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14. ABSTRACT Neurofibromatosis type 1 (NFI) is characterized by benign but disfiguring skin tumors, pigmentation defects and learning disabilities, as well as increased risk of brain tumors. The NF1 tumor suppressor protein (neurofibromin) inhibits Ras, a protein that is overactive in a wide variety of human cancers. NF1 also controls levels of cyclic AMP, an important intracellular messenger involved in cell growth and learning. We have used an Affymetrix whole genome chip, containing all 13,500 genes of the fruit fly Drosophila, to identify 93 genes with altered expression patterns in flies that have no NF1 protein compared to normal wild type flies. Importantly, expression of half these genes is restored to normal levels when human NF1 is expressed in flies with no NF1. At least a quarter of these genes are involved in nervous system function or development.					
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INTRODUCTION

Neurofibromatosis type 1 (NF1) is characterized by benign but disfiguring skin tumors, pigmentation defects and learning disabilities, as well as increased risk of brain tumors. The NF1 tumor suppressor protein (neurofibromin) inhibits Ras, a protein that is overactive in a wide variety of human cancers. NF1 also controls levels of cyclic AMP, an important intracellular messenger involved in cell growth and learning. Microarray chips can be used to identify changes in gene expression under particular circumstances, such as within a tumor. We have used an Affymetrix whole genome chip, containing all 13,500 genes of the fruit fly *Drosophila*, to identify 93 genes with altered expression patterns in flies that have no NF1 protein compared to normal wild type flies. Importantly, expression of half these genes is restored to normal levels when human NF1 is expressed in flies with no NF1. At least a quarter of these genes are involved in nervous system function or development.

BODY

Task 1. Identification of genes whose expression pattern is dependent on NF1 function in *Drosophila*

- a. Isolate mRNA from heads of NF1 mutant and wild type flies to probe *Drosophila* whole genome oligonucleotide microarrays. (Completed)**
- b. Finish crossing existing transgenic lines into NF1 mutant and wild type backgrounds (Completed)**
- c. Perform further mutagenesis of human NF1 gene and select transgenic *Drosophila* lines with inserts on second chromosome to cross into NF1 mutant and wild type backgrounds (Completed)**
- d. Isolate mRNA from heads of transgenic flies expressing both wild type and mutant human neurofibromin to probe whole genome oligonucleotide microarrays (Partially completed)**

The microarray analysis was performed using head mRNA from two *Nf1* null mutants, compared with wild type flies (**Task 1a**), as well as for *Nf1* null mutant flies expressing human NF1 in all tissues (**Task 1d**) as detailed below. Transgenic lines carrying additional human NF1 (hNF1) mutant isoforms were established (**Task 1c**). These new hNF1 mutant lines and existing hNF1 mutant lines were crossed into the *Nf1* null mutant background and wild type background (**Tasks 1b & 1c**).

Nf1 mutant flies used in microarray experiments include the *Nf1^{P1}* mutant, which has a deletion of the entire *Nf1* gene, from the first exon onwards, and several downstream genes from the *Enhancer of Split* complex, and the *Nf1^{P2}* mutant that has a P-element inserted in the first intron of the *Nf1* gene. Both mutations abolish NF1 protein production (The et al., 1997). The k33 wild type control line is the parental line that was used to generate the two *Nf1* null alleles by local hopping of a P-element (The et al., 1997). *Nf1* mutant flies expressing human NF1 (hNF1; *Nf1^{P2}*) were generated by crossing arm-Gal4; *Nf1^{P2}* flies (expressing Gal4 in all tissues at all stages) to UAS-hNF1; *Nf1^{P2}* flies and collecting 1st generation progeny (**Figure 1C**, Supporting Data).

Flies of each genotype were raised under identical conditions on standard cornmeal medium at 25°C in a humidified incubator. Flies were collected and frozen in liquid nitrogen at the same time of day to minimize circadian variations, and RNA isolations and chip hybridizations were performed in parallel for all genotypes in each replicate (n=5, except *Nf1^{P1}* n=3). For each independent RNA isolation heads were separated from ~1000 bodies per

genotype on dry ice using a 710 mm sieve, then ground in a liquid nitrogen chilled pestle prior to total RNA extraction with Trizol (Invitrogen). Messenger RNA was isolated using magnetic beads (Dyna) and double stranded cDNA was prepared using a Superscript II kit (Invitrogen). Biotin-labeled cRNA probes were prepared using an Enzo-Bioarray kit, then fragmented prior to hybridizing to a Gene Chip microarray at 55°C overnight. The chips were washed at high stringency in a Fluidics Station and scanned.

We proposed to perform microarray analysis of Nf1 mutant flies expressing four existing mutant human NF1, and six additional mutant human NF1 as detailed in **Table 1**. Previously established transgenic lines containing four different mutant human NF1s (L847P, R1276P, K1423E and L1932P) were crossed into the Nf1 mutant and wild type backgrounds. Three additional mutants (S818A, 991delM, and R1391S) were created by site directed mutagenesis and verified by PCR and sequencing. Two other proposed mutants (1658delIY, and 2366delNF) were not made due to failure of site directed mutagenesis. Creation of a third proposed mutant (R1391K) was not attempted. Transgenic lines were established for S818A and R1391S and crossed into the Nf1 mutant background. The 991delM construct proved refractory to transformation and no insertions were recovered after two separate rounds of embryo injections. The location of the six point mutations that were generated and successfully introduced into transgenic flies is diagrammed in **Figure 1A** (Supporting Data).

Table 1: Transgenic *Drosophila* lines carrying mutant human NF1 genes.

Amino Acid Change	Clinical Occurrence	Functional Effect	Number of Fly Lines	Expression Confirmed	Body Size	Learning	MAPK Activity
S818A	0	remove PKA site	3	RT-PCR	rescue	nd	nd
L847P	2	unknown	2	RT-PCR	rescue	rescue	rescue
991delM	2	unknown	0	nd	nd	nd	nd
R1276P	3	disable GAP activity	4	RT-PCR	rescue	rescue	no rescue
R1391S	1	reduce GAP activity	2	RT-PCR	rescue	rescue	no rescue
R1391K	0	increase Ras affinity	0	nd	nd	nd	nd
K1423E	5	reduce GAP activity	3	RT-PCR western	rescue	rescue	no rescue
1658delIY	1	unknown	0	nd	nd	nd	nd
L1932P	2	unknown	4	RT-PCR	rescue	rescue	rescue
2366delNF	4	unknown	0	nd	nd	nd	nd

Nf1 mutant flies have smaller body size, reduced adenylyl cyclase (AC) activity, increased MAP Kinase (MAPK) activity, and learning defects when compared to wild type flies (Guo et al., 1997; 2000; The et al., 1997, Tong et al., 2002). Before assaying the effect of normal and mutated human NF1 on the expression of genes in Nf1 mutants (**Task 1d**), we felt it was important to first establish their effect on the phenotypes seen in Nf1 mutant flies. During the reporting period, we examined the ability of the six human NF1 point mutations (**Figure 1A**, Supporting Data) to rescue these phenotypes as summarized in **Table 1** and detailed below. Expression of normal and mutant human NF1s was controlled using the yeast Gal4-UAS

system. The crosses performed to generate flies expressing the constructs in all tissues, or just in the nervous system are shown in **Figures 1B & 1C** (Supporting Data). Expression of the constructs was confirmed using RT-PCR and in some instances Western blots, as summarized in **Table 1**. Detailed methods describing the assays for body size, MAPK activity, AC activity and olfactory learning can be found in the attached publications (Ge et al., 2004; Hannan et al., 2006).

Body size defects in Nf1 mutant flies can be rescued by activation of the cAMP pathway (The et al., 1997). Expression of all six point mutants in Nf1 mutant flies was able to significantly rescue body size defects in both males and females, as shown here for representative lines for each transgenic construct (**Figure 2**, Supporting Data). Rescue is only partial even with full length human NF1 but all point mutants rescue to a similar extent as normal human NF1 (**Figure 2**, Supporting Data). The three established S818A transgenic lines are all X-linked so we were unable to assay males expressing the S818A point mutation. It will be necessary to generate additional lines with S818A inserted on the second or third chromosome in order to fully assess the effect of this mutation on body size and other phenotypes, particularly since expression of X-linked genes is subject to dosage compensation. A comparable but more extensive analysis of body size for normal human NF1 and four point mutants (R1276P, R1391S, K1423E, L847P) has recently been published (Hannan et al., 2006).

Phosphorylation of MAPK is a direct indicator of Ras pathway activation. Briefly, it is assayed by probing Western blots with antibodies to phosphorylated and unphosphorylated MAPK, then comparing the ratio of phospho-MAPK to total MAPK. NF1 is a Ras-GAP that promotes the hydrolysis of Ras-GTP to Ras-GDP, thereby inactivating Ras. Since Nf1 mutants have increased levels of active Ras-GTP, the levels of phospho-MAPK are greatly elevated in the mutants (Williams et al., 2001). We established that expression of normal human NF1 in Nf1 mutant flies is able to significantly reduce phospho-MAPK levels (**Figure 3**, Supporting Data). Expression of NF1 point mutants that disrupt GAP activity (R1276P, R1391S, K1423E) is unable to reduce phospho-MAPK levels, while constructs with point mutations outside the GRD region (L847P, L1932P) can reduce phospho-MAPK to a similar extent as normal human NF1 (**Figure 3**, Supporting Data). The S818A mutant was not assayed for reasons discussed above. Data for normal human NF1 and four of the point mutants (R1276P, R1391S, K1423E, L847P) has recently been published (Hannan et al., 2006).

AC activity is controlled by a number of different neurotransmitters acting through G-protein coupled receptors in both mammals and flies. We have recently established a novel alternate pathway for AC activation in flies that is stimulated by growth factors such as EGF and TGF α and is dependent on both Ras and NF1 GAP activity (Hannan et al., 2006). AC activity was assayed in the larval nervous system of Nf1 mutants expressing normal and mutant human NF1 (**Figures 4A & 4B**, Supporting Data). Expression of the three NF1 point mutants that disrupt GAP activity (R12776P, R1391S, K1423E) is unable to support EGF stimulation of AC (**Figure 4A**, Supporting Data). Neurotransmitter stimulated AC activity is normal in these larvae, and comparable to that seen in larvae expressing full length human NF1 and in wild type controls (**Figure 4A**, Supporting Data). Expression of normal human NF1 and the non-GRD mutants (L847P, L1932P) in larvae can restore both EGF and neurotransmitter stimulated AC activity (**Figures 4A & 4B**, Supporting Data). S818A has not been assayed. The data for normal human NF1 and four point mutants (R1276P, R1391S, K1423E, L847P) is published (Hannan et al., 2006).

Olfactory learning deficits in Nf1 mutant flies can also be rescued by activation of the cAMP pathway (Guo et al., 2000). A preliminary analysis of olfactory learning in Nf1 mutants

expressing normal human NF1 and five point mutants was conducted during the reporting period. Significant, yet partial, rescue of learning defects was observed for all constructs when expressed globally in all tissues (**Figure 5A**, Supporting Data), as well as when expression was restricted to nervous system tissues (**Figure 5B**, Supporting Data). We have since conducted a more comprehensive analysis of both learning & memory defects in flies expressing these constructs, showing that the cAMP pathway is required for learning while the Ras pathway is required for long term memory (Ho et al., accepted).

As described above we have completed microarray expression profiling for the Nf1 mutant flies, and for Nf1 mutants expressing normal human NF1 (**Task 1d**). We felt that it would be impractical to also perform microarray analysis on each of the six lines expressing human NF1 point mutants. To gain the required statistical power an additional 45-50 analyses (n=5 for each genotype plus genetic controls) would be required at a cost of ~\$500 each which seemed prohibitive. Since we would be performing Quantitative Real Time PCR (QPCR) for the already identified candidate NF1-regulated genes, in order to confirm their altered expression profiles (see below), it was decided to restrict the analysis of the mutant human NF1 lines to QPCR of the confirmed candidate NF1 regulated genes.

Task 2. Analysis and confirmation of microarray data

- a. Perform hierarchical and K-means cluster analysis of expression profiles to identify candidate genes whose expression is linked to NF1 function (Partially Completed)**
- b. Confirm alterations in candidate gene expression patterns using quantitative RT-PCR and Northern analysis (Not Completed)**

During the statistical analysis phase of this project our colleagues at Cold Spring Harbor Laboratory developed and rigorously tested an algorithm to analyze their own Affymetrix Drosophila microarray data. We adopted this approach since it offered a much more statistically powerful approach than the current commercially available software for hierarchical and K-means cluster analysis, such as Gene Spring. Average difference values were calculated using Affymetrix software. These values were then normalized to the mean average difference and subjected to BoxCox transformation using a two-way ANOVA, and the statistical significance was then calculated by bootstrapping, as described by Dubnau and colleagues (Dubnau et al., 2003), except they used and n=10 while for our microarrays n=5.

This statistical analysis reveals 53 transcripts that are consistently up-regulated in *Nf1* mutants ($p < 0.05$), and 40 transcripts which are consistently down-regulated ($p < 0.05$). Most importantly, more than half of these transcripts (29/53, 18/40) are restored to wild type levels by expression of a normal human NF1 transgene in *Nf1* mutant flies. Amongst those genes with known or inferred function are 24 genes potentially involved in nervous system function. The mean average difference values for these genes (compared with k33 controls) are diagrammed in **Figure 6** (Supporting Data). There are several serine/threonine kinases (PKA-C1, PKCd, PK17e), a tyrosine kinase (cad96ca), two EGF-like proteins (dsd, CG31665), some G-protein coupled receptors (Rh5, 5HT2, NepYr), a Ca²⁺ channel (trpl), and a number of transcription factors with functions in nervous system development (tfl1b, unc4, sima). Thirteen of these transcripts are restored to wild type levels by expression of human NF1 in *Nf1* mutant flies (**Figure 6**, Supporting Data). Other known genes include various metabolic enzymes (15), proteases (10), transporters (10), bacterial defense proteins (5) and other enzymes (4), with no immediately obvious link to NF1 or nervous system function. In addition there are 25 genes with as yet unknown function.

Several difficulties were encountered when performing QPCR, including a lot of failed negative controls. Due to lack of time this phase of the proposal was not completed during the reporting period (**Task 2b**). A reliable QPCR protocol has since been established and is currently being utilized for further studies of these genes with the support of Army Grant W81XWH-04-1-025 to Dr Hannan.

