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Dissection of Neurofibromatosis type 1-regulated pathways and its role in distinct phases of memory

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Abstract of the Dissertation

Dissection of Neurofibromatosis type 1-regulated pathways and its role in distinct phases of memory

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Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder characterized by benign tumors of the peripheral nervous system called neurofibromas, café au lait spots, and extreme freckling. In addition, at least 40% of afflicted children have learning difficulties. The NF1 protein contains a highly conserved GTPase-activating protein (GAP) domain that inhibits Ras activity, and the G-terminal region regulates G protein-dependent activation of adenylyl cyclase (AC). Behavioral analysis has indicated that learning and memory is also disrupted in *Drosophila* and mouse NF1 models, however, the learning defect in flies is attributed to altered activation of the cAMP pathway, whereas the mouse learning deficit results from increased Ras activity. Because of the enormous difference in the time scale involved in training paradigms for

mice (water maze) and flies (odor-foot shock association), we suspected that different components of memory were being affected. In this study I first show that NF1 regulates two separate signaling pathways that lead to adenylyl cyclase (AC) stimulation. Interestingly, different regions of the NF1 protein are required for mediating each of these pathways. The GAP-related domain, together with the Ras protein, is required for mediating growth factor stimulating AC, while the C-terminal region is essential for conferring neurotransmitter signaling for AC stimulation. Here, I also show for the first time that not only short-term memory but also long-term memory was defective in *Drosophila Nf1* mutants. The underlying signaling mechanisms for these two behavioral phenotypes of the NF1 mutants are also examined. I found that the GAP-related domain with its GAP activity and binding with Ras was necessary and sufficient for long-term memory, while the C-terminal domain of NF1 that is required for G protein-dependent activation of AC was critical for learning. Thus, this study shows that two functional domains of the same protein participate independently in two distinct signaling pathways, as well as the formation of two memory components.

Table of Contents

List of Abbreviations

5-HT	serontonin
AC	adenylyl cyclase
AL	antennal lobe
amn	amnesiac
ARM	anesthesia-resistant memory
ATP	adenosine 5'-triphosphate
cAMP	cyclic-3',5'adenosine mono-phosphate
CASK	calcium/calmodulin-dependent serine protein kinase
CC	central complex
cDNA	complimentary deoxyribonucleic acid
CREB	cAMP response element binding protein
CSRD	cysteine serine-rich domain
Cterm	C-terminal
CXM	cycloheximide
DCO	catalytic subunit of cAMP-dependent protein kinase
dCREB2	*Drosophila* cAMP reponse element binding protein
DDAH	N(G),N(G)-dimethylarginine dimethylaminohydrolase
DER	*Drosophila* epidermal growth factor
Df	deficiency
DNA	deoxyribonucleic acid

dnc	dunce
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular-activated protein kinase
E(spl)	enhancer of split
FMRFamide	Phe-Met-Arg-Phe-amide
Gas	alpha subunit of stimulatory G protein
GAP	guanosine triphosphatase-activating protein
GPCR	G protein-coupled receptor
GRD	guanosine triphosphatase-activating protein related domain
GTPase	guanosine triphosphatase
GTP?S	guanosine triphosphate-gamma sulphate
hNF1	human neurofibromatosis type 1 gene
HRP	horse radish peroxidase
hsNF1	heat shock promoter driven *Drosophila* neurofibromatosis type 1 gene
IPTG	isopropyl thiogalactoside
Leo	leonardo
LRD	leucine-rich domain
LRN	learning
LTM	long-term memory

MAPK	mitogen-activated protein kinase
MB	mushroom body
MgCl	magnesium chloride
mRNA	messenger ribonucleic acid
MTM	middle-term memory
µg	microgram
µl	microliter
µM	micromolar
NF1	neurofibromatosis type 1
NF1-GRD-GST	guanosine triphosphatase-activating protein-related domain of neurofibromatosis type 1 tagged with glutathione
Nterm	N-terminal
ng	nanogram
nM	nanomolar
PACAP38	pituitary adenylyl cyclase activating peptide 38
PCR	polymerase chain reaction
Phospho-MAPK	phosphorylated mitogen-activated protein kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
rad	radish
RasGAP	guanosine triphosphatase-activating protein of Ras
RNA	ribonucleic acid

RNAi	ribonucleic acid interference
rpm	revolution per minute
RT	room temperature
RTK	receptor tyrosine kinase
Rut	rutabaga
Rut-AC	rutabaga encoded adenylyl cyclase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
STM	short-term memory
TBS	tris-buffered saline
TBST	tris-buffered saline with tween
TGFα	transforming growth factor alpha
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride

List of Figures and Tables

Chapter 2

Chapter 3

Appendix

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Publications

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Introduction

Neurofibromatosis type 1 and the NF1 gene

NF1 is one of the most common autosomal dominant genetic disorder with a prevalence of 1 in 3,500 regardless of gender and ethnic background (Friedman et al., 1999). It is a multi-system disorder with complications affecting the eyes, skeleton, blood vessels, endocrine system, and peripheral and central nervous systems (North, 2000). It has been categorized as a neurocutaneous syndrome since individuals with NF1 usually present with café au lait spots, skinfold freckles, and Lish nodules (iris hamartomas). In addition to cutaneous features, individuals with the syndrome may also develop benign tumor growth, such as neurofibromas, plexiform neurofibromas, and low-grade brain tumors. Benign neurofibroma can develop into maglinant peripheral nerve sheath tumors (MPNSTs). *NF1* mutations in the brain can result in astrogliosis and astrogliomas, and cognitive defects, such as mental retardation in 4 – 8% and learning disability in 30 – 60% of afflicted children (North, 2000). Among the afflicted, visual-spatial function seems to be most severely affected, but compromised language skills, executive function, attention and motor coordination are also common.

The NF1 gene was identified in 1990 by positional cloning and mapped to chromosome 17q11.2 (Cawthon et al., 1990). Four genes have been shown to localize in this locus, but mutations in genomic DNA from afflicted individuals involved only one of the four genes (Ballester et al., 1990; Riccardi, 1993; North, 2000). The size of the gene is about 350kb and it is composed of 60 exons with three splice variants (Viskochil et al.,

1993). The full-length messenger RNA transcript is about 11 to 13 kb in length with a 3.5 kb 3' un-translated region. The messenger RNA encodes a protein that is composed of 2818 amino acids named neurofibromin. Soon after the gene had been identified and cloned, the only known function of the NF1 gene is the down-regulation of Ras signal transduction pathway (Viskochil et al., 1993).

Neurofibromas are one of the most common type of tumors occurred in individuals afflicted with NF1. This type of tumors is associated with the peripheral nerves, and seen most commonly close to the spinal nerve roots. Neurofibromas are composed of multiple cell types including Schwanna cells, mast cells, perineurial cells, fibroblasts, and endothelial cells. Studies have shown 40 to 80% of the cells in neurofibromas are Schwann cells, which, when isolated from the tumors, exhibit invasive behavior and promote angiogenesis (Sheela et al., 1990; Tucker and Friedman, 2002). In most mammalian cells, the cAMP-PKA pathway promotes cell growth arrest and differentiation. On the other hand, cAMP induces proliferative response in Schwann cells and NF1 acts to antagonizes the accumulation of cAMP within these cells (Kim et al., 2001). This finding suggests that increased level of Ras biological activity may not be the only mechanism that contributes to tumor formation in NF1 patients. The study of NF1's relationship with the cAMP pathway may help elucidate the intricate molecular mechanism underlying NF1 pathology.

Neurofibromin as a GAP and its interaction with the Ras protein

The NF1 gene codes for a protein, named neurofibromin (NF1), that is 2,818 amino acids in size (Marchuk et al., 1991). The central part of the protein sequence, a

segment of 250 amino acids, show high homology to Ras-specific GTPase activating proteins such as p120GAP (Trahey and McCormick, 1987) and its yeast homologues IRA1 and IRA2 (Tanaka et al., 1990). Numerous studies in yeast have shown that this central segment of NF1 is a functional GAP-related domain (NF1-GRD) that can catalyze the intrinsic GTPase activity of GTP-bound Ras (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). Since then, various other studies show the importance of NF1 for the regulation of Ras activity, and that in cell lines the absence of NF1 leads to an increase in GTP-bound form, the active form of Ras (DeClue et al., 1992). Interestingly, there has also been evidence showing that NF1 can regulate Ras-dependent growth in cell lines by a mechanism that is independent of its GAP activity (Johnson et al., 1994).

Structural studies have been conducted in elucidating the mechanisms involved in the interaction that occur between Ras and p120GAP, as well as Ras and NF1 (Scheffzek et al., 1997; Scheffzek et al., 1998). Comparing to human p120GAP, the minimal domain with full catalytic activity is smaller for NF1; 230 residues for NF1, and 270 residues for p120 GAP (Ahmadian et al., 1996). In addition, the affinity of NF1 for GTP-bound Ras is about 50 to 100 fold higher than that of p120GAP, and the kinetics for association and dissociation for p120GAP is much faster (Ahmadian et al., 1997). When the crystallographic structure of NF1/Ras interaction is examined, Scheffzek et al. identified residues within the Ras-binding groove of the NF1-GRD that are essential for their interaction (Scheffzek et al., 1998). Amongst these residues, three of them were analyzed in our biochemical and behavioral assays (Figures 2.4, 2.5, and 3.3; See Figure A.1 for 3D view of hypothetical NF1/Ras proteins interaction). Arg1276 residue was found to have been mutated to proline in an NF1 patient with malignant schwannoma, and analysis

with the substitution show that although NF1 with the R1276P mutation can bind to Ras, its GAP activity has been compromised 8000-fold (Klose et al., 1998). Lys1423 have been shown to be the most frequently mutated residue in solid tumors (Li et al., 1992; Upadhyaya et al., 1997), and decreased Ras affinity seems to be the major effect of this residue mutated to glutamate (Poullet et al., 1994). Arg1391 residue is important for catalysis and the stability of the finger loop of the GRD (Figure A.1), and it is situated within the most conserved of GAP catalytic domain, the FLR motif (Brownbridge et al., 1993; Scheffzek et al., 1998). The effects of these point mutations on NF1's ability to interact with Ras or perform GAP activity in my study will be discussed in the following chapters.

Ras signaling and learning and memory

MAPK signaling cascade is one of the downstream targets of Ras signaling. This signaling cascade is responsible for the regulation of gene expression, protein synthesis, receptor insertion, modulation of ion channels, and dendritic spine stabilization (Figure A.2) (Sweatt, 2004). For the activation of the MAPK cascade, there are several upstream signaling receptors identified and one of them is the receptor tyrosine kinases (RTKs), which can bind to growth factors such as EGF. EGFR is a well-studied RTK that has mostly been known for its role in development in *Drosophila* (Shilo, 2003). A single gene has been identified in the fly genome that codes for EGFR, called DER. The overall protein sequence identity between DER and the mammalian ErbB family members is about 40% (Bogdan and Klambt, 2001). The extracellular portion of DER contains three cysteine-rich motifs that comprise the ligand-binding domain (Figure A.3). The surface

receptor is present as a monomer without any stimulation. Upon binding of ligand, DER will dimerize and transautophosphorylation will take place on its cytoplasmic domain (Bogdan and Klambt, 2001; Jorissen et al., 2003; Shilo, 2003).

Numerous studies have established the role of Ras signaling in learning and memory as well as synaptic plasticity (Brambilla et al., 1997; Atkins et al., 1998; Ohno et al., 2001). Spatial learning defect is observed in heterozygous *Nf1* knockout mice, and this phenotype can be remedied by reducing Ras biological activity through genetic or pharmacologic manipulations (Costa et al., 2002). Additionally, pharmacologic agents that inhibit PI3K and MEK, two of the Ras effectors, can perturb learning and memory in rodents (Lin et al., 2001; Ohno et al., 2001). Moreover, when the Ras activator tyrosine kinase receptor B receptor or the Ras effector B-raf were specifically disrupted in mouse neuron, synaptic plasticity and learning and memory are affected (Minichiello et al., 1999; Chen et al., 2006). These findings all point to the importance of Ras and its effectors in synaptic plasticity and learning and memory.

Adenylyl cyclases and the cAMP pathway

Adenylyl cyclases (AC) are integral membrane proteins consisting of twelve transmembrane (TM) domains in two sets of six with its intracellular loop and long cytoplasmic tail shown to be important for its stimulation and enzyme activity (Figure A.4) (Girault and Greengard, 2004). Nine membrane-bound isoforms and one soluble form of these enzymes have so far been identified in the mammalian system (Patel et al., 2001). The reaction that ACs catalyze is the conversion of ATP to 3',5'-cyclic AMP These enzymes are mostly known to be regulated by G-protein mediated pathways,

Ca^{2+}/calmodulin, and other protein kinases (Patel et al., 2001). cAMP is involved in metabolism, cell proliferation, gene transcription (Sutherland, 1972), muscle contraction (Harvey and Belevych, 2003), direct regulation of ion channels (Kopperud et al., 2003), and learning and memory (Alberini et al., 1995). Studying the regulation of cAMP production, therefore, is essential to understanding these mechanisms.

In learning and memory, several identified mutants are intimately related to the cAMP pathway. One of the first and the most well-known of them is *rutabaga* (*rut*) (Dudai et al., 1976). *Rut* mutant is defective in *Drosophila* olfactory conditioning paradigm and its gene codes for a calcium- and calmodulin-responsive AC (Zars et al., 2000). This AC is a homologue of the mammalian AC I and VIII subtypes, which has been shown to be involved in learning and memory (Wu et al., 1995; Wong et al., 1999). Temporally and spatially controlled expression of a rutabaga transgene within the mushroom body of *rut* mutants is able to rescued the learning defect observed in *rut* mutant, indicating this particular AC is required in the MB to confer learning behavior (McGuire et al., 2003). The transcription factor CREB is another protein within the cAMP pathway that is involved in olfactory conditioning, particularly in LTM (Yin et al., 1994). Interestingly, dCREB gene codes for at least seven isoforms and two of these isoforms, dCREB2-a and dCREB2-b, are activator and blocker of CREB-dependent gene expression, respectively (Yin et al., 1995). Overexpression of dCREB2-b can block the formation of LTM, while overexpression of dCREB2-a can enhance it (Yin et al., 1994; Yin et al., 1995). From the two genes that mentioned above, cAMP pathway is heavily entwined with learning and memory in *Drosophila*. Therefore, a deeper understanding of

the pathway will let us gain insights into the molecular mechanisms that govern learning and memory processes.

NF1's involvement with both the Ras and cAMP pathways

NF1 has been shown to be a tumor suppressor protein with its central GRD region shown to accelerate inactivation of Ras by stimulating its GTPase activity (Ballester et al., 1990). NF1 can also associate with microtubules through its GRD and bind with syndecan to form a complex with CASK (Xu and Gutmann, 1997; Hsueh et al., 2001). In human patients, mutation that abolish RasGAP function of NF1 leads to multiple symptoms including cognitive dysfunction, suggesting that loss of the GAP function underlies learning defect (Klose et al., 1998). Similar to NF1 patients, mice and *Drosophila* NF1 mutants display learning defects; in mice this learning defect is due to increased Ras activity, and in *Drosophila* this defect involves NF1's modulation of the cAMP pathway (Costa et al. 2002; Guo et al. 2000). These reports indicate NF1 is involved in a variety of pathways. The first of my research goals focuses on genetically dissecting the pathways that lead to NF1 modulation of AC activity in *Drosophila*.

Previous study in *Drosophila* has shown that NF1 and *rutabaga* (rut) are involved in mediating pituitary adenylyl cyclase-activating peptide (PACAP)-like stimulation of K^+ current at the larval neuromuscular junction (NMJ) (Guo et al., 1997). Later study shows that, in addition to learning defects, NF1 mutant flies also exhibit smaller body size in larval, pupal, and adult stages (The et al., 1997). Both the learning and body size defects can be rescued by expressing a heat-shock inducible constitutively active mutant catalytic subunit of cAMP-dependent protein kinase (PKA) transgene. Biochemical

studies have shown that, similar to *rut* mutant, AC activity stimulated by G-protein in adult fly head membrane fraction is attenuated in NF1 mutants compared to wild type, suggesting that NF1 plays an important role in regulating AC activation in adult flies (Guo et al., 1997). These studies show that NF1 is involved in the cAMP pathway to govern learning, control body size, and mediate neuropeptide signaling.

Behavioral defects in two NF1 model systems are attributed to different pathways

In *Drosophila,* homozygous deletion of NF1 leads to compromised performance in the Pavlovian olfactory conditioning paradigm. This defect is attributed to the disruption of the *rutabaga*-encoded adenylyl cyclase (Rut-AC) pathway. Mice with a heterozygous ablation of NF1 exhibit learning defects in the Morris water maze, and this phenotype can be remedied by genetically or pharmacologically reducing the biological activity of the Ras protein (Costa et al., 2002). Although both reports showed learning deficiency, mice need to be trained for many days in the water maze to perform at an acceptable level, while training for flies only takes minutes. In addition, injection of a protein synthesis inhibitor to the lateral ventricle significantly reduces performance in the water maze, suggesting the learning phenotype exhibited by the $Nf1^{+/-}$ mice may actually be a form of protein synthesis-dependent memory rather than learning *per se*. We hypothesize that the NF1 protein maybe involved in different phases of memory through its activation of different signaling pathways.

Several studies that have attempted to evaluate genotype-phenotype correlations of NF1 with patient samples, have not established any functional significance of mutations in different regions of the NF1 protein. Aside from regulating Ras activity,

NF1 has been shown to regulate cAMP levels in both *Drosophila* and mouse models (Tong et al., 2002). The first part of this study demonstrates that distinct regions of the NF1 protein regulate two different AC signaling pathways; the GRD is necessary and sufficient for mediating growth factor stimulation of AC through the Ras pathway, while sequences within the C-terminal region are required for neuropeptide and neurotransmitter stimulated activation of AC in cooperation with $G\alpha_s$ and Rut-AC. This prompted us to investigate whether these distinct structure-function relationships at the biochemical level would have a correspondingly distinct effect on behavioral output.

Memory phases in Drosophila

Researchers have established *Drosophila melanogaster* as one of the most versatile animal model system to study learning behavior using numerous paradigms. These paradigms include courtship suppression (Gailey et al., 1982), visual learning (Wolf and Heisenberg, 1997), aggression (Chen et al., 2002), appetitive reward learning (Tempel et al., 1983), and olfactory conditioning (Tully and Quinn, 1985). Among them, the Pavlovian olfactory conditioning is one of the most well-established and robust. By exposing a group of a hundred flies to an aversive odor (conditioned stimulus, CS) paired with footshocks (unconditioned stimulus, US), flies can learn to associate the punishing US with the odor. This "cycle" training can be repeated ten times to generate different kinds of long lasting memory. When the cycles are repeated continuously without rest, anesthesia-resistant memory (ARM) will be formed. This form of memory lasts for 3 days and it is not dependent on protein synthesis. However, if the cycles are repeated with rest, a type of protein-synthesis dependent memory, long-term memory (LTM), will

be generated and will last for more than seven days (Tully et al., 1994). In addition, analyses of different behavioral mutants, such as *rut, dnc, DCO,* and *amn,* lead to the identification of short-term memory (STM) (Dudai et al., 1976; Byers et al., 1981; Livingstone et al., 1984; Tully and Quinn, 1985) and postulation of middle-term memory (MTM) (Tully et al., 1990; Skoulakis et al., 1993; Li et al., 1996; Tully et al., 1996). The accumulated data from these studies culminate into a memory decay curve (Figure A.5).

