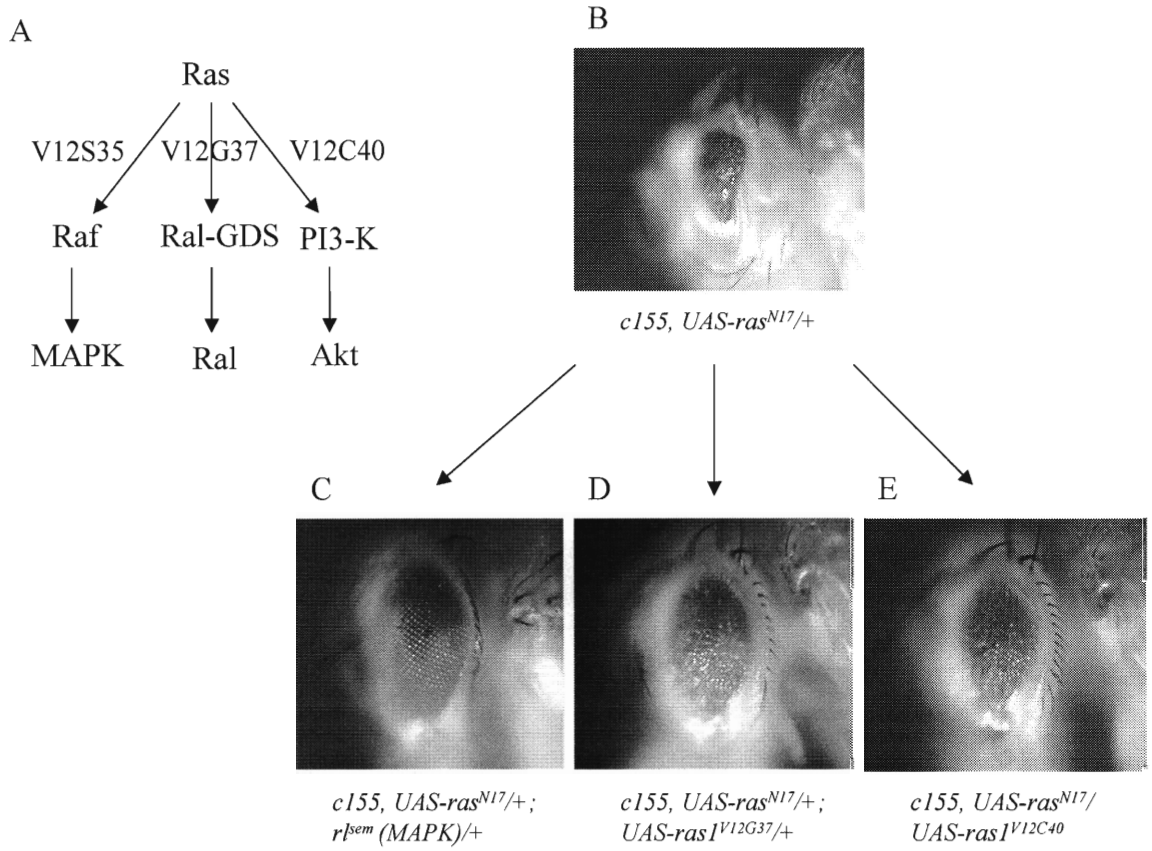


Figure 4-5. Activation of any one of Ras downstream effectors results in suppression of the small eye phenotype of *c155, UAS-ras^{N17}*. (A) Selective activation of Ras downstream effectors by constitutively-active Ras1 with second mutations in the effector-loop domain. (C) Because *UAS-ras1^{V12S35}* flies were lethal when crossed to *c155, UAS-ras^{N17}*, a gain-of-function (activating) mutation in the *Drosophila MAPK* gene (*rl*) was used instead to test if activation of the Raf/MAPK branch can suppress the small eye phenotype of *c155, UAS-ras^{N17}* (B). (D and E) Expression of *UAS-ras1^{V12G37}* or *UAS-ras1^{V12C40}* also resulted in suppression.