KEY RESEARCH ACCOMPLISHMENTS

1. Microarray data collected from wild type control flies, Nf1 mutant flies and human NF1 transgenic flies
2. Microarray data analysed statistically to identify 93 genes with significant changes in expression levels
3. Genes with nervous system function targeted for further study
4. Transgenic lines expressing mutant human NF1 established and assayed for effect on body size, learning, AC activity and MAPK activity.
5. Novel pathway identified for growth factor stimulated AC activity dependent on Ras and NF1.

REPORTABLE OUTCOMES

Presentations:

- Hannan, F (November 2002). Neurofibromin (NF1) and Ras Regulate Adenylyl Cyclase (AC) and Learning in *Drosophila*. Biochemistry Department, Latrobe University, Melbourne, Australia.
- Hannan, F, Guo, H-F, Ho, I, Zhong, Y (June 2003). A *Drosophila* Model for Neurofibromatosis Type I. National Neurofibromatosis Foundation Annual Meeting, Aspen, Colorado USA.
- Hannan, F (September 2003). *Drosophila* Model for Neurofibromatosis Type I (NF1). Department of Cell Biology and Anatomy, New York Medical College, Valhalla, New York, USA.
- Zhong, Y (June 2004). Genetic Dissection of Adenylyl Cyclase Stimulation Requiring G α , Ras and Neurofibromin in *Drosophila*. National Neurofibromatosis Foundation Annual Meeting, Aspen, Colorado, USA.
- Hannan, F (June 2005). Direct Activation of Adenylyl Cyclase by NF1 and Ras. Children's Tumor Foundation Annual Meeting, Aspen, Colorado, USA.
- Hannan, F (July 2005) *Drosophila* Model for Neurofibromatosis Type I (NF1). Genetics Department, Monash University, Melbourne, Australia.
- Zhong, Y (February 2006). *Drosophila* Models. Children's Tumor Foundation Learning & Memory Meeting. Los Angeles, California, USA.

Funding applications:

- US Army 2003. Functional Analysis of Human NF1 by Expression in *Drosophila*. (Hannan)
- NIH R01 2003. Functional Analysis of Human NF1 Mutations in *Drosophila* (Hannan)
- Sinsheimer Award 2004. Functional Analysis of Human Neurofibromatosis Type I (NF1) Mutations in *Drosophila* (Hannan)
- US Army 2004. Functional Analysis of Human NF1 in *Drosophila* (Zhong)
- McKnight Scholar Award 2005. Dual Roles for NF1 in Learning Versus Memory (Hannan)
- Brain Tumor Society 2005. Neurofibromin (NF1) Regulated Genes (Hannan)
- Sontag Foundation 2005. Neurofibromin (NF1) Regulated Genes (Hannan)
- Whitehall Foundation 2005. Dual Roles for NF1 in Learning Versus Memory (Hannan)

Employment opportunities:

Frances Hannan:

Lecturer, Latrobe University, Melbourne, Australia

Assistant Professor, New York Medical College, Valhalla, NY USA

Lecturer, Level C, Monash University, Melbourne, Australia

CONCLUSIONS

The identification of 93 candidate NF1-regulated genes (NRG) by microarray analysis is an important first step for future investigations of the interactions between NF1 and these NRGs. Further comprehensive analysis of these genes is ongoing in our laboratory and the laboratory of Dr Frances Hannan. Reliable QPCR protocols have been established and confirmation of the initial microarray data is ongoing. This work is now being supported by US Army Grant W81XWH-04-1-025 to Dr Hannan.

Studies of the mutant human NF1s generated during this study have identified a novel pathway for growth factor stimulated regulation of NF1 that is dependent on NF1 and Ras (Hannan et al., 2006), and have identified distinct regions of the NF1 protein that control learning versus long-term memory in *Drosophila* (Ho et al., accepted, *J.Neurosci.*). Having defined the effect of the point mutations on the phenotypes of Nf1 mutants, we are now in a strong position to use QPCR to readily identify differences in expression of the candidate NRGs that are related to activation of the Ras versus cAMP pathways. We expect to publish a manuscript based on these analyses in the future. This new information regarding molecules and pathways downstream of neurofibromin function, may eventually lead to novel targets for treatment strategies in NF1 patients.

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- Ge X, Hannan F, Xie Z, Feng C, Tully T, Zhou H, Xie Z, Zhong Y. (2004). Notch signaling in *Drosophila* long-term memory formation. *Proc Natl Acad Sci U S A*, 101(27):10172-10176.
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- Ho, I. S.*, Hannan, F.*, Guo, H.-F., Hakker, I., Zhong, Y. (accepted, *J.Neurosci.*). Distinct functional domains of NF1 regulate immediate versus long-term memory formation.
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Williams, J.A., Su, H.S., Bernards, A., Field, J., and Sehgal, A. (2001). A circadian output in *Drosophila* mediated by neurofibromatosis-1 and Ras/MAPK. *Science* 293(5538):2251-2256.

APPENDICES

Appendix 1. Supporting Data.

Figure 1. NF1 Point Mutants and Crossing Schemes.

Figure 2. Rescue of Body Size.

Figure 3. Restoration of MAPK Activity.

Figure 4. Rescue of Growth Factor and Neurotransmitter Stimulated AC Activity.

Figure 5. Rescue of Learning.

Figure 6. Candidate NF1-Regulated Genes with Potential Nervous System Function.

Appendix 2. Manuscripts.

1. Ge X, Hannan F, Xie Z, Feng C, Tully T, Zhou H, Xie Z, Zhong Y. (2004). Notch signaling in *Drosophila* long-term memory formation. *Proc Natl Acad Sci U S A*, 101(27):10172-10176.
2. Hannan, F. *, Ho, I. *, Tong, J.*, Zhu, Y., Nurnberg, P. & Zhong, Y. (2006). Effect of Neurofibromatosis Type I mutations on a novel pathway for Adenylyl Cyclase activation requiring Neurofibromin and Ras. *Hum. Mol. Genet.*, 15, 1087-1098.

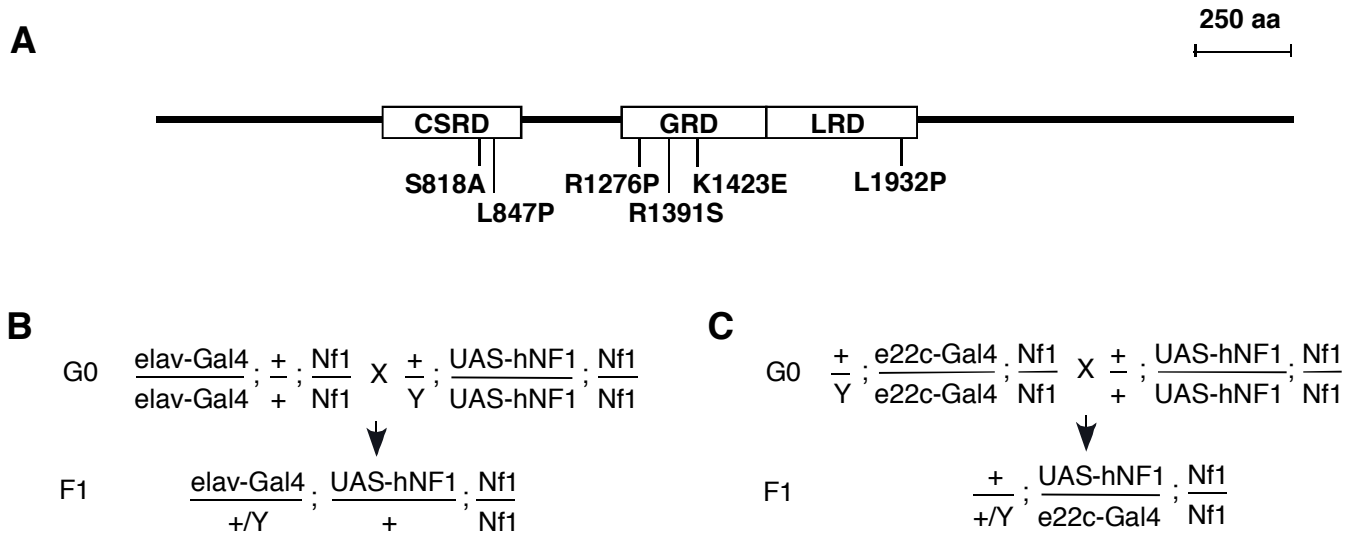


Figure 1. NF1 Point Mutations and Crossing Schemes. (A) Location of human NF1 missense mutations relative to Cys-Ser Rich Domain (CSRD), GAP Related Domain (GRD), and Leu Rich Domain (LRD). Crosses to express human NF1 constructs: (B) in nervous system alone using the elav-Gal4 X chromosome driver, or (C) in all tissues using e22c-Gal4 or arm-Gal4 second chromosome drivers.

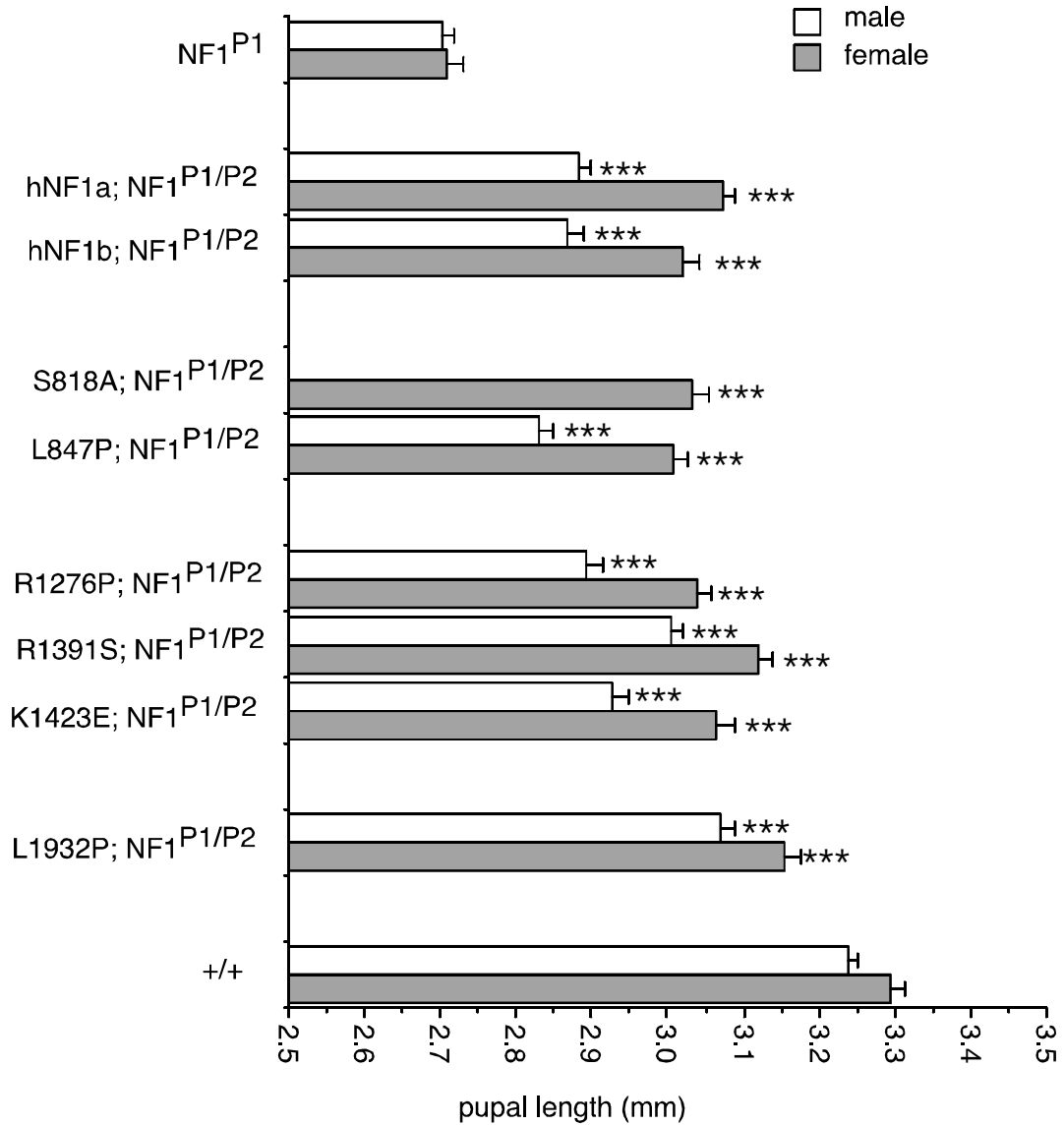


Figure 2. Rescue of Body Size. Expression of normal and mutant human NF1 in the nervous system using the elav-Gal4 driver significantly rescues body size compared to NF1^{P1} mutant, but does not fully restore body size to K33 wild type (+/+) level (n=50, ***p<0.0005).

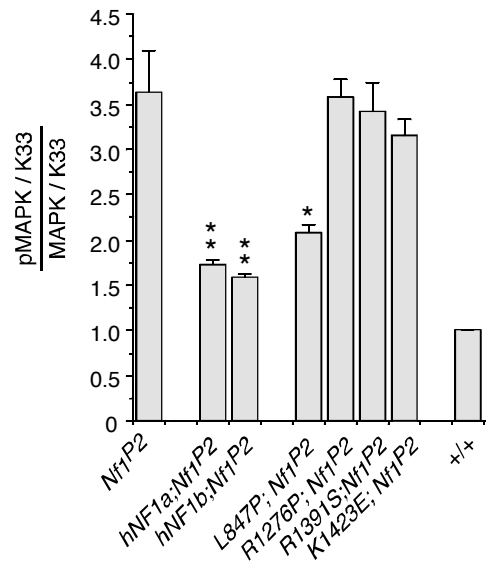


Figure 3. Restoration of MAPK Activity. Expression of normal human NF1 (hNF1a, hNF1b) and the L847P mutant can significantly reduce phospho-MAPK in Nf1 mutants towards wild type (+/+) levels (* $p < 0.05$, ** $p < 0.01$). In contrast, the three GRD domain mutants (R1276P, R1391S and K1423E) cannot reduce phospho-MAPK levels (n=4 to 6). Expression in all tissues using e22c-Gal4.