Pavlovian olfactory conditioning paradigm represents a powerful tool for measuring flies' ability to learn and remember in my behavioral analysis described in Chapter 3. Mutants defective in each of the distinct phases of memory have been isolated, and NF1 has been classified as a learning/STM mutant in the fly model. In this study we revealed that NF1 is also involved in LTM. In order to further dissect the role of NF1 in learning versus LTM, we expressed the highly-conserved human NF1 (hNF1) protein in *Nf1* null mutant flies, including variants containing clinically relevant missense mutations as well as large deletions, to identify the structural and/or functional requisites for these behaviors. These analyses revealed that the GRD is required for LTM, while sequences in the C-terminal region mediate learning.

The anatomical site of learning and memory in flies has been mostly attributed to the antennal lobes and the mushroom body of the *Drosophila* brain (Liu and Davis, 2006). Central complex is another fly brain structure that has been associated with behavioral output such as courtship suppression (Popov et al., 2003) and visual (Liu et al., 2006) memory, but its role in olfactory learning had only been implicated (Davis, 1996). To understand the brain structures where NF1 may function to mediate behavioral output, we use several Gal4 lines that have specific expression in antennal lobes,

mushroom body, and central complex to express hNF1 in *Nf1* null mutant background to rescue learning or LTM. Together our structure-function analysis, this will allow us to structurally and anatomically dissect NF1's function in the fly brain.

Study aims and dissertation layout

In this study, I sought to dissect NF1's functional role in signaling pathways as well as behavioral output in *Drosophila*. In Chapter 2 I first demonstrated that NF1 mediate two separate signaling pathways for stimulation of AC. One NF1-dependent pathway involves EGFR and Ras, while the other requires GPCR and Rut-AC. Secondly, I established that different regions of the NF1 protein are required for each of these pathways. The GRD is necessary and sufficient in mediating EGFR stimulation of AC, while sequences in the C-terminal region are essential for GPCR stimulation of Rut-AC. In Chapter 3, I revealed that aside from learning, NF1 is required for long-term memory formation in the *Drosophila* olfactory conditioning paradigm. In addition, by expressing the human NF1 gene with partial deletions as well as clinically relevant point mutations I am able to identify regions that are required for learning versus long-term memory. These findings will allow other researchers to understand the role of NF1 in signaling transduction pathways as well as gain insight into the mechanism underlying learning and long-term memory.

Chapter 2

A novel pathway for Adenylyl Cyclase activation requiring Neurofibromin and Ras

Introduction

Mutations in the human NF1 gene are characterized by benign but disfiguring tumors of the peripheral nervous system, as well as increased incidence of malignant peripheral nerve sheath tumors and central nervous system tumors (Riccardi, 1993). About 40% of children with NF1 exhibit learning deficits (North et al., 1997; Gutmann, 1999), and mouse models of NF1 recapitulate both the tumor and learning phenotypes (Cichowski and Jacks, 2001; Zhu and Parada, 2001; Costa and Silva, 2003). In *Drosophila*, *Nf1* mutations affect circadian rhythms (Williams et al., 2001), body size (The et al., 1997), responses to neuropeptides (Guo et al., 1997), and olfactory learning (Guo et al., 2000). Thus, the NF1 protein is essential for normal neural development and plasticity in both vertebrates and invertebrates.

Gaining insights into the molecular mechanisms of NF1 function requires the identification of cellular signal transduction pathways that are disrupted by NF1 mutations. Biochemical and genetic analysis in mammals and *Drosophila* has revealed that NF1 both inhibits Ras activity (Cichowski and Jacks, 2001; Williams et al., 2001; Zhu and Parada, 2001; Costa and Silva, 2003), and regulates AC activity (Guo et al., 1997; The et al., 1997; Tong et al., 2002; Dasgupta et al., 2003). The NF1 protein has a central GTPase Activating Protein (GAP)-Related Domain (GRD), which catalyses the intrinsic GTPase activity of Ras (Viskochil et al., 1993). Many of the tumor phenotypes

observed in NF1 patients and animal models have been attributed to hyperactivation of Ras, that is observed for example in Schwann cells (DeClue et al., 1992) and mast cells (Ingram et al., 2001). Learning deficits seen in *Nf1*+/- heterozygous mice can also be rescued by manipulation of Ras levels (Costa et al., 2001; Costa et al., 2002), however the NF1-regulated AC/cAMP pathway is important for controlling learning (Guo et al., 2000) and neuropeptide responses (Guo et al., 1997) in flies, as well as neuropeptide stimulated AC activity in both flies and mammals (Tong et al., 2002; Dasgupta et al., 2003). The NF1-dependent activation of AC versus down-regulation of Ras may therefore have important phenotypic consequences, but the molecular mechanism whereby NF1 regulates AC activity has not yet been determined.

The product of the *Drosophila Ras1* gene is functionally equivalent to vertebrate H-Ras, K-Ras, or N-Ras that are mutated in 30% of human cancers (Bos, 1989). Ras signaling is down-regulated by the activity of GAPs, which catalyze the hydroysis of Ras-GTP to Ras-GDP. Five genes are reported to encode Ras-specific GAPs in *Drosophila* (Bernards, 2003). The *Gap1* and *Nf1* genes each encode a GRD that can bind with Ras and catalyze GTPase activity (Gaul et al., 1992; The et al., 1997), however, the Gap1 protein requires regions outside the GRD to achieve full catalytic activity (Powe et al., 1999). Guanine exchange factors (GEFs) promote the exchange of GDP for GTP to activate Ras, thereby enabling intereaction with downstream effectors such as Raf-1 and PI3 kinase (Rommel and Hafen, 1998; Quilliam et al., 2002). GEF activation of Ras is controlled by signaling through RTKs such as *sevenless* and the *Drosophila* EGFR (Simon et al., 1991; Schlessinger, 2002; Jorissen et al., 2003). Classical genetic studies in *Drosophila* identified the *sevenless* RTK and its GEF *son-of-sevenless* (SOS) through

their effect on eye development (Simon et al., 1991). Mutations in the *Gap1*, *Ras1*, *sevenless*, and *EGFR* genes also lead to defects in eye development and embryo patterning (Simon et al., 1991; Gaul et al., 1992; Schlessinger, 2002). The *Nf1* gene product does not perform a critical function in either of these pathways, probably owing to redundancy of Gap1 and NF1 activity, as *Gap1;Nf1* double mutants are lethal (The et al., 1997).

Our study identifies three distinct AC signalling pathways in the *Drosophila* brain, including a novel NF1/Ras-dependent AC pathway activated by growth factors that remarkably does not involve Gas, as well as two separate neurotransmitter stimulated AC pathways, one requiring NF1 and Gas, while the other requires Gas alone. Analysis of the effect of human NF1 mutants and partial deletions, expressed in flies with no NF1, shows that separate domains of NF1 control the different AC pathways. In particular we show that RasGAP activity of NF1 is necessary for Ras/NF1- but not NF1/Gas-dependent AC signaling, while part of the C-terminal region is sufficient for NF1/Gas-dependent AC signaling and regulation of body size.

Materials and Methods

D. melanogaster media, strains and heat shock conditions

Flies were raised at room temperature (22°C-24°C) on standard cornmeal medium. The *Nf1* mutants $Nf1^{P1}$ and $Nf1^{P2}$, together with the parental K33 line and $hsNf1;Nf1^{P2}$ flies were obtained from A. Bernards (Massachusetts General Hospital, Boston, MA). K33 flies used as wild type controls have a P-element inserted 1.5kb downstream of the *Nf1* locus, that was mobilized to generate the $Nf1^{P1}$ and $Nf1^{P2}$ null mutant alleles (The et al., 1997). $Nf1^{P1}$ deletes most of the *Nf1* gene and several downstream genes from the *Enhancer of Split* locus, while $Nf1^{P2}$ carries a P-element insertion within the first intron of the *Nf1* gene, and neither allele produces any detectable NF1 protein (The et al., 1997). Heat shock induction of NF1 was performed at 35°C for 2 hr, then flies were rested at 21-23°C for 1hr. The Ras^{e1B} and Ras^{e2F} mutants are from the *Drosophila* Stock Center (Bloomington, IA). Each has an amino acid substitution in either the Switch II or Switch I effector domains, respectively (Simon et al., 1991). Both affect Ras activation and binding to downstream effectors and are homozygous lethal. The EGF receptor mutants are also from the Bloomington Stock Center. $Egfr^{t1}$ is a hypomorph and *Df(2R)Egfr18* is a homozygous lethal deficiency (Clifford and Schupbach, 1994). Ras^{e1B}, Ras^{e2F} and *Df(2R)Egfr18* heterozygotes carrying a balancer (wild type) chromosome (TM3 or CyO) (Lindsley and Zimm, 1992) were used for all assays. Gsa^{B19} is a hypomorphic mutant (Wolfgang et al., 2001) provided by M. Forte (Vollum Institute, Portland, OR). Gal4 driver lines: *elav*-Gal4;$Nf1^{P1}$ (Williams et al., 2001) was obtained from A. Sehgal (University of Pennsylvania, Philadelphia, PA);

e22c-Gal4 (Duffy et al., 1998) was from N. Perrimon (Harvard Medical School, Boston, MA). White118(isoCJ1) (Yin et al., 1994) was obtained from T. Tully (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Adult head AC activity assay

The previously described AC activity assay (Guo et al., 2000) was modified as follows. To prepare fly head membrane extracts, 20 male heads were homogenized in 850µl lysis buffer. The membrane fraction was then extracted by centrifugation at 178,000*g* for 10 min at 4oC. Total protein concentration was assayed (BioRad Bradford Assay) and adjusted to the range of 1-2µg/µl. Supernatant was mixed with AC assay buffer, and Ras or Rab protein, at 1nM to 1µM concentrations, was then added to the sample at different time points prior to starting the AC reaction: 0min, 10min, 30min, 60min or 90min. H-Ras and Rab3a were purchased from Sigma. K-Ras was from Merck.

GST fusion protein preparation

Wild type and mutant NF1-GRD-GST fusion proteins (Kim and Tamanoi, 1998) and GST alone were purified using glutathione beads as follows: One liter cultures of *Escherischia coli* DH5a cells carrying GST fusion plasmids were grown in LB plus 100µg/ml ampicillin at 37^{o}C to log phase and treated with 1mM isopropyl-ß-Dthiogalactopyranoside for 1hr. Cells were collected and lysed by sonication, at 4^{o}C for 6 cycles with 20 sec each cycle, in 40ml sonication buffer containing 1mM EDTA, 1mM EGTA 0.1% lubrol, 0.1mM dithiothreitol and protease inhibitor cocktail (Roche). After centrifugation at 10,000rpm for 30min at 4^{o}C, ~30ml of supernatant was added to 1ml of

50% glutathione beads (Sigma), rotated for 1-2hr at 4°C followed by centrifugation at 3000 rpm for 5min at 4°C. Beads were washed with 10ml sonication buffer with protease inhibitor cocktail and then washed with 10ml elution buffer containing 50mM Tris, 0.5mM MgCl2, and 0.5mM dithiothreitol. For elution of the protein, 3ml of elution buffer was added plus 4.2mg/ml glutathione (Sigma) and supernatants were collected by centrifugation. Proteins were added to the head membrane extracts, at 1µM concentration, at different time points as described above for Ras. NF1-GRD-GST fusion constructs were provided by F. Tamanoi (University of California, Los Angeles, CA).

Larval brain AC activity assay

In order to mimic physiological conditions as closely as possible, larval brains were dissected in saline and manually dissociated into individual neurons, then separated into control and experimental groups. To further minimize variability, control and experimental groups in comparison were always assayed in the same batch. Results generated from such experiments were highly consistent. Controls were treated with 1M Tris buffer while experimental groups were treated for 5min (2µM, growth factors, and insulin) or for 2min (0.2µM, neurotransmitters). Growth factors (mouse EGF, rat TGFa), insulin, and neurotransmitters (dopamine, FMRFamide, histamine and serotonin) were purchased from Sigma. The samples were spun down at 1,800*g* then lysis buffer was added for homogenization. The same procedure as the head membrane preparation was then followed. All statistical analyses were performed using the Paired Student's t-Test.

Mutagenesis of hNF1 and cloning of deletion constructs

Clones containing normal hNF1 were obtained from A. Bernards (Massachusetts General Hospital, Boston, MA). The 88:12 clone is a NotI-SalI fragment that contains the entire hNF1 cDNA cloned into NotI-SalI sites of pBluescript (pBSK; Stratagene). The UAS-hNF1 clone contains the NotI-SalI fragment of 88:12 cloned into NotI-XhoI sites of the pUAST vector, destroying both the SalI and XhoI site. For this study a NotI-XhoI fragment of 88:12 was subcloned into NotI-XhoI cut pBluescript, and a XhoI-KpnI fragment of 88:12 was subcloned into XhoI-KpnI cut pBluescript. Site-directed mutagenesis of the subclones used the Stratagene Chameleon kit with a pBSK specific phosphorylated selection primer (5'-pCCGCCACCGCGATGTAGCTCCAATTCGC-3') and mutation specific mutagenesis primers, that altered a restriction enzyme site in addition to creating the desired clinically identified amino acid mutation (Table 1). Clones were selected by restriction analysis and verified by PCR and sequencing, then mutagenized fragments were digested, gel-purified, and ligated into the UAS-hNF1 construct. Deletion constructs (Figure 2.4A) were generated using restriction digests and other enzymes as noted below, and verified by sequencing and PCR. The UAS-GRD2 construct (residues 986-1746, bases 3153-5432) was prepared by subcloning an NheI fragment into the XbaI site of pUAST. The UAS-? GRD2 construct (deletion 986-1746) was generated by digesting the UAS-hNF1 clone with NheI to remove bases 3153-5432, then digesting single stranded ends with Mung Bean nuclease (New England Biolabs) and re-ligating to restore the hNF1 reading frame. The UAS-GRD1 construct (residues 1241–1746, bases 3918-5432) was prepared by digesting the UAS-GRD2 construct with XhoI and re-ligating to remove the NheI-XhoI fragment (bases 3153-3917). The UAS-Nterm clone (residues 1-985, bases 198-3152) was prepared by digesting UAS-hNF1

with NheI and Xba I and religating to remove the GRD and C-terminal regions. The UAS-Cterm clone (residues 1748-2843, bases 5433-8717) was prepared by digesting with NotI and NheI, end-filling with Klenow and re-ligating the blunt ends to remove the N-terminal and GRD regions.

Transgenic flies

P-element mediated transformations were performed by injecting the mutated UAS-hNF1 cDNAs and deletion constructs into white118(isoCJ1) (Yin et al., 1994) *Drosophila* embryos together with pTURBO as a source of transposase (Rubin and Spradling, 1982). DNA used for injection was prepared using Qiagen kits and checked by PCR and restriction analysis. F1 transformants were identified by eye color and the location of insertions was assayed by crossing to the double balancer line *w/Y;CyO/Sp;TM3Ser/Sb* (Lindsley and Zimm, 1992). Transcription of UAS-hNF1 transgenes in flies was controlled using the global Gal4 driver, *e22c*-Gal4, and a nervous system specific X chromosome line, *elav*-Gal4 (see above). Second chromosome hNF1 insertion lines and Gal4 driver lines were crossed into the $Nf1^{P1}$ or $Nf1^{P2}$ mutant background using *w/Y;CyO/Sp;TM3Ser/Sb* (Lindsley and Zimm, 1992) to create doubly homozygous lines with normal or mutant UAS-*hNf1;*$Nf1^{P1}$ or Gal4 driver;$Nf1^{P2}$. The crossing schemes designed to generate progeny carrying one copy of the transgene and one copy of the Gal4 driver in the *Nf1* mutant background are outlined (Figure 4B and 4C). Each of the mutant hNF1s and deletion constructs was tested using multiple Gal4 driver lines (in addition to the two presented here) and multiple insertion lines, except for R1276P for which only one transgenic line could be generated.

MAPK activity

Flies were collected at the same time each day to minimize circadian differences in phospho-MAPK levels (Williams et al., 2001). For each genotype 10 heads were homogenized in75µl 1xSDS loading buffer (Invitrogen) plus 0.5mM dithiothreitol and protease inhibitor cocktail (Roche). Samples were run on precast 10% Tris-glycine gels (Novex, Invitrogen) in 1xTris-glycine-SDS buffer at 125V for 2hours. Proteins were transferred to nitrocellulose in 1xNovex buffer plus 20% methanol for 2hours at 25V. Transfer was verified by Ponceau staining, then blots were blocked in 5%milk/TBST for 1hour at room temperature (RT), rinsed for 3x5min in TBST, then probed with primary antibody diluted 1/500 in 5%milk/TBST overnight at 40^{o}C. Rabbit polyclonal antibodies to phosphorylated and non-phosphorylated human p44/42 MAPK were obtained from Cell Signaling Technology. Following rinses of 3x5min in TBST, blots were incubated with 1/10,000 donkey anti-rabbit HRP conjugated secondary antibody (Amersham) diluted in 5%milk/TBST for 1 hour at RT, then rinsed again for 3x5min in TBST, followed by 5min TBS, prior to detection of signal using the ECL kit (Amersham) and multiple timed exposures to X-ray film. Blots were stripped for reprobing using ReBlot (Chemicon). A representative Western blot probed with an anti-phospho-MAPK antibody and then stripped and reprobed with anti-MAPK antibody is shown (Figure 2.4D). Levels of phospho-MAPK and total MAPK were quantified using the densitometric function of the FluorChem imager (Alpha Innotech). After subtraction of in-lane background, levels of phospho-MAPK and total MAPK were normalized relative to control K33 wild type samples (+/+) run in parallel on each gel (Figure 2.4D). The ratio of phospho-MAPK to

MAPK was determined and the results of 4 to 6 independent experiments are graphed (Figure 2.4E). Expression of full length hNF1 was confirmed by Western blot using a rabbit polyclonal antibody *sc-68* (Santa Cruz) directed against the carboxy terminal domain of human NF1 (data not shown).

Body size measurement

The normal hNF1 gene has been shown to partially rescue AC-dependent small body size defects when expressed in the *Nf1* mutant background, using the global Gal4 drivers *armadillo*-Gal4 and *e22c*-Gal4 (Tong et al., 2002). In order to improve the statistical power of our body size analysis, we separated males and females for pupal size measurements in this study, since the large difference in body size between the sexes may mask the effects of the transgenes. Body size was assayed by measuring the length of late stage 10 pupae (eye pigments visible) (Bainbridge and Bownes, 1981) with a digital micrometer (Mitutoyo). Pupae were placed into a 96 well plate and their sex determined after eclosion of adults. At least 50 pupae of each sex were measured and statistical significance was assessed using a Paired Student's T-test.