Figure 4-6

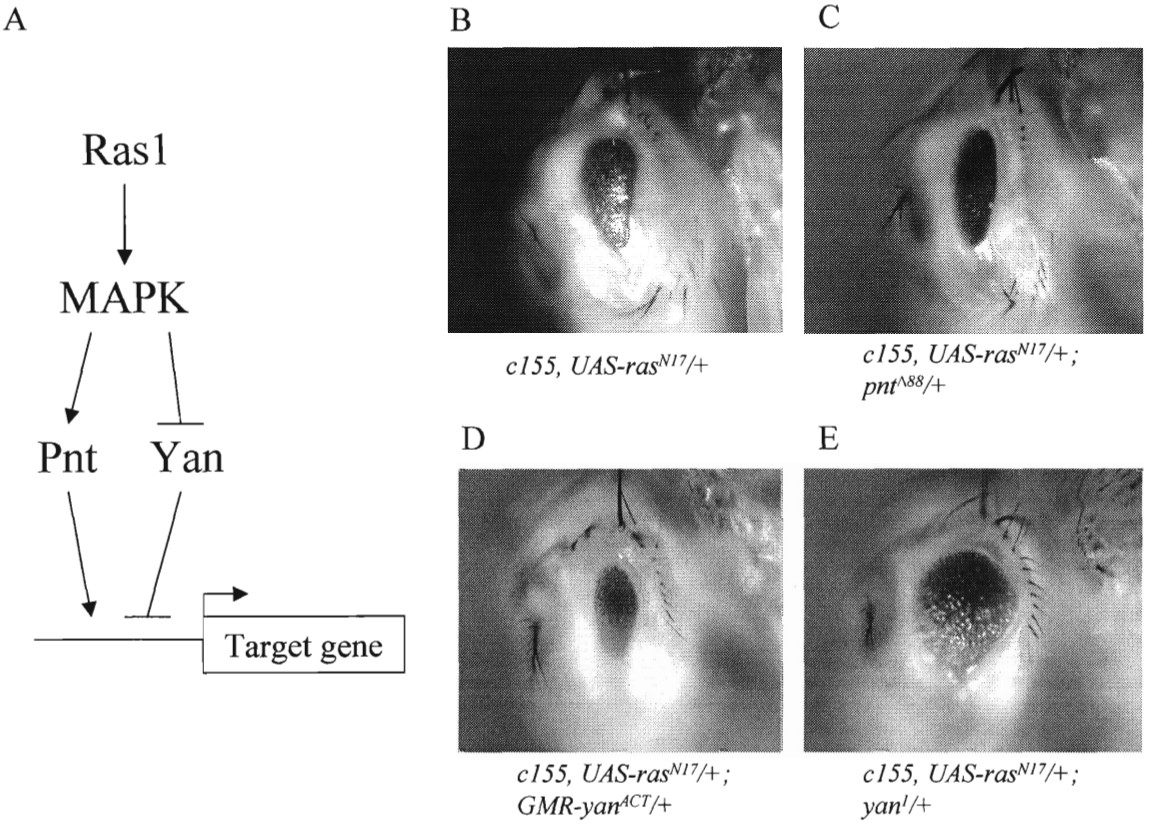


Figure 4-6. MAPK-regulated transcription factors are modifiers of the small eye phenotype of *c155, UAS-ras^{N17}*. (A) The two transcription factors Pnt (P2 isoform) and Yan are regulated by MAPK. Yan antagonizes the function of the Ras/MAPK/Pnt pathway. (B-D) The small eye phenotype of *c155, UAS-ras^{N17}* (B) was enhanced by a loss-of-function mutation in the *pnt* gene (C) or expression of an activated form of *yan* (D). Conversely, a loss-of-function mutation in the *yan* gene suppressed the phenotype (E).