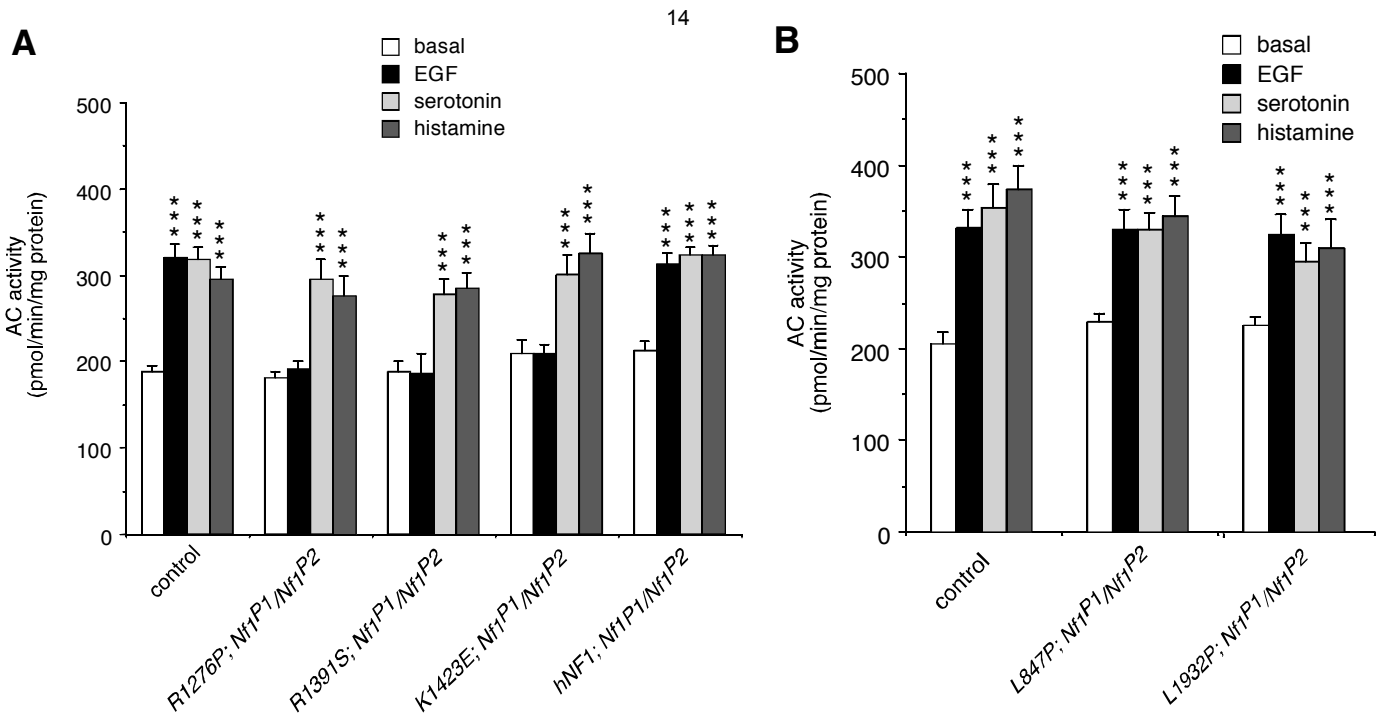


Figure 4. Rescue of Growth Factor and Neurotransmitter Stimulated AC Activity. Normal and mutant human NF1 were expressed in the nervous system using the elav-Gal4 driver. **(A)** The three GAP domain mutants (R1276P, R1391S, K1423E) are unable to rescue EGF stimulated AC activity, but neurotransmitter stimulated AC activity is normal and comparable to full length human NF1 (hNF1) and wild type controls. **(B)** The L847P and L1932P mutants rescue both EGF and neurotransmitter stimulated AC activity. Values are mean \pm SEM (n=4, ***p<0.001).

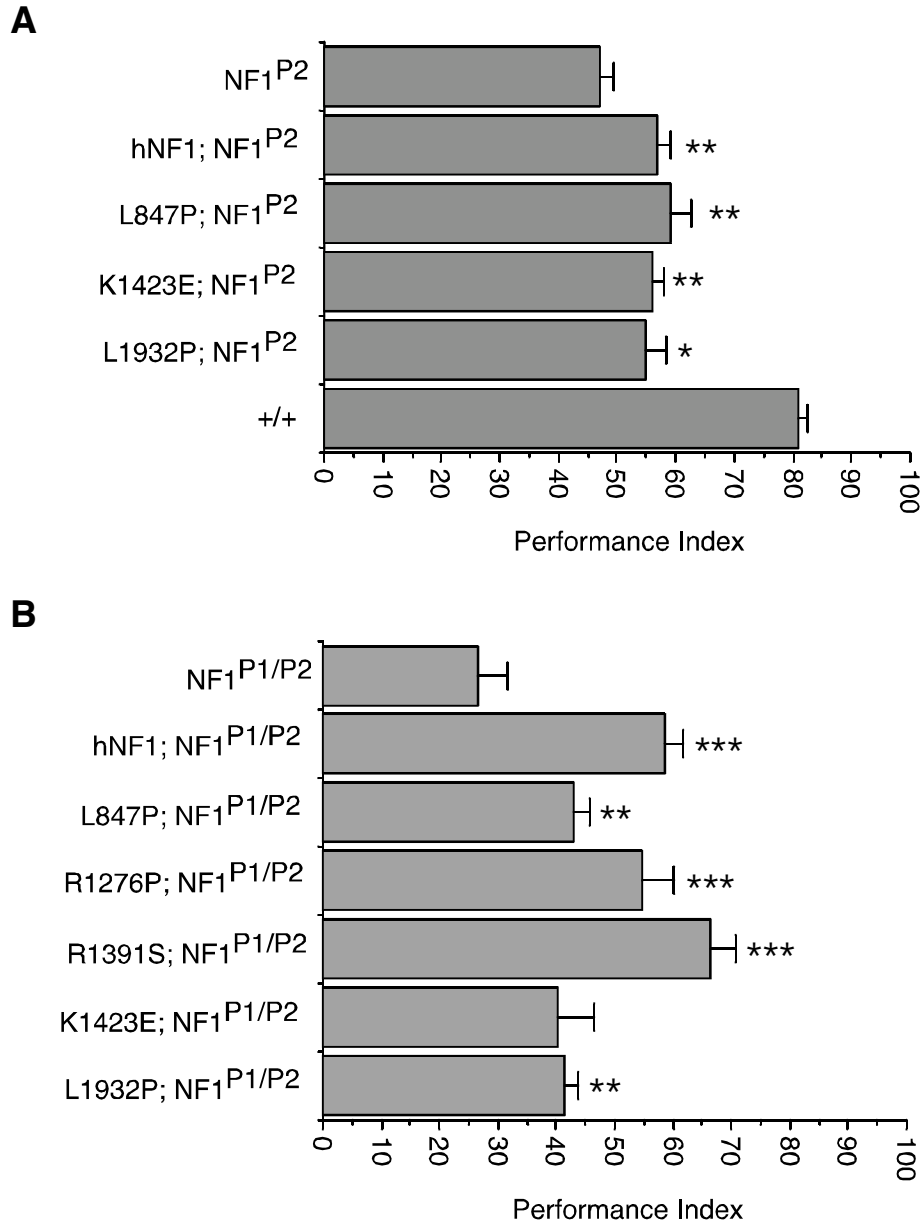


Figure 5. Rescue of Learning. Expression of normal and mutant human NF1 significantly rescues learning as compared to NF1^{P2} or NF1^{P1/P2} mutant controls, but does not fully restore learning to wild type (+/+) levels (n=8, ***p<0.0001, **p<0.001 *p<0.01). Expression in all tissues using arm-Gal4 driver (**A**) or using nervous system specific elav-Gal4 driver (**B**).

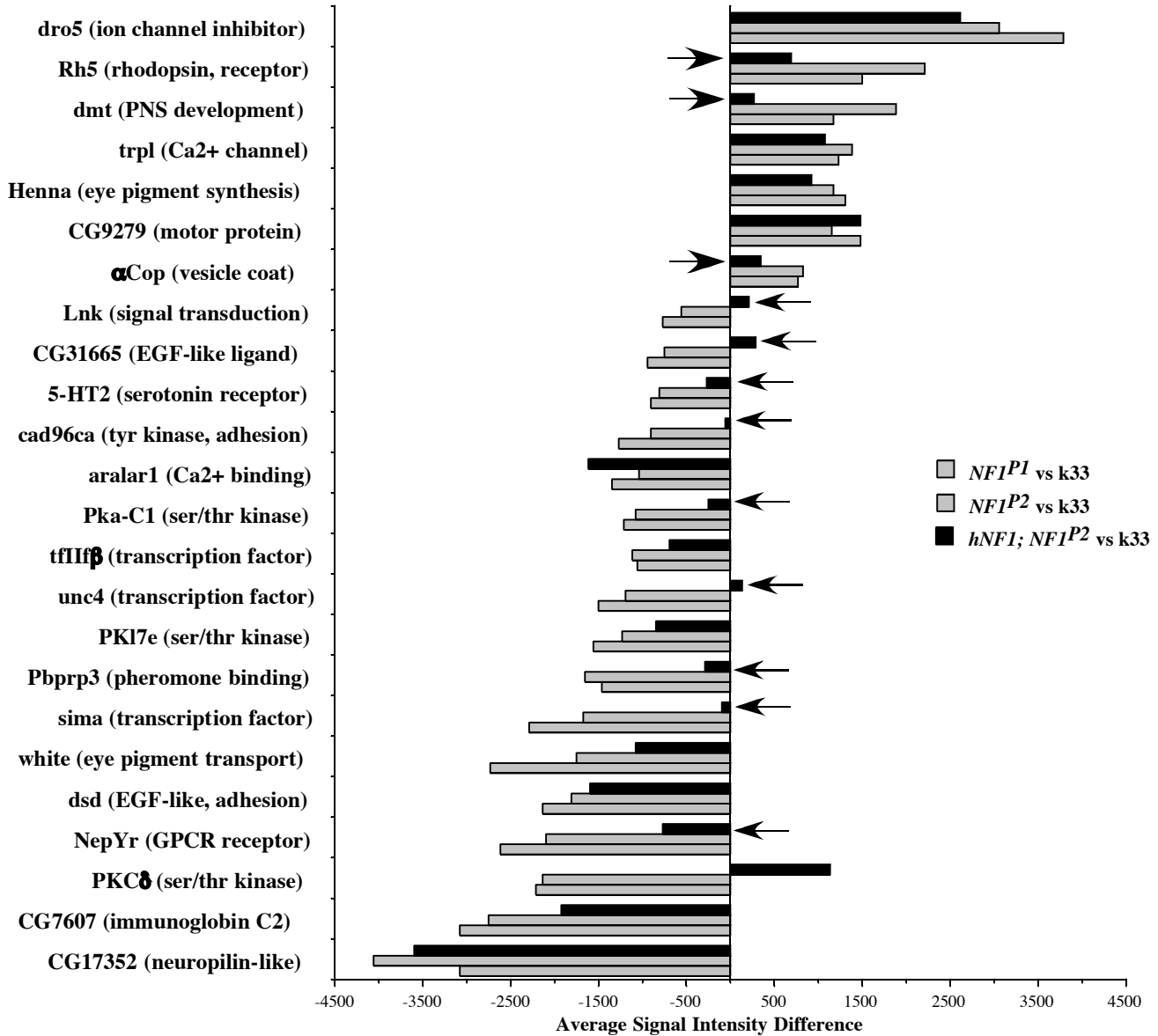


Figure 6. Candidate NF1 Regulated Genes with Potential Nervous System Function. The expression profiles of 24 genes were significantly ($p < 0.05$) altered when comparing NF1 mutant flies to wild type (k33) flies. Expression levels of 13 genes were restored to wild type levels (arrowheads) by transgenic expression of human NF1 (hNF1) in NF1 mutant flies. (n=3 for *NF1^{P1}*; n=5 otherwise).

Notch signaling in *Drosophila* long-term memory formation

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Notch (N) is a cell surface receptor that mediates an evolutionarily ancient signaling pathway to control an extraordinarily broad spectrum of cell fates and developmental processes. To gain insights into the functions of N signaling in the adult brain, we examined the involvement of N in *Drosophila* olfactory learning and memory. Long-term memory (LTM) was disrupted by blocking N signaling in conditional mutants or by acutely induced expression of a dominant-negative N transgene. In contrast, neither learning nor early memory were affected. Furthermore, induced overexpression of a wild-type (normal) N transgene specifically enhanced LTM formation. These experiments demonstrate that N signaling contributes to LTM formation in the *Drosophila* adult brain.

Activation of Notch (N) receptors has been linked to the specification of many cell types in both vertebrates and invertebrates (1). Binding of ligands such as Delta (2) causes cleavage of the intracellular domain of the N protein (3–5). The cleaved cytoplasmic domain of N (Nintra) enters the nucleus, in which it regulates expression of target genes (1). Intercellular communication mediated by N signaling consists of two different modes: lateral inhibitory signaling and inductive signaling. A prototypic example of lateral inhibitory signaling is that loss of N function causes a cluster of equivalent proneural cells to all assume the default neuronal fate rather than an epidermal fate (6). Inductive N signaling, on the other hand, mediates interactions between cells that are nonequivalent before the signal initiates, such as induction of cone cells by photoreceptor cells and specification of midline cells in the embryo (7–9).

In contrast to the extensive understanding of the vital role of N in development, the significance of N signaling in adult brains has yet to be revealed, although there is continuous presence of N protein and its ligands in the adult vertebrate nervous system (10). It has been reported that N signaling regulates neurite outgrowth in mammals (11), and chronic reduction of N activity in adult fruit flies also leads to progressive neurological syndromes (12). Processing of N requires γ -secretase activity, whereas presenilin (PS) is a critical component of the γ -secretase complex (13). Mutations in the PS genes are associated with the early onset of Alzheimer's disease (14). Conditional knockout of the *PS1* gene in mice is also associated with reduced clearance of hippocampal memory traces (15). Although involvement of N in these PS mutant phenotypes remains to be determined, it has been shown that N plays a role in PS-mediated formation of neural projections in postmitotic neurons necessary for learned thermotaxis in *Caenorhabditis elegans* (16). In the present study, we examined the role of N signaling in learning and memory in *Drosophila*.