Results

NF1 and Ras directly activate AC

Our first indication that Ras may directly activate AC was shown by incubation of human H-Ras with *Drosophila* head membrane extracts to produce a dose- and time-dependent increase in AC activity, as measured by increases in cAMP levels (Figure 1A). AC activity was also stimulated by human K-Ras (Figure 2.1B), but not Rab3a (Figure 2.1C), suggesting activation is specific to the Ras family of small GTPases, and not due to depletion of GTP or other factors. Second, this stimulation was shown to be NF1-dependent, since it was eliminated in *Nf1* homozygous null mutant flies, $Nf1^{P1}$ and $Nf1^{P2}$ (Figure 2.1D), that do not express any detectable NF1 protein (The et al., 1997). Furthermore, acute expression of a wild type *Nf1* transgene in the mutant background, controlled by a heat shock promoter (hsNF1;$Nf1^{P2}$), was able to fully restore the H-Ras-stimulated AC activation to wild type levels (Figure 2.1E). The acute nature of the response to NF1 indicates this is not a developmental effect, and that NF1 is a critical component of the Ras-stimulated AC activity. To further define the role of NF1 in Ras-stimulated AC activity we examined the effect of a purified GST-fusion protein containing an NF1-GRD fragment that retains GAP activity (Kim and Tamanoi, 1998). Significant increases in AC activity, measured by increased cAMP levels, were shown in wild type extracts treated with NF1-GRD fusion protein in the absence of H-Ras (Figure 2.1E). This effect is specific to the GAP activity of the NF1-GRD fragment since it is abolished in two NF1-GRD mutants (R1391S; K1419Q; Figure 2.1E) with reduced GAP activity, found in NF1 patients (Upadhyaya et al., 1987; Gutmann et al., 1993; Kim and

Tamanoi, 1998). The NF1-GRD fragment was also unable to stimulate AC activity above control levels in Ras^{e2F}/+ heterozygotes (Figure 2.1F), which have an inactivating mutation in the Switch I region of Ras (Simon et al., 1991) that normally activates Ras and interacts with downstream effectors. This suggests that levels of active Ras in these heterozygous flies are insufficient to stimulate AC activity. Thus, both Ras and NF1 are absolutely required for stimulation of AC, indicating that NF1 is surprisingly both a negative regulator of Ras, and plays an essential role in AC activation.

Growth factors stimulate the novel NF1/Ras-dependent AC pathway

To evaluate the functional significance of this novel pathway, we developed an assay to examine effects of neurotransmitters and growth factors on Ras stimulation of AC activity *in vivo.* Significant stimulation of AC activity was observed in wild type larval brains treated with Epidermal Growth Factor (EGF) or Transforming Growth Factor a (TGFa; Figure 2.2A and 2.2B). Stimulation of AC activity was abolished in *Drosophila* EGF receptor mutants (Figure 2.2A and 2.2B), including the *Egfrt1* hypomorphic mutant and the *Df(2R)Egfr18*/+ deficiency heterozygote (Clifford and Schupbach, 1994), demonstrating that these growth factors are acting directly on the *Drosophila* EGF receptor to stimulate AC activity. The stimulation of AC activity by growth factors is also abolished in both *Nf1* homozygous null mutants and in Ras^{e1B}/+ and Ras^{e2F}/+ heterozygotes (Figure 2.2A and 2.2B). The Ras^{e1B} mutation affects the Switch II activator/effector domain of Ras (Simon et al., 1991) that contacts R1391S of NF1. Again, this demonstrates a requirement for both Ras and NF1 in the stimulation of AC activity.

To ensure there is no crosstalk between EGF receptors and Gas, we assayed growth factor stimulation of AC in *Gsa*^{B19} hypomorphic mutants (Wolfgang et al., 2001). Normal levels of stimulation of AC activity by both EGF and TGFa growth factors were seen in larval brains of *Gsa*^{B19} mutants (Figure 2.2C and 2.2D), consistent with the fact that the *Drosophila* EGF receptor does not contain the juxtamembrane domain that facilitates crosstalk in vertebrate EGF receptors (Bogdan and Klambt, 2001). Stimulation by GTP?S is very low in the *Gsa*^{B19} mutants (Figure 2.2C and 2.2D), indicating that Gas is indeed the major stimulatory G-protein in larval brains. Control treatment of larval brains with insulin did not stimulate AC activity (Figure 2.2E). Thus stimulation of AC by both EGF and TGFa growth factors requires the EGF receptor, Ras and NF1, but does not involve Gas. Since the identified ligands for the *Drosophila* EGF receptor are members of the TGFa family (Bogdan and Klambt, 2001), stimulation of the Ras/NF1-dependent AC pathway by TGFa suggests that EGF receptor signaling activates this pathway in flies using endogenous ligands.

Neurotransmitters stimulate two additional AC pathways

We next examined the effects of neurotransmitters and neuromodulators that are ligands for G-protein coupled receptors. Stimulation of AC by the neuropeptide Phe-Met-Arg-Phe-amide (FMRFamide), and by the neurotransmitter dopamine was not affected in *Nf1* null mutants or *Ras*/+ heterozygotes, however it was abolished in *Gsa*^{B19} mutants that disturb the classical G-protein signaling pathway (Figure 2.3A and 2.3B). Thus, alterations in NF1 or Ras that disrupt growth factor dependent stimulation of AC activity (see Figure 2.2A and 2.2B), do not affect classical G-protein dependent stimulation of

AC. In contrast, stimulation of AC by the neurotransmitters serotonin and histamine was disrupted in both *Nf1* null mutants and $Gs\alpha^{B19}$ mutants but not in *Ras*/+ heterozygotes (Figure 2.3C and 2.3D), demonstrating an NF1/Gas-dependent pathway for stimulation of AC activity that does not require Ras. A number of other neurotransmitters and neuromodulators had no effect on AC activity, including the neuropeptide pituitary AC activating polypeptide neuropeptide (PACAP38) (data not shown), suggesting there are no receptors for these ligands in the larval brain.

Human NF1 mutations affect MAPK activity in Nf1 mutant flies

To address the possibility that NF1-dependent activation of AC versus downregulation of Ras activity is responsible for the variety of phenotypes seen in NF1 patients and animal models, we examined clinically relevant missense mutations from NFI patients that are scattered throughout the length of the hNF1 protein (Fahsold et al., 2000; Messiaen et al., 2000; Serra et al., 2001), as well as deletions of hNF1. We expressed hNF1 containing four different missense mutations and five partial deletions (Figure 2.4A) in the *Drosophila Nf1* mutant background, and assayed their effect on growth factor and neurotransmitter stimulated AC activity. The mutations chosen for this study occur in multiple patients and affect conserved amino acids. When assayed in yeast, the GRD domain mutants R1391S and K1423E mutants drastically reduce GAP activity (Upadhyaya et al., 1987; Gutmann et al., 1993; Poullet et al., 1994), while the R1276P mutant completely abolishes GAP activity (Klose et al., 1998). Transcription of UAS-hNF1 transgenes in flies was controlled using Gal4 drivers (Brand and Perrimon, 1993), including one that is expressed globally (*e22c*-Gal4) (Duffy et al., 1998) and a

nervous system specific driver (*elav*-Gal4) (Lin and Goodman, 1994). Assays were performed on flies that carry one copy of the normal or mutant UAS-*hNF1* transgene and one copy of the Gal4 driver in the *Nf1* mutant background (Figure 2.4B and 2.4C), showing that hNF1 functions in *Drosophila*, and defining two separate domains that mediate activation of distinct AC pathways.

Phosphorylation of mitogen activated protein kinase (MAPK) is elevated in *Drosophila Nf1* mutants due to increased Ras activity (Williams et al., 2001). We first showed that normal hNF1 is able to inhibit Ras by showing that phospho-MAPK is reduced to wild type levels when hNF1 (two independent lines; hNF1a and hNF1b) is expressed in *Nf1* mutant flies under control of the *e22c*-Gal4 global driver (Figure 2.4D and 2.4E). As expected, mutant hNF1s with defective RasGAP activity (R1276P, R1391S, K1423E) or lacking the GRD (?GRD2, Cterm) cannot reduce phospho-MAPK levels (Figure 2.4E). The GRD fragments alone (GRD1, GRD2) were able to restore phospho-MAPK to wild type levels, and the L847P mutation did not affect the RasGAP activity of full length hNF1 (Figure 2.4E).

Human NF1 mutations affect AC activity in Nf1 mutant flies

We then demonstrated that the RasGAP activity of hNF1 was required for growth factor stimulated AC activity, by expressing the mutant hNF1s or deletions under control of the nervous system specific *elav*-Gal4 driver for larval brain assays. Mutant hNF1s with defective RasGAP activity, or lacking the GRD, did not respond to EGF stimulation (Figure 2.5A and 2.5C). However, the L847P mutant and the GRD fragments responded

normally to EGF (Figure 2.5B and 2.5C), indicating that the RasGAP activity of the GRD is indeed required for growth factor stimulated NF1/Ras-dependent AC activity.

We next examined serotonin and histamine stimulated AC activity to see whether RasGAP activity of NF1 was required for the NF1/Gas-dependent AC pathway. Stimulation of AC was normal for mutant hNF1s with or without RasGAP activity (Figure 2.5A and 2.5B), indicating that NF1/Gas-dependent AC activity does not require RasGAP activity. Consistent with this, the GRD fragments alone were not sufficient to restore NF1/Gas-dependent AC activity (Figure 2.5C). We then asked whether any other region of NF1 is required for NF1/Gas-dependent AC activity. Constructs lacking the GRD (? GRD2, Cterm) were able to restore neurotransmitter stimulated AC activity (Figure 2.5C), demonstrating that sequences in the C-terminal region, common to ? GRD2 and Cterm (see Figure 2.4A), are essential for NF1/Gasdependent AC activity.

Human NF1 mutations also affect body size in Nf1 mutant flies.

To further confirm that RasGAP activity is not required for NF1/Gasdependent AC activity, we examined the effect of expressing the hNF1 mutants and deletions on the small body size phenotype previously seen in adult flies (The et al., 1997). This phenotype can be rescued by supplying cAMP, but not by decreasing Ras activity (The et al., 1997). We first confirmed that normal hNF1 is able to rescue the small body size of males and females using both *elav*-Gal4 and *e22c*-Gal4 drivers (Figure 2.5D). All four clinically relevant missense mutants, including those with defective RasGAP activity, are able to rescue body size just as effectively as normal hNF1 (Figure 2.5E) and neither of the GRD fragments was able to rescue body size (Figure 2.5F). Thus, the RasGAP

activity of hNF1 is not required for rescue of body size. Both the GRD deletion and C-terminal fragment were effective at rescuing body size, but not the N-terminal fragment (Figure 2.5F). The L847P mutation in the region upstream of the GRD can still rescue MAPK activity (Figure 2.4D and 2.4E), AC activity (Figure 5B) and small body size (Figure 2.5E). This mutation may affect other aspects of NF1 function such as regulation or localization, rather than activity.

Conclusions

Three separate pathways for AC activation defined in this study are depicted in Figure 2.6. First, a novel pathway for AC activation, downstream of growth factor stimulation of EGF receptors that requires both Ras and NF1, but not Gas. Second, an NF1/Gas-dependent AC pathway operating through the Rut-AC and stimulated by serotonin and histamine, as observed here in the larval brain. The Rut-AC pathway may also be stimulated by PACAP38 at the larval neuromuscular junction and in adult heads as shown in previous studies (Guo et al., 1997; Tong et al., 2002). Third, a classical heterotrimeric G-protein stimulated AC pathway (Sunahara and Taussig, 2002) operating through Gas. The ACs activated by NF1/Ras (AC-X) or Gas (AC-Y) have not yet been identified.

This study shows for the first time that Ras can stimulate AC in an NF1-dependent manner in higher organisms. The functionality of human NF1 in the fly system, and the high degree of identity between human and fly NF1 (60%, The *et al.*, 1997), suggests that similar pathways for AC activation may also operate in mammals. Previous studies failed to detect stimulation of AC by Ras in cultured vertebrate cell lines

and in *Xenopus* oocytes (Birchmeier et al., 1985), however these cell types may not contain sufficient NF1 to support NF1/Ras-dependent AC activation.

Our experiments with human NF1 mutants show that the GRD domain and the RasGAP activity of NF1 are both necessary and sufficient for growth factor stimulated NF1/Ras-dependent AC activity. We also conclude that C-terminal residues downstream of the GRD are critical for both body size regulation and neurotransmitter stimulated NF1/Gas-dependent AC activity, thus defining for the first time a region outside the GRD that contributes to this pathway.

Thus NF1, while being a negative regulator of Ras, is also actively involved in stimulation of AC activity. Moreover, it regulates AC activity through at least two different mechanisms, one of which depends on the RasGAP activity of NF1. It is tempting to speculate that discrepancies between the learning and memory phenotypes of mice versus flies, where spatial learning in $Nf1^{+/-}$ mice can be rescued by decreasing Ras activity (Costa et al., 2002), while olfactory learning in *Nf1* null flies is cAMP – dependent (Guo et al., 2000), may depend on the activation of separate AC pathways. The multifunctional nature of the NF1 protein illuminates its importance in nervous system development, tumor formation and behavioral plasticity, and may also explain the wide range of clinical manifestations in Neurofibromatosis Type 1.

Figures and Legends

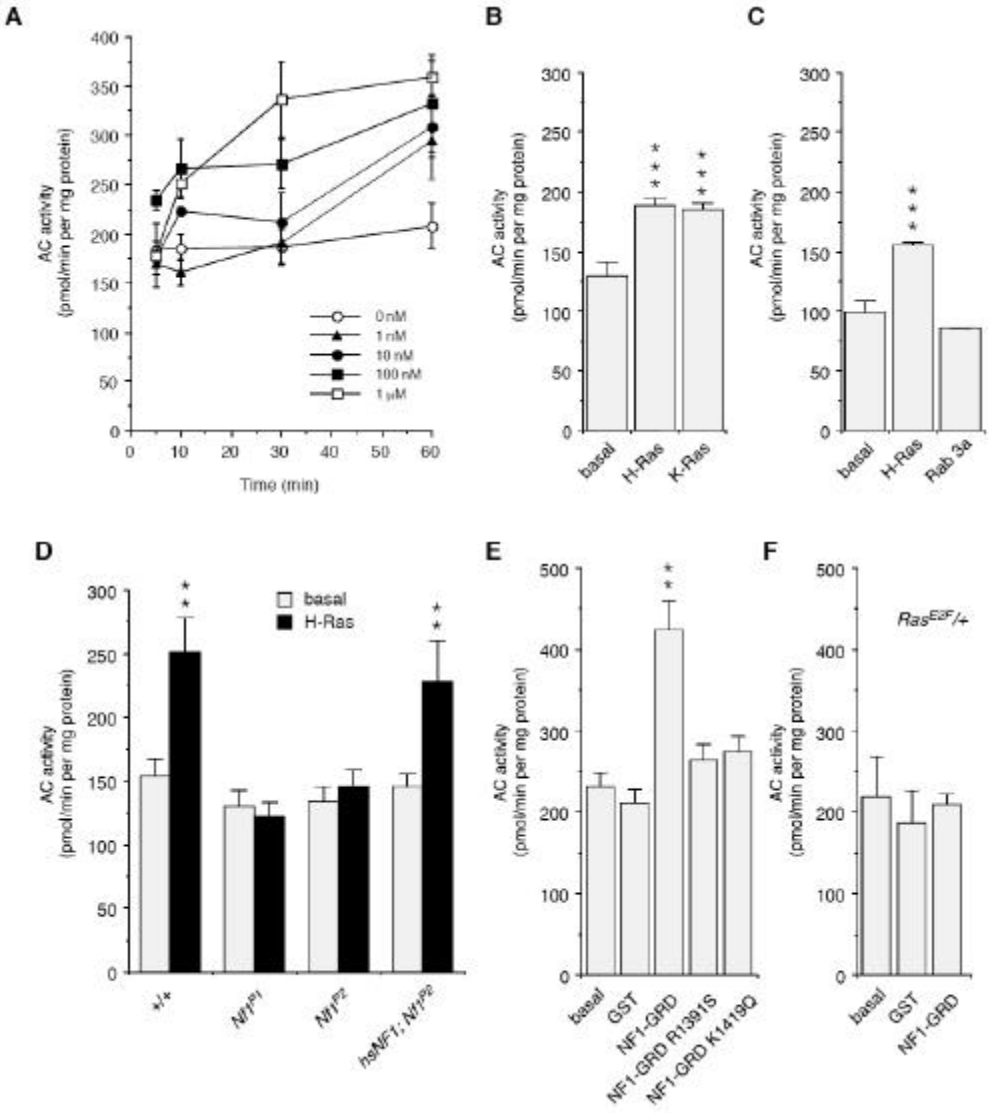

Figure 2.1. NF1 and Ras directly activate AC. (**A**) Significant increases in AC activation were observed after 10min to 60 min incubation with human H-Ras at different concentrations ($p<0.05$; n=3). (**B**) Both H-Ras and K-Ras stimulate AC activity (1µM; t=30 min; n=4). (**C**) Rab3a does not stimulate AC activity (1µM; t=30 min; n=4). (**D**) H-Ras stimulation of AC was eliminated in *Nf1*^{P1} and *Nf1*^{P2} mutant flies, and restored by heat shock induced expression of a fly *Nf1* transgene in *hsNf1;Nf1*^{P2} flies (1µM; t=60 min; n=8,8,8,3). (**E**) A human NF1-GRD-GST fusion protein is able to stimulate AC, in

the absence of H-Ras. There was no stimulation by GST alone, or by NF1-GRD-GST missense mutants, R1391S and K1419Q, that reduce RasGAP activity (1µM; t=30 min; n=4). (**F**) Stimulation by human NF1-GRD-GST was abolished in Ras^{e2F}/+ heterozygotes (1µM; t=30 min; n=2). (A-F) Values are mean ± s.e.m. (**p<0.05, ***p<0.01).