Figure 4-7

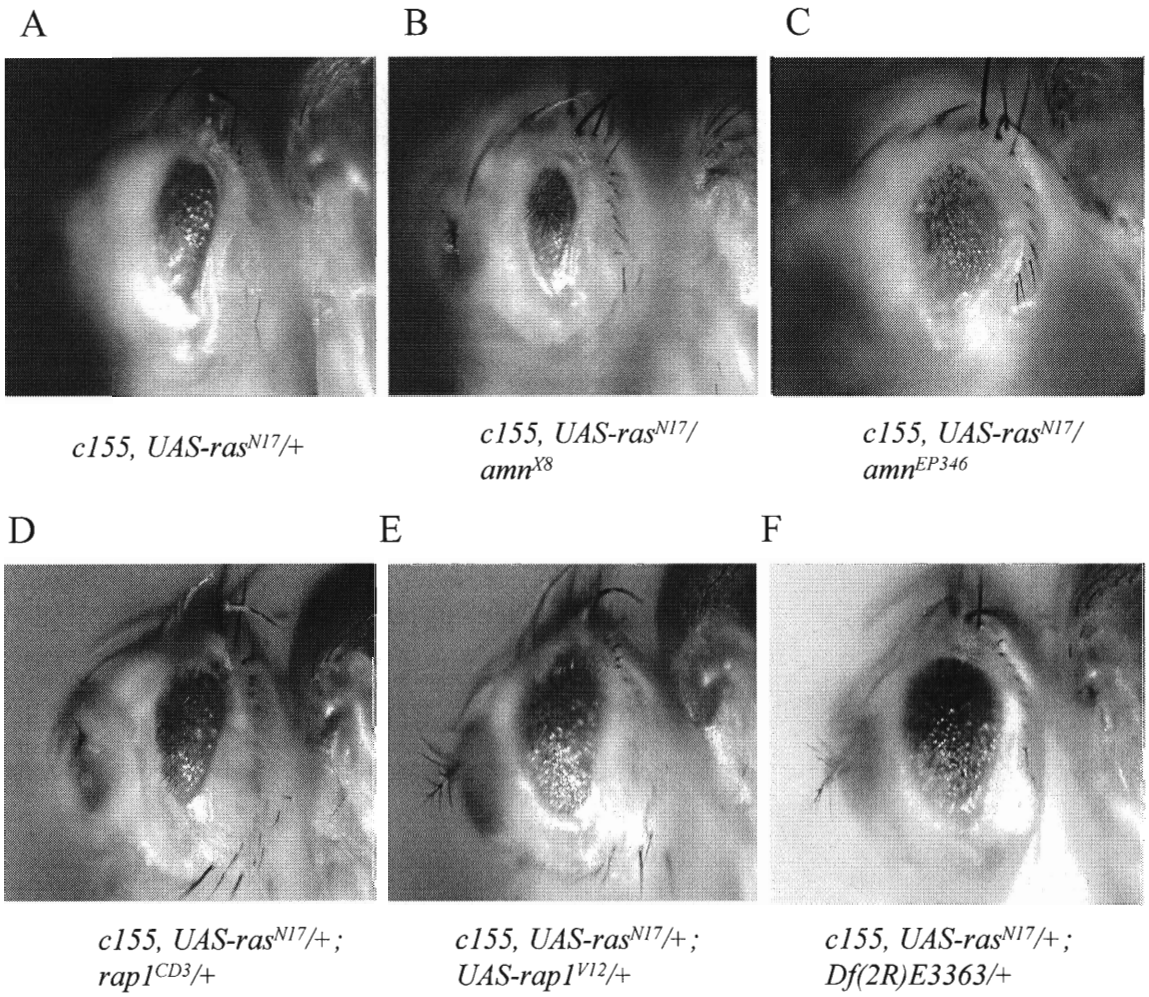


Figure 4-7. The small eye phenotype of *c155, UAS-ras^{N17}* (A) is modified with alterations in Amnesiac (Amn) or Rap signaling. (B) Enhancement by a loss-of-function mutation in the *amn* gene. (C) Suppression by overexpression of wild-type *amn*. (D and E) Although a loss-of-function mutation did not modify the phenotype (D), expression of a dominant-active form of *rap1* resulted in suppression (E). In addition to *amn* and *rap1*, the deletion line *Df(2R)E3363* was also identified as a modifier (F).