A Pavlovian procedure that pairs odors with foot-shock (17) was used in this study to assess the effects of N signaling on adult behavioral plasticity. Genetic analyses have demonstrated that memory formation after such Pavlovian training occurs in functionally distinct temporal phases (18). Two of these memory phases, short-term memory and middle-term memory, are labile

and short-lived, whereas another two phases, anesthesia-resistant memory (ARM) and long-term memory (LTM), are resistant to various disruptive treatments and persist for several days. LTM and ARM have been dissected genetically. Disruptions of two transcription factors, cAMP-response element-binding protein (CREB) or alcohol dehydrogenase factor-1, abolish LTM without affecting ARM (19, 20). Conversely, ARM is disrupted but LTM is normal in *radish* mutants and in transgenic flies expressing a dominant-negative form of atypical protein kinase C designated PKM (18, 21, 22). Given these observations and the established role for N signaling in gene regulation (1), we focused our experiments on LTM formation in adult flies carrying a conditional mutation of N or expressing N transgenes.

Materials and Methods

Fly Stocks. *N^{ts2}* were obtained from the Bloomington Stock Center (Bloomington, IN) and were outcrossed with FM7 balancer flies for five generations. The presence of the *N^{ts2}* mutation was confirmed by embryonic lethality at restrictive temperature (30°C). Transgenic flies, heat-shock N⁺ (*hs-N⁺*) and *hs-N Δ ^{cdc10rpts}* were gifts from M. Young's laboratory at The Rockefeller University (New York) (4, 23) and were outcrossed for five generations with our standard wild-type strain, *w¹¹¹⁸(isoCJI)* (19). For all behavioral analyses, *w¹¹¹⁸(isoCJI)* serves as the control.

Pavlovian Learning and Memory. The training and testing procedures were the same as described in refs. 17–19. Briefly, a group of \approx 100 flies was sequentially exposed to two odors (60 s for each with 45 s of rest in between). During exposure to the first odor, flies were simultaneously subjected to electric shock (twelve 1.5-s pulses with 3.5-s intervals, 60 V); this constitutes a single training cycle. To measure "learning," flies were transferred immediately after training to the choice point of a T maze and forced to choose between the two odors. For 24-h memory, flies were subjected to multiple spaced training sessions (1, 2, or 10 training sessions with a 15-min rest between each) or to massed training (10 sessions with no rest interval). After training, flies were transferred to food vials and stored at 18°C for 24 h before testing for their distribution in the T-maze arms. The performance index (PI) was calculated on the basis of the distribution of flies in each arm (17). A PI of 0 represented a 50:50 distribution, whereas a PI of 100 represented 100% avoidance of the shock-paired odor. For determining PIs in *N^{ts2}* at restrictive temperatures, flies were incubated at 30°C for

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Abbreviations: N, Notch; PS, presenilin; ARM, anesthesia-resistant memory; LTM, long-term memory; CREB, cAMP-response element-binding protein; hs-N, heat-shock N; PI, performance index; rp, ribosomal protein.

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2 days, trained, stored during the retention interval, and tested at 30°C.

Sensorimotor Responses. Odor-avoidance responses were quantified by exposing naive flies to each odor (octanol or methylcyclohexanol) versus air in the T maze. After 120 s, the number of flies in each arm of the T maze was counted, and the PI was calculated for each odor individually as reported (17).

The ability to sense and escape from electric shock was quantified by inserting electrifiable grids into both arms of the T maze; shock pulses were delivered to one of the arms. Flies were transported to the choice point of the T maze, allowing them to choose between the two arms. After 60 s, the center compartment was closed, trapping flies in their respective arms. Individual PIs were calculated as for odor acuity. For determining PIs in *N^{ts2}*, flies were incubated at 30°C for 2 days, and the measurements were also carried out at 30°C.

Drug Feeding. The drug-feeding protocol is the same as described in ref. 18. Flies were fed 35 mM cycloheximide in 5% glucose dissolved in 3% ethanol at 25°C for 12 h before heat-shock treatment. After heat-shock treatment, drug was fed for 3 h before training and for 24 h at 18°C after training during the retention period.

Heat-Shock Treatment. Flies subjected to heat shock were placed in empty vials in a water bath that was maintained at 37°C for 30 min. Flies then were transferred back to bottles with food and rested for 3 h at room temperature (20–24°C) before the training sessions. To minimize leaky expression for the groups not subjected to heat shock, flies were incubated at 18°C overnight before training, which was performed at room temperature.

RT-PCR of Induced N Expression. Flies were raised at either 18 or 25°C. One group of flies was shifted from 18 to 37°C for 30 min and returned to 18°C for 3 h before freezing flies at –70°C. Total RNA was prepared from 200 mg of frozen flies by using the RNeasy minikit (Qiagen, Valencia, CA), and mRNA was isolated by using the Dynabeads mRNA Direct microkit (Dyna, Madison, WI). Three independent RNA preparations were made for each temperature condition. First-strand cDNA was synthesized directly from the mRNA by using the Superscript first-strand synthesis system (Invitrogen). PCR amplification with a 25:1 mixture of *Taq* DNA polymerase (Invitrogen) and *Pfu* DNA polymerase (Stratagene) was carried out in Invitrogen 10× PCR buffer using 1.5 mM MgCl₂ with 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by extension at 72°C for 10 min. Three pairs of N-specific primers were designed against regions of the *Drosophila* N cDNA encoding the intracellular domain (GenBank accession no. NM_057511) (24). Primers N1 (5'-gatccaattgtcaggataac-3') and N2 (5'-gaatgtaccgcttcggtat-3') amplify a 285-bp fragment between bases 6722 and 7004; primers N3 (5'-agcgaatggagtegggtcccg-3') and N4 (5'-gaattgctctgctgtgtggca-3') amplify a 268-bp fragment between bases 7932 and 8197; primers N5 (5'-ctcggaggcctggagtctggttc-3') and N6 (5'-ggatagctatccaacgtttggac-3') amplify a 256-bp fragment between bases 8501 and 8754. The N3–N4 and N5–N6 primer pairs span regions of genomic DNA that contain introns, thus allowing distinction between cDNA products and potential genomic contaminants. Lack of genomic DNA contamination was confirmed by the absence of any PCR products when RT-PCR was carried out in control reactions without reverse transcriptase. Ribosomal protein 49 (rp49)-specific primers (5'-atgaccatccgccagcatac-3' and 5'-gagaacgcaggcagcctgtgg-3') were designed to amplify a 391-bp fragment between bases 1 and 391 of the rp49-coding region (GenBank accession no.

Table 1. Sensorimotor responses of different genotypes

Genotype (temp)	OA (MCH)	OA (OCT)	SR
<i>w¹¹¹⁸(isoCJ1)</i>	70 ± 3	69 ± 1	85 ± 2
HS- <i>w¹¹¹⁸(isoCJ1)</i>	73 ± 4	67 ± 3	84 ± 2
<i>N^{Δcdc10rpts}</i>	76 ± 4	74 ± 4	83 ± 1
HS- <i>N^{Δcdc10rpts}</i>	75 ± 2	76 ± 2	83 ± 2
<i>hsN⁺</i>	74 ± 5	73 ± 4	83 ± 1
HS- <i>hsN⁺</i>	70 ± 5	71 ± 5	83 ± 2
<i>w¹¹¹⁸(isoCJ1)</i> (30°C)	58 ± 6	42 ± 9	52 ± 7
<i>N^{ts2}</i> (30°C)	50 ± 9	35 ± 8	39 ± 11

There is no significant difference for “task-relevant” sensorimotor responses among all comparisons between the control and experimental groups except olfactory acuity for octanol between HS-*w¹¹¹⁸(isoCJ1)* and HS-*N^{Δcdc10rpts}*. All experiments were conducted at room temperature except those indicated as (30°C). OA, olfactory acuity; MCH, methylcyclohexanol; OCT, octanol; SR, electric-shock reactivity; HS, 37°C heat shock.

Y13939). The control rp49 mRNA should be expressed at equal levels in all cells at all stages (25).

Results

Consistent with the idea that N may affect LTM, we found that alteration of N activity did not affect learning or short-term memory. A temperature-sensitive N allele, *N^{ts2}*, is viable at permissive temperature but embryonically lethal at restrictive temperature (26). As adults, these flies were behaviorally normal (shock reactivity and olfactory acuity) (Table 1) and showed normal learning scores (Fig. 1a) after 2 days of incubation at the restrictive temperature (30°C). Furthermore, learning was also not affected in transgenic flies that express dominant-negative N, *hs-N^{Δcdc10rpts}* (Fig. 1c). Expression of *hs-N^{Δcdc10rpts}* was induced acutely via heat-shock treatment (30 min at 37°C, with 3 h of recovery before training; see *Materials and Methods*). The *N^{Δcdc10rpts}* protein lacks the intracellular domain that diffuses into the nucleus after N activation (4). Without the N intracellular domain, the *N^{Δcdc10rpts}* protein binds ligands normally but is unable to regulate gene expression (27). All mutants tested were outcrossed to control lines to remove potential modifiers.

In contrast, reduction of N activity disrupted 1-day memory. One-day memory after spaced training (10 training sessions with a 15-min rest interval between each) normally is composed of roughly equal amounts of ARM and LTM, whereas 1-day memory after massed training (10 training sessions with no rest interval) is composed only of ARM (18). One-day memory was significantly reduced in *N^{ts2}* mutants at a restrictive temperature (30°C) when compared with the permissive temperature (18°C) (Fig. 1b). There was also a slight but statistically insignificant reduction in memory scores in the control group when comparing the higher temperature with the lower temperature (Fig. 1b). Induced expression of dominant-negative N in *hs-N^{Δcdc10rpts}* flies also significantly reduced 1-day memory after spaced training (Fig. 1d). Flies were given a 30-min heat shock and subjected to spaced training 3 h afterward. To determine which components (i.e., ARM or LTM) were affected, we also assayed 1-day memory after massed training in *hs-N^{Δcdc10rpts}* transgenic flies (Fig. 1d). There was no difference for 1-day memory after massed training, indicating disruption of LTM but not ARM. The heat-shock treatment did not exert any detectable effect on control flies (Fig. 1d). Thus, reduced N activity, resulting either from a conditional mutation or induced expression of a dominant-negative N, specifically blocked the formation of LTM without affecting early memory or ARM.

We then evaluated the effects of overexpression of wild-type N⁺ in *hs-N⁺* transgenic flies. Flies were given a 30-min heat shock and trained and tested 3 h afterward. Overexpression of

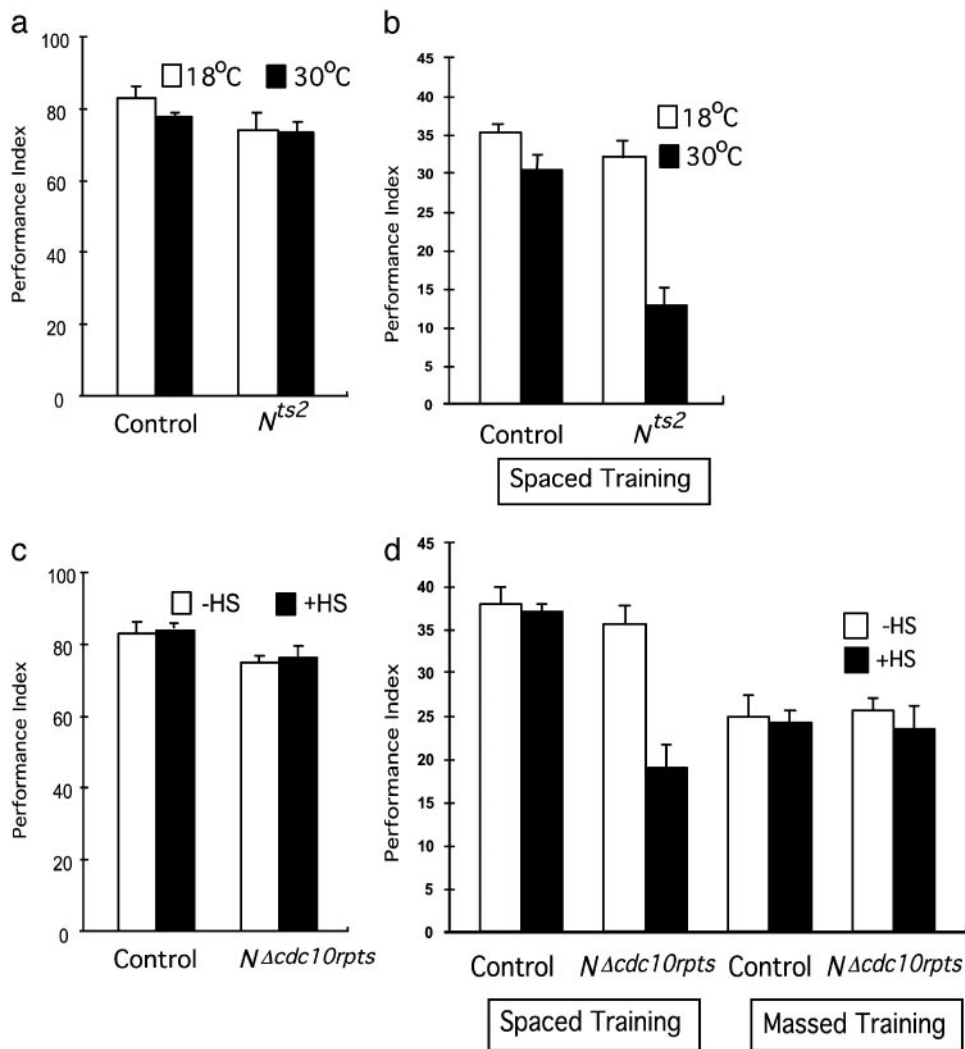


Fig. 1. Blockade of LTM formation by disruption of N signaling. (a) Learning is not affected in temperature-sensitive N mutants. Learning scores were obtained at permissive (18°C) and restrictive (30°C) temperatures (see *Materials and Methods*) for the temperature-sensitive N mutant, *N^{ts2}*, and for control flies. In this and all following figures, the isogenic line, *w¹¹¹⁸(isoCj1)* (19), was used as a control ($n = 7, 2, 8,$ and 7 for data points from left to right). (b) Reduced 24-h memory at the restrictive temperature for *N^{ts2}*. Twenty-four-hour memory was determined after spaced training (10 training sessions with a 15-min rest interval between each). Spaced training induces formation of persistent memory that consists of two distinct components: protein synthesis-dependent LTM and ARM ($n = 4$ for all data points). (c) Learning is not affected by acutely induced expression of a dominant-negative N transgene (*N^{Δcdc10rpts}*). Expression of *N^{Δcdc10rpts}* was induced by 37°C heat shock for 1 h followed by 3 h of rest before training ($n = 6$ for all data points). (d) Blockade of LTM but not ARM by acutely induced expression of dominant-negative *N^{Δcdc10rpts}*. The LTM component of persistent memory induced by spaced training is blocked, but ARM induced by massed (or spaced) training is not affected ($n = 8, 10, 8, 8, 15, 15, 17,$ and 17 for data points from left to right).