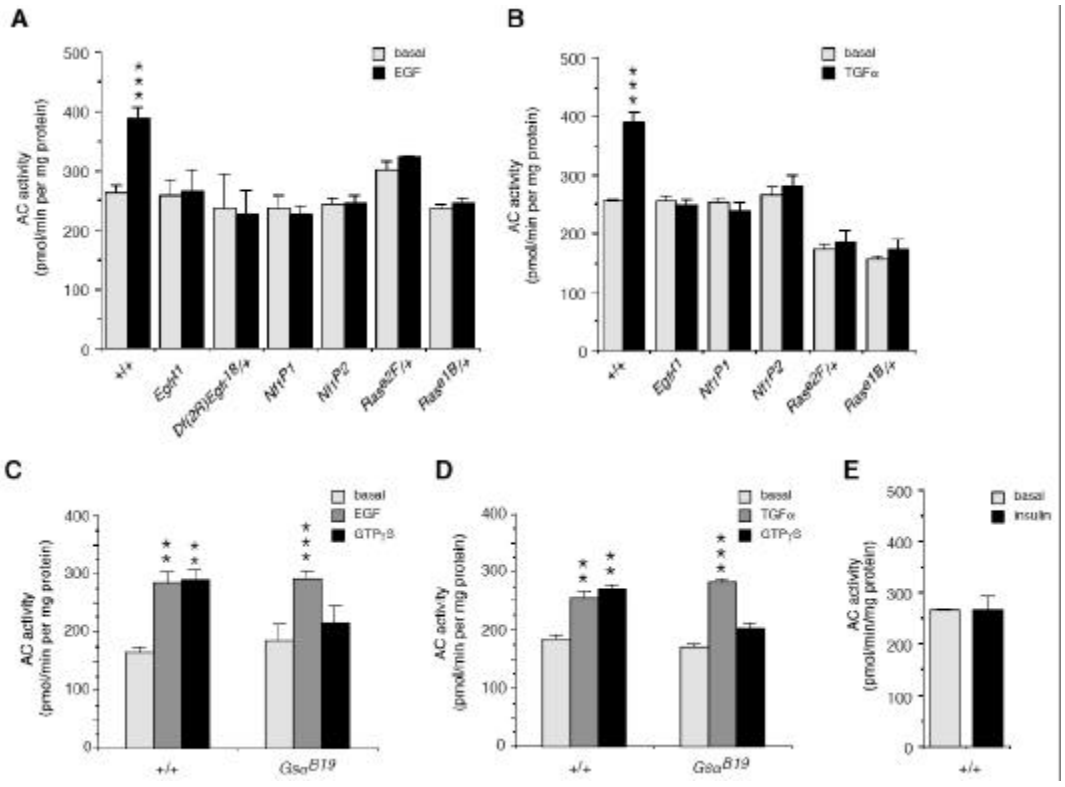

Figure 2.2. Growth factors stimulate the novel NF1/Ras-dependent AC pathway. (**A**) AC activity was significantly increased by treatment of larval brains with 2µM EGF (n=18). This stimulation was abolished in EGF receptor mutants, *Egfr*^{t1}, and heterozygotes, *Df(2R)Egfr18*/CyO; in *Nf1* null mutants, *Nf1*^{P1} and *Nf1*^{P2}; and in *Ras* heterozygotes, *Ras*^{e2F}*/TM3* and *Ras*^{e1B}*/TM3*. (n=4). (**B**) Stimulation of AC by 2µM TGFa was similarly abolished in the *Egfr*^{t1} mutant, *Nf1* mutants and *Ras* heterozygotes. (n=4). Stimulation of AC by 2µM EGF (**C**) or TGFa (**D**) is not affected in a hypomorphic *G*as mutant, *Gs*a^{B19}, whereas stimulation by 20µM GTP?S is perturbed (n=3). (**E**) There was no stimulation of AC by 2µM insulin (n=3). (A-E) Values are mean ± s.e.m. (**p<0.01, ***p<0.005).

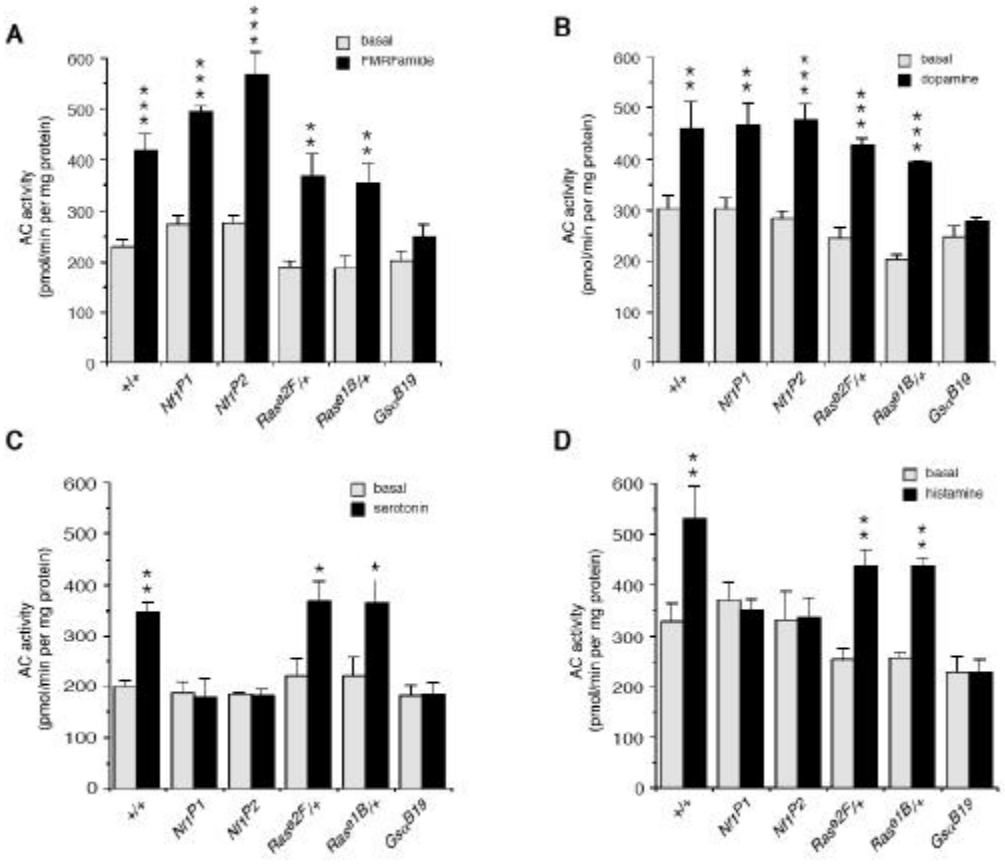

Figure 2.3. Neurotransmitters and neuromodulators stimulate two additional AC pathways. FMRFamide and dopamine stimulate Gas-dependent AC: activation of AC by 200nM FMRFamide (**A**) and dopamine (**B**) is disrupted in *G*as mutants, but not in *Nf1* mutants or *Ras* heterozygotes. (n=3-4). Serotonin and histamine however, stimulate NF1/Gas-dependent AC: activation of AC by 200nM serotonin (**C**) and histamine (**D**) is disrupted in *G*as and *Nf1* mutants but not in *Ras* heterozygotes (n=4). (A-D) Values are mean ± s.e.m. (**p*<0.05; ***p*<0.01, ****p*<0.005).

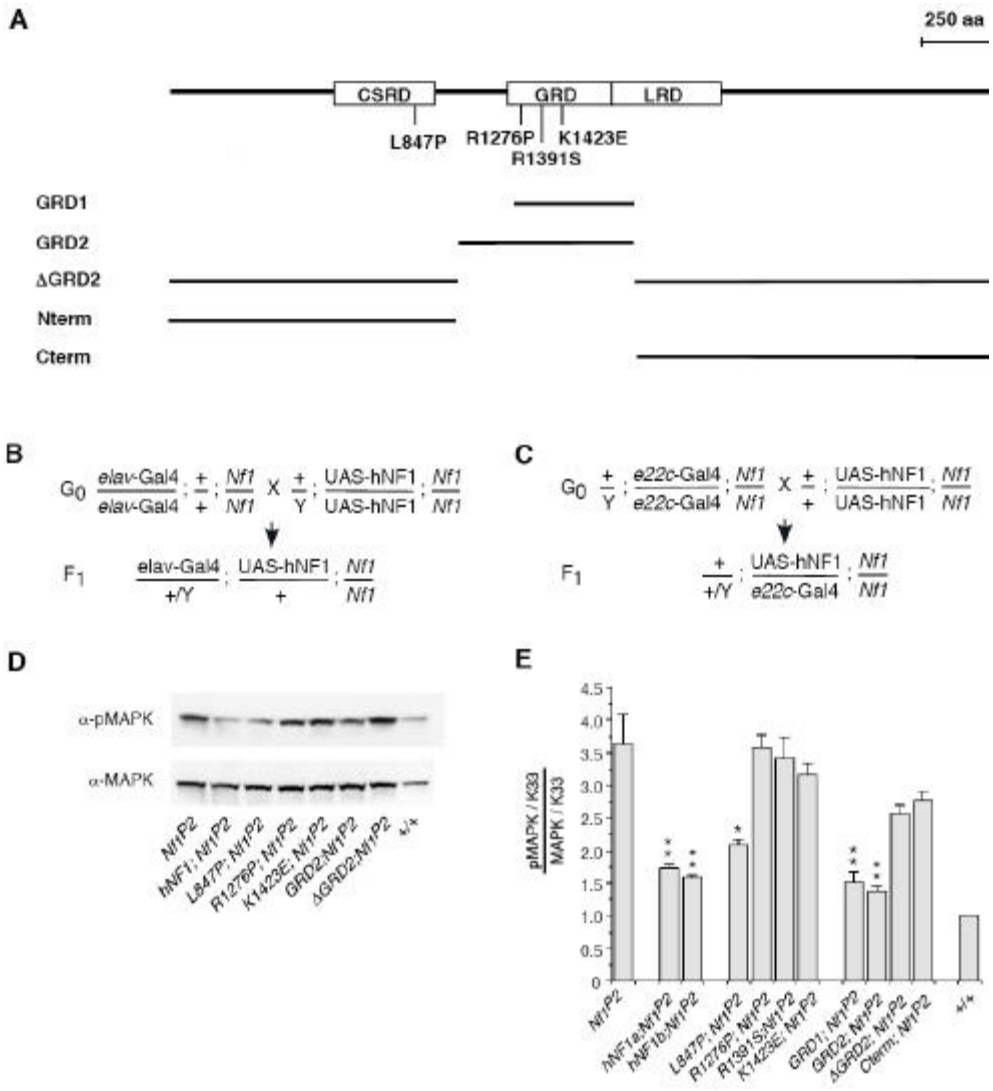

Figure 2.4. Missense mutations and deletions of human NF1 modulate *Drosophila* MAPK activity. (**A**) Position of four hNF1 missense mutations, and size of five hNF1 deletion constructs, that have been expressed and analyzed in *Drosophila Nf1* null mutants (CSRD, CysSer rich domain; GRD, GAP related domain; LRD, Leu rich domain). Crosses required to generate F1 progeny expressing UAS-hNF1 mutants or deletion constructs under control of the nervous system specific *elav*-Gal4 driver (**B**) on the X chromosome or the globally expressing *e22c*-Gal4 driver (**C**) on the second chromosome. (**D**) Representative Western blot of head extracts from flies expressing

normal and mutant hNF1s and deletions, probed with anti-phospho-MAPK then stripped and reprobed with anti-MAPK antibodies. (**E**) Levels of phospho-MAPK versus total MAPK levels in flies expressing hNF1 mutants and deletions, normalized to K33 wild type (+/+) control values (see Methods). (D, E) Expression is under control of the *e22c*-Gal4 driver. (E) Values are mean ± s.e.m. (n=4-6, **p*<0.05, ***p*<0.01).

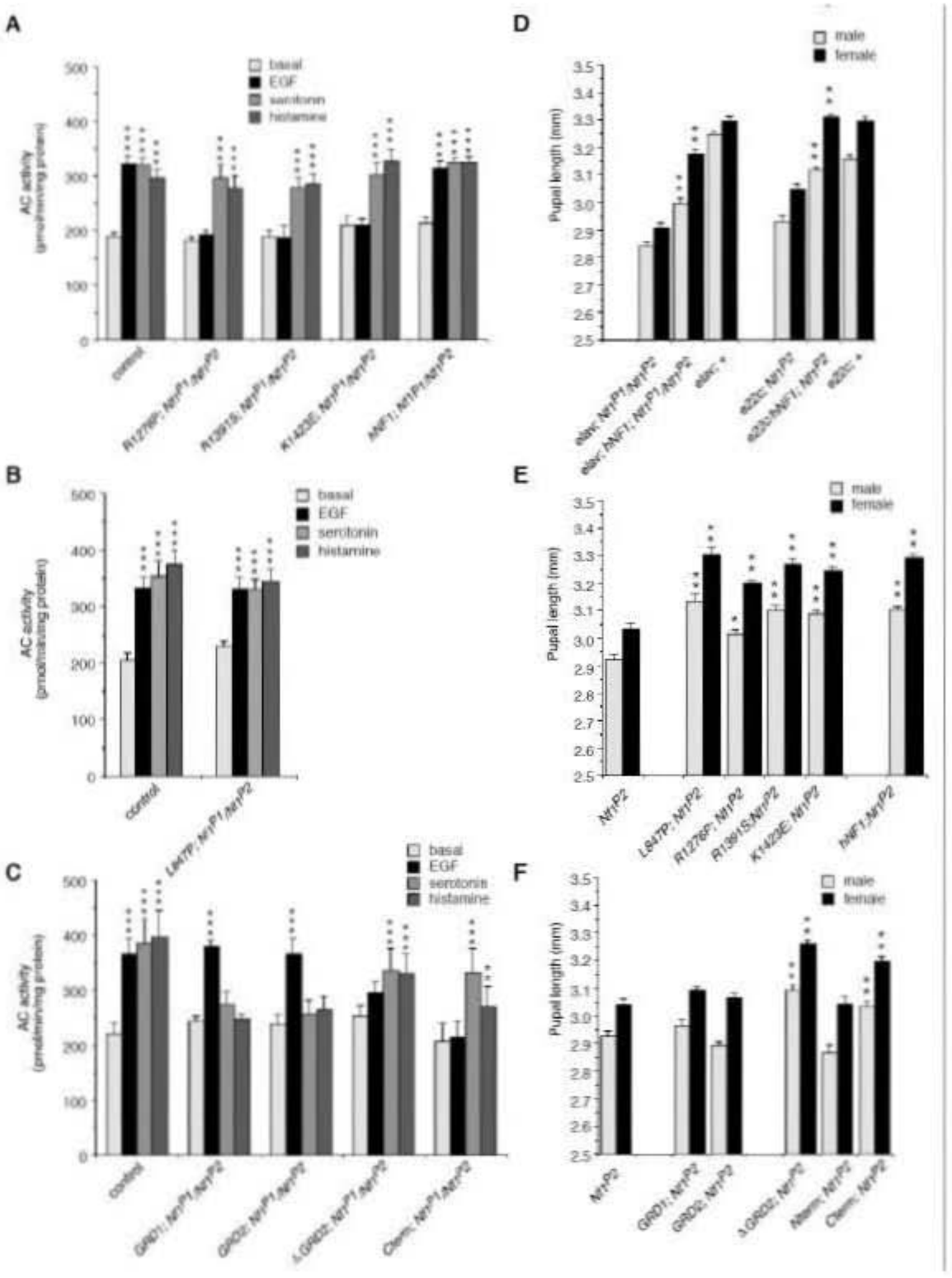

Figure 2.5. Separate domains of human NF1 mediate activation of different AC pathways. (**A**) EGF does not stimulate AC activity in flies expressing RasGAP-defective

mutant hNF1s (R1276P, R1391S, K1423E), compared to K33 (control) flies or flies expressing normal hNF1, however serotonin and histamine stimulated AC activity is fully restored. (**B**) Stimulation of AC activity by EGF, serotonin and histamine is restored in flies expressing the L847P hNF1 mutation. (**C**) EGF stimulated AC activity is restored in lines expressing GRD fragments (GRD1; GRD2), but serotonin and histamine stimulated AC activity is absent. Conversely, serotonin and histamine, but not EGF, stimulate AC activity in flies expressing a GRD deletion (?GRD2) or a C-terminal fragment (Cterm) alone. (**D**) Pupal length is increased in flies expressing normal hNF1 using *elav*-Gal4 or *e22c*-Gal4 drivers compared to *Nf1* mutant and K33 wild type (+) controls expressing driver alone. (**E**) Pupal length is also increased in flies expressing all four missense mutations (L847P, R1276P, R1391S or K1423E) compared to *Nf1* mutants expressing driver alone. (**F**) Pupal length is not increased in flies expressing GRD fragments (GRD1; GRD2) or an N-terminal fragment (Nterm), however it is increased in flies expressing a GRD deletion (?GRD2) or a C-terminal fragment (Cterm). (A-C) Expression is under control of the *elav*-Gal4 driver, values are mean ± s.e.m. (n=4, $^{**}p<0.01$, $^{***}p<0.001$). (D-F) Expression is under control of the *e22c*-Gal4 driver except where otherwise indicated, values are mean ± s.e.m. (n>50, $^{*}p<0.01$, $^{**}p<0.001$).

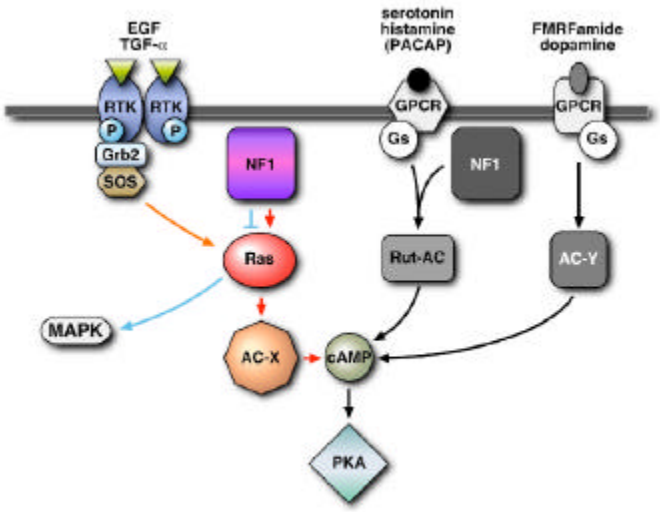

Figure 2.6. AC can be activated by at least three distinct pathways: First, a novel NF1/Ras-dependent pathway stimulated by growth factors such as EGF and TGFa that activates an unidentified AC (AC-X), and does not involve Ga_s; Second, an NF1/Gas-dependent pathway, acting through Rut-AC, stimulated by serotonin and histamine, and possibly PACAP38 (see discussion), that does not require Ras; Third, a classical NF1-independent pathway, involving Gas but not NF1 or Ras, stimulated by FMRFamide and dopamine that activates an unidentified AC (AC-Y).