Figure 4-8

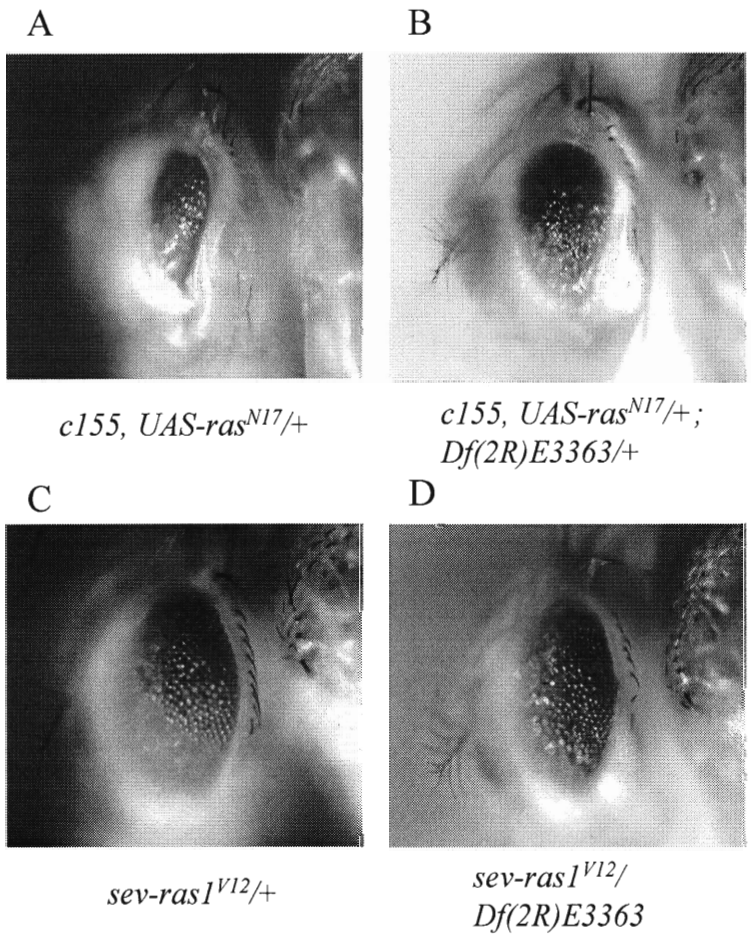
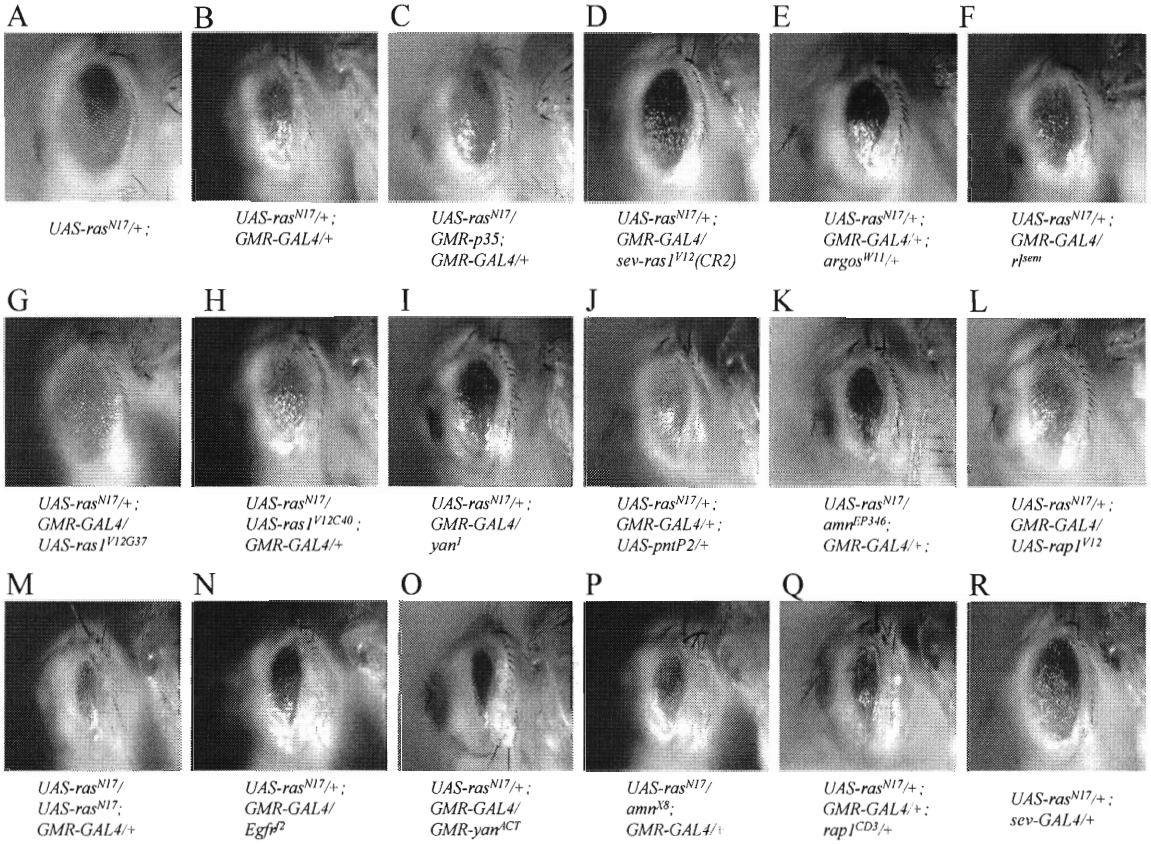


Figure 4-8. *Df(2R)E3363* was identified as a strong modifier in a screen using the small eye phenotype (A and B), while being only a weak one for the *sev-ras1*^{V12} rough eye (C and D). This illustrates the sensitivity of the small eye-based screen using *c155, UAS-ras*^{N17} flies.