N^+ had no significant effect on learning (Fig. 2a), consistent with our observations that disruption of N does not affect learning. Overexpression of N^+ after heat-shock treatment was confirmed by RT-PCR of adult head mRNA (Fig. 2b). Acutely induced overexpression of N^+ also did not affect 1-day memory for flies subjected to the normal spaced training procedure (10 training cycles) (Fig. 3a). However, 1-day memory was enhanced significantly for flies overexpressing N^+ that had only one or two spaced training sessions (Fig. 3a). To characterize the nature of this enhancement in 1-day memory, we showed that enhanced memory elicited by one or two spaced training sessions in flies overexpressing N^+ could be blocked by feeding of cycloheximide, a drug blocking protein synthesis (18), to flies (Fig. 3b). It has been shown that feeding cycloheximide to flies can block formation of protein-synthesis-dependent LTM (18). There was no significant difference for sensory modalities necessary for performing the learning task (Table 1). Thus, overexpression of the N^+ gene facilitates LTM formation.

Discussion

In the present study, our data revealed that one function for N in the adult brain is to mediate the formation of LTM. Three different lines of evidence support this conclusion. First, 1-day memory was reduced in a temperature-sensitive N mutant at the restrictive temperature (30°C) at which N receptor function is supposedly defective. Second, 1-day memory was diminished in

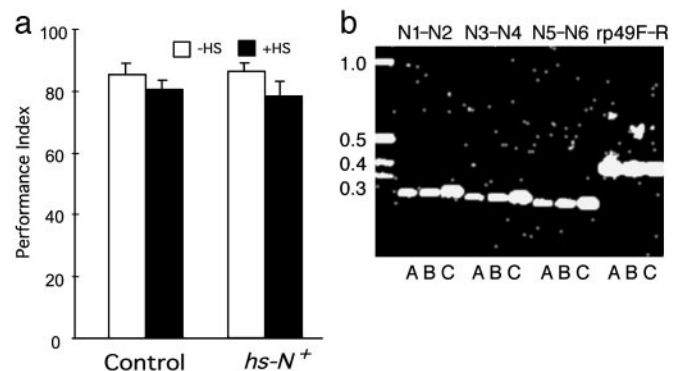


Fig. 2. Learning is not affected by overexpression of wild-type N^+ (*hs-N⁺*). Flies were subjected to 30 min of heat shock at 37°C followed by 3 h of rest. (a) Learning scores were similar for controls and *hs-N⁺* flies regardless of whether they were subjected to heat-shock treatment ($n = 2, 4, 2,$ and 4 for data points from left to right). (b) Heat shock induced expression of *hs-N⁺* cDNA in adult heads. Semiquantitative RT-PCR using N primer pairs N1-N2, N3-N4, and N5-N6 shows induction of the *hs-N⁺* transgene by 30 min of heat shock at 37°C followed by 3 h of rest (lane C) when compared with PCR from control flies that were kept at 18°C (lane A) or 25°C (lane B). The rp49F-R control primers show no temperature-induced increase in expression of rp49. Details of PCR primer pairs and expected products are given in *Materials and Methods*. Three separate mRNA isolations showed the same pattern of increased expression of the *hs-N⁺* transgene after 37°C heat shock.

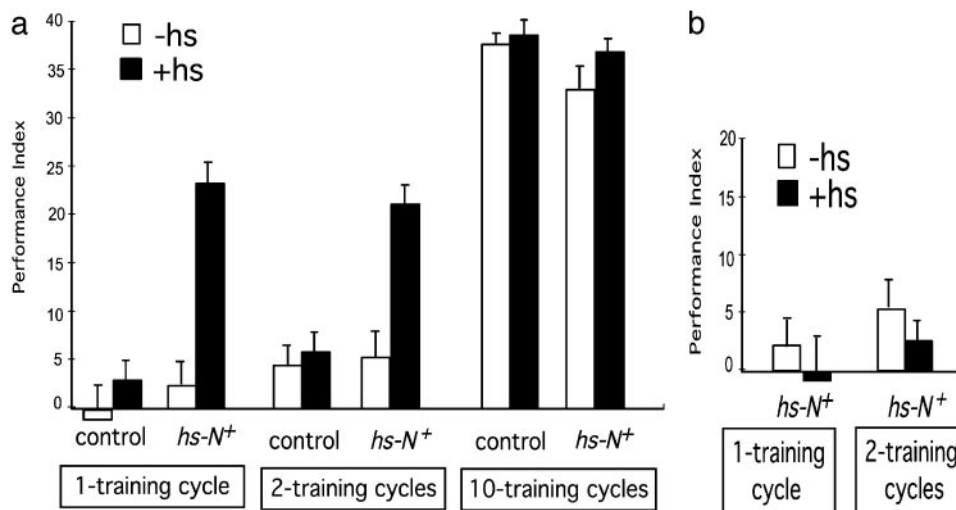


Fig. 3. LTM is enhanced by overexpression of wild-type N⁺ (*hs-N⁺*). (a) Enhanced LTM after acutely induced overexpression of wild-type N⁺. Flies were subjected to the same heat-shock treatment as described above. Twenty-four-hour memory was compared between *hs-N⁺* flies with and without heat shock for 1, 2, and 10 spaced training cycles, respectively ($n = 6, 6, 5, 7, 7, 9, 5, 5, 6, 6, 7$, and 8 for data points from left to right). (b) Blockade of enhanced LTM by an inhibitor of protein synthesis (cycloheximide). Flies were subjected to the same heat-shock treatment as described for a. Twenty-four-hour memory was compared between *hs-N⁺* flies with and without heat shock for one and two spaced training cycles, respectively ($n = 7, 9, 5$, and 5 for data points from left to right; see *Materials and Methods* for drug-feeding conditions).

transgenic flies that express a dominant-negative N $\Delta^{cdc10rpts}$. Third, 1-day memory formation was facilitated by overexpression of N⁺. Although known for its crucial role in development of the nervous system, the use of temperature-sensitive mutations and of transgenic flies carrying inducible genes through acute treatment have allowed us to disassociate the role of N in adult physiology from its role in development. For all experiments that led to the observations noted above, flies were allowed to develop under relatively normal conditions and N function was perturbed acutely at the adult stage before the training. Moreover, we also showed that temporary disruption of N function at the adult stage exerted no significant effects on sensorimotor responses as well as on learning. Thus, the observed effects can be attributed specifically to memory, confirming that 1-day memory can be affected by manipulation of N function. Our finding is corroborated by an independent observation in which 1-day memory is reduced by disturbing N function through the use of RNA interference and *N^{ts1}* loss of function in adult flies (28).

One-day memory consists of both ARM and LTM phases (18). Our observation suggests a specific effect of manipulation of N function on LTM. One-day memory elicited by massed training (ARM only) (18) was not affected by expression of dominant-negative N $\Delta^{cdc10rpts}$, whereas 1-day memory after spaced training (ARM and LTM) was reduced significantly. Consistent with this observation, enhanced memory induced by one or two spaced training sessions was blocked by a drug that inhibits protein synthesis. Such drugs are known to block LTM specifically (18).

Over the last several decades, a large number of genes in *Drosophila* have been identified to affect learning and memory through both forward and reverse genetic approaches (for review see ref. 29). Many signal transduction pathways have been

implicated, such as cAMP, Ca²⁺/CaM-dependent kinase, protein kinase C, and mitogen-activated protein kinase pathways. However, none of the components of the N signaling pathway have been implicated by previous studies, which may reflect a crucial role of N signaling in development for which most mutations in this pathway are lethal and therefore unsuitable for behavioral studies.

It is notable that in flies with overexpression of N⁺, only one training session was required to elicit LTM instead of the usual 10 spaced training cycles. This enhancement is very similar to that reported for CREB (30). Induced expression of *hs-dCREB2-activator* also reduced the number of spaced training sessions, required to yield the maximal level of LTM in normal flies, from 10 sessions to 1 session (30). Future experiments may examine specific roles for the CREB and N pathways in the formation of LTM. Involvement of the CREB pathway in LTM formation is a mechanism observed in a wide range of organisms including *Drosophila*, *Aplysia*, and vertebrates (19, 30–32). The N signaling pathway is also highly conserved evolutionarily (1). In fact, a recent report showed that learning and memory are defective in a heterozygous *Notch1^{+/-}* mouse knockout (33), although a developmental etiology could not be ruled out for such knockout mice. Our results extend this observation and the evolutionary implications thereof by revealing an acute role for N signaling during memory formation.

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Effect of neurofibromatosis type I mutations on a novel pathway for adenylyl cyclase activation requiring neurofibromin and Ras

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Neurofibromatosis type I (NFI) is a common genetic disorder that causes nervous system tumors, and learning and memory defects in humans, and animal models. We identify a novel growth factor stimulated adenylyl cyclase (AC) pathway in the *Drosophila* brain, which is disrupted by mutations in the epidermal growth factor receptor (EGFR), neurofibromin (NF1) and Ras, but not $G\alpha_s$. This is the first demonstration in a metazoan that a receptor tyrosine kinase (RTK) pathway, acting independently of the heterotrimeric G-protein subunit $G\alpha_s$, can activate AC. We also show that $G\alpha_s$ is the major $G\alpha$ isoform in fly brains, and define a second AC pathway stimulated by serotonin and histamine requiring NF1 and $G\alpha_s$, as well as a third, classical $G\alpha_s$ -dependent AC pathway, which is stimulated by Phe-Met-Arg-Phe-amide (FMRamide) and dopamine. Using mutations and deletions of the human NF1 protein (hNF1) expressed in *Nf1* mutant flies, we show that Ras activation by hNF1 is essential for growth factor stimulation of AC activity. Further, we demonstrate that sequences in the C-terminal region of hNF1 are sufficient for NF1/ $G\alpha_s$ -dependent neurotransmitter stimulated AC activity, and for rescue of body size defects in *Nf1* mutant flies.

INTRODUCTION

Mutations in the human *NFI* 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 (1). About 40% of children with NFI exhibit learning deficits (2,3), and mouse models of NFI recapitulate both the tumor and learning phenotypes (4–6). In *Drosophila*, *Nf1* mutations affect circadian rhythms (7), body size (8), responses to neuropeptides (9) and olfactory learning (10). 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 *NFI* mutations. Biochemical and genetic analysis in mammals and *Drosophila* has revealed that NF1 inhibits Ras activity (4–7), and regulates AC activity and cAMP levels (8–13). The NF1 protein has a central GTPase activating protein (GAP)-related domain (GRD), which catalyzes the intrinsic GTPase activity of Ras (14). Many of the tumor phenotypes and learning deficits observed in NFI patients and animal models have been attributed to hyperactivation of Ras, that is observed, for example, in Schwann cells and mast cells (15–18). However, the NF1-regulated AC/cAMP pathway is important for controlling neuropeptide responses (9) and learning (10) in flies, as well as neuropeptide-stimulated AC activity in both flies and mammals (12,13). The NF1-dependent activation of AC versus downregulation of Ras may therefore

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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 (19). Ras signaling is down-regulated by the activity of GAPs, which catalyze the hydrolysis of Ras-GTP to Ras-GDP. Five genes are reported to encode Ras-specific GAPs in *Drosophila* (20). The *Gap1* and *Nf1* genes each encode a GRD that can bind with Ras and catalyze GTPase activity (8,21), however, the *Gap1* protein requires regions outside the GRD to achieve full catalytic activity (22). Guanine exchange factors (GEFs) promote the exchange of GDP for GTP to activate Ras, thereby enabling interaction with downstream effectors such as Raf-1 and PI3 kinase (23,24). GEF activation of Ras is controlled by signaling through RTKs such as *sevenless* and the *Drosophila* EGFR (25–27). Classical genetic studies in *Drosophila* identified the *sevenless* RTK and its GEF *son-of-sevenless* (SOS) through their effect on eye development (25). Mutations in the *Gap1*, *Ras1*, *sevenless* and *EGFR* genes also lead to defects in eye development and embryo patterning (21,25,26). 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 (8).

Our study identifies three distinct AC signaling pathways in the *Drosophila* brain, including a novel growth factor activated NF1/Ras-dependent AC, that remarkably does not require $G\alpha_s$, as well as two separate neurotransmitter-stimulated AC pathways, one requiring NF1 and $G\alpha_s$, whereas the other requires $G\alpha_s$ alone. Analysis of the effect of human NF1 mutations 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-dependent AC signaling but not NF1/ $G\alpha_s$ -dependent AC signaling, whereas part of the C-terminal region is sufficient for NF1/ $G\alpha_s$ -dependent AC signaling and regulation of body size.

RESULTS

NF1 and Ras activate AC

The first indication that Ras may 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 (Fig. 1A). AC activity was also stimulated by human K-Ras (Fig. 1B), but not Rab3a (Fig. 1C), suggesting that activation is specific to the Ras family of small GTPases, and not because of depletion of GTP or other factors. Secondly, this stimulation was shown to be NF1-dependent, as it was eliminated in *Nf1* homozygous null mutant flies, *Nf1^{P1}* and *Nf1^{P2}* (Fig. 1D), which do not express any detectable NF1 protein (8). 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 (Fig. 1D). The acute nature

of the response to NF1 indicates that 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 (28). 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 (Fig. 1E). This effect is specific to the GAP activity of the NF1-GRD fragment, as it is abolished in two NF1-GRD mutants (R1391S; K1419Q; Fig. 1E) with reduced GAP activity, found in NF1 patients (28–30). The NF1-GRD fragment was also unable to stimulate AC activity above control levels in *Ras^{e2F/+}* heterozygotes (Fig. 1F), which have an inactivating mutation in the Switch I region of Ras (25) 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, and that endogenous *Drosophila* Ras can interact with human NF1.