Chapter 3

Distinct functional domains of NF1 regulate immediate versus long-term memory formation

Introduction

Neurofibromatosis type 1 (NF1) is one of the most common neurogenetic disorders with a prevalence of 1 in 3,500 (Stephens et al., 1987). NF1 is predominantly identified by neurofibromas, benign tumors of the peripheral nervous system, as well as malignant peripheral nerve sheath tumors (Stephens et al., 1987). Learning disabilities are commonly observed in 30 – 60% of afflicted children (North, 2000). The NF1 protein has a central GRD that accelerates inactivation of Ras (Ballester et al., 1990). Although no direct correlation has been established between specific mutations and phenotypes, a missense mutation that abolishes the RasGAP function of NF1 was found in human patients with multiple symptoms including learning disability and mental retardation, suggesting that loss of NF1's GAP function may underlie cognitive dysfunction (Klose et al., 1998). In addition to regulating Ras activity, NF1 has been shown to regulate cAMP levels in both *Drosophila* and mouse models (Guo et al., 1997; The et al., 1997; Guo et al., 2000; Tong et al., 2002; Dasgupta et al., 2003; Hannan et al., 2006). Interestingly, while no specific region of the protein has been associated with any NF1 disease phenotypes (Fahsold et al., 2000; Messiaen et al., 2000; Mattocks et al., 2004), our recent report demonstrated that the GRD is sufficient for mediating Ras-dependent regulation of

signal transduction pathways, while the C-terminal region is required for G-protein-dependent AC activation (Hannan et al., 2006).

In *Drosophila*, *Nf1* null mutants exhibit compromised learning, or immediate memory, in the Pavlovian olfactory conditioning paradigm. This behavioral phenotype is attributed to disruption in the *rutabaga*-encoded adenylyl cyclase (Rut-AC) pathway (Guo et al., 2000). In the Morris water maze, $Nf1^{+/-}$ mice exhibit a spatial learning defect that is due to increased Ras activity (Costa et al., 2001; Costa et al., 2002; Li et al., 2005). Such discrepancy is likely caused by the vast temporal difference between the two training paradigms. It only takes minutes to train and test flies (Tully and Quinn, 1985), while for mice it takes two training sessions per day and six days to complete the training (Morris, 1984). In addition, injection of a protein synthesis inhibitor to the lateral ventricle of the mice significantly reduces their performance in the water maze (Meiri and Rosenblum, 1998). This suggests that the behavioral phenotype exhibited by the $Nf1^{+/-}$ mice may actually be a form of long-lasting memory that requires repetitive training sessions and is dependent on protein synthesis. In this report we demonstrated that *Nf1* mutant flies also exhibit abolished long-term memory (LTM). Expressing the highly-conserved human NF1 (hNF1) protein in *Nf1* null mutant flies, including variants containing clinically relevant missense mutations as well as large deletions, allowed us to identify the structural and/or functional requisites for these behaviors. Our analyses revealed that the GRD is required for LTM, while sequences in the G-terminal region regulate immediate memory.

Materials and Methods

Fly Stocks

Flies were raised at room temperature (22°C to 24°C) on standard cornmeal medium. The *Nf1* mutants $Nf1^{P1}$ and $Nf1^{P2}$, together with the parental K33 line were obtained from A. Bernards (Massachusetts General Hospital, Boston, MA). The Gal4 driver line *elav*-Gal4;$Nf1^{P1}$ (Williams et al., 2001) was obtained from A. Sehgal (University of Pennsylvania, Philadelphia, PA). Construction of UAS-*hNF1* transgenes and generation of transgenic fly lines carrying normal human NF1 (hNF1), and human NF1 point mutants and deletion mutants was described previously (Hannan et al., 2006). Transcription of UAS-*hNF1* transgenes in flies was controlled using a nervous system specific X chromosome line, *elav*-Gal4 (see above). The crossing schemes designed to generate progeny carrying one copy of the transgene and one copy of the Gal4 driver in the *Nf1* mutant background are outlined (Fig. 3.2A).

Transgenic flies

All MB-Gal4;$Nf1^{P1}$ double lines used for anatomical analysis were generated by performing crosses using double balancers; *w/Y;CyO/Sp;TM3Ser/Sb* for crossing c747 and 201Y, and *FM7/Y;+/+;TM3Ser/Sb* for crossing c107 and Feb170 into $Nf1^{P1}$. Originally the introduction of OK107-*Gal4* into NF1 mutant background was mistakenly done using *w/Y;CyO/Sp;TM3Ser/Sb*, the X and 3rd chromosome double balancer flies. However, it was later found that OK107 is on the 4th chromosome. The correct genotype was then generated using the original stock of $Nf1^{P1}$;OK107-*Gal4*, which consists of

Nf1^{P1};OK107-*Gal4*, *Nf1*^{P1};OK107-*Gal4/+*, and *Nf1*^{P1} flies. Flies were selected from just-eclosed, at which point flies that contained OK107 insertion displayed darker eye color than those without the insertion. These flies were used for single pair mating. After egg-laying, these parents were used to perform single fly PCR to determine whether they had one or two copies of OK107. Progenies from eight pair of mating parents that both contained two copies of OK107 insertion were mixed to establish *Nf1*^{P1};OK107-*Gal4* flies. Three generations of each line of flies were confirmed by PCR for their homozygosity of the Gal4 insertion as well as NF1 null mutation.

One-Cycle Training

Flies were trained and tested with the classical (Pavlovian) conditioning protocol of Tully and Quinn (Tully and Quinn, 1985). Briefly, around 100 flies were trapped in a training chamber that is lined with an electrifiable copper grid. Two odors were then delivered to the flies sequentially through air current, with the first odor (CS+) delivery paired with electric shock (US) but no shock was received with the delivery of the second odor (CS-). Each odor was delivered in an interval of one minute, with a 45s of fresh air after each odor's delivery. This procedure constituted one training cycle. To test for learning, flies were transferred to a choice point where the two odors were presented to them by two converging air currents. Flies were given 120s to choose between the two arms of the T-maze, from which odors were delivered. At the end of this period flies were trapped inside individual arms, anesthetized, and counted. To eliminate odor bias, the concentrations of the two odors, which are aversive to untrained flies, were calibrated such that untrained flies distributed themselves 50:50 in the T-maze.

Performance Index

Two groups of flies always were trained and tested in one complete experiment; for one group methylcyclohexanol (MCH) was CS+ and benzaldehyde (BA) was CS-, while for the second group BA was CS+ and MCH was CS-. The "probability correct" of each reciprocal group was calculated as the number of flies avoiding CS+ minus those avoiding CS- divided by the total number of flies in the T-maze arms. The resulting two probability corrects are then averaged and normalized to become one performance index (PI), which can range from 0 (a 50:50 distribution reflecting no learning) to 100 (all flies learned to avoid shock-paired odor).

Long-Term Memory

This training paradigm is in accordance to previous report (Yin et al., 1994). Extended training procedures were performed with an automated training system in which fresh air was bubbled at 750 ml/min through one of the three channels in a "bubbler manifold" (custom built by General Valve Corp.). One channel was for "fresh" air, a second was for BA, and the third was for MCH. Each channel contained two vials, one with 10 ml of distilled water and the other with either pure heavy mineral oil (Fisher) alone or with a particular dilution of BA or MCH (Fluka). Switching of bubbler channels and of a relay to deliver electric shock pulses to the flies was computer controlled (system custom designed by Island Motion Inc.). During massed training flies received ten training cycles (as above) delivered one right after the other. For spaced training flies received ten training cycles with a 15 min rest interval between each cycle. To assay

memory retention, flies were tapped gently from the training chamber into their usual food vials and stored at 18^{O}C for the duration of 24 hrs. Flies were then transferred to the choice point of the T-maze where the usual 2 min test trial was performed.

Cycloheximide Feeding and Heat-Shock Treatment

The cycloheximide (CXM) feeding regimen was as reported (Yin et al., 1994). Briefly, groups of about 100 flies were placed in feeding tubes that contained one Whatmann filter paper strip soaked with 125µL of solution mixture. Solution mixture contained 35mM (CXM+) in 4% sucrose or 4% sucrose (CXM-), and was fed to the flies at 25^{O}C for 12 – 15 hrs before training and again at 18^{O}C during the 24-hour retention period. Flies were allowed to clean themselves in standard food vials 30 min before training.

The heat-shock protocol is similar to that previous reported (Guo et al., 2000). Heterozygous transgenic flies (*hsNF1/+;Nf1*^{P2}) were used to avoid potential recessive effects of the insertion on behavior. Flies were raised at 18^{o}C and moved to 30^{o}C for 30 min. After a 2-hour resting period at room temperature (20^{o}C – 24^{o}C), flies were subjected to spaced training and tested 24 hours later at 25^{o}C.

Olfactory Acuity and Shock Reactivity

Odor-avoidance responses to BA, or MCH were quantified with the method of Boynton and Tully (Boynton and Tully, 1992). Briefly, groups of about 100 untrained flies received a 2-min test trial in the T-maze. Different groups were given a choice

between either BA or MCH versus fresh room air. PIs were calculated as above. Shock-avoidance responses to 60V were quantified with the method of Dura *et al*. (Dura et al., 1993). Briefly, groups of about 100 untrained flies received a 2-min test trial in the T-maze. Each arm of the T-maze contained an electric shock grid, and different groups of flies were given a choice between shock versus no shock. PIs were calculated as above. Both olfactory acuity and shock reactivity were normal for all genotypes (Table 3.1).

Results

Expression of human NF1 transgene in Nf1 null mutants can rescue learning and LTM defects

To dissect the long-term memory phenotype of *Nf1* mutants we subjected flies to massed (10 cycles with no rest interval) or spaced (10 training cycles with 15-minute rest intervals) training protocols before we tested for their memory 24 hours later (see Materials and Methods). At the time of testing, spaced-trained flies exhibit two memory components, anesthesia-resistant memory (ARM) and long-term memory (LTM). LTM is protein-synthesis dependent while ARM is not. On the other hand, flies that received massed training will only exhibit ARM (Tully et al., 1994; Dubnau and Tully, 1998). In our analyses, mutants that are defective in LTM but exhibit normal ARM performance will be categorized as LTM mutants. All flies in this study are able to detect odors and shock (see Table 3.1).

Two *Nf1* null mutants were used in this study, $Nf1^{P1}$ and $Nf1^{P2}$, neither of which showed any detectable NF1 protein expression and both are defective in olfactory associative learning (The et al., 1997; Guo et al., 2000). K33 flies, the parental line of the *Nf1* mutants, were used as a wild type control. We first confirmed the *Nf1* mutant learning phenotype by testing them immediately after one cycle of training (see Materials and Methods). Consistent with our previous report (Guo et al., 2000), these mutants exhibit significantly lower learning performance when compared to wild type control flies (Figure 3.1A). Both *Nf1* null mutants also display compromised 24-hour memory after spaced training compared to the parental line (Figure 3.1A), whilst they exhibit normal ARM, measured 24 hours after massed training (Figure 3.1B). This indicates that

NF1 is specifically affecting the LTM component of 24 hour memory, in addition to its effect on learning.

The *Drosophila* NF1 protein has 60% identity with the human NF1 ortholog (The et al., 1997), and previous experiments show that the human protein can function in place of the fly protein to rescue body size and stimulation of AC activity (Tong et al., 2002; Hannan et al., 2006). Amino acid residues that are normally conserved between the two species are found mutated in NF1 patient samples, suggesting their potential functional significance in the fly (Hannan et al., 2006). We hypothesized that the human protein would be able to rescue the behavioral phenotypes encountered in *Nf1* mutants. To examine whether hNF1 can rescue fly *Nf1* mutant behavioral phenotypes, we expressed the hNF1 protein in the null mutant background using the *elav-GAL4* driver, which has a pan-neuronal expression pattern (see Figure 3.2A for crossing scheme). The transgenic parental lines *elav;Nf1*^{P1} and *UAS-hNF1;Nf1*^{P2} were generated using an isogenic line *2202u*, which displays similar LTM performance to K33 (Figure 3.2D). The *2202u* line is used as the wild type control in Figure 3.2 and Figure 3.4. When compared to the parental control lines (*elav;Nf1*^{P1} and *UAS-hNF1;Nf1*^{P2}), the expression of hNF1 in the *hNF1;Nf1*$^{P1/P2}$ progeny (*elav/+/Y;UAS-hNF1/+;Nf1*$^{P1/P2}$) significantly rescued both learning and LTM to wild type level (Figure 3.2B) and also retained normal level of ARM (Figure 3.2C). Thus human NF1 is also conserved for behavioral function with the Drosophila ortholog. The rescue of LTM by hNF1 suggests that NF1 is essential for the formation of LTM, in addition to its established role in learning.

In order to rule out any developmental abnormalities that may contribute to the LTM defect observed in *Nf1* mutants, we used a heat shock promoter to induce

expression of the *Drosophila Nf1* gene in the $Nf1^{P2}$ mutant background by temperature shifting before training (See Materials and Methods). According to our previous study, this heat shock-induced expression was enough to rescue the learning phenotype (Guo et al., 2000). Acute expression of the *Nf1* gene before spaced training significantly rescued the LTM defect in the $Nf1^{P2}$ mutant background (Figure 3.2D). These results indicate that NF1 is required acutely for the formation of LTM.

The GRD region of NF1 is required for its function in LTM

To gain insights into the underlying mechanisms of the LTM phenotype, we examined various point mutations observed in NF1 patients that selectively disrupt NF1-regulated signal transduction pathways (Figure 3.3A). Two of the clinically identified hNF1 mutations, R1391S and K1423E exhibit greatly reduced affinity for Ras (Gutmann et al., 1993; Poullet et al., 1994), while R1276P has more than 8,000-fold reduced GAP activity compared to wild type NF1 protein (Klose et al., 1998). Flies expressing any of the three hNF1 point mutations (*elav/+/Y;UAS-R1276P/+;*$Nf1^{P1/P2}$; *elav/+/Y;UAS-R1391S/+;*$Nf1^{P1/P2}$; *elav/+/Y;UAS-K1423E/+;*$Nf1^{P1/P2}$) display normal learning (Figure 3.3B) and ARM (Figure 3.3D), but defective LTM performance (Figure 3.3B). This suggests that the GAP activity of NF1 as well as its interaction with the Ras protein is extremely important for NF1-dependent LTM.

To further evaluate the importance of the GRD for the NF1 behavioral phenotypes, we generated transgenic flies expressing hNF1 protein fragments of different sizes that contain the GRD, named GRD1 (*elav/+/Y;UAS-GRD1;*$Nf1^{P1/P2}$) and GRD2 (*elav/+/Y;UAS-GRD2;*$Nf1^{P1/P2}$), as well as an hNF1 protein that has a deletion of the

GRD, named GRDdel (*elav/+/Y;UAS-GRDdel;Nf1*$^{P1/P2}$; Fig. 3A). Expression of GRDdel rescues learning of *Nf1* mutant flies to wild type level, while both GRD1 and GRD2 flies (Figure 3.3C) show similar learning performance to *Nf1* null mutants (Figure 3.1A), suggesting that the GRD is not important for NF1-dependent learning. We also tested GRDdel, GRD1 and GRD2 flies for 24-hour memory after spaced training. LTM is rescued, although partially, in flies expressing GRD1 and GRD2 but not GRDdel (Figure 3.3C) while ARM is not affected by any of the fragments tested (Figure 3.3D). This verifies that the GRD fragment is indeed functional for behavioral rescue, and further illustrates that the GRD is an essential region for NF1-dependent LTM.

In order to verify that rescue of 24-hour memory after spaced training by hNF1, or the GRD fragment, is indeed rescue of the LTM defect, we fed flies with a protein synthesis inhibitor, cycloheximide (CXM), before and after spaced training (see Materials and Methods). As mentioned above, LTM is protein synthesis-dependent while ARM is not. Therefore, LTM should be sensitive to CXM treatment, as shown previously (Tully et al., 1994; Yin et al., 1994). 24-hour memory performance was compromised when wild type control flies, and flies expressing hNF1 (*hNF1;Nf1*$^{P1/P2}$) or GRD fragment alone (*GRD1;Nf1*$^{P1/P2}$), were subjected to CXM treatment (Figure 3.4). This indicates that NF1, and especially the GRD, is indeed required for protein synthesis-dependent memory, LTM.

The C-terminal region of NF1 is essential for learning

Since expression of the GRDdel fragment rescues learning as shown above (Figure 3.3C), we hypothesized that regions important for NF1-dependent learning lie

outside of the GRD. Two different truncated hNF1 transgenes were used to test this hypothesis; the Nterm (*elav/+/Y;UAS-Nterm;Nf1*$^{P1/P2}$) construct contains regions upstream of the GRD, while the Cterm (*elav/+/Y;UAS-Cterm;Nf1*$^{P1/P2}$) construct contains regions downstream of the GRD (Fig. 3A). Biochemical assays indicate that Cterm is functional for NF1-dependent neuropeptide and neurotransmitter stimulation of AC activity (Hannan et al., 2006). The Cterm fragment also rescues the cAMP-dependent *Nf1* mutant body size defect, while the N-terminal region and the GRD do not rescue body size (Hannan et al., 2006). Neither transgene was able to rescue the LTM defect in the null mutant background (Figure 3.5), consistent with the absence of the GRD region in these constructs. The Cterm fragment, however, rescues learning significantly (Figure 3.5), suggesting that elements within this region are crucial for NF1 to mediate learning. Take together, these data indicate that the different structural/functional relationships revealed by biochemical assays in our previous study (Hannan et al., 2006), also have a correspondingly distinct effect on the role of NF1 in different phases of learning and memory.

NF1 may mediate different phases of memory in distinct fly brain regions

The anatomical site of learning and memory in flies has been mostly attributed to the antennal lobes (AL) and the mushroom body (MB) of the *Drosophila* brain (Liu and Davis, 2006). Central complex (CC) is another fly brain structure that has been associated with behavioral output such as courtship suppression (Popov et al., 2003) and visual (Liu et al., 2006) memory, but its role in olfactory learning had only been implicated (Davis, 1996). To examine the brain structures where NF1 may function to mediate olfactory

conditioning, we use several Gal4 lines that have specific expression in the AL, MB, and CC to express hNF1 in *Nf1* null mutant background to rescue learning or LTM.

All 3 MB-Gal4 lines, c747, 201Y and OK107 show significantly rescue in learning when used to express hNF1 (Fig. 3.6A, left). On the other hand, the CC-Gal4 lines, c107 and Feb170, are not able to rescue the learning defect in NF1 mutants. These transgenic flies were also subjected to spaced training and test for their performance in LTM. All MB lines show partial rescues of 24-hour memory compared to the negative controls (Fig. 3.6A, right). Interestingly, the CC-Gal4 lines also rescued 24-hour memory partially after spaced training (Fig. 3.6B, right). These data suggest that NF1 is required to function in both the MB and central complex to mediate LTM, while only in the MB to mediate learning.

It was later found that the genotype of the $Nf1^{P1}$*;OK107-Gal4* fly stock was heterozygous for the OK107 insertion due to an error in crossing. The correct double transgenic line was then generated by single pair mating and PCR (See Materials and Methods). Afterward, spaced training experiments were repeated using the newly generated flies along with another MB-*Gal4* line c747. The re-generated $Nf1^{P1}$*;OK107-Gal4*, when crossed with *UAS-hNF1;*$Nf1^{P2}$, was able to partially rescue the 24-hour memory defect found in *Nf1* null mutants (Figure 3.6D). The level of partial rescue is comparable to that demonstrated by c747 (Figure 3.6D), and both of these repeated data are similar to the original results (Figure 3.6A). The similarity in the level of partial rescue between heterozygous and homozygous OK107 insertion may be attributed to a combination of the nature of partial rescue and variation of performance inherent in spaced training.