Figure 4-9


A
UAS-ras^{N17}/+;
B
UAS-ras^{N17}/+;
GMR-GAL4/+
C
UAS-ras^{N17}/
GMR-p35;
GMR-GAL4/+
D
UAS-ras^{N17}/+;
GMR-GAL4/
sev-ras1^{V12}(CR2)
E
UAS-ras^{N17}/+;
GMR-GAL4/+;
argos^{W11}/+
F
UAS-ras^{N17}/+;
GMR-GAL4/
rl^{Sem}
G
UAS-ras^{N17}/+;
GMR-GAL4/
UAS-ras1^{V12G37}
H
UAS-ras^{N17}/
UAS-ras1^{V12C40};
GMR-GAL4/+
I
UAS-ras^{N17}/+;
GMR-GAL4/
yan^{1}
J
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GMR-GAL4/+;
UAS-pntP2/+
K
UAS-ras^{N17}/
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GMR-GAL4/
UAS-rap1^{V12}
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GMR-GAL4/+
N
UAS-ras^{N17}/+;
GMR-GAL4/
Egfr^{f2}
O
UAS-ras^{N17}/+;
GMR-GAL4/
GMR-yan^{ACT}
P
UAS-ras^{N17}/
amn^{X8};
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R
UAS-ras^{N17}/+;
sev-GAL4/+

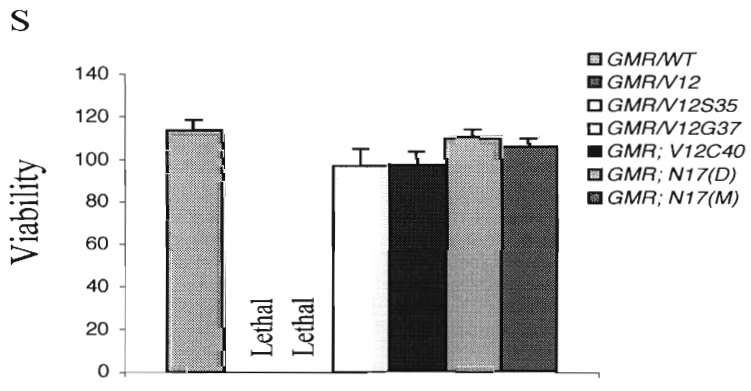
S
Viability
140
120
100
80
60
40
20
0
Lethal
Lethal
GMR/WT
GMR/V12
GMR/V12S35
GMR/V12G37
GMR; V12C40
GMR; N17(D)
GMR; N17(M)

Figure 4-9. Expression of *ras*^{N17} also generates a small eye phenotype when driven by the *GMR-GAL4* driver. (A) *UAS-ras*^{N17} itself as a control. (B) The small eye of *GMR*-driven *ras*^{N17} flies. The phenotype was modified in a similar way to *c155, UAS-ras*^{N17}. (C-L) Shown is suppression of the phenotype. (M-Q) Enhancement of the phenotype. (R) Expression of *ras*^{N17} by *sev-GAL4* did not result in small eyes, but only resulted in rough eyes. (S) *GMR*-driven *ras*^{N17} flies were as viable as other *GMR*-driven *ras* flies with normal eye size (Various *UAS-ras* flies were crossed to *GMR-GAL4* flies). As in the case with the *c155* driver (Figure 4-1G), *UAS-ras1*^{V12} and *UAS-ras1*^{V12S35} were lethal when crossed to *GMR-GAL4*. *UAS-ras1*^{V12G37}, *UAS-ras1*^{V12C40}, and *UAS-ras1*^{N17} (*Drosophila* N17) only showed rough eyes when crossed to *GMR-GAL4*, same as the results with *c155*.