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 EGF or TGF α (Fig. 2A and B). Stimulation of AC activity was abolished in *Drosophila* EGFR mutants (Fig. 2A and B), including the *Egfr^{tl}* hypomorphic mutant and the *Df(2R)Egfr^{18/+}* deficiency heterozygote (31), demonstrating that these growth factors are acting directly on the *Drosophila* EGFR 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 (Fig. 2A and B). The *Ras^{e1B}* mutation affects the Switch II activator/effector domain of Ras (25) that contacts R1391S of NF1. Again, this demonstrates a requirement for both Ras and NF1 in the stimulation of AC activity.

To ensure that there is no crosstalk between EGFR and $G\alpha_s$, we assayed growth factor stimulation of AC in *Gsa^{B19}* hypomorphic mutants (32). Normal levels of stimulation of AC activity by both EGF and TGF α growth factors were seen in larval brains of *Gsa^{B19}* mutants (Fig. 2C and D), consistent with the fact that the *Drosophila* EGFR does not contain the juxtamembrane domain that facilitates crosstalk in vertebrate EGFRs (33). Stimulation by GTP γ S is very low in the *Gsa^{B19}* mutants (Fig. 2C and D), indicating that $G\alpha_s$ is indeed the major stimulatory G-protein in larval brains. Control treatment of larval brains with insulin did not stimulate AC activity (Fig. 2E). Thus, stimulation of AC by both EGF and TGF α growth factors require EGFR, Ras and NF1, but does not involve $G\alpha_s$. The identified ligands for the *Drosophila* EGFR are members of the TGF α family (33). This suggests that stimulation of the Ras/NF1-dependent AC pathway in flies may be activated by binding of endogenous ligands to the EGFR.

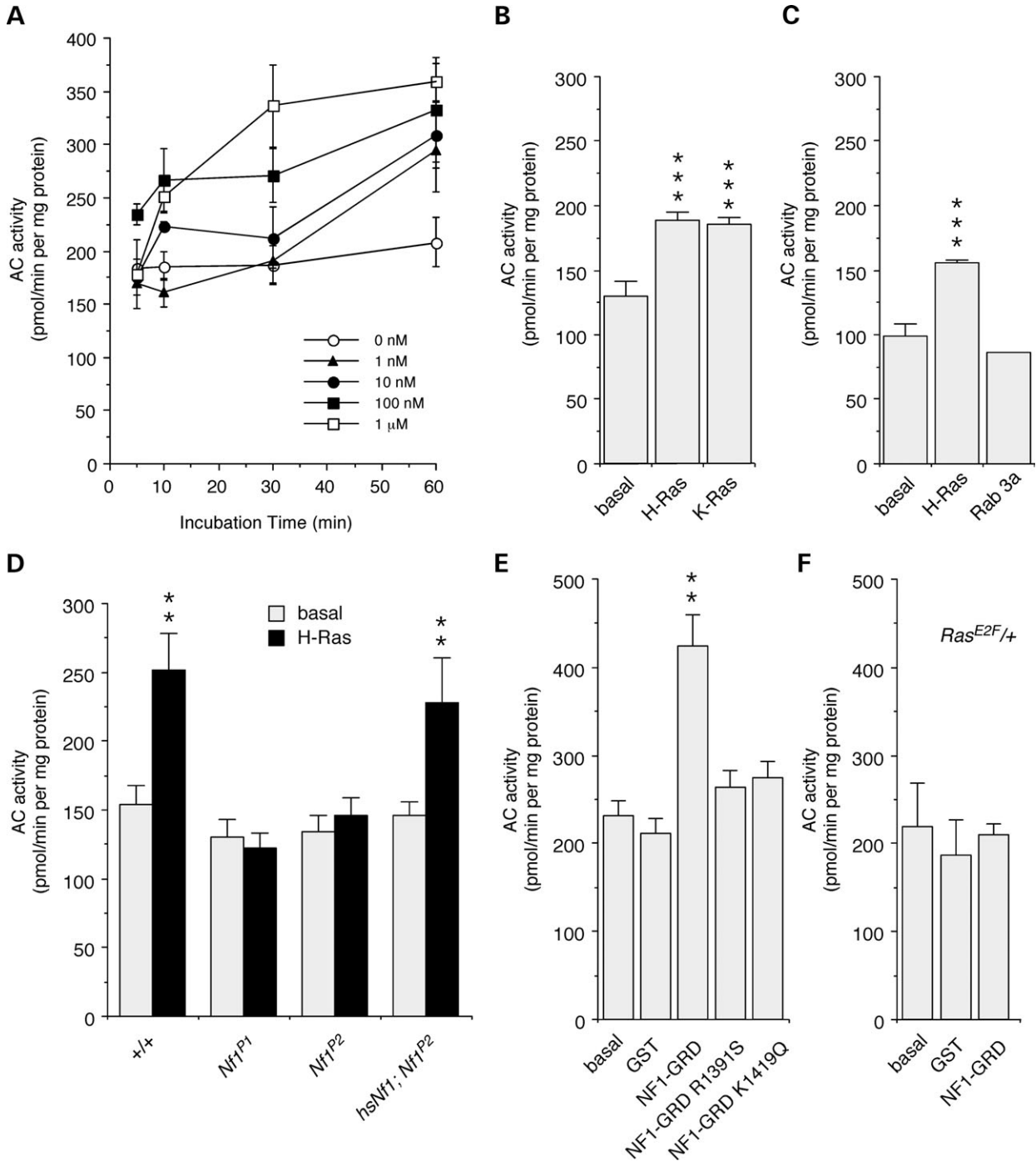


Figure 1. NF1 and Ras activate AC. (A) Significant increases in AC activation were observed after 10 min to 60 min incubation with human H-Ras at different concentrations ($P < 0.05$; $n = 3$). (B) Both H-Ras and K-Ras stimulate AC activity (1 mM; $t = 30$ min; $n = 4$). (C) Rab3a does not stimulate AC activity (1 mM; $t = 30$ 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 (1 mM; $t = 60$ 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 (1 mM; $t = 30$ min; $n = 4$). (F) Stimulation by human NF1-GRD-GST was abolished in *Ras^{E2F/+}* heterozygotes (1 mM; $t = 30$ min; $n = 2$). (A-F) Values are mean \pm SEM (** $P < 0.05$; *** $P < 0.01$).

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 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

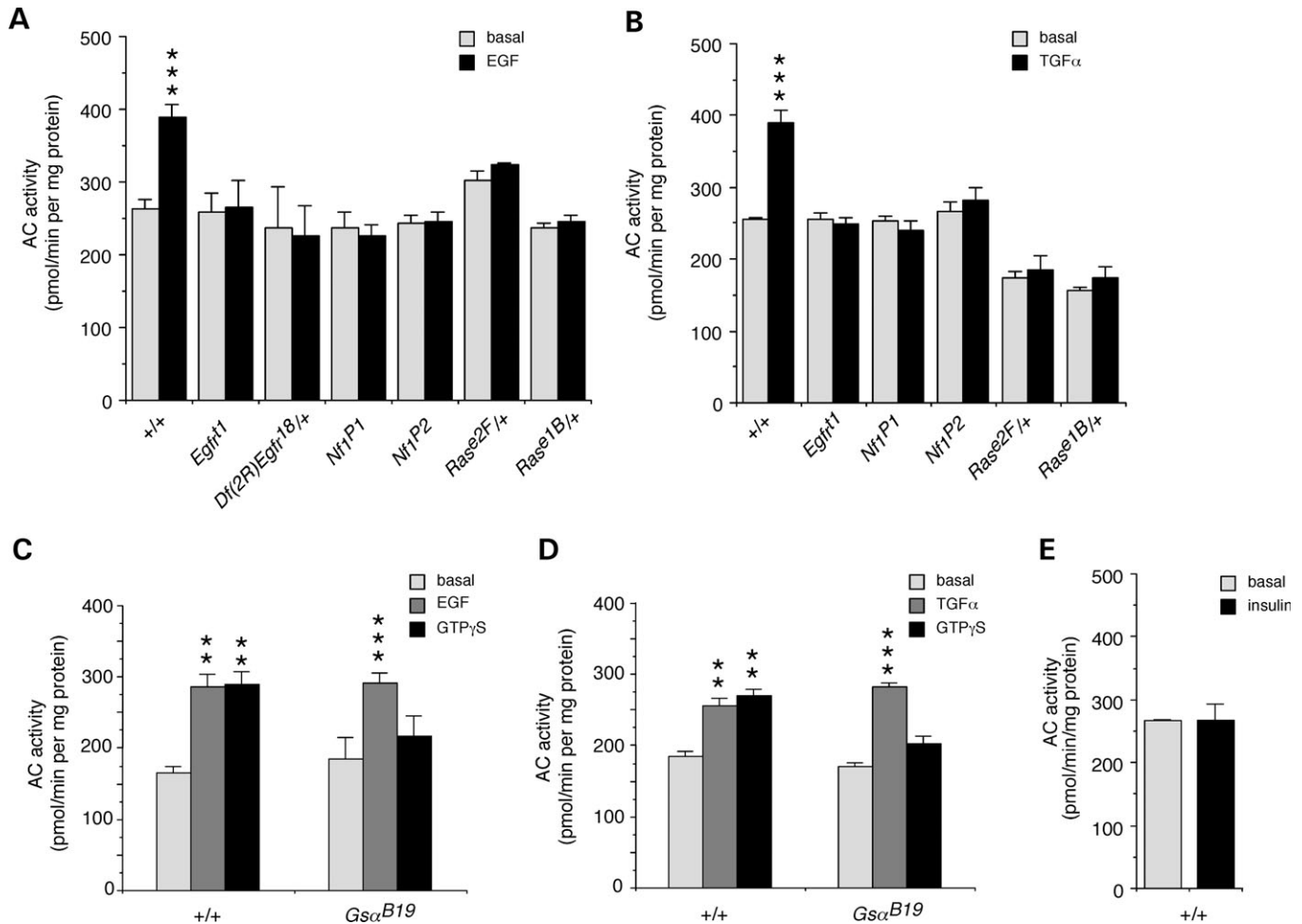


Figure 2. Growth factors stimulate the novel NF1/Ras-dependent AC pathway. (A) AC activity was significantly increased by treatment of larval brains with 2 mM EGF ($n = 18$). This stimulation was abolished in EGFR mutants, *Egfr¹*, and heterozygotes, *Df(2R)Egfr¹⁸/CyO*; in *Nf1* null mutants, *Nf1^{P1}* and *Nf1^{P2}*; and in *Ras* heterozygotes, *Ras^{e2F}/TM3* and *Ras^{1B}/TM3* ($n = 4$). (B) Stimulation of AC by 2 mM TGFα was similarly abolished in the *Egfr¹* mutant, *Nf1* mutants and *Ras* heterozygotes ($n = 4$). Stimulation of AC by 2 mM EGF (C) or TGFα (D) is not affected in a hypomorphic *Gα_s* mutant, *Gsa^{B19}*, whereas stimulation by 20 mM GTPγS is perturbed ($n = 3$). (E) There was no stimulation of AC by 2 mM insulin ($n = 3$). (A–E) Values are mean \pm SEM (** $P < 0.01$; *** $P < 0.005$).

pathway (Fig. 3A and B). Thus, alterations in NF1 or Ras that disrupt growth factor-dependent stimulation of AC activity (Fig. 2A and B), 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 *Gsa^{B19}* mutants but not in *Ras*/+ heterozygotes (Fig. 3C and D), demonstrating an NF1/*Gα_s*-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 that 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 NFI patients and animal models, we examined clinically relevant missense mutations from NFI patients that are scattered throughout the length of the hNF1 protein (34–36), as well as deletions of hNF1. We report here the effect of expressing hNF1 containing four different missense mutations and five partial deletions (Fig. 4A) in the *Drosophila Nf1* mutant background, which were assayed for 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 (Table 1). When assayed in yeast, the GRD domain mutants R1391S and K1423E drastically reduce GAP activity (29,30,37), whereas the R1276P mutant completely abolishes GAP activity (38). Transcription of UAS-*hNF1* transgenes in flies was controlled using Gal4 drivers (39), including the one that is expressed globally (*e22c-Gal4*); (40) and a nervous system specific driver (*elav-Gal4*); (41). 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 (Fig. 4B and C), showing that

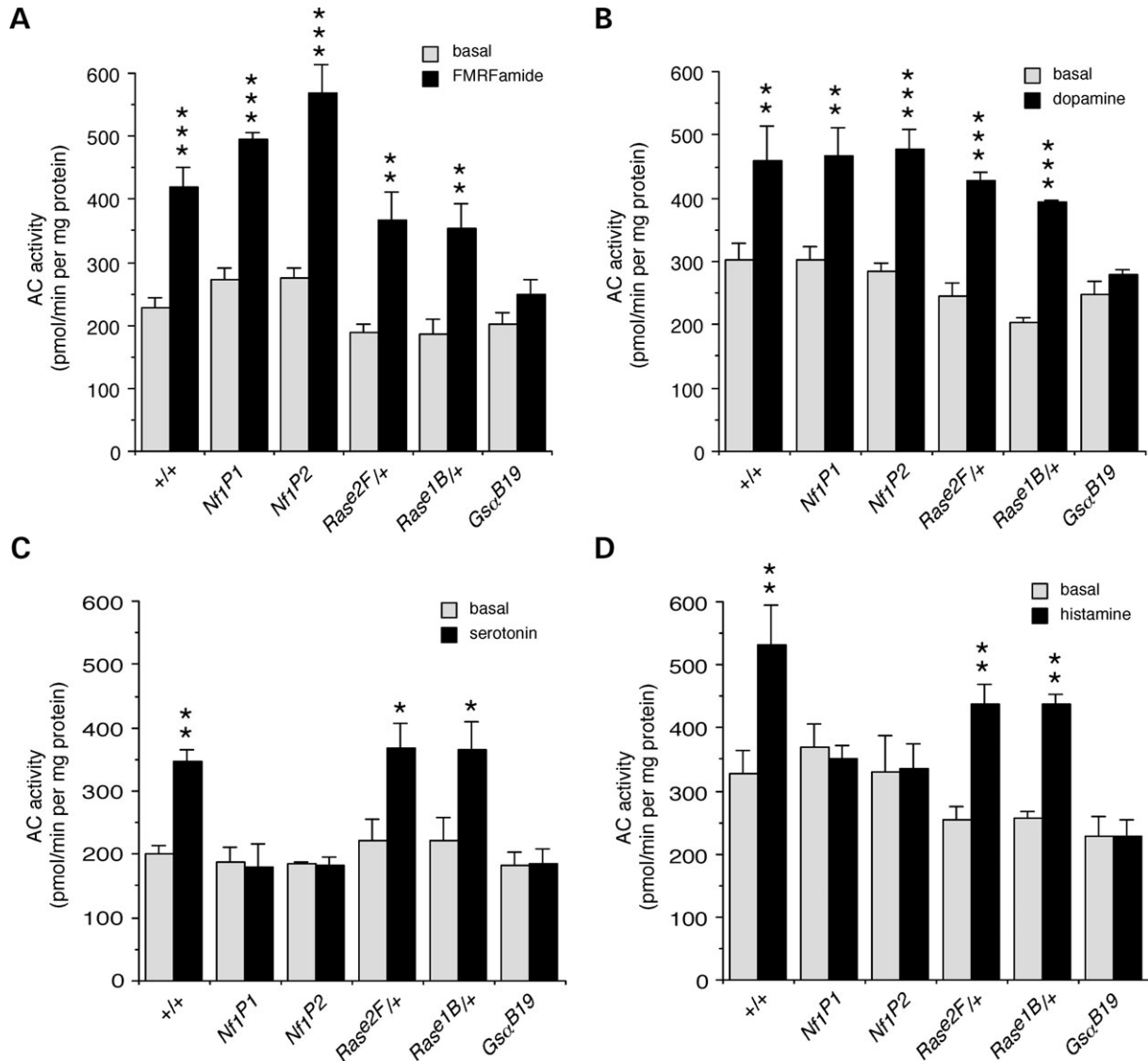


Figure 3. Neurotransmitters and neuromodulators stimulate two additional AC pathways. FMRFamide and dopamine stimulate $G\alpha_s$ -dependent AC: activation of AC by 200 nM FMRFamide (A) and dopamine (B) is disrupted in $G\alpha_s$ mutants, but not in *Nf1* mutants or *Ras* heterozygotes ($n = 3-4$).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