In order to verify that the portion of 24-hour memory rescue by the MB- and CC-lines is protein synthesis-dependent LTM, we treated one line from each type of Gal4 lines with the protein synthesis inhibitor CXM. This treatment decreased the 24-hour memory level of either the MB or the CC line similar to the wild type control (Fig. 3.6C), indicating what has been rescued by the MB and CC Gal4 lines is indeed protein synthesis-dependent LTM.

Conclusion

In this study, we have dissected the functional significance of two NF1 protein regions using the Pavlovian olfactory conditioning paradigm in *Drosophila*. The C-terminal region contains sequences that are essential for immediate memory, while the GRD is required for LTM formation. These two regions also possess distinct biochemical properties by which they individually mediate different signaling pathways (Hannan et al., 2006). These unique properties of NF1 suggest that different signal transduction pathways contribute to distinct phases of memory.

The Morris water maze, for testing spatial learning performance in mice, requires the subject to find a platform submerged under water by using spatial cues in the environment. This task requires two training sessions per day and, in the case of $Nf1^{+/-}$ mice, six days to complete the training regimen (Silva et al., 1997). The amount of time for this task is significantly longer than the four minutes required for training flies in the Pavlovian olfactory learning task (Tully and Quinn, 1985; Guo et al., 2000). In fact the water maze paradigm is strikingly similar to the spaced training we used for LTM induction in flies, both of which have repetitive training as well as resting components. This similarity is indeed valid as both paradigms have been shown to produce protein synthesis-dependent memory (Tully et al., 1994; Meiri and Rosenblum, 1998). This study resolves the discrepancy of different pathways underlying the behavioral phenotypes exhibited by these two NF1 animal model systems. Our results indicate that different phases of memory were examined in previous reports.

According to earlier findings, the GRD deletion and point mutants used in this study are also defective in mediating growth factor-stimulated Ras-dependent AC activity

(Hannan et al., 2006). The three GRD point mutants have been shown to be essential for NF1's affinity for Ras, as well as GAP activity (Gutmann et al., 1993; Poullet et al., 1994; Klose et al., 1998). In the mammalian system, growth factor receptors have been demonstrated to be an essential component for the maintenance of long term potentiation (LTP), an electrophysiological phenomenon suggested to be the underlying mechanism for learning and memory (Bramham and Messaoudi, 2005). Ras signaling has also been shown to play an important role in synaptic plasticity, as well as learning and memory (Brambilla et al., 1997; Atkins et al., 1998). The EGFR was shown to be important for Ras-mediated AC stimulation in our previous study (Hannan et al., 2006). The GRD point mutants' effects on LTM suggest that the epidermal growth factor receptor (EGFR) and Ras pathway may be an important mechanism for LTM in flies, as illustrated in our working model (see Figure 3.6). Further experiments assaying the LTM performance of Ras and EGFR mutants will be needed to confirm this hypothesis.

Combining our present behavioral data together with the former biochemical analysis (Hannan et al., 2006), we proposed a working model as shown in Figure 3.6. Two independent pathways are mediated by different regions of the NF1 protein. The C-terminal region controls the G protein-dependent AC pathway, which can be stimulated by neurotransmitters such as serotonin and histamine (Hannan et al., 2006). This NF1-cAMP pathway is important for learning (Figure 3.5) (Guo et al., 2000). The GRD region regulates Ras activity, which can be stimulated by growth factors such as EGF to induce cAMP production (Hannan et al., 2006). This NF1-Ras pathway is essential for LTM formation. This requires NF1-GRD's normal GAP activity and interaction with the Ras protein (Figure 3.3B). Although our data showed that fragments containing the GRD can

only partially rescue the LTM defect, this may be due to insufficient conformational support of the GRD fragments to fully restore wild type function. The fact that deletion of the GRD from the NF1 protein (Figure 3.3C) eliminates the ability to rescue the LTM defect suggests the importance of the GRD in NF1's role in regulating LTM formation.

This report is the first step in gaining insight into the nature of the cognitive defects found in NF1 patients using the *Drosophila* model system. Interestingly, the NF1 protein presents a unique case of having distinct regions governing two independent steps of an important cognitive process. These NF1 protein regions that are involved in different phases of learning and memory contain different types of post-translational modification sites, such as phosphorylation sites for protein kinase A and protein kinase C (Mangoura et al., 2006), and binding sites for proteins such as syndecan (Hsueh et al., 2001). It will be interesting to investigate the role these sites play in governing the behavioral outputs assayed in this report, to find out the exact mechanisms and pathways that govern the distinct behaviors of learning and memory.

Figures and Legends

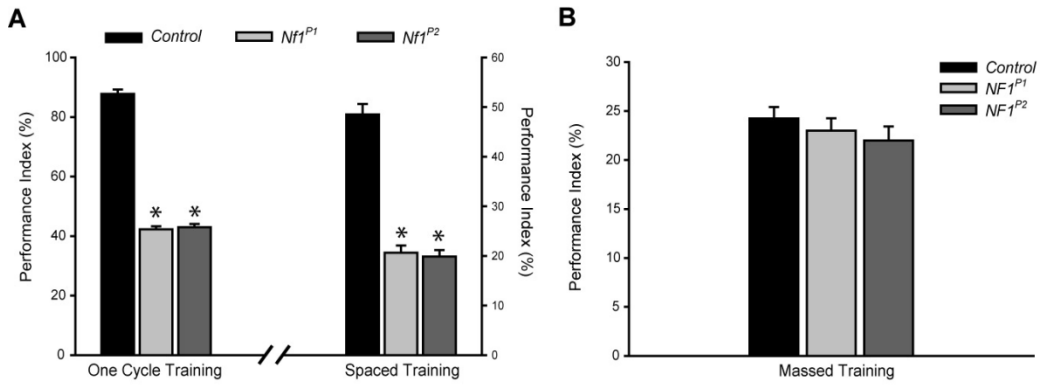

Figure 3.1. Learning and long-term memory (LTM) defects, but normal anesthesia-resistant memory (ARM) in *Nf1* null mutants. (**A)** Learning and LTM defects in *Nf1* mutants. Compared to K33 (control) parental group, the $Nf1^{P1}$ and $Nf1^{P2}$ mutants display significantly lower performance (*, $p < 0.001$) in learning (one cycle training) and in LTM (spaced training; refer to Materials and Methods for details). **(B)** Normal ARM performance in *Nf1* mutants. Memory performance was tested 24 hours after massed training. *Nf1* mutants perform similar to the parental *K33* (control) flies after massed training. This indicates that the 24-hour memory defect observed in these mutants is in fact an LTM defect since ARM is normal. PI scores are expressed as mean ± s.e.m., $n = 8$.

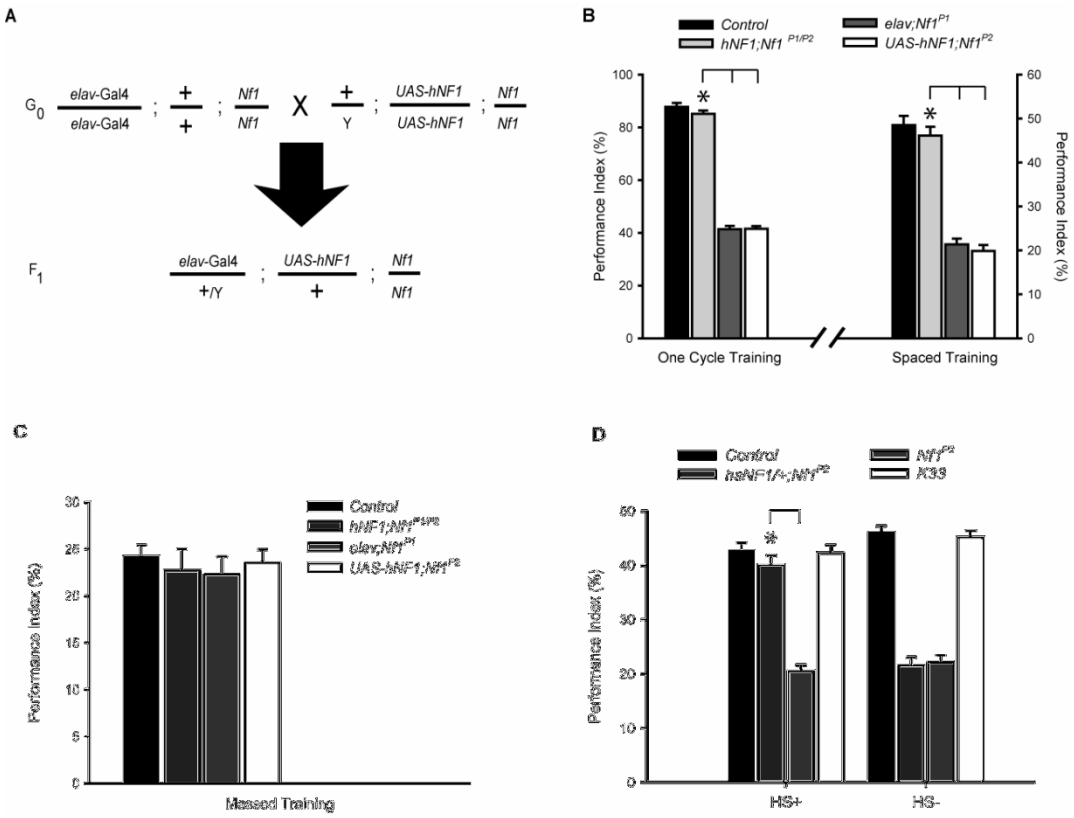

Figure 3.2. Rescue of learning and LTM defects by expressing human NF1 (hNF1) as well as heat-shock NF1 (hsNF1) transgene in *Nf1* null mutants. **(A)** Crosses performed to generate F1 progeny expressing *UAS-hNF1* constructs under the control of the pan-neuronal *elav*-Gal4 driver. **(B)** Rescue of learning and LTM by expressing hNF1 transgene in *Nf1* null mutants. Transgenic flies expressing hNF1 pan-neuronally (*elav/+/Y;UAS-hNF1/+;Nf1*$^{P1/P2}$) exhibit significant increases (*, $p < 0.001$) in both learning (left) and LTM (right) from parental lines (*elav;Nf1*^{P1} and *UAS_hNF1;Nf1*^{P2}). The wild type control is *2202u*, an isogenic line from which transgenic parental lines were generated (Hannan et al., 2006). **(C)** Normal ARM performance in all transgenic lines. None of the transgenes shows any non-specific effect on ARM (n = 4 PIs per group). **(D)** Acute expression of NF1 rescues LTM. Heat-shock induced expression of NF1 (*hsNF1/+;Nf1*^{P2}) before spaced training significantly rescues (*, $p < 0.001$) the LTM

defect found in *Nf1* mutants when compared to both *2202u* (control) and *K33* wild type flies. This indicates the importance of NF1 in LTM formation. HS+, raised at 18^{o}C, and shifted 30^{o}C for 30 min 2 hours before training; HS-, no heat-shock treatment. PI scores are expressed as mean ± s.e.m., n = 8 unless otherwise indicated.

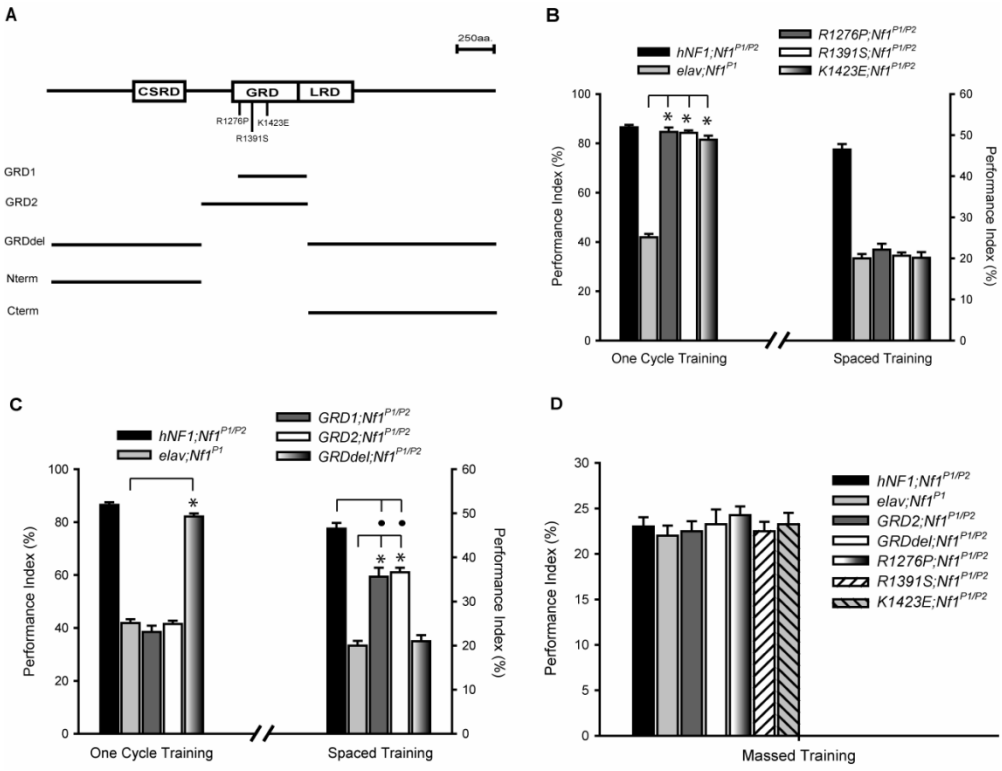

Figure 3.3. The GRD domain and GAP activity are necessary and sufficient for LTM formation, while NF1 without the GRD domain rescues learning. **(A)** Positions of three hNF1 missense mutations and size of five hNF1 deletion constructs that have been expressed in *Drosophila Nf1* null mutants. Refer to text for detailed description of these mutants. (CSRD, CysSer rich domain; GRD, GAP-related domain; LRD, Leu rich domain; GRD1 and GRD2, GRD fragments of different sizes; GRDdel, NF1 protein with the GRD domain deleted; Nterm, N-terminal fragment; Cterm, C-terminal fragment). **(B)** GRD point mutations restore learning to wild type level but fail to rescue LTM. The three GRD point mutations are able to significantly rescue (*, $p < 0.001$) the learning defect in the *Nf1* mutant (*elav;Nf1*^{P1}), to the same extent as the full length human NF1 transgene. However, the three point mutations are not able to rescue the LTM defect of *Nf1* mutants (right). **(C)** Rescue of LTM but not learning by GRD fragments. Flies expressing GRDdel significantly rescue (*, $p < 0.001$) learning to the wild type level, while flies

expressing the GRD fragments, GRD1 and GRD2, do not rescue learning (left). Mutant flies expressing both GRD fragments exhibit partial yet significant rescue (*, $p < 0.001$) of LTM compared to the *Nf1* mutant (right). When compared to flies expressing full length hNF1 transgene, mutants expressing the GRD fragments are significantly lower in LTM performance (•, $p < 0.001$), indicating only partial rescue of LTM. In contrast, mutants expressing the GRD-deleted protein show no rescue of LTM (right). **(D)** Normal ARM performance in wild type and mutant transgenic lines. None of the transgenes shows any non-specific effect on ARM ($n = 4$ PIs per group), indicating that NF1 is only involved in LTM. PI scores are expressed as mean ± s.e.m., $n = 8$ unless otherwise indicated.

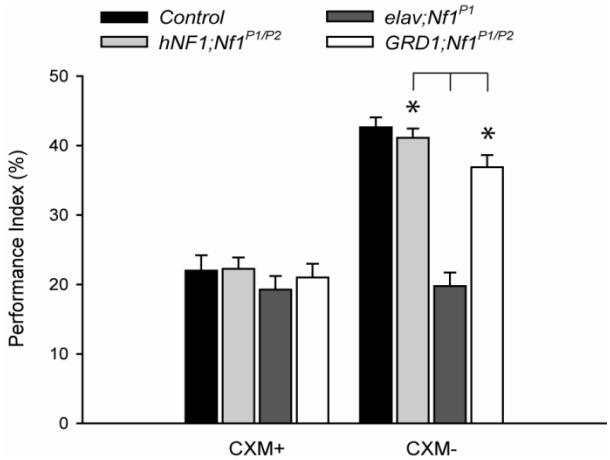

Figure 3.4. Cycloheximide (CXM) abolished LTM performance in wild type and hNF1 transgenic flies. CXM, a protein synthesis inhibitor, was fed to the wild type, hNF1, and GRD1 flies before spaced training and again during the 24-hour retention period (See Materials and Methods). For the CXM-treated group (CXM+), LTM for wild type control, hNF1 and GRD1 flies was reduced to $Nf1^{P1}$ mutant levels. However when hNF1 and GRD1 flies were treated with vehicle, they still displayed significant rescue compared to the *elav;*$Nf1^{P1}$ parental control (right; *, $p < 0.001$). These results indicate NF1 is indeed important for the formation of protein synthesis-dependent memory, and that the GRD fragment specifically rescues LTM after spaced training. PI scores are expressed as mean ± s.e.m., $n = 8$; $p < 0.001$.

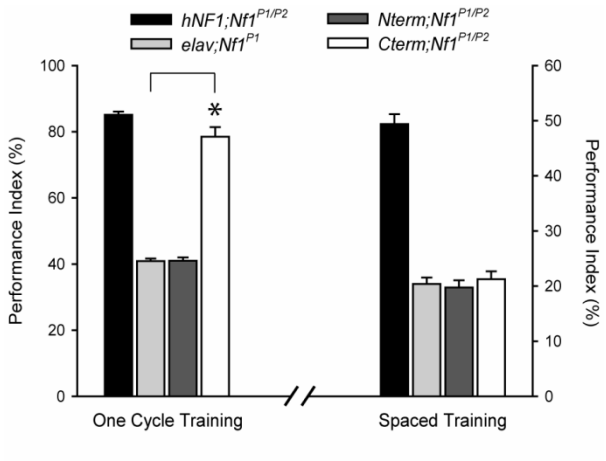

Figure 3.5. Rescue of learning by Cterm fragment. Flies expressing Cterm (see fig. **3A**) exhibit complete rescue of learning compared to the wild type transgene (*, $p < 0.001$), while the N-terminal fragment (Nterm, fig. **3A**) has no effect on the learning score (left). Both fragments are unable to restore LTM performance (right). These data indicate region-specific functionality of the NF1 protein for distinct memory phases; i.e. the GRD is required for LTM, and the C-terminal is essential for learning. PI scores are expressed as mean ± s.e.m., $n = 8$.