Figure 4-10

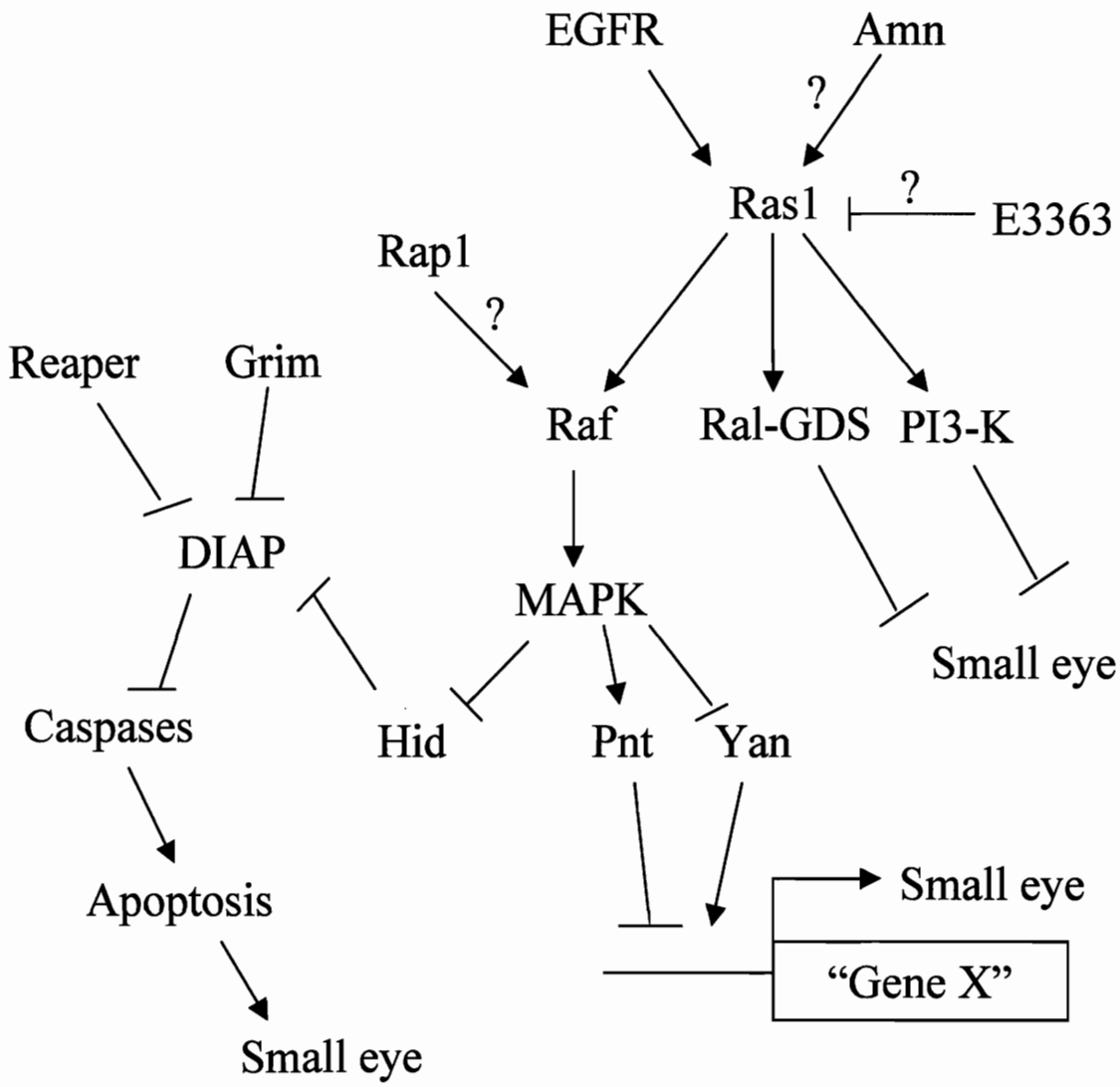

Figure 4-10. A model for signaling pathways associated with the small eye phenotype resulting from inhibition of Ras1. The targets of Amn, Rap1, and E3363 remain to be determined, as well as those of the Ral-GDS, PI3-K, Pnt, and Yan pathways.

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CHAPTER 5

Discussion and Conclusion

5-1. Summary of the findings

In the first section of Chapter 1, I provided a comprehensive survey on Ras research. Ras plays an important role as a signaling molecule to transduce extracellular signals to intracellular effectors. I illustrated that Ras regulates a variety of cellular events, such as cell proliferation, differentiation, and apoptosis. I also highlighted recent findings that suggest additional roles of Ras in neuronal and behavioral plasticity. I noted that deregulation of Ras signaling has been implicated in human cancers, including neurofibromatosis type 1 (NF1) disease that can impair cognitive functions. In the second section, I made an extensive review on olfactory learning and memory in *Drosophila*. I introduced the behavioral paradigm based on classical conditioning used for my studies. A synthetic overview that integrates genetic, neuronal, and behavioral findings was subsequently provided based on classical and current literatures. My primary purpose in this thesis research was to examine the role of Ras signaling in olfactory learning and memory in *Drosophila*.