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 because of increased Ras activity (7). 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 (Fig. 4D and E). As expected, mutant hNF1s with defective RasGAP activity (R1276P, R1391S, K1423E) or lacking the GRD (Δ GRD2, Cterm) cannot reduce phospho-MAPK levels (Fig. 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 (Fig. 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 (Fig. 5A and C). However, the L847P mutant and the GRD fragments responded normally to EGF (Fig. 5B and C), 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

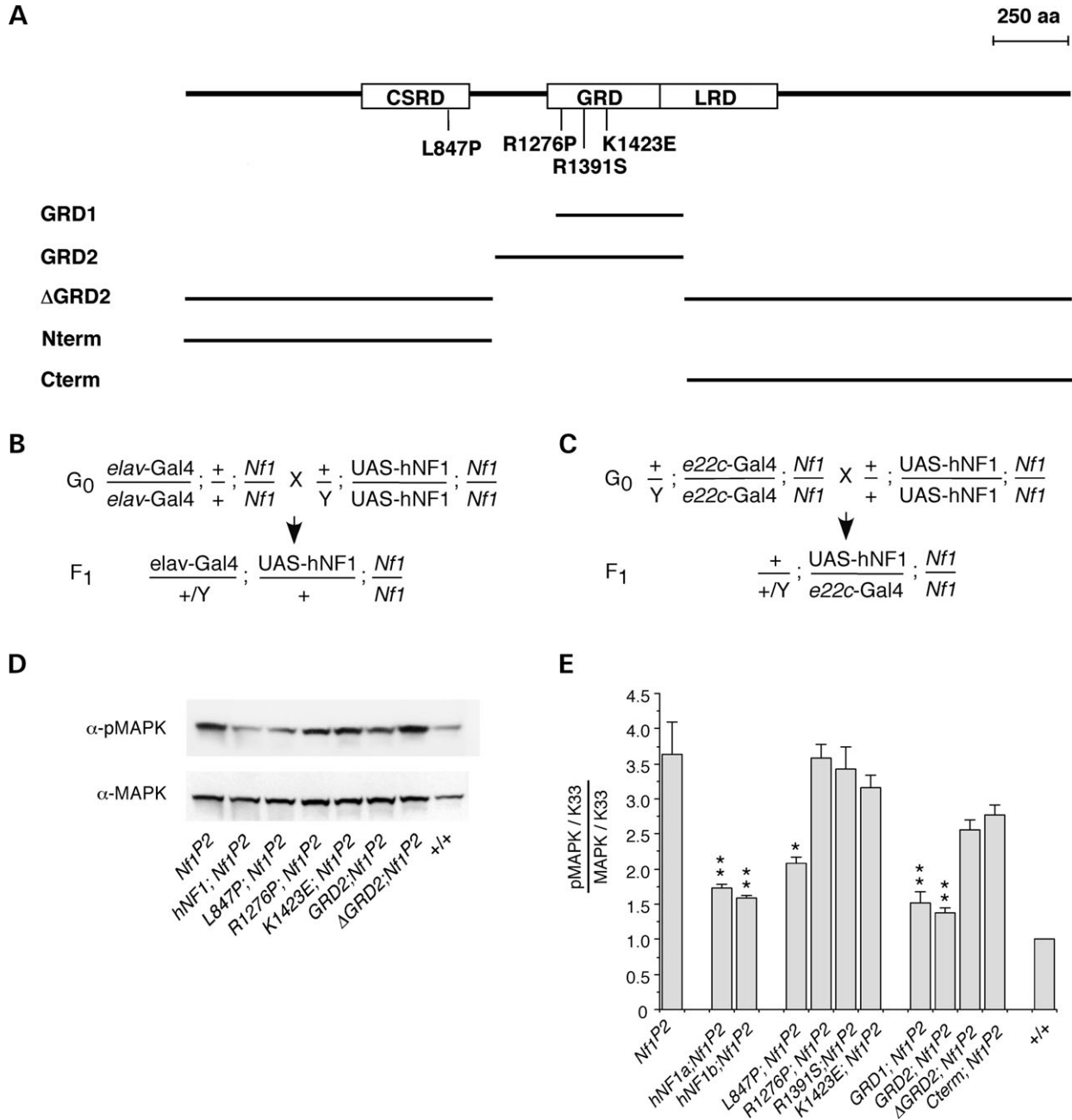


Figure 4. Missense mutations and deletions of human NF1 modulate *Drosophila* MAPK activity. (A) Position of four hNF1 missense mutations, and size of five hNF1 deletion constructs, that have been expressed and analyzed in *Drosophila Nf1* null mutants (CSR, Cys-Ser-rich domain; GRD, GAP-related domain; 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 re-probed 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 Materials and Methods). (D and E) Expression is under control of the *e22c*-Gal4 driver. (E) Values are mean \pm SEM (* $P < 0.05$; ** $P < 0.01$; $n = 4-6$).

for the NF1/Gα_s-dependent AC pathway. Stimulation of AC was normal for mutant hNF1s with or without RasGAP activity (Fig. 5A and B), indicating that NF1/Gα_s-dependent AC activity does not require RasGAP activity. Consistent with this, the GRD fragments alone were not sufficient to restore NF1/Gα_s-dependent AC activity (Fig. 5C). We then asked

whether any other region of NF1 is required for NF1/Gα_s-dependent AC activity. Constructs lacking the GRD (ΔGRD2, Cterm) were able to restore neurotransmitter-stimulated AC activity (Fig. 5C), demonstrating that sequences in the C-terminal region, common to ΔGRD2 and Cterm (Fig. 4A), are essential for NF1/Gα_s-dependent AC activity.

Table 1. Human *NF1* missense mutations expressed in *Drosophila*

Mutation ^a	Amino acids conserved ^b	Effect	Mutagenic primer ^c	Site added	Number of lines
Human NF1	—	Normal	—	—	4
L847P (2)	gFLc <u>AL</u> GGVC	Not known	5'pTGGAGGCACACTCCCCAGGT <u>GCACAAAGGAAGCCAGTC3'</u>	<u>Apa</u> LI	2
R1276P (1)	MQTLFR <u>GN</u> SL	Abolish GAP activity	5'pGGCCAAGCTGTTGCCCGGGAA GAGAGTCTGC3'	<u>Sma</u> I	1
R1391S (1)	mFLR <u>FIN</u> PAI	Reduce GAP activity	5'pGGCAGGATTGATA <u>AA</u> GCTTAG GAACATGGC3'	<u>Hind</u> III	2
K1423E (5)	kLMSK <u>IL</u> QsI	Reduce GAP activity	5'pGATTGGCAATACTCTGCA <u>AG</u> AT <u>CTCGGACATTA</u> ACTTC3'	<u>Bgl</u> II	3

^aNumber of clinical occurrences is bracketed (30,35,38,39).

^bAmino acids that are identical in human, mouse and fly are capitalized and the mutated amino acid is underlined.

^cRestriction site added is underlined and mutated bases are in bold. Primers are complementary to the coding strand of hNF1 and 5'-phosphorylated.

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

In order to confirm the physiological relevance of the NF1/ $G\alpha_s$ -dependent AC activity, and to verify that RasGAP activity is not required, we examined the effect of expressing the hNF1 mutants and deletions on the small body size phenotype previously seen in adult flies (8). This phenotype can be rescued by supplying cAMP, but not by decreasing Ras activity (8). We first showed that normal hNF1 is able to rescue the small body size of males and females using both *elav*-Gal4 and *e22c*-Gal4 drivers (Fig. 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 (Fig. 5E) and neither of the GRD fragments was able to rescue body size (Fig. 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 (Fig. 5F). The L847P mutation in the region upstream of the GRD can still rescue MAPK activity (Fig. 4D and E), AC activity (Fig. 5B) and small body size (Fig. 5E). This mutation may affect other aspects of NF1 function such as regulation or localization, rather than activity.

DISCUSSION

Three separate pathways for AC activation defined in this study are depicted in Figure 6. First, a novel pathway for AC activation, downstream of growth factor stimulation of EGFR that requires both Ras and NF1, but not $G\alpha_s$. Secondly, an NF1/ $G\alpha_s$ -dependent AC pathway operating through the Rutabaga-AC (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 (9,10,12). Thirdly, a classical G-protein coupled receptor-stimulated AC pathway (42) operating through $G\alpha_s$ alone. The AC activated by NF1/Ras (AC-X), or $G\alpha_s$ (AC-Y), has not yet been identified.

This study shows for the first time that Ras can stimulate AC in an NF1-dependent manner in higher organisms, via an RTK-coupled pathway that is independent of the $G\alpha_s$

G-protein. The functionality of human NF1 in the fly system, and the high degree of identity between human and fly NF1 (60%); (8), 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 (43), and in *Xenopus* oocytes (44), however, these cell types may not contain sufficient NF1 to support NF1/Ras-dependent AC activation. This is consistent with our observation that levels of both Ras and NF1 are critical for stimulation of AC activity in adult head membranes. The reported EGF activation of AC in cardiac myocytes and other tissues requires both $G\alpha_s$, and the juxtamembrane domain of the EGFR (45,46), which is not present in the *Drosophila* EGFR (33).

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/ $G\alpha_s$ -dependent AC activity, thus defining for the first time a region outside the GRD that contributes to this pathway. Interestingly, expression of a human NF1 GRD fragment in *Nf1*^{-/-} astrocytes results in only partial restoration of NF1-mediated increases in cAMP levels in response to PACAP (13). Thus, regions outside the GRD also seem to be necessary for activation of AC in these mammalian cells.

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. 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 I.

MATERIALS AND METHODS

Drosophila melanogaster media, strains and heat-shock conditions

Flies were raised at room temperature (22–24°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

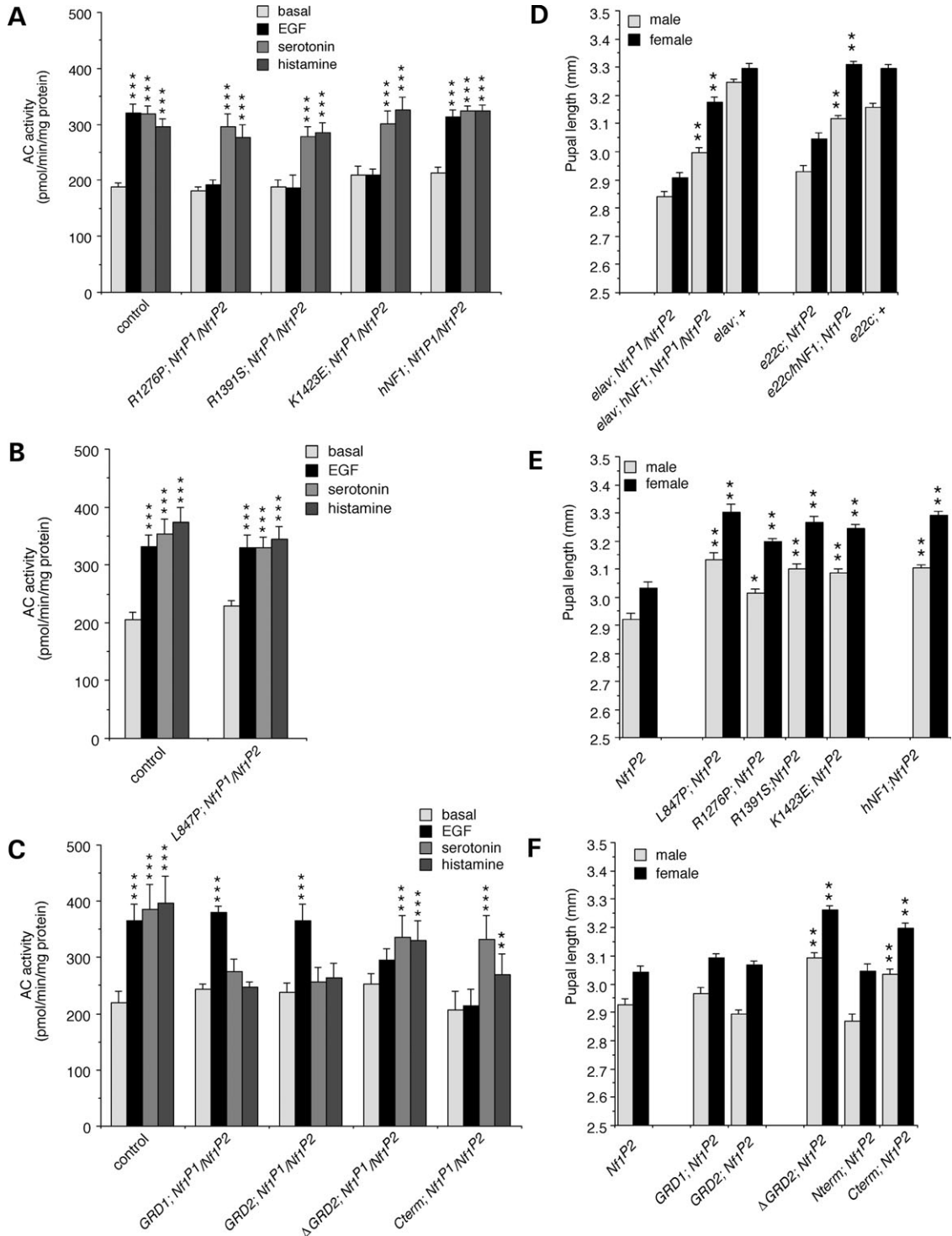


Figure 5. Separate domains of human NF1 mediate activation of different AC pathways. (A) EGF does not stimulate AC activity in flies expressing RasGAP-defective mutant hNF1s (R1276P, R1391S, K1423E), compared with K33 (control) flies or flies expressing normal hNF1, however, serotonin- and histamine-stimulated 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 (Δ 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 with *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 with *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 (Δ GRD2) or a C-terminal fragment (Cterm). (A–C) Expression is under control of the *elav-Gal4* driver, values are mean \pm SEM (** $P < 0.01$; *** $P < 0.001$; $n = 4$). (D–F) Expression is under control of the *e22c-Gal4* driver except where otherwise indicated, values are mean \pm SEM (* $P < 0.01$; ** $P < 0.001$; $n > 50$).