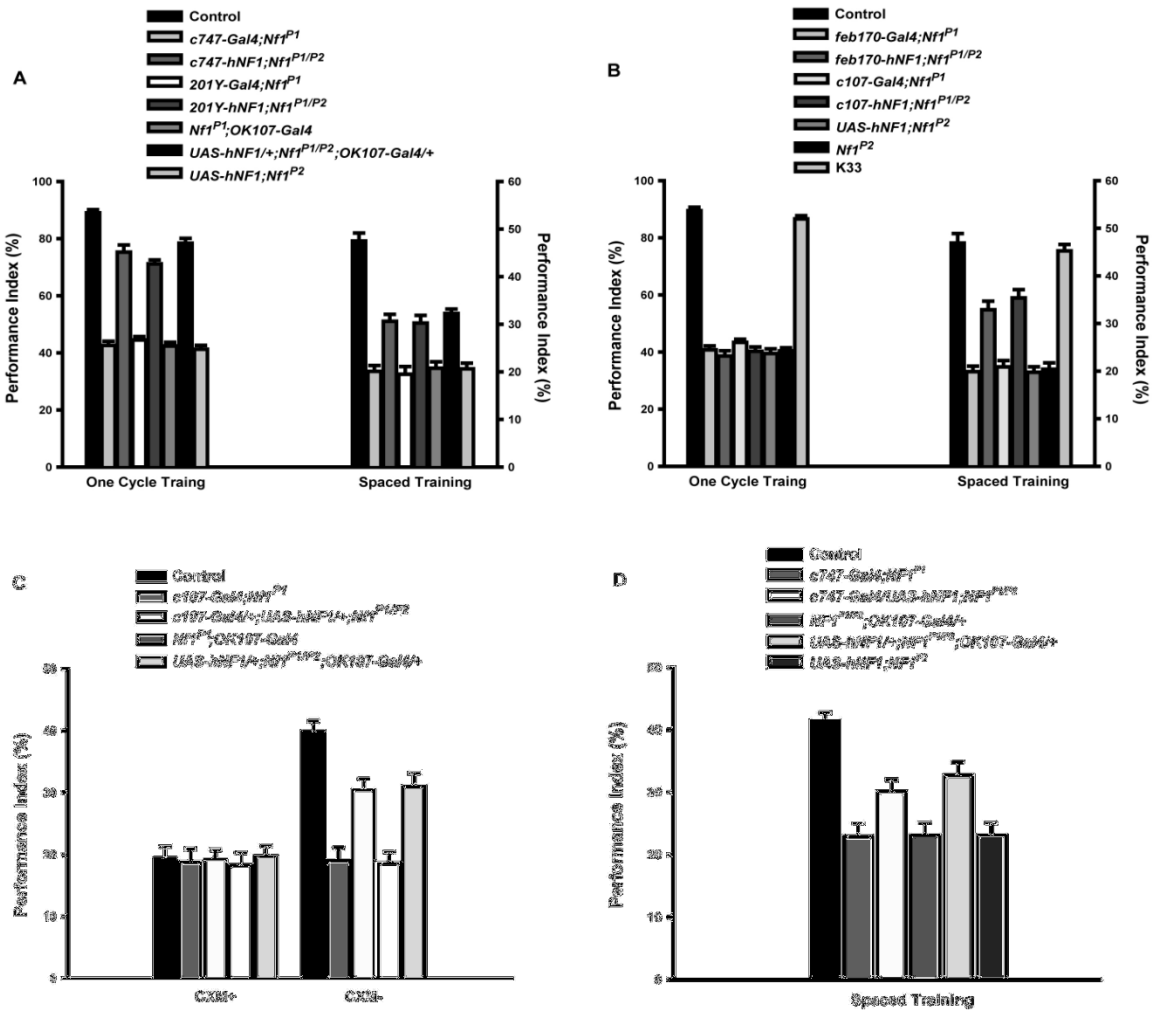

Figure 3.6. NF1-mediated learning occurs in mushroom body, while NF1-mediated LTM occurs in both mushroom body and central complex. **(A)** hNF1 expression in mushroom body rescues learning defect, and LTM defect partially. A total of 3 mushroom body (MB)-specific Gal4 lines were used to analyzed, namely *c747-hNF1;Nf1*$^{P1/P2}$ (*c747-Gal4/+/UAS-hNF1/+;Nf1*$^{P1/P2}$), *201Y-hNF1;Nf1*$^{P1/P2}$ (*201Y-Gal4/+/UAS-hNF1;Nf1*$^{P1/P2}$), *UAS-hNF1/+;Nf1*$^{P1/P2}$*;OK107/+* (*+/Y;UAS-hNF1/+;Nf1*$^{P1/P2}$*;OK107/+*). Expressing wild type hNF1 in the MB leads to a significant increase of learning performance compared to negative controls (left). The same anatomical location also allows hNF1 to partially rescue NF1-dependent LTM (right). **(B)** hNF1 functions in central complex to rescue

LTM defect specifically. *Feb170-hNF1;Nf1*$^{P1/P2}$ (*Feb170/+/Y;UAS-hNF1/+;Nf1*$^{P1/P2}$) and *c107-hNF1;Nf1*$^{P1/P2}$ (*c107/+/Y;UAS-hNF1/+;Nf1*$^{P1/P2}$) were used to test hNF1's ability to rescue learning or LTM in the central complex (CC). Both Gal4 lines are able to rescue LTM partially (right), but not learning (left). **(C)** Cycloheximide treatment abolish rescues of LTM presented by hNF1 expressed in MB or CC. Partial rescue presented by hNF1 expressed in MB (*UAS-hNF1/+;Nf1*$^{P1/P2}$*;OK107-Gal4/+*) or CC (*c107/+/Y;UAS-hNF1/+;Nf1*$^{P1/P2}$) can be abolished by the treatment of CXM pre- and post-spaced training. This indicates the portion that is rescued by the two different anatomical Gal4 lines is indeed protein synthesis-dependent LTM. **(D)** Re-generated OK107-driven hNF1 displays partial rescue in 24-hour memory. PI = mean ± s.e.m., $n = 8$; $p < 0.001$

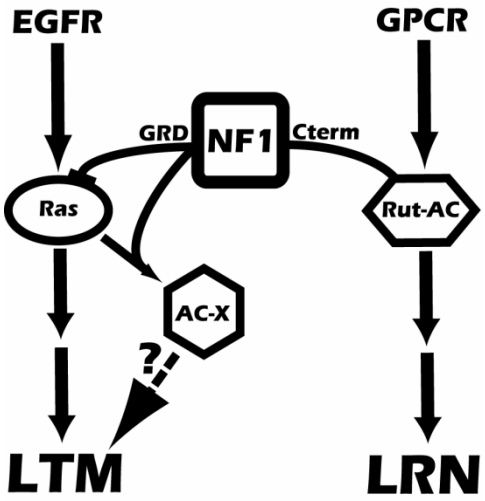

Figure 3.7. Working model for regulation of distinct memory processes by different domains of NF1. In this model, two different signaling pathways underlie distinct phases of memory formation, which are both mediated by NF1. The GRD domain, with its GAP activity and interaction with the Ras protein, is necessary and sufficient for mediating EGFR signaling (Hannan et al., 2006), as well as LTM (Fig. 3C). Thus EGFR may be an essential signaling mechanism to mediate LTM in flies. Also shown is the synergistic stimulation of an unknown AC (AC-X) by NF1 and Ras proteins (Hannan et al., 2006). This AC-X may be the downstream target of Ras and NF1 governing LTM formation. The C-terminal of the NF1 protein has been shown to mediate G-protein signaling (Hannan et al., 2006), and is essential to regulate learning, or immediate memory (Fig. 5). Therefore, signaling molecules such as serotonin and histamine, whose downstream signaling pathways are mediated by NF1 (Hannan et al., 2006), may be important for learning or immediate memory. EGFR, epidermal growth factor receptor; GPCR, G-protein coupled receptor; Rut-AC, *rutabaga*-encoded adenylyl cyclase; LTM, long-term memory; LRN, learning.

Table 3.1 Performance Indexes for shock reactivity and olfactory avoidance

Genotypes	Shock Reactivity (60V)	Odor Avoidance	
		BA	MCH
2202u	85 ± 3	79 ± 3	82 ± 3
K33	78 ± 3	79 ± 3	77 ± 4
elav/+/Y;UAS-hNF1/+;Nf1$^{P1/P2}$	83 ± 2	80 ± 2	80 ± 3
Nf1^{P1}	79 ± 2	76 ± 2	76 ± 3
Nf1^{P2}	79 ± 3	77 ± 2	77 ± 3
elav/+/Y;UAS-GRD1;Nf1$^{P1/P2}$	83 ± 4	79 ± 3	83 ± 2
elav/+/Y;UAS-GRD2;Nf1$^{P1/P2}$	81 ± 2	79 ± 2	82 ± 3
elav/+/Y;UAS-GRDdel;Nf1$^{P1/P2}$	80 ± 3	79 ± 3	78 ± 2
elav/+/Y;UAS-R1276P;Nf1$^{P1/P2}$	80 ± 2	80 ± 3	77 ± 2
elav/+/Y;UAS-R1391S;Nf1$^{P1/P2}$	81 ± 2	80 ± 3	81 ± 2
elav/+/Y;UAS-K1423E;Nf1$^{P1/P2}$	82 ± 2	81 ± 3	80 ± 4
elav/+/Y;UAS-Nterm;Nf1$^{P1/P2}$	81 ± 3	79 ± 3	80 ± 3
elav/+/Y;UAS-Cterm;Nf1$^{P1/P2}$	79 ± 2	80 ± 3	78 ± 4

All wild type, transgenic, and mutants flies used in this study have normal response to aversive odors and electric shocks. All scores are expressed as mean PI ± s.e.m. For all shock reactivity and odor avoidance assays, n = 4. No statistical difference at the level of a = 0.05 is detected for sensorimotor activities and odor avoidance.

Chapter 4

Conclusions and Perspectives

Our understanding of the NF1 protein's functionality has been largely led by it's sequence similarity with RasGAP proteins when the NF1 gene was first cloned in 1990 (Ballester et al., 1990; Cawthon et al., 1990; Martin et al., 1990; Xu et al., 1990). Since then many studies have been trying to associate regions of the NF1 protein with the variety of phenotypes of the disease with no avail (Fahsold et al., 2000; Messiaen et al., 2000; Serra et al., 2001; Mattocks et al., 2004). There are also reports of a few proteins that interact with NF1, but the functional significant of these interactions are not always readily elucidated (Aravind et al., 1999; Hsueh et al., 2001; Tokuo et al., 2001; Feng et al., 2004). Our former studies with NF1 in biochemical, electrophysiological, and behavioral paradigms have linked the NF1 protein to the cAMP pathway for the first time (Guo et al., 1997; Guo et al., 2000; Tong et al., 2002). In this study, we try to examine how NF1 are involved in both the Ras and the cAMP pathways, dissect the structure-function relationship of the NF1 protein, as well as explore the role of NF1 in different phases of memory in the Pavlovian olfactory conditioning paradigm.

Distinct regions of NF1 mediate EGFR and GPCR stimulation AC activity via two separate biochemical pathways

In my study, I first identified two NF1-dependent and one NF1-independent signaling pathways that lead to the activation of AC. One of these pathways relies on the

interaction between NF1 and Ras, and is stimulated by the growth factor EGF. The other NF1-dependent pathway is stimulated by neurotransmitters such as serotonin and histamine, and is mediated by GPCR, NF1 and Rut-AC. Distinct signaling molecules that can stimulate these two separate pathways allow us to dissect the structure-function relationship of the NF1 protein. With our biochemical analyses detailed in Chapter 2, we were able to approximate regions of the NF1 protein which are responsible for mediating different signaling pathways for AC activation. This is the first study where different functional domains of NF1 are identified biochemical.

Yeast was the first model system that revealed the relationship between Ras protein and AC (Broek et al., 1985; Toda et al., 1985). This relationship seems to be conserved in the *Drosophila* system as shown in Chapter 2. However, in *Drosophila* both Ras and NF1 proteins are required for the stimulation of AC as mutations that eliminate NF1/Ras interaction as well as NF1's RasGAP activity abolishes this stimulation completely (Figure 2.1E). NF1 mutants have also been shown to disrupt neuropeptide's ability to enhance K^+ current in electrophysiological analyses, and this can be remedied by supplying cAMP before recordings (Guo et al., 1997). These two findings led us to explore the physiological significance of NF1 being involved in two different biochemical pathways that both lead to the activation of AC. My biochemical analysis showed that EGFR can stimulate the production of cAMP and this stimulation is mediated by the cooperation of NF1 and Ras proteins. In addition, GPCR can stimulate AC activity and this is conferred by NF1 and Rut-AC. These results indicate the dual role of NF1 in mediating different signaling molecules for the stimulation of AC *in vivo* (Figure 2.6). Interestingly, there is as yet unidentified AC that is stimulated by the EGF

pathway. This is in contrast to the NF1-dependent GPCR pathway, in which Rut-AC is the AC producing cAMP. RNAi experiments may help further elucidate the exact identity of this EGF-stimulated AC.

With our working model of NF1 being responsible for mediating stimulation of AC by distinct groups of signaling molecules, I gain the tool for dissecting the function of different regions of NF1 in mediating signaling pathways. Our results indicate the importance of the GRD region for the EGF stimulation of AC. Furthermore, NF1's RasGAP activity and interactivity with Ras is essential for this stimulation to occur (Figure 2.5A). These results beg one to speculate that the NF1/Ras complex, transient as it may be, is able to stimulate AC. There has never been evidence of direct contact of NF1 with AC or Ras with AC. It should be interesting to examine whether there is indeed physical interaction between the three proteins. The limiting factor will be the potentially extremely transient nature of the complex.

The C-terminal of the NF1 protein has been shown to interact with the transmembrane glycoprotein syndecan (Hsueh et al., 2001), DDAH (Tokuo et al., 2001), and 14-3-3 (Feng et al., 2004). No functional significance has been shown on the binding between NF1 and syndecan, although syndecan is important in the regulating cell migration and axon guidance in *C. elegans* (Rhiner et al., 2005). DDAH is a nitric oxide synthase regulator and its interaction with NF1 through the Cterm and CSRD is important for NF1's phosphorylation by PKA (Tokuo et al., 2001). Increased activity of PKA is able to rescue the learning defect exhibited by *Nf1* null mutants in the olfactory learning paradigm (Guo et al., 2000); it maybe interesting to examine DDAH's and PKA's role in regulating NF1 activity to understand *Nf1* mutants' behavioral phenotype. Another

important signaling protein that interacts with the Cterm of NF1 is 14-3-3. PKA's phosphorylation on several sites of the Cterm is required for this interaction to occur (Feng et al., 2004). Interestingly, a *Drosophila* learning mutant called *leo* gene also codes for a 14-3-3 protein (Skoulakis and Davis, 1996). One may speculate that the Cterm may be important for mediating learning, as shown in Chapter 3 of this thesis.

During the screening of neurotransmitters, we identified dopamine as a signaling molecule that is dependent only on Gs_a to stimulate AC activity (Figure 2.3B). Later, by using *rut* mutant to perform biochemical analysis, we also found that dopamine can stimulate AC independent of Rut-AC. Interestingly, dopamine has been shown to be involved in aversive olfactory memory in *Drosophila* (Schwaerzel et al., 2003), similar to *rut*. It is relatively surprising that our study revealed serotonin and histamine to be the neurotransmitters that can induce cAMP production by stimulating Rut-AC (Figure 2.3 C and D). One possibility for this difference may be attributed to the serotonin circuit overlapping with tyrosine hydroxylase (TH) circuit knocked out by the targeted *shibire*^{ts} expression in the dopamine study (Schwaerzel et al., 2003). However, a previous study examined the expression profile of dopaminergic and serotonergic neurons in the larvae and revealed that the two types of neurons seldom overlapped in the CNS (Lundell and Hirsh, 1994). In *Drospohila*, there are several serotonin receptors and they are 5HT-dro1, 5HT-dro2A, and 5HT-dro2B. Among them, 5HT-dro1 is the receptor that stimulates AC activity while the other two inhibits it (Witz et al., 1990; Saudou et al., 1992). Recent study has established the expression pattern of 5HT-dro2 receptors in the adult fly CNS to be ubiquitous with increased level in ellipsoid body and antennal lobe (Nichols, 2007). However, the expression pattern of 5HT-dro1 receptor in the adult CNS has not been

thoroughly studied. Until the pattern of 5HT-dro1 expression in the adult CNS, it can be hypothesized that the expression of this particular 5HT receptor is ubiquitous and that in addition to dopamine, serotonin may also play a role in modulating aversive olfactory learning. Another possibility is that there may be a difference in terms of dopamine downstream signaling targets between larvae and adult stages.

Structure-function relationship of the NF1 protein also corresponds to its role in olfactory learning

In the mouse model, complete knockout of the NF1 gene is lethal due to a defect of heart formation by E14.5 (Brannan et al., 1994). For analyzing behavioral performance in the mouse model, heterozygous NF1 knockout (*Nf1*$^{+/-}$) mice were used (Silva et al., 1997). In the Morris water maze learning paradigm, a mouse is trained in a circular water tank to navigate for a platform placed in a specific quadrant. The water tank is situated in a room with different spatial cues on the walls for the mouse to associate with the placement of the platform. After four days of training, with two training sessions per day, the mouse is tested for the length time it takes to find and the duration it lingers in the quadrant where the platform was place but has been removed for testing (Morris, 1984). *Nf1*$^{+/-}$ mice are defective in this behavioral paradigm, and this phenotype can be rescued by decreasing Ras biological activity genetically or pharmacologically (Costa et al., 2002). On the other hand, olfactory learning defect observed in *Nf1* null mutants in flies was rescued by increasing PKA activity, and thereby manipulating the cAMP pathway (Guo et al., 2000). Although the paradigms are different, both studies claimed the phenotype exhibited by their own NF1 mutants was learning. Interestingly, the water

maze training protocol closely resembles that of the spaced training protocol in flies. Spaced training in flies (see Materials and Methods) generate two forms of long-lasting memory: ARM, which does not require protein synthesis, and LTM, which does require protein synthesis (Tully et al., 1994). When the Morris water maze paradigm was examined more closely by the Rosenblum group, it was revealed that the learning that occurred in the water maze was actually a form of memory that is protein synthesis-dependent (Meiri and Rosenblum, 1998). This prompted us to address whether NF1 is involved in both learning and LTM in the flies, and what may be the underlying mechanism that mediate these phases of memory formation.