In Chapter 2, I presented my own findings on the learning and memory behavior of flies carrying hypomorphic mutations in the *ras1* gene, the *Drosophila* homolog of mammalian *H*-, *K*-, or *N-ras*. The *ras1* mutant flies showed a significant memory deficit, while learning tested immediately after training sessions was unaffected. The severity of

the memory impairment correlated with molecular lesions in the *ras1* gene. The sensorimotor functions required to perform the behavioral task were normal in the *ras1* mutant, so was the overall morphology of the mushroom bodies (MBs), the center for the olfactory learning and memory. These results suggested a direct role of Ras signaling in maintaining olfactory memory that was acquired previously. This notion was further supported by additional evidence that the memory impairment of the *ras1* mutant could be rescued by pan-neural expression of wild-type *ras1*.

In the rest of the chapter, I also presented my findings on the brain tissue-specific effects of inhibition of Ras signaling on olfactory learning and memory in *Drosophila*. I further presented the presynaptic morphology of the memory-impaired hypomorphic *ras1* mutant at the larval neuromuscular junction (NMJ). Expression of a dominant-negative form of *ras1* ($ras1^{N17}$) in MB neurons resulted in a significant learning impairment, without affecting simple sensorimotor functions and overall MB morphology. The presynaptic terminal morphology of the hypomorphic *ras1* mutant was altered so that the number of presynaptic varicosities was increased significantly. These findings in this chapter collectively led me to the conclusion that Ras is required for olfactory learning and memory in *Drosophila*.

The theme of Chapter 3 carried an implication for cognitive impairments when Ras signaling is deregulated as in NF1 disease. I demonstrated that enhanced Ras signaling can impair learning behavior in a tissue-specific manner in *Drosophila*. Flies expressing an activated-form of a *ras1* transgene in a subset of the intrinsic MB neurons exhibited an olfactory learning impairment, when tested right after associative training. Flies expressing the same dominant-active *ras1* in neurons in the central complex showed

normal learning, indicating that the effect of the transgene expression on learning was tissue-specific. Again, simple sensorimotor functions and overall MB morphology were normal in the learning-impaired transgenic flies. These results suggested that enhanced Ras signaling disrupts acute processes required for CS-US association. Indeed, consistent with this notion, acute induction of dominant-active *ras1* expression before training resulted in a learning impairment.

Although the Ras/Raf/MAPK pathway has been recently implicated in neuronal and behavioral plasticity, expression of a dominant-active form of *raf* in the same set of the MB neurons did not result in a learning impairment as in the case of activated-*ras1* expression. Because activation of other known Ras downstream pathways (i.e., the Ral-GDS and PI3-K pathways) did not result in a learning impairment, the effect of enhanced Ras signaling in the MBs on learning might be mediated in a cooperative way by the known downstream effectors or as yet unknown effectors of Ras.

Last in Chapter 4, I described the results of a preliminary characterization of a small eye phenotype resulting from inhibition of Ras signaling in *Drosophila*. During the course of behavioral studies, I unexpectedly found that the compound eye is ablated when a dominant-negative form of mammalian *ras* is expressed in cells in the developing eye. The affected eye was severely reduced in size and largely missing normal ommatidial morphology. The reduced eye size was modified in a way sensitive to the level of Ras signaling. Hence, the phenotype was postulated to result from inhibition of Ras signaling by the expression of dominant-negative *ras*. The phenotype was based on massive cell death in the developing eye resulting from inhibition of EGFR/Ras-dependent signaling pathways, including the MAPK and PI3-K pathways. Additional components such as

Amnesiac and Rap appeared to modulate the signaling machinery implicated in the small eye phenotype. Becasue *ras* has been highly implicated in tumorigenesis in humans, the Ras-associated small eye phenotype was considered to provide a powerful tool to help develop therapeutic strategies for human cancers, as well as further understand the complexity of Ras signaling in basic biological events.

5-2. Problems to be pursued

Although, through my research, *ras* was defined as a gene that controls olfactory learning and memory in *Drosophila*, many important questions are still outstanding to refine our understanding. For example, tissue-specific roles of Ras in memory should be further studied. In a study described in Chapter 2, the memory deficit of the hypomorphic *ras1* mutant (*ix12a/ΔC40B*) was rescued only when a wild-type *ras1* transgene was expressed by a pan-neural *GAL4* driver. However, based on my observations, flies carrying *GAL4-UAS* transgenes in the *ix12a/ΔC40B* background were in general much less viable to adult than *ix12a/ΔC40B* alone (10~50% of *ix12a/ΔC40B*). They also appeared to be much weaker in adulthood. Therefore, the failure of memory rescue by MB or DPM expression can be due to toxic effects of transgene inclusion in the severely hypomorphic *ras1* background. Given such problems, the experiment to test the memory rescue by wild-type *ras1* may be refined by using milder *ras1* mutants such as *ix12a* homozygotes. Also, if I could add temporal control on transgene expression (e.g., by making it temperature-dependent), the acute role of Ras signaling in memory could be tested more rigorously in a tissue-specific manner (for an available technology, see McGuire et al., 2003; McGuire et al., 2004). Similarly, by improving the genetic background and

transgenic technology, the effects of tissue-specific suppression of Ras signaling on learning and memory might be fully uncovered.