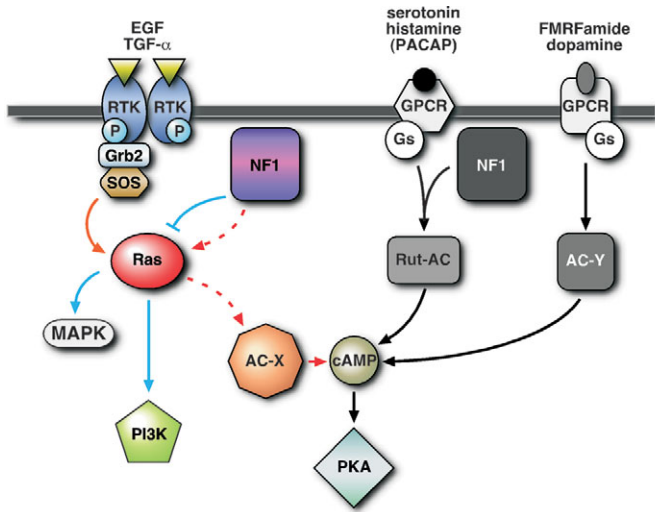


Figure 6. AC can be activated by at least three distinct pathways: First, a novel NF1/Ras-dependent pathway stimulated by growth factors such as EGF and TGF α that activates an unidentified AC (AC-X), and does not involve G α_s ; secondly, an NF1/G α_s -dependent pathway, acting through Rutabaga-AC (Rut-AC), stimulated by serotonin and histamine, and possibly PACAP38 (see discussion), that does not require Ras; thirdly, a classical NF1-independent pathway, involving G α_s but not NF1 or Ras, stimulated by FMRFamide and dopamine that activates an unidentified AC (AC-Y).

from A. Bernards (Massachusetts General Hospital, Boston, MA, USA). K33 flies used as wild-type controls have a P-element inserted 1.5 kb downstream of the *Nf1* locus, that was mobilized to generate the *Nf1*^{P1} and *Nf1*^{P2} null mutant alleles (8). *Nf1*^{P1} deletes most of the *Nf1* gene and several downstream genes from the *Enhancer of Split* locus, whereas *Nf1*^{P2} carries a P-element insertion within the first intron of the *Nf1* gene, and neither allele produces any detectable NF1 protein (8). Heat-shock induction of NF1 was performed at 35°C for 2 h, then flies were rested at 21–23°C for 1 h. The *Ras*^{e1B} and *Ras*^{e2F} mutants are from the *Drosophila* Stock Center (Bloomington, IA, USA). Each has an amino acid substitution in either the Switch II or Switch I effector domains, respectively (25). Both affect Ras activation and binding to downstream effectors and are homozygous lethal. The EGFR mutants are also from the Bloomington Stock Center. *Egfr*¹¹ is a hypomorph and *Df(2R)Egfr*¹⁸ is a homozygous lethal deficiency (31). *Ras*^{e1B}, *Ras*^{e2F} and *Df(2R)Egfr*¹⁸ heterozygotes carrying a balancer (wild-type) chromosome (*TM3* or *CyO*; 47) were used for all assays. *Gs* α ^{B19} is a hypomorphic mutant (32) provided by M. Forte (Vollum Institute, Portland, OR, USA). Gal4 driver lines: *elav-Gal4*/*Nf1*^{P1} (7) was obtained from A. Sehgal (University of Pennsylvania, Philadelphia, PA, USA); *e22c-Gal4* (40) was from N. Perrimon (Harvard Medical School, Boston, MA, USA). White^{118(isoCJ1)} (48) was obtained from T. Tully (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA).

Adult head AC activity assay

The AC activity assay was performed as detailed here, essentially as described by Livingstone (49). About 20 heads/genotype of adult male *Drosophila*, anaesthetized by chilling, were

homogenized in 850 μ l lysis buffer (25 mM Tris–acetate buffer at pH 7.5, 1 mM dithiothreitol, 0.01 mg/ml aprotinin and 0.01 mg/ml pepstatin) using a glass pestle. Membranes were recovered by centrifugation at 178 000g for 10 min at 4°C, then re-suspended in 800 μ l lysis buffer on ice. Protein concentration (typically 1.5–2.5 mg/ml) was determined using the Bradford Protein Assay (BioRad), and adjusted to 1 mg/ml. Fifty microlitres of 2 \times assay buffer (50 mM Tris–acetate buffer at pH 7.5, 20 mM MgCl₂, 2 mM dithiothreitol, 10 mM creatine phosphate, 200 units/ml creatinine kinase, 0.1 mM cAMP at pH 7.5, 0.2 mg/ml bovine serum albumin, 0.02 mg/ml aprotinin, 0.02 mg/ml pepstatin and fresh 0.2 mg/ml PMSF) was added to 20 μ l samples in glass vials on ice. Twenty microlitres Ca²⁺ solution (2.5 mM CaCl₂, 0.25 mg/ml calmodulin and 5 mM EGTA) was then added to samples, bringing the final [Ca²⁺] to 10⁻⁷ M (as calculated using MaxChelator v1.31). Samples were equilibrated at 25°C for 2 min, then H-Ras, K-Ras, Rab3a or GST-NF1-GRD were added (1 nM to 1 μ M) to the membrane preparation. After 5–60 min incubation at 25°C, 10 μ l radioactive substrate (10 μ Ci α -P³²-ATP, 2 mM ATP) was added, and each sample was incubated at 25°C for a further 10 min. Reactions were stopped by adding 150 μ l of stop solution (33 pCi/ μ l ³H-cAMP, 1.3% SDS, 30 mM ATP, 0.9 mM cAMP at pH 7.5). Then 750 μ l water was added and samples were loaded onto 1.5 ml Dowex AG 50W-X4 columns (pre-washed with 10 ml 1 M HCl followed by 10 ml water) and washed with 2 ml of water. Samples were eluted with 4 ml water onto Alumina columns (pre-washed with 10 ml 0.1 M imidazole at pH 7.5) and washed with 1 ml 0.1 M imidazole at pH 7.5. Then samples were eluted with 4 ml 0.1 M imidazole into scintillation vials, and 4 ml scintillation cocktail (Ultima Flow M) was added to each vial. Samples were counted for 5 min in a Beckman LS600IC using two windows 0–400 and 400–1000. Column efficiency was determined by recovery of ³H-cAMP, and the amount of ³²P-cAMP produced (pmol/min/mg protein) was calculated taking column efficiency into account. H-Ras and Rab3a were purchased from Sigma. K-Ras was from Merck. Radionucleotides were from Amersham and other chemicals from Sigma. All statistical analyses were performed using the paired student's *t*-test. Buffers for all experiments were prepared using Milli-Q purified water (Millipore).

GST fusion protein preparation

Wild-type and mutant NF1-GRD-GST fusion proteins (28) and GST alone were purified using glutathione beads as follows: 1 l cultures of *Escherichia coli* DH5 α cells carrying GST-fusion plasmids were grown in LB plus 100 mg/ml ampicillin at 37°C to log-phase and treated with 1 mM isopropyl- β -D-thiogalactopyranoside for 1 h. Cells were collected and lysed by sonication, at 4°C for six cycles with 20 s each cycle, in 40 ml sonication buffer containing 1 mM EDTA, 1 mM EGTA 0.1% lubrol, 0.1 mM dithiothreitol and protease inhibitor cocktail (Roche). After centrifugation at 15 700g (Beckman JS-13-1) for 30 min at 4°C, ~30 ml of supernatant was added to 1 ml of 50% glutathione beads (Sigma), rotated for 1–2 h at 4°C followed by centrifugation at 735g for 5 min at 4°C. Beads were washed with 10 ml sonication buffer with protease inhibitor cocktail and then washed

with 10 ml elution buffer containing 50 mM Tris, 0.5 mM MgCl₂ and 0.5 mM dithiothreitol. For elution of the protein, 3 ml of elution buffer was added plus 4.2 mg/ml glutathione (Sigma) and supernatants were collected by centrifugation. Proteins were added to the head membrane extracts, at 1 mM concentration, at different time points as described above for Ras. GST-NF1-GRD fusion constructs were provided by F. Tamanoi (University of California, Los Angeles, CA, USA).

Larval brain AC activity assay

About 30 brains per genotype of third instar larvae were dissected in *Drosophila* larval saline solution (70 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 115 mM sucrose, 5 mM HEPES, 5 mM trehalose, 1.5 mM CaCl₂, 20 mM MgCl₂ at pH 7.1) at room temperature. In order to mimic physiological conditions as closely as possible, brains were dissociated manually into individual neurons in 100 µl larval saline, using forceps and pipetting, than separated into control and experimental groups that were kept on ice while other genotypes were dissected. To minimize variability, control and experimental groups in each comparison were always assayed in the same batch. Results generated from such experiments were highly consistent. Experimental groups were incubated at room temperature, with 2 mM growth factors in 10 mM Tris-acetate (pH 7.5) for 5 min, or with 0.2 mM neurotransmitters in 10 mM Tris-acetate for 2 min, as indicated in methods. Controls were treated with 10 mM Tris-acetate alone for 2–5 min. After incubation, cells were centrifuged at low speed (1800g), then re-suspended in 180 µl lysis buffer (see above), and homogenized for 2 min on ice in an Eppendorf tube using a plastic pestle (Kontes). Protein concentration (typically 2–2.5 mg/ml) was determined using the Bradford Protein Assay (BioRad). Fifty microlitres 2× assay buffer (see above) and 20 µl Ca²⁺ solution (see above) was added to 20 µl samples in glass vials on ice. Samples were equilibrated at 25°C for 2 min, then 10 µl radioactive substrate (see above) was added, and reactions were incubated at 25°C for a further 10 min (growth factors) or 5 min (neurotransmitters). Reactions were stopped, then applied to columns and counted exactly as described for adult heads (see above). Growth factors (mouse EGF, rat TGFα), insulin and neurotransmitters (dopamine, FMRFamide, histamine and serotonin) were purchased from Sigma.

Mutagenesis of hNF1 and cloning of deletion constructs

Clones containing the human *NF1* gene were obtained from A. Bernards (Massachusetts General Hospital). The 88:12 clone is a *NotI*–*SalI* fragment that contains the entire human *NF1* 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' pCGCCACCGCGATGTAGCTCCAATTCG 3')

and mutation-specific mutagenesis primers, which 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 (Fig. 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-ΔGRD2 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 *XbaI* and re-ligating 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 white^{118(isoCJ1)} (48) *Drosophila* embryos together with pTURBO as a source of transposase (50). 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* (47). 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*^{P2} mutant background using *w/Y;CyO/Sp;TM3Ser/Sb* (47) to create doubly homozygous lines with normal or mutant *hNF1*; *Nf1*^{P2} 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 (Fig. 4B and C). 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 (Table 1).

MAP kinase activity

Flies were collected at the same time each day to minimize circadian differences in phospho-MAPK levels (7). For each genotype, 10 heads were homogenized in 75 µl 1× SDS loading buffer (Invitrogen) plus 0.5 mM dithiothreitol and protease inhibitor cocktail (Roche). Samples were run on precast

10% Tris–glycine gels (Novex, Invitrogen) in 1× Tris–glycine–SDS buffer at 125 V for 2 h. Proteins were transferred to nitrocellulose in 1× Novex buffer plus 20% methanol for 2 h at 25 V. Transfer was verified by Ponceau staining, then blots were blocked in 5% milk/TBST for 1 h at room temperature, rinsed for 3 × 5 min in TBST, then probed with primary antibody diluted 1/500 in 5% milk/TBST overnight at 4°C. Rabbit polyclonal antibodies to phosphorylated and non-phosphorylated human p44/42 MAPK were obtained from Cell Signaling Technology. Following rinses of 3 × 5 min in TBST, blots were incubated with 1/10 000 donkey anti-rabbit HRP-conjugated secondary antibody (Amersham) diluted in 5% milk/TBST for 1 h at room temperature, then rinsed again for 3 × 5 min in TBST, followed by 5 min TBS, prior to detection of signal using the ECL kit (Amersham) and multiple timed exposures to X-ray film. Blots were stripped for re-probing using ReBlot (Chemicon). A representative western blot probed with an anti-phospho-MAPK antibody and then stripped and re-probed with anti-MAPK antibody as shown in Figure 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 (Fig. 4D). The ratio of phospho-MAPK to MAPK was determined and the results of four to six independent experiments are graphed (Fig. 4E). Expression of full-length hNF1 was confirmed by western blot using a rabbit polyclonal antibody *sc-68* (Santa Cruz) directed against the C-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* (12). In order to improve the statistical power of our body size analysis, we separated males and females for pupal size measurements in this study, as 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); (51) 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.

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Conflict of Interest statement. The authors declare that they have no conflict of interest regarding this manuscript.

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