Our behavioral analyses shown in Chapter 3 of this thesis indicate that NF1 is not only required for learning but also LTM in the *Drosophila* olfactory conditioning paradigm. In addition, distinct functional domains of the NF1 protein confer different phases of memory formation. Comparing the biochemical with the behavioral analyses in this thesis, it's not hard for one to make the correlation of the NF1-dependent signal transduction pathways with the different phases of memory that require the NF1 protein. The point mutations within the GRD were able to abolish 24-hour memory performance to mutant level while rescuing learning defect to the wild type level (Figure 3.3B). These data indicate that the importance of NF1/Ras interaction and the RasGAP activity of NF1 to mediate LTM. This is not unlike the stimulation of AC by EGF mediated by the cooperation of NF1 and Ras (Figure 2.5E). Since growth factors receptors have been shown to be required for the maintenance of LTP (Bramham and Messaoudi, 2005) and there is evidence that EGF can facilitate LTP (Terlau and Seifert, 1989; Ishiyama et al., 1991), the DER may be involved in LTM in *Drosophila*. It will be interesting to test the

DER mutants used in the biochemical analysis to evaluate whether they have normal 24 hour memory after massed and spaced trained.

As mentioned above, the Cterm of the NF1 protein can interact with a signaling protein 14-3-3 (Feng et al., 2004), coded by the *leonardo* gene in *Drosophila*, that has been demonstrated to be important in olfactory learning (Skoulakis and Davis, 1996; Philip et al., 2001). 14-3-3 proteins have been shown to interact with PKC, which can phosphorylate NF1 at various sites (Mangoura et al., 2006). The significance of PKC phosphorylation of NF1 remains to be seen in the context of governing learning, but PKC has been demonstrated in both flies and mouse to be an essential protein for learning and memory, as well as LTP (Abeliovich et al., 1993b; Abeliovich et al., 1993a; Kane et al., 1997; Weeber et al., 2000; Drier et al., 2002). The Cterm can also interact with the NOS regulator DDAH, with this interaction being required for PKA phosphorylation of the NF1 protein (Tokuo et al., 2001). Homologue of DDAH is found in the *Drosophila* genome with annotation CG1764 and no characterization. It should be interesting to examine whether this gene conserved interaction with NF1 and whether disruption of this gene will lead to learning defect.

Our preliminary anatomical analysis of NF1 suggests that the NF1 protein functions in both the mushroom body and, surprisingly, the central complex of the adult brain for learning and memory. Until now mushroom body has been the only *Drosophila* brain structure shown to play a central role for olfactory learning. Our preliminary data suggest that NF1 functions in these two brain structures for different phases of learning and memory. NF1 functions in the mushroom body to mediate learning, and partially LTM (Figure 3.1), while it is required for mediating LTM in the central complex (Figure

3.2). These data suggest that there are sites extrinsic to the MB for mediating learning, while both the MB and CC are required for governing LTM. In addition, the data also lead us to hypothesize that there are connections between the MB and the CC, and that there may even be direct connection between the antennal lobes to the CC, assuming learning and LTM are sequential processes as was proposed (Dubnau and Tully, 1998). Further analysis with expressing different functional domains of NF1 in these two structures will further clarify the pathways underlying these two distinct phases of memory in the two brain structures.

Future Directions

The functional sites of the NF1 protein in the brain for its mediation of learning and memory should be further examined in the future. This can be achieved by expressing hNF1 using various Gal4 lines that have unique expression patterns in different subsets of neurons in the fly brain. We can further improve this correlation study by introducing GFP into the hNF1 protein and examine the expression pattern of the hNF1 protein tagged with GFP (hNF1-GFP) under the Gal4 drivers using imaging analysis. Another approach to further our understanding of NF1 functionality is to express hNF1-GFP using a more general Gal4 driver, such as *armadillo-Gal4* or *elav-Gal4*, in the null mutant background. Using confocal laser scanning microscopy we may be able to see sub-cellular localization of NF1 in the neurons that populate different regions of the brain. Since NF1 is essential in MB and CC to mediate learning and LTM, it will also be interesting to see the distribution of NF1 protein in these structures. A corollary of the confocal imaging experiment will be to electrically stimulate specifically

the antennal nerve and image live changes of NF1 protein distribution in the MB, if any. Since NF1 is a cytoplasmic protein and has been shown to modulate AC, which is a membrane protein, we may see a change in subcellular distribution when the olfactory circuitry is stimulated directly. This series of experiments may allow us to shed more lights on the functional significance of the NF1 protein in the context of neural circuitry.

Summary

The research presented in this thesis focuses on dissecting NF1-dependent signal transduction pathways and associating these pathways with different phases of memory formation. The discrepancy between the underlying mechanisms of mouse and fly NF1 model in learning has also been resolved. The conclusions presented herein have provided a relative mapping of the NF1 protein domains that can mediate two different signaling pathways and two distinct phases of learning and memory. Though some questions remain unanswered and new ones have been raised, these studies have provided many hypotheses that may provide insights into pathways that may underlie NF1 pathogenesis and formation of different learning and memory phases.

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Appendix

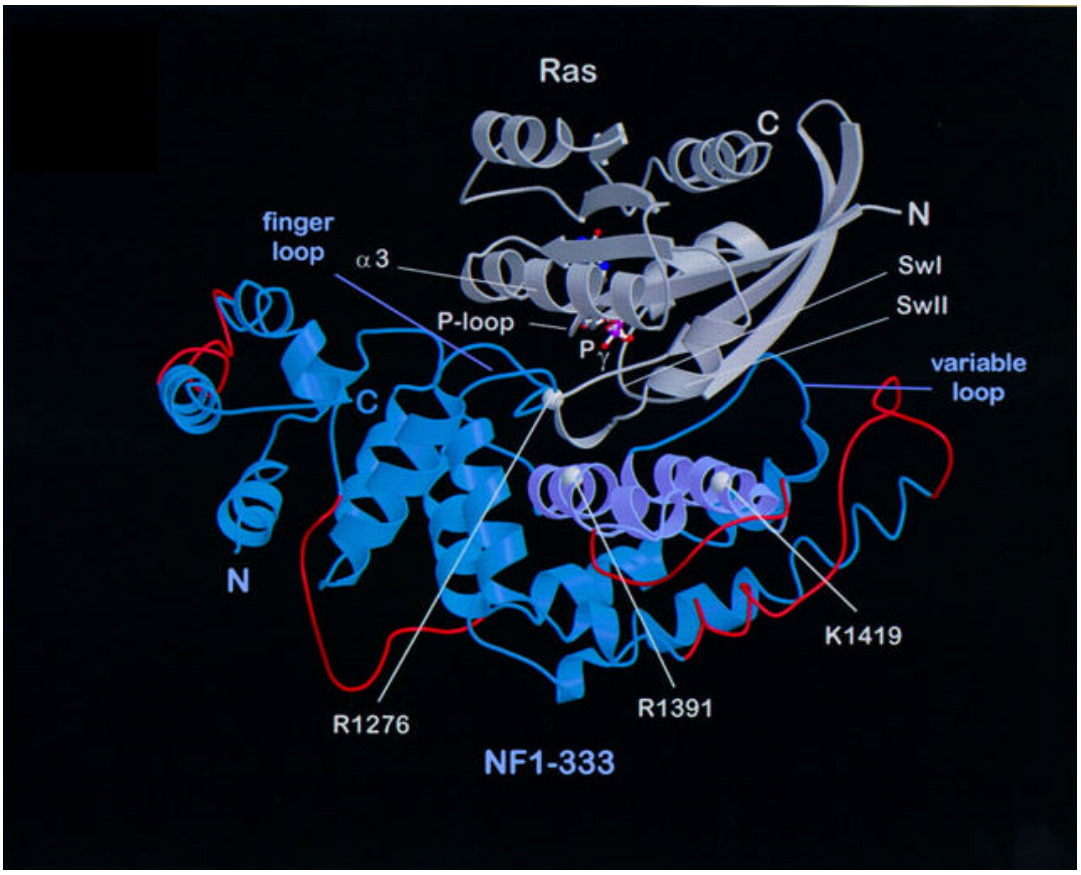

Figure A.1. On the Ras–NF1GRD interaction. Hypothetical complex between Ras (grey) and NF1-333 (blue), modelled according to the structure of the Ras-GDP–AlF3–GAP-334 complex. Segments coloured in red are derived from the GAP-334 model and correspond to regions of presumed high mobility in NF1-GRD. SwI, Switch I; SwII, Switch II. Positions of patient mutations affecting the interaction with Ras are indicated by grey spheres. Adapted from Scheffzek et al. (Scheffzek et al., 1998)

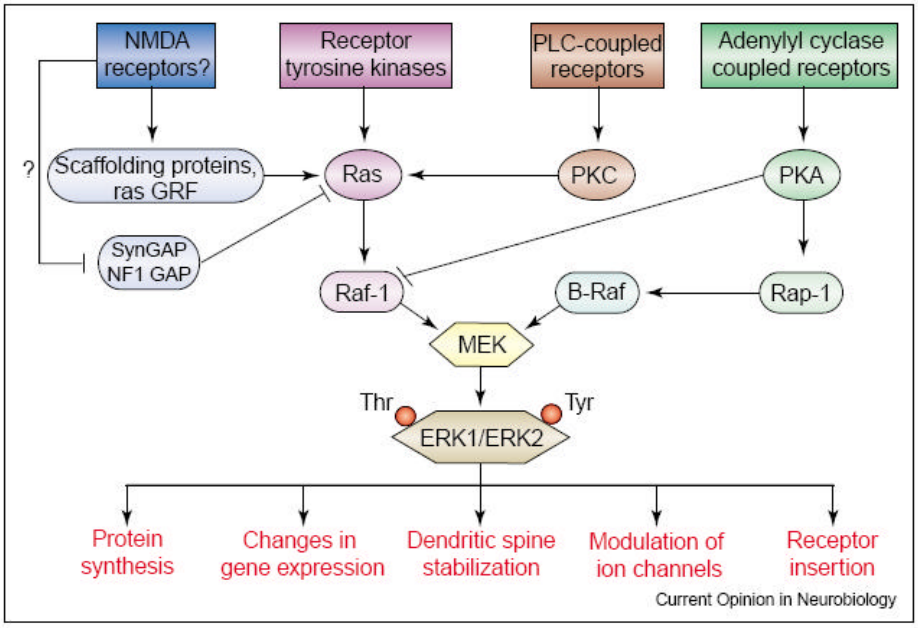

Figure A.2. Regulation and targets of the ras/ERK pathway in neurons. The ERK/MAP kinase cascade can be activated by a number of receptors and protein kinases within the hippocampus. As such, it can integrate a wide variety of signals and result in a final common output. The ERK cascade is initiated by the activation of Raf kinase through the small GTP-binding protein, ras, or the ras-related protein, rap-1. Activated Raf then phosphorylates MEK (mitogen extracellular regulating kinase), a dual specific kinase. MEK phosphorylates ERK 1 and 2 on a tyrosine and threonine residue. Once activated, ERK exerts many downstream effects, including the regulation of cellular excitability and the activation of transcription factors leading to altered gene expression. Each MAP kinase cascade (ERK, JNK, and p38 MAPK) is composed of three distinct kinases activated in sequence, and despite the fact that many separate MAP kinase families exist, there is limited crosstalk between these highly homologous cascades. Although many of the steps of the ERK cascade have been elucidated, the mechanisms by which the components of the MAP kinase cascade come into physical contact have not been

investigated. In this context it is interesting to note that there are multiple upstream regulators of ERK in the hippocampus: NE, DA, nicotinic ACh, muscarinic ACh, histamine, estrogen, serotonin, brain derived neurotrophic factor (BDNF), NMDA receptors, metabotropic glutamate receptors, AMPA receptors, voltage-gated calcium channels, reactive oxygen species, various PKC isoforms, PKA, nitric oxide (NO), NF1, and multiple ras isoforms and homologs. Adapted from Sweatt. (Sweatt, 2004)

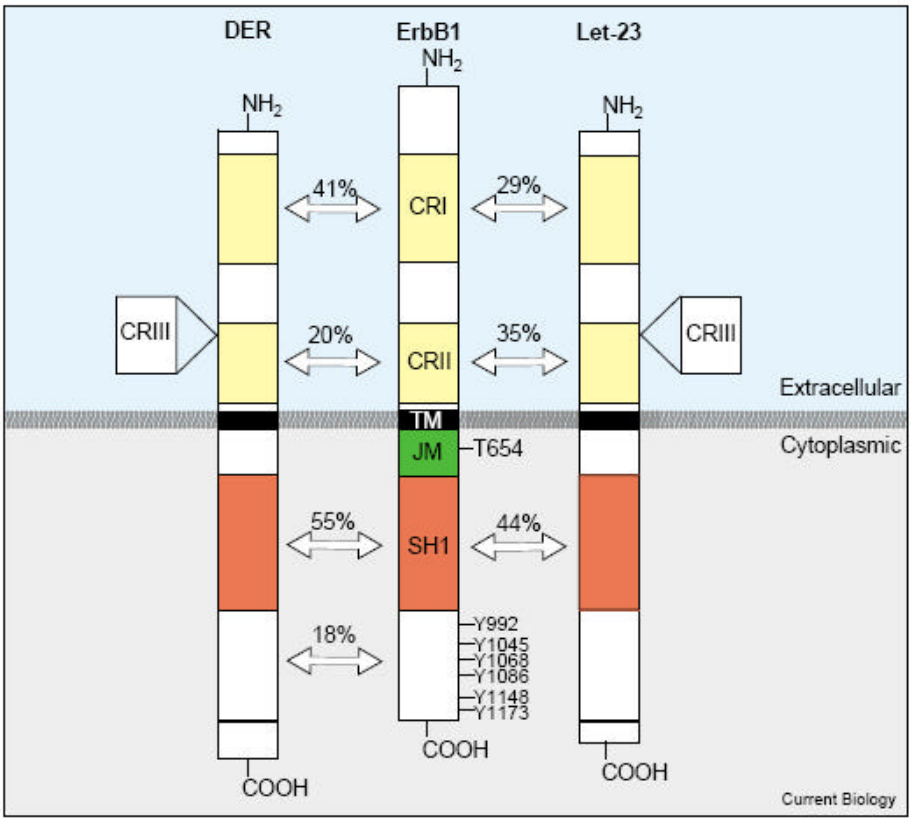

Figure A.3 EGFR structure. The EGFR is a monomer consisting of 1186 amino acids. Its extracellular domain is characterized by two cysteine-rich motifs (CR I and CRII). DER and Let-23 have an additional CRIII domain. The overall amino acid identity between the human ErbB1 and Let-23 proteins is 29% and that between DER and ErbB1 is 38%. Amino acids in EGFR that can be phosphorylated are indicated. TM, transmembrane domain; JM, juxtamembrane region. Adapted from Bogdan and Klambt. (Bogdan and Klambt, 2001)

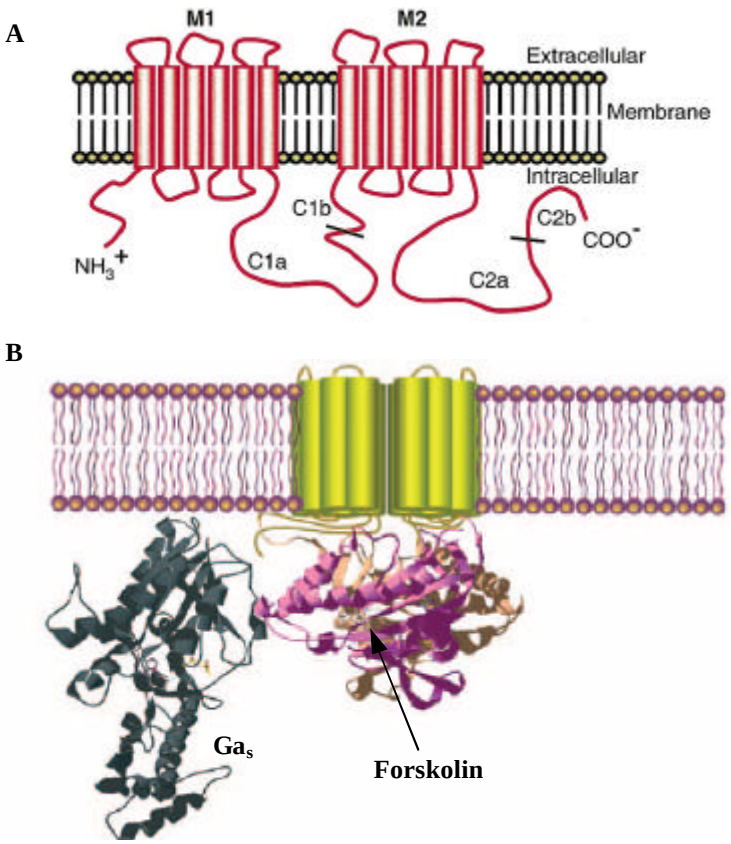

Figure A.4. Schematic and 3D topography of membrane-bound AC. **(A)** the putative topology of AC isoforms. The location of the major cytosolic regions C1 and C2 are shown in reference to the whole molecule. M1 and M2 denotes the regions in the AC molecule which span the membrane 6 times each. Adapted from Patel et al. (Patel et al., 2001) **(B)** Structure of membrane-bound mammalian adenylyl cyclase bound to the activator Ga_s. Illustration of the crystal structure of the catalytic domain of adenylyl cyclase bound to Ga_s and superimposed onto the membrane-spanning region of mammalian adenylyl cyclase. Ga_s •GTP?–S in its activated form is demarcated in gray. The cyclase domains, C1 (tan) and C2 (mauve) interact and form the binding sites for forskolin and the substrate, ATP. Adapted from Tesmer et al. and Sunahara et al. (Sunahara et al., 1997; Tesmer et al., 1997)

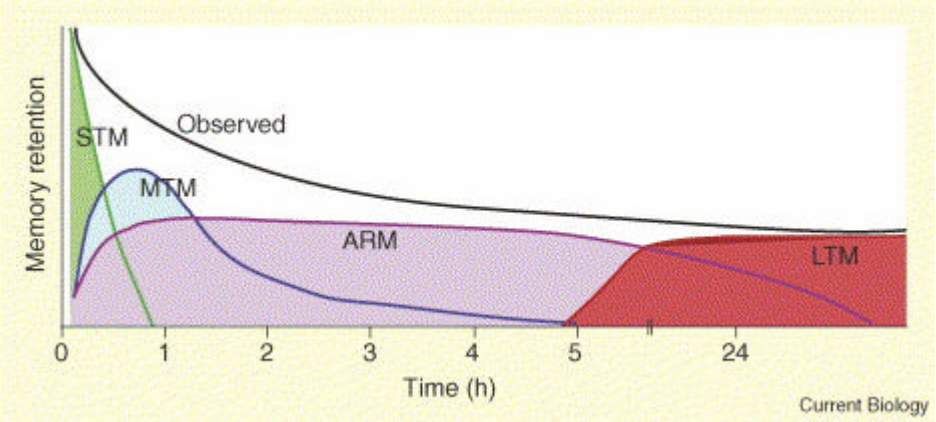

Figure A.5. Dissection of memory phases. At the behavioral level, the observed decay of memory appears relatively seamless (black). In *Drosophila*, at least four mechanistically distinct phases have been described. These phases are short-term memory (STM; green), middle-term memory (MTM; blue) anesthesia-resistant memory (ARM; purple) and long-term memory (LTM; red). Adapted from Margulies et al. (Margulies et al., 2005)