What signaling mechanisms underlie the role of Ras in learning and memory is another important question to pursue. It is well demonstrated that in many cell types, Ras mediates growth factor/receptor tyrosine kinase signaling to MAPK (see Chapter 1). However, it is still not clear how Ras is activated in the context of neuronal or behavioral plasticity. It is entirely an open question how Ras functions, for example, in MB neurons in mediating olfactory learning and memory in *Drosophila*.

Previous studies have demonstrated that genes that are implicated in olfactory learning, such as *dunce* (cAMP phosphodiesterase), *rutabega* (adenylyl cyclase), and *DCO* (protein kinase A), are expressed preferentially in MB neurons (Han et al., 1992; Nighorn et al., 1991; Skoulakis et al., 1993). *amnesiac*, a gene that is implicated in memory consolidation, encodes a neuropeptide that can activate the cAMP pathway and is expressed in the DPM neurons that project to axons of MB neurons (Waddell et al., 2000). Interestingly, in Chapter 4, I found that the ablation of the *Drosophila* compound eye that results from inhibition of Ras signaling was substantially suppressed by overexpression of *amnesiac*. Furthermore, in the *Drosophila* larval NMJ, Ras can be activated by an adenylyl cyclase-activating peptide (Zhong, 1995). With these observations, it is tempting to speculate that as well as the cAMP pathway, Ras in MB axons may be activated by Amnesiac secreted from the DPM neurons to maintain memory. This hypothesis can be tested by overexpressing *amnesiac* in the DPM neurons in the hypomorphic *ras1* background. If overexpressed Amnesiac augments Ras signaling in MB axons, the memory deficit of the *ras1* mutant may be suppressed.

In Chapter 3, my finding that enhanced Ras signaling in the MBs impairs olfactory learning led to the further experiments that test what downstream effector pathway(s) mediate the Ras signaling. I tested three known pathways, including the Raf/MAPK and the PI3-K/Akt pathways, which have recently been implicated in neuronal and behavioral plasticity. Intriguingly, however, the obtained data did not show any single pathway solely mediating the Ras signaling. That is, enhanced activation of any single pathway in the MBs did not result in mimicking the learning phenotype resulting from activation of Ras. This does not imply that these pathways do not contribute to mediating Ras signaling for learning. They may be activated by Ras in a synergistic way. I am currently pursuing this possibility by activating them simultaneously in various combinations.

Now, I am also pursuing another possibility that as yet unknown downstream pathways mediate Ras signaling in the MBs in *Drosophila*. *247; UAS-ras1*^{V12} flies express an activated-form of *ras1* in MB neurons. In Chapter 3, I showed that they are severely impaired in olfactory learning. Taking advantage of their learning phenotype, I am pursuing a screen for genes that may mediate Ras signaling in MB neurons. Identification of mutations that suppress the learning deficit of *247; UAS-ras1*^{V12} would provide clues to delineate the nature of Ras signaling in the MBs for olfactory learning and memory.

The small eye phenotype that I described in Chapter 4 also provides an opportunity to identify novel components that are involved in the Ras pathway. With the ease of scoring the phenotype (i.e., scanning of eye size under a dissection microscope) and the demonstrated sensitivity to the level of Ras signaling, it provides a very efficient

and powerful system for genetic screens. Genes that are identified associated with the Ras pathway through this system could be readily tested for olfactory learning and memory. Such systematic approach using multiple assays should facilitate to uncover how Ras mediates olfactory learning and memory in *Drosophila*.

The small eye phenotype that results from inhibition of Ras signaling is by no means only useful for gene identification for learning and memory. It is also a potentially powerful tool to help understand the nature of Ras signaling in other basic biological events, such as cell proliferation, cell differentiation and apoptosis. It also appears to have a potential to serve as a tool for drug discovery for human cancers. In this context, further characterizations of the Ras-associated small eye and extensive genetic or drug screens based on this phenotype should be included in the list of future projects that are urgent to pursue.

5-3. References